Insight into the underlying immune interactions of *Rickettsia* infection in the vector-pathogen-host interface

Natacha Alexandra Korni da Fonseca Milhano

Tese apresentada à Universidade de Évora
para obtenção do Grau de Doutor em Ciências Veterinárias

ORIENTADORES: *Doutora Rita de Sousa*
Professor David H. Walker
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ÉVORA, Setembro 2014

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The research work for this dissertation was performed at the Center for Vectors and Infectious Diseases Research, National Institute of Health (CEVDI/INSA), Lisbon, Portugal, and at the Department of Pathology, University of Texas Medical Branch, Galveston, Texas, USA.

The project was financed by the fellowship SFRH/BD/45174/2008 granted by the Fundação para a Ciência e Tecnologia, Lisbon, Portugal.

ACKNOWLEDGEMENTS

This incredible life experience would not have been possible, conceivable, or made sense without the presence and constant support of many, many valuable beyond words people who, in one way or another, contributed immeasurably towards spawning this thesis into existence.

First and foremost, I would like to thank my supervisors, Dr. Rita de Sousa, Dr. David Walker and Prof. Manuela Vilhena.

Dr. Rita de Sousa, for initiating me into this fascinating rickettsial world and sharing her vast knowledge with me, for her unconditional support, patience and vivaciousness at all times, for always readily giving me very sound advice on various lab dilemmas, and also a harsh word when needed, in a sentence, for bearing with me for so long -Thank you.

Dr. David Walker, for accepting to become my supervisor and opening his lab to me, for his invaluable insights regarding this project, for sharing his thoughts and wisdom on Rickettsiology during our many meetings where ideas flowed, suggestions were made, and experiments designed, for always having a kind word, it was a pleasure and a great honour to work with you.

To Prof. Manuela Vilhena, who has been limitless in her support and advice on many topics, and for always putting my mind at ease at stressing moments.

To Sherrill, for always knowing what to do in all situations and for being such a jolly person, spreading happiness wherever she goes.

To Dr. Donald Bouyer, for all his advice in many situations, and constant support when needed.

To Dr. Vsevolod Popov, for teaching me all I know about EM.

To all the lab members of the Walker and Bouyer lab at UTMB, for helping me so generously in many facets of my work, in particular Dr. Tais Saito, who helped me tremendously throughout my stay in the lab, and with whom I learned a whole world more about ticks than I could ever expect, Dr. Rong Fang, for her invaluable insights into immunology, and Jeremy, thank you so much for everything.

I would also like to thank Dr. Francisco Alarcon-Chaidez, who started me out in the direction of dissecting ticks and all analyses thereafter, with great friendship.

To all lab members in Centro de Estudos de Vectores e Doenças Infecciosas, with a special thanks to my close bunker colleagues, who have always made the daily toil incredible.
To my special Patricias and Catarina for being awesome friends.
To my unique Galveston family at the Yellow house, without whom life will never be the same again.
To my mother, who has been my light throughout.
To all present and gone - Thank you.

I would also like to extend my acknowledgements to the institutions where the research towards the completion of this dissertation was performed, namely Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisbon, Portugal, and the Department of Pathology, University of Texas Medical Branch, Galveston, Texas, USA.

Finally, I am extremely grateful to Fundação para a Ciência e Tecnologia for awarding me a PhD fellowship which allowed this work to come to fruition.

ABSTRACT

Ixodid ticks are second only to mosquitos in their notorious role as vectors of pathogens to both animals and humans. Rickettsioses are among the most important tick-borne diseases in Europe, Mediterranean spotted fever in particular. To date, many studies have been performed in order to uncover the underlying mechanisms of this disease, in terms of the interactions among its constituents, i.e., the pathogen, *Rickettsia conorii*, its vector, *Rhipicephalus sanguineus* tick, and a vertebrate host. However, important gaps remain in this knowledge, among them studies of the relationship of its vector and pathogen, and also the role of tick saliva in vector-host interactions. Thus, in order to approach these limitations, in the first part of this dissertation three studies were performed in order to analyze the vector-pathogen-host interface in natural settings (chapter 2). As a result of the first of these studies, co-infections of *Borrelia lusitaniae* with *R. helvetica* and *R. slovaca* were found in ticks collected from a natural safari park in the south of Portugal; new host-pathogen associations were found in the second study described, performed in Madeira Island, namely lizards infected with *R. monacensis*, as well as detection of *R. helvetica* in ticks, which was a first occurrence in this island; and in the last study a new species of *Rickettsia* was isolated from soft ticks collected from pig pens from Alentejo, Portugal. In chapter 3 experimental studies on the vector were performed through analysis of *R. massiliae* in *Rh. sanguineus* tick organs. We performed a quantitative analysis of *R. massiliae* in the salivary glands of feeding *Rh. sanguineus* ticks, and observed a statistically significant increase in bacterial load during the first two days, followed by a plateau up to day 6 of feeding. An ultrastructural study was also performed on the salivary glands, ovaries and midgut of *R. massiliae* infected-*Rh. sanguineus*, where we observed the reactivation phenomenon of *Rickettsia* in the salivary glands of fed ticks as a result of tick feeding. In the final part of this dissertation the role of tick saliva was ascertained in terms

of bacterial burden and immune responses in a murine susceptible host, using uninfected *Rh. sanguineus* ticks and C3H/HeJ mice. No statistically significant differences in bacterial load were observed between the two groups of R*. conorii*-infected animals, one of which infested with ticks. However, host cytokine analysis of both groups of animals revealed statistically significant differences, suggesting an inhibitory effect of tick saliva on host pro-inflammatory responses (chapter 4).

RESUMO

Estudo das interacções imunes do hospedeiro e vector resultantes da infecção por *Rickettsia* spp.

Os ixodídeos desempenham um papel fundamental como vectores de agentes patogénicos tanto em animais como em humanos. As rickettsioses, com destaque para a febre escaro-nodular, encontram-se entre as doenças transmitidas por carraças mais importantes na Europa. Até à data muitos estudos foram efectuados de modo a descortinar os mecanismos subjacentes a esta doença, em termos das interacções entre os seus constituintes, i.e., agente patogénico, *Rickettsia conorii*, o seu vector, o ixodídeo *Rhipicephalus sanguineus*, e um hospedeiro vertebrado. Todavia, existem ainda lacunas neste conhecimento a nível da relação vector-agente patogénico, para além do papel da saliva do vector nas interacções agente patogénico-hospedeiro. Assim, numa tentativa de colmatar estas limitações, na primeira parte desta dissertação foram realizados três estudos para análise dos fenómenos que ocorrem naturalmente na interface vector-agente patogénico-hospedeiro ocorrentes na natureza (capítulo 2). Como resultado do primeiro destes estudos, foram encontradas co-infecções de *Borrelia lusitaniae* com *R. helvetica* e *R. slovaca* em ixodídeos capturados num parque safari no sul de Portugal. No segundo estudo descrito, efectuado na ilha da Madeira, foram encontradas novas associações hospedeiro-agente patogénico, nomeadamente lagartixas infectadas com *R. monacensis*, detectou-se *R. helvetica* em carraças, pela primeira vez nesta ilha, e foi possível isolar uma nova espécie de *Rickettsia* a partir de carraças de corpo mole capturados em pocilgas no Alentejo, Portugal. O capítulo 3 aborda os resultados de estudos experimentais efetuados no vector, através da análise de *R. massiliae* em orgãos de *Rh. sanguineus*. Realizámos uma análise quantitativa da *R. massiliae* em glândulas salivares de *Rh. sanguineus* durante a sua refeição sanguínea, tendo-se observado um aumento estatisticamente significativo da carga bacteriana nos

dois primeiros dias de alimentação, seguido de um patamar até ao dia 6 da alimentação sanguínea. Foi também efectuado um estudo ultraestrutural nas glândulas salivares, ovários e intestino médio de *Rh. sanguineus* infectados com *R. massiliae*, onde observámos o fenómeno de reactivação da *Rickettsia* nas glândulas salivares dos ixodídeos alimentados, resultante do processo de alimentação. Na parte final desta dissertação analisámos o papel da saliva de ixodídeos em termos da carga bacteriana e respostas imunes num hospedeiro murino susceptível, usando *Rh. sanguineus* não infectados e ratinhos C3H/HeJ. Não verificámos diferenças estatisticamente significativas entre as cargas bacterianas de dois grupos de animais infectados com *R. conorii*, em que apenas um dos grupos estava infestado com carraças. Todavia, a análise de citoquinas no hospedeiro em ambos os grupos experimentais revelou diferenças estatisticamente significativas, sugerindo um efeito inibitório da saliva dos ixodídeos nas respostas pro-inflamatórias do hospedeiro (capítulo 4).

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CD	Cluster of differentiation
CEVDI	Centro de Estudos de Vectores e Doenças Infecciosas Dr. Francisco Cambournac
CTL	Cytotoxic T lymphocytes
DCs	Dendritic cells
DEBONEL	*Dermacentor*-borne necrosis erythema lymphadenopathy
DNA	Deoxyribonucleic acid
gltA	Citrate synthase
ID	Intradermal
IFA	Immunofluorescent assay
IFN	Interferon
IGS	Intergenic spacer
IL	Interleukin
INSA	Instituto Nacional de Saude Dr. Ricardo Jorge
ISF	Israeli spotted fever
ha	Hectares
htr	Rickettsial 17KDa protein gene
LB	Lyme borreliosis
LPS	Lipopolysaccharide
mRNA	Messenger ribonucleic acid
MSF	Mediterranean spotted fever
NF-kB	Nuclear factor
NK	Natural killer
NTC	Non-template control
NO	Nitric oxide
OmpA	Outer membrane protein A
OmpB	Outer membrane protein B

OSP	Outer surface protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Phosphoinisitide
PTK	Protein tyrosine kinase
qPCR	Quantitative polymerase chain reaction
RC	*Rickettsia conorii*
RMSF	Rocky Mountain spotted fever
rrs	16S ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
Sca	Surface cell antigen
SFG	Spotted fever group
SGE	Salivary gland extracts
s.l.	sensu lato
s.s.	sensu stricto
sp. nov.	Species nova
TEM	Transmission electron microscopy
TG	Typhus group
Th1/2	T helper 1 and 2 cells
TIBOLA	Tickborne lymphadenitis
TNF	Tumor necrosis factor
TLR	Toll-like receptor
UTMB	University of Texas Medical Branch

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CHAPTER 1

LITERATURE REVIEW AND AIMS

1. 1. Introduction

Ticks are obligate hematophagous ectoparasitic arthropods parasitizing all vertebrate classes, at some stage of their life cycle (Sonenshine 1991). Not only can they damage human and animal skin, but they also play a pivotal role in transmitting zoonotic pathogens. In fact, ticks are second only to mosquitos in their importance as vectors of human pathogens, including bacteria, viruses and protozoa, and represent the most critical group of arthropods capable of transferring pathogens between animal species (Balashov 1972). Their biomedical importance and significant impact on the economy, resulting from massive losses in the cattle industry, have been paramount in the increasing interest in the study of these arthropods (Paddock and Telford 2010).

1.2. Ticks

1.2.1. Tick classification

The first reports on tick phylogenetic trees appear to have been made by Hoogstraal and Aeschlimann (1982), inferred from intuition on relative 'primitiveness' of tick morphology, life cycles and of their hosts. However, preliminary hypotheses about evolutionary relationships of ticks had already been suggested long before (Pomerantsev 1948, Camicas and Morel 1977). It was only later, in the 1990s, that molecular characters were taken into account in phylogeny, paving the way to an ever increasing number of papers on molecular phylogeny and evolution of ticks (Wesson and Collins 1992, Black and Piesman 1994, Rich *et al*. 1997, Klompen *et al.* 2000, among many others).

Ticks belong to the class Arachnida, subclass Acari, order Parasitiformes and suborder Ixodida (Sonenshine 1991), and are divided into three families: Ixodidae, or hard ticks, containing 720 species, Argasidae, or soft body ticks, comprising 186 species, and Nutalliellidae, represented by a single species confined to southern Africa (Table 1.1).

Table 1.1. Taxonomy within the order Ixodidae (adapted from Barker and Murrell 2008)

Family	Genus
Ixodidae	*Amblyomma* (143 species)
	Anomalohimalaya (3 species)
	Bothriocroton (6 species)
	Cosmiomma (1 species)
	Cornupalpatum (1 species)
	Margaropus (3 species)
	Nosomma (1 species)
	Dermacentor (36 species)
	Haemaphysalis (166 species)
	Hyalomma (27 species)
	Ixodes (249 species)
	Rhipicentor (2 species)
	Rhipicephalus (79 species)
Argasidae	*Argas* (58 species)
	Antricola (3 species)
	Carios (88 species)
	Ornithodoros (37 species)
	Otobius (3 species)
Nuttalliellidae	*Nuttalliella*

The family Ixodidae comprises two major groups, the Prostriata and the Metastriata, mainly distinguished based upon their mating habits. The reproductive strategy of the Metastriate group is closely linked to their feeding, i.e., they only mate while feeding on the host, and the Prostriata mate as readily on or off the host (Sonenshine 1991). The Prostriata include 249 species in a single genus *Ixodes*, whereas the Metastriata are divided into four subfamilies: the Amblyomminae, including the genus *Amblyomma*; the Hyalomminae, including the genus *Hyalomma*; Haemaphysalinae, including the genus *Haemaphysalis*; and the Rhipicephalinae, including the genera *Dermacentor*, *Cosmiomma*, *Margaropus*, *Nosomma*, *Anomalohimalaya*, *Rhipicentor* and *Rhipicephalus* (Mullen and Durden 2002).

Argasidae are divided in two main lineages, the subfamilies Argasinae, which include the genera *Argas* and *Antricola*, and Ornithodorinae, including genera *Carios*, *Ornithodoros* and *Otobius* (Klompen, 1992, Klompen and Oliver 1993).

1.2.2. Tick morphology

Ticks are structured in two parts which are fused together: the capitulum, or gnathosoma, where the mouthparts are included, and the body, or idiosoma, which is formed by the fusion of the podosoma and opisthosoma. The podosoma contains the legs and genital pore and the opisthosoma, located on the posterior region from the leg coxae, the spiracles and the anal aperture (Sonenshine 1991). The mouthparts contain the segmented palps, the basis capituli, from which the segmented chelicerae extend, and the hypostome (Anderson and Magnarelli 2008). It is the movement of both chelicerae, resulting in the rip and tear of the host's skin, that allows for the formation of a blood pool from which the tick then sucks the blood using the food canal in its hypostome (Sonenshine 1991).

Ixodid ticks possess a very distinct external morphology from that of Argasid ticks, in that a hard sclerotized plate, or scutum, covers the entire body in male ixodid ticks, and the dorsal anterior part of females and immature forms (Fig.1.1A). The synthesis of new alloscutum, the folded cuticle posterior to the anterior dorsal body in females, nymphs and larvae, allows their expansion upon feeding, which in females can reach up to 100 times their unfed size, depending on species (Sonenshine 1991). As a result, upon full engorgement, the scutum occupies a very small percentage on the anterior region of the largely extended body (Fig.1.1B). Males, on the other hand, are limited in their physical expansion when feeding due to the complete coverage of the idiosoma by the scutum (Fig.1.1A). Immature forms resemble adults, except they lack the external genital pores, porose areas and foveal pore clusters present in adults. In addition, larvae possess only three pairs of legs, whereas nymphs and adults possess four (Sonenshine 1991).

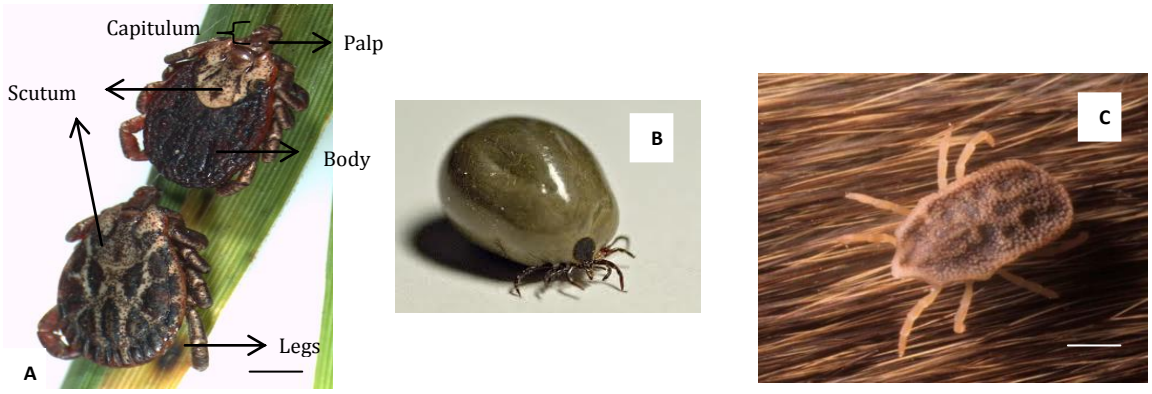

Fig.1.1. Hard and soft tick morphology. (A) Dorsal-lateral view of male (below) and female (above) *Dermacentor marginatus*. (B) Engorged female *Ixodes* sp. (C) Dorsal view of argasid tick. Bar 1.5mm.

Argasid ticks possess a leathery and flexible cuticle, with no scutum (Fig.1.1C). Males and females are distinguished only by their genital pore. Their capitulum is located in a subterminal position, with the exception of larvae, where it is positioned anteriorly, and no dorsal shield is present (Anderson and Magnarelli 2008). In contrast to ixodid ticks, argasids do not swell immeasurably during feeding, extensive stretching does occur, owing to the folded nature of the integument; however, no additional growth takes place (Sonenshine 1991). The nymphal instar number may vary from 2-8, depending on bloodmeal sizes in preceding instars, and adults have multiple gonadotrophic cycles, as they can feed repeatedly with females laying small eggs masses after each feeding (Sonenshine 1991).

1.2.3. Ixodid tick life cycle and feeding behaviour

The ixodid tick life cycle includes four stages, namely, the embryonated egg, larva, nymphs and adults (Fig.1.2).

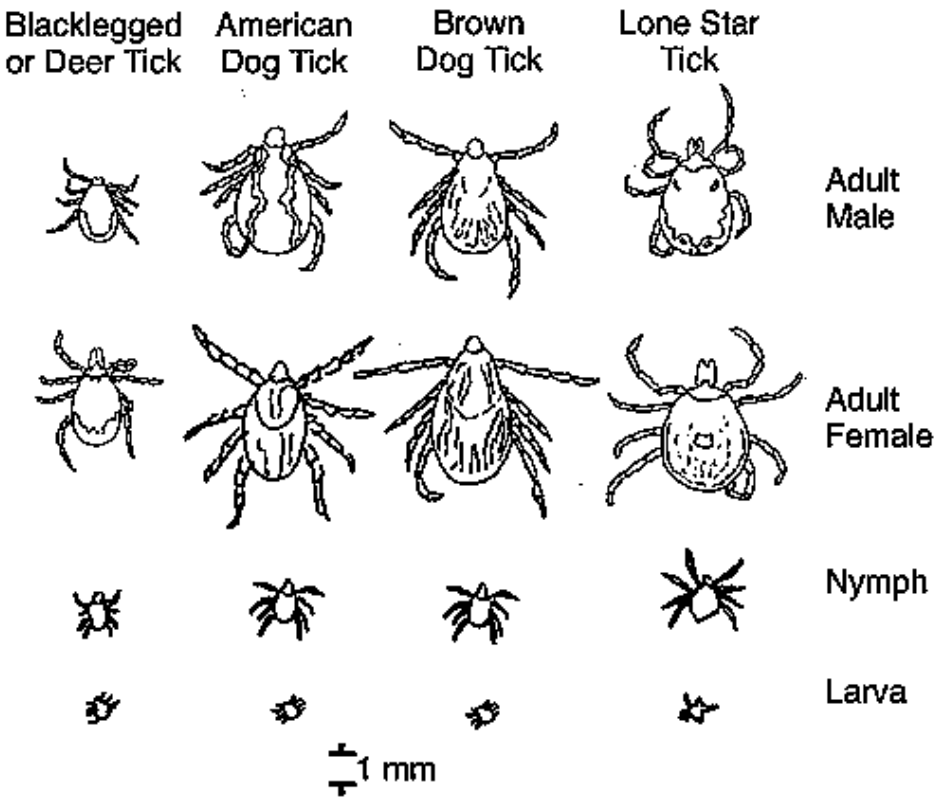

Fig.1.2. Relative sizes of four different species of ticks in three life stages: larvae, nymphs and adults (courtesy of Mary Predny from Virginia Cooperative Extension Publication).

The majority of ixodid species require a blood meal in order for the immature stages, i.e., larvae and nymphs, to transition to the subsequent development stage, the same being true of adults for reproduction (Sonenshine 1993).

The life cycle of ixodid ticks is classified according to the number of times the development stages change hosts, and whether or not immature stages molt on their host, thus giving rise to one-host, two-host or three-host ticks (Balashov 1972, Sonenshine 1993). In general, larvae remain in areas of high humidity, usually close to the ground, where they quest for small mammals, on which they feed and drop off (or stay on, in one or two host tick cases) once engorged. After molting into nymphs the cycle begins again until they engorge and drop off (or stay on, in one host ticks). Engorged larvae and nymphs weigh about 10 to 20 times their unfed weight. Once nymphs molt into adults, they will then seek, feed and mate on their hosts, although mating may also occur on vegetation, a less

common occurrence, after which the engorged females drop off and lay eggs (Fig.1.3). Engorged females can weigh up to 100 times their unfed weight (Sauer *et al.* 1995, Goodman *et al.* 2005).

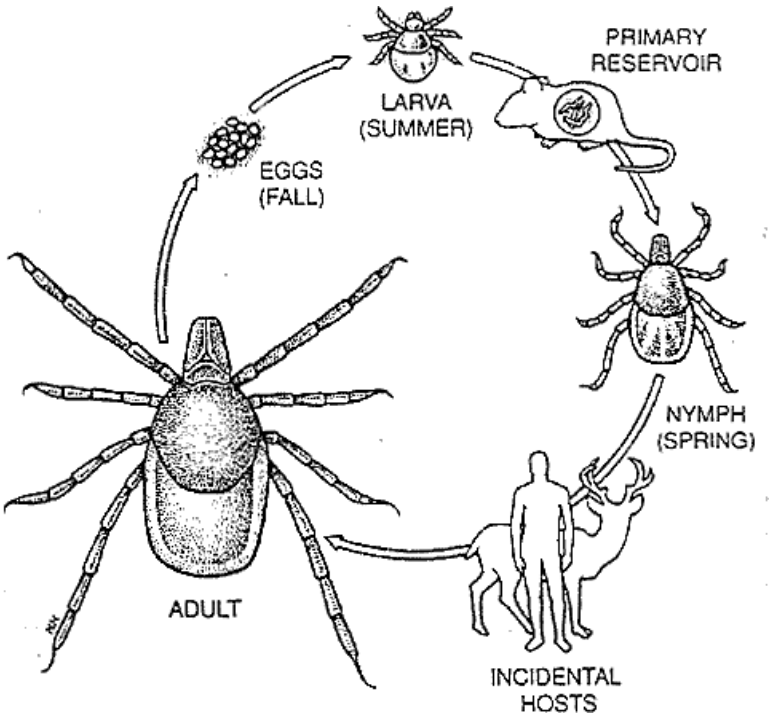

Fig.1.3. Life cycle of ixodid ticks (adapted from www.wwhd.org).

Depending on the species, females can lay up to 3000 eggs or more, after which they dies. The males can feed more than once, and remain on the host seeking to mate with other females. Sexual dimorphism only exists in the adult stage. The time period between feeding periods can range from weeks to several months. The complete life cycle of a tick depends on the host availability and microenvironment, i.e., temperature and humidity, and it may take between one and two years, although some species can go live to six years (Anderson and Magnarelli 2008).

Ticks depend on bloodmeals for their survival, molting and reproduction. According to Anderson and Magnarelli (2008), a successful bloodmeal depends on a series of sequential

steps, which they divided as appetence, engagement, exploration, penetration, attachment, ingestion, engorgement, detachment and disengagement. The main host seeking pattern ixodid ticks adopt is the ambush strategy, where ticks crawl onto vegetation and wait for unsuspecting hosts to pass by (Sonenshine 1993, Goodman *et al*. 2005). Once this occurs, host stimuli such as odor, carbon dioxide, heat and vibration trigger the tick to then cling on to the host, be it hair, fur, or cloth. Once the attachment site is selected, usually in sheltered locations of the host's body, a process aided by tactile stimuli, the tick prepares itself by placing its body at an angle relative to the host's and uses its chelicerae to start cutting through the epidermis and dermis, inserting its hypostome into the lesion. Cement secretion in the tick saliva occurs soon afterwards, pooling around the mouthparts and quickly hardening, thus securing the tick firmly onto the feeding site (Anderson and Magnarelli 2008). An alternating pattern of imbibing blood and injecting pharmacologically active ingredients through tick saliva then takes place, and occurs throughout the whole bloodmeal. Ixodid ticks, in contrast to argasid ticks, are slow feeders. They usually remain attached for an extended period of time, ranging from a few days to a couple of weeks, depending on their life cycle and species involved. In general larvae can feed from 2 to 4 days, nymphs up to 8 days, and adults can feed for as long as two weeks. The prolonged period of the bloodmeal is required for the production of new cuticle which permits the progressively increasing size of the tick's body while feeding (Anderson and Magnarelli 2008). Once engorged, the ticks drop off and seek a safe haven for the molting process. Unfed ticks are able to survive long periods without feeding due to their ability to absorb water from unsaturated air (Needham and Teel 1991).

1.2.3.1. *Rhipicephalus sanguineus* tick

Rhipicephalus sanguineus, also known as the brown dog tick, is the most geographically widespread tick species in the world, particularly in tropical and subtropical regions (Fig. 1.4).

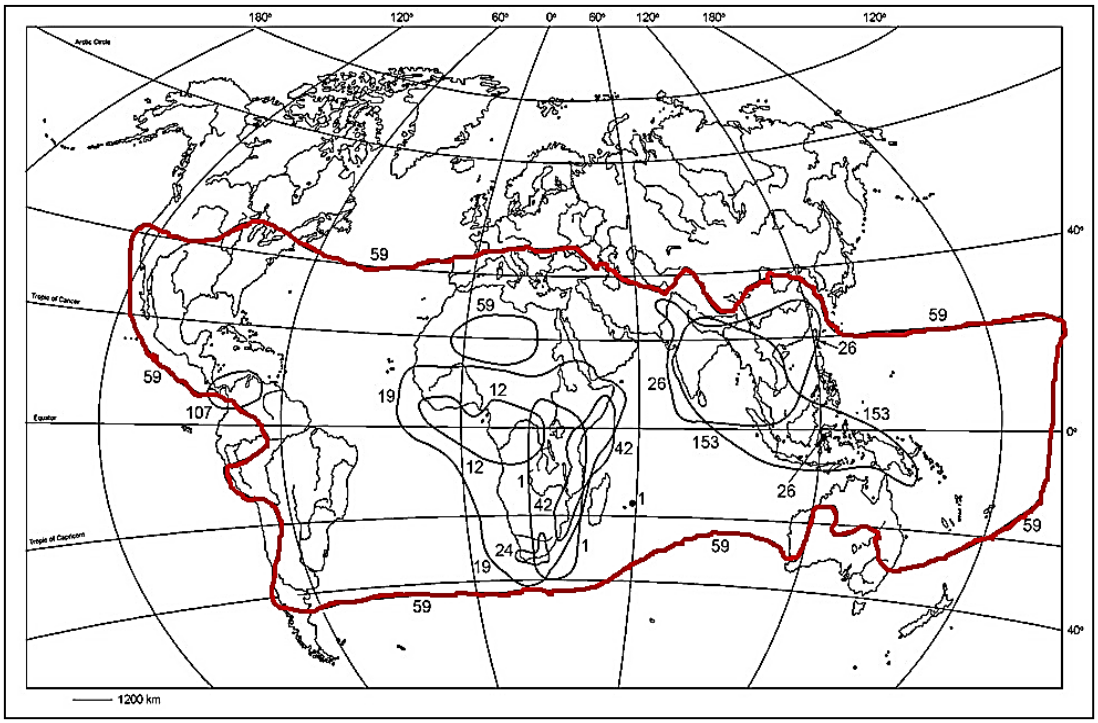

Fig.1.4. World distribution of *Rh. sanguineus* (Latreille, 1806) (Kolonin 2009)

It was originally described by Latreille (1806) as *Ixodes sanguineus*, and was later placed in the genus *Rhipicephalus* (Koch 1884). Many attempts to classify the species have since then been undertaken, and the taxonomic debate remains to this day (Camicas *et al.* 1998, Szabo *et al.* 2005, Dantas-Torres *et al.* 2013).

Rhipicephalus sanguineus ticks feed primarily on dogs, but are also known to parasitize a wide range of wild and domestic animals, including cats, rodents, birds, and also humans (Dantas-Torres 2010). *Rhipicephalus sanguineus* play a fundamental role in veterinary and human medicine, as it is a known vector and reservoir of many pathogens, including

Rickettsia, *Ehrlichia*, *Babesia*, and *Coxiella burnetii* (Dantas-Torres 2008). It exhibits a primarily endophilic nature, but can also survive in outdoor environments, particularly in refuges and areas where dogs are found (Demma *et al.* 2005, Parola *et al.* 2008). *Rhipicephalus sanguineus* mainly displays a host-seeking behaviour; however, it also adopts the questing behaviour (Dantas-Torres 2010). As a metastriate, this species reaches sexual maturity and mates only while on the host, with females not engorging fully until mating occurs (Dantas-Torres 2010). *Rhipicephalus sanguineus* is a three-host tick, requiring a new host per feeding stage, upon which larvae typically feed for around 2 days, nymphs for 4-5 days, and females can feed for weeks, depending on host and environmental factors (Koch 1982, Troughton and Levin 2007). The process of oviposition may last several weeks, and the number of eggs laid, varying from 1500-4000, depends on the weight and period of oviposition (Koch 1982).

1.2.4. Ixodid tick salivary glands and role of tick saliva

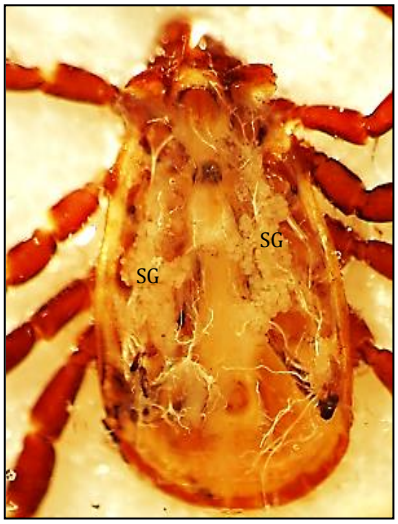

Fig. 1.5. *Rhipicephalus sanguineus* salivary glands (SG)

The salivary glands of ixodid ticks are the largest glands in the tick's body, and undergo remarkably complex cytological changes necessary to accommodate the tick's physiological requirements (Bowman *et al.* 2008). They consist of grape-like clusters of acini, granular and agranular cells, and are localized anterolaterally on both sides of the tick's body, occupying one third to one half of the hemocoel (Fig. 1.5). Salivary glands are composed of three acinar types in females (I-III) and four in males (I-IV), attached to a main and branching ducts

(Till 1961, Chinery 1965). Type I acini are localized along the main duct, near the anterior end of the salivary gland, whilst the remaining types are found posteriorly along a ramifying system of intralobular ducts (Fig. 1.6). Each acinar type consists of multiple cell types: type I acini are agranular, and possess one central cell type surrounded by pyramidal cells, a constrictor cell and peritubular cells surrounding the acinar duct; type II contain granular 'a', 'b', 'c1-c4', agranular ablumenal interstitial cells and an adlumenal interstitial cell, and type III has only 3 cell types, 'd', 'e' and 'f' (Fig.1.6). Type IV acini are present only in male ticks, containing only one type of secretory cells, type G. They are small in undifferentiated ticks, becoming hypertrophied during feeding and as large or even larger than types II and III acini (Sonenshine 1991).

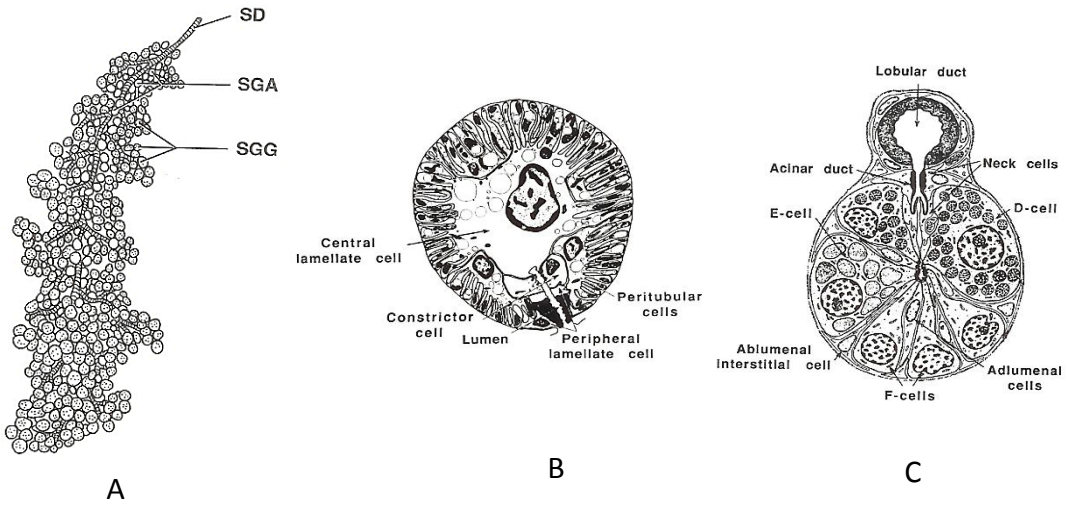

Fig. 1.6. Diagrams illustrating (A) General view of salivary gland; (B) Agranular acinus; (C) Granular acinus. SD- Main salivary duct; SGA- agranular salivary gland acini (type I); SGG- granular salivary gland acini (types II, III) (adapted from Sonenshine 1991).

The acini undergo extensive differentiation during tick feeding, with major changes in cellular differentiation.

Type I acini are involved in osmoregulation, particularly during the unfed periods of the tick, where the cells secrete highly concentrated solutions which are excreted via salivary ducts onto the hypostome surface (Gill and Walker 1987). Moisture from the atmosphere is then condensed on these hygroscopic salty deposits, which are then imbibed by the tick, keeping it hydrated. Various repetitions of this cycle facilitate the maintenance of the tick's water balance (Sonenshine 1991). Types II and III granular acini are mainly involved in secretion of bioactive compounds during feeding periods, including cement and anticoagulants. The role of acini type IV in males is not clear, an involvement in the spermatophore transfer to the female genital pore has been suggested, along with observations of intense salivation occurring during this process (Feldman-Muhsam *et al.* 1970).

After engorgement, the female tick detaches and, after a few days, the salivary glands start to degenerate through a highly regulated process of programmed cell death. The increased levels of ecdysteroids in the salivary glands post-detachment have been associated with the degenerative process of the tick salivary gland (Harris and Kaufman 1985, Lomas *et al.* 1998).

The salivary glands play a fundamental role both as sites of development and subsequent transmission of infectious agents as well as secretion of bioactive products released into the saliva during the bloodmeal.

Throughout the course of a bloodmeal, ticks remain attached to a host for extended periods of time, ranging from a few days to a couple of weeks, depending on their stage in the life cycle and species involved. A normal host response to the mechanical damage caused by the tick bite would be an immediate activation of hemostasis, preventing blood loss; inflammation, producing itch or pain, thus potentially triggering defensive behavior of the host, and immunity (cellular and humoral) (Francischetti *et al.* 2010)(Fig.1.7). However, ticks have circumvented these potential problems by developing a wide variety

of pharmacologically active molecules in their saliva, which are injected into their host, allowing them to remain essentially undetected during their blood meal, feed successfully and transmit infectious agents (Ribeiro *et al.* 2006, Wikel 2013). In particular, tick saliva plays vital functions in the immunomodulation by the host via: a) increasing blood flow in the bite site through secretion of vasoactive agents, b) inoculating anticoagulants that keep the host's blood in the fluid form, c) inhibiting the inflammatory process in the host, and d) immunosuppressing the host and enabling the attachment of ticks, making their rejection by the host difficult (Sauer *et al.* 2000).

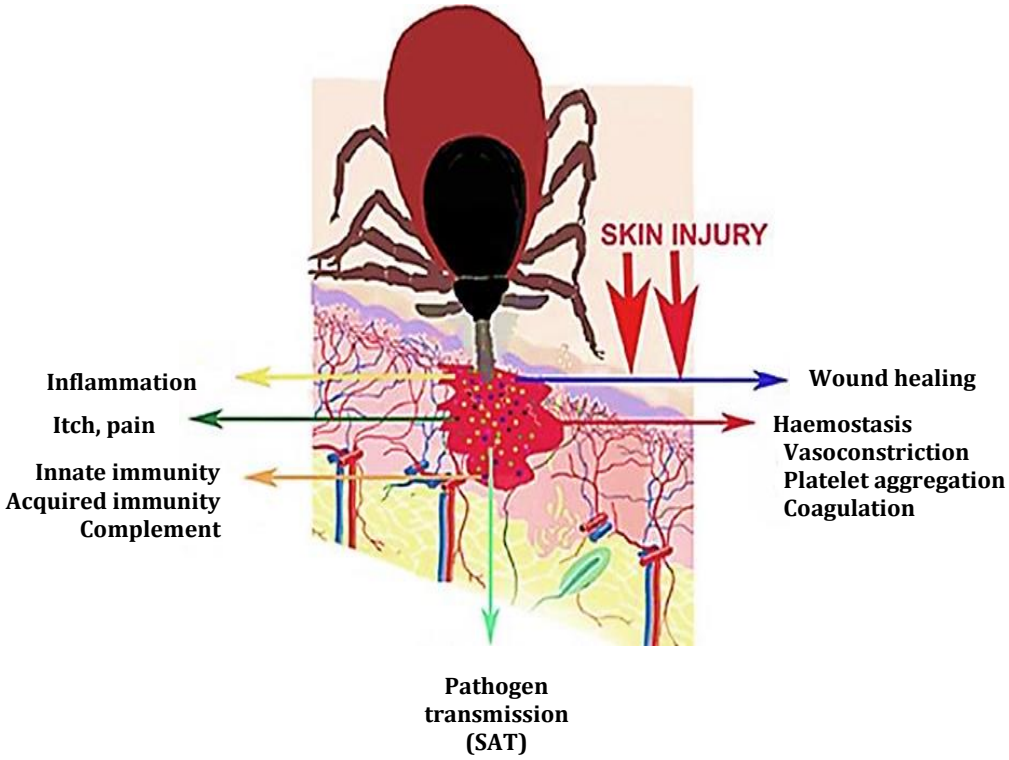

Fig.1.7. Ixodid tick saliva constituents modulate host defence responses (itch, pain, haemostasis, inflammation, immune reactions) (adapted from Kazimirova and Stibraniova 2013).

The hundreds of proteins contained in tick saliva are differentially expressed throughout the arthropod's bloodmeal, stimulated by the continuous tick/host interplay. An increasing amount of studies have been and are being focused on genomic and proteomic

approaches in order to identify and characterize these proteins, and many compounds have now been identified (Table 1.2).

Table 1.2. Genomic and proteomic studies (adapted from Liu and Bonnet 2014).

Studies/tick species	Tick organs	Tickborne pathogens	Technique used	No. diff. expressed transcripts proteins	References
Genomic					
D. variabilis female	Salivary glands Midgut Ovaries	*R. montanensis*	DD-PCR	54	Macaluso et al. 2003
I. scapularis nymph	Salivary glands	*B. burgdorferi*	LCS	10	Ribeiro et al. 2006
I. scapularis nymph	Whole tick	*Langat virus*	MH	48	McNally et al. 2012
I. scapularis embryos	IDE8 tick cells	*A. marginale*	SSH	35	De la Fuente et al. 2007
I. ricinus female	Whole tick	*B. burgdorferi*	SH	11	Rudenko et al. 2005
R. appendiculatus female	Salivary glands	*T. parva*	LCS	3	Nene et al. 2004
R. microplus male	Salivary glands	*A. marginale*	SSH	99	Zivkovik et al. 2010
Proteomic					
I. scapularis embryos	IDE8 tick cells	*A. marginale*	2D-DIGE; MALDI-TOF MS	3	De la Fuente et al. 2007
I. scapularis embryos	ISE6 tick cells	*A. phagocytophilum*	IEF, 2D-DIGE, MALDI-TOF MS, RP-LC MS/MS	5	Villar et al. 2010a
R. bursa female	Whole internal organs	*T. annulata*	2D-DIGE, RP-LC MS/MS, MALDI-TOF MS	16	Villar et al. 2010b
R. microplus female	Ovaries	*Babesia bovis*	IEF, 1/2DGE, HPLC-ESI-MS/MS	19	Rachinsky et al. 2007
R. microplus female	Midgut	*B. bovis*	EF, 1/2DGE, HPLC-ESI-MS/MS	20	Rachinsky et al. 2008
Rh. sanguineus female	Whole internal organs	*R. conorii*	2D-DIGE, RP-LC MS/MS, MALDI-TOF MS	10	Villar et al. 2010b
Rh. sanguineus female	Whole internal organs	*E. canis*	2D-DIGE, RP-LC MS/MS, MALDI-TOF MS	6	Villar et al. 2010b
	Whole ticks	*A. ovis*	IEF, 2D-DIGE, MALDI-TOF MS, RP-LC MS/MS	50	Villar et al. 2010a
R. turanicus female	Whole internal organs	*A. ovis*	2D-DIGE, RP-LC MS/MS, MALDI-TOF MS	9	Villar et al. 2010b

DD-PCR: differential-display polymerase chain reaction, LCS: cDNA library clones sequencing, MH: microarray hybridization, SH: subtractive hybridization, SSH: suppression-subtractive hybridization; D: dimensional, DIGE: differential in-gel electrophoresis, DGE: dimensional gel electrophoresis, ESI: tandem electrospray, HPLC: high-performance liquid chromatography, IEF: isoelectric focusing, MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight, MS: mass spectrometry, RPLC: reversed phase liquid chromatography

Cytokines play a fundamental role in regulating immune and inflammatory responses, including innate immunity, antigen presentation, and cellular recruitment and activation, among others (Borish and Steinke 2003). Studies have shown that ticks are able to polarize murine host cytokine expression from a Th1-mediated immune reaction, predominant at the early stages of feeding, towards a Th2 profile several days into the bloodmeal (Ramachandra and Wikel 1992, Ramachandra and Wikel 1995, Ferreira and Silva 1998). Th1 cells produce interleukin-2 (IL-2) and interferon-γ (IFN-γ), for control of cell mediated immunity, and Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 for mediation of Ab responses (McGhee 2005). Tick saliva or salivary gland extracts have been shown to severely impair T-cell functions, due, in part to reduced local production of IL-2 and IFN-γ (Ribeiro *et al.* 1985, Ramachandra and Wikel 1992, Urioste *et al.* 1994, Inokuma *et al.* 1994).

Some constituents in tick saliva may have more than one biological activity, an example of which are prostaglandins. These are well known immunosuppressants, and can suppress IL-2 and IFN-γ production, thus inhibiting T-cell function (Betz and Fox 1991). Prostaglandin E2, contained in *Boophilus microplus* saliva, has been shown to inhibit T-cell proliferation (Inokuma *et al.* 1994). On the other hand, increased prostaglandin levels in the saliva of *Amblyomma americanum* ticks inhibit platelet aggregation by preventing ADP secretion during platelet activation (Ribeiro *et al.* 1992, Bowman *et al.* 1995). Another possible function of tick prostaglandins is a vasodilatory one, potentially important at the tick feeding site (Bowman *et al.* 1996). Apyrase is another example of a multifunctional component, inhibiting platetet aggregation (Titus and Ribeiro 1990) and also preventing neutrophil aggregation and degranulation of mast cells (Ribeiro *et al.* 1985).

The tick immunosuppressive effects on the host enable a favourable environment for the transmission and establishment of tick-borne pathogens (Wikel and Bergman 1997,

Frischknecht 2007). Early studies using insects showed that salivary gland homogenates of the sandfly *Lutzomyia longipalpis* increased *Leishmania major* up to 5000 times in cutaneous lesions, and the size of these lesions grew 10-fold after injecting of a mixture of salivary gland homogenate and promastigotes into mouse footpads (Titus and Ribeiro 1988). Further studies showed that treatment of C3H/HeJ mice with cytokines suppressed by salivary gland extracts, namely TNF-α, IFN-γ and IL-2, conferred resistance to infestation of *B. burgdorferi* infected *I. scapularis* nymphs (Wikel *et al*. 1997). To date, the transmission of at least 10 tick-borne agents has been shown to be potentiated by tick saliva, in a phenomenon called saliva-assisted transmission, or SAT (Nuttall and Labuda 2008).

Even though significant advances in technology have taken place in terms of those approaches, the complexity of working with ticks, particularly infected ones, added to the limited amounts of protein obtained from ticks, have makes this area of study challenging to conduct (Valenzuela 2002, Alarcon-Chaidez and Wikel 2004, Madden *et al*. 2004, Oleaga *et al*. 2007, Rachinsky *et al*. 2007, Villar *et al*. 2010a).

1.3. Ixodid ticks as vectors of disease

Ixodid ticks play a crucial role as vectors of human and veterinary diseases, and their importance in public health has been highlighted with the emergence of new vector-borne infectious agents as well as re-emergence of previously known ones (Gubler 1998) (Table 1.3).

Table 1.3. Tick-borne diseases of humans (adapted from Dantas-Torres *et al*. 2012).

Diseases	Pathogens	Vectors	Distribution
African tick bite fever	*Rickettsia africae*	*Amblyomma hebraeum, A. variegatum*	Africa, West Indies
Human granulocytic anaplasmosis	*Anaplasma phagocytophilum*	*Haemaphysalis concinna, H. punctata, Ixodes ricinus, I. pacificus, I. scapularis, Rhipicephalus bursa*	Europe, North America, Asia
Human monocytic ehrlichiosis	*Ehrlichia chaffeensis*	*A. americanum*	North America
Lyme borreliosis	*Borrelia burgdorferi sensu lato*	*I. hexagonus, I. pacificus, I. persulcatus, I. ricinus, I. scapularis*	Asia, Europe, North America
Mediterranean spotted fever	*Rickettsia conorii*	*Rh. sanguineus, R. turanicus*	Africa, Asia, Europe
Relapsing fever	*Borrelia spp.*	*Ornithodoros spp.*	Africa, Asia, Europe, North America, South America
Rocky Mountain spotted fever	*R. rickettsii*	*A. aureolatum, A. cajennense, Dermacentor andersoni, D. variabilis, Rh. sanguineus*	North, South and Central America
Tularemia	*Francisella tularensis*	*Many species of different genera*	Asia, Europe, North America
Babesiosis	*Babesia divergens, B. microti*	*I. ricinus, I. scapularis*	Europe, North America
Crimean-Congo hemorragic fever	Nairovirus	*A. variegatum, H. punctata, Hyalomma anatolicum, H. marginatum, H. truncatum, R. bursa*	Africa, Asia, Europe
Tick-borne encephalitis	Flavivirus	*I. persulcatus, I. ricinus, H. concinna, H. punctata*	Asia, Europe

The global number of epidemiologically important tick-borne diseases has increased dramatically in the last 30 years, examples of which are the more than ten newly

recognized spotted fever rickettsioses identified since 1984 (Raoult *et al.* 1986, Parola *et al*. 2005, Paddock *et al*. 2008, Shapiro *et al.* 2010).

Malaria and dengue are among the most important vector-borne diseases in the world; however, in Europe, tick-borne infections prevail as the main vector-borne diseases, particularly rickettsioses such as Mediterranean spotted fever, MSF (Randolph 2010).

Rickettsioses are caused by obligate intracellular bacteria belonging to the order Rickettsiales, and are transmitted by arthropod vectors such ticks, lice, mites and fleas, which may act as vectors, reservoirs or/and amplifiers (Parola *et al*. 2005). Rickettsial diseases have a worldwide distribution and are among the oldest infectious diseases known to man, causing mild to severe or even fatal human cases. With the advances in molecular biology and cell culture techniques, new and re-emerging rickettsioses are continuously being described, many of which have been shown to play a role in human pathology (Vitale *et al.* 2006, Jado *et al.* 2007, Mediannikov *et al.* 2008, Nilsson 2009).

In 1899, Edward E. Maxey reported the first clinical description of Rocky Mountain spotted fever. The initial description of typhus fever was published by Fracastoro in 1546. The genus *Rickettsia* was named after Howard T. Ricketts (1871-1910), a medical scientist trained in Pathology and Microbiology, who identified the causative agent of Rocky Mountain spotted fever, *Rickettsia rickettsii*, in 1906. This bacterium was identified for the first time in the blood of experimentally infected guinea pigs and monkeys, and also in tissues and eggs of *Dermacentor variabilis* ticks (Ricketts 1906a,b). While in Mexico City investigating the origin of an epidemic typhus outbreak, Howard T. Ricketts was infected during his attempts to isolate the organism, succumbing from the infection in 1910. Later on other *Rickettsia* came to be discovered, namely *R. conorii*, *R. typhi*, *R. parkeri*, *R. montanensis*, and *R. rhipicephali*, in 1910, 1929, 1939, 1963 and 1978, respectively, of which the last two are non-pathogenic (Parola *et al.* 2005).

1.3.1. Rickettsial morphology

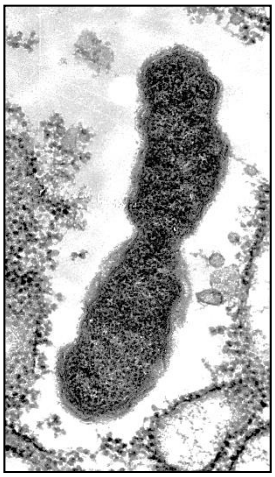

Fig. 1.8. Electron micrograph of binary fission of *Rickettsia* sp. (Courtesy of Dr. Popov)

Rickettsiae are small, gram-negative, obligate intracellular bacteria belonging to the order Rickettsiales, family Rickettsiaceae and genus *Rickettsia*. Phenotypically they grow in the cytoplasm of host cells, unbound by membranes, with some species also growing in the nucleus, and divide by binary fission (McDade 1998). Morphologically they are short, rod-like coccobacillary microorganisms, ranging from 0.3 to 0.5μm in width and 0.8 to 2 μm in length, with no flagella or pili (McDade 1998) (Fig. 1.8). The *Rickettsia* are surrounded by an outer cell wall, similar in appearance to that of other gram-negative bacteria, containing lipopolyssacharide (LPS), its precursor 2-keto-3-deoxyoctulosonic acid (KDO), and peptidoglycan (Schramek *et al.* 1976, Smith and Wrinkler 1979). External to the cell wall, they are often surrounded by a protein S-layer and electron lucent halo zone of undetermined composition (Silverman *et al.* 1978a,b).

1.3.2. *Rickettsia* classification

Rickettsia are phylogenetically divided into four groups, based on whole-genome analysis and antigenic characteristics: spotted fever group (SFG), including most of the rickettsial species, among many others; typhus group (TG), including *Rickettsia typhi* and *R. prowazekii*; an ancestral group which includes *R. bellii* and *R. canadensis*; and a transitional group, in which are included *R. australis*, *R. felis* and *R. akari* (Gillespie *et al.* 2007, 2008). Classification of *Rickettsia* within each group is an ever changing process, with shifts of certain *Rickettsia* spp. between groups occurring with the advent of new

evaluation methods. Traditionally *Rickettsia* were classified into 3 groups: typhus, spotted fever and ancestral group. Proposal of the new transitional group came with the phylogenomic and bioinformatics evaluation of nine *Rickettsia* spp. (Gillespie *et al*. 2007). The discovery of rickettsial plasmids allows for potential transfer of genetic material between groups of *Rickettsia* (Baldridge *et al.* 2010). An example of this re-classification is *R. felis*, formerly a member of the SFG *Rickettsia*, now in the transitional group (Ogata *et al.* 2005; Gillespie *et al.* 2008). Antibody cross-reactivity to LPS antigens occurs among members of the same biogroup, being uncommon to occur between groups (Vishwanath 1991).

Outer membranes proteins A (OmpA) and B (OmpB), members of the surface cell antigen (Sca) autotransporter (AT) protein family of *Rickettsia*, also known as Sca0 and Sca5, respectively, play a fundamental role in adhesion of *Rickettsia* to host cells. An additional 15 Sca orthologs (Sca1-Sca4, Sca6-Sca16) have been identified in nine rickettsial genomes (Blanc *et al.* 2005); however, only four of these (Sca0, Sca1, Sca2 and Sca5) have been shown to have specific functions (Li and Walker 1998, Feng *et al.* 2004, Uchiyama *et al.* 2006, Cardwell and Martinez 2009). All *Rickettsia* possess OmpB, but only the SFG rickettsiae possess OmpA.

Rickettsiae of the SFG possess several distinct characteristics that set them apart from other species, including expression of OmpA which, along with LPS and heat shock proteins, are recognized by the host humoral immune response; they reside in tick vectors, and use actin-based motility for intracellular locomotion (Gillespie 2007, Walker and Ismail 2008). Members of this group include both pathogenic and apparently non-pathogenic *Rickettsia* (Table 1.4).

Table 1.4. Main spotted fever group *Rickettsia* (adapted from Renvoise *et al*. 2009).

Species	Disease
R. aeschlimannii	Not designated
R. africae	African tick-bite fever
R. amblyommii	Not designated
R. conorii	Mediterranean spotted fever, Israeli spotted fever, Astrakhan fever, Indian tick-bite typhus
R. heilongjiangensis	Far Eastern tick-borne rickettsiosis
R. helvetica	Aneruptive fever
R. honei	Flinders Island spotted fever
R. japonica	Japanese or oriental spotted fever
R. massiliae	Not designated
R. monacensis	Not designated
R. parkeri	Not designated
R. raoultii	DEBONEL-TIBOLA*
R. rickettsii	Rocky Mountain spotted fever
R. sibirica	Siberian tick typhus
R. sibirica mongolitimonae	Lymphangitis-associated rickettsiosis
R. slovaca	DEBONEL-TIBOLA*
R. tamurae	Suggested spotted fever

*DEBONEL: Dermacentor Borne Necrosis Erythema Lymphadenopathy; TIBOLA: TIck-Borne Lymphadenitis.

The incidence of SFG *Rickettsia* is determined by the geographic location of the tick vector, whose activity then determines the incidence of disease. The two main SFG diseases in Europe are MSF, caused by *R. conorii*, and DEBONEL-TIBOLA, caused by *R. slovaca*; however, the discovery of new pathogenic species is steadily increasing the list (de Sousa *et al*. 2006, Vitale *et al*. 2006, Jado *et al*. 2007).

1.3.2.1. Mediterranean spotted fever

Mediterranean spotted fever is one of the oldest recognized vector-borne diseases, and its etiologic agent is *R. conorii*. The disease was described for the first time in Tunisia by Conor and Bruch (1910), and was from then onwards known as boutouneuse fever, due to macular-papular skin eruptions (Parola *et al*. 2005). The inoculation eschar at the site of

the bite, the 'tache noire', was later described in 1925 by Boinet and Pieri in Marseilles (Olmer 1925). Later on, Durand and Conseil confirmed *Rh. sanguineus*, the brown dog tick, as the vector of the disease (Durand and Conseil 1930). In 1932 Brumpt described the organism in tick samples and named it *R. conorii*, in honor of Conor (Brumpt 1932).

Mediterranean spotted fever is endemic in the Mediterranean area, including northern Africa, Middle East, India and Pakistan (Raoult and Roux 1997) (Fig. 1.9). Cases of infection by *R. conorii*, mostly attributed to travelling to endemic areas, have also been reported in northern and central Europe, Japan, United Kingdom and United States (Anderson *et al.* 1981, Lambert *et al.* 1984, Mc Donald *et al.* 1988, Yoshikawa *et al.* 2005, Chai *et al.* 2008).

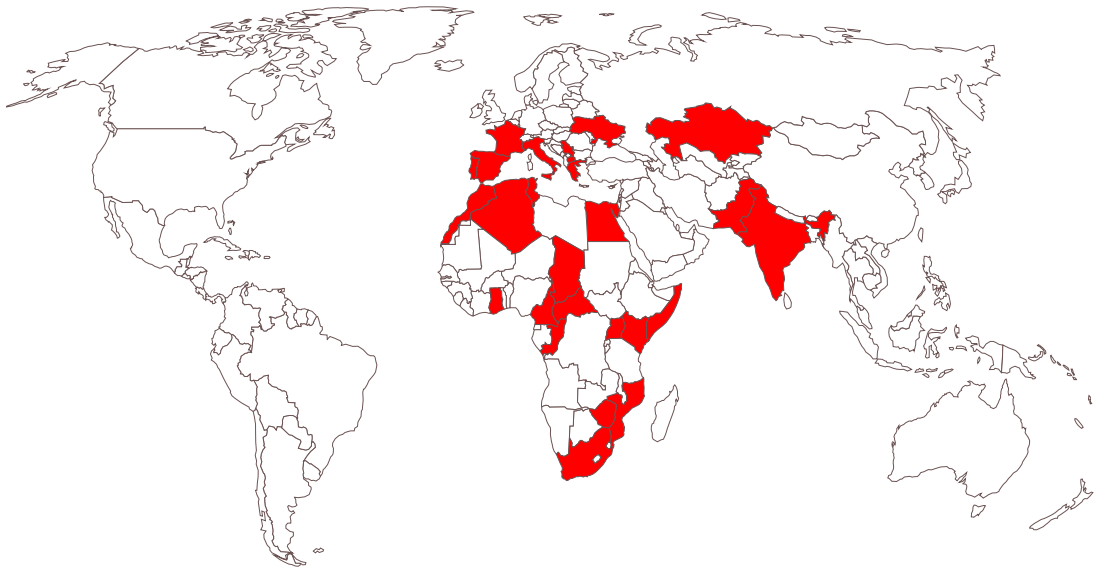

Fig.1.9. Distribution of MSF endemic countries.

Early reports of MSF portrayed it as a benign disease (Olmer 1957); however, from the 1980s onwards, an increasing recognition of severe cases has been reported in Europe (Raoult *et al*. 1982, Mansueto *et al.* 1986, de Sousa *et al.* 2003a, Mouffok *et al*. 2006). Portugal has one of the highest incidence rates of the Mediterranean basin, 8.4 per 100 000 inhabitants (de Sousa *et al*. 2008), and in 1997 the fatality rate was as high as 32% (de Sousa *et al.* 2003b). The etiologic agents in this country are *R. conorii* Israeli spotted fever (ISF) strain and *R. conorii* Malish strain, both transmitted by the brown dog tick *Rh. sanguineus* (de Sousa *et al.* 2003b).

The clinical signs of MSF at onset, in common with other rickettsioses, are fever, headaches and myalgias. A typical maculopapular rash appears 3 to 5 days later; an eschar may be present at the bite site. Severe forms of the disease may involve multiorgan failure and a fatal outcome (Yagupski 1993). Treatment is available with a course of antibiotics, most commonly doxycycline.

Diagnostic methods for detection of *R. conorii,* as well as for other rickettsial species, include serology, mainly by indirect immunofluorescent assay (IFA), molecular biologic detection techniques, including conventional and real-time polymerase chain reaction (PCR), and cell culture isolation. Serology is insufficient to determine the causative *Rickettsia* sp., as there is serological cross-reactivity among the SFG *Rickettsia*, warranting the need for further molecular or cell culture techniques for confirmation.

1.3.3. Ecology of *Rickettsia*

The ecology of SFG *Rickettsia* is not completely elucidated yet. It is believed that some *Rickettsia* circulate between wild vertebrates and arthropod vectors in enzootic or epizootic cycles (McDade and Newhouse 1986, Telford and Parola 2007). Ticks are thought to be vectors or reservoirs of SFG *Rickettsia* in nature, as these organisms are able

to remain perpetually in ticks and also be transmitted transovarially and transtadially, however, with varying degrees of efficiency (Table 1.5).

Table 1.5. Rates of infection and transovarial transmission (TOT) for infected ticks (Socolovschi *et al.* 2009).

Rickettsia	Tick	Infection rate (%)	TOT (%)
R. conorii	*Rh. sanguineus*	0-1.4%	100
R. rickettsii	*D. andersoni,*	0.26-1.5	100
	D. variabilis	0.0143-1.3	30-40
R. africae	*A. hebraeum*	20-30	100
	A. variegatum	27-100	Yes
R. massiliae	*R. turanicus, R. sanguineus*	0.7-50	100
R. slovaca	*D. marginatus*	7.2-40.6	100
R. rhipicephali	*Dermacentor sp.*	1.26-1.32	38-100
R. sibirica	*D. nutalii*	12	100
R. bellii	*Amblyomma sp.*	1.4-17.4	NS*
	I. loricatus	60.9	100
	Dermacentor sp.	1.3-2.2	NS
R. helvetica	*I. ricinus*	0.6-46.45	100
R. peacockii	*D. andersoni*	66	73.3
R. monacensis	*I. ricinus*	2.4-52.9	NS*
R. aeschlimannii	*H. marginatum marginatum*	1.8-57.9	Yes
R. amblyommii	*Amblyomma americanum*	3.7-23.6	Yes
R. raoultii	*D. reticulatus*	5.6-23	NS
	D. marginatus	22.5-83.3	86.4-100

*NS Not studied

Humans are accidental or dead-end hosts, playing no role in the acquisition and maintenance of SFG *Rickettsia* in nature.

The life cycle of a SFG *Rickettsia* starts with infection of the tick vector, which may occur either when pathogen-free ticks feed on rickettsemic animals, or when they co-feed with infected ticks (Fig. 1.10).

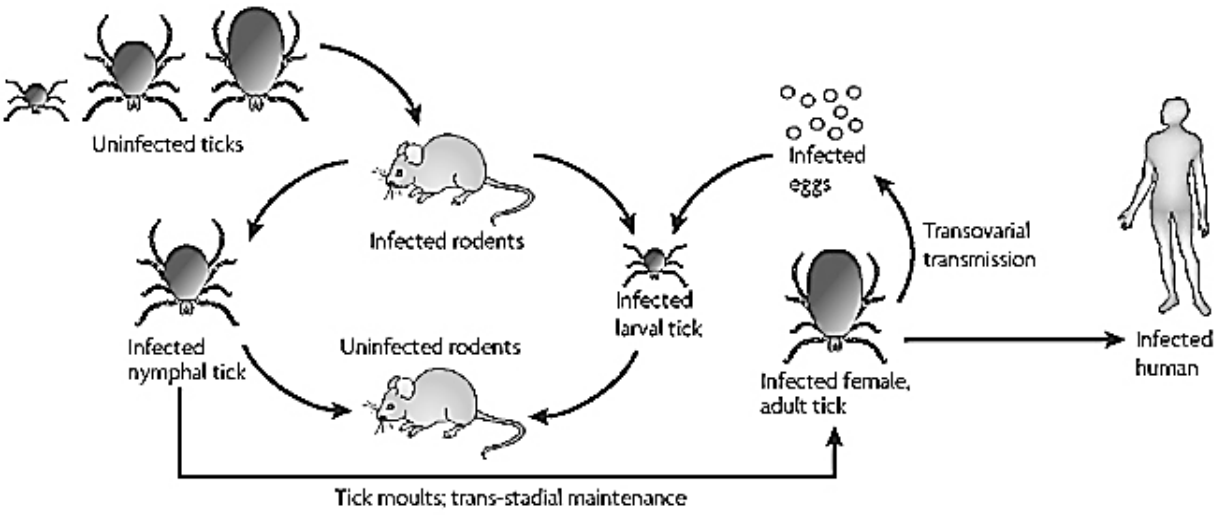

Fig.1.10. Life cycle of tickborne rickettsiae (adapted from Walker and Ismail 2008).

In either case, sufficient host blood must be imbibed in order to have a sufficient amount of infecting *Rickettsia,* i.e., a larger quantity of blood imbibed by the tick will result in higher rickettsial loads ingested and longer periods of feeding will also increase the chance of infection. Once in the tick vector, *Rickettsia* invade and multiply in gut epithelial cells, later escaping and invading the hemocoel and infecting hemocytes, which will transport them to the remaining tick organs causing a generalized infection (Walker 1988a, Piesman and Gage 1996). Depending on the tick/*Rickettsia* species, all or the majority of tick organs are infected (Hayes and Burgdorfer 1979, 1982, Santos *et al*. 2002). Once infected, transtadial and transovarial transmission to the progeny can occur (Burgdorfer and Brinton 1975, Rehacek 1989). During long tick starvation periods, the *Rickettsia* lay dormant in the organs until its vector finds a host to feed on, whereby the rickettsiae are then reactivated. It has been shown that *R. rickettsii* loses its virulence in guinea pigs when ticks are starved. This situation is reversed upon feeding (Spencer and Parker 1923). This phenomenon is known as reactivation, and may be explained as an

adaptation mechanism of the *Rickettsia* to the physiological state of its vector (Hayes and Burgdorfer 1982, Walker 1988a). In the co-feeding case, the neighbouring feeding ticks need to be at a close enough distance for the spread of bacteria to occur (Phillip 1959).

Not much is known about the effects of rickettsial infection on the host ticks in nature. Burgdorfer *et al*. showed a reduction in tick fertility as a result of rickettsial infection (Burgdorfer and Varma 1967, Burgdorfer and Brinton 1975). Hayes *et al*. (1979) showed minor cytopathological effects in salivary glands and ovaries of *Rh. sanguineus* infected with *R. montanensis*. In another study death after molting and severe malformations were observed in surviving adult *Rh. sanguineus* intracelomically inoculated with *R. conorii* (Santos *et al*. 2002).

1.3.4. Rickettsial pathogenesis and host immune response

Rickettsia of the SFG are transmitted to humans via the bite of all stages of infected ticks. Transmission of *Rickettsia* occurs several hours after attachment of the tick vector and, from the portal of entry in the skin, the organisms initially most likely spread via lymphatic vessels to the lymph nodes, and subsequently through the bloodstream to various host organs such as brain, lungs, liver, spleen, lymph nodes and heart. At each site they attach through OmpA, Sca1, Sca2 (present only in the SFG), and OmpB (present in all *Rickettsia*), enter and proliferate in vascular endothelial cells, the main targets of rickettsial infection (Walker 1996, Martinez *et al.* 2005). The proliferation of *Rickettsia* at the bite site results in the typical dermal and epidermal necrosis commonly known as 'eschar' or 'tache noire' (Walker *et al.* 1988b). Monocytes, macrophages and hepatocytes are also targets of rickettsial infection, albeit at a lesser degree (Mansueto *et al.* 2012). At the surface of the endothelial cells numerous Ku70 cellular receptors are expressed to which rickettsial OmpB attaches, and, in the case of SFG *Rickettsia,* OmpA also attaches to the integrin $\alpha_2\beta_1$. It has been hypothesized that Sca 1 and Sca2 act in concert with other

rickettsial proteins, such as OmpA and OmpB, to interact with target mammalian cells, mainly endothelial cells (ECs), during the infection process (Mansueto *et al.* 2012). Other adhesins have been proposed to be involved in rickettsial adhesion and entry into the cells, namely Adr1 and Adr2 in *R. conorii* (Renesto *et al.* 2006). *Rickettsia* internalization subsequently occurs through many signal transduction pathways, involving Cdc42, phosphoinositide 3-kinase, c-Src and other protein tyrosine kinase activities, inducing phagocytosis as a result of a zipper mechanism with alteration of cytoskeletal actin at the entry site (Martinez and Cossart 2004). Once in the cytosol, *Rickettsia* escapes from the phagocytic vacuole by secreting phospholipase A2, phospholipase D and hemolysin C, which disrupt the phagosomal membrane, thus avoiding phagolysosomal fusion and death (Whitworth *et al.* 2005). RickA, another rickettsial surface protein, activates the actin nucleating complex, Arp2/3, which in turn induces the polymerization of a network of actin filaments propelling the rickettsia towards the host cell membrane (Gouin *et al.* 2004, Jeng *et al.* 2004). Sca2 has recently been shown to also be involved in the actin-based motility of rickettsiae, at a later stage of infection than RickA (Kleba *et al.* 2010, Reed *et al.* 2014). The membrane is then deformed outwards, and invagination occurs into an adjacent cell. Disruption of both membranes frees the *Rickettsia* into the adjoining cell without exposure to the extracellular environment (Walker 2007). Typhus group *Rickettsia* do not polymerize actin filaments for mobility, instead they accumulate massively in the endothelial cells until these burst, releasing the organisms into the bloodstream (Walker 2007). As rickettsiae rapidly spread from cell to cell by actin-based motility, early cell death *in vitro* occurs (Heinzen *et al.* 1993). Studies have shown the involvement of reactive oxygen species (ROS) as mediators of cell injury (Eremeeva and Silverman, 1998, Rydkina *et al.* 2002, 2004). Rickettsial phospholipase activity, namely phospholipase D and A2 have also been pointed out as possible mediators of direct cell injury (Walker *et al.* 2001a, Renesto *et al.* 2003). With progression of rickettsial disease

further endothelial damage, resulting in increased vascular permeability, leads to widespread vascular dysfunction, edema, hypovolemia and hypoperfusion (Bechah *et al.* 2008). The main organs affected in severe rickettsial infections are lungs and brain, with noncardiogenic pulmonary edema and cerebral edema as principal causes of morbidity and mortality (Walker 1998, Walker *et al.* 2003).

Rickettsia primarily target endothelial cells which, once infected, trigger activation of the innate immune response, including cytokine production, stimulation of acute phase response and also activation of phagocytes, namely neutrophils and monocytes, and natural killer (NK) cells (Valbuena *et al.* 2002). NK cells are among the first to respond to rickettsial infection, and their cytotoxicity is mediated through release of granules containing perforin and granzymes or through induction of death receptor-mediated apoptosis (Ismail *et al.* 2002), undergoing notorious expansion two days post infection (Billings *et al.* 2001). They kill rickettsiae either via cytotoxic attack of infected cells or activation of macrophages and endothelial cells through production of gamma interferon (IFN-γ) (Ismail *et al.* 2002). NK cell depletion has been shown to enhance mouse susceptibility to rickettsial infection (Billings *et al.* 2001, Fang *et al.* 2012). Tumor necrosis factor-α (TNF- α), in concert with IFN-γ, mediates intracellular rickettsicidal activity by stimulation of inducible synthesis of nitric oxide (Feng *et al.* 1994, Walker *et al.* 1997). Other rickettsicidal effectors are reactive oxygen species and tryptophan degradation by indoleamine 2,3-dixoygenase. Proinflammatory cytokines are produced by macrophages, among other target cells, along with chemokines (Table 1.6).

Table 1.6. Cytokines and chemokines produced during rickettsial infections (adapted from Valbuena *et al.* 2002).

Cytokines and chemokines	Mouse tissues (IHC)	Mouse sera	Human sera	Endothelial cells *in vitro*	PBMCs *in vitro*
IL-1	-	Early	Not detected	Early	-
IL-6	-	Early	Early	Early	-
IFN-α/β	-	Early	-	-	-
IFN-γ	-	Early	Early	-	-
TNF-α	-	Early	Early	-	Early
IL-12	-	Early	-	-	Early
IL-10	-	Late	Early	-	-
CXCL-8	-	-	Not detected	Early	-
CXCL-9	Early	-	-	Early	-
CXCL-10	Early	-	-	Early	-
CX3CL1	Early	-	-	-	-
CCL-2	-	-	-	Early	-

IHC- Immunohistochemistry; PBMCs- Peripheral blood mononuclear cells

A study performed on human skin biopsy samples from mild to moderate MSF cases showed high mRNA levels of TNF, IFN-γ, IL-10, RANTES, indoleamine-2,3-dioxygenase and inducible nitric oxide synthase (de Sousa *et al.* 2008). Significantly high levels of intralesional IL-10 were shown to be inversely correlated with low levels of IFN-γ and TNF (de Sousa *et al.* 2008).

The critical roles of both host TNF-α and IFN-γ enhance the importance of cellular immunity of the T helper 1 (Th1) type against rickettsial infections (Valbuena *et al.* 2002). CD8+T cells have been shown to be fundamental in immune response against rickettsiae (Feng *et al.* 1997; Walker *et al.* 2000). Dendritic cells (DC) are the most potent antigen presenting cells, and have been suggested to play a major role in innate and acquired immunity. Studies have shown that rickettsiae stimulate DCs to develop protective Th1 responses in resistant hosts; however, suppressive adaptive immunity is induced in susceptible hosts (Fang *et al.* 2007).

Further studies involving both *in vitro* investigation and *in vivo* animal models are essential in order to understand the complex pathophysiological mechanisms in rickettsioses.

1.4. Animal models of rickettsial infection

The development of animal models which closely mimic the pathological characteristics of human rickettsial disease, in terms of portal of entry, route of spread, target cells, pathogenic mechanisms and immune response, are extremely important for the development of innovative interventions in vaccine and treatment research. The mechanisms of pathogenesis of many *Rickettsia* remain largely uncharacterized, partly due to a lack of reproducible animal models (Bechah *et al.* 2008). To date, three animal models of pathogenesis of rickettsioses have been developed, which faithfully reproduce the pathological features occurring in rickettsial disease, namely C3H/HeN mice for the study of *R. conorii* (Walker *et al*. 1994) and *R. typhi* (Walker *et al*. 2000), and BALB/c and C57Bl/6 mice for *R. australis* (Feng *et al*. 1993 and Walker et al. 2001b). C3H/HeJ mice have recently been shown to be a more susceptible model for the study of *R. conorii* (Jordan *et al*. 2009).

Despite the increase in animal model development studies, a gap still remains regarding the natural route of infection, i.e., the role of the tick in rickettsial dissemination and progression of disease.

Of particular interest in this project are the interactions among *Rickettsia spp.* (*R. massiliae a*nd *R. conorii*), the tick vector *Rh. sanguineus* and a susceptible murine host, C3H/HeJ.

1.5. Aims

To date many studies have been undertaken using animal models for the study of rickettsial mechanisms of pathogenesis in the host, as well as studies on the tick vector to uncover the identities of tick salivary proteins acting as immunomodulatory compounds in the host. However, many gaps remain, among them incisive studies on the vector, regarding its relationship with its infecting pathogens, and also the role of tick saliva in the pathogenesis of *Rickettsia* in animal models. In the present project our main goal was to approach these gaps, in order to broaden the knowledge of rickettsial diseases, of particular interest Mediterranean spotted fever, an endemic disease in Portugal, by analyzing the different interfaces of the vector-host-pathogen triad. The aims of this dissertation were thus divided into three main parts:

1) To analyze vector-pathogen-host relationships as they occur in nature, thus gaining insight into the diversity and prevalences of rickettsial organisms in different hosts and tick species (chapter 2).

2) To study the vector-*Rickettsia* interface through characterization of *Rickettsia* in the vector, namely quantification of *R. massiliae* in the salivary glands of *Rh. sanguineus* ticks during feeding, as well as ultrastructural analysis of the bacteria in salivary glands, midgut and ovaries of the tick vector (chapter 3).

3) To study the host-vector-rickettsia interface through analysis of the role of tick saliva in the intradermal dissemination of *R. conorii* ISF as well as the immune response in a susceptible murine host, C3H/HeJ mice (chapter 4).

This dissertation is organized into six chapters: chapter 1 pertains to a literature review and aims of the dissertation; in chapter 2 we analyze the vector-pathogen-host

relationships in nature; in chapter 3 we performed an experimental approach focusing on the vector, in order to analyze the vector-pathogen interactions; in chapter 4 we focused on the host, analyzing the role of tick saliva in the vector-pathogen-host interaction; and finally chapter 5 pertains to the general discussion including future directions.

CHAPTER 2

RICKETTSIAL DIVERSITY IN PORTUGAL

2. RICKETTSIAL DIVERSITY IN PORTUGAL

This chapter is based on the research papers:

Milhano N, Lopes de Carvalho I, Alves AS, Arroube S, Soares J, Rodriguez P, Carolino M, Núncio MS, Piesman J, De Sousa R (2010) Coinfections of *Rickettsia slovaca* and *Rickettsia helvetica* with *Borrelia lusitaniae* in ticks collected in a Safari Park, Portugal. Tick and Tick-Borne Diseases 1: 172-177.

De Sousa R, Lopes de Carvalho I, Santos AS, Bernardes C, **Milhano N**, Jesus J, Menezes D, Núncio MS (2012) Role of the lizard *Teira dugesii* as a potential host for *Ixodes ricinus* tick-borne pathogens. Appl Env Microbiol 78: 3767-3769.

Milhano N, Palma N*,* Nuncio MS, Marcilli *A,* Lopes de Carvalho I, Walker DH, de Sousa R (2014) *Rickettsia lusitaniae* sp. nov. isolated from the soft tick *Ornithodoros erraticus* (Acarina:Argasidae). Comp Immunol Microbiol Infect Dis 37: 189-193.

2.1. INTRODUCTION

The interface between vector-pathogen-host as it occurs in nature was analyzed in this chapter, through studies of prevalence of *Rickettsia spp.,* along with *Borrelia spp.* and *Anaplasma phagocytophilum* in various tick species and hosts, collected from rural areas of mainland Portugal and Madeira Island.

2.2. COINFECTIONS OF *RICKETTSIA SLOVACA* AND *RICKETTSIA HELVETICA* WITH *BORRELIA LUSITANIAE* IN TICKS COLLECTED IN A SAFARI PARK, PORTUGAL

Abstract

Borrelia and *Rickettsia* bacteria are the most important tick-borne agents causing disease in Portugal. The identification and characterization of these circulating agents, mainly in recreational areas, is crucial for the development of preventive measures in response to the gradually increasing exposure of humans to tick vectors. A total of 677 questing ticks including *Dermacentor marginatus*, *Rhipicephalus sanguineus, Ixodes ricinus*, *Hyalomma lusitanicum*, *H. marginatum* and *Haemaphysalis punctata* were collected in a Safari Park in Alentejo, Portugal, to investigate the prevalences of infection and characterize *Borrelia* and *Rickettsia* species. From a total of 371 ticks tested by PCR for *Borrelia burgdorferi* sensu lato (s.l.), of which 247 were tested for *Rickettsia*, an infection prevalence of 18.3% was found for *B. lusitaniae* and 55.1% for *Rickettsia* spp. Sequence analysis of positive amplicons identified the presence of *B. lusitaniae* (18.3%), *R. monacensis* strain IRS3 (51.7%) and *R. helvetica* (48.3%) in *I. ricinus. R. slovaca* (41.5 %), *R. raoultii* (58.5 %) and also *B. lusitaniae* (21%) were identified in *D. marginatus* ticks. One (5.9%) *Hyalomma lusitanicum* was infected with *B. lusitaniae,* and *R. massiliae* was found in one *Rhipicephalus sanguineus.* Co-infection was found in 7 (20%) *I. ricinus* and 34 (23.3%) *D. marginatus* ticks. We report, for the first time, simultaneous infection with *R. helvetica* and *B. lusitaniae* and also *R. slovaca,* the agent of TIBOLA/DEBONEL, with *B. lusitaniae*.

Additionally, six isolates of *B. lusitaniae* were established, and isolates of *Rickettsia* were also obtained for the detected species using tick macerates cultured in mammalian and mosquito cell lines. This report describes the detection and isolation of tick-borne agents from a Portuguese Safari Park, highlighting the increased likelihood of infection with multiple agents to potential visitors or staff.

Introduction

Mediterranean spotted fever (MSF) and Lyme borreliosis (LB) are the two main tick-borne diseases included in the nationally notifiable diseases of the Portuguese Ministry of Health in Portugal that pose major concern to public health. However, *R. conorii*, which causes MSF, is not the only pathogenic *Rickettsia* species circulating in Portugal. Other members of spotted fever group, capable of causing disease, have been detected in Portuguese patients, such as *R. sibirica mongolotimonae* strain, and in ticks, such as *R. slovaca, R. aeschlimannii*, *R. helvetica, R. massiliae, R. monacensis and R. raoultii* (De Sousa *et al*. 2006, Bacellar *et al*. 1995a, Lopes de Carvalho *et al*. 2008a). Some of these spotted fever group rickettsiae seem to have low pathogenicity, namely *R. massiliae* (Beati and Raoult, 1993, Vitale *et al*. 2006), *R. monacensis* (Simser *et al*. 2002, Jado *et al*. 2007) and *R. raoultii* (Parola *et al*. 2009). Nevertheless, they were found to be involved in human disease.

The first human case of LB, caused by *Borrelia burgdorferi* sensu lato (s.l.) was detected in Portugal in 1989; however, it was only recognised as a notifiable disease in 1999 (David de Morais *et al*. 1989). Analysis of laboratory data has confirmed LB as being underreported, with an estimated incidence rate of 0.04 per 100 000 inhabitants (Lopes de Carvalho et al. 2008b). Using molecular biological assays, *B. afzelii, B. valaisiana, B. garinii* and *B. burgdorferi* sensu stricto (s.s.) were detected in *I. ricinus* collected on Madeira Island (Matuschka *et al*. 1998, Lopes de Carvalho *et al*. 2008a). In other studies

conducted in Portugal, *I. ricinus* and *H. marginatum* ticks were found to be infected with *B. lusitaniae, B. afzelii, B. valaisiana, B. garinii* and *B. burgdorferi s.s.* (De Michelis *et al*. 2000). *B. lusitaniae*, the most prevalent *Borrelia* species in Portugal, was isolated from a human patient in 2004 and 2008, confirming its role in the pathogenesis of LB (Collares-Pereira *et al*. 2004, Lopes de Carvalho *et al*. 2008b).

Safari parks are popular tourist attractions with thousands of visitors each year, and the possibility of tick bites is considerable in these rich ecological niches. In this study, the main objective was to identify which *Rickettsia* and *Borrelia* species are present in questing ticks from one of the largest Safari Parks (approximately 90 ha) in Portugal, harbouring more than 40 species of exotic animals. Furthermore, as this park has imported exotic animals, this study may help to determine the presence of any imported tick species.

An initial screening survey was performed on ticks, based on molecular detection, in order to evaluate the prevalence of infection and assess the potential agents that may be tick-transmitted to humans in this park. Subsequently, the main goal was to isolate *Rickettsia* and *Borrelia* species for agent characterization and future use for diagnostic purposes.

Materials and methods

Tick samples

Ticks were collected by flagging in a Safari Park in Alentejo, Portugal (38° 03' 03.45'' N), in areas utilized by vertebrate hosts. The identification was performed using morphological characters with standard taxonomic keys. The sampling was performed non-systematically from December 2006 to April 2009.

DNA extraction and amplification

The ticks were washed in 70% ethanol and sterile distilled water, dried on sterile paper and subsequently boiled in 25% ammonium hydroxide solution, as described previously by Schouls *et al*. (1999). A negative control was included for every 10 DNA extractions to monitor the occurrence of false-positives. The tick lysate was stored at -20°C until further use. Amplification of rickettsial DNA was performed targeting the gene for citrate synthase (*gltA),* outer membrane protein A (*ompA*) and B (*ompB*) as described previously (De Sousa *et al*. 2006). PCR amplification of the 5S (rrf)-23S (rrl) intergenic spacer (Rijpkema *et al*. 1995) was performed for the detection and isolation of *Borrelia*. To characterize the isolates, the outer surface protein A (*ospA*) (Lopes de Carvalho *et al*. 2008a,b) and *ospC* genes were also performed. The *ospC* primers were designed (OspC1F 5'ACGGATCATTGTTAGCAGGAG3'/OspC1R-5'GCATCAGTAGCAGCACCAG), using multiple alignments of published *ospC* sequences of *B. lusitaniae* within GenBank (EF179585; EF179575) (Vitorino et al. 2008). All amplification reactions included positive and negative controls.

DNA sequencing

Amplicons were purified using Jetquick Purification PCR Product Spin Kit (Genomed, Inc.) and sequenced with the Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 377 DNA sequencer, according to the manufacturer's recommendations. The sequencing reactions were performed with the forward and reverse primers used for the PCR amplifications. The sequences were assembled by combining the sequences generated by each primer, using the Lasergene software (DNASTAR). For identity determination, primer sequences were removed, and the analysis was conducted with PAUP 4.0b10 software.

Cultivation of Rickettsiae

Adult *I. ricinus, Rhipicephalus sanguineus,* and *Dermacentor marginatus* ticks collected from vegetation were washed for 5 min in 10% bleach, 70% ethanol and dried with sterile paper. All these tick species were inoculated in Vero cell line (African green monkey fibroblast cells). *Dermacentor marginatus* and *I. ricinus* were also inoculated in mosquito C6/36 cell line (derived from *Aedes albopictus)*, where half the number of ticks per isolation attempt was randomly inoculated in Vero cells, and the other half in C6/36 cells. When inoculating in Vero cell-coated shell-vials, ticks were first triturated in Eagle's minimal essential medium (MEM, Gibco, Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum and 1% glutamine. For inoculation in C6/36 cell-coated shell-vials, L-15 medium (Leibovitz, Gibco, Invitrogen, Paisley, UK) supplemented with 3% fetal bovine serum was used. Both Vero and C6/36 cell-coated shell-vials were incubated at 2 temperatures: half the number of shell-vials coated with each cell type was incubated at 28°C and the other half at 32°C. After 6-7 days the cells were scraped with glass beads, transferred onto a confluent monolayer of the same cell type in 25-cm^2 flasks, and incubated at the respective temperatures for a further 6 days. The monolayers were scraped every 6-8 days for *Rickettsia* detection by Gimenez staining (Gimenez 1964). Positive isolates were established after 3 successful passages, and samples were stored at -80° C. Isolate characterization was performed by PCR and sequencing.

Cultivation of *Borrelia* spirochetes

Adult *I. ricinus* ticks were disinfected by successive immersion in iodine, 70% ethanol, and distilled water and inoculated in 8 mL of complete Barbour-Stoenner-Kelly (BSK) II medium (Sigma).

Cultures were maintained at 34°C for 3 months and examined weekly by dark-field microscopy to monitor the presence of spirochetes. After 6 weeks, 1 mL of each culture

was centrifuged for 15 min at 7,000 x g, and DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen Hilden, Germany) according to the manufacturer's recommendations.

Confirmation of the identity of *Borrelia* isolates was performed by PCR amplification of cultured spirochetes and subsequent sequencing. Positive isolates were collected and stored at -80°C, after 2 successful passages.

Results

A total of 677 ticks, 291 *D. marginatus*, 168 *Rh. sanguineus*, 163 *I. ricinus*, 30 *H. lusitanicum*, 17 *Haemaphysalis punctata* and 8 *Hyalomma marginatum*, were collected from vegetation in a Safari Park in Alentejo, Portugal (Table 2.1). No new tick species that might have been introduced by exotic animals were detected.

Of all the collected ticks, 371 (54.8%) were tested by PCR for *B. burgdorferi* s.l., of which 247 (66.6%) were then tested for *Rickettsia* (Table 2.1).

Table 2.1. Prevalence and isolates of *Rickettsia* spp and *Borrelia lusitaniae* from questing ticks of a Safari Park, Portugal.

			Rickettsia				*Borrelia*			
			PCR		Culture		PCR		Culture	
Tick species	**No. of ticks collected**	**Gender/stage**	**No. pos/ analyzed**	***Rickettsia* spp (Infection rate in positive tick species)**	**No. pos/ analyzed**	**Isolated species (n)**	**No. pos/ analyzed**	***Borrelia* spp (Infection rate in positive tick species)**	**No. pos/ analyzed**	**Isolated species**
D. marginatus	291	161 F 130 M	106/146	*R. slovaca* (41.5%) *R. raoultii* (58.5%)	13/73	*R. slovaca (*13)	59/280	*B. lusitaniae* (21%)		
Rh. sanguineus	168	75 F 72 M 21 N	1/36	*R. massiliae*	3/15	*R. massiliae* (3)	0/16			*NP*
I. ricinus	163	101 F 62 M	29/35	*R. monacensis* (51.7%) *R. helvetica* (48.3%)	10/78	*R. monacensis* (5) *R. helvetica* (5)	8/35	*B. lusitaniae* (22,9%)	6/39	*B. lusitaniae*
H. lusitanicum	30	11 F 19 M	0/9				1/17	*B. lusitaniae*		*NP*[a]
H. punctata	17	8 F 9M	0/13				0/15			
H. marginatum	8	2 F 4 M 2 N	0/8				0/8			
Total	677		136/247		26/166		68/371		6/39	

[a]NP, culture was not performed; F, female; M, male; N, nymph.

Rickettsial DNA was detected in 136 (55.1%) ticks. Sequence analyses of *Rickettsia*-positive amplicons from *I. ricinus* showed that 14 (48.3%) samples have 100% sequence identity with *gltA* and *ompB* sequences of *R. helvetica* (U59723 and AF123725) and 15 (51.7%) samples have 100% identity with *gltA* and *ompA* of *R. monacensis* strain IRS3 (EF501755 and EU078962).

Forty-four (41.5%) *D. marginatus* positive tick samples showed > 99% and 100% identity with *gltA* and *ompA* of *R. slovaca* (U59725 and U43808), respectively, and 62 (58.5%) tick samples (named PoTiR7dt) showed 100% identity with *gltA* and *ompA* of *R. raoultii* (DQ365804 and GQ404429), previously detected in *Dermacentor* ticks collected in Russia and Spain, respectively. In *I. ricinus,* there was no difference in tick infection by gender for both *R. helvetica* and *R. monacensis*. However, in *Dermacentor,* we found a significantly higher percentage of infection in female ticks (65%) for both *Rickettsia* species, when compared with males. One *Rh. sanguineus* was infected with *R. massiliae* showing 100% of identity with *gltA* and *ompA* of a previous strain of *R. massiliae* (DQ459393 and DQ459388).

B. lusitaniae was detected in 68 (18.3%) ticks using 5S (*rrf*)-23S (*rrl*) intergenic spacer, with 100% sequence identity with PoTiB1 (DQ111065). Infection percentages of 21% were found in *D. marginatus*, 22.9% in *I. ricinus,* and 5.9% in *H. lusitanicum*. Co-infection of *Borrelia* and *Rickettsia* was observed in 41 (16.6%) ticks, 7 (20%) *I. ricinus* and 34 (23.3%) *D. marginatus.* Co-infection was found in *I. ricinus* and *D. marginatus* with *R. helvetica* and *R. slovaca,* respectively, each of which was infected with *B. lusitaniae.* Concerning isolation attempts performed in this study, *Rickettsia* isolation attempts were undertaken using 2 cell lines, Vero and C6/36 cells, incubated at both 28° and 32°C. *R. monacensis* strain IRS3 and *R. helvetica* were each isolated from 5 *I. ricinus*, *R. massiliae* from 3 *Rh. sanguineus* and *R. slovaca* from 13 *D. marginatus* ticks (Table 2.2). All 5 *R. monacensis* strain IRS3 isolations were achieved at 28°C, four proliferated in Vero cells

and one in C6/36 cells after a mean of 14 days. Three out of 5 successful isolates of *R. helvetica* were detected in C6/36 cells at 28°C, the other 2 isolates were obtained at 32°C, one in C6/36 cells and the other in Vero cells, after a mean of 14 days. *R. massiliae* was detected in Vero cells after 35 days, 2 at 28°C and one at 32°C. One isolate of *R. slovaca* was detected in C6/36 cells incubated at 32°C, and the other 12 isolates in Vero cells, 4 of which were incubated at 28°C and the remaining 8 at 32°C. *Rickettsiae* were visualised by Gimenez staining after a mean of 15 days incubation at either temperature. All *Rickettsia* isolates were characterized by PCR and sequencing as described for the prevalence of infection. The sequences of the fragments *gltA*, *ompA* and *ompB* genes amplified from rickettsial isolates were submitted to the Genbank and their accession numbers were obtained (Table 2.2).

Table 2.2. *Rickettsia* isolates, culture conditions and GenBank accessing numbers.

Rickettsia species isolates	Studied genes	Accession no.	Number of isolates and culture conditions			
			Vero cells 28ºC	Vero cells 32ºC	C6/36 cells 28ºC	C6/36 cells 32ºC
***R. monacensis* strain IRS3 (PoTiR634)**	*gltA;ompA*	HM149283;HM149288	4	-	1	-
***R. slovaca* (PoTiR443)**	*gltA;ompA*	HM149281;HM149286	4	8	-	1
***R. helvetica* (PoTiR32)**	*gltA;ompB*	HM149280;HM149285	-	1	3	1
***R. massiliae* (PoTiR600)**	*gltA;ompA*	HM149282;HM149287	2	1	-	-

Six (15%) isolates of *B. lusitaniae* were obtained from 39 *I. ricinus* ticks.

Five identical isolates named PoTiB7(prototype isolate) showed to be 100% identical to *ospA* gene of *B. lusitaniae* previously deposited in GenBank as PoHUB1 (EU863625). Identity of 100% was also obtained for *ospC* and IGS sequences of *B. lusitaniae*

PoTiBmfP220 (EF179581) and TT928 (AY575767), respectively. One isolate of *B. lusitaniae*, named PoTiB8, different from the other isolates, showed 100% identity for the *ospA* gene and IGS sequences with PoTiBGr41 (EF179568) and PoTiB1 (DQ111065), respectively. Fragment sequences of the 5S(rrf)-23S(rrl) intergenic spacer, *ospA*, and *ospC* genes from PoTiB7 have been deposited in GenBank with the accession numbers HM193536, HM193537, and HN193538, and for the PoTiB8 isolate, the sequences of the 5S (rrf)-23S (rrl) intergenic spacer and *ospA* genes were HM193539 and HM193540, respectively.

Discussion

This report describes the detection of 5 *Rickettsia* species and *B. lusitaniae,* capable causing disease in humans, from ticks collected in a Portuguese Safari Park. With the exception of *R. raoultii,* the other 4 rickettsia species detected in this study were also isolated from ticks, namely *R. monacensis* strain IRS3 and *R. helvetica* from *I. ricinus* ticks, *R. massiliae* from *Rh, sanguineus* and *R. slovaca* from *D. marginatus*. *B. lusitaniae* was isolated from *I. ricinus* only because it was the sole tick species used for culture attempts. Among the SFG rickettsiae present in *I. ricinus,* our study showed a high prevalence of *R. helvetica* (48.3%) and *R. monacensis* (51.7%). Similar prevalences for both agents was found in studies from Bulgaria (59%), the northeastern part of Italy and Slovenia (55.8%-58.5%) (Christova *et al*. 2003, Floris *et al*. 2008). *R. helvetica* was detected for the first time in Swiss *I. ricinus* in 1979 and confirmed as a new member of SFG *Rickettsia* in 1993 (Beati *et al*. 1993). Since then, it has been isolated in many European countries, and in Portugal it has been found in *I. ricinus* and *I. ventalloi* (1.8%) parasitizing birds (Bacellar *et al*. 1995a, Santos-Silva *et al*. 2006). *R. helvetica* infection is progressively becoming a clinical entity of its own: it was associated with periomyocarditis in 1999, has been serologically associated with patients in Europe and

Asia, and was recently reported in 2 Swedish patients with meningitis and septicemia (Nilsson *et al*. 1999, Fournier *et al*. 2000).

Rickettsia monacensis was isolated from *I. ricinus* for the first time in Germany, but most European countries which have reported the prevalence of this agent used molecular detection (Simser *et al*. 2002, Sreter-Lancz *et al*. 2005). In this study we describe, for the first time, the isolation of *R. monacensis* IRS3 from *I. ricinus* in Portugal. This agent had already been previously detected by PCR in Madeira Island, albeit with a lower prevalence (7%), when compared to the present study (Lopes de Carvalho *et al*. 2008a). *R. monacensis* was recently associated with febrile disease in humans in northern Spain (Jado *et al*. 2007).

In this study, both *R. raoultii* and *R. slovaca* were detected in *D. marginatus* by PCR, with prevalences of 58.5% and 41.5%, respectively. *R. raoultii* (formerly genotypes RpA4, DnS14, DnS28) was described for the first time in *R. pumilio* and *D. nutallii* in Russia, and later in other European countries (Rydkina *et al*. 1999, De Sousa *et al*. 2006). Recently, this rickettsia species was found in *Dermacentor* removed from Spanish and French patients and associated with cases of TIBOLA/DEBONEL (Ibarra *et al*. 2006, Parola *et al*. 2009). Nevertheless, *R. slovaca* remains the main etiological agent responsible for the majority of TIBOLA/DEBONEL cases causing a mild rickettsiosis, with low fever, regional lymphoadenopathy, and eschar occurring mostly on the head. *R. slovaca* has been identified in *D. marginatus* and *D. reticulatus* in Portugal and in most European countries (Bacellar *et al*. 1995b, Parola *et al*. 2009). The rates of infection of *D. marginatus* ticks with *R. slovaca* in Europe ranged from 21% to 45.4% in Hungary and Switzerland, respectively (Beati *et al*. 1994, Lakos and Raoult, 1999). In our study, *D. marginatus* ticks showed high prevalence of infection with *R. slovaca* (41.5%), very similar to the prevalence found in previous studies from Portugal (34.5%), Switzerland (45.4%), and La Rioja, Spain (40.6%) (Vitorino *et al*. 2007, Beati *et al*. 1993, Oteo *et al*. 2006). In Portugal *D. marginatus* is

present in all districts with a higher density from September to April. Surprisingly, up to now no cases of TIBOLA/DEBONEL have been described or notified in Portuguese patients. Based on the high prevalence of *R. slovaca* in *Dermacentor* ticks in our country, we assume that the cases in Portuguese patients are probably misdiagnosed or diagnosed as SFG *Rickettsia* infection based only on serology. As there is cross-reaction between SFG rickettsiae, species identification is not possible.

The different conditions (Vero and C6/36 cells, maintained at 28°C and 32°C) used in this study for isolation attempts of *Rickettsia* had the intent of increasing the chances of successful isolation. In this study, *R. monacensis* was easily propagated and isolated in Vero cell lines at 28°C. This strain was isolated for the first time in Germany using *I. scapularis* cell line (ISE6), and was later replicated in other tick (IRE11, DAE100) and mammalian (L-929 and Vero) cell lines at 34°C (Simser *et al*., 2002). In the study by Simser et a. (2002), *Rickettsia* coccobacilli were observed after 26 days (ISE6 cell line), a longer period of time compared to the present study, where rickettsiae were detected after a mean of 14 days. The authors also mention that it took more than 26 days to grow *R. monacensis* in the mammalian cell line. In our study, the incubating temperature seemed more determinant for rickettsial growth than the type of cell line. More isolates of some species of *Rickettsia* were obtained at 28°C, when compared to their counterparts cultivated at 32°C, as was the case in 3 isolates of *R. helvetica* in C6/36 cell line. *R. slovaca* seemed to have an optimal growth at 32°C. Based on the high prevalence of infection with *R. raoultii* by PCR, we would have expected to have isolated this species; however, for reasons unknown to us, this was not the case.

B. lusitaniae is the most prevalent *Borrelia* genospecies in Portugal and was isolated for the first time in *I. ricinus* ticks collected in the Alentejo region (Nuncio *et al*. 1993). In this study, the prevalence of infection with *B. lusitaniae* in *D. marginatus* was similar to that found in *I. ricinus* ticks, the main vector of *B. burgdorferi* s.l. Due to the fact that *D.*

marginatus were immediately frozen at -80°C, *B. burgdorferi* s.l. was only detected by PCR, and no isolation attempts were possible. It remains unknown whether *D. marginatus* is a vector of *Borrelia*; therefore, further studies need to be undertaken. Previous studies conducted by other researchers using mouse models and *D. variabilis* ticks have shown that this species can acquire *B. burgdorferi* spirochetes; however, transmission could not be demonstrated as has been the case with *I. scapularis*, the main vector of *B. burgdorferi* s.s. in the United States (Soares et al. 2006). However, our results could be explained by the fact that in some ecosystems *D. marginatus* may be implicated as a secondary vector in *B. burgdorferi* s.l. transmission, as has been the case in other countries (Angelov *et al*. 1996). Furthermore, in another study conducted in Portugal, this hard tick species was also highly infected with *B. burgdorferi* s.l. in Grândola region (Baptista *et al*. 2004). Interestingly, Grândola is approximately 34 km from the Safari park where the ticks analysed in this study were collected.

Another important finding achieved using 'Recombination Detection Program, version 3' (Heath *et al.* 2006) was the fact that the sequence analysis obtained here seems to indicate that the positive isolates from *I. ricinus* may be recombinant strains, as was recently described for another *B. lusitaniae* strain found to have an intersection in the allelic profiles of the *B. lusitaniae* populations from 2 regions in Portugal, Mafra and Grândola (Vitorino *et al*. 2008). Moreover, an isolate of this genospecies was recently obtained from an *Apodemus sylvaticus* also captured in Alentejo region, indicating that this mammal could be a reservoir of *B. lusitaniae* (Lopes de Carvalho *et al*. 2009). Further studies using housekeeping genes should be undertaken to clarify the phylogeographic structure of these *B. lusitaniae* strains.

Previous studies have confirmed the presence of several species of *Borrelia* (*B. lusitaniae*, *B. garinii*, *B. afzelii*, *B. valaisiana and B. burgdorferi* s.s.) in ticks from mainland Portugal

and Madeira Island, and the infection prevalence varied between 2.7% and 34.7% (de Michelis *et al*. 2000, Baptista *et al*. 2004, Lopes de Carvalho *et al*. 2008a).

Studies on the frequency of co-infection of ticks with several pathogens vary depending on tick species and countries. However, it is always very important to evaluate the existence of co-infections as it may facilitate the prognosis of simultaneous infections in humans. To our knowledge, this is the first report of dual infections of *R. helvetica* and *R. slovaca*, each with a coinfection of *B. lusitaniae*. The most common bacterial coinfections in *Ixodes* ticks reported in previous studies include dual or multiple agents such as *B. burgdorferi* s.l, *Anaplasma phagocytophylum*, *Babesia* spp and *R. monacensis* (Fernandez-Soto *et al*. 2004, Swanson *et al*. 2006). To date, no description of dual infection with *R. helvetica* has been made, with the exception of the report by Fernández-Soto, which mentions infection with *R. helvetica* and *B. burgdorferi* s.l. in an *I. ricinus* collected from an asymptomatic patient (Fernandez-Soto *et al*. 2004). In Portugal co-infections of *B. lusitaniae* and *R. monacensis* were found in *I. ricinus* ticks in Madeira Island, and previous studies based on serological data have also suggested dual infections in LB patients with *A. phagocytophilum* (Lopes de Carvalho *et al*. 2008, Santos *et al*. 2004).

Although reports have shown exacerbated illness in patients coinfected with *A. phagocytophilum* and *Borrelia,* little is known about the dynamics and transmission of dual infections (Thomas *et al*. 2001, Krause *et al*. 2002). Animal models have also shown that co-infection can enhance bacterial burden, transmission and disease progression (Thomas *et al*. 2001). The occurrence and implications of a simultaneous infection by *R. helvetica* or *R. slovaca* with other pathogenic agents, such as *B. lusitaniae,* in patients has to be further evaluated, and clinicians need to be aware of these possible differential diagnoses in Portuguese patients exposed to tick bites. Coinfections may show variable clinical symptoms.

Considering the prevalence of single and dual rates of infection found in this study, there is a considerable risk of tick exposure to both staff and visitors to this park. The Safari Park has a restricted area, where visitors may only circulate in a vehicle, and also areas outside of this restricted area where people are allowed to circulate freely by foot and have contact with domestic animals. Moreover, the park has a new area with an artificial river for rafting activities surrounded by vegetation. Our flagging locations encompassed both areas (restricted and not restricted areas), where infected ticks were found. Areas are separated by fences; however, it is possible that rodents and birds may pass those fences carrying ticks with them. We therefore think it would be advisable and instructive to institute flyers mostly for children, adapted to different ages, informing visitors of the Safari Park about preventive measures against tick-borne diseases.

2.3. THE ROLE OF *TEIRA DUGESII* LIZARD AS A POTENTIAL HOST FOR *IXODES RICINUS* TICK-BORNE PATHOGENS

Abstract

PCR screening of ticks and tissue samples collected from 151 *Teira dugesii* seems to indicate a potential role of this lizard species in the maintenance and transmission cycle of some *Ixodes ricinus* tick-borne agents, such as *Rickettsia monacensis*, *R. helvetica* and *Borrelia lusitaniae*, that are circulating in Madeira Island.

Introduction

Lizards have been identified as vertebrate hosts of ixodid ticks (Dantas-Torres *et al*. 2008). The existence of an endemic lizard population of *Teira dugesii* that has become one of the most prevalent vertebrate species in Madeira Island and is known to host immature stages of *Ixodes ricinus* drew our attention. Additionally, in this island, *I. ricinus* has been shown to be infected with different genospecies of *Borrelia* spp*, Rickettsia monacensis* and *Anaplasma phagocytophilum* (Lopes de Carvalho *et al*. 2008a, Matuschka *et al*. 1994, 1998, Santos *et al*. 2009). A comprehensive investigation was thus undertaken to study the potential role of *T. dugesii* in the maintenance and transmission of *I. ricinus* tick-borne agents.

One hundred and fifty one tissue samples from lizard's tail and 211 *I. ricinus* removed from 100 lizards were collected monthly from March 2009 to February 2010 in Calheta and Campanario areas on Madeira Island. DNA samples were analyzed by PCR for *Rickettsia*, *Borrelia* and *A. phagocytophilum* using specific primers pairs targeting two genes for each agent, as previously described (Choi *et al*. 2005, de Sousa *et al*. 2005,

Massung *et al*. 1998, Rijpkema *et al*. 1995, Sumner *et al*. 1997). Additionally, 60 DNA samples extracted from lizard blood prints were tested for *A. phagocytophilum*.

Ticks were present only in lizards captured in Calheta (n=130) and were mostly found in the forelimb axial region. An average of 2.1 ticks per animal was observed. Nymphs were detected in all studied periods, but larvae were only present from early April to November (Fig.2.1).

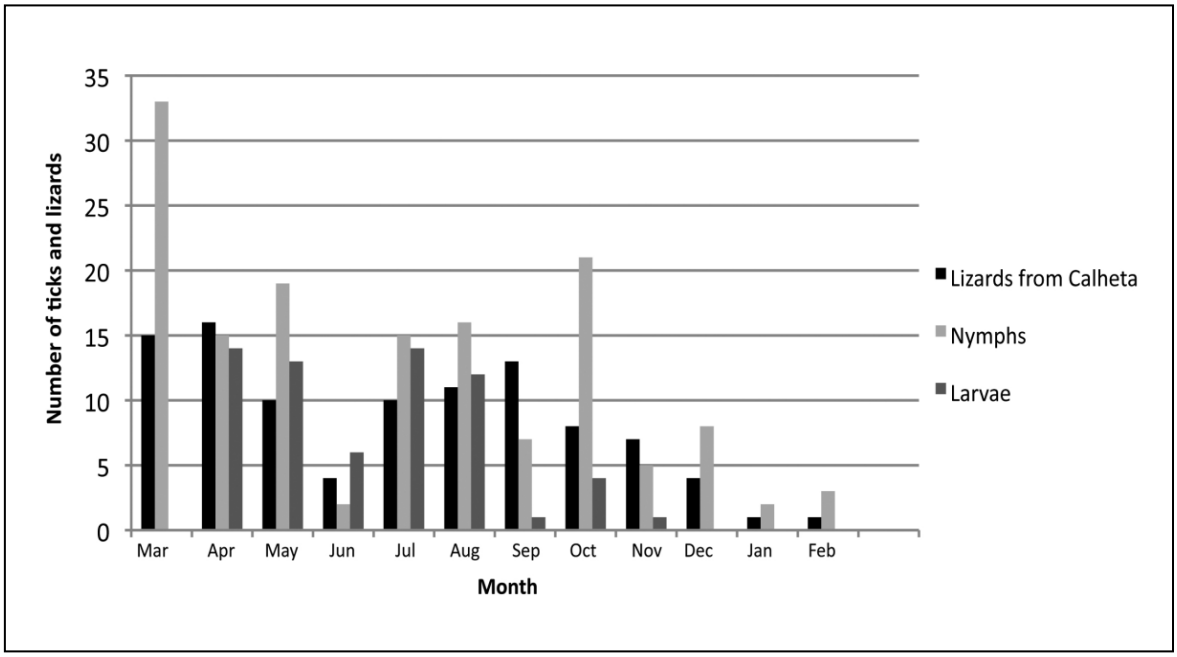

Fig.2.1. Numbers of lizards and their ticks (nymphs and larvae) collected in Calheta.

Lizards and ticks infected with *B. lusitaniae, R. monacensis*, *R. helvetica* and *A. phagocytophilum* were only found in Calheta*,* as shown in table 2.3. *Rickettsia* positive amplicons showed 100% sequence identity with *gltA* (341/341) and *ompB* (384/384) of *R. helvetica* (GenBank accession number U59723 and HQ232251) and 100% identity with *gltA* (341/341 bp) and *ompB* (384/384 bp) of *R. monacensis* (HM149283 and EU883092), previously detected in Portuguese ticks (Lopes de Carvalho *et al*. 2008a, Milhano *et al*. 2010). Two main genetic variants of *B. lusitaniae* were detected in the intergenic spacer (IGS) partial sequence from 5S *(rrf)* to 23S *(rrl)*: variant 1, presenting

100% identity to PotiB6, a tick isolate previously reported in Madeira Island (EU078961) (Lopes de Carvalho *et al*. 2008a), and variant 2, detected in only one tick and showing 100% identity to PoAnB1, a Portuguese isolate from *Apodemus sylvaticus* (EF647595) (Lopes de Carvalho *et al*. 2009).

A. phagocytophilum positive amplicons showed 100% sequence identity to previously described *rrs* sequences from Portuguese ticks (EU098006) (Rijpkema *et al*. 1995). However, *groESL* gene analysis revealed two main polymorphic positions in the encoding sequence, enabling the identification of three genetic variants: variant A, found in one lizard and eight ticks and showing 100% identity to a North American strain isolated from humans (U96728), variant B (nucleotide 401, G→A; nucleotide 497, T→C), detected in five ticks, and variant C (nucleotide 401, G→A), found in two ticks and 100% identical to *A. phagocytophilum* partial sequence detected in an *I. ricinus* species from Madeira Island (EU004826) (Santos *et al*. 2009).

Table 2.3. Prevalence of *Borrelia burgdorferi* sensu lato, *Rickettsia* spp., and *Anaplasma phagocytophylum* infection in lizard tissue samples and ticks collected from lizards

Sample type and stage (n)	*Rickettsia monacensis* No. (%)	*Rickettsia helvetica* No. (%)	*R. monacensis* or *R. helvetica* No. (%)	*Borrelia lusitaniae* No. (%)	*Anaplasma phagocytophilum* No. (%)	Coinfections (No. %)[a]
Ticks						
Larvae (65)	20 (30.8)	0	20 (30.8)	3 (4.6)	0	1(1.5)Bl + Rm
Nymphs (146)	67 (45.9)	2 (1.4)	69 (47.3)	22 (15.0)	15 (10.3)	10(6.8) Bl + Rm; 5(3.4) Ap+ Rm
Total (211)	87 (41.2)	2 (1.4)	89 (42.2)	25 (11.8)	15 (7.1)	16 (7.6)
Lizard tissue						
Adults (83)	6 (7.2)		6 (7.2)	3 (3.6)		0
Juveniles (68)	4 (5.9)	2 (1.3)	6 (7.2)	4 (5.9)	1 (0.7)	0
Total (151)	10 (6.6)	2 (1.3)	12 (7.9)	7 (4.6)	1 (0.7)	0
Lizard bloodprints						
Total					0	

[a] Bl+Rm, *Borrelia lusitaniae* and *Rickettsia monacensis* coinfection; Ap+Rm, *Anaplasma phagocytophilum* and *Rickettsia monacensis* coinfection

Rickettsia was the most prevalent agent detected both in lizards and ticks, with a higher infection in nymphs. An interesting finding was the presence of *R. monacensis* and *R. helvetica* in lizard tissues because the latter had never been detected on the island. These results suggest not only that disseminated infections can occur in *T. dugesii* but also that this species may be a potential or transitory reservoir infecting ticks. The fact that all the ticks collected from lizards were localized on the lizard forelimb axial area and that sample tissue for PCR detection was removed from the tail seems to indicate that disseminated infection may occur. *R. monacensis* had previously been detected by PCR on Madeira ticks, albeit with a lower prevalence (20%) than that of the present study (41.1%) (Lopes de Carvalho *et al*. 2008a). This may be due to the screening of different areas and the fact that ticks were collected in vegetation and not in lizards. The detection of *B. lusitaniae* in lizard tissues and the high level of infected parasitizing ticks also support the importance of *T. dugesii* in the maintenance of this genospecies on Madeira Island, which is in agreement with studies carried out in other lizard species (Amore *et al*. 2007, Dsouli *et al*. 2006, Majlathova *et al*. 2006, Richter and Matuschka 2006). *B. lusitaniae* has also been associated with other reservoirs such as rodents (*A. sylvaticus*) and migratory birds (Lopes de Carvalho *et al*. 2009, Poupon *et al*. 2006). It is suggested by several authors that bird ticks may be responsible for the geographic dispersion of *B. lusitaniae,* and both rodents and lizards may be responsible for perpetuating this genospecies in enzootic cycles where they are the major vertebrate hosts for *I. ricinus* (Lopes de Carvalho *et al*. 2009, Richter and Matuschka 2006). As described for *R. monancensis*, a higher prevalence (11.8%) of *B. lusitaniae* in ticks from lizards was found in this work than in our previous work (2.7%) in Madeira Island (Lopes de Carvalho *et al*. 2008a). Another important finding in this study is the presence of polymorphisms that reveal the presence of genetic variants of *B. lusitaniae,* which appear to be specifically associated with lizard hosts, in some sequences. Further studies need to address the

eventual evolutionary forces responsible for this genetic diversity and the presence of *B. lusitaniae* subpopulations associated with specific reservoirs. Other *Borrelia* genospecies, such as *B. afzelii, B. garinii,* and *B. valaisiana*, that were previously detected in ticks in Madeira Island in the same area were not detected in either ticks attached to lizards or in lizard tissues (Matuschka *et al*. 1998). Currently, we are testing the hypothesis that *T. dugesii* might have a borrelicidal effect on some *Borrelia* genospecies other than *B. lusitaniae*.

Regarding *A. phagocytophilum*, the data obtained thus far suggest that Madeira Island lizards are exposed to infected ticks, but they do not seem to be a primary reservoir host for this agent. In fact, the sole *T. dugesi* positive tail-snip detected contrasts with the number of studied lizards and with the much higher number of positive parasitizing nymphs found in the same area. Moreover, no DNA was found in the lizard blood prints tested, even with the blood-borne nature of this agent. Since *A. phagocytophilum* is not transovarially transmitted, the absence of *A. phagocytophilum* infection in all attached larvae suggests that lizards were not infectious for ticks and raises the hypothesis that positive nymphs may have resulted from previous infections as larvae when feeding on other reservoir hosts. These results are in accordance with previous findings in an experimental infection study of *A. phagocytophilum* in lizards (Nieto *et al*. 2009). Studies performed by Matuschka and collaborators (1994, 1998) suggest that rats are potential reservoir hosts for *Borrelia* in this island. This could also be true for *A. phagocytophilum*. Nevertheless, a preliminary study performed on Madeira Island in rodents did not find *A. phagocytophilum* infection in two of the three species present on the island (Santos *et al*. 2009). During the present study, field work was also directed toward the capture of rodents, but unfortunately, no animals were captured, a result which might be related to recent massive campaigns for rodent control in the area.

In conclusion, Madeira Island harbors a large population of *T. dugesii* lizards that seems to be involved in the maintenance and transmission cycle of at least two tick-borne agents. Future experimental studies involving *R. monacensis* maintenance in lizards and its transmission to feeding ticks are crucial to evaluate whether lizards are transitory reservoirs and how long they can sustain the infection. The study of other potential hosts is also important to understand the complex interaction between ticks and hosts and pathogens on this island.

2.4. *RICKETTSIA LUSITANIAE* SP. NOV. ISOLATED FROM THE SOFT TICK *ORNITHODOROS ERRATICUS* (ACARINA:ARGASIDAE)

Abstract

In this study a novel *Rickettsia* of the spotted fever group, isolated from *Ornithodoros erraticus* soft ticks collected from pigpens in the south of Portugal, is described. After initial screening revealed *Rickettsia*-positive ticks, isolation attempts were then performed. Successful isolates were achieved by shell-vial technique using Vero E6 cells at 28ºC. Molecular characterization of the isolate was performed based on analysis of five rickettsial genes *gltA*, *ompA*, *ompB*, *sca1* and *htr* with their subsequent concatenation along with other rickettsial species resulting in a clustering of the new isolate with *R. felis* and *R. hoogstraalii*. The degree of nucleotide sequence similarity with other rickettsiae fulfills the criteria for classification of our isolate as a novel species. The name *R. lusitaniae* sp. nov. (= CEVDI PoTiRo) is proposed for this new species found in *O. erraticus*.

Introduction

Rickettsia are obligately intracellular Gram-negative microorganisms belonging to the order Rickettsiales. Several species have been shown to cause disease in humans and are transmitted by hematophagous ticks, fleas and lice (Parola *et al*. 2005). The majority of *Rickettsia* species are transmitted by ixodid or hard ticks; however, a few studies have associated these agents with argasid or soft ticks. *Rickettsia felis*, as well as other *Rickettsia* spp., have been described in *Carios capensis* soft ticks collected from nests of brown pelicans in Charleston County, USA (Reeves *et al*. 2006). This cosmopolitan tick of seabirds has also been described as being infected with *Candidatus* 'R. hoogstraalii', in coastal Georgia, USA, and was later characterized and named *R. hoogstraalii* sp. nov.

(Keirans *et al*. 1992, Matilla *et al*. 2007, Duh *et al*. 2010). A similar *Rickettsia* has also been detected in Africa, in *Ornithodoros moubata* ticks in Tanzania, and more recently a closely related *Rickettsia* was detected in *Argas persicus* from Ethiopia (Cutler *et al*. 2006, Pader *et al*. 2012).

In Portugal three species of argasid ticks have been identified: *Argas vespertilionis*, *O. maritimus* and *O. erraticus* (Lucas 1849), the latter having the most prominent impact in terms of public health. It has been shown to play a role in transmitting *Borrelia hispanica* and *B. crocidurae*, causing tick-borne relapsing fever in humans, as well as African swine fever virus in animals, both in the Iberian Peninsula and Africa (Basto *et al*. 2006, Vial *et al*. 2006, Sarih *et al*. 2009, Palma *et al*. 2012, Ravaomanana *et al*. 2011).

Until the present time the main focus for the analysis of *Rickettsia* in Portugal has been ixodid ticks, with several species having been detected including *R. conorii*, *R. aeschlimannii*, *R. sibirica*, *R. helvetica*, *R. monacensis*, *R. slovaca*, *R. massiliae* and *R. raoultii* (Bacellar *et al*. 1991, De Sousa *et al* 2006, Santos-Silva *et al*. 2006b, Milhano *et al*. 2010). Given the increasing evidence of soft ticks harbouring rickettsial agents, in this study we decided to analyse *O. erraticus* ticks, already known for their role as vectors of *Borrelia* sp.

Materials and Methods

Tick samples

The tick samples used in this study were selected from an original collection of 390,476 *O. erraticus* ticks from a project evaluating the prevalence of *B. hispanica* in Portugal (Palma *et al*. 2012). Ticks were collected overnight with CO_2 traps from 11 pigpens, in south Alentejo, Portugal, from April to September, 2009, and June to August, 2010, stored in flasks in the dark with circulating air at room temperature and 63% relative humidity for a

period of up to three months. They were subsequently identified by gender and developmental stage using morphological characters with standard taxonomic keys.

DNA amplification

Ornithodoros erraticus pools were randomly selected from 2009 (48 pools) and 2010 (74 pools), and screened by PCR, for the 11 pigpens studied. Each pool tested consisted of a mixture of five DNA samples extracted individually from five ticks. *Ornithodoros erraticus* ticks were washed for 5 min in sterile water, followed by 10% bleach and 70% ethanol, and subsequently dried on sterile filter paper. DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

Amplification of rickettsial DNA was performed targeting the gene for citrate synthase (*gltA)* [23] (Roux *et al*., 1997), outer membrane protein A (*ompA*) and B (*ompB)* (De Sousa *et al*. 2006). For rickettsial characterization, in addition to these genes, amplification targeting Sca1 autotransporter protein (*sca1*) and 17kDa protein (*htr*) was also performed as previously described (Ngwamidiba *et al*. 2006, Williams *et al*. 1992).

Molecular characterization

Amplicons were purified using Jetquick Purification PCR Product Spin Kit (Genomed, Inc.), and the products were sequenced with the Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 377 DNA sequencer, according to the manufacturer's recommendations. The sequencing reactions were performed with the forward and reverse primers used for PCR amplification. The sequences were assembled combining sequences generated by each primer using the Lasergene software v.7.0 (DNASTAR). For identity determination, primer sequences were removed. The sequences (*gltA*, *htr, ompA*, *sca1 and ompB*) were concatenated and aligned with corresponding sequences of other *Rickettsia* species available in GenBank using CLUSTAL algorithm and

manually aligned using GeneDoc software (Nicholas & Nicholas, 1997). Phylogenetic trees were inferred by the maximum-parsimony methods performed with PAUP 4.0b10 software (Kumar *et al*. 2004) with 1,000 replicates of random-addition taxa and tree bisection and reconnection branch swapping; all positions were equally weighed. Bayesian analysis was performed using Mr Bayes v3.1.2 (Ronquist *et al*. 2003). The tree searches employed GTR+GAMMA and proportion of invariable sites. The first 25% of the trees from 100,000,000 generations were discarded as burn-in.

Cultivation of Rickettsiae

Eleven live female *O. erraticus* and 8 frozen nymphs from the 2010 collection were selected from PCR-positive pens and washed for 5 min in 10% bleach, 70% ethanol and allowed to air dry. The adults were cut longitudinally, with one half immediately frozen at -80ºC and the other half tested by PCR for *Rickettsia*. The corresponding halves of the PCR-positive ones, along with the eight nymphs, were used for *Rickettsia* isolation attempts in Vero E6 cells. Each tick was then triturated in Eagle's minimal essential medium (MEM, Gibco, Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum and 1% glutamine, inoculated into Vero E6 cell-coated shell vials and incubated at 28°C. After 6-7 days incubation the cells were removed with glass beads, transferred onto a confluent monolayer of the same cell type in 25 cm^2 flasks and incubated at the same temperature for another 6 days. Monolayers were scraped after 6-7 days for *Rickettsia* detection by Gimenez staining (Gimenez, 1964). When microscopy showed rickettsial infection by Gimenez staining, a new slide was prepared to identify the bacteria by indirect immunofluorescence assay (IFA), using sera from two patients with confirmed diagnosis of rickettsioses of the spotted fever group (Bacellar *et al*. 2003). Positive isolates were established after three successful passages, after which the cells were harvested and stored at -80° C. Characterization of isolates was performed by PCR and sequencing,

targeting the genes described above. Successful isolates were then also propagated in a mosquito C6/36 cell line (derived from *Aedes albopictus)*, supplemented with 3% fetal bovine serum containing L-15 medium (Leibovitz, Gibco, Invitrogen, Paisley, UK).

Results

A total of 122 pools of *O. erraticus,* 48 from 2009 and 74 from 2010*,* selected from an original collection of soft ticks collected from 11 pigpens in south Alentejo, Portugal, were used for an initial PCR screening of *Rickettsia.* Seven out of the total of 11 pig pens tested were positive. Thirteen (27.1%) pools out of the total tested for 2009 and five (6.8%) pools out of the total for 2010 were positive (Table 2.4)*.*

Table 2.4. PCR detection of *Rickettsia* sp. in *O. erraticus* pools from Portugal in 2009 and 2010.

Pig pens	Months (2009; 2010)	Pools tested (positive/total)	Tick life stage by pool
P1	April	3/5	Female
	July	n.a.	
P2	April	0/5	
	July	0/10	
P3	April	2/3	Nymph
	July	0/6	
P4	April	3/5	1 male, 2 nymph
	July	n.a.	
P5	June	1/5	Nymph
	June	0/8	
P6	June	0/5	
	July	0/10	
P7	August	1/3	Nymph
	August	0/5	
P8	August	1/5	Nymph
	June	3/10	Nymph
P9	August	0/2	
	July	0/10	
P10	August	1/5	Nymph
	July	2/5	1 female, 1 nymph
P11	September	0/5	
	July	0/10	

n.a. not available

Eleven live female *O. erraticus* from one of the two positive pigpens from the 2010 collection (Table 2.4) were washed, cut in half and tested by PCR for *Rickettsia*. Six of these were positive, so the corresponding half was inoculated into Vero cells, along with eight frozen nymphs collected from the same pigpen. In total, 14 isolation attempts were thus performed using the shell-vial technique at 28°C. After 14 days incubation, *Rickettsia* was visualized by Gimenez staining in one adult and one nymph *O. erraticus* culture. Further propagation of the *Rickettsia* in a mosquito C6/36 cell line was successful, with visualization of coccobacilli after 12 days incubation at 28°C. Immunofluorescent rickettsiae were visualized by IFA using the sera from one patient with Mediterranean spotted fever (MSF) and other patient with lymphangitis associated rickettsiosis (LAR). PCR and isolate characterization, performed by BLAST analysis, revealed that the novel isolate was closest to the corresponding sequence of *R. hoogstraalii*, 97.7% (803/822) bp for *gltA* and 99.5% (392/394) for *htr,* of *R. australis* strain Cutlack with 92.9% (444/478) for *ompA*, and of *R. felis* with 92.5% (765/827) for *ompB* and 98.3% (475/483) for *sca1*. Concatenation of the five genes showed a clustering of our isolate, PoTiRo, with *R. felis* and *R. hoogstraalii* (Figure 2.2).

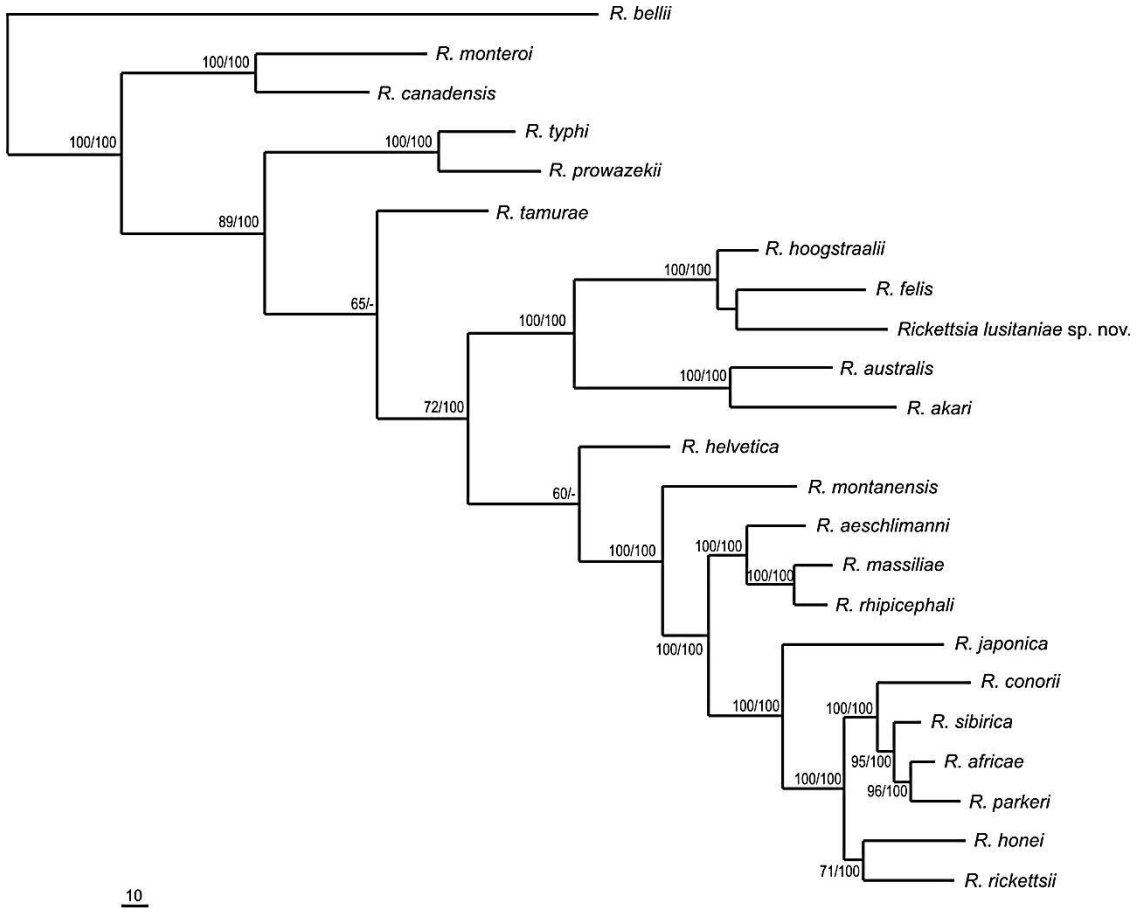

Figure 2.2. Molecular phylogenetic analysis of *R. lusitaniae* sp. nov. isolated from the tick *O. erraticus* from Portugal. A total of 3003 aligned sequences corresponding to *gltA*, *htr*, *ompA*, *sca1* and *ompB* genes (820, 394, 488, 483, 818 nt, respectively) were concatenated and subjected to maximum-parsimony inference analysis. Bootstrap values are shown at the nodes. Bar, 10 substitutions.

The partial sequences of the rickettsial isolate generated in this study were submitted to GenBank; JQ771933 for *gltA* gene, JQ771934 for *htr* gene, JQ771935 for *ompA,* JQ771936 for *sca1* and JQ771936 for *ompB* (Table 2.5).

Table 2.5. Accession numbers of *Rickettsia* species used in the phylogenetic analysis.

Rickettsia species	GenBank accession numbers				
	gltA	*htrA*	*sca1*	*ompA*	*ompB*
R. bellii	CP00849	DQ517289	DQ306904	-	-
R. monteiroi	FJ269035	FJ269036	JF347727	-	-
R. canadensis	AB297809	AF445381	DQ306905	-	-
R. typhi	AE017197	JX198507	DQ306919	-	-
P. prowazekii	AJ235273	CP004889	DQ306914	-	-
R. tamurae	AF394896	AB114825	-	DQ103259	-
R. hoogstraalii	FJ767737	FJ767736	-	-	-
R. felis	CP000053	CP000053	DQ306907	-	AF182279
R. lusitaniae	**JQ771933**	**JQ771934**	**JQ771936**	**JQ771935**	**JQ771937**
R. australis	U59718	M74042	DQ306903	AF149108	AF123709
R. akari	CP000847	CP000847	DQ306902	-	AF123707
R. helvetica	U59723	AF181036	DQ306908	-	-
R. montanensis	U74786	U11017	DQ306912	FM883670	AF123716
R. aeschlimanni	HM050276	DQ379979	DQ306900	GQ180862	AF123705
R. massiliae	U59719	CP000683	DQ306911	CP000683	AF123714
R. rhipicephali	DQ865206	DQ865207	DQ306915	U43803	DQ865209
R. japonica	U59724	D16515	DQ306910	DQ019319	AF123713
R. conorii	AE006914	AE006914	DQ306906	RCU43794	AY643093
R. sibirica	U597734	AF445384	DQ306918	AF179635	AY331393
R. africae	U597733	CP001612	DQ306901	RSU43805	AF123720
R. parkeri	EF102236	EF102237	DQ306913	EF102238	EF102239
R. honei	U59726	AF060706	DQ306909	DQ19319	AF123713
R. rickettsii	CP000848	CP000766	DQ306916	CP000848	CP000848

Discussion

Ixodid ticks are the main vectors and reservoirs of *Rickettsia*, and many genera are known to transmit human rickettsial pathogens, including *Rhipicephalus*, *Dermacentor*, *Hyalomma*, *Ixodes* and *Amblyomma* (Azad & Beard 1998). To date little is known about the role of soft ticks in the transmission of *Rickettsia*. In this study a novel *Rickettsia* was detected and isolated from *O. erraticus* ticks collected from pigpens in south Alentejo, Portugal. The majority of infected ticks were in the nymphal stage; however, no relationship was established between the tick stage and infection rate. Phylogenetic

analysis of isolates from *O. erraticus* was performed using the concatenated sequence set of 820, 394, 488, 483 and 818 nt of *gltA*, *htr*, *ompA*, *sca1* and *ompB* genes, respectively, showing a close segregation of our rickettsial isolates with *R. felis* and *R. hoogstraalii*, supported by high bootstrap values (100%). Serological evidence that our isolate belongs to the spotted fever group was also confirmed by a reaction by IFA when *O. erraticus* antigen was tested with sera from two patients with confirmed diagnosis of MSF and LAR. Given the similarity of our isolate with *R. hoogstraalii* and *R. felis*, and the fact that the latter grows more efficiently at low temperatures in amphibian and mosquito cells (Raoult *et al*. 2001, Horta *et al*. 2006), propagation of our isolate was attempted, and achieved, on a mosquito C6/36 cell line. *R. hoogstraalii* was initially isolated from *Haemaphysalis sulcata* ticks from Croatia (Duh *et al*. 2010). In that study the tick triturates were first inoculated onto C6/36 cells, and *Rickettsia* visualized after 12 days shell-vial cultivation. No cytopathic effect was observed. They were then passaged onto Vero cells, with a 90% infection rate after 15 days, when a severe cytopathic effect was observed. Similarly, in our study, *Rickettsia* were detected at days 14 and 12 of cultivation in Vero and C6/36 cells, respectively; however, no cytopathic effect was apparent in either cell type.

Recent studies have detected two closely related *Rickettsia* in Africa, one in *O. moubata* collected in Tanzania and the other in *A. persicus* collected in Ethiopia (Cutler *et al*. 2006, Pader *et al*. 2012). As was the case in our study, those species also cluster closely to *R. hoogstraalii.* Adaptation to the different tick vectors has been pointed out as a possible reason for the divergence observed between the *Rickettsia* strains, which could also explain the differential clustering of the rickettsial isolate obtained in our study. The guidelines for taxonomic classification and identification of a new rickettsial species state that an isolate should not show more than 99.8 and 99.9% degrees of nucleotide similarity with *rrs* and *gltA* genes of the most homologous validated species, and also no more than 98.8, 99.2 and 99.3% for *ompA*, *ompB* and *sca4*, respectively (Fournier *et al*. 2003). Our

isolate, PoTiRo, is genetically distinct from other *Rickettsia,* showing 97.7% homology for *gltA*, 92.9% for *ompA*, 92.5% for *ompB*, thereby abiding by the guidelines for those genes, and thus classified as a new species, hereby named as *Rickettsia lusitaniae* sp. nov.

Further studies regarding the potential pathogenicity of *R. lusitaniae* sp. nov. should be addressed, considering its close phylogenetic relationship with *R. felis*, a recognised human pathogen. Additionally, *O. erraticus* are not only long-lived ticks, capable of surviving up to 15-20 years (Encinas-Grandes *et al*. 1993), but have also been identified as vectors of human disease (Estrada-Pena *et al*. 1999).

Description of *Rickettsia lusitaniae* sp. nov.

Rickettsia lusitaniae (lu.si.tan'i.ae. L. gen. fem. adj. *lusitaniae*, referring to Lusitania [Portugal], where the organism was isolated).

An obligately intracellular, Gram-negative bacterium which grows in Vero E6 cells at 28ºC in minimum essential medium supplemented with 5% fetal bovine serum. This species also grows in mosquito C6/36 cells supplemented with 3% FBS at 28°C. The *gltA*, *ompA*, *ompB*, *sca1* and *htr* gene sequence analysis indicates that *R. lusitaniae* sp. nov. is a separate species from other spotted fever group *Rickettsia*, being most closely related to *R. felis* and *R. hoogstraalii* . No information is available on the possible pathogenicity of these bacteria for vertebrate hosts.

The type strain, PoTiRo, was isolated from *O. erraticus* ticks collected in south Alentejo, Portugal, in 2010, and has been deposited in the Rickettsial Collection of the Center for Vector and Infectious Diseases Dr. Francisco Cambournac, National Institute of Health Águas de Moura, Portugal.

CHAPTER 3

STUDIES IN THE VECTOR

3. STUDIES IN THE VECTOR

This chapter is based on the research paper:

Milhano N, Popov V, Vilhena M, de Sousa R, Bouyer D, Walker D (2014) A quantitative study of *Rickettsia massiliae* dissemination in *Rhipicephalus sanguineus* organs. Ticks and Tickborne Dis 5: 709-714.

3.1. INTRODUCTION

Rhipicephalus sanguineus is the most widespread ixodid tick around the world, and its role as vector of rickettsial species, namely *R. conorii*, *R. massiliae* and *R. rickettsia*, is well documented. An increasing number of studies are being performed in order to advance the knowledge of rickettsial diseases using this vector; however, many gaps still remain, namely in terms of the vector-pathogen interface. Thus, in order to address these, a set of preliminary studies as well as a study resulting in publication are described in this chapter.

3.2. PRELIMINARY STUDIES

In order to analyse the vector-pathogen interface, proteomic studies of the salivary gland contents of *Rh. sanguineus* in different life and nutritional statuses, as well as infection studies of this tick species with *R. conorii* were performed, as described below.

3.2.1. Proteomic analysis of *Rh. sanguineus* salivary glands

The rationale behind this study was that, given the complexity of tick saliva content and its importance in subverting the host immune response, the proteomic analysis of saliva under different feeding statuses and life stages of *Rh. sanguineus* would identify a set of proteins allowing a further understanding of the mechanisms underlying the tick, the pathogen, and ultimately, a vertebrate host.

A first experiment was thus undertaken using salivary glands from both adult and nymphal *Rh. sanguineus*, in order to ascertain different proteins between these two life stages which may account for differential rickettsial transmission between life stages. Thus, salivary glands were dissected from both unfed nymphs and adult female *Rh.*

sanguineus, quantified, cleaned, and subsequently the proteins of SGE separated on 2D gels. Out of 414 spots analyzed, 158 were classified as qualifying for protein identification, based on an intensity fold difference ≥ 2. From the 158 spots, 20 differentially expressed spots were selected for further processing; however, insufficient sample availability led to poor protein identification through matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/TOF) analysis. Nevertheless, bioinformatics analysis of the gels revealed significant differences between the two sets of gels, translating into proteomic salivary differences between nymphs and adults, thereby justifying future use of these different tick life stages in subsequent studies.

A second set of experiments focused on adult *Rh. sanguineus* under different nutritional statuses. Female ticks were allowed to feed on a guinea pig for a period of 48 to 72 h, after which they were detached and their salivary glands were dissected, together with a group of unfed adult female *Rh. sanguineus,* and processed as above. After the 2D gel separation, 94 spots out of 1144 spots analyzed were classified as qualifying for identification, based upon differential expression criteria of p-value ≤ 0.05 and intensity fold difference ≥ 3.0, from which 12 spots were selected and processed for MALDI-TOF/TOF identification. Six spots were identified as tick proteins, two of which were specifically from *Rh. sanguineus* ticks (Fig. 3.1).

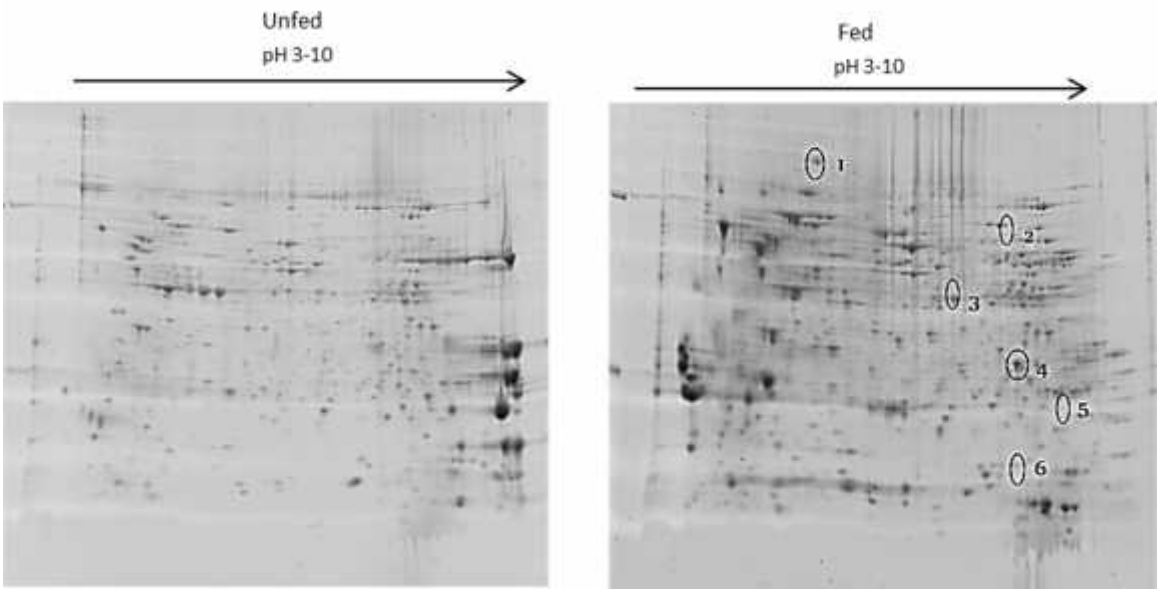

Fig. 3.1. 2D gels of unfed versus fed adult female *Rh. sanguineus.* The greatest similarities were: 1- *Ixodes scapularis* hypoxia upregulated protein, putative; 2 - *I. scapularis* putative GMP synthase; 3- *Amblyomma americanum* hypothetical protein; 4- *Rh. sanguineus* protein disulfide isomerase; 5- *Rh. sanguineus* putative cement protein; 6- *A. americanum* hypothetical protein.

Two spots were identified as *Rh. sanguineus* proteins, namely spot 4 (Expected molecular weight (EMW) 29kDa/ Measured molecular weight (MMW) 27 kDa) identified as a protein disulfide isomerase, catalyzes protein folding; and spot 5 (EMW 20kDa/MMW20kDa), identified as putative cement protein, used for the formation of the cement cone which attaches the tick to the host.

Another two spots were identified as *I. scapularis* proteins, namely spot 1 (EMW 141 kDa/MMW 90 kDa) identified as a putative hypoxia upregulated protein, possibly an oxidative stress-induced NADPH: quinone oxireductase with an antioxidant function; and spot 2 (EMW 71 kDa/MMW 77 kDa): Putative GMP synthase. Involved in the *de novo* synthesis of purine nucleotides.

The last two spots were identified as *Amblyomma americanum* proteins, specifically spot 3 (EMW 39 KDa/MMW 45 KDa), identified as a hypothetical protein, similar to glutamine

synthetase, with a described role in formation of glutamine; and spot 6 (EMW 16kDa/MMW 20 kDa), as a hypothetical protein, role related to missfolding proteins.

The proteins identified in this preliminary study, located in the alkaline region of the gel, are similar to proteins mainly involved in protein folding, purine synthesis, or with an antioxidant effect.

These studies were not pursued as significant protein quantities would have been necessary in order to obtain robust reproducible results, meaning unsurmountable quantities of ticks and animals.

3.2.2. Infection of *Rh. sanguineus* ticks with *R. conorii* ISF

In order to analyze the interactions between *Rh. sanguineus* and *R. conorii*, our first step was to establish a *R. conorii*-infected colony of *Rh. sanguineus*, which was our aim in this study. Experimental infection studies of *Rh. sanguineus* with *R. conorii* ISF and Malish strains have shown high tick mortality, particularly with the latter species of *Rickettsia* (Levin *et al.* 2009). Based on these results, we attempted to establish an infected colony using the strain *R. conorii ISF*. Two methods of infection were tested: immersion of *Rh. sanguineus* nymphs in a *R. conorii* ISF suspension and subsequent mouse infestation, which resulted in an infection success rate of 9%; and feeding on rickettsemic mice by uninfected *Rh. sanguineus* larvae, which resulted in a 13% success rate. Based on these results, where a large number of ticks and animals would be necessary in order to establish an infected colony, in addition to time constraints issues, we decided to perform subsequent vector-pathogen studies using a *R. massiliae*-infected *Rh. sanguineus* colony maintained at UTMB, as described in the following study.

3.3. A QUANTITATIVE STUDY OF *RICKETTSIA MASSILIAE* IN *RHIPICEPHALUS SANGUINEUS* ORGANS

Abstract

Rickettsia massiliae, belonging to the spotted fever group of *Rickettsia,* is a human pathogen causing a similar course of disease to that caused by *R. conorii*, the originally recognized etiologic agent of Mediterranean spotted fever. In view of this similarity, we performed an ultrastructural study of *R. massiliae* in organs of *Rhipicephalus sanguineus* ticks, in order to advance knowledge of the complex dynamics at the tick-pathogen interface in rickettsioses.

Adult *R. massiliae*-infected *Rh. sanguineus* ticks were fed on uninfected Hartley strain guinea pigs, and five females were collected daily throughout their feeding period up to day 6, and analyzed by quantitative real-time PCR and electron microscopy. An increase in rickettsial content was observed in the salivary glands, particularly in the first two days of feeding, and a plateau was observed between days 3 and 6. Rickettsial organisms were observed in all tick organs analyzed, in higher numbers in the fed state, and statistically significant differences were observed in measurements of the periplasmic layer of *R. massiliae* in salivary glands of fed and unfed *Rh. sanguineus* ticks, with increased thickness in the former case.

This study provides insight into the interface between *R. massiliae* and *Rh. sanguineus* ticks, highlighting the need for analysis of *R. massiliae* to fully ascertain its place as an important pathogenic agent of a spotted fever rickettsiosis.

Introduction

Mediterranean spotted fever (MSF), caused by *Rickettsia conorii*, is one of the oldest rickettsioses known to man, and was described for the first time at the beginning of the 20th century (Conor and Bruch, 1910). With the advent of new molecular techniques, an increase of newly discovered spotted fever group *Rickettsia* has occurred in the past decade, with new pathogenic species continuously being isolated. *R. massiliae* was recently associated with human disease in Italy, France and Argentina (Vitale *et al.* 2006, Cascio *et al.* 2013, Parola *et al.* 2008, Garcia-Garcia *et al.* 2010). The signs and symptoms of human infection by this *Rickettsia* are very similar to those caused by *R. conorii*, whereby the illness may be misdiagnosed for other MSF-like illnesses, particularly in geographical regions where both rickettsial species are present (Fernandez-Soto *et al.* 2006a). This situation was observed in the first reported human *R. massiliae* infection, confirmed 20 years after the initial isolate was obtained from the patient, as it had been presumed at the time to be an MSF case, based solely on serology (Vitale *et al.* 2006). As there is cross-reactivity among spotted fever group *Rickettsia* when using serology as the diagnostic tool, further confirmation by molecular methods or isolation is required to ascertain the rickettsial species responsible for the infection. This leads to the possibility that some MSF-like infections, being diagnosed as such, are in fact caused by *R. massiliae*, accentuating the potentially important role this *Rickettsia* plays in the etiology of spotted fever group rickettioses. In addition, several studies have shown that wild caught *Rhipicephalus sanguineus* ticks, the common vector for both rickettsial species, have a higher prevalence of infection with *R. massiliae* than with *R. conorii* (Bacellar *et al.* 1995a, Márquez *et al.*, 2008, Fernandez-Soto *et al.* 2006b), or are infected with only *R. massiliae* (Beeler *et al.* 2011, Milhano *et al.* 2010, Eremeeva *et al.* 2006, Chochlakis *et al.* 2012). A recent study described cases of spotted fever in an urban area of southern France, where co-infection of *Rh. sanguineus* ticks with both *Rickettsia* species was detected (Renvoise

et al. 2012). Experimental studies have shown that *R. conorii* has a deleterious effect upon its vector (Levin *et al.* 2009) and that low temperatures have a negative effect on the viability of *Rh. sanguineus* ticks naturally infected with *R. conorii* (Socolovschi *et al.* 2012), Such an effect could account for the higher prevalence of *R. massiliae* in ticks found in nature. These observations serve as further emphasis on the possibility that *R. massiliae* may in fact be the cause of disease in many cases diagnosed as MSF, and for the prevalence of antibodies to spotted fever group rickettsiae in some populations, thereby accentuating the importance of developing incisive studies focused on this species in order to broaden what is currently known about spotted fever group rickettsial pathogenesis.

Very little is known about the physiological relationship between *R. massiliae* and its vector, *Rh. sanguineus*. The majority of reports regarding this species include detection in humans and ticks and few experimental studies (Matsumoto *et al.* 2005, Levin *et al.* 2014). Furthermore, its clinical similarity to *R. conorii* infection, added to the fact that our laboratory has a colony of *Rh. sanguineus* ticks that are naturally infected with *R. massiliae*, make it a good candidate for study focusing on the dynamics of the vector-pathogen relationship, which can be used as a model of spotted fever group rickettsioses. With this purpose, we analyzed the daily changes in salivary gland rickettsial content of blood feeding *R. massiliae*-infected *Rh. sanguineus* ticks, as well as the distribution and ultrastructure of this *Rickettsia* in organs of unfed and fed ticks.

Materials and Methods

Ticks and animal infestations

Rickettsia massiliae-infected adult *Rh. sanguineus* ticks were used in this study, belonging to a colony maintained in the Level 3 Arthropod Containment Laboratory in the Department of Pathology of the University of Texas Medical Branch (UTMB). The ticks

were originally collected from dogs in North Carolina. A sample of tick couples were sent to UTMB for establishment of a tick colony and fed on male Hartley strain guinea pigs for at least three generations. The animals were purchased from Charles River Laboratories and housed in an Animal Biosafety Level 3 facility at UTMB.

All experiments and procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at UTMB. The animal care and use program at UTMB conducts reviews involving animals in accordance with the *Guide for the Care and Use of Laboratory Animals* (2011) published by the National Research Council. The Animal Resource Center provides a comprehensive program of veterinary care, animal husbandry, technical advice, assistance and facilities to advance research at UTMB.

Rickettsia massiliae identification in *Rh. sanguineus* ticks

Adult female *R. massiliae*-infected *Rh. sanguineus* ticks were washed for 5 min in 10% bleach, 70% ethanol, sterilized distilled water and allowed to air dry. Each tick was individually macerated in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, inoculated into shell-vials coated with a monolayer of Vero cells, and incubated at 32°C. After 5 days, the monolayer was scraped and transferred to a confluent monolayer of the same cell type in 25-cm^2 flasks, and incubated at the same temperature. Monolayers were scraped every 5 days for *Rickettsia* detection using Diff-Quik staining. Positive isolates were established after three successful passages and stored at -80°C. DNA of the isolates was extracted using the Qiagen DNeasy Blood and Tissue Kit (Valencia, CA) according to the manufacturer's instructions. Amplification was performed by conventional PCR targeting the 806 bp fragment of citrate synthase (*gltA*) gene, a 618 bp fragment of outer membrane protein B (*ompB*) gene, and 428 bp of RecA recombinant protein (*recA*) gene, as previously described (de Sousa *et al.* 2006, de Mera *et al.* 2009).

All reactions included negative and positive controls. To confirm identification, the amplicons were subsequently purified using the Qiagen MinElute Reaction Cleanup Kit (Valencia, CA), and sent to the UTMB core facility for sequence determination. The sequences were analysed by combining the sequences generated by each primer using Lasergene software v.7.0 (DNASTAR).

Tick organ dissections

Forty pairs of adult *Rh. sanguineus* ticks were allowed to feed on Hartley strain guinea pigs for the duration of up to six days, and five female ticks were forcibly detached on a daily basis, from day 1 until day 6 of the bloodmeal. Salivary glands were dissected from unfed and fed *Rh. sanguineus*, and washed in phosphate buffered saline (PBS), pH 7.2, and half of each organ was then individually stored in PBS and frozen at -20ºC until DNA extraction. The other half was placed in Ito's fixative and stored at 4ºC until processed for electron microscopy. Midgut was collected from unfed ticks and ovaries from 6 day-fed ticks, and processed in a similar manner as described for salivary glands.

DNA extraction from tick salivary glands and amplification

Tick salivary glands were stored separately in PBS and processed for DNA extraction using the Qiagen DNeasy Blood and Tissue Kit (Valencia, CA) according to the manufacturer's instructions. All samples were individually analyzed by real-time quantitative PCR targeting *Rickettsia* spp. citrate synthase gene, using primers C5 (forward) and C6 (reverse) in a 25 µL final reaction volume in a Biorad iQ SYBR Green Supermix (Hercules, CA), as described previously (Labruna *et al.* 2004). Real-time PCR assays were performed using an iCycler IQ from Biorad (Hercules, CA), with each reaction having a non-template control. To quantify copies of *R. massiliae* genes in salivary gland

samples, serial dilutions of a plasmid containing a single-copy portion of citrate synthase gene of *R. conorii* was used to construct a standard curve. The results are expressed as the daily mean rickettsial load (citrate synthase copy number) detected per salivary gland. The assays were performed in triplicate.

Transmission electron microscopy

Ovaries, salivary glands and gut samples for transmission electron microscopy (TEM) were individually fixed and stored in tubes containing Ito's fixative (2.5% formaldehyde, 0.1% glutaraldehyde, 0.03% picric acid, 0.03% $CaCl_2$ and 0.05 M cacodylate buffer at pH 7.3) (Ito and Rikihisa 1981) as modified by Berryman and Rodewald (1990), post fixed in 1% osmium tetroxide for 1 h, stained *en bloc* in 2% aqueous uranyl acetate at 60C for 20 min, dehydrated in a graded series of ethanol concentrations, and embedded in epoxy resin, Poly/Bed 812. Ultrathin sections were cut on a Leica EM UC7 (Leica Microsystems, Buffalo Grove, IL), placed on Formvar-carbon copper 200 mesh grids, stained with lead citrate and examined in a Philips (FEI) CM-100 electron microscope at 60 kV. Ultrastructural changes in *R. massiliae* in salivary glands of unfed and fed *Rh. sanguineus* were analyzed by measuring distances between the outer osmiophilic layer of the cell wall and the plasma membrane membrane in ten different areas per rickettsia in at least five organisms per nutritional status using Image J. The magnification used for the measurements was 89,000x. Microcapsule thickness was measured in the areas of the section where the cell wall and cytoplasmic membrane were cut perpendicularly, i.e., trilaminar structure of both was clearly visible.

Statistical analysis

GraphPad Prism 6 one-way analysis of variance (ANOVA) and Student's t-test were used to examine differences between the daily rickettsial loads in tick salivary glands during the bloodmeal and the trilaminar structure distances, respectively. P-values ≤ 0.05 were considered significantly different for each analysis.

Results

Two successful isolates were established, and rickettsial identification was confirmed by conventional PCR and DNA sequence determinations targeting *gltA, ompB* and *recA* genes. Sequence analysis revealed 100% sequence identity with homologous sequences of *gltA* (CP003319; U59720), *ompB* (DQ503428; AF123710) and *recA* (CP003319; AY124750) with both *R. massiliae* strain AZT80 and *R. massiliae* strain Bar29. The organism is designated North Carolina isolate.

The salivary glands, midgut and ovaries of adult female *R. massiliae*-infected *Rh. sanguineus* ticks evaluated in this study were analyzed by qPCR and electron microscopy. All the salivary gland halves tested contained *Rickettsia* DNA detected by qPCR, and there was a progressive increase in rickettsial titers in the salivary glands, ranging from 5.34 x 10^5 rickettsial citrate synthase copies per *Rh. sanguineus* salivary gland in unfed ticks to 2.05 x 10^7 copies in ticks fed for 6 days. A statistically significant difference ($P \leq 0.05$) according to ANOVA analysis was observed throughout the six day period of blood feeding (Fig. 3.2). A statistically significant difference was also observed between days 1 and 2 using t-test analysis (Fig. 3.2).

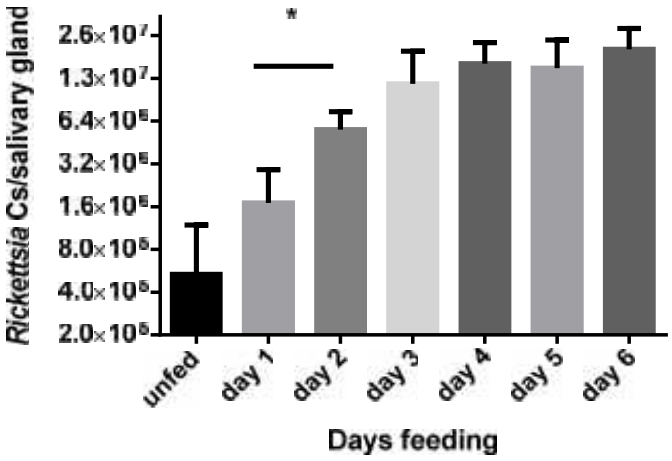

Fig.3.2. Real time qPCR of rickettsial citrate synthase (Cs) gene per tick salivary gland.

The salivary gland halves corresponding to the samples with the highest rickettsial loads, in both unfed ticks (Fig. 3.3) and ticks fed up to day 6 (Fig. 3.4), were then processed for TEM, along with midgut and ovary samples from unfed and 6 day-fed ticks, respectively. In all samples *Rickettsia* were visualized, overall showing a bacillary morphology, on average from 0.3 to 0.4 μm in diameter and 0.6 to 0.9 μm in length. Microcapsular layer was inapparent on many rickettsiae in salivary glands of unfed ticks. If present, its thickness did not exceed 12 nm. In fed ticks, microcapsular layer was 6-10 nm thick. An electron lucent 'halo zone' was visible surrounding most organisms, particularly in ticks in the fed state, externally adjacent to the trilaminar cell wall (Fig. 3.4). Interior to the trilaminar cell wall, a periplasmic space, whose thickness appeared to vary according to the nutritional status of the tick, and a plasma membrane were observed.

In the unfed *Rh. sanguineus*, few *Rickettsia* were detected in the cytoplasm of interstitial cells of the salivary gland along with mitochondria, with no organisms detected in the secretory cells (Fig. 3.3).

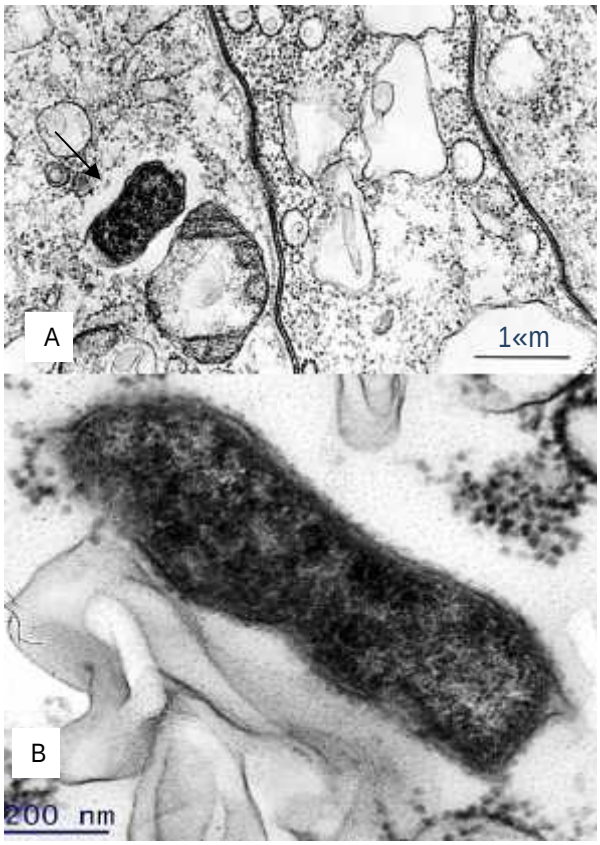

Fig.3.3. Electron micrographs of *R. massiliae* (arrow) in unfed *Rh. sanguineus* salivary gland.

The rickettsial cell wall was observed as an even structure, closely associated with the plasma membrane when compared with the fed state, where a more irregular pattern was observed, accompanied by a larger periplasmic space and surrounding 'halo zone' (Fig. 3.4).

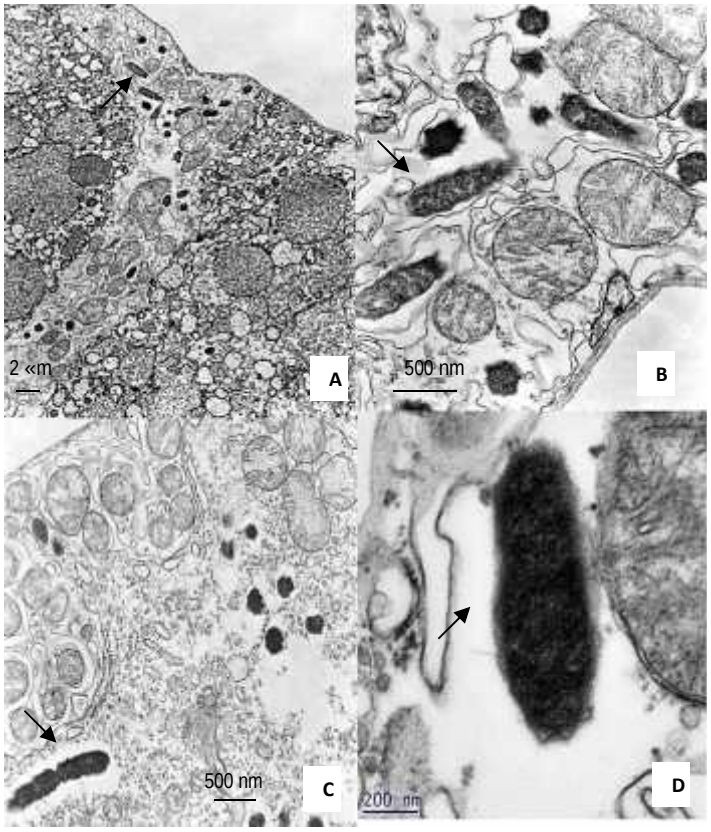

Fig.3.4. Electron micrographs of *R. massiliae* (arrows) in 6-day fed *Rh. sanguineus* salivary glands. (A) *R. massiliae* in interstitial cells.(B) Detail of interstitial cell showing numerous *Rickettsia* and mitochondria. (C) Binary fission of *Rickettsia* in interstitial cell. (D) *R. massiliae* amongst secretory vesicles.

In the salivary glands of fed *Rh. sanguineus*, *Rickettsia* were observed distributed individually or in clusters in the cytoplasm of interstitial and secretory cells of type III granular acini (Fig. 3.4 A, B, D).

Numerous mitochondria were identified interspersed with the *Rickettsia* in the interstitial cells (Fig. 3.4 A, B). In the secretory cells, *Rickettsia* were tightly aggregated in some areas of the cytoplasm and were present between secretory vesicles (Fig. 3.4 D). Rickettsial binary fission was observed in the salivary interstitial cell cytoplasm, with rickettsiae surrounded by a clear space or 'halo zone' (Fig. 3.4 C).

The average distances measured between the two trilaminar structures, the cell membrane and the cell wall of *R. massiliae* in the salivary glands of unfed and fed *Rh. sanguineus*, were 16 nm (± 0.31 nm) and 25 nm (± 0.32 nm), respectively, a statistically significant difference ($P < 0.05$, Student's test).

In the ovaries of *Rh. sanguineus* ticks fed for 6 days, *Rickettsia* were visualized free in the cytoplasm of oocyte type IV cells, among the yolk granules and varied sized lipid droplets, and surrounded, in some cases, by a 'halo zone' (Fig. 3.5 A). In oocytes we also observed gram-negative bacteria with morphology different from *Rickettsia*. They were shorter, with oval shape, mostly within membrane-bound vacuoles (Fig. 3.5 B). Some of them were dividing. Presumably they were tick endosymbionts.

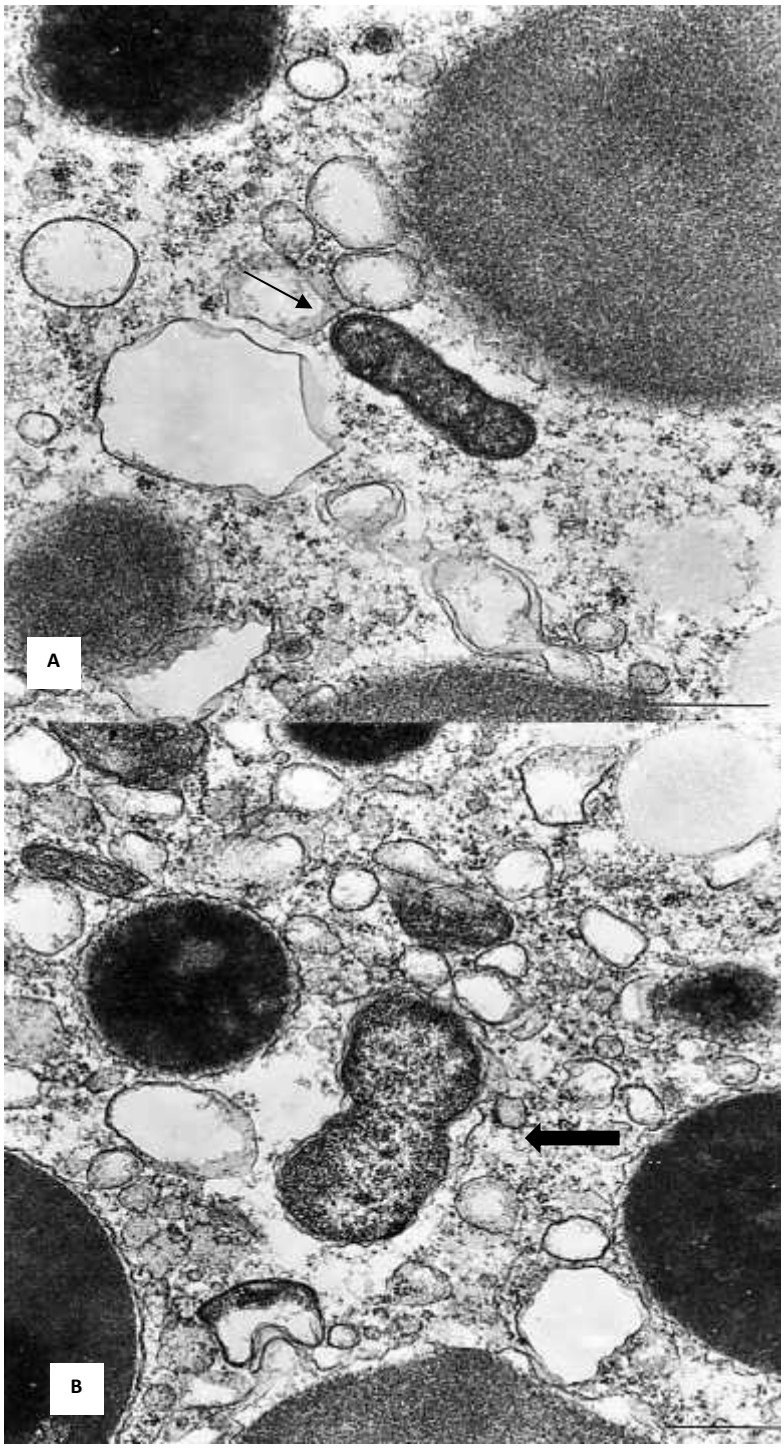

Fig.3.5. Electron micrographs of *R. massiliae* (arrows) and gram-negative bacterial symbionts (bold arrow) in the ovaries of 6-day fed *Rh. sanguineus*. (A) *R. massiliae* in cytoplasm of oocyte. (B) Gram-negative bacterium in the cytoplasm of oocyte. Bar 1 μm.

In the midgut of unfed ticks, only one type of cell was observed, residual digestive cells, possibly resulting from the previous feeding stage preceding the molt. A large number of vesicles were present in the cytoplasm of these cells, along with endosomes and lipid granules, and interspersed among them were *R. massiliae* organisms, some of which manifested a 'halo zone' (Fig. 3.6).

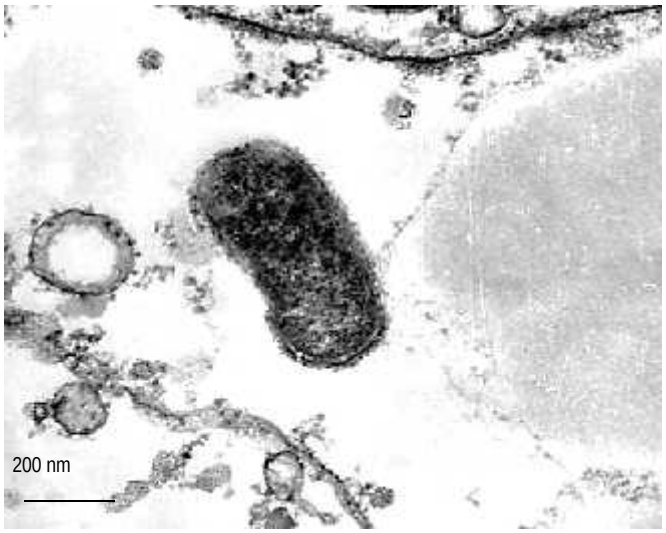

Fig.3.6. Electron micrographs of *R. massiliae* in the midgut of unfed *Rh. sanguineus.*

Discussion

To this day, not much is known about *R. massiliae* in terms of its relationship to its vector, *Rh. sanguineus;* therefore, we undertook a study at the vector-pathogen interface using this *Rickettsia* species as a model. With this aim, we analyzed the changes in *R. massiliae* content in salivary glands of *Rh. sanguineus* ticks during blood feeding, and complemented this with an ultrastructural study of infected tick organs. In the first part of this study, a progressive and statistically significant increase in *R. massiliae* content in *Rh. sanguineus* salivary glands was observed during feeding on the warm-blooded host, particularly in the first two days of feeding, when a statistically significant difference in rickettsial content was observed, reaching a plateau on days 3 to 6. This is consistent with

activation and proliferation of rickettsial organisms as a result of tick feeding, with rapid multiplication occurring at the beginning of the bloodmeal. The plateau reached on days 3 to 6 could be indicative of a resulting equilibrium between rickettsial secretion into the host and rickettsial growth in the tick salivary gland, or possibly the result of host immune response to the tick and/or the rickettsia. This study was not extended throughout the entire period of *Rh. sanguineus* feeding, which usually lasts for approximately 10 days, due to the fact that in previous experiments when this was attempted, the experiment was deleterious for the animals. We observed a two log increase in *R. massiliae* titers between the unfed and day 6-fed ticks, with rickettsial titers reaching a maximum of more than 10^7 copies per salivary gland. A recent study was performed on *Amblyomma americanum* ticks to quantify the rickettsial content in salivary glands, midgut and ovaries of feeding ticks at 12 h intervals during 9 days (Zanetti *et al.* 2008). No distinct shift in the growth of *Rickettsia* was observed in any of the tissues analyzed, and none maintained the highest burden of *Rickettsia* throughout the course of tick feeding. Overall, the highest rickettsial content in that study was detected in the salivary glands and gut samples, followed by ovaries (Zanetti *et al.* 2008). Considering the general feeding process of ticks, an increase of rickettsial content in the salivary glands during the course of the bloodmeal is expected, particularly at the beginning of this process. Throughout the duration of the tick bloodmeal a constant alternating pattern of imbibing blood and secretion of saliva takes place, along with the greater temperature on the skin of the animal than in the ambient environment, which triggers metabolic activation of *Rickettsia* with subsequent increase in proliferation in the salivary glands glands (Munderloh and Kurti, 1995). A recent study reported that blood feeding and increased temperature affected the transcriptional profile of *R. rickettsii*. The blood feeding exerted more distinctive effects when compared to temperature shift, such as an increase in bacterial loads, which seems to indicate that blood constituents are

involved in the modulation of rickettsial gene expression (Galletti *et al.* 2013). This effect on rickettsial activation was observed in our ultrastructural study, where the increased amount of *Rickettsia* in many salivary gland cell types in the fed ticks was observed compared to the unfed ticks. The reactivation phenomenon, described by Hayes and Burgdorfer (1982), where the *Rickettsia* develop different ultrastructural characteristics, including variations in cytoplasmic density, periplasmic space, electron-lucent halo zone, microcapsular layer, ribosomal appearance and degree of reticulation, according to the physiological state of the tick, were also observed in our study, as a possible means of adaptation of these bacteria to the physiological state of the vector. In the fed state, the trilaminar cell wall manifestated, in general, a more sinuous appearance, seemingly due to widening of the periplasmic space. A statistically significant difference was verified upon measuring the distance between the plasma membrane and the outer osmiophilic layer of the cell wall at various points within each organism, in at least five organisms per nutritional state. Interestingly, in another study using *R. conorii*-infected *Rh. sanguineus* ticks, no ultrastructural changes in the *Rickettsia* were observed between the unfed and fed status (Santos *et al.* 2002). However, in that study the ticks were artificially infected with Vero-cultivated *R. conorii* as nymphs, and the resulting infected adults were then used to perform the ultrastructural experiment. This situation may not have allowed *Rickettsia* to adapt to their new environment. In our study, *R. massiliae* had been transtadially and transovarially maintained in *Rh. sanguineus* for many generations, whereby the rickettsiae sensed and adapted to changes in the vector and altered their physiology accordingly. Also, in that study, although a generalized infection was observed, no *Rickettsia* were observed in secretory cells of the salivary glands, as was observed in this study, which again could be the result of the recent artificial infection.

The presence of a 'halo zone' or electron lucent area around the outer membrane of many *Rickettsia* has been suggested as possibly acting as a protective mechanism of the

organisms against host cell enzymes and host defense mechanisms (Hayes and Burgdorfer 1979). Such 'halo zones' were observed surrounding many *Rickettsia* in this study, particularly in the fed state, and were most prominent surrounding dividing bacteria in the salivary glands of fed *Rh. sanguineus*, which agrees with Hayes and Burgdorfer's observations, as binary fission is a crucial step for the dissemination of *Rickettsia*.

Rickettsia massiliae were also detected in the midgut and ovaries of unfed and fed *Rh. sanguineus*, respectively, showing their widespread dispersal throughout the tick. Overall no cytopathic effect was observed in the vector's cells, which has been the case with other species of *Rickettsia* (Hayes and Burgdorfer 1979, Costa *et al.* 2011). This observation would fit with the successful transtadial and transovarial maintenance of *R. massiliae*, and could explain why this species is prevalent in *Rh. sanguineus* in nature, when compared to the rarity of *R. conorii*, which is known to have a deleterious effect on its tick vector.

In conclusion, this study provides quantitative and qualitative insight into the *R. massiliae-Rh. sanguineus* interface, which is particularly relevant because the rickettsia is a human pathogen, and emphasizes the need for further studies in order to fully ascertain the place of *R. massiliae* as an agent of the spotted fever rickettsioses.

CHAPTER 4

STUDIES IN THE HOST

4. STUDIES IN THE HOST

This chapter is based on the research paper:

Milhano N, Saito TB, Bechelli J, Fang R, Vilhena M, de Sousa R, Walker DH (2014) The role of *Rhipicephalus sanguineus* saliva in the dissemination of *Rickettsia conorii* in C3H/HeJ mice. Accepted in Medical and Veterinary Entomology.

4.1. INTRODUCTION

In this chapter an analysis of the vector saliva on pathogen dissemination in the host is performed, thus giving an insight into the vector-pathogen-host interactions, using uninfected *Rh. sanguineus* ticks, *R. conorii* and a susceptible murine host, C3H/HeJ mice.

4.2. THE ROLE OF *RHIPICEPHALUS SANGUINEUS* SALIVA IN THE DISSEMINATION OF *RICKETTSIA CONORII* IN C3H/HEJ MICE

Abstract

Several animal models have been characterized for the study of rickettsial pathogenesis; however, the role of tick saliva in these models remains unclear. Furthermore, the major route of inoculation used in these models has been intravenous, whereas intradermal inoculation of the skin would mimic the more natural route of infection. In an attempt to address these gaps, we analyzed the role of tick saliva in the transmission of *Rickettsia conorii* in a murine host by intradermally inoculating two groups of susceptible C3H/HeJ mice with this rickettsial species, and infesting one group with nymphal *Rhipicephalus sanguineus* ticks, allowing them to feed to completion. Quantification of bacterial loads and mRNA levels of IL-1β, IL-10 and NF-κB was performed in C3H/HeJ lung samples by real-time PCR and reverse transcriptase real-time PCR, respectively. Lung histology was examined to evaluate the pathological manifestations of infection. No statistically significant difference was observed in bacterial loads in the lungs of mice between these two groups; however, a statistically significant difference was observed in the levels of IL-1β and NF-κB, which were both higher in the rickettsiae-inoculated without tick feeding group. Lung histology from both groups of animals revealed infiltration of inflammatory cells. Overall, this study showed that intradermal inoculation of *R. conorii*

caused infection in lungs of C3H/HeJ mice, and tick saliva inhibited proinflammatory effects.

Introduction

Ticks are obligately hematophagous ectoparasites, and their saliva plays a fundamental role in thwarting the host immune system during their prolonged feeding period, thus facilitating the transmission of pathogens (Liu & Bonnet, 2014; Wikel, 2013). Pharmacologically active components contained in tick saliva inhibit hemostasis, reduce inflammation, and suppress or modulate host immune responses (Kazimírová & Stibraniova, 2013; Ribeiro, 1987, 1995). This ability to circumvent immunity is essential for the tick's success in feeding and has been shown to enhance efficient pathogen transmission (Lieskovska & Kopecky 2012; Chen *et al.,* 2012). A prime example is the brown dog tick *Rhipicephalus sanguineus,* the most widely distributed tick globally, and amongst the most important vectors of pathogenic agents to both humans and animals (Dantas-Torres, 2010; Otranto *et al.,* 2014). *Rhipicephalus sanguineus* is the vector of *Rickettsia conorii,* responsible for Mediterranean spotted fever (MSF), an endemic rickettsial disease in the Mediterranean basin, India, Russia and Africa. Many studies have been undertaken in order to understand rickettsial pathogenesis in the host, stemming from the development of three endothelial target murine models of rickettsioses, namely C3H/HeN mice for the study of *R. conorii* (Walker *et al.,* 1994) and *R. typhi* (Walker *et al.,* 2000), and BALB/c mice or C57Bl/6 mice for *R. australis* (Feng *et al.,* 1993; Walker *et al.,* 2001a). However, there remains a gap in knowledge regarding the role of the tick saliva in the natural route of rickettsial transmission to the host. Furthermore, the models developed thus far have employed the intravenous route of infection, whereas intradermal (ID) inoculation of the skin, which would more closely

resemble the natural route of infection, has yet to be described. We performed preliminary experiments to evaluate the ID route of infection in C3H/HeJ mice, and observed consistent infection in the lungs. Thus, in the present study we focused on this organ only. In order to address the limitations mentioned, in the present study we used non-infested and *Rh. sanguineus* nymph-infested *R. conorii*-ID inoculated C3H/HeJ mice, a strain shown to be a more susceptible model for the study of *R. conorii* pathogenesis (Eisemann *et al.,* 1984), to analyze the role of tick saliva in rickettsial transmission.

Materials and Methods

Mice and ticks

All experiments were performed using 4-6 week old C3H/HeJ mice (Jackson Laboratories) housed in an Animal Biosafety Level 3 facility at the University of Texas Medical Branch (UTMB).

Rhipicephalus sanguineus nymphs were obtained from the Oklahoma State University Tick Rearing Facility (Stillwater, Oklahoma, USA). The ticks were placed in small glass vials sealed with a mesh and plastic lid pierced with holes in order to allow for air exchange, and kept in desiccators at room temperature and approximately 90% humidity.

All experiments and procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at UTMB. The animal care and use program at UTMB conducts reviews involving animals in accordance with the *Guide for the Care and Use of Laboratory Animals* (2011) published by the National Research Council. The Animal Resource Center provides a comprehensive program of veterinary care, animal husbandry, technical advice, assistance and facilities.

Tick infestation

A capsule made from a cut-off 2 mL microtube (Sarstedt, Germany) was attached to the shaved back of each sedated mouse (2-4% isoflurane inhalation) using Kamar adhesive (Kamar Products, Zionville, IN), which is routinely used in livestock for adhesion of heat detector pads. On the following day, 10 *Rh. sanguineus* nymphs were counted and introduced into the capsule on each mouse, and allowed to feed to repletion.

Rickettsial preparation

The *R. conorii* Israeli spotted fever (ISF) strain was obtained from the American Type Culture Collection (ATCC). The rickettsial stock was passaged and maintained in specific pathogen-free embryonated chicken egg yolk sacs, and was passaged three times in Vero cell culture before animal inoculation. Stocks were prepared from homogenization of infected cells in sucrose-phosphate-glutamate buffer (218 mM sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4, 4.9 mM monosodium glutamic acid, pH 7.0) and stored at -80°C. The concentration of *Rickettsia* in the inoculum was determined by real-time quantitative polymerase chain reaction (qPCR), and diluted in phosphate buffered saline (PBS), pH 7.2 prior to animal inoculation. The mouse inoculum dose, 1 x 10^7 copies *R. conorii* ISF /animal, was based on results from our preliminary infection studies (unpub. data) in C3H/HeJ mice, where 1 x 10^7 copies *R. conorii* ISF /animal were intradermally inoculated and resulted in consistent infection in the lungs of all animals.

Experimental design

C3H/HeJ mice were divided into two groups, containing five experimental animals and three controls each: in one group of animals, 8 animals were infested with 10 *Rh.*

sanguineus nymphs each, and when all or the majority of ticks had attached, five mice were each inoculated ID with $1x10^7$ copies of *R. conorii* ISF strain (RC/tick infestation group) and the controls with PBS; in the other group, five mice were inoculated ID with 1×10^7 copies of *R. conorii* ISF strain (RC group), and the other three (controls) with PBS. The rickettsial inoculation site was in the region of the capsule containing the ticks. The animals were monitored daily for signs of disease and sacrificed at five days post inoculation, previously shown as a rickettsial peak (Walker *et al.,* 1994), and also coinciding with engorgement of the majority of the nymphs. Lung samples were collected and processed for analysis of rickettsial loads, cytokine and transcriptional factor levels and histology.

Quantification of bacterial loads by quantitative real-time PCR

For rickettsial quantification in the mouse lung samples and *R. conorii* inoculum, DNA was extracted using the Qiagen DNeasy Blood and Tissue Extraction Kit (Valencia, CA). Samples were then analyzed by quantitative real-time PCR targeting *Rickettsia* spp. citrate synthase gene, *gltA*, using primers C5 (forward) and C6 (reverse), as previously described (Labruna *et al.,* 2004). Serial dilutions of plasmids containing a single copy of a fragment of *R. conorii* Malish strain *gltA* gene and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were used for quantification and normalization, respectively. Real-time PCR assays were performed in triplicate using an iCycler IQ from Bio-Rad (Hercules, CA), and controls were included in the absence of DNA template (NTC). The results are expressed as rickettsial *gltA* copies per 10^6 GAPDH.

Cytokine analysis by real time RT-PCR

Mouse lungs, for both experimental groups and controls, were collected in RNA Later, and RNA extraction was performed using Qiagen RNeasy Mini kit (Valencia, CA). cDNA was synthesized using Bio-Rad iScript cDNA synthesis kit (Hercules, CA), and the RT-PCR reactions were performed in a 25 uL final reaction mix, using specific primers to amplify IL-10, IL-1β and NF-κB genes. β–actin gene was used as the housekeeping gene as a control for variations in RNA concentrations between samples. The RT-PCR assays were performed using an iCycler IQ from Bio-Rad (Hercules, CA) with each reaction including a non-template control (NTC). The ΔΔCt method was used for quantification with results expressed as the mRNA relative ratio ($2^{-\Delta\Delta Ct}$) (Livak & Schmittgen, 2001).

Histology

Lung tissue samples from the mice infected with *R. conorii*, and infested with ticks or only inoculated with rickettsiae, were fixed in 10% neutral buffered formaldehyde for a minimum period of 24h, and embedded in paraffin. Tissues were then sectioned at 5 μM and stained with hematoxylin and eosin for histological analysis.

Statistical analysis

GraphPad Prism 6 one-way analysis of variance (ANOVA) and Student's t-test were used to examine statistically significant differences between rickettsial loads, and cytokine and transcription factor levels between mice infected with *R conorii* and infested with ticks or only inoculated with rickettsiae. P-values < 0.05 were considered significantly different for each analysis.

Results

All mice were sacrificed on day five post inoculation, at which time the majority of nymphal *Rh. sanguineus* had engorged and detached. At necropsy splenomegaly was observed in *R. conorii*-infected mice, both with and without tick infestation. The controls did not have enlarged spleens. We observed no statistical difference between the lung bacterial loads of both groups of animals (Fig. 4.1). The controls were negative for *R. conorii* infection.

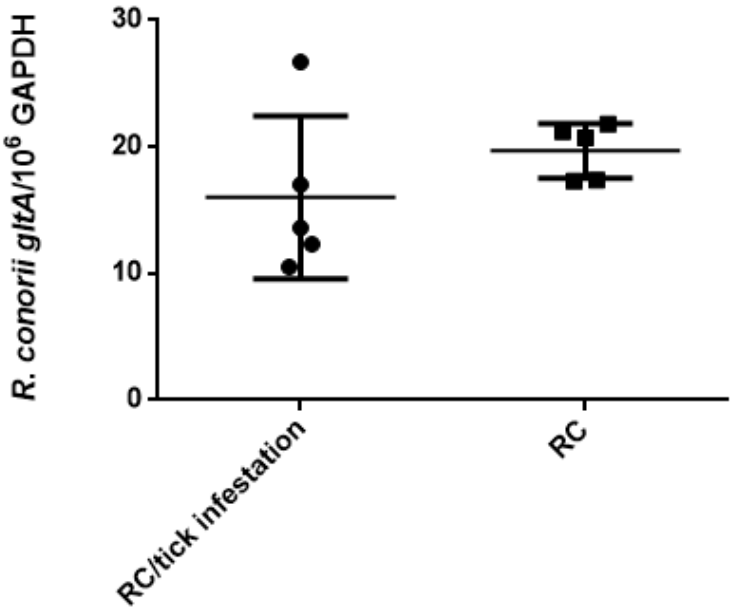

Fig.4.1. Real time qPCR of rickettsial citrate synthase (*gltA*) gene. RC/tick infestation- Group of C3H/HeJ mice infested with ticks and inoculated intradermally with *R. conorii* ISF; RC- Group of C3H/HeJ mice inoculated intradermally with *R. conorii* ISF, no tick infestation.

Analysis of cytokine and transcription factor levels in the lungs revealed that *R. conorii* inoculation alone induced higher levels of IL-1β and NK-κB than in *R. conorii*-infected mice with tick infestation, with a statistically significant difference ($p < 0.05$) (Fig. 4.2).

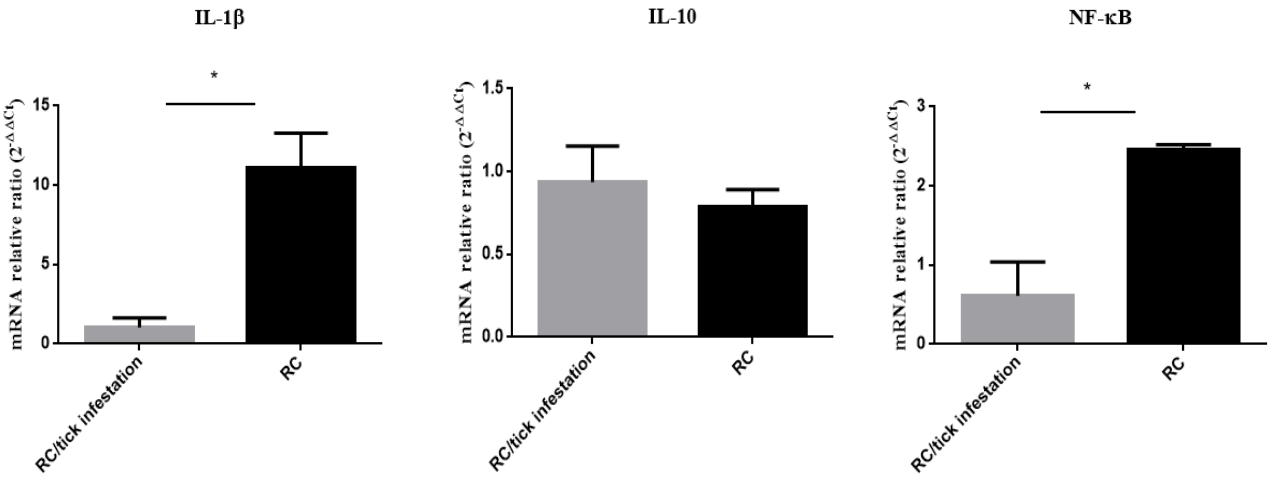

Fig.4.2. Reverse transcriptase qPCR of mRNA relative ratios ($2^{-\Delta\Delta Ct}$) of IL-1β, IL-10 and NF-κB.
* $p < 0.05$.

On the other hand, the tick infested group had higher IL-10 levels than the infected mice without tick infestation. There was, however, no statistically significant difference in the latter case ($p > 0.05$). Histologic sections of the lungs of both groups of *R. conorii*-infected animals showed foci of vasculitis with subendothelial infiltration of inflammatory cells (Fig. 4.3).

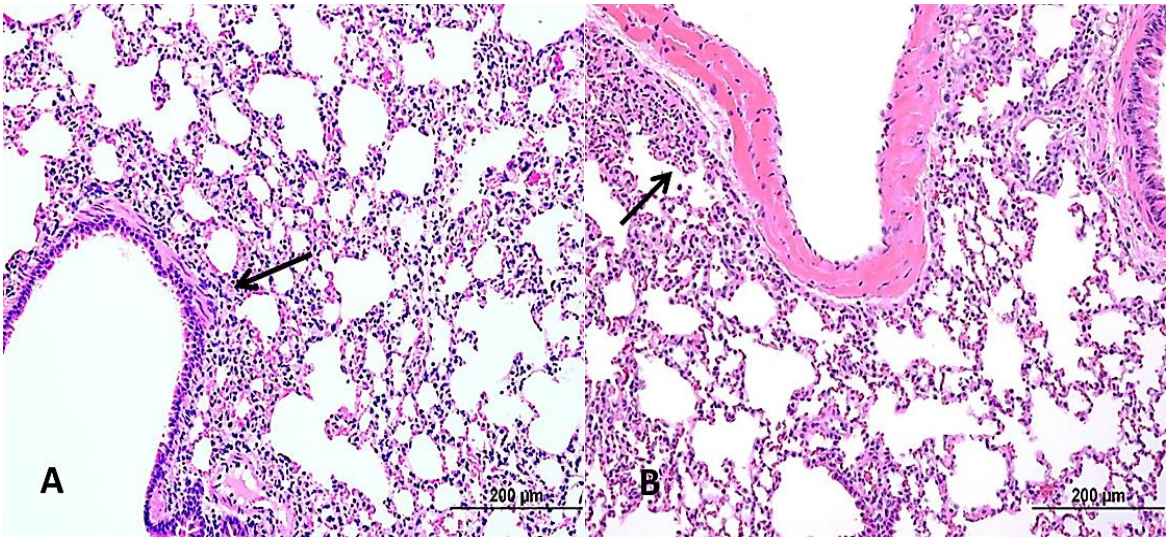

Fig.4.3. Photomicrographs of lung of mice inoculated intradermally with *R. conorii* adjacent to feeding *Rh. sanguineus* ticks (A) and inoculated only with *R. conorii* (no ticks feeding) (B). Mild multifocal perivascular and alveolar septal thickening by interstitial infiltration by mononuclear cells can be observed in both images. 20x. Hematoxylin-eosin stain.

Discussion

The main objective of this study was to analyze the effects of *Rh. sanguineus* saliva on the dissemination of *R. conorii* in the host, using an intradermal route of rickettsial inoculation in order to mimic the natural tick bite route of inoculation. With this purpose, we used a previously demonstrated susceptible mouse strain, C3H/HeJ, for this *Rickettsia* species (Eisemann *et al.,* 1984) in order to favor establishment of infection. This strain of mice, possessing a missense mutation in the TLR4 gene which leads to a single amino acid change in the cytoplasmic portion of TLR4 (Hoshino *et al.,* 1999), is highly susceptible to *R. conorii*, succumbing to an overwhelming infection at doses which are non-lethal to TLR4-competent C3H/HeN mice (Jordan *et al.,* 2009). As a result, in our study, all lung samples from both experimental groups tested were found to contain rickettsiae; however, no statistically significant differences in bacterial loads were found between them. The pathologic response was confirmed by histological analysis of the lungs, where mild subendothelial cellular infiltration was observed in the alveolar septa.

It has been shown that the burden of some inoculated pathogens increases in the host as a result of tick saliva, a phenomenon described as saliva-activated transmission (Nuttall & Labuda, 2004; Wikel, 1999; Titus *et al.,* 2006). A recent study described experiments using *R. parkeri* and infestations with *Amblyomma maculatum* ticks to ascertain the role of tick feeding in rickettsial survival and proliferation at the tick feeding site, and showed a marked increase in the rickettsial load in the tick-infested group versus the inoculated-only group (Grasperge *et al.,* 2012). In that study the mice were inoculated on the nape of the neck, and 20 minutes later infested with nymphal ticks at the inoculation site. Our aim was to study the systemic proliferation of *R. conorii*. Therefore, in preliminary experiments (unpub. data) we analyzed spleen, lung, liver and lymph nodes for bacterial burdens and detected consistent infection in the lungs. In the present study we thus

decided to focus on this organ. Our rationale in allowing the ticks to attach first, and some salivation to occur into the feeding site and only then to inoculate the animals with *Rickettsia*, was to mimic what might happen during tick feeding. Once a tick attaches, saliva is injected into the wound, immediately initiating anti-inflammatory responses which may then allow for the enhanced transmission of the pathogen (Wikel, 2013). Based on the established C3H/HeN model for *R. conorii*, where the peak of rickettsial infections was verified on days three and five (Walker *et al.,* 1994), we determined that day five would be appropriate for animal studies after an ID inoculation that would likely result in a delay to dissemination than with intravenous inoculation. Also, by day five most of the nymphal ticks had completed their bloodmeal, allowing the saliva to exert its effects throughout the whole feeding period. The fact that there were no significant differences in rickettsial burdens between the *R. conorii* inoculated-only group and the tick-infested *R. conorii* inoculated group in our study suggests several possibilities, among them that day five is either too soon for the saliva to exert its effects on the dissemination of the rickettsiae, or too late, and an equilibrium between the anti-inflammatory components of the saliva and the immune responses of the host could have already been reached. A further experiment to elucidate this point would be to study more timepoints, and ascertain whether significant differences would be observed between the experimental groups.

Interestingly, even though there was no indication of a tick saliva effect on rickettsial burdens in the lung, the analysis of mRNA levels of cytokines and the transcription factor NF-κB showed statistically significant differences for IL-1β and NF-κB levels between infected tick-infested mice and infected-only animals. Both IL-1β and NF-κB were higher in the rickettsiae-inoculated only mice. It has been shown that *I. scapularis* ticks suppress host macrophage and Th1 lymphocyte cytokine production (Wikel & Bergman, 1997; Urioste *et al.,* 1994) and polarize the immune responses towards a Th2 cytokine profile

(Ferreira & Silva, 1999; Schoeler *et al.,* 1999), thus increasing the likelihood of successful feeding and pathogen transmission (Wikel & Bergman, 1997). Proinflammatory cytokine production, including IL-1β, depends upon the NF-κB pathway (Li & Verma, 2002). Our results, showing a decrease in both IL-1β and NF-κB in the tick-infested infected mice, clearly indicate a role of tick feeding on inhibition of proinflammatory host responses initiated by a tick-borne pathogen. Another study also showed that extracts from the salivary gland of *Dermacentor andersoni* ticks reduced the IL-1 levels during the early phases of tick feeding (Ramachandra & Wikel, 1992). In addition, we observed higher mRNA levels of IL-10 in the tick infested mice; however, without a statistically significant difference. This situation has also been observed in previous studies, where a concomitant increase in the Th2 cytokine IL-10 in the spleen of C3H/HeJ mice was observed, under different infectious statuses (Zeidner *et al.,* 1997; Ferreira & Silva, 1999; Boppana *et al.,* 2009).

In conclusion, our study demonstrated that nymphal *Rh. sanguineus* tick saliva suppresses the proinflammatory response in mice intradermally inoculated with *R. conorii* ISF.

It would be interesting to extend these studies in other mouse strains with differing levels of susceptibility to *R. conorii*: susceptible C3H/HeN, less susceptible Balb/c, and highly resistant C57BL/6 mice, including additional timepoints and analyzing other anti-inflammatory and pro-inflammatory cytokines.

CHAPTER 5

GENERAL DISCUSSION

The incidence of tick-borne diseases is steadily increasing around the world, highlighting the importance of ticks as the main arthropod vectors of pathogens of both animals and humans (Nicholson *et al.* 2010, Dantas-Torres *et al.* 2012). The recent advances in molecular biology have facilitated the global detection of an ever growing number of tick-borne pathogenic microorganisms, including several species of *Rickettsia* (Paddock 2009, Duh *et al.* 2010, Pacheco *et al.* 2011).

Until around two decades ago MSF, caused by *R. conorii*, was the only diagnosed spotted fever group rickettsiosis in Europe (Raoult *et al.* 1986, Punda-Polic *et al.* 2003, Colomba *et al.* 2006, Dzelalija *et al.* 2007, Rovery *et al.* 2008). However, it is recognized that some previously claimed MSF cases may have in fact been caused by other rickettsial species, which have since then been proven to be pathogenic to humans and cause similar clinical manifestations, such as *R. monacensis*, *R. sibirica mongolotimoniae*, *R. massiliae,* and *R. akari* (de Sousa *et al.* 2006, Vitale *et al.* 2006, Jado *et al.* 2007, Parola *et al.* 2009).

In Portugal, the two main rickettsial strains causing MSF are *R. conorii* Malish and ISF (de Sousa *et al.* 2003), and recent reports have described additional *Rickettsia* species causing human disease in our country, namely *R. sibirica mongolotimoniae* and *R. slovaca* (de Sousa *et al.* 2006, de Sousa *et al.* 2013). These species have also been detected in ticks in Portugal, in addition to *R. helvetica*, *R. aeschlimannii*, *R. raoultii*, and *R. monacensis* (de Sousa *et al.* 2006, Santos-Silva *et al.* 2006, Santos-Silva *et al.* 2006b, Vitorino *et al.* 2007, Lopes de Carvalho *et al.* 2008a).

In order to expand the vast knowledge of tick and rickettsial diversity in Portugal, we performed detection studies of *Rickettsia* as well as to other tick-borne agents, in Ixodid and Argasid ticks collected from several biotopes. These studies are described in the first part of the dissertation as an introductory analysis of the intricate relationships in the tick vector-pathogen-host triad, as they occur in their natural setting. Thus, in the first study described in chapter 2, pertaining to the collection and analysis of ticks in the largest safari

park in Portugal, five rickettsial species were detected by an initial PCR screening and further identified by sequencing as *R. helvetica*, *R. slovaca*, *R. monacensis*, *R. massiliae* and *R. raoultii*. Subsequent cultivation of those rickettsiae in cell culture was performed, resulting in the isolation of the first four species mentioned above. All *Rickettsia* isolated from the ticks are known human pathogens, and it would be an interesting project to follow up the studies described in the subsequent chapters of this dissertation using these rickettsial species. *Borrelia lusitaniae* was also detected and isolated from ticks collected in the safari park and, to our knowledge, this was the first report on dual tick infections of *B. lusitaniae* with *R. slovaca* and *R. helvetica.* Human tick-borne co-infections have been described, with LB and babesioses being the most common cases, resulting in prolonged and more intense clinical manifestations than those verified in LB alone (Krause *et al.* 1996, Krause *et al.* 2002). A recent report also described human co-infections of LB and rickettsioses (Xuefei *et al.* 2013). The results of our study serve as an alert to visitors and staff alike regarding awareness of ticks and tickborne pathogens at parks, as they highlight the potential risk of acquiring multiple infections through tick bites.

In the second study described in chapter 2, PCR screening for *Rickettsia*, *Borrelia* and *Anaplasma phagocytophilum* was performed on tissue samples and ticks collected from *Teira dugesii* lizards from Madeira Island, with the purpose of ascertaining their role in the transmission and maintenance of pathogenic agents. Only immature stages were found parasitizing the lizards, with nymphal stages predominantly present throughout all collection periods, and larvae only present from early April to November. Ticks and lizard samples were collected from both areas studied, Calheta and Campanario; however, infection with all agents was only detected in samples from the former region. *Rickettsia* was found to be the most prevalent agent in both ticks and lizards, with detection of *R. monacensis* and *R. helvetica*, the latter never having been found before on the island. Both ticks and lizard tissue samples were found to be infected with *B. lusitaniae.* Interestingly,

polymorphisms were detected in some *B. lusitaniae* sequences, pointing to the existence of genetic variants of *Borrelia*, in some cases strictly associated with the lizards in Madeira Island. *Anaplasma phagocytophilum* was detected in ticks and in only one tail sample, possibly suggesting a transtadial transmission rather than infection via the infected host lizards. The results obtained in this study seem to indicate a disseminated infection of *Rickettsia* and *Borrelia* in the lizard, as the infected ticks and tail samples were taken from opposite longitudinal sites of the lizards, i.e., the ticks were collected from the forelimb axial area. These observations point to the possibility that these vertebrates may act as transient reservoirs for tick infection. To sum up, the results obtained in this study suggest that *T. dugesii* lizards in Madeira Island appear to be involved in the maintenance and transmission cycle of *Rickettsia* spp. and *B. lusitaniae*; however, to define their role as reservoirs, further studies will need to be undertaken.

In the last study described in chapter 2, we focused on Argasid ticks for detection of rickettsial species, namely *O. erraticus* ticks, responsible for transmitting pathogenic agents to both humans and animals (Basto *et al.* 2006, Vial *et al.* 2006, Sarih *et al.* 2009). Ixodid ticks are the most common vectors of rickettsial species; however, reports have detected *Rickettsia* in several Argasid ticks, such as *Carios capensis*, *Argas persicus*, and *O. moubata* (Cutler *et al.* 2006, Reeves *et al.* 2006, Pader *et al.* 2012).

In our study, 122 DNA pools from *O. erraticus* soft ticks collected from 11 pig pens in the south of Portugal were screened by PCR, and ticks from positive pools were subsequently cultivated in two cell lines at two different temperatures, to increase the chances of a successful isolation. PCR-positive samples and isolate characterization revealed a new rickettsial species, phylogenetically close to *R. felis* and *R. hoogstraalii*. The latter species has also been described in soft ticks (Mattila *et al.* 2007, Duh *et al.* 2010, Pader *et al.* 2012). Serological evidence confirmed this new rickettsial species, named *Rickettsia lusitaniae* sp. nov., as belonging to the SFG *Rickettsia*. It would be interesting to follow up this study to

address the potential pathogenicity of this new *Rickettsia*, given its close phylogenetic relationship with *R. felis.*

In conclusion, these studies have revealed novel discoveries such as the detection of *R. helvetica and R. monacensis* in a new host involving *Teira dugesii* lizards and, and also a new rickettsial species infecting an argasid tick.

Having explored the association between ticks, host and their pathogens in nature, our next aim was to break down the natural triad interface and focus on the vector-pathogen level, specifically the analysis of interactions between *Rickettsia* and its ixodid vector. Given the public health impact of MSF in Portugal, our initial intent was to study the relationship between *Rhipicephalus sanguineus* ticks and *R. conorii* strains; therefore, the first step was to establish a *R. conorii*-infected *Rh. sanguineus* colony. Both strains of *R. conorii*, Malish and ISF, are etiologic agents in MSF in Portugal; however, bearing in mind that *R. conorii* Malish strain to be more lethal to ticks (Levin *et al.* 2009), the subsequent studies were performed using *R. conorii* ISF strain. Two methods of infection were tested: immersion of *Rh. sanguineus* nymphs in a *R. conorii* ISF suspension and subsequent mouse infestation, in a similar manner to previous studies (Policastro and Schwan 2003, Fiserova *et al.* 2008, Levin *et al.* 2009) resulting in an infection acquisition rate of 9%; and infestation of rickettsemic mice with uninfected *Rh. sanguineus* larvae, which resulted in a 13% acquisition rate. Our results were lower than other rates of infection studies of *Rh. sanguineus* ticks with *R. conorii,* where prevalences of infection between 35-66% were observed (Levin *et al.* 2009). These preliminary experiments led us to conclude that in order to establish and maintain a colony of *Rh. sanguineus* infected with *R. conorii*, an unsurmountable amount of animals and ticks would be needed for the tick infestation, in addition to long molting periods, and given the time frame for this project the decision was made to perform the study described in this chapter using a colony of *R. massiliae*-infected

Rh. sanguineus ticks from UTMB. *Rickettsia massiliae* is a human pathogen that causes similar clinical symptomatology as *R. conorii* and other SFG rickettsioses, and is also less pathogenic for ticks, therefore providing a good agent for our proposed studies.

Thus, the study in chapter 3 describes a quantitative insight into the feeding process of *R. massiliae-Rh. sanguineus* female ticks up to day 6, by analyzing the bacterial burden evolution in the tick salivary glands by qPCR, and electron microscopic analysis of morphological changes in unfed and fed salivary glands, unfed midgut and fed ovaries. A gradual increase in rickettsial burden was observed in the first two days of feeding in the salivary glands, followed by a plateau from day three to six. *Rickettsia* are metabolically activated upon vector feeding and start multiplying and proliferating in the tick tissues, which was confirmed with EM by the increase in rickettsial organisms observed between unfed and fed salivary glands. In addition, a significantly wider periplasmic space in rickettsial morphology was observed in the fed status versus the unfed, which has been pointed out as a result of the reactivation phenomenon (Hayes and Burgdorfer 1982). Another study performed using *Rh. sanguineus* ticks infected with *R. conorii* did not observe any changes in the periplasmic space between unfed and fed ticks (Santos *et al.* 2002). In that study artificially infected ticks were used, whereas in our study the ticks had been infected with *R. massiliae* for many generations. This would have allowed the rickettsial organisms to become established in the tick over time and adapt their cellular physiology according to physiological changes in the tick.

In conclusion, with this study we verified that *R. massiliae* is well adapted to its vector, is able to multiply in various tick organs without causing cytological damage to its vector, which could explain how these bacteria are perpetually maintained in nature, with 100% transovarial and transstadial transmission to its progeny.

Preliminary studies were performed on *Rh. sanguineus* tick salivary glands to determine differential protein expression between different stages of the life cycle (nymphs versus adults) and feeding stage (unfed versus fed) using 2D gels, followed by MALDI/TOF/TOF, and infectious status (uninfected versus infected ticks with *R. massiliae*), using SDS-PAGE followed by LC/MS/MS. As a high tick salivary protein amount is needed to perform these studies, in order to obtain replicable results, which would imply a large number of ticks and also of animals for feeding purposes, we decided against pursuing this line of research for this dissertation. However, it would be interesting to pursue this line of work in future experiments, in order to uncover further potential salivary tick proteins and analyze their role in host models.

Many studies have been undertaken to understand the complex mechanisms involved in tick-borne rickettsioses, and the development of animal models that mimic the course of rickettsial pathogenesis observed in humans has greatly enhanced our knowledge of *Rickettsia* and rickettsial diseases. To date, four main animal models have been characterized, namely C3H/HeN mice for the study of *R. conorii* (Walker *et al.* 1994) and *R. typhi* (Walker *et al.* 2000), and BALB/c and C57Bl/6 mice for the study of *R. australis* (Feng *et al.* 1993, Walker *et al.* 2001). A recent study has described C3H/HeJ mice as a more susceptible model for *R. conorii* (Jordan *et al.* 2009), and these mice were also used for the study of *R. parkeri* (Grasperge *et al.* 2012).

Despite the significant advances in the knowledge of the rickettsiae-host interface resulting from the extensive use of these models, many of the studies performed to this date have been *in vitro*, thereby possibly bypassing important *in vivo* considerations, such as the role of tick feeding in these complex interactions. The pharmacological compounds contained in tick saliva have been shown to be crucial in modulating many aspects of the host responses, including hemostatic, immune and inflammatory events, thus facilitating the transmission of pathogens (Kazimirova and Stibraniova 2013, Wikel 2013).

The last aim of this project had the intent of approaching this gap by studying the role of tick saliva in rickettsial infection in a murine host. We undertook preliminary experiments using the more susceptible mouse model, C3H/HeJ mice, to ensure infection with a previously untested inoculation mode, the intradermal route. Intradermal inoculations of *R. conorii* were thus performed on the posterior region on the back (where the tick capsule would be placed in subsequent experiments), and we collected lungs, liver, spleen and lymph nodes for bacterial burden analysis by qPCR. A consistent infection in the lungs was observed in all replicate animals, whereas the other organs were inconsistently infected. As the purpose was to have consistent infection to allow for comparison of potential differences with addition of tick saliva, by way of tick infestation, and thereby infer the effects of tick saliva, the subsequent study described in chapter 4 focused on the lungs.

Thus, C3H/HeJ mice, having been proved to be an appropriate model for the purposes of the study in hand, were divided in two groups, a *R. conorii*-ID inoculated only group, and a group subjected to tick infestation and then ID inoculated with *R. conorii*. Bacterial load analysis of mouse lungs by qPCR did not reveal statistically significant differences between the two groups. This could have been due to either to a premature timepoint, where the effects of tick saliva had not yet had time to exert their immunomodulatory effects, or the reverse situation, where the host immune response had been triggered against either the tick or rickettsial infection. On the other hand, significant results were obtained when analyzing cytokine and transcription factor levels in the lungs. Levels of IL-1β and NF-κB were significantly lower in the tick infested group than the *R. conorii*-inoculated only group, indicating an inhibitory effect of tick saliva on pro-inflammatory cytokines, in accordance with other studies, where a Th2 profile is enhanced with tick infestation with skewing of the host response away from Th1 (Ferreira and Silva 1999, Schoeler *et al.* 1999). Levels of IL-10 were higher in the tick-infested group versus the *R. conorii*-

inoculated only group. Even though this result was not statistically significant, a reduction of IL-10 has also been observed in other tick infestation studies (Zeidner *et al.* 1997, Ferreira and Silva 1999, Boppana *et al.* 2009), and has also been observed in other studies of rickettsial infection, where a decrease of IL-10 is observed (Cillari *et al.* 1996, Forte *et al.* 2009). IL-10 is capable of suppressing the immune response.

To confirm the results in this study, further studies should be performed addressing:

a) Further timepoints for organ collection, in order ascertain whether there is a point where a statistical difference between inoculated-only and tick-infested groups can be observed, as has been the case in other studies (Grasperge *et al.* 2012).

b) Additional cytokines to further analyze host immune responses.

c) Analysis of host skin samples, at earlier timepoints, to ascertain immune events at the start of tick feeding.

d) Inoculation at the site of feeding. It could be that the results observed could have been a result of *R. conorii* inoculation having been as close as possible to, but not in, the infestation site.

A preliminary study was performed using C3H/HeN and C3H/HeJ mice infested with *R. massiliae*-infected *Rh. sanguineus* nymphs to analyze the role of TLR4, absent in C3H/HeJ mice, in the host immune response against infection with this tick-transmitted *Rickettsia.* The results showed a statistically significant difference in bacterial loads between these two groups, where the more susceptible model, C3H/HeJ mice developed higher bacterial loads, confirming a protective role of TLR4 against *R. massiliae* infection, as had been shown for *R. conorii* in previous experiments (Jordan *et al.* 2008). In their experiment, they observed a decrease in protective Th1 responses in the TLR4-deficient mice. However, in that study the mice were infected by intravenous inoculation with *R. conorii*, whereas in our case the mice were infected by infestation with *R. massiliae*-infected ticks.

Ticks are shown to skew host immunity toward a Th2 response in detriment of Th1, whereby it would be interesting to follow up this preliminary experiment adding *R-massiliae*-inoculated only animals, and ascertaining not only the tick influence on Th2/Th1 responses, but also examining the role of TLR4 in the different experimental groups, through cytokine analysis.

The studies described in this dissertation have not only contributed to further insights into the complexity of vector-pathogen-host interactions, but also advanced the knowledge of rickettsial pathogenesis in terms of highlighting the role of tick saliva in this intricate relationship, helping to pave the way for additional studies in this field.

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