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Xiangye Liu

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DOCTEUR DE L'UNIVERSITÉ PARIS-EST

Spécialité : Sciences du vivant

Présentée et soutenue publiquement par

Xiangye LIU

Le 15 Novembre 2013

**Identification of *Ixodes ricinus* female salivary glands factors
involved in *Bartonella henselae* transmission**

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I. INTRODUCTION

Ticks are obligate blood-feeding ecto-parasites of many hosts including mammals, birds and reptiles. Currently, 31 genera of ticks, and around 900 tick species have been identified all over the world [1]. The ticks are harmful for their hosts both directly and indirectly. Directly, they are responsible in skin wounds, blood loss, as well as tick toxicosis [2]. Simultaneously, their bites could also be the sites of secondary microbial infections. Indirectly, ticks are high competent vectors of several pathogens, responsible for high morbidity and mortality both in humans and animals all over the world [3]. They are effectively the most important vectors worldwide after mosquitoes for humans, and the ones that transmit the highest variety of pathogens including viruses, bacteria and parasites [3].

Recently, due to the intensification of human and animal movements and socio-economic and environmental changes, the geographical distribution of several tick species has expanded. The list of potential or known tick-borne pathogens (TBPs) is constantly evolving, and emergence or re-emergence of tick-borne diseases (TBDs) is increasingly becoming a problem [4]. For example, novel vectors invading different locations as well as human and animal reservoir movements may lead to the development of unknown risks, particularly for zoonosis. In this context, it is essential to clearly identify pathogens associated with ticks, as well as to understand the complex interactions between ticks and the pathogens they transmit, in order to develop efficient control strategies.

Because of the limited success and disadvantages (resistance, environmental hazard, increased cost) of controlling ticks via acaricides, new approaches are effectively urgently needed. In light of limited understanding of immunity to TBPs, TBP strain diversity, and more generally the transmission of multiple TBPs by the same tick species, vaccine strategies that target conserved components of ticks that play key roles in vector infestation and vector capacity have become particularly

attractive [5]. The primary rate-limiting step in development of anti-tick vaccines is identification of protective antigenic targets [6]. To identify tick components with a direct effect on pathogen transmission for inclusion in anti-tick vaccines, screening should ideally be focused on genes that are highly-expressed in tick saliva, and more particularly on genes whose expression is induced during salivary gland (SG) infection. Therefore, research on molecular interactions among ticks, hosts, and pathogens as well as the identification of suitable antigenic targets is a major challenge for the implementation of tick and TBDs control strategies. Here, we focus our research on the analysis of the interaction between the tick *Ixodes ricinus* and the bacteria *Bartonella henselae*.

Ixodes ricinus (Acari: Ixodidae) is a three-life stage hard tick that is the most common tick species in Europe. It is frequently associated with bites in humans, and can transmit Tick-Borne Encephalitis virus, *Babesia* spp., *Borrelia burgdorferi* s. l., *Rickettsia* spp., *Anaplasma* spp., and in a lesser extent *Bartonella* spp. [7].

Bartonella spp. are facultative intracellular bacteria associated with a number of emerging diseases in humans and animals [8]. One of that, *B. henselae*, causes cat scratch disease as well as being increasingly associated with a number of other syndromes, particularly ocular infections and endocarditis [9]. To date, no vaccine is available. The main reservoir for *B. henselae* is cats and transmission occurs from cat to cat by cat fleas [10]. However, new potential vectors, in particular ticks of *Ixodes* species, have been recently implicated. The potential for involvement of ticks in transmission of *Bartonella* spp. has been heartily debated for many years because of the numerous but indirect proofs of its existence (see reviews by [11-13]). However, our laboratory has recently demonstrated that *I. ricinus* is a competent vector both for *B. henselae* *in vitro* and for *B. birtlesii* *in vivo* [14,15]. By coupling these results with those of the epidemiological studies on the subject, we can now assert that *I. ricinus* can transmit some *Bartonella* spp. in the field, although the importance of such a transmission still need to be evaluated.

Therefore, in the present work, our aim is to study the molecular interactions that may occur between *B. henselae* and *I. ricinus* in order to identify *I. ricinus* salivary gland factors implicated in the process of bacteria transmission, and that may provide new targets to impair this transmission. The choice of such a model was motivated by several reasons. First, *B. henselae* corresponds to the most common human pathogen transmitted by pets in industrialized countries, as mentioned no vaccine exists, and more and more human cases are reported after a tick bite. Secondly, this bacterium is studied since several years in our laboratory and represents a good model of TBPs that can be easily manipulated in laboratory. At last, but not least, even if we know now that *B. henselae* transmission by ticks may occur in the field, we also know that it is probably not the main way of transmission. Indeed, this model may not represent a couple with strong co-evolutionary relationships between the bacteria and the vector. This may help to identify very general mechanisms associated with pathogen exploitation of tick vector and may lead to the identification of blocking mechanisms that could be apply to a broad range of TBPs. However, it should be of course necessary to verify in the future if molecules identified here are also implicated in coevolved systems as those representing by *I. ricinus* and *B. burgdorferi* as example.

After a general introduction on *I. ricinus* and *Bartonella* spp., the background concerning various methods used to feed ticks and infect them with their associated pathogens, as well as hard tick factors reported as implicated in TBP transmission, are presented. Then, the results obtained during my PhD are presented in two parts. The first one corresponds to a comparison of feeding methods (animal and artificial membrane feeding system), blood origin (sheep and chicken blood), and blood status (*Bartonella* spp. infected and uninfected) on *I. ricinus* engorgement. The second part reports the identification of *I. ricinus* salivary glands differentially expressed transcripts in response to *B. henselae* infection and the role of one of them in tick feeding and salivary gland infection by the bacteria.

II. BACKGROUND

II.1. *Ixodes ricinus*

II. 1.1. Taxonomy and morphology

Ticks are arthropods that belong to arachnids and the subclass of Acarida. They are composed of four families, *Ixodidae* and *Amblyommidae* (the hard ticks), *Argasidae* (the soft ticks) and the *Nuttalielidae* (*Nuttalielidae namaqua*), according to the classification established by Camicas (Figure 1) [1]. The hard ticks (more than 700 species) are distinguished from the soft ones (around 200 species) by the presence of a scutum or hard shield. The family *Nuttalielidae* contains only a single species, a tick found in southern Africa with a morphology that is between hard and soft ticks. The *Argasidae* can be found all over the world, feed rapidly compared to hard ticks, primarily on birds, and are rarely found to parasitize land animals or humans. It is on the other hand the case for the hard ticks to whom belongs *I. ricinus*.

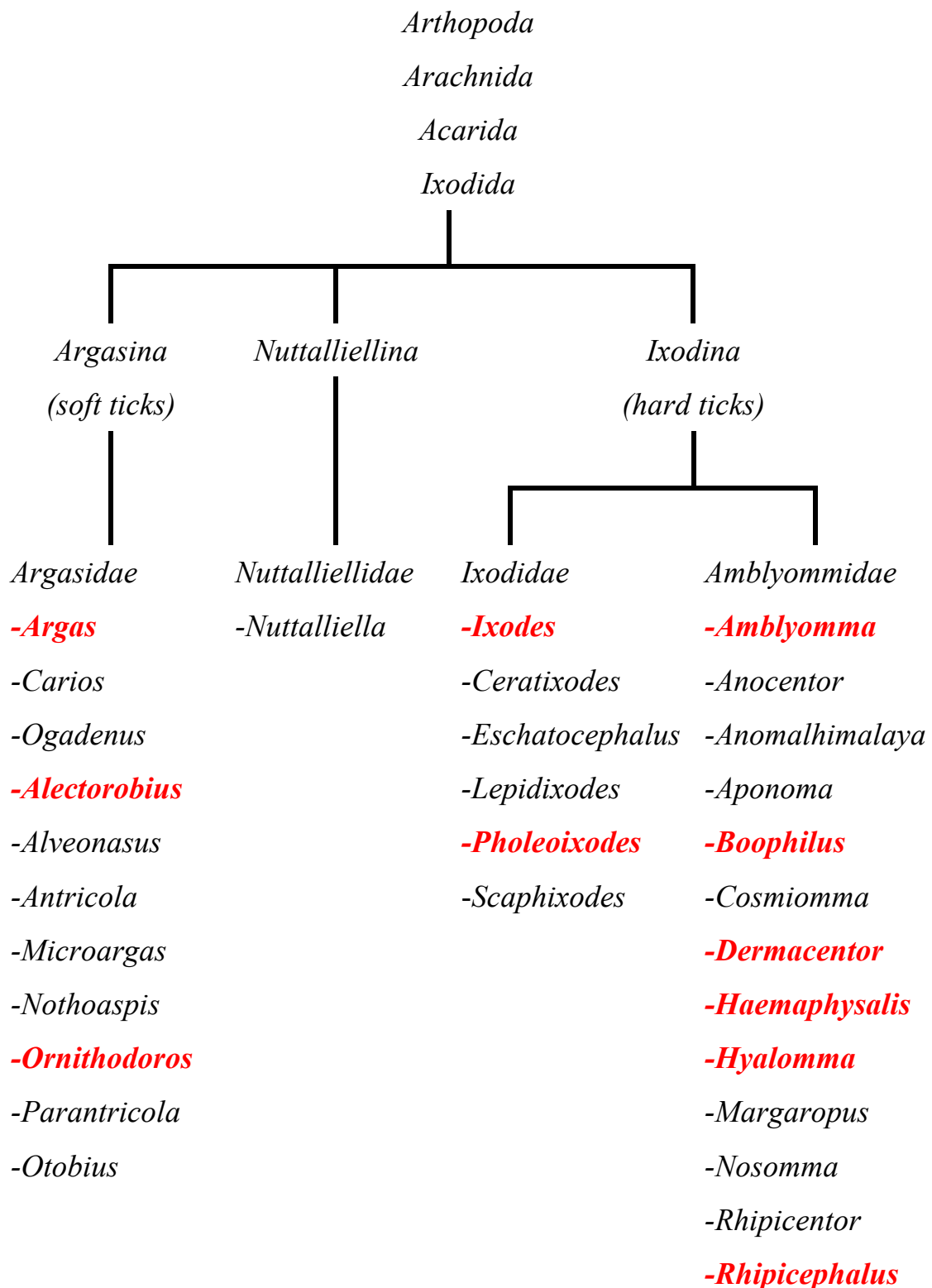


Figure 1. Classification of the ticks (from Camicas J, *et al.* 1998). Tick genera mentioned in red correspond to those with species implicated in pathogen transmission.

The general morphological description of the three main tick families was schematized in Figure 2. As all *ixodidae*, *I. ricinus* has a sclerotized scutum without eyes, and is characterized by the apical position of its mouthparts on their hypostome and the arch shape of its anal fissure [16].

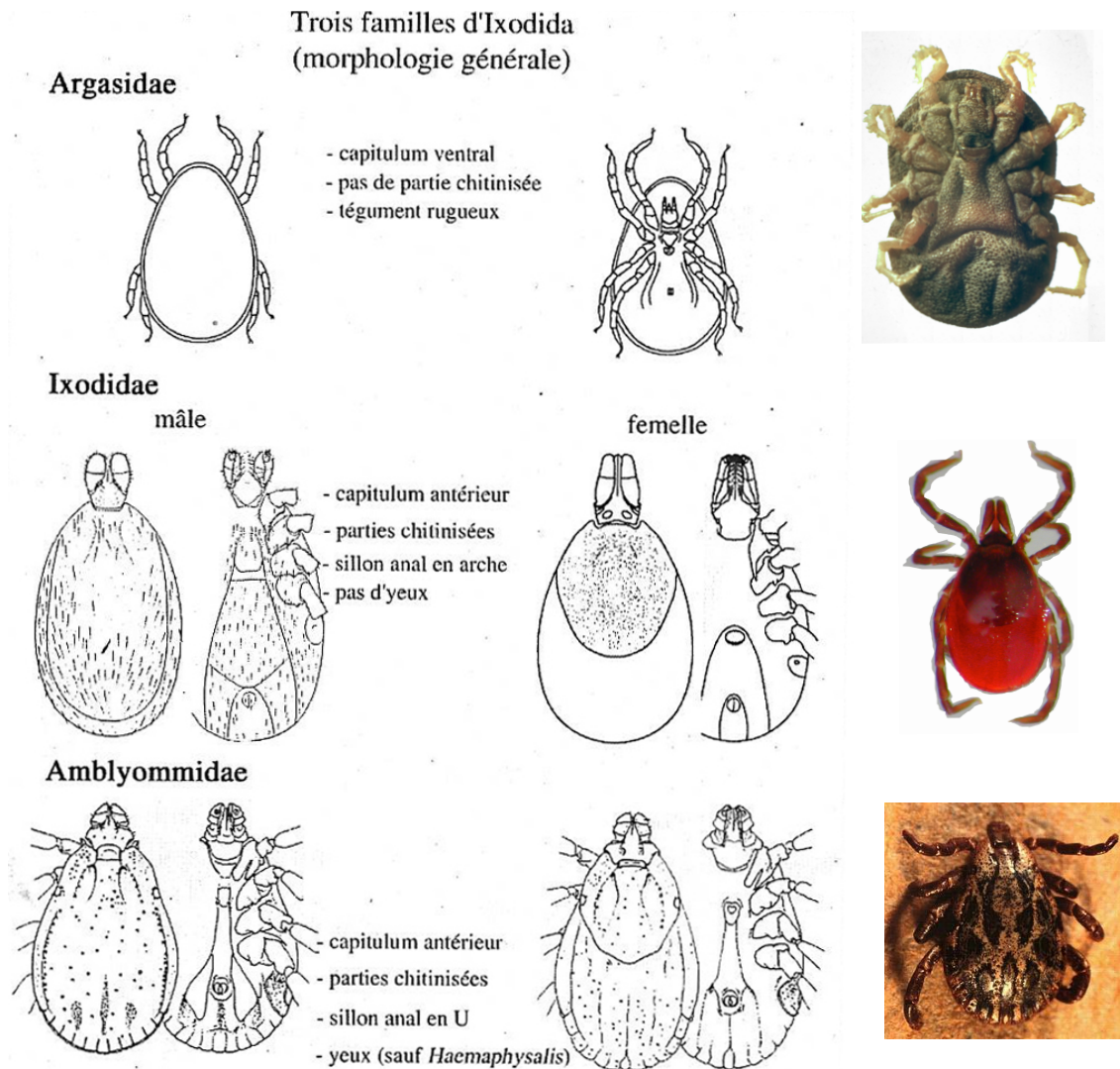


Figure 2. Schematic representation of general morphological description of the three main tick families (from Pérez-Eid C, 2007).

II. 1.2. Geographical distribution

I. ricinus, often called castor bean tick or sheep tick, is the most common tick species in Europe. It is widely distributed in Northwestern Europe, from Ireland to Central Asia (Iran) and from Scandinavia to North Africa. It is present in relatively dry Mediterranean habitats in Northern Africa and in the Iberian Peninsula, in damp sheep pastures of Ireland, Scotland, Wales and England, and in relatively humid, mixed coniferous/deciduous woodland biotopes throughout most of Europe including Scandinavia and western Russia (Figure 3) [17].

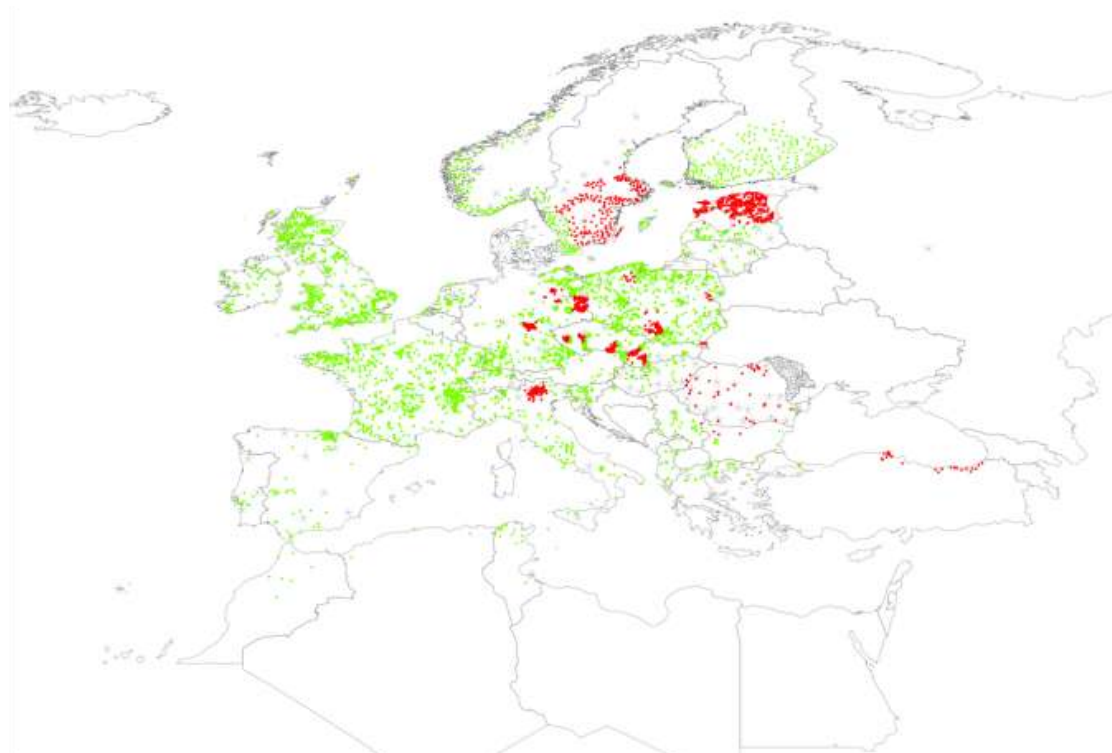


Figure 3. Geographical distribution of *I. ricinus* ticks (from EFSA Journal 2010).

- * Smallest administrative region or territorial unit for statistics, data from last 10 years,
- Coordinate (latitude/longitude), data from last 10 years,
- Coordinate (latitude/longitude), historical data (before 2000).

II. 1.3. Biological cycle

I. ricinus has a three-host life cycle: larva, nymph and adult, with a size vary from 2mm to 30mm (Figure 4). As arachnids, all stages posses 8 legs with the exception of larvae that harbors 6 legs.



Figure 4. Three life cycle stages of *I. ricinus*. From left to right: larva, nymph, female adult, male adult (by Stanek G, *et al.* 2012).

In the field, the life cycle takes approximately 1.5 to 2 years to complete and the length of this cycle vary according to the environmental conditions and the availability of hosts. To complete its cycle, *I. ricinus* requires three hosts. Blood feeding occurs once in each stage except male adult, and takes two to ten days, depending on the life stage (Figure 5) [18]. Humans can be parasitized by all tick life stage.

The larvae emerge from eggs laid by engorged female adults in 5-6 months. They usually feed on small animals such as rodent and bird. After feeding during 2-3 days, they detach from their host and 3-4 months later, molt into nymphs on the ground (Figure 5).

The nymph usually feeds in the following year of molting, and on larger animals such as bird or squirrel. After 5 or 6 days feeding, they detach from their host and 3-4 months later, molt into adult, either males or females (Figure 5).

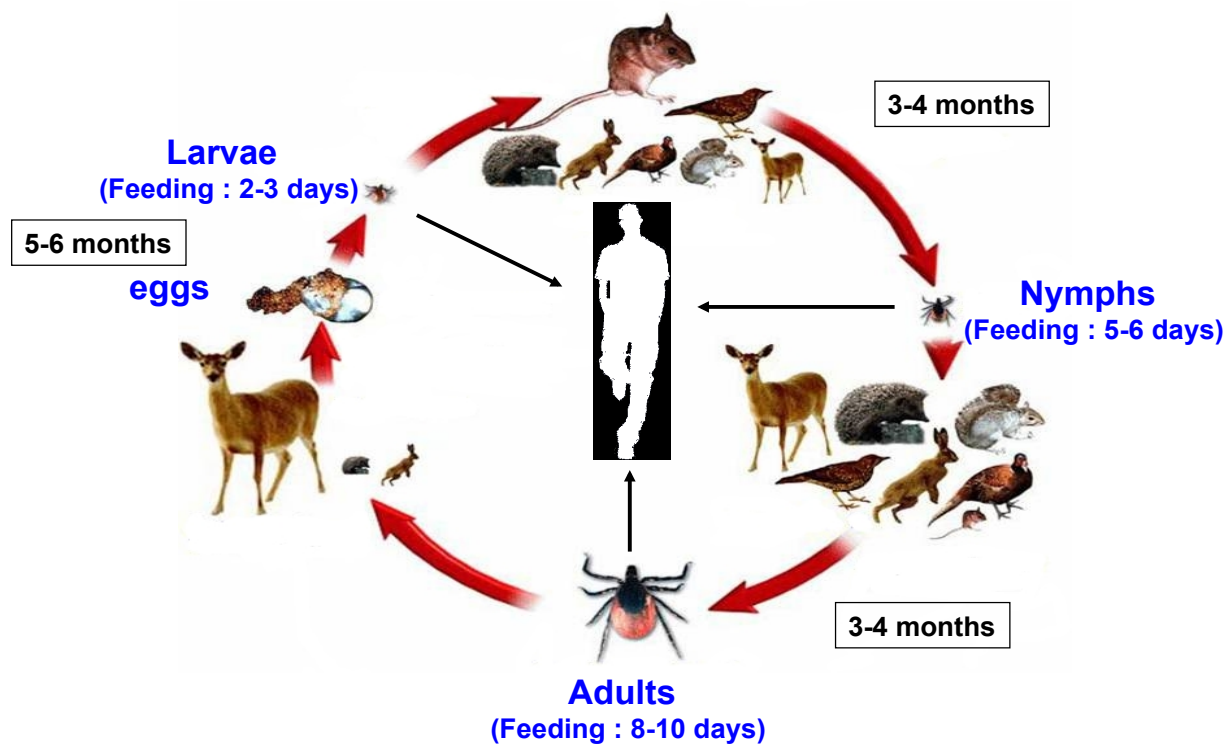


Figure 5. Biological cycle of *I. ricinus*: the size of animals is a function of preferences of each stage (after Gray J. and Kaye B., 2011).

Female adults attach to larger hosts such as deer or livestock. Male adults don't take blood meal or a sporadic one; but they can stay on the host for a long period waiting for female adults. Mating can occur on the ground or on the host and is necessary for the female to achieve her blood meal. Females take a large volume of blood during 8-10 days and grow to the size of a small bean, their weight increasing 100 times or more (Figure 6). After two or more weeks, up to 3,000 eggs are laid on the ground by an adult female tick [19].



Figure 6. View of engorged *I. ricinus* female.

II.1.4. Pathogens transmitted by *I. ricinus*

The incidence of TBDs has increased in the recent years, and many important TBDs, transmitted by *I. ricinus* such as anaplasmosis, babesiosis, and Lyme borreliosis are gaining more and more attention [4]. Moreover, with the development of molecular biology, it is now possible to identify many agents, which can be transmitted by ticks to humans and animals. In Europe, *I. ricinus* is the most important reservoir of medical and veterinarian TBPs including bacteria, parasites, and viruses. A listing of pathogens recognized as transmitted by *I. ricinus* and associated vertebrate hosts, is presented in table 1. Some new pathogens will be undoubtedly reported and characterized in the future, and this list of pathogens transmitted by *I. ricinus* will be prolonged.

Pathogens	Diseases	Principal reservoirs
<i>Babesia</i> genus		
<i>B. divergens</i>	Cattle babesiosis*	Cattle
<i>Babesia</i> sp. EU1	Human babesiosis*	Roe deer
<i>B. microti</i>	Human babesiosis*	Rodents
<i>Borrelia</i> genus		
<i>B. afzelii</i>	Lyme disease*	Rodents
<i>B. bavariensis</i>	Lyme disease*	Rodents
<i>B. bissettii</i>	Unknown	Rodents
<i>B. burgdorferi</i> sensu stricto	Lyme disease*	Rodents, birds
<i>B. finlandensis</i>	Non-pathogenic	Mountain hares
<i>B. garinii</i>	Lyme disease*	Birds, rodents
<i>B. lusitaniae</i>	Unknown	Lizards, rodents
<i>B. spielmanii</i>	Lyme disease*	Dormice, rodents
<i>B. valaisiana</i>	Unknown	Birds, lizards
<i>Bartonella</i> genus		
<i>B. henselae</i>	Cat scratch disease*	Cats
<i>B. birtlesii</i>	Unknown	Rodents
Flaviviridae, Flavivirus		
Tick-borne encephalitis virus	Tick-borne encephalitis *	Rodents
Louping ill virus	Louping ill	Sheep
<i>Rickettsia</i> genus		
<i>R. helvetica</i>	Non-eruptive fever*	Deer
<i>R. monacensis</i>	Mediterranean spotted fever like*	Unknown
Others		
<i>A. phagocytophilum</i>	Human granulocytic anaplasmosis*	Sheep, dogs, cattle
Crimean-Congo Hemorrhagic Fever virus	Crimean-Congo Hemorrhagic Fever*	European hares
Eyach virus	Encephalitis*	Unknown
<i>Francisella Tularensis</i>	Tularemia*	Rabbits, hares, muskrats
<i>Neohrllichia mikurensis</i>	Unknown	Rodents

Table 1. List of microorganisms known to be transmitted by *I. ricinus*, their principal vertebrate reservoirs, and diseases they are responsible for ([14,15,20-28]).

* proved zoonotic diseases.

II.2. *Bartonella* species and tick borne transmission

Bartonella spp. are small, curved, pleomorphic, hemotropic Gram-negative bacteria that are responsible for several diseases in humans and animals [29,30]. Currently, over 20 *Bartonella* species or subspecies have been associated with a large spectrum of clinical syndromes in humans, including Carrion's disease, trench fever, cat scratch disease [9,30]. Few blood-feeding arthropods have been confirmed to be competent vectors for transmission of *Bartonella* spp.: the louse *Pediculus humanus humanus* transmits *Bartonella quintana* [31], the cat flea *Ctenocephalides felis* is responsible for the transmission of *B. henselae* [10], the sand fly *Lutzomyia verrucarum* is the vector of *Bartonella bacilliformis* [32], and the flea *Ctenophthalmus nobilis* is implicated in the transmission of *Bartonella grahamii* and *Bartonella taylorii* to bank voles [33]. However, an increasing number of *Bartonella* spp. have been isolated or detected within the last decade years from a wide range of hematophagous arthropods, including human fleas *Pulex irritans*, various hard tick species, such as *Ixodes* spp., *Dermacentor* spp., *Haemaphysalis* spp., or several species of biting flies [34]. *Bartonella* spp. detection in arthropod vectors was mainly performed by PCR amplification and sequencing of *Bartonella* specific genes as *gltA*, *ftsZ* or 16SrRNA [13]. However, the detection of DNA in these arthropods does not imply that they are vectors of the corresponding pathogens and the role of these ectoparasites in transmission of *Bartonella* spp. among vertebrate hosts needed to be confirmed.

Bartonella spp. transmission by ticks has been heartily debated for many years (see reviews by [11-13]). However, some indirect evidence, which are molecular and serological epidemiological surveys in humans and animals, support *Bartonella* spp. transmission by ticks. *Bartonella* spp. have been associated with several tick species around the world (Figure 7) and numerous data have been published to date regarding identification of *Bartonella* DNA in both engorged ticks collected from their natural

hosts and questing ticks collected from the environment (see reviews [11,13]). As various *Bartonella* spp. are common in wild and domestic animals, acquisition of these erythrocyte associated microorganisms by feeding ticks with a blood meal can be expected, and thus detection of bacterial DNA in engorged or partially engorged ticks does not add to the debate. However, positive PCR results in questing ticks do indicate that the bacterium (or at least its DNA) can survive in the tick through the molt from one life stage to another. In addition, a number of studies have reported co-infections in both humans and animals with *Bartonella* spp. and known TBP's such as *Borrelia* spp., *Anaplasma* spp. or *Babesia* spp., suggesting that these might be co-transmitted by the same vectors [35-45]. *Bartonella* spp. have also been detected by either PCR, serology, or culture in humans and animals after tick bites without any known contact with other arthropods [41,46-48]. Recently, Angelakis *et al.* reported detection of *B. henselae* infection in three patients, who developed scalp eschar and neck lymphadenopathy following tick bites [49]. A *Dermacentor* sp. tick removed from one of these patients contained DNA of *B. henselae*, although it is unclear whether the person acquired an infection from the tick, or the tick from the person.

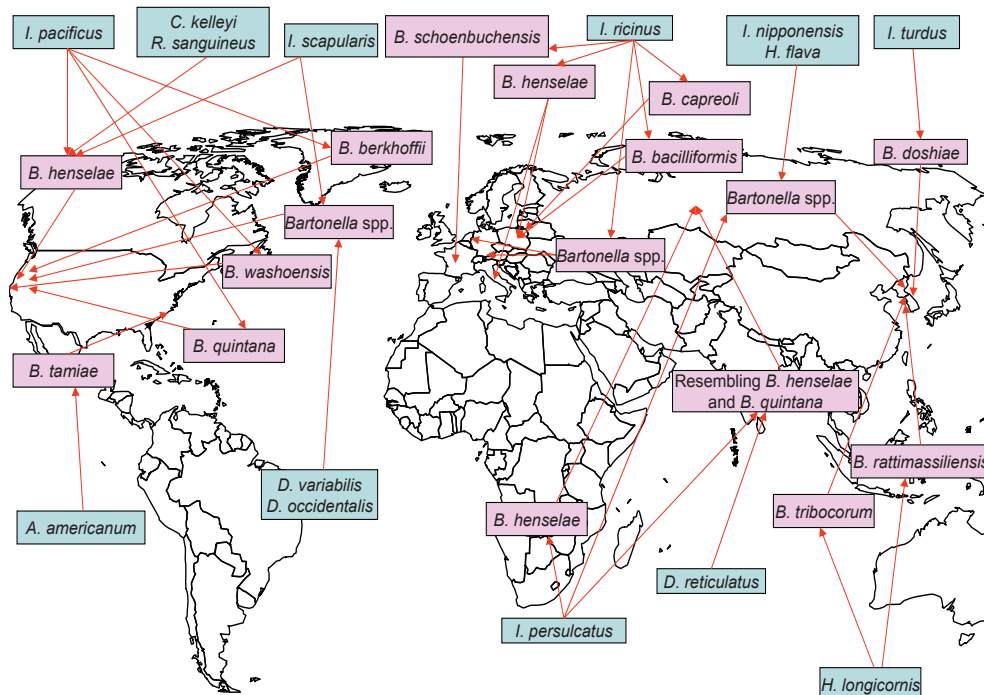


Figure 7. Worldwide locations of ticks (blue boxes) identification with *Bartonella* spp. (pink boxes) (by Angelakis E, *et al.* 2010).

The direct evidence of transmission of *Bartonella* spp. by ticks to a susceptible animal was firstly reported in 1926 by Noguchi [50]. In this study, adult *Dermacentor andersoni* ticks fed for several days on *B. bacilliformis*-infected monkeys, were removed and then allowed to reattach on naïve animals. Although the naïve animals became infected, it may correspond to mechanical transfer of the pathogen by blood-contaminated mouthparts of the tick. It didn't assess either the tick's vector competence or bacterial transstadial transmission throughout the tick life's cycle.

In 2008, our laboratory demonstrated, via artificial membrane-feeding system, that ticks are competent vectors for *B. henselae* [14]. This study reported that immature *I. ricinus* ticks can acquire *B. henselae* via artificially infected blood feeding, maintain the bacteria through molting, and secret it into blood during another new artificial feeding. Moreover, the bacteria infected tick salivary glands were inoculated in cats, which developed a typical *B. henselae* infection. This study represented the first experimental data on *Bartonella* spp. transmission by ticks but results obtained needed *in vivo* confirmation with an animal model.

With this aim in view, and because of biosafety concerns associated with tick feeding upon cats infected with *B. henselae*, a murine model of bartonellosis: *Bartonella birtlesii* infecting mice, was used [15]. In this trial, *I. ricinus* larvae and nymph were fed on a *B. birtlesii*-infected mouse. The nymph, which had molt from infected larvae, can successfully transmit the bacteria to naïve mice during a new blood meal. Additionally, the female adults, which had molted from the infected nymphs, can successfully emit *B. birtlesii* into uninfected blood via artificial membrane feeding, and the bacteria has been successfully recovered into tick salivary glands and muscle tissues. This work represented the first *in vivo* demonstration of a *Bartonella* species transmission by a tick. It did not claim that ticks are principal vectors of *Bartonella* spp., but it does corroborate a prospect that ticks play a role in the natural cycles of some of the *Bartonellae* including those pathogenic for humans.

This statement was effectively confirmed by the fact that some bartonellosis cases have been reported in patients after a tick bite [49,51]. Consequently, bartonellosis should now be included in the differential diagnosis for patients exposed to tick bites.

II.3. Ticks rearing and infection methods

In spite of the importance of TBDs, our knowledge of the transmission of pathogens by the ticks remains incomplete. Study of tick-host-pathogen interactions appears to be essential for controlling tick-borne diseases. For that purpose, large numbers of live ticks are required, which should be raised under controlled conditions in order to perform experimental infections. However, rearing ticks, and in particular hard ticks, is not easy due to their complex biological cycle and feeding process [52]. Some tick-feeding methods have been developed for that purpose, including feeding ticks directly on animals and feeding ticks via animal or artificial membranes.

Moreover, various methods have also been developed and used to infect hard ticks with pathogens in order to study pathogen transmission. These methods include feeding ticks on infected animals, injecting pathogens through the cuticle, using of capillary tubes filled with infectious suspensions to feed ticks, and feeding them on artificial or animal-derived membranes. Among them, artificial membrane feeding systems mimic the natural conditions of tick infection more closely than other methods, because pathogens are mixed in blood and absorbed throughout the blood meal via the digestive tract. In addition, it allows standardized blood meals with large number of ticks and without the need of animals.

Feeding and infection techniques of hard ticks are presented and discussed in the following review that was published in the journal “Acarologia” in 2012.

LABORATORY ARTIFICIAL INFECTION OF HARD TICKS: A TOOL FOR THE ANALYSIS OF TICK-BORNE PATHOGEN TRANSMISSION

Sarah BONNET and Xiang Ye LIU

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ABSTRACT — Despite its importance, our knowledge of pathogen transmission by ticks is incomplete. Detailed studies on the transmission, maintenance, infectivity, virulence, and pathogenicity of tick-borne microparasites all require the use of large numbers of live ticks raised under controlled conditions and difficulties in rearing ticks in the laboratory could partly explain the current lack of data. The most complex part in maintaining tick colonies doubtlessly lies in their engorgement, as ticks are strict haematophagous arthropods. Indeed, relatively few research teams have worked on artificial feeding systems for ticks due to the long, complex, and poorly understood feeding patterns of these arthropods. It is nonetheless essential to investigate the mechanisms underlying tick infection and infectiousness in order to better understand parasite-host-vector relationships and elaborate new control strategies for transmitted pathogens. The various methods used to date to feed ticks and infect them with their associated pathogens are reviewed here and their advantages and inconveniences are discussed.

KEYWORDS — ticks; artificial feeding; in vitro infection

INTRODUCTION

Ticks are among the most important vectors of human and animal diseases and surpass all other arthropods in the variety of pathogenic organisms they can transmit: including fungi, viruses, bacteria and protozoa. To study the biology of ticks or their interactions with associated pathogens, it is indispensable to be able to maintain tick colonies under laboratory conditions and to have efficient techniques to artificially infect them. In addition, it is widely recognized that the dynamics, pathogenesis and symptoms of infection, as well as the subsequent immune response, strongly depend on the route of pathogen introduction into a suscepti-

ble vertebrate host (Crippa *et al.* 2002; de Souza *et al.* 1993; Gern *et al.* 1993; Massung *et al.* 2004; Motameni *et al.* 2005; Piesman 1993). During natural transmission, tick-borne pathogens are injected into the vertebrate host at the same time as tick saliva, which favors infection by interfering with host immunological responses (Nuttall 1999). This means that studying tick-borne pathogen transmission to vertebrate hosts requires that ticks be infected under laboratory conditions.

However, rearing ticks, and in particular hard ticks, is not easy due to their complex biological cycle. The problems encountered in the maintenance of productive laboratory colonies doubtlessly explain a significant proportion of the existing

gaps in our knowledge of tick vector competence and transmission pathways. The *Ixodidae* likely possess the most complex feeding biology of all hematophagous arthropods. Indeed, the fact that they only feed on blood, and do so for an extended period of time (3-12 days), greatly limits our ability to set up artificial feeding systems which can function over the required time intervals. Several methods have been developed and used to infect hard ticks with pathogens, including feeding ticks on infected animals, injecting pathogens through the cuticle, using capillary tubes filled with infectious suspensions to feed ticks, and feeding them on artificial or animal-derived membranes. In this review, we summarize each of these techniques, discuss their application to pathogen transmission, and present their strengths and weaknesses (summarized in the Table 1). However, we first start with a brief outline of the Ixodid tick life cycle and highlight the importance of considering tick biology and ecology when attempting artificial feeding and tick rearing in the laboratory.

Overview of the hard tick life cycle

The general life cycle for hard tick species can be found in Sonenshine (1991). Here, we summarise the main points that are important to consider for artificial tick infections. Hard ticks have larval, nymphal and adult forms, all of which require a blood meal. Adult ticks tend to be restricted to feed on large-bodied animal hosts, whereas larval and nymphal stages also exploit smaller animals. For many species, Humans can be incidental hosts to the three life stages. A three-host life cycle, which includes host-seeking, feeding and off-host moulting (or egg-laying) in each life stage, is the most common developmental pattern for the majority of hard ticks of medical and veterinary interest. After feeding on a rather substantial quantity of host blood, females drop from the hosts and commence oviposition in a sheltered microenvironment, laying up to several thousand eggs. During each life stage, ticks may enter diapause for a variable amount of time depending on environmental conditions. Under favorable conditions in the natural environment, the life cycle of three-host tick species, from

larval hatching to the hatching of the next larval generation, can be completed in less than one year, but is typically longer (2-3 years). Compared with other haematophagous arthropods, feeding ixodid ticks is therefore a slow and complex process, taking several days to several weeks for repletion and detachment alone. In addition, successful host attachment depends on the presence of an appropriate array of chemical and physical stimuli that entice ticks into feeding.

Laboratory-adapted conditions for tick feeding

Most species of non-nidicolous ticks (or exophilic ticks, *i.e.* that occupy open, exposed habitats) have a clear, well-defined seasonal period of activity, during which time they engage in questing, a behavior expressed by the willingness of the tick to crawl or climb to favorable locations where they may attach to passing hosts. This active period can vary within the zoogeographic range of the species and among life stages (Sonenshine, 1991). Under laboratory conditions, photoperiod and ambient temperature can be adapted to the specific tick species being studied in order to induce this active period, thereby stimulating the desire to eat and accelerating the biological cycle. In 1979, Doube and Kemp (1979) reported that environmental factors, *e.g.*, variation in temperature or relative humidity, influence tick attachment behavior and survival, but do not generally affect feeding duration once ticks are attached to warm-blooded vertebrates. However, since their study, it has been demonstrated that diurnal rhythms and other environmental factors can, in fact, affect engorgement and detachment patterns. For example, mated females of the southern cattle tick *Rhipicephalus (Boophilus) microplus* engorge most rapidly at night but do not drop-off until the animals begin leaving the cattle sheds, typically in early morning, facilitating the dispersal of the replete female ticks in the host's habitat (Bianchi and Barre 2003). Similarly, nidicolous ticks tend to concentrate their feeding activities during the period when the host is resting or sleeping in the nest or burrow (Olivier 1989). Recently, experiments performed on birds reported the capacity of *I. arboricola* to extend the duration of attachment when the

TABLE 1: Summary of the major strengths and weaknesses of techniques used to artificially infect ticks with pathogens. Only key models (ticks and pathogens) and associated references are mentioned here. More specific information can be found in the main text.

Infection method	Frequency of use	Tick species	Pathogens studied	Key references	Major strengths	Major weaknesses
Direct feeding on the host	Many studies	<i>I. ricinus</i>	<i>B. divergens</i>	Joyner <i>et al.</i> , 1963	Physiologically realistic; Relatively easy set-up; Ability to infect a large quantity of ticks	Expensive; Ethical considerations; Inability to quantify infective dose; Restricted use for wild hosts
		<i>D. andersoni</i>	<i>A. marginale</i>	Kocan <i>et al.</i> , 1986		
		<i>R. appendiculatus</i>	<i>T. parva</i>	Bailey, 1960		
		<i>A. variegatum</i>	<i>T. mutans</i>	Young <i>et al.</i> , 1996		
		<i>A. hebraeum</i>	<i>C. ruminantium</i>	Heyne <i>et al.</i> , 1987		
		<i>I. ricinus</i>	<i>B. birtlesii</i>	Reis <i>et al.</i> , 2011a		
Injection	Few studies	<i>R. appendiculatus</i>	<i>T. parva</i>	Jongejan <i>et al.</i> , 1980	Ability to quantify infective dose	Physiologically unrealistic; High tick mortality; Live animals needed (ethical and logistical considerations)
		<i>D. andersoni</i>	<i>A. marginale</i>	Kocan <i>et al.</i> , 1996		
		<i>I. scapularis</i>	<i>B. burgdorferi</i>	Kariu <i>et al.</i> , 2011		
Capillary	Many studies	<i>D. andersoni</i>	<i>L. pomona</i>	Burgdorfer, 1957	Natural infection route; Ability to quantify infective dose	Difficult set-up; Live animals needed (ethical and logistical considerations); Ingestion of blood and pathogen not simultaneous
		<i>R. appendiculatus</i>	<i>T. parva</i>	Purnel et Joyner, 1967		
		<i>I. ricinus</i>	<i>B. burgdorferi</i>	Monin <i>et al.</i> , 1989		
		<i>A. variegatum</i>	Dugbee virus	Booth <i>et al.</i> , 1991		
		<i>R. sanguineus</i>	<i>E. chaffeensis</i>	Rechav <i>et al.</i> , 1999		
		<i>D. variabilis</i>	<i>A. marginale</i>	Kocan <i>et al.</i> , 2005		
		<i>D. variabilis</i>	<i>R. montana</i>	Macaluso <i>et al.</i> , 2011		
Membrane (animal skin or silicone membrane)	Many studies	<i>A. variegatum</i>	<i>T. mutans</i>	Voigt <i>et al.</i> , 1993	Natural infection route; Ingestion of blood and pathogen simultaneous; Ability to quantify infective dose; No need for live animals; Ability to infect a large quantity of ticks	Daily change of the blood (and risk of contamination); Membrane preparation required; Olfactory stimuli sometimes required (for non-animal membranes)
		<i>R. appendiculatus</i>	<i>B. ruminantium</i>	Young <i>et al.</i> , 1996		
		<i>R. appendiculatus</i>	<i>T. parva</i>	Wallade <i>et al.</i> , 1993		
		<i>I. ricinus</i>	<i>B. divergens</i>	Bonnet <i>et al.</i> , 2007		
		<i>I. ricinus</i>	<i>B. henselae</i>	Cotté <i>et al.</i> , 2008		

host bird did not return to a suitable environment for the tick, with no apparent costs of prolonged attachment (White *et al.* 2012).

Host-seeking ticks recognize a variety of stimuli from prospective hosts which, in turn, excites their host-finding behavior. Among these, odors are undoubtedly the most important and best-studied stimuli (Waladde and Rice 1982). Carbon dioxide represents one of the most important host-derived

odorants and can be used in the laboratory to stimulate tick activity. Radiant heat, such as host's body heat, also acts as a stimulus and acts synergistically with odors (Lees 1948). Other stimuli which ticks may potentially use in host-finding activities have received little attention, especially visual cues and vibrations. Finally, in some instances, tick-derived rather than host-derived stimuli are of critical importance in tick host-seeking behavior. For exam-

ple, *Amblyomma variegatum* and *A. hebraeum* are excited by the CO₂ produced by cattle but select tick-infested animals when they detect the aggregation-attachment pheromone emitted by attached, feeding ticks (Norval *et al.* 1989). Based on these studies, stimuli from hosts and pheromones produced by ticks can therefore be used in the laboratory to promote tick feeding.

Some tick species feed only on specific hosts, or on a narrow range of closely related hosts, whereas others may be categorized as opportunistic (Sonenshine 1975). Host specificity results from a process of selective host recognition and the ability of ticks to avoid host rejection (Ribeiro 1987) and can differ between life stages for a given tick species. To a certain extent, host choice is influenced by the height at which ticks seek hosts on the vegetation, but this is by no means the sole determinant of host specificity. Macro- and micro-habitat distribution also influences host selection by favoring encounters between ticks and their hosts. Host selection also requires tick recognition of specific host characteristics, such as host odors, for example. Unfortunately, our understanding of host selection in ticks and the functional basis of host specificity is extremely limited and can be a significant obstacle for tick rearing and study. As some hosts can not being maintained under laboratory conditions, the implementation of specifically-adapted artificial feeding systems may be necessary. For example, as mentioned above, odor from the appropriate host animal may be used to stimulate tick feeding on artificial membranes. Sweat and exfoliated skin collected from horses naturally infested with *A. cajennense*, has been used to stimulate feeding in this tick species (de Moura *et al.* 1997). In the same way, shredded bovine hair and hair extract (*i.e.*, lipid extracted from freshly shaven hair dissolved in dichloromethane) have been used, respectively, on silicone membranes in order to entice feeding in *A. hebraeum* and *I. ricinus*, tick species that readily exploit cattle (Krober and Guerin 2007a; Kuhnert *et al.* 1995).

In conclusion, the general environmental conditions that a tick is exposed to in its natural habitat, including temperature, humidity, diurnal rhythms, activity periods, questing behavior, sensory modal-

ities and stimuli, must all be considered carefully when setting up a laboratory system. In many cases, laboratory conditions will need to be adapted to the specific needs of the tick species of interest in order to increase the chances of successful tick feeding and colony establishment.

Systems for maintaining and/or infecting ticks

Feeding and infection directly on the host

Despite the constraints associated with host specificity, some tick species can be readily fed in the laboratory on easily handled animals. Indeed, rabbits are classically used to feed ticks in laboratory: immature stages of *Rhipicephalus evertsi evertsi* (Londt and Van der Bijl 1977), all life stages of *I. scapularis*, *I. pacificus*, *A. americanum*, *Dermacentor occidentalis*, *D. variabilis*, *Haemaphysalis leporispalustris* and *R. sanguineus* (Troughton and Levin 2007), *R. appendiculatus* (Bailey 1960), *A. variegatum* (Voigt *et al.* 1993), *D. andersoni* (Howarth and Hokama 1983), *A. hebraeum* (Heyne *et al.* 1987), *I. ricinus* (Bonnet *et al.* 2007). In these cases, the typical way to engorge ticks is to use feeding bags or capsules glued to clean-shaven skin on the back of the animal (Figure 1). Sometimes, larger animals have been used as blood sources, such as calves for *R. evertsi evertsi* (Londt and Van der Bijl 1977), *R. appendiculatus* (Musyoki *et al.* 2004), and *D. andersoni* (Kocan *et al.* 1986), or sheep for *A. hebraeum* (Heyne *et al.* 1987) and *I. ricinus* (Bonnet *et al.* 2007).

The use of natural hosts for tick feeding and methods of direct infection on infectious animals is the method of choice to obtain conditions that are closest to the physiological reality of transmission. However, acquisition, housing, and handling of animal hosts can be complicated, expensive and sometimes even impossible. In fact, in some cases and in particular for wildlife, maintaining the natural host of a specific tick-borne pathogen is impossible in laboratory. There are some examples where wild animals were used as the blood meal source, such as groundhogs for feeding *Ixodes cookei* (Farkas and Surgeoner, 1991) or tortoises for feeding *A. hebraeum* (Heyne *et al.*, 1987) but this practice remains exceptional. The most commonly used model of tick infection directly on animals involves pathogens in-

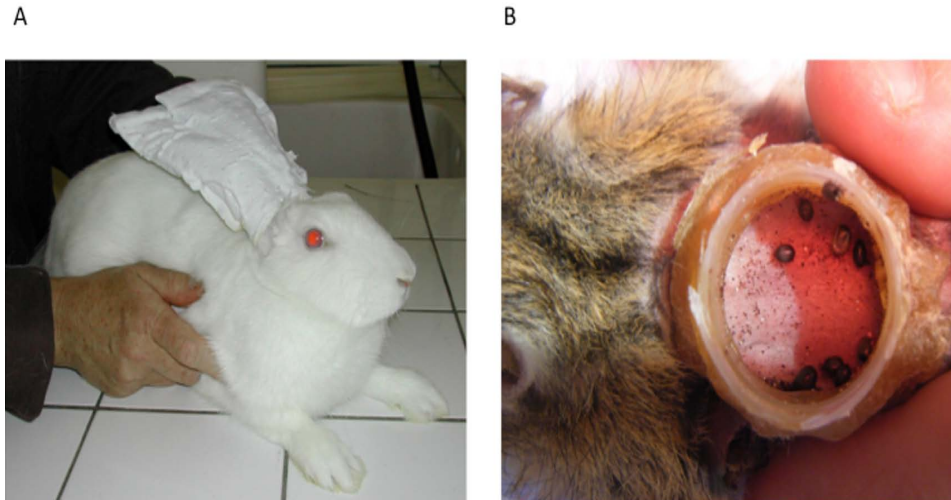


FIGURE 1: Views of *Ixodes ricinus* feeding in the laboratory on A – rabbit, and B – Siberian chipmunks (*Tamias sibiricus barberi*). On rabbits, ticks were put in an ear-bag which was placed on shaved ear skin and sealed with tape at the base. Ticks were checked daily until repletion, and were then collected and stored under standardized conditions. For chipmunks, animals were briefly anaesthetized with 3 % Isoflorane and a plastic cap, open at both ends, was glued onto their shaved back with wax. Hungry larvae and nymphs were placed in the cap, which was then sealed with tape. Ticks were allowed to feed until repletion for 5-6 days. At this time, the cap was opened, and the engorged ticks were collected and stored under standardized conditions.

fecting cattle, such as *Babesia divergens* transmitted by *I. ricinus* (Donnelly and Peirce 1975; Joyner *et al.* 1963; Lewis and Young 1980), *Anaplasma marginale* by *D. andersoni* (Kocan *et al.* 1986), *Theileria parva* by *Rhipicephalus appendiculatus* (Bailey 1960; Musyoki *et al.* 2004), or *T. mutans* and *Cowdria ruminantium* transmitted by *A. variegatum* (Young *et al.* 1996). Sheep were used to infect *A. hebraeum* with *C. ruminantium* (Heyne *et al.* 1987). Infectious gerbils have been used in order to infect *I. ricinus* with *B. divergens* (Lewis and Young 1980; Mackenstedt *et al.* 1990). Finally, laboratory mice have also been used for studying *Bartonella birtlesii* transmission by *I. ricinus* (Reis *et al.* 2011a), or *Borrelia burgdorferi* by *I. scapularis* (Burkot *et al.* 2001).

However, with the direct feeding technique, it is impossible to quantify the pathogen dose received by the tick during feeding and thus to standardize the experimental conditions. Even if a venous blood sample is simultaneously analyzed, it may have different pathogen concentrations than the tick's biting site and tick blood meals are too long to monitor temporal changes in pathogen concentrations with any precision. Likewise, for some pathogens, the parasitaemia, bacteraemia or viral peak in the host

can be short (1-4 days) and it may be difficult to synchronize it with tick feeding. Finally, for ethical considerations, it is always desirable to limit the use of laboratory animals and thus to find alternative artificial systems.

Infection by injection

In a few studies, ticks have been infected by direct injection of a suspension containing the pathogen through the cuticle. *R. appendiculatus* have been successfully infected after inoculation with fresh or cryopreserved blood containing *T. parva* (Jongejan *et al.* 1980; Walker *et al.* 1979), whereas attempts to infect ticks by inoculating cultured stages of *T. parva* failed (Jongejan *et al.* 1980). Another study reported that *D. andersoni* exposed percutaneously as nymphs to *Anaplasma marginale*, transmitted the pathogen to calves as feeding adults, even though no bacterial colonies were detected in gut tissues of the inoculated ticks (maybe because the infective dose was too low or because the bacteria developed in tissues other than gut) (Kocan *et al.*, 1986). In a study aimed at infecting *A. americanum*, *D. variabilis* and *R. sanguineus* with *Ehrlichia chaffensis*, Rechav *et al.* (1999) concluded that the inoculation technique by injection

tion is not accurate or practical for routine infection of ticks with pathogens because of the low survival rate of inoculated ticks. Recently, a procedure for infecting *I. scapularis* with *B. burgdorferi* via a microinjection by the anal aperture was reported and seems more satisfactory in terms of tick survival (Kariu *et al.* 2011).

When using direct inoculations by injection, the exact assessment of the pathogen dose received by the tick is possible. However, in addition to the high tick mortality previously mentioned (Rechav *et al.* 1999), this technique does not enable the experimenter to avoid the use of animals for feeding ticks post-infection. Finally, and more importantly, it does not correspond to the normal infection pathway used by the pathogen to infect ticks, which naturally occurs via the mouthpart and the digestive tract during the blood meal. This difference can have important consequences for pathogen development, particularly when the parasite in question undergoes several developmental stages in the tick gut (Chauvin *et al.* 2009). It has also been demonstrated that bacteria, such as *Borrelia burgdorferi*, express different molecules depending on the engorgement status of the vector (Hovius *et al.* 2007). Consequently, the results obtained with direct inoculation systems may be difficult to extrapolate and apply to natural infections.

Infection by capillary feeding

The use of blood-filled capillary tubes placed over the mouthparts of the tick was first reported in 1938 by Gregson who used this technique to collect saliva from *D. andersoni* (Gregson 1938). Later, in 1950, Chabaud used it for engorging *Haemaphysalis excavatum*, *H. dromedarii* and *R. sanguineus* with different nutriment combinations as a means of studying tick nutrition (Chabaud 1950). In Chabaud's study, ticks were pre-fed on the host, removed, and the capillary tube containing various substrates was placed over the tick's mouthparts. Since these initial studies, capillary tubes filled with infectious suspensions have been used for feeding ticks in several biological models: *Leptospira pomona* / *A. maculatum*-*D. andersoni* (Burgdorfer 1957), *T. parva* / *R. appendiculatus* (Purnell and Joyner 1967; Walker *et al.* 1979),

B. burgdorferi / *I. ricinus* (Kurtenbach *et al.* 1994; Monin *et al.* 1989), *B. burgdorferi* / *I. scapularis* (Broadwater *et al.* 2002; Korshus *et al.* 2004), dugbee virus / *A. variegatum* (Booth *et al.* 1991), *E. chaffeensis* / *A. americanum*-*D. variabilis*-*R. sanguineus* (Rechav *et al.* 1999), *A. marginale* / *D. variabilis* (Kocan *et al.* 2005) or *R. montana*-*R. rhipicephali* / *D. variabilis* (Macaluso *et al.* 2001). In these studies, capillary feeding was performed either before or after feeding on the animal host, the animal host being necessary in order to feed ticks to repletion.

Tick infection by capillary feeding presents the advantage of using the natural infection route via the mouthparts and the digestive tract. It also permits one to control the amount of fluid ingested by the tick and the titer of the pathogen that enters the tick. However, tick manipulation during the pre- or post-feeding period on the natural host with a forced removal from the host is delicate in practice. Similarly, only very small amounts of fluid (0.01-0.03 ml) can be ingested by ticks with this technique (Burgdorfer 1957; Rechav *et al.* 1999) because ticks feed in an unnatural manner. Finally, and most importantly, natural transmission conditions are poorly replicated using this method, as the tick acquires the pathogen in large quantities and without blood. Normally, the pathogen is absorbed by the tick throughout the blood meal period during which time the tick has already begun digestion and the pathogen has started the next step its developmental cycle.

Membrane feeding systems

The membrane feeding technique consists in feeding ticks through a membrane on blood taken from animals or culture media. It is the most frequently used feeding technique for ticks as demonstrated by two previously published reviews on the subject (Krober and Guerin 2007b; Waladde *et al.* 1996). In 1956, Pierce and Pierce used air cell membranes from embryonated hen eggs in order to feed *R. microplus* larvae and nymphs (Pierce and Pierce 1956). Since then, several membranes from different animal origins have been used with variable success to engorge ticks, including pieces of cattle skin for *R. microplus* (Kemp *et al.* 1975) and *A. variegatum*



FIGURE 2: View of attached *I. ricinus* nymphs on a rabbit skin used in the membrane feeding system.

(Voigt *et al.* 1993; Young *et al.* 1996), calf mesentery and modified Baudruche membranes for *R. microplus* (Kemp *et al.* 1975; Waladde *et al.* 1979) and *R. appendiculatus* (Waladde *et al.* 1991; Young *et al.* 1996), rabbit skin for *A. variegatum* (Voigt *et al.* 1993; Young *et al.* 1996), *D. andersoni* (Howarth and Hokama 1983), *R. appendiculatus* (Musyoki *et al.* 2004) and *I. ricinus* (Bonnet *et al.* 2007) (Figure 2), mouse skin for *D. andersoni* (Howarth and Hokama 1983; Paine *et al.* 1983) and *I. scapularis* (Burkot *et al.* 2001), and gerbil skin for *I. ricinus* (Bonnet *et al.* 2007). Membranes of non-animal origin made from silicone have also been used with success, particularly for feeding the different instars of *A. hebraeum* (Kuhnert *et al.* 1995), *I. ricinus* females (Krober and Guerin 2007b), *A. cajennense* adults (de Moura *et al.* 1997), and recently *H. anatolicum anatolicum* and *H. dromedarii* (Tajeri and Razmi 2011). However, without the addition of specific stimuli, the use of such membranes has proved ineffective for ticks such as *A. variegatum* (Voigt *et al.* 1993). This is related to the fact that one of the greatest difficulties is to encourage the attachment of unfed ticks (see above). It is for this reason that attachment stimuli are always required with silicone membranes, and/or why some authors use these membranes after a pre-feeding step on live animals. This was the case, for example, for *I. holocyclus* where the authors wanted to collect tick-produced toxins to study tick paralysis (Stone *et al.* 1983). In addition to being logistically difficult, the major disadvantage of arti-

ficial membrane systems that employ pre-feeding is the low reattachment success on the membrane (Howarth and Hokama 1983).

However, regardless of the limitations associated with artificial membrane techniques, this method has proved successful in infecting feeding ticks. Howarth and Hokama (1983) were able to obtain infectious adults of *D. andersoni* when the preceding nymphal stages were infected with *Anaplasma marginale* via an animal skin membrane and after a pre-feeding step on a rabbit. An almost similar protocol was used by Burkot *et al.* (2001) for successfully infecting *I. scapularis* ticks with *B. burgdorferi*. Here, ticks were pre-fed on a mouse and the mouse skin was harvested with *I. scapularis* still attached. The skin was then fixed to a glass membrane feeder containing bacterial infected blood (Burkot *et al.* 2001). In other studies, animal skin membranes have been used with success and without the need of a pre-feeding step on a living animal. *A. variegatum* was infected with *T. mutans* and *Cowdria ruminantium* in this way (Voigt *et al.* 1993; Young *et al.* 1996), as was *R. appendiculatus* with the same pathogens and a modified Baudruche membrane (Young *et al.* 1996). In 1993, Waladde *et al.* succeeded in transmitting *T. parva* to susceptible cattle via adult *R. appendiculatus* infected as nymphs through a Baudruche membrane that was made attractive to ticks by the addition of a combination of tactile and olfactory stimuli (Waladde *et al.* 1993). The same experiment was then reproduced

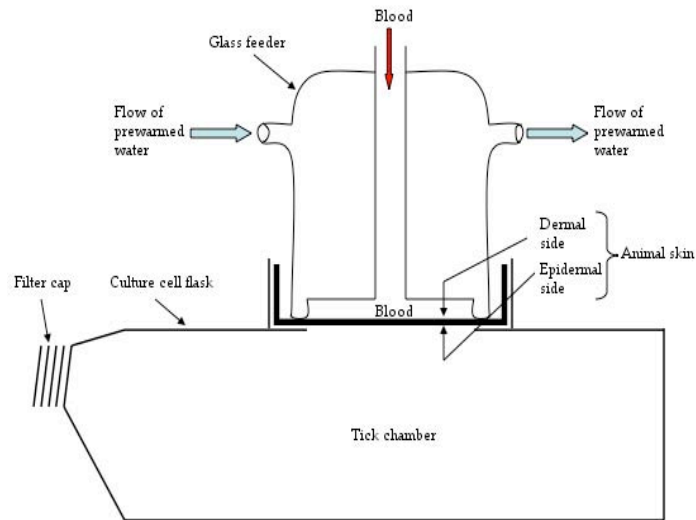


FIGURE 3: Diagram of the membrane feeding apparatus used in the experimental feeding of *Ixodes ricinus* ticks (adapted from Bonnet et al. 2007).

successfully using rabbit skin membranes (Musyoki et al. 2004). Finally, gerbil (for immature life stages) and rabbit (for adults) skin membranes have been used in order to infect *I. ricinus* with both *B. divergens* and *Bartonella henselae* without the need for additional stimuli (Bonnet et al. 2007; Cotte et al. 2008).

The membrane feeding apparatus consists of a blood container with a membrane placed either on the top (Bonnet et al. 2007; Burkot et al. 2001; Musyoki et al. 2004; Voigt et al. 1993; Young et al. 1996) or the bottom (Howarth and Hokama 1983; Kuhnert et al. 1995; Paine et al. 1983; Waladde et al. 1991) of the tick containment unit. Placing the blood above the membrane favors a continuous gravitational pressure on the membrane and is essential for infection with intraerythrocytic pathogens because of the rapid sedimentation of the red blood cells. Several tick-feeding devices with different blood containment units have been explored and tested, including plastic cylinders (Young et al. 1996), plate wells (Howarth and Hokama 1983; Krober and Guerin 2007a), honey jars (Kuhnert et al. 1995) or glass feeders (Bonnet et al. 2007) as represented on figure 3.

In order to mimic the host environment more closely, a temperature (35 – 39°C) adapted to the

tick species of interest should be applied either to the incubator, where the whole apparatus is placed, or just to the blood. As already mentioned, olfactory stimuli for attachment and feeding are sometimes required and are indispensable in the case of membranes from non-animal origins. The required stimuli could differ depending on the species and genera of ixodid ticks under study. A carbon dioxide atmosphere has been used as stimulant for tick attachment, between 5 and 10 % CO₂ for *A. variegatum* for example (Voigt et al. 1993; Young et al. 1996). Host hair, tick feces, animal fur extracts and synthetic aggregation-attachment pheromone mixtures have all been used for stimulating the attachment of *A. hebraeum* (Kuhnert et al. 1995). For stimulating *R. appendiculatus* feeding, Young et al. (1996) also used cattle/tick washes and tick feces. However, de Moura et al. (1997) demonstrated that for *A. cajennense* silicone membranes treated with blood vestiges was more efficient than other tested phagostimulants. Finally, adenosine triphosphate (ATP) and reduced glutathione have also been used as phagostimulants (Kuhnert et al. 1995; Paine et al. 1983). Finally, membrane thickness must be adapted to the size of the tick's mouthparts which can be short or long depending on the genera and the tick life stage.

Membrane feeding techniques mimic the natural conditions of tick infection more closely than other methods because pathogens are mixed in blood and are absorbed throughout the blood meal via the digestive tract. In most cases, using animal skin membranes has the important advantage that no tick pre-feeding is required for attachment and engorgement on the membrane, and no attachment stimuli are required. It is, on the other hand, necessary to sacrifice laboratory animals in order to obtain the skins and to carefully prepare them (Bonnet *et al.* 2007; Musyoki *et al.* 2004) to avoid any biodegradation and blood contamination. The final engorgement weight of membrane-fed ticks also tends to be lower (or equal) than that of animal fed ticks (Musyoki *et al.* 2004; Voigt *et al.* 1993; Young *et al.* 1996), even if molting and egg-laying success are generally comparable. Membrane feeding permits a direct assessment of pathogen concentration in the blood sample ingested by the ticks. Repeated assays with large tick numbers are also possible with this system. Finally, membrane-feeding techniques can allow one to evaluate the effects of drugs or transmission-blocking blood components, as well as helping to elucidate attachment stimuli, feeding stimuli and nutritional requirements of ticks. Feeding immature stages presents less difficulty than for adults because of their shorter feeding times. Indeed, the principal difficulty with this technique resides in maintaining a continuous bloodmeal without contamination by bacteria or fungi during the slow blood-feeding process and the required daily changes of the blood. In addition, the mouthparts and oral secretions of the ticks can also contaminate the blood in the feeding device because of the absence of host defense mechanisms. However, the addition of antibiotic and antifungal products to the circulating blood can prevent this problem. To favor feeding, anticoagulants should also be used and it was reported that heparinized blood was found to be the most suitable for tick feeding (Voigt *et al.* 1993; Waladde *et al.* 1993; Young *et al.* 1996). Consequently, within the framework of experimental pathogen transmission, it is necessary to test pathogen viability under the tick feeding conditions beforehand.

CONCLUSIONS

Ticks possess many unusual features that contribute to their remarkable success and vector potential. One of the most outstanding is their longevity and their reproductive potential (*i.e.*, ability to produce large numbers of eggs), which makes them substantial pathogen reservoirs in the field. Another is the fact that they are pool feeders (*i.e.*, sucking all the fluids and potential pathogens that are exuded into the wound generated by the bite). During feeding, they absorb a very large quantity of blood and over a relatively long period of time, thereby increasing the chance of ingesting a pathogen. It is this last parameter that makes them particularly difficult to study in the laboratory because these natural conditions are complicated to replicate. Nevertheless, there is no doubt that effective *in vitro* feeding systems for Ixodid ticks of medical and veterinary importance have major benefits. Even if feeding ticks on live experimental animals seems the simplest, it is not always practicable according to the biological model and may be considered as ethically debatable. Various methods have therefore been elaborated to feed and infect ticks artificially, among which the membrane feeding technique mimics reality more closely than the other techniques. However, each technique has strengths and weaknesses and the chosen method will depend on the question addressed. In all cases, infecting ticks under controlled conditions enables one to test a great spectrum of biological questions, including the ability to study the development of pathogens inside their vectors, to uncover transmission pathways, and to evaluate the influence of biologically active substances exchanged between host and vector. For now, these techniques all tend to have long and difficult set-up periods, giving sometimes unpredictable results. Efforts to standardize and simplify laboratory protocols, which would greatly improve our ability to exploit these methods, should now be the aim of future work.

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II.4. Tick-borne pathogen transmission: modalities and molecular mechanisms

II.4.1. General introduction

As already mentioned, ticks can transmit a high variety of pathogens including bacteria, viruses and parasites, and many veterinary and human diseases, are due to pathogens that are transmitted by ticks all over the world [3,4].

Pathogen transmission by hard ticks can be briefly summarized as follows. Each of the three life stages of a hard tick, larva, nymph and adult, requires a blood meal. For most hard ticks of medical and veterinary importance (including *Ixodes* spp., *Dermacentor* spp., *Amblyomma* spp.), a three-stage life cycle including host-seeking, feeding and off-host molting (or egg-laying), is the most common developmental pattern, when there was some of them like *Rhipicephalus microplus* (formerly *Boophilus microplus*) harbor a single host cycle. When ticks feed on a pathogen-infected vertebrate host, they imbibe the host blood with contaminated TBPs. Once ingested, the pathogen life cycle differs depending on the pathogen (see Figure 1 of the following review). The pathogen invades the tick body via the haemolymph and colonizes tick's organs such as the salivary glands or the ovaries with or without the stimulus of a new blood meal. Finally, pathogens are re-transmitted to new vertebrate hosts during tick blood feeding via saliva and, for some of them, they can be transferred to the next tick generation via transovarial transmission.

II.4.2. Tick molecules implicated in pathogen transmission

During ixodid ticks slow, long and complex feeding process [52], ticks face the problem of host haemostasis, inflammation and adaptive immunity, and therefore, have evolved a complex and sophisticated pharmacological armamentarium against these barriers. Accordingly, various components of tick saliva, including anti-clotting, anti-platelet aggregation, vasodilator, anti-inflammatory and immunomodulatory molecules allow them to successfully feed (see reviews by [53-55]). For almost all TBPs, their transmission occurs during the blood feeding process, and they are injected into the vertebrate host at the same time via tick saliva during the blood meal. Indeed, ticks act not only like a syringe in the transmission of TBPs but tick saliva factors can facilitate pathogen transmission and infection at the blood feeding sites, a phenomenon named saliva-activated transmission (SAT) [56]. Much direct and indirect evidence has reported SAT for bacteria, parasites and viruses transmitted by ixodid tick species [56]. During tick infection and transmission, TBPs must also adapt to tick-specific physiological and behavioral characteristics, particularly with regard to blood feeding, blood meal digestion, molting and immune responses [57,58]. They also have to cross many tick barriers such as intestinal, salivary or ovarian ones when ingested by ticks and multiple distinct cell types must be invaded for pathogenic multiplication to occur. All these events imply that there is inevitably a molecular dialogue between the pathogen and its vector.

Therefore, modulation of tick protein expression during tick feeding, particularly in salivary glands, is not only implied in blood meal acquisition, but is also linked to pathogen acquisition, multiplication, transmission. Several studies have reported that tick salivary glands produce differentially expressed proteins in response to pathogen

infections, which may correspond to factors implicated in transmission [59-66]. Indeed, some tick salivary gland factors have been identified as able to enhance the acquisition or transmission of pathogens, whereas others are able to inhibit tick-borne pathogen acquisition and transmission.

All the hard tick molecules identified to date as being implicated in pathogen transmission are presented in detail in the following review, which is in press in the journal “PLoS Neglected Tropical Diseases”.

Hard tick factors implicated in pathogen transmission: a review

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Abstract

Ticks are the most common arthropod vector after mosquitoes, and are capable of transmitting the greatest variety of pathogens. For both humans and animals, the worldwide emergence or re-emergence of tick-borne disease is becoming increasingly problematic. Despite being such an important issue, our knowledge of pathogen transmission by ticks is incomplete. Several recent studies, reviewed here, have reported that the expression of some tick factors can be modulated in response to pathogen infection, and that some of these factors can impact on the pathogenic life cycle. Delineating the specific tick factors required for tick-borne pathogen transmission should lead to new strategies in the disruption of pathogen life cycles to combat emerging tick-borne disease.

Introduction

Ticks are obligate blood-feeding ecto-parasites of many hosts including mammals, birds and reptiles, and are also vectors for several bacterial, parasitic or viral pathogens. After mosquitoes, ticks are the second most common arthropod pathogen vector [1]. Recent intensification of human and animal movements, combined with socio-economic and environmental changes, as well as the expanding geographical distribution of several tick species, have all contributed to the growing global threat of emerging or re-emerging tick-borne disease (TBD), along with increasing numbers of potential tick-borne pathogens [2]. Despite an urgent requirement for in-depth information, the existing knowledge of tick pathogen transmission pathways is incomplete. *Ixodidae* possess the most complex feeding biology of all hematophagous arthropods [3], therefore the resulting difficulties in maintaining productive laboratory colonies doubtlessly explain a significant proportion of the gaps in our knowledge [4]. Moreover, because of the disadvantages of current TBD control methods (resistance, environmental hazard, increased cost), new approaches are urgently needed. Among these, vaccine strategies targeting those molecules that play key roles in vector competence are particularly promising [5,6]. Consequently, research on molecular interactions between ticks and pathogens as well as the identification of suitable antigenic targets is a major challenge for the implementation of new TBD control strategies.

During the blood feeding process, ticks confront diverse host immune responses,

and have evolved a complex and sophisticated pharmacological armament in order to successfully feed. These include anti-clotting, anti-platelet aggregation, vasodilator, anti-inflammatory and immuno-modulatory systems [7]. For most TBP, transmission via the saliva occurs during blood feeding (Figure 1), in addition, many tick adaptations exist which may promote TBP transmission, notably by interfering with the host immune response [8-10]. Moreover, during their development within the tick and their subsequent transmission to the vertebrate host, pathogens undergo several developmental transitions and suffer population losses, to which tick factors presumably contribute. Several studies have clearly reported that pathogens can influence tick gene expression, demonstrating molecular interaction between the vector and pathogen [11-24]. Our review briefly outlines TBP transmission, highlights evidence of molecular interactions between hard ticks and TBP, and describes several tick molecules implicated in pathogen transmission.

Tick-borne pathogen transmission

Hard ticks progress through larval, nymphal and adult stages, all of which require a blood meal. For the majority of hard ticks of medical and veterinary relevance (including *Ixodes* spp., *Dermacentor* spp., *Amblyomma* spp.) a three-stage life cycle including host-seeking, feeding and off-host molting (or egg-laying), is the most common developmental pattern, whereas some ticks, such as *Rhipicephalus microplus* (formerly *Boophilus microplus*) undergo a single host cycle. Ticks feeding

on a pathogen-infected vertebrate host also imbibe these pathogenic microorganisms and, once ingested, the pathogen's life cycle differs depending on the pathogen (Figure 1). In the midgut, pathogens such as *Anaplasma marginale* can undergo initial multiplication within membrane-bound vacuoles [25,26]. *Borrelia* spp. or *Bartonella* spp. remain in the midgut during tick molting and only invade the salivary glands after a new blood meal stimulus [27,28], whereas *Babesia* spp. and *Rickettsia* spp. immediately invade both the tick ovaries and salivary glands via the hemolymph [29,30]. *Theileria* spp. parasites exhibit a similar cycle in the vector but without ovarian invasion [31]. *Anaplasma* spp. and some arboviruses also migrate from the gut to salivary glands where they remain during molting, up until the next tick life stage and blood feeding episode [32,33]. Once inside the tick, intestinal, salivary or ovarian barriers must be crossed, and multiple distinct cell types must be invaded for pathogenic multiplication to occur. During tick infection and transmission, TBP must also adapt to tick-specific physiological and behavioral characteristics, particularly with regard to blood feeding, blood meal digestion, molting and immune responses [34]. Finally, pathogens are re-transmitted to new vertebrate hosts during tick blood feeding via the saliva, and for certain pathogens, they can be transferred to the next tick generation via transovarial transmission (Figure 1). This vertical transmission is an absolute necessity for those TBP infecting single host ticks species such as the *R. microplus*-transmitted *Babesia bovis*.

Functional transcriptomic/proteomic studies of tick and tick-borne pathogen interactions

Several investigations performed in different models with varying approaches are summarized in Table 1. In general, they report that tick gene or protein expression can be regulated in response to pathogen infection. Most of the modulated transcripts or proteins were not associated with a known protein or an assigned function, however some were able to be annotated as putative proteins.

Transcriptomic studies

Macaluso *et al.* used differential-display PCR (DD-PCR) to identify *Dermacentor variabilis* tick transcripts, which were variably expressed in response to *Rickettsia montanensis* infection [11]. Among identified transcripts, nine were down-regulated in the infected tick midgut; five transcripts (clathrin-coated vesicle ATPase, peroxisomal farnesylated protein, α -catenin, salivary gland protein SGS-3 precursor, and glycine-rich protein) were also down-regulated in the tick salivary glands; whereas six (clathrin-coated vesicle ATPase, peroxisomal farnesylated protein, Ena/vasodilator-stimulated phosphoprotein-like protein, α -catenin, tubulin α -chain, and copper-transporting ATPase) were up-regulated in infected tick ovaries. However, it was clearly demonstrated that the DD-PCR technique poses serious problems in the re-amplification of selected transcripts and generates many false positives [35], consequently, this method is rarely used today.

EST (Expressed Sequence Tag) sequences derived from cDNA libraries have

also been used to analyze and compare gene expression in *Rhipicephalus appendiculatus* ticks infected with *Theileria parva*. Results suggested an up-regulation in the expression of some glycine-rich proteins named TC1268, TC1278 and TC1272, in infected salivary glands [12].

Subtractive hybridization libraries have also been used in order to investigate the response of *Ixodes ricinus* whole ticks to blood feeding and to infection with *Borrelia burgdorferi*, the agent for Lyme disease [13]. This study showed that 11 genes were specifically induced after a blood meal on *B. burgdorferi*-infected guinea pigs, which included several thioredoxin peroxidases, glutathione S-transferase and defensins.

The response to *A. marginale* infection was also analyzed in male *R. microplus* salivary glands by subtractive hybridization libraries [16]. Based on EST sequences, 43 unique transcripts (such as proline- or glycine-rich proteins) were up-regulated, whereas 56 were down-regulated (including histamine binding protein, immunoglobulin G binding protein or the Kunitz-like protease inhibitor).

When analyzing the response of *Ixodes scapularis* nymphal ticks to *B. burgdorferi* infection via the sequencing of cDNA library clones, Ribeiro, J.M. *et al* showed that ten salivary gland genes were significantly differentially expressed during bacterial infection [14]. Among these ten genes, seven were overrepresented in the *B. burgdorferi* infected nymphs, including those coding for the 5.3-kDa peptide family, basic tail family and histamine-binding protein (HBP) family, however three

genes coding for HBP family proteins were overexpressed in the non-infected nymphs.

To investigate the effect of feeding and flavivirus infection on the salivary gland transcript expression profile in *I. scapularis* ticks, a first-generation microarray was developed using ESTs from a salivary gland-derived cDNA library [17]. Among the 48 salivary gland transcripts presenting differential expression after virus infection, three were statistically differentially regulated during the three analyzed post-feeding periods, two were up-regulated and one down-regulated. One of the up-regulated genes belonged to the 25-kDa salivary gland protein family presenting homology to lipocalins, whose function is the transportation of small molecules.

Finally, several differentially regulated genes were identified by using suppression-subtractive hybridization analyses of cultured IDE8 *I. scapularis* tick cells in response to *A. marginale* infection [15]. Twenty-three genes were up-regulated, including glutathione S-transferase, vATPase or selenoprotein W2a; whereas six were down-regulated (including β -tubulin, ferritin or R2 retrotransposon reverse transcriptase-like protein).

All approaches used in the above-mentioned studies led to the identification of differentially expressed tick transcripts in response to TBP infection. Some of the observed discrepancies between models may be due both to the models themselves but also to the differing sensitivity of specific techniques. In future, transcriptomic analysis may be performed by using new powerful NGS techniques that harbor high

sensitivity. Moreover, using the same technique, to analyze transcripts in *A. marginale*-infected IDE8 tick cells [15,16] and *A. marginale* infected *R. microplus* demonstrated that more differentially regulated transcripts were identified *in vivo* (Table 1), suggesting that *in vitro* models should be used with caution. In any case, the lack of genomic information for almost all tick species (the only available tick genome is that of *I. scapularis*) leads to difficulties in data analysis. The analysis of mRNA expression levels is undoubtedly an effective method to identify tick gene expression during TBP infection, but the level of mRNA and the concentration of corresponding proteins only have a correlative, rather than a causative association. Therefore, the quantities of translated proteins in ticks in response to TBP infection should also be assessed.

Proteomic studies

Proteomic profiling of *B. bovis*-infected *R. microplus* ticks demonstrated that ten proteins were differentially up-regulated in ovaries, including endoplasmic reticulum protein, glutamine synthetase, and a family of Kunitz-type serine protease inhibitors and nine proteins were down-regulated, including tick lysozyme and a hemoglobin subunit [18]. In the midgut, 15 proteins were up-regulated, including gamma-glutamyltransferase1 and a putative ATP synthase-like protein; five proteins were down-regulated, including heat shock cognate 70 protein, putative heat shock-related protein and signal sequence receptor beta [19].

The proteomic profile of *I. scapularis* embryonic tick cells was investigated in response to *Anaplasma sp.* Infection [15,20]. Results showed that the translation elongation factor 1 γ was up-regulated, whereas GST (glutathione-S-transferase) and a putative high-mobility group-like protein were under-expressed in *A. marginale* infected IDE8 tick cells [15]. HSP70 (heat shock protein 70) was over-expressed, but other putative HSPs were under-expressed in *Anaplasma phagocytophilum* infected ISE6 tick cells [20].

Differentially expressed proteins were also identified in *Rhipicephalus* spp. ticks infected with *Anaplasma ovis*, *Theileria annulata*, *Rickettsia conorii*, or *Ehrlichia canis* by comparing them with non-infected ticks [20,21]. Results showed that the protein expression profile (among which actin, enolase or guanine nucleotide-binding protein were identified) varied according to the analyzed models. Fifty-nine proteins have been identified as differentially expressed in *A. ovis*-infected *Rhipicephalus turanicus* ticks, sixteen in *T. annulata*-infected *Rhipicephalus bursa*, ten in *R. conorii*-infected *Rhipicephalus sanguineus*, and six in *E. canis*-infected *R. sanguineus*.

Thus, relatively few studies have focused on the proteome, reflecting the relative difficulty of studying the subject compared to research on transcripts. However, analyzing protein expression allows to take into account any translational modifications that may occur.

Tick factors implicated in tick-borne pathogen transmission

As reported above, the expression of some tick factors can be modulated by TBP infection during stages of acquisition, multiplication/migration in the vector, and/or transmission to hosts. These factors correspond to two types of molecules: those facilitating pathogen development, and those which limit it, i.e. the molecules from the tick's own immune system. However, based on the afore mentioned studies, it is difficult to confirm whether the identified molecules are specific to the studied microorganisms. Therefore functional studies are required to validate their implication in pathogen development. Antibodies can be used for this purpose, but the most widely used method currently is RNA interference (RNAi), a gene-silencing technique suited to tick analysis when other methods of genetic manipulation are rare [36]. Tick factors that have been identified as implicated in TBPs life cycles are summarized in Table 2 and described below.

Tick factors contributing to tick-borne pathogen acquisition

The host skin site, to which the tick attaches during feeding, is a critical interface between ticks, hosts and the TBP [37]. For ticks, it is the location of their indispensable blood meal; for hosts, it acts as the barrier preventing blood loss and pathogen invasion; however for pathogens, it is an ecologically privileged niche that should be exploited.

Salp16, an *I. scapularis* salivary protein, facilitates *A. phagocytophilum* acquisition [38]. In Salp16-deficient ticks, infection of tick salivary glands by *A.*

phagocytophilum is strongly decreased. Interestingly, silencing Salp16 does not affect *B. burgdorferi* acquisition, indicating pathogen specificity [38]. Salp16 is implicated in vertebrate host blood-cell membrane digestion, facilitating the escape of *A. phagocytophilum* from host-cell vacuoles and then its subsequent dissemination throughout the tick's body, including salivary glands [39,40].

Salp25D, an antioxidant protein identified in both the midgut and salivary glands of *I. scapularis*, is up-regulated following blood meals [41,42]. Injecting Salp25D-specific dsRNA into the tick body silences Salp25D salivary gland expression and impairs *B. burgdorferi* acquisition. However silencing midgut Salp25D expression by injecting dsRNA into the tick anal pore does not impact on *B. burgdorferi* acquisition, suggesting that the same protein may play different roles according to the organ concerned [42].

Defensins are components of the tick's innate immune system, protecting ticks from both Gram-negative and Gram-positive bacteria [43]. Accordingly, defensins are up-regulated in *R. montanensis*-infected *D. variabilis* [43]. Interestingly, varisin, a specific *D. variabilis* defensin, is also over-expressed in *A. marginale*-infected tick salivary glands, but is under-expressed in the midgut after feeding on pathogen-infected sheep, suggesting that *A. marginale* might down-regulate varisin expression to establish gut infection [44]. Silencing varisin expression via RNAi was predicted to increase tick bacterial infection levels. However silencing produced the opposite result, as levels of *A. marginale* were significantly reduced in tick midgut

after feeding on an infected calf [44].

Subolesin, another tick protective molecule discovered in *I. scapularis* [45], was proven to be up-regulated in *A. marginale*-infected ticks [46]. Both gene silencing or immunization with a subolesin recombinant protein results in lower *A. marginale*, *A. phagocytophilum* and *Babesia bigemina* infection levels in hard ticks, demonstrating no TBP species specificity [47-49]. In addition, oral vaccination of mice with vv-sub (vaccinia virus-expressed subolesin) reduces *B. burgdorferi* acquisition by *I. scapularis* larval ticks from infected mice, *B. burgdorferi* transmission to uninfected mice, as well as numbers of tick that have fully engorged [50]. Consequently, subolesin not only plays an important role in the acquisition and transmission of several pathogens, but also contributes to effective tick blood feeding. The correlation between tick subolesin expression and pathogen infection highlights subolesin's role in innate tick immune responses [51]. Alternatively, subolesin could up-regulate factors facilitating tick pathogen acquisition. Indeed, inhibiting subolesin expression results in lower pathogen infection levels, which could perhaps be influenced by other molecular pathways such as those required for gut and salivary gland function and development, resulting in the ingestion of less infected blood [48]. On the other hand, such inhibition may suppress the expression of other subolesin-regulated genes required for pathogen infection and multiplication [46].

During *A. phagocytophilum* acquisition by *I. scapularis*, α 1,3-fucosyltransferases expression is up-regulated in ticks [52]. Silencing three α 1,3-fucosyltransferases in *I.*

scapularis nymphs significantly decreases *A. phagocytophilum* acquisition from infected mice, but not tick engorgement and bacteria transmission from infected ticks to mice [52]. This strongly suggests that *A. phagocytophilum* modulates α 1,3-fucosyltransferase expression and utilizes α 1,3-fucose to colonize ticks during acquisition.

At the tick bite site, a strong innate immune response is initiated by the host's complement cascade [8]. Schuijt *et al* discovered that TSLPI (tick salivary lectin pathway inhibitor) interferes with the human lectin complement cascade, leading to decrease *Borrelia* lysis [53]. They suggest that TSLPI could play a crucial role in successful acquisition of *Borrelia* by *I. scapularis* from *Borrelia*-infected hosts. When pathogen-free *I. scapularis* larvae were engorged on *B. burgdorferi*-infected mice, which had been immunized with recombinant TSLPI protein, *Borrelia* acquisition by the larval ticks was effectively impaired, strengthening TSLPI's predicted role [53].

Silencing putative GST (glutathione S-transferase) and vATPase (H^+ transporting lysosomal vacuolar proton pump) genes in *D. variabilis* ticks, inhibits *A. marginale* infection after tick feeding on infected calves [51]. It was hypothesized that GST may protect tick gut cells from oxidative stress caused by *A. marginale* infection, and vATPase might facilitate *A. marginale* infection in tick gut and salivary glands by receptor-mediated endocytosis.

Tick factors contributing to tick-borne pathogen multiplication or migration within ticks

The tick midgut is the first major defensive barrier against pathogen infection [54,55]. In order to first establish an infection and then promote transmission, pathogens need to be able to successfully overcome this barrier (by colonizing cells, or by passing through or between cells) [56]. Pathogens imbibed during the blood meal must contend with heterophagic blood meal digestion, escape the midgut, and then migrate via the hemolymph to the salivary glands, where a second round of multiplication often occurs, culminating during transmission feeding and often dependent upon resumption of tick feeding. Following multiplication, TBP are transmitted via the saliva to the new host; the efficiency of this process can be influenced by the replication level [56]. These complex migration/multiplication processes are sure to require diverse molecular interactions between the TBP and the vector.

To date, only the tick protein TROSPA (tick receptor outer surface protein A), identified in *I. scapularis* ticks infected with *B. burgdorferi*, is thought to influence the TBP life-cycle in the midgut [23]. TROSPA is a specific ligand for *B. burgdorferi* OspA, and is required for successful spirochetes colonization of tick midgut [23]. Blocking TROSPA with antisera, or silencing TROSPA expression via RNAi, reduced the ability of *B. burgdorferi* to adhere to the tick gut *in vivo*, thereby preventing efficient colonization of the vector and reducing pathogen transmission to the mammalian host [23].

The TRE31 *I. scapularis* tick gut protein is involved in *B. burgdorferi* migration

from tick midgut to salivary glands [24]. Knocking down TRE31 expression by directly injecting TRE31-dsRNA into the gut of *B. burgdorferi*-infected *I. scapularis* nymphs, results in unchanged numbers of gut *B. burgdorferi*, but significantly fewer spirochetes in tick hemolymph and salivary glands [24], suggesting that TRE31 likely enables spirochetes migration from tick midgut to salivary glands. Interestingly, it was demonstrated that *B. burgdorferi* outer-surface lipoprotein BBE31 can interact with TRE31, and that anti-BBE31 antibodies also decreases numbers of *Borrelia* entering the hemolymph [24].

P11, an *I. scapularis* salivary gland secreted protein, is up-regulated in response to *A. phagocytophilum* infection and facilitates migration of *A. phagocytophilum* from tick midgut to salivary glands [57]. Silencing P11 impairs effectively *A. phagocytophilum* infection of tick haemocytes *in vivo* and consequently, decreases pathogen infection levels both in haemolymph and in salivary glands [57]. P11 is thought to enable haemocyte infection by *A. phagocytophilum*, permitting pathogen dissemination into the tick body [57].

Silencing *D. variabilis* tick GST and SelM (salivary selenoprotein M) genes showed that *A. marginale* multiplication was inhibited in salivary glands after tick TBP acquisition from infected calves [51]. *A. marginale* may increase GST and SelM expression to reduce oxidative stress caused by pathogen infection that may help pathogen multiplication in tick cells.

Finally, the *I. scapularis* protein TSLPI previously mentioned, is also thought to

be implicated in spirochetal multiplication within ticks [53]. Indeed, when some larvae were fed on *Borrelia*-infected mice passively immunized with rTSPLI antiserum, the succeeding nymphal stage had lower spirochetal loads than control group [53].

Tick factors contributing to tick-borne pathogen transmission to vertebrate hosts

In most transmission cases, pathogens present in tick salivary gland cells invade vertebrate hosts at the skin site where ticks have salivated during blood feeding [8]. Some factors present in the saliva are then used by microorganisms to increase their pathogenicity and evade host immune responses [8-10]. A few of these factors have been identified and are listed below.

Salp15 is a salivary gland protein expressed by both *I. scapularis* and *I. ricinus* ticks during engorgement [41,58]. During blood feeding, *B. burgdorferi* induces and usurps Salp15 to facilitate murine infection [22]. Silencing Salp15 in *I. scapularis* drastically reduces the capacity of *B. burgdorferi* to infect mice [22]. Salp15 affects T-cell proliferation by binding to the CD4 (+) co-receptor [59] and inhibits dendritic cell activation by binding to the C-type lectin DC-SIGN [60]. When binding to *B. burgdorferi* outer surface protein C (OspC) [22], Salp15 protects the bacteria from antibody-mediated killing, and inhibits keratinocyte inflammation [61].

I. scapularis tick histamine release factor (tHRF) also contributes to tick engorgement and host-transmission of *B. burgdorferi* [62]. Silencing tHRF by RNAi significantly decreases *B. burgdorferi* burden in mouse heart and joints, and markedly

impairs tick feeding. Moreover, the *B. burgdorferi* tick burden is substantially lower in *I. scapularis* fed on tHRF antiserum-immunized mice, and the spirochete burden is markedly reduced in these mice [62].

During the rapid tick-feeding phase, tick sensitivity to histamine declines [63,64], and expression of HBPs (histamine binding proteins) decreases from 48 to 72 h post-tick attachment, whereas tHRF increases from 0 to 48 h post-tick attachment [62]. It has been speculated that the reciprocal expression of HBPs and tHRF may augment local histamine concentration at the tick-feeding site during the rapid feeding phase, thereby modulating vascular permeability and enhancing blood flow which in turn facilitates tick engorgement [62]. Moreover, the vasodilatory effect of histamine might contribute to the efficient dissemination of *Borrelia* from the original tick-feeding site to distal sites [62].

To determine TSPLI's role in *B. burgdorferi* transmission from tick to host, TSLPI-dsRNA was injected into *B. burgdorferi*-infected *I. scapularis* nymphs, or rTSLPI rabbit antiserum was used to immunize mice [53]. *Borrelia* transmission to mice was impaired via TSLPI-silenced nymphs, as well as from nymphs to rTSLPI antiserum-immunized mice demonstrating that TSLPI plays a significant role in the transmission of *Borrelia* from arthropod vectors to vertebrate hosts [53]. Indeed, in each case, the spirochete burden was significantly lower after seven days in mice skin and heart, and after 21 days in mice joints. It is known that both classical and alternative complement pathways are involved in complement-dependent killing of

Borrelia [65]. Schuijt *et al* demonstrated that TSLPI inhibits direct killing of *B. burgdorferi* by the complement system, inhibits phagocytosis of *B. burgdorferi* by human neutrophils, as well as *Borrelia*-induced complement-mediated chemotaxis, by directly inhibiting the activation of the MBL (mannose-binding lectin) complement pathway [53].

Tick factors inhibiting tick-borne pathogen acquisition and transmission

An *I. scapularis* salivary gland gene family encoding 5.3-kD proteins, which are up-regulated by the tick signaling transducer activator of transcription (STAT) pathway and by *A. phagocytophilum* infection, might belong to a novel antimicrobial peptide (AMP) gene family [66,67]. When silencing a member of 5.3-kD protein gene family (gene-15), the *A. phagocytophilum* infection of tick salivary glands and transmission to mammalian host were significantly increased [67]. Therefore, the salivary gland gene family encoding 5.3-kD proteins is involved in anti-*A. phagocytophilum* defense. It is the only reported tick factor which can both inhibit tick-borne pathogen acquisition and transmission. This function probably contributes to its regulation by the tick's STAT pathway, which also plays a role in controlling *A. phagocytophilum* infection in ticks and transmission to the host [67].

Finally, one *D. variabilis* kunitz protease inhibitor (DvKPI) was found to be up-regulated both by blood feeding and *Rickettsia montanensis* infection [68]. When silencing DvKPI, the bacterial colonization of tick midgut was increased to 90% [69], suggesting that this molecule can limit *R. montanensis* acquisition by ticks, possibly

by limiting bacterial host cell invasion.

Conclusion

The interactions existing between ticks and tick-borne pathogens are complex. Interacting tick factors function in a finely tuned equilibrium to influence pathogen transmission. Several tick immune factors impede pathogen expansion, whereas some factors promote pathogen infection during their transmission from one infected host to another. It is now firmly established that tick-borne pathogen infection induces differential expression of tick genes. However, a global analysis both at the transcriptional or protein levels, similar to those presented in this review, does not enable us to differentiate whether tick responses are due to a specific pathogen that has co-evolved with the tick, or whether such tick responses may belong to an innate immune response to any invading organism. Moreover, genes that are thought to be regulated during pathogen development need to be confirmed with functional studies. Therefore, with the development of newer and more efficient biological techniques, such as RNAi, we expect rapid progress in the elucidation of the molecular mechanisms governing pathogen transmission by ticks.

Delineating the specific pathogen and tick ligands required for TBP acquisition, development and transmission, should lead to the development of new TBP-targeting strategies. Such factors could become candidates for anti-tick and anti-TBP vaccines, providing novel approaches to preventing tick-borne diseases. Indeed, in light of our

limited understanding of immunity to TBPs, TBP strain diversity, and more generally the transmission of multiple TBPs by the same tick species, vaccine strategies that target conserved tick components playing key roles in vector infestation and vector capacity have become particularly attractive [5]. Anti-tick vaccines based on recombinant antigens are environmentally safe, are less likely to select for resistant strains compared to acaricides, and can incorporate multiple antigens to target a broad range of tick species and their associated TBPs [6]. Anti-tick vaccines could potentially indirectly reduce TBD transmission by reducing the tick burden, or directly, through interference with tick components that enhance TBP transmission. For vaccines acting indirectly, reduction in tick burden is unlikely to be achieved unless the targeted tick species feeds principally on the host species for which the vaccine is intended. While this holds true for *R. microplus* and cattle [70], it does not for several species of ticks responsible for important TBD, such as *Ixodes* sp, for which a direct effect on transmission must be sought.

Key Learning Points

- The route of tick-borne pathogens from an infected vertebrate host to a new host via hard ticks is composed of three major steps; 1) acquisition of the pathogen by ticks, 2) pathogen expansion and movement within ticks, and 3) pathogen transmission from an infected tick to a vertebrate host.
- The expression of some tick factors can be modulated in response to pathogen infection, and these factors can impact on the pathogenic life cycle.
- Tick factors contributing to tick-borne pathogen transmission are potential vaccine candidates for controlling tick-borne disease.

Key Papers in the Field

- McNally KL, Mitzel DN, Anderson JM, Ribeiro JM, Valenzuela JG, et al. (2012) Differential salivary gland transcript expression profile in *Ixodes scapularis* nymphs upon feeding or flavivirus infection. *Ticks Tick Borne Dis* 3: 18-26.
- Rachinsky A, Guerrero FD, Scoles GA (2007) Differential protein expression in ovaries of uninfected and *Babesia*-infected southern cattle ticks, *Rhipicephalus (Boophilus) microplus*. *Insect Biochem Mol Biol* 37: 1291-1308.
- Ramamoorthi N, Narasimhan S, Pal U, Bao F, Yang XF, et al. (2005) The Lyme disease agent exploits a tick protein to infect the mammalian host. *Nature* 436: 573-577.
- Pal U, Li X, Wang T, Montgomery RR, Ramamoorthi N, et al. (2004) TROSPA, an *Ixodes scapularis* receptor for *Borrelia burgdorferi*. *Cell* 119: 457-468.

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Figure Legend

Figure 1. Possible TBP transmission route from an infected host to a new host, via hard ticks.

Note that pathogen multiplication can occur in both the tick midgut or salivary glands, depending on the pathogen. Arrows indicate migrating pathogen pathways.

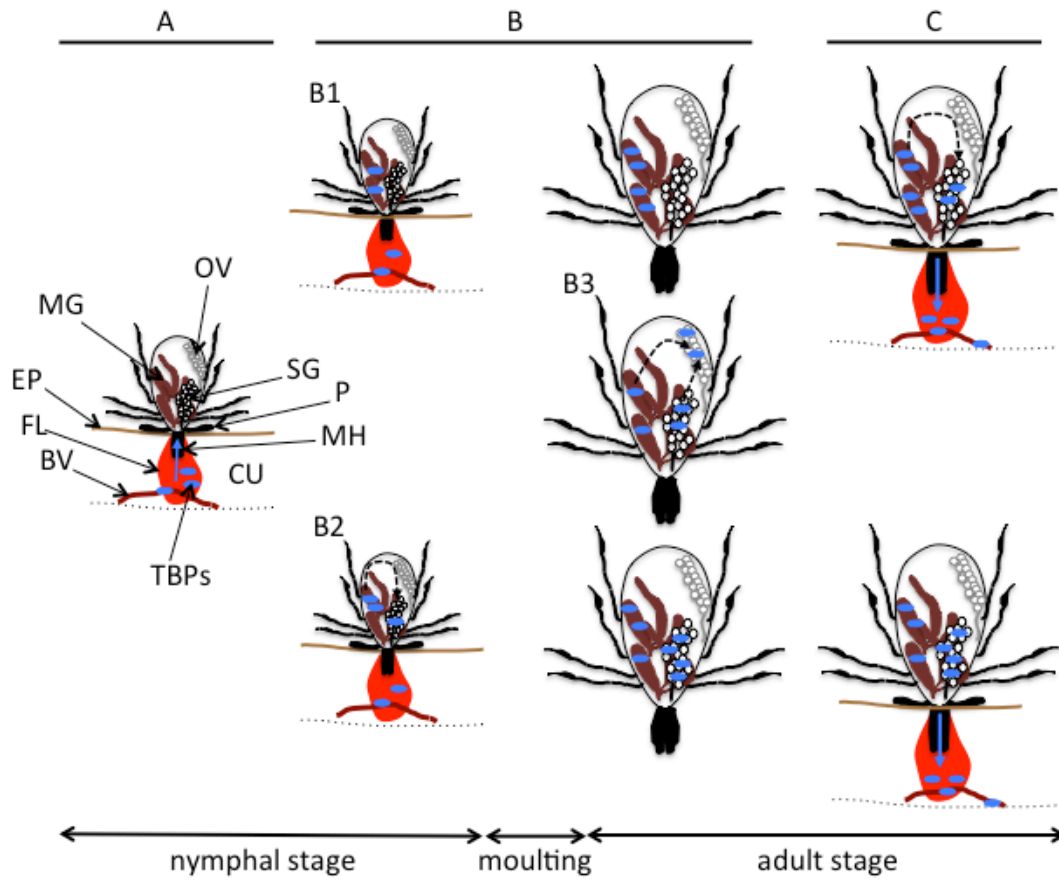
A: Acquisition of TBP by a nymphal stage tick during blood feeding;

B: TBP development within the tick; preservation in the tick gut (B1); dissemination into the hemolymph and migration to the salivary glands, which can occur either immediately after acquisition (B2) or after the stimulus of a new blood meal (C); dissemination into the hemolymph and migration to the ovaries (B3), which may or may not occur, and which can lead to transovarial transmission and infection of the succeeding generation;

C: TBP transmission from the subsequent adult tick stage to a new vertebrate host during blood feeding;

BV: blood vessel; CU: cutis; EP: epidermis; FL: feeding lesion; MG: midgut; MH: mouthparts (chelicera and hypostome); OV: ovaries; P: palp; TBP: tick-borne pathogens; SG: salivary glands. Small blue ovals represent TBP.

Figure 1.



Tables

Table 1. Functional transcriptomic/proteomic tick and TBP interaction studies.

Tick species	Tick organs	Tick-borne pathogens	Technique used	Number of differentially expressed transcripts/proteins	Refs
Transcriptomic studies					
<i>D. variabilis</i> female	SG, MG, OV	<i>R. montanensis</i>	DD-PCR	54	[11]
<i>I. scapularis</i> nymph	SG	<i>B. burgdorferi</i>	LCS	10	[14]
<i>I. scapularis</i> nymph	WT	<i>Langat virus</i>	MH	48	[17]
<i>I. scapularis</i> embryos	IDE8 tick cells	<i>A. marginale</i>	SSH	35	[15]
<i>I. ricinus</i> female	WT	<i>B. burgdorferi</i>	SH	11	[13]
<i>R. appendiculatus</i> female	SG	<i>T. parva</i>	LCS	3	[12]
<i>R. microplus</i> male	SG	<i>A. marginale</i>	SSH	99	[16]
Proteomic studies					
<i>I. scapularis</i> embryos	IDE8 tick cells	<i>A. marginale</i>	2D-DIGE, MALDI-TOF MS	3	[15]
<i>I. scapularis</i> embryos	ISE6 tick cells	<i>A. phagocytophilum</i>	IEF, 2D-DIGE, MALDI-TOF MS, RP-LC MS/MS	5	[20]
<i>R. bursa</i> female	WIO	<i>T. annulata</i>	2D-DIGE, RP-LC MS/MS, MALDI-TOF MS	16	[21]
<i>R. microplus</i> female	OV	<i>B. bovis</i>	IEF, 1/2DGE, HPLC-ESI-MS/MS	19	[18]
<i>R. microplus</i> female	MG	<i>B. bovis</i>	IEF, 1/2DGE, HPLC-ESI-MS/MS	20	[19]
<i>R. sanguineus</i> female	WIO	<i>R. conorii</i>	2D-DIGE, RP-LC MS/MS, MALDI-TOF MS	10	[21]
<i>R. sanguineus</i> female	WIO	<i>E. canis</i>	2D-DIGE, RP-LC MS/MS, MALDI-TOF MS	6	[21]
<i>R. turanicus</i> female	WT	<i>A. ovis</i>	IEF, 2D-DIGE, MALDI-TOF MS, RP-LC MS/MS	50	[20]

<i>R. turanicus</i> female	WIO	<i>A. ovis</i>	2D-DIGE, RP-LC MALDI-TOF MS	MS/MS,	9	[21]
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SG: salivary glands, MG: midgut, OV: ovaries, WT: whole ticks, WIO: whole internal organs;
 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.

Table 2. Hard tick factors, which contribute to/ inhibit TBP acquisition, multiplication and migration, and transmission.

Tick species	Tick factors	Genbank accession number	Tick-borne pathogens	Expression level in pathogen infected ticks	Pathogen life cycle modified	Refs
<i>D. variabilis</i>	GST	DQ224235	<i>A. marginale</i>	Up-regulation	Acquisition, multiplication	[15,51]
	Subolesin	AY652657	<i>A. marginale</i>	Up-regulation	Acquisition, transmission	[46,47,51]
	varisin	AY181027	<i>A. marginale</i>	Down-regulation (MD), Up-regulation (SG)	Acquisition, multiplication	[44]
<i>I. scapularis</i>	vATPase	ES429091	<i>A. marginale</i>	Up-regulation	Acquisition	[15,51]
	SelM	ES429105	<i>A. marginale</i>	Up-regulation	Multiplication	[15,51]
	P11	DQ066011	<i>A. phagocytophilum</i>	Up-regulation	Acquisition, migration	[57]
	Salp15	AF209914	<i>B. burgdorferi</i>	Up-regulation	Transmission	[22,58]
	Salp16	AF061845	<i>A. phagocytophilum</i>	Up-regulation	Acquisition	[38]
	Salp25D	AF209911	<i>B. burgdorferi</i>	No-change	Acquisition	[22,42]
	Subolesin	AY652654	<i>A. phagocytophilum</i>	No-change	Acquisition	[47,49]
	Subolesin	AY652654	<i>B. burgdorferi</i>	Unknown	Acquisition, transmission	[50]
	tHRF	DQ066335	<i>B. burgdorferi</i>	Up-regulation	Transmission	[62]
	TROSPA	AY189148	<i>B. burgdorferi</i>	Up-regulation	Multiplication	[23]
	TRE31	HQ998856	<i>B. burgdorferi</i>	Up-regulation	Migration	[24]
TSLPI	AEE89466	<i>B. burgdorferi</i>	Up-regulation, then down-regulation	Acquisition, transmission, multiplication	[53]	
<i>R. microplus</i>	α 1, 3-fucosyltransferases	XM_002401196	<i>A. phagocytophilum</i>	Up-regulation	Acquisition	[52]
		XM_002404622				
		XM_002406085				
		XM_002415522				
<i>R. microplus</i>	Subolesin	DQ159966	<i>A. marginale, B. bigemina</i>	Up-regulation	Acquisition	[46,48]
<i>D. variabilis</i>	DvKPI	EU265775	<i>R. montanensis</i>	Up-regulation	Acquisition	[68,69]
<i>I. scapularis</i>	5.3-kD protein	EEC00268	<i>A. phagocytophilum</i>	Up-regulation	Acquisition and transmission	[67]

SG: salivary glands, MG: midgut.

II.5. TBD vaccine strategies based on tick molecules

Currently, tick control is essentially based on acaricides, while their use has generated a lot of problems such as the selection of acaricide-resistant ticks, environmental contamination and contamination of milk and meat products with drug residues (review in [67]). New approaches that are environmentally sustainable and that provide broad protection against current and future TBPs are then urgently needed and vaccines against tick molecules are promising in this purpose [67]. For controlling TBP transmission, such vaccines could possibly act directly or indirectly; directly through interference with tick components that enhance TBP transmission; indirectly through a reduction of tick population.

To date, TickGARD, which is made of a *Rhipicephalus (Boophilus) microplus* tick midgut protein, Bm86, is the only commercially available anti-tick vaccine (in Australia and Cuba), acting only against *R. microplus* [68]. This vaccine is believed to lyse the tick gut wall, thus interfering with feeding and subsequent egg production. Thus, the vaccination impact on TBDs is secondary to its effect on tick viability or infestation. However, reduction in tick burden and hence incidence of TBDs are unlikely to be achieved unless the targeted tick species feeds only on the host species for which the vaccine is intended. While this holds true for *R. microplus* and cattle, it does not for several species of ticks responsible for important TBDs, such as *Ixodes* spp., for which a direct effect on vector capacity must thus be sought.

In light of these considerations, the great achievement will probably become true to best reduce TBP infection with good candidate antigens, which have the function of both controlling tick infestations and several TBP transmission. Recent application of reverse vaccinology to the development of anti-tick vaccines has led to discover promising candidate antigens, which are subolesin and its orthologs [69].

Nevertheless, it is difficult to get a high efficacy of both controlling tick infestations and several TBPs transmission with only one type of antigen. Therefore, vaccine efficacy would be increased by the use of multiple antigens (“cocktails”). To identify such tick components, screening should ideally be focused on proteins highly-expressed in tick saliva, and more particularly on proteins whose expression is induced during tick salivary gland in response to TBP infection.

III. OBJECTIVES

The general objective of this thesis is to identify molecular interactions between *I. ricinus* and *B. henselae*, and find some targets that may be used as vaccines against ticks and TBPs in the future. More precisely, the first objective is to identify *I. ricinus* salivary gland differentially expressed transcripts in response to *B. henselae* infection with next generation sequencing techniques (454 pyrosequencing and HiSeq 2000). The second objective is to identify the role of one of the proteins coded by these transcripts in tick feeding and *B. henselae* transmission processes.

For this purpose, we used the membrane-feeding technique to infect *I. ricinus* with *B. henselae*. Thus, in the first part of my PhD, I evaluated the use of this technique for *I. ricinus* infection by *B. henselae*. Additionally, the influence of blood origin and feeding system on tick feeding were also evaluated.

IV. EXPERIMENTAL STUDIES

IV.1. Evaluation of membrane feeding for infecting *I. ricinus* with *Bartonella* spp.

IV.1.1. Introduction to article 1

In previous studies, it has been demonstrated that molting and egg-laying success of membrane-fed ticks are comparable to animal fed ticks, and that the final engorgement weight of membrane-fed ticks tends to be equal or lower than that of animal fed ticks [70-72]. That demonstrates that membrane-feeding technique is an effective tool for tick rearing but few statistical comparison has been done between both techniques until now. Moreover, it was reported that ticks could be well infected by TBPs via artificial membrane feeding technique [14,22,38,70-72], but no study was interested in the impact of blood infection by pathogens nor blood origin on tick feeding.

Thus, in the first part of my PhD, I focused my interest on evaluating the impact of several factors including feeding systems, origin and infectious status of the blood meal on *I. ricinus* feeding behavior. In order to compare the effects of feeding method on several tick engorgement parameters, *I. ricinus* ticks were separately fed on an artificial membrane feeding system and on mice. Sheep and chicken blood were also used to analyze the effects of blood origin on tick engorgement via membrane feeding. Finally, to investigate the effects of infectious status of blood on tick engorgement, ticks were fed with *Bartonella* spp.-infected versus uninfected blood, both via membrane feeding technique and on mice.

This study has been submitted to the journal “Tick and Tick Borne Diseases”, and is presented below with the format required by the journal.

IV.1.2. Article 1

Impact of feeding system and infection status on *Ixodes ricinus* feeding

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Abstract

Artificial membrane feeding systems are effective tools for both tick rearing and studying tick-borne pathogen transmission. In order to compare the effects of the type of feeding system on tick engorgement, *Ixodes ricinus* ticks were either fed on an artificial membrane feeding system, or live mice. Sheep and chicken blood were used with the membrane system to assess the effects of blood origin on tick engorgement. To investigate the effects of blood meal infection on tick engorgement, ticks were either fed with *Bartonella*-infected or uninfected blood, both via membrane feeding and on mice. The proportion of engorged ticks, the duration of tick feeding, and the weight of engorged ticks were assessed. Feeding on the artificial system led to a longer duration of tick feeding and a lower proportion of engorged ticks than when fed on mice, however, the weight of engorged ticks was unaffected. The proportion and weight of engorged ticks, as well as the duration of feeding were not affected by blood origin. Feeding on an infected blood meal or on infected mice decreased the proportion and the weight of engorged ticks, but did not affect tick feeding duration.

Keywords

Ixodes ricinus, *Bartonella* spp., *in vitro/vivo* feeding

Introduction

Ticks are haematophagous arthropods that feed on mammals, birds and reptiles; and many tick species are also vectors for bacteria, parasites and viruses (de la Fuente et al., 2008). The emergence or re-emergence of tick-borne diseases is becoming an increasing problem for both humans and livestock (Dantas-Torres et al., 2012), however, current knowledge of tick-borne pathogen transmission is incomplete. Therefore the study of tick-host-pathogen interaction is of increasing importance in order to control tick-borne diseases. These types of studies require large numbers of live ticks, which need to be raised under controlled conditions in order to perform experimental infections.

The most popular tick infection model is direct feeding on animals infected with pathogens (Bonnet and Liu, 2012). The use of natural infectious hosts to infect ticks is, of course, the method closest to the physiological reality. However, the acquisition, housing, and handling of animal hosts can be complicated, expensive and infeasible. In fact, in some cases, and for wildlife studies in particular, maintaining the natural host of a specific tick-borne pathogen in the laboratory is impossible. For this reason, artificial infection systems have been developed. Several different artificial infection methods exist, such as infection by injection, capillary feeding, or artificial membrane feeding systems (see review by (Bonnet and Liu, 2012)). Of these techniques, artificial membrane feeding systems more closely mimic the natural conditions of tick

infection than other methods as pathogens are added to the blood meal and subsequently infect the tick via the natural route (Bonnet and Liu, 2012).

However, very few studies have aimed to compare tick engorgement via membrane feeding systems with directly feeding on the animal, and none of them concerned *Ixodes ricinus* ticks. For *Rhipicephalus* and *Amblyomma* genus, it has been reported that molting and egg-laying success of membrane-fed ticks is comparable to animal-fed ticks, but that the final engorgement weight of membrane-fed ticks tends to be equal to or lower than that of animal-fed ticks (Musyoki et al., 2004; Voigt et al., 1993; Young et al., 1996). In addition, ticks have successfully been infected with tick-borne pathogens via the artificial membrane feeding technique (see review by (Bonnet and Liu, 2012)), suggesting that this technique is an effective tool for tick infection. However, no studies have been performed to evaluate the effects of pathogen-infected blood on tick feeding, by comparing the proportion of engorged ticks, the duration of tick feeding, or the weight of engorged ticks between ticks engorged with infected or non-infected blood in the same conditions.

In this study, in order to compare the effects of feeding methods on such several tick engorgement parameters, *Ixodes ricinus* ticks were either fed on an artificial membrane feeding system or on mice. Blood of both sheep and chicken, which are among the preferential hosts of *I. ricinus* and from which sufficient quantities of blood can be taken without making the animals suffer, was used to determine the effects of blood origin on tick engorgement via membrane feeding. Finally, to

investigate the effects of pathogen-infected blood on tick engorgement, ticks were fed with *Bartonella* spp.-infected, versus uninfected blood, a model of transmission validated and routinely used in our laboratory (Bonnet et al., 2007; Cotte et al., 2008). *Bartonella henselae*, responsible for cat scratch disease, was used for experiments involving the membrane feeding system whereas, because of biosafety concerns associated with tick feeding upon cats infected with *B. henselae*, a murine model of bartonellosis: *Bartonella birtlesii* infecting mouse, was used for *in vivo* experiments.

Materials and methods

Animals and ethics statement

In order to obtain avian blood, six-month old chickens were housed in an avian facility of the CRBM (*Centre de Recherche Biomedicale*) based at the Alfort Veterinary School. Blood from the wing vein was collected into heparin-containing Venoject tubes at 10KU/mL (Terumo Europe, Leuven, Belgium) and maintained at 4°C until use in feeding experiments.

Four-week old OF1 female mice (Charles River Laboratories, L'Arbresle, France) were infected with *Bartonella birtlesii* by intravenously injecting 5×10^8 CFU in 100µL phosphate-buffered saline (PBS) directly into the tail vein of each mouse. Mouse infection status was confirmed by semi-nested PCR as previously described (Reis et al., 2011).

This study was carried out in strict accordance with good animal care practices recommended by the European guidelines. The protocol was approved by the Ethics Committee for Animal Experiments of ENVA (*Ecole Nationale Vétérinaire d'Alfort*) (Permit Number: 2008-11).

Bacterial strains

Bartonella birtlesii (IBS325^T) or *Bartonella henselae* (Houston-1 ATCC 49882) were grown on 5% defibrinated sheep blood Columbia agar plates incubated at 35°C in an atmosphere of 5% CO₂. After five to seven days of incubation, *B. birtlesii* and *B. henselae* were separately harvested and resuspended in sterile PBS before being used to inoculate mice or artificial feeding media.

Ticks

All experiments were performed with *I. ricinus* pathogen-free laboratory colony ticks, reared at 22°C with 95% relative humidity and with a 12h light/dark cycle as previously described (Bonnet et al., 2007).

Tick feeding

Ticks were checked each 12 hours and engorged nymphs were harvested, counted, weighed and maintained at 22°C and 95% relative humidity for molting. Each feeding process was performed in triplicate under the same conditions.

Nymphs feeding on artificial membrane feeding system

Groups of 250 nymphs were placed in an artificial membrane feeding system chamber as previously described (Bonnet et al., 2007) (Figure 1A). Briefly, the feeder

apparatus was closed with Parafilm® membrane at the top and with a rabbit skin membrane at the bottom. In order to attract the ticks, a constant temperature (37°C) was maintained by use of a water-jacket circulation system through the glass feeder. The culture box containing the ticks was placed under the feeding apparatus and 5 mL of blood, changed twice a day, were introduced until the ticks were replete. Each group of nymphal ticks was separately fed with either sheep blood (SB) (defibrinated, BioMérieux, Lyon, France), chicken blood (CB) or *B. henselae*-infected sheep blood (ISB). For this last sample, five µL of the *B. henselae* suspension at a concentration of 10⁹ CFU/mL in PBS was added to five mL sheep blood to reach a concentration of 10⁶ CFU/ml of blood in membrane feeders, a concentration that could be encountered in infected cats. All blood samples were treated with fosfomycin (100µg/mL), amphotericin B (250µg/mL) and heparin (10KU/mL) as previously described (Cotte et al., 2008).

Nymphs feeding on mice

At day 14 post-inoculation of mice, 25 nymphs were placed into a capsule on the back of each three *B. birtlesii*-infected mouse (IM) or three pathogen-free mice (M) as previously described (Reis et al., 2011) (Figure 1B).

Monitoring criteria

Three criteria were monitored: the proportion of engorged ticks, duration of tick feeding, and the weight of engorged ticks. The proportion of engorged ticks represents the number of nymphs successfully engorged versus the total number of nymphs, i.e.

the proportion of engorged ticks, which detached alone at the end of the blood meal. In order to analyze tick feeding duration, the feeding was divided into two phases (Anderson and Magnarelli, 2008). Phase I encompassed from the beginning of tick engorgement to the first evacuation of feces. This several-day period includes tick host-seeking, attachment to the membrane/animal skin, initiation of feeding, blood digestion and the evacuation of feces. Phase II corresponded to the time between the first fecal evacuation and subsequent tick detachment, indicating repletion. For all experiments, the time was noted when at least one tick had carried out the defined criteria. The weight of engorged ticks reflects blood meal volume; therefore 27 engorged ticks were weighed for each experimental condition.

Statistical analysis

The proportion of engorged ticks was analyzed by fitting a Generalized Linear Model (GLM) with binomial error structure (i.e. a logistic regression). As the data were over-dispersed, a dispersion parameter was estimated. The means and standard error of the mean (Van Den Abbeele et al., 2010) presented in the figures are those calculated after fitting to the model. Feeding duration was analyzed by fitting a GLM with Poisson error structure (i.e. log linear regression) and engorged tick weight was analyzed by fitting a GLM with normal error structure. Analyses were carried out using GenStat version 14.1. (VSN International Ltd., Hemel Hempstead, Hertfordshire, UK).

Results

Effects of feeding system on tick engorgement

The proportion of engorged ticks was higher when fed directly on mice ($88.6 \pm 3.7\%$, $n=75$) than when fed with sheep blood via membrane feeding ($47.7 \pm 1.7\%$, $n=750$) ($F_{1,4} = 47.2$, $P= 0.002$) (Figure 2A). In addition, the first phase of tick feeding was significantly longer ($F_{1,4}=28.2$, $P=0.006$) for nymphs fed on an artificial membrane system (5.3 ± 0.3 days, $n=750$) than for nymphs fed on mice (2.7 ± 0.3 days, $n=75$). The second phase did not significantly vary ($F_{1,4}=3.9$, $P=0.12$), where nymphs fed on mice took one day, and nymphs fed on the artificial membrane system took two days to detach (Figure 2B). The weight of engorged nymphs on the artificial membrane system was slightly, but not significantly, lower (3.38 ± 0.16 mg, $n=27$) than that of engorged nymphs on mice (3.61 ± 0.13 mg, $n=27$) ($F_{1,52} = 1.18$, $P=0.28$) (Figure 2C).

Effects of blood origin on tick engorgement

There were no significant differences between the proportion of ticks that became engorged when fed on sheep ($47.7 \pm 1.7\%$, $n=750$) vs. chicken blood ($55.0 \pm 3.3\%$, $n=750$) ($F_{1,4} = 3.74$, $P= 0.13$) via the membrane feeding system (Figure 3A). The duration of feeding (Phase I or Phase II) was not significantly different between sheep and chicken blood with a mean of 7.3 and 6.7 days, respectively ($P>0.4$) (Figure 3B), nor were subsequent tick weights different ($P>0.1$) (weight of engorged ticks, SB= 3.38 ± 0.16 mg, CB= 3.05 ± 0.20 mg, $n=27$ in each case), (Figure

3C).

Effects of blood meal infection on tick engorgement

We again found a significant increase in the proportion of ticks engorged on mice (M=88.6±3.7%, IM=83.3±1.9%, n=75) vs. membrane feeding with sheep blood (SB=47.7±1.7%, ISB=41.5±1.7%, n=750) ($F_{1,9} = 80.3$, $P < 0.001$) whether the blood is infected or not. The infection status of the blood meal (infected with *Bartonella* or not) resulted in a small but significant decrease in the proportion of engorged ticks ($F_{1,9} = 5.34$, $P = 0.046$) (Figure 4A). Phase I and Phase II tick feeding durations were not influenced by infection status of the blood meal ($F_{1,9} = 0.24$, $P = 0.64$ and $F_{1,9} = 0.19$, $P = 0.68$ respectively) either by membrane feeding or on mice (Figure 4B). By contrast, feeding on an infected blood meal resulted in a marginally significant decrease in weight ($F_{1,106} = 4.09$, $P = 0.046$, $n = 27$) (Figure 4C). There were no significant interaction effects between infection status and blood source for any of the measured outcome variables.

Discussion

Few previous studies have addressed the differences between *in vitro* and *in vivo* tick-feeding systems (Musyoki et al., 2004; Voigt et al., 1993; Young et al., 1996), and no study has focused on differences that could exist due to the type of blood used to feed ticks. In addition, there is little information concerning the difference between ticks fed with pathogen-infected blood compared to uninfected blood. In order to evaluate such differences, the proportion of engorged ticks, duration of tick feeding, and weight of engorged ticks was monitored in this study under several experimental conditions.

Here, we show that the proportion of engorged ticks is higher in mouse-fed compared to membrane-fed ticks. This has been previously observed in other tick species fed on membrane *vs.* bovine (Musyoki et al., 2004; Voigt et al., 1993; Young et al., 1996). Such a difference in feeding success may be attributed to the fact that host responses and stimuli are not present with artificial membrane feeding; in addition, the use antibiotic and antifungal components may also have an impact. Compared to other haematophagous arthropods, ixodid ticks feed at a slower rate, taking from three to ten days depending on the life stage (Krober and Guerin, 2007). An array of chemical and physical stimuli can facilitate tick attachment at feeding sites on the host (Guerenstein et al., 2000). For membrane feeding systems, several live animal stimuli have been used with success to encourage tick attachment and feeding (Bonnet and Liu, 2012). However, in this study, no animal stimulus was used, perhaps explaining why phase I of membrane-fed ticks is nearly three days longer

than that of mouse-fed ticks. Indeed, in phase II, there were no differences between mouse-fed and artificial membrane-fed ticks. Concerning the mean weight of engorged nymphs, we did not find any difference between mouse-fed and membrane-fed ticks, indicating that ticks retain a similar capacity to draw and digest blood either via a membrane or from an animal. In light of these results, we can then suppose that if the presence of antibiotics (in the *in vitro* system) did have an effect on tick feeding success, this effect would occur at the beginning of the blood meal (i.e. the motivation to continue with a blood meal) and not during the digestion phase.

We know from previous work that tick fitness and engorgement vary with the host spp. selected and that host blood quality may influence tick size (Brunner et al., 2011; Venzal and Estrada-Peña, 2006). For example, it has been reported that the mean weight of nymphs fed on mice was 3.5 mg, whereas the mean weight of bird-fed *I. ricinus* nymphs has been reported as 4.2 mg (Heylen et al., 2010). Such a difference linked to host characteristics may be due to both nutritive resources present in the blood, or to host immune responses which may reduce blood meal quality and therefore tick size (Bize et al., 2008). However, and although *I. ricinus* nymphs may have a preference for avian blood, we found no differences in any of the feeding variables resulting from either avian or mammalian blood.

It has also been reported that some vector-borne pathogens are capable of altering the feeding behavior of their vector, in order to increase pathogen acquisition and transmission (Cornet et al., 2013; Ferguson and Read, 2004; Koella et al., 1998;

Lacroix et al., 2005; Scholte et al., 2006; Van Den Abbeele et al., 2010). In our study, we found the opposite: feeding on *Bartonella*-infected blood decreased the proportion of engorged nymphs and reduced their subsequent weight. However the comparison between the ticks and the other models mentioned here must be taken with caution. In fact, and compared with other haematophagous arthropods, the feeding process of ixodid ticks is slow and complex, taking several days to several weeks for repletion and detachment alone (Sojka et al., 2013). This effect occurred in both the *in vivo* and *in vitro* systems, suggesting that the presence of the pathogen may directly reduce the motivation to blood-feed, rather than ticks responding indirectly to host cues of infection.

Conclusion

Even though artificial membrane feeding systems are less effective than animal feeding systems with regards to duration of tick feeding and proportion of engorged ticks, they do have many obvious advantages. For example, they permit the direct assessment of pathogen concentration in blood samples, facilitate repeated assays with large tick numbers, and most importantly, they can be used to infect ticks with particular pathogens in the absence of a live animal. Using this method, we were able to evaluate the influence of blood origin and pathogen presence. Whilst the former had no impact on tick feeding, the presence of *Bartonella* had a small but significant negative impact on feeding success. The reasons for this remain to be explored, and if elucidated, may have epidemiological significance.

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Figure Legends

Figure 1. View of the artificial membrane feeding system (A) and animal feeding model (B) used to engorge *I. ricinus* ticks.

Figure 2. Effect of feeding system on tick engorgement

I. ricinus nymphs were engorged both via artificial membrane system with sheep blood (SB) and on mice (M). Proportion of engorged ticks, duration of tick feeding and weight of engorged ticks were compared. Mean \pm SEM (Standard Error of the Mean), n: number of ticks.

Figure 3. Effects of blood origin on tick engorgement

I. ricinus nymphs were engorged via artificial membrane system with sheep blood (SB) and chicken blood (CB). Proportion of engorged ticks, duration of tick feeding and weight of engorged ticks were compared. Mean \pm SEM (Standard Error of the Mean), n: number of ticks.

Figure 4. Effects of blood meal infection on tick engorgement

I. ricinus nymphs were engorged via artificial membrane system with *B. henselae*-infected sheep (ISB) or uninfected blood (SB), and on *B. birtlesii*-infected mice (IM) and uninfected mice (M). Proportion of engorged ticks, duration of tick feeding and weight of engorged ticks were compared. Mean \pm SEM (Standard Error of the Mean), n: number of ticks

Figure 1. View of the artificial membrane feeding system and animal feeding model used in this study

A



B



Figure 2. Effect of feeding systems on tick engorgement

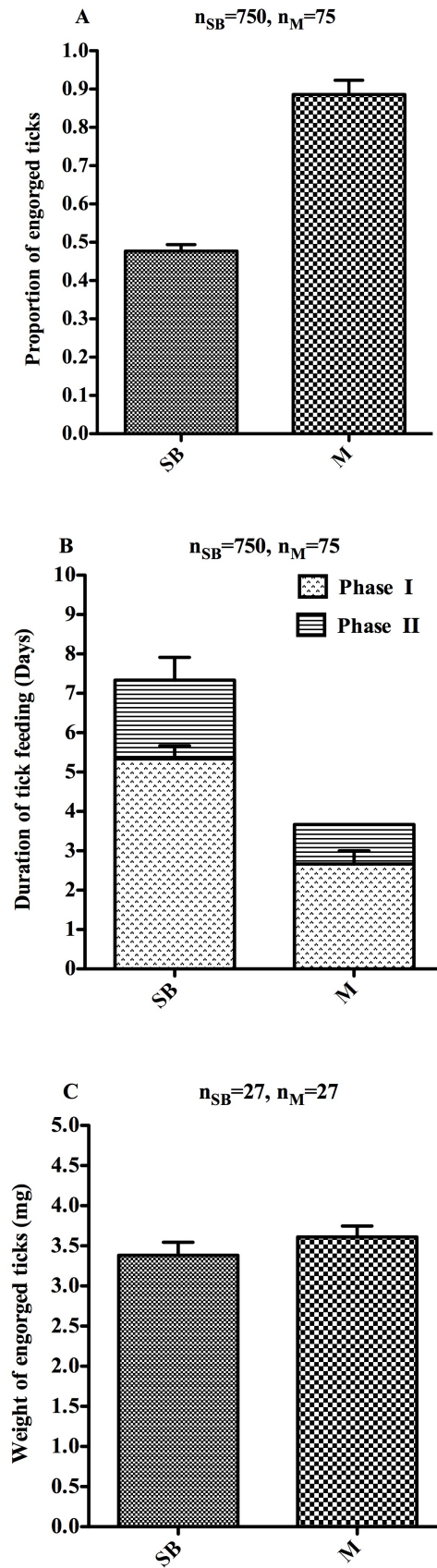


Figure 3. Effects of blood origin on tick engorgement

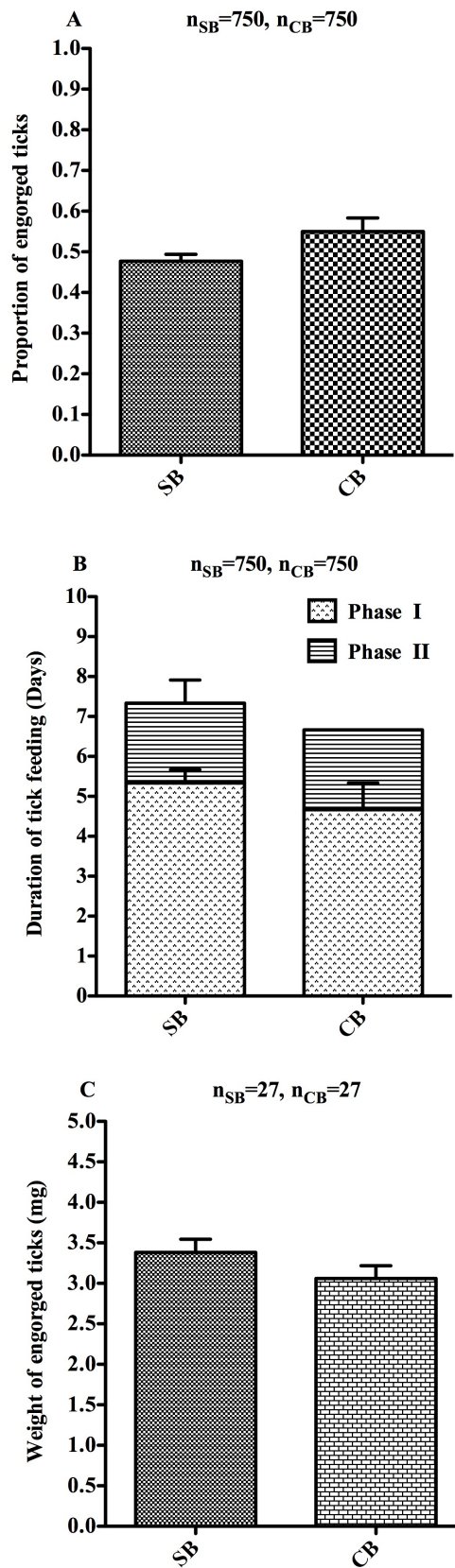
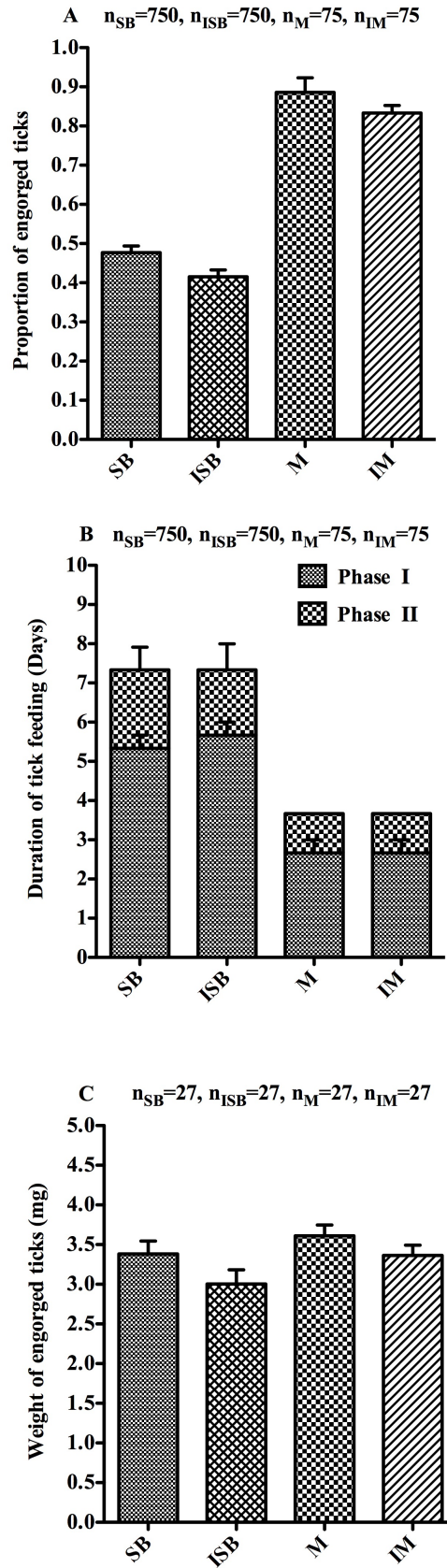


Figure 4. Effects of blood meal infection on tick engorgement



IV.1.3. Conclusion of article 1

Results obtained in this study confirmed that even if artificial membrane feeding led to a lower proportion of engorged ticks and a longer duration of tick feeding than direct feeding on animal, the weight of engorged ticks was unaffected. In addition, tick-feeding success was not affected by blood origin. At last, the proportion and weight of engorged ticks are decreased by *B. henselae* infection of the blood meal when tick-feeding duration was not affected. Taken together, these results show that membrane-feeding technique is an efficient tool for laboratory infection of *I. ricinus* by *B. henselae*, and was thus used for the continuation of our experiments.

IV.2. Analysis of *B. henselae*-infected *I. ricinus* salivary gland transcripts

IV.2.1. Introduction to article 2

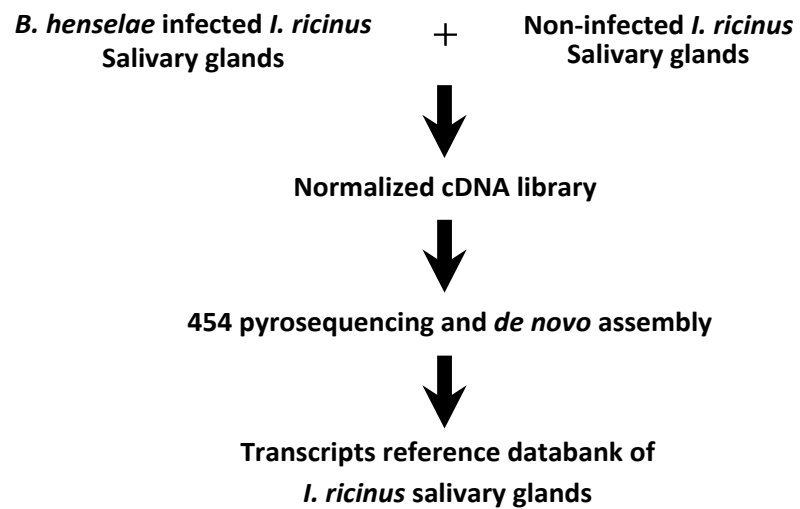
As mentioned in the introduction of this manuscript, new control strategies of tick populations and TBP transmission are urgently needed. Among them, those based on identification of novel transmission blocking target and specific molecules playing key roles in pathogen pathogenicity and/or survival, should be strongly focused on. In the past years, several studies have reported that tick salivary glands produce differentially expressed transcripts or proteins in response to pathogen infection, which may correspond to factors implicated in the transmission [59-66]. Indeed, some of proteins have been identified as able to enhance the transmission or acquisition of pathogens such as *Borrelia burgdorferi* [73-77], *Anaplasma phagocytophilum* and *Anaplasma marginale* [78].

The second part of my thesis aims to identify *I. ricinus* tick salivary gland transcripts that are over or under expressed in response to *B. henselae* infection. The strategy used to identify differentially expressed transcripts during *Bartonella* infection is presented in Figure 8. The tick sialome (transcripts expressed in the salivary glands) of *I. ricinus* infected and non-infected by *B. henselae* was sequenced with next generation sequencing techniques. In order to construct a transcriptomic reference databank of female *I. ricinus* SGs, the 454 pyrosequencing technique (leading to long sequences) was first used to sequence transcripts from *B. henselae*-infected and non-infected ticks. *De novo* assembly of all the obtained reads was performed and the result of assembly was reported for contigs and isotigs. The HiSeq2000 was then used for sequencing the transcriptome in both infected and non-infected ticks (leading to small fragments of around 50pb length) and their comparison allowed to select reads corresponding to the mRNA differentially expressed in response to the bacteria infection. Then, the alignment of HiSeq2000

reads against the transcriptomic reference databank obtained by 454 pyrosequencing, digital expression level calculation and bioinformatics analysis allowed the identification of *I. ricinus* SGs gene families significantly differentially expressed in response to infection with *B. henselae*. The expression profile of five representative transcripts was then validated using quantitative RT-PCR under the two different conditions. In addition, effective tool for investigating tick gene role, RNA interference (RNAi), was used to investigate the role in tick feeding and *B. henselae* transmission process of *IrSPI* (*Ixodes ricinus* Serine Protease Inhibitor) that belongs to the BPTI/Kunitz family of serine protease inhibitor, and which is the most highly expressed transcript in *I. ricinus* salivary glands during *B. henselae* infection.

This study and the results obtained are presented below as a manuscript in preparation.

1) Transcripts sequencing by 454: construction of a reference databank



2) Transcripts sequencing by HiSeq2000: identification of differentially expressed transcripts

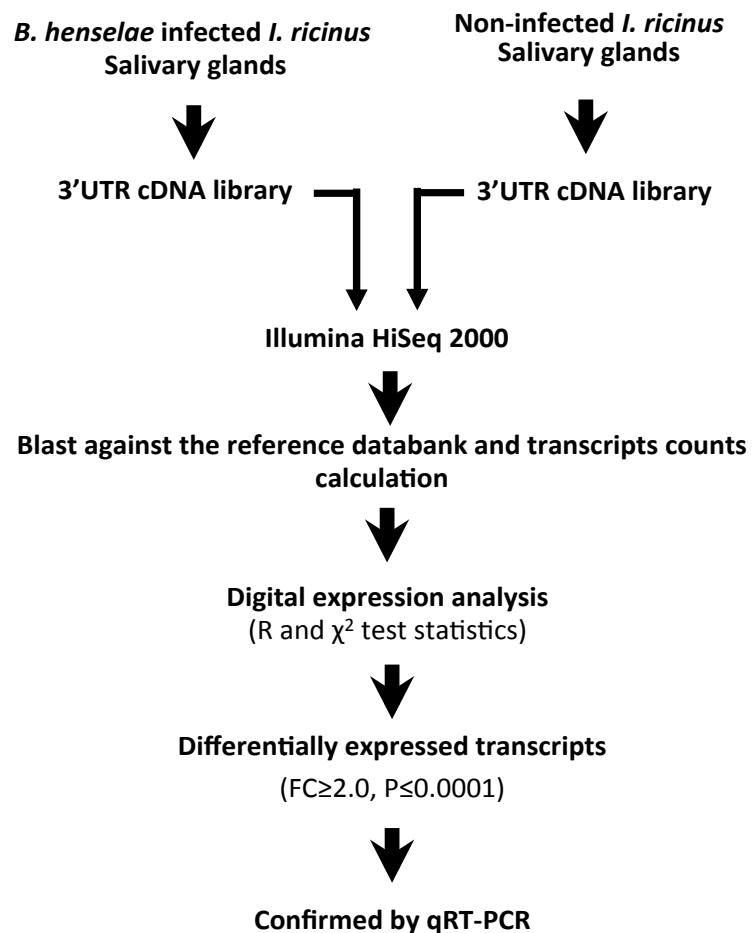


Figure 8. Diagram representation of the strategy used to identify *I. ricinus* differentially expressed transcripts during *B. henselae* infection.

IV.2.2. Article 2

High throughput sequencing of *Ixodes ricinus* salivary gland transcriptome analysis and identification of a tick serine protease inhibitor involved in tick feeding and *Bartonella henselae* infection

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Abstract

Ixodes ricinus is the most widespread and abundant tick in Europe, bites frequently humans, and is the vector of several pathogens including those responsible for Lyme disease, Tick Borne Encephalitis, anaplasmosis, babesiosis and bartonellosis. These tick-borne pathogens are transmitted to vertebrate hosts by saliva during the blood meal, and tick salivary gland factors are necessarily implicated in transmission. In order to identify such tick factors, the transcriptome of female *I. ricinus* salivary glands was sequenced by next generation sequencing techniques and compared between *Bartonella henselae* infected and non-infected ticks. The implication of the most up-regulated gene (*IrSPI*), in blood feeding and salivary glands infection by *B. henselae* was characterized by using RNA interference. The high throughput sequencing of *I. ricinus* salivary gland transcriptome led to 24,539 isotigs. 829 and 517 transcripts were significantly up- and down-regulated in response to bacteria infection, respectively. Sequence homologies researches showed that, among them, 161 transcripts corresponded to 9 groups of tick salivary gland gene families already described, while the other ones corresponded to genes of unknown function. The expression of five selected genes belong to BPTI/Kunitz family of serine protease inhibitor (including *IrSPI*), tick salivary peptide group 1 protein (20kDa), salp15 super-family protein (for two genes), and arthropod defensins, was validated by qRT-PCR. Silencing the most up-regulated gene (*IrSPI*) resulted in reduction of tick feeding and bacteria loaded in tick salivary glands. This study increases the available genomic information for *I. ricinus*, improves the knowledge to understand of the molecular interaction between tick and tick-borne pathogens, and provides a potential vaccine candidate to control tick-borne diseases.

Author summary

I. ricinus is the most common tick species in Europe, and acts as vector for several pathogens including bacteria from *Bartonella* genus. The mechanisms by which ticks modulate their gene expression in response to pathogen infection are poorly understood. In this report, we compared differentially expressed genes of tick salivary glands during *B. henselae* infection by using next generation sequencing techniques. This approach identified 829 and 517 transcripts significantly up- and down-regulated in response to bacteria infection, respectively. Among them 161 corresponded to 9 groups of ticks salivary gland gene families already described. By silencing the most up-regulated transcript (*IrSPI*), we demonstrated its implication in both tick feeding and bacteria infection of the salivary glands. This study demonstrated molecular dialogue existing between pathogen and its vector and provides, with *IrSPI*, a potential vaccine candidate to control bacteria transmission by ticks.

Introduction

Ticks are obligate blood-feeding ectoparasites of vertebrate hosts that transmit pathogens to humans and animals such as viruses, bacteria and protozoa. *Ixodes ricinus* (Acari: Ixodidae) is a three-life stage hard tick (larvae, nymphs and adult males and females; all of which require a blood meal except the adult male) that is one of the most common tick species in Western Europe. It is frequently associated with bites in humans, and is, among others, the vector of Tick-Borne Encephalitis virus, *Babesia* spp., *Borrelia burgdorferi* s.l., *Rickettsia* spp., and *Anaplasma phagocytophilum* [1]. The potential for the involvement of ticks in the transmission of *Bartonella* spp. has been heartily debated for many years because of the numerous, but indirect, evidence of its existence (see reviews by [2-4]). However, we have demonstrated that *I. ricinus* is a competent vector both for *Bartonella henselae* *in vitro* and for *Bartonella birtlesii* *in vivo* and that it corresponds to a good model to study the modalities of pathogen transmission by ticks [5,6]. *Bartonella* species are facultative intracellular gram-negative bacteria that are responsible for several diseases in humans and animals [7]. Currently, 13 *Bartonella* species or subspecies have been associated with a large spectrum of clinical syndromes in humans and among them, *B. henselae* is responsible for cat-scratch disease for which no vaccine exists to date [8]. This disease, possibly the most common zoonosis acquired from domestic animals in industrialized countries, is becoming increasingly associated with other symptoms, particularly ocular infections and endocarditis [9-11].

Compared with other haematophagous arthropods, feeding ixodid ticks is a slow and complex process, taking several days to several weeks for repletion and detachment alone [12]. This prolonged period of attachment has sparked great interest in studying tick salivary gland (SG) secretions during feeding. During the

blood-feeding process, ticks face effectively the problem of host haemostasis, inflammation and adaptive immunity and have evolved a complex and sophisticated pharmacological armamentarium against these barriers. Accordingly, saliva of blood-sucking ticks contains anti-clotting, anti-platelet aggregation, vasodilator, anti-inflammatory and immunomodulatory components that allow ticks to successfully feed (see reviews by [13-15]). Tick-borne pathogens are injected into the vertebrate host at the same time as tick saliva during the blood meal. Therefore, modulation of tick SGs protein expression during feeding is also linked to pathogen transmission and favor infection by interfering with host immunological responses [16]. In addition, several studies have reported that tick SGs produce differentially expressed transcripts in response to pathogen infection, some of them corresponding to factors implicated in pathogen transmission [17].

The first tick SGs gene expression analysis was performed in *Amblyomma variegatum* tick by sequencing about 4,000 cDNA clones [18]. Since then, many SGs transcriptome analysis have been performed with traditional sequencing based on the Sanger method, and for several tick species including *Dermacentor andersoni*, *Amblyomma americanum*, *A. cajennense*, *Rhipicephalus (Boophilus) microplus*, *I. pacificus*, *I. ricinus*, *I. scapularis*, *Ornithodoros coriaceus*, and *R. sanguineus* [17]. More recently, with the development of the next generation sequencing (NGS) techniques, higher transcriptome coverage and deeper insight into rare transcripts can be obtained and Schwarz A, *et al.* reported 272,220 contigs sequenced from SG transcriptomes of early- and late-feeding nymphs or adults *I. ricinus* [19]. As the primary rate-limiting step in the development of anti-tick vaccines is identification of protective antigenic targets [20], NGS techniques will provide a huge contribution in the investigation of vector and pathogen interactions, accelerating the process of

antigen discovery and thus vaccine development. Indeed, new approaches that are environmentally safe and that provide broad protection against current and future tick-borne pathogens are urgently needed, and one attractive solution is the development of vaccine strategies that target conserved components of ticks that play key roles in vector infestation or vector capacity [21].

The aim of this study is to identify tick genes involved in bacterial development and transmission to the vertebrate host in order to improve the understanding of the molecular interaction between tick and tick-borne pathogens, and to provide potential vaccine candidates to control tick-borne diseases. The model of *B. henselae* transmission by *Ixodes ricinus* was chosen for this purpose. Basing on the hypothesis that genes, which are regulated by the bacteria in the tick's SGs are implicated in such a transmission, the transcriptomes of SGs from infected and non-infected ticks were compared after high-throughput sequencing. Sequences of differentially expressed genes were then analyzed and compared to genes known to be implicated in tick-borne pathogen transmission in other models. The most up-regulated one was then chosen to validate its involvement in *B. henselae* infection and tick feeding.

Materials and methods

Ticks and bacterial strain

All the pathogen-free *I. ricinus* larvae derived from a laboratory colony reared at 22°C and 95% relative humidity with 12 h light/dark cycles [5]. *B. henselae* (Houston-1 ATCC 49882) was grown in 5% defibrinated sheep blood Columbia agar (CBA) plates incubated at 35°C in an atmosphere of 5% CO₂. After 7 days,

bacteria were harvested and suspended in sterile phosphate-buffered saline (PBS) before being used for artificial feeding of ticks [5].

Tick sample preparation

The method of artificial feeding used in this study was previously described [5]. Briefly, 5 mL of sheep blood (BioMérieux, Lyon, France) were added into feeders and changed twice every day until tick repletion. For *B. henselae* infected sheep blood feeder, 5 µL of the *B. henselae* suspension at a concentration of 10^9 CFU/mL in PBS was added to 5 mL sheep blood. After engorgement and infection, larvae were allowed to molt into nymphs. The same protocol was then applied in order to engorge *B. henselae*-infected nymphs with *B. henselae*-infected blood. Nymphs were then allowed to molt into adult females or males. For the multiplication and/or migration of *B. henselae* into the SGs [5], the resulting females were partially engorged 4 days with bacteria free blood before being dissected for the two groups of samples: *B. henselae*-infected *I. ricinus* (BIr) and non-infected *I. ricinus* (NIr). SGs were dissected on ice under a magnifying glass in sterile ice-cold 1X PBS. All the SGs were briefly washed in sterile ice-cold 1X PBS and immediately stored at -80°C until total RNA extraction and sequencing. All ticks from control groups were engorged following the same protocol without any infection of blood meals. The same protocol was used for RNAi experiments except that females were allowed to feed for 7 days before analysis.

Total RNA extraction

Total RNA was isolated from SGs using TRIzol[®] Reagent (Invitrogen, USA), RQ1 RNase-free DNase (Promega, USA) and RNasin[®] Ribonuclease Inhibitor

(Promega, USA) following the manufacturer's description. All RNA samples were pooled for each condition (B_{Ir} and N_{Ir}) and quality and quantity of total RNA was assessed with Nanodrop 2000 (Thermo SCIENTIFIC, USA). Thirty µg total RNA per sample, corresponding to 69 pairs of salivary glands, was sent to GATC Biotech AG (Konstanz, Germany) for cDNA synthesis and sequencing. Same extraction protocol was followed for RNA samples used in qRT-PCR.

***B. henselae*-infected and non-infected *I. ricinus* salivary gland transcript sequencing**

To generate the *I. ricinus* SGs reference transcriptome, the two total RNA samples (SGs from B_{Ir} and N_{Ir}) were pooled at equimolar concentrations and cDNA libraries were constructed and normalized before sequencing with GS FLX Titanium platform (454 pyrosequencing, Roche, CT, USA). After the sequencing primers and adapters were trimmed, *de novo* assembly of all the reads was performed with GS *De Novo* Assembler Software version V2.5.3 (454 Life Science Corp, CT, USA) and the result of assembly was reported for contigs and isotigs.

For comparison of the two transcriptome, B_{Ir}-SGs and N_{Ir}-SGs 3'UTR cDNA libraries were separately sequenced on the HiSeq2000 at GATC Biotech AG (Konstanz, Germany). The reads (50 bp length) data from all runs per sample were concatenated and polyA trimmed.

Transcript annotation

All the isotigs were imported into the BLAST2GO version 2.5.0 (www.blast2go.org) program for homology searches and Gene Ontology (GO) annotation. In the homology searches, the isotigs were compared against the NCBI nr

protein database using BlastX with E-value cutoff 1.0E-10. The blast results were used for mapping the consensus sequencing into GO terms and to summarize the distribution of the sequences into three main categories: Biological Process (BP), Cellular Components (CC) and Molecular Functions (MF).

The KEGG (Kyoto Encyclopedia of Genes and Genomes) automatic annotation server was used for gene ortholog assignment and pathway mapping for all the isotigs. Depending on the similarity hit against KEGG database using BlastX, the isotigs were assigned with the unique enzyme commission (EC) numbers. Distribution of isotigs under the respective EC numbers was used to map them to the KEGG biochemical pathway.

Analysis of differentially expressed transcripts between *B. henselae*-infected and non-infected *I. ricinus* salivary glands

Burrows-Wheeler Transform Aligner (BWA) [22] was used to align polyA trimmed HiSeq2000 reads against the *I. ricinus* SGs reference transcriptome, i.e. the isotigs data produced by 454 pyrosequencing. The resulting sequence alignment/map was used to calculate counts (number of reads that have mapped to reference).

The counts per isotigs were counted in BIr-SGs and NIr-SGs samples. Isotigs having counts lower than 5 were eliminated. To calculate relative expression profiles in infected ticks, relative abundance (RA) values were computed for each isotig per sample by dividing its sequence count by the total sequence count in the sample. Differentially expressed isotigs between infected and non-infected ticks were detected by using the R [23] and χ^2 test statistics with Bonferroni correction using the IDEG6 software (http://telethon.bio.unipd.it/bioinfo/IDEG6_form/) [24]. An isotig was considered to be significantly differentially expressed in response to *B. henselae*

infection when its RA had a fold change (FC) ≥ 2.0 and both statistical tests yielded significant values at $P \leq 0.0001$.

The open reading frame (ORF) of differentially expressed isotigs was determined by using the ORF finder webserver at www.ncbi.nlm.nih.gov/projects/gorf and the conserved domains searching for each differentially expressed isotig was done using conserved domains database (CDD) web sever version (CDD v3.03) at www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml.

Real time quantitative PCR

Validation of the expression profiles of some selected genes was performed by real time quantitative RT-PCR (qRT-PCR) on different SG samples obtained following the same protocol as for the NGS sequencing. First-strand cDNA was synthesized with SuperScriptTM III First-Strand Synthesis system for RT-PCR kit (Invitrogen) from 400ng total RNA. Each qPCR reaction was performed in 12 μ L with 0.2X LightCycler[®] 480 DNA SYBR Green I Master Mix (Roche), 1X of each primer and 2 μ L of template. Reactions were run with Roche LightCycler[®] 480 System under the following conditions: 95 °C 5 min; 95 °C, 10 s, 60 °C 15 s, 72 °C 15 s, 45cycles. Each sample was run in triplicate with results generated by Roche LightCycler[®] 480 Software V1.5.0. Relative quantification of gene expression was calculated by using the comparative Ct method [25]. The results were normalized using *I. ricinus* actin gene, and the sequence-specific primers used for qPCR are listed in Table 1. The statistical analysis was performed by two-tailed Student's *t* tests and significant values at $p \leq 0.0001$. Data analysis was performed with Prism 5.0 (GraphPad Software, Inc. USA), and results were expressed as mean \pm SEM (standard error of the mean).

***I. ricinus* serine protease inhibitor gene silencing by RNA interference**

The most up-regulated tick gene after *B. henselae* infection, which is a BPTI/Kunitz type serine protease inhibitor, was called *I. ricinus* serine protease inhibitor (*IrSPI*) (GenBank accession number: KF531922) and selected for functional analysis in ticks. Small interference RNA (siRNA) target sites were designed using the E-RNAi Webservice (www.dkfz.de/signaling/e-rnai3/idseq.php). No modification was done for siRNA sequences (Table 1), and they were synthesized in vitro using Stealth RNAi™ siRNA construction kit (Life technologies, France). The injection protocol was performed as previously described [26]. A total of 4nL (25µM) of siRNA (~10¹³ molecules) was microinjected into the body of female ticks. The control ticks received 4nL of nuclease free water (Life technologies, France).

To evaluate the influence of *IrSPI* gene silencing on tick feeding and *B. henselae* infection in SGs, some control and *IrSPI*-siRNA injected *B. henselae*-infected female ticks were fed on non-infected sheep blood via artificial membrane feeding system. Eight ticks were used in each group (control and siRNA injection). Ticks were weighted individually after a meal of 7 days and weight was compared between siRNA-injected group and control by Student's *t* test with unequal variance. Ticks were then dissected and one SG was used for total RNA (TRIzol® Reagent, Invitrogen, USA) extraction to confirm gene silencing by qRT-PCR with specific primers, while the other one was used for DNA (Wizard® genomic DNA purification kit, Promega, USA) extraction to detect *B. henselae* presence by qPCR with *B. henselae* 16S-23S intergenic spacer (ITS) gene primers [27] (Table 1). Quantitative PCR results were assessed by extrapolation from the standard curve and normalized to the *I. ricinus* actin. The statistical analysis of qPCR was performed by two-tailed Student's *t* tests.

A p value <0.05 was scored as a significant difference. Data analysis was performed with Prism 5.0 (GraphPad Software, Inc. USA), qPCR were performed in triplicate and results expressed as mean \pm SEM

Results

Tick samples

After engorgement of 4,548 larvae and resulting nymphs with *B. henselae* infected or non-infected sheep blood, 110 *B. henselae* infected *I. ricinus* females and 109 non-infected *I. ricinus* females were obtained. After partially feeding on sheep blood, 69 *B. henselae* infected females and 69 non-infected females were dissected for SGs preparation and total RNA isolation. A mean of 590 ng total RNA per SG was obtained.

***I. ricinus* salivary gland transcriptome analysis**

In order to obtain as many as possible transcripts from BIr-SGs and NIr-SGs, the normalized cDNA library was sequenced twice using GS FLX titanium platform. After trimming off the additional sequences (primers and adapters), all the reads were used for transcripts assembly, generating 30,853 contigs and 15,756 isogroups, which were composed of 24,539 isotigs (Table 2). The size description of the contigs and isotigs are shown in Figures 1A and 1B, respectively.

Sequence homologies between translated *I. ricinus* SGs isotigs and the nr protein database were identified with BlastX using Blast2GO software. Out of the 24,539 assembled isotig sequences, 14,736 sequences (60.1%) had significant similarity (E-value $\leq 1E-10$) with sequences present in the Genbank. Among them, 10,713 (72.7%) had their best alignment with *I. scapularis* sequences, 1,332 (9.0%) with *A.*

maculatum sequences, 568 (3.9%) with *I. ricinus*, 481 (3.3%) with *I. pacificus* sequences, and 63 (0.4%) with *I. persulcatus* sequences (Figure 2).

Blast results database was then used to annotate the isotigs with GO terms. Isotigs were classified according to the categories of biological process (BP) in which they may be implicated, cellular components (CC) in which they can be classified, and molecular function (MF) they may be related to. One or more GO IDs were assigned to 10,859 (44.3%) isotigs. The number of isotigs that could be annotated in BP, CC and MF categories were 5,308, 7,213 and 9,283, respectively. In the BP category, oxidation reduction (12.8%) was the most abundant GO term, followed by proteolysis (9.7%) (Figure 3A). In the CC category, the most abundant term was integral to membrane (11.4%), followed by nucleus (8.1%), cytosol (7.7%) and cytoplasm localization (7.4%) (Figure 3B). In the MF category, the most abundant term was binding proteins (63.2%) (Figure 3C).

The determination of the various biological pathways in which the obtained isotigs may be implied was performed using KEGG server. Some isotigs were assigned to more than one biological pathway. Out of the 24,539 isotigs analyzed, 2,465 may be implicated in metabolism pathways such as C5-Branched dibasic acid, Ether lipid, Starch and sucrose, or Fatty acid metabolism. 936 mapped isotigs were suspected to be implicated in biosynthesis pathways such as Cutin, suberine, wax, Glycosylphosphatidylinositol (GPI)-anchor, Novobiocin, phenylalanine, tyrosine and tryptophan biosynthesis. Additionally, 1,095 mapped isotigs may be implicated in 33 others pathways such as Glycolysis/Gluconeogenesis, Benzoate degradation, and Synthesis and degradation of ketone bodies.

Analysis of differently expressed transcripts between *B. henselae*-infected tick

salivary glands and non-infected ones

In order to investigate the differential expression of transcripts between BIr-SGs and NIr-SGs, the corresponding 3'UTR cDNA libraries were sequenced and generate 210 and 150 millions raw sequences reads, respectively. Isotigs with RA fold change (FC) ≥ 2 and $\chi^2 \leq 0.0001$ were selected as significantly differentially expressed leading to a percentage of 5.5% (1,346/24,539) of isotigs varied in their expression level during *B. henselae* infection. Of them, 829 isotigs were up-regulated in *B. henselae*-infected ticks and 517 isotigs were down-regulated after bacteria infection. Based on their sequence homologies with databases, these isotigs were classified in 3 groups of (a) proteins with homology to proteins of known function, (b) proteins with homology to proteins of unknown function and (c) proteins without homology (Table 3). Among the first group, proteins were classified into nine families of proteins, out of which four contained both up-regulated and down-regulated transcripts in response to pathogen infection, while five of them corresponded to transcripts that were down-regulated in response to infection (Table 3).

The expression of five selected transcripts was validated by qRT-PCR (Figure 4). The expression of 2 transcripts, which belong to BPTI/Kuntiz family of serine protease inhibitor (GenBank accession number: KF531922) and Salp15 superfamily protein (GenBank accession number: KF531924), was induced by *B. henselae* infection; and the expression of 3 transcripts, which belong to tick salivary peptide group1 protein (GenBank accession number: KF531923), Salp15 superfamily protein (GenBank accession number: KF531925), and arthropod defensins (GenBank accession number: KF531926), was reduced by *B. henselae* infection. The fold change (FC) calculation and statistical analysis ($p \leq 0.0001$) indicate a good correlation between the transcripts expression profile revealed by next generation

sequencing based data and transcripts abundance analysed by qRT-PCR (Figure 4).

Silencing *IrSPI* gene decreases tick feeding capacity as well as tick's SGs infection by *B. henselae*

RNAi was used to evaluate the effect of *IrSPI* silencing on tick feeding and tick salivary gland infection by *B. henselae*. In *B. henselae* infected ticks, *IrSPI* transcript abundance was suppressed 90% ($p=0.001$) in ticks that received *IrSPI*-siRNA oligonucleotide compared to that ticks that received control injection (Figure 5A). The mean weights of siRNA-injected *B. henselae*-infected female were significantly decreased 1.6-fold ($12.7\text{mg} \pm 1.7$ vs. $20.3\text{mg} \pm 2.1$), when compared to controls (Figure 5B). *B. henselae* loaded within SGs was significantly reduced 2.5-fold in *IrSPI*-siRNA injected tick when compared to controls ($1.6 \times 10^{-4} \pm 0.1$ and $3.9 \times 10^{-4} \pm 0.2$ per actin gene copy, respectively) (Figure 5C).

Discussion

In this study, the transcriptome of bacteria-infected *I. ricinus* female SGs was characterized for the first time by using next generation sequencing techniques, leading to a very important source of new data on this medically important vector and its molecular relationships with TBPs. Major groups of identified genes included those encoding for proteins involved in protein binding, oxidation reduction or proteolysis, and that are integral to membrane, nuclear or cytoplasmic. These results provided a reference databank for the *I. ricinus* SG transcriptome, which is particularly important in the absence of *I. ricinus* genome sequence, and abundant genetic information about *I. ricinus* response to pathogen infection. Until now, the studies of tick SGs transcriptome contained hundreds or thousands of expressed sequence tags (ESTs) sequences [17], except for the most recent study of *I. ricinus* SGs transcriptome analysis performed using next-generation sequencing on early- and late-feeding nymphs or adults [19]. In this latest study, all ticks analyzed were collected from nature, fed on various animals, and without any indication of the sanitary status of the animals on which the ticks were able to feed. Indeed, even if this study, as ours, confirms a higher transcriptome coverage than classical methodologies and increases the available genomic information for *I. ricinus*, results on transcriptome dynamics during attachment to the host that are reported should be considered with precaution in the absence of data on the infected status of ticks that were compared.

In fact, in *I. ricinus* SGs, we reported that 5.5% of the identified isotigs varied in their expression level during *B. henselae* infection, reflecting probable molecular interactions between the pathogen and the vector. Balance between up and down-regulated genes suggested a co-evolutionary mechanism to guarantee both

pathogen and vector survival. Up-regulated genes may reflect tick responses to bacteria infection while down-regulated transcripts may reflect a manipulation by the bacterium with the aim of multiplying in the SGs and establishing an infection of the tick. After searching for sequence homologies in databases, some proteins with homology to proteins of known function were classified into nine families, which are discussed here, although we should keep in mind that having the same domain would not necessarily imply having the same function.

Ten isotigs which are down-regulated in response to *B. henselae* infection, presented high similarity with IxAC (*Ixodes* anti-complement) proteins that are implicated in tick blood feeding process [28]. None of these showed any functional domain, GO terms or implication in a biology pathway, but their high similarity with anti-complement proteins of ixodid ticks (82-100%) suggested an anti-complement activity. The alternative pathway of complement activation is an important defense mechanism in vertebrates and it has been demonstrated that SGs extracts of ixodid ticks can inhibit this pathway activity [29]. For blood feeding ectoparasites such as ticks, it is crucial to inhibit host complement alternative pathway to achieve blood feeding. Several studies have reported anti-complement proteins in ixodid ticks [28-32], some of which are up-regulated during blood feeding [32]. In our study, we found that the isotigs annotated as anti-complement proteins were down-regulated in response to *B. henselae* infection. As both conditions compared here corresponded to engorged ticks, anti-complement proteins could have been up-regulated in both infected and uninfected ticks but at a lesser extent in *B. henselae* infected ticks. It could be surprising that the bacterium down regulated anti-complement proteins because of the fatal impact of the complement on *Bartonella* spp., but *Bartonella* spp. possess their own defense system against the complement that may explain such a

regulation [33].

Four isotigs, all of them being down-regulated in response to *B. henselae* infection, harbored an arthropod defensin domain (Acc CDD: c103093) and are implicated in tick defense response process. Defensins are 3-4 KDa cationic antimicrobial peptides (AMPs) which contain six disulfide-paired cysteines [34]. The antimicrobial activity of defensins is mainly directed against Gram-positive bacteria, but some defensins have anti-Gram-negative bacteria activity [35-37]. In ticks, defensins are mainly expressed in the midgut after blood feeding [36,38-40], and sometimes in other organs such as SGs and ovaries [39]. It has been reported that defensins are up-regulated in the midgut of *O. moubata* after injection of *Escherichia coli* and *Micrococcus luteus* [41,42]. In the same way, in *D. variabilis* naturally infected with *A. marginale*, defensins are up-regulated after an injection of *E. coli*, *Bacillus subtilis* and *M. luteus* [43]. Interestingly, when ticks are infected with tick-borne pathogens, tick defensins present variable expression levels during blood feeding. In *R. montanensis* infected *D. variabilis* ticks, one defensin presented a down-regulation at 18 hours post feeding, an up-regulation between 24 and 48 hours, and a down-regulation at 72 hours in the midgut, whereas in the fat body, a down-regulation before 48 hours and an up-regulation at 72 hours post feeding was observed [44]. It was also reported that one contig annotated as defensin precursor was down-regulated in Langat virus (LGTV) infected *I. scapularis* ticks [45]. Thus, variable regulation including down-regulation of defensin expression was observed in the presence of pathogens that are transmitted by ticks as for *B. henselae* in this study. It could be hypothesized that defensins are up-regulated as a tick protective response to infection with non tick-borne pathogens. However, in the presence of tick-borne pathogens that have co-evolved with the tick vector, these pathogens can manipulate

defensin expression in order to suppress tick immune response for their survival, multiplication and transmission.

Six isotigs down-regulated in response to *B. henselae* infection presented high similarity with *I. pacificus* collagen-like salivary secreted peptide (CLSP) (70-92%). Functional domains, GO terms or implication in specific biological pathways were not identified for these isotigs. As the CLSP identified in *I. pacificus* are relatively glycine and proline rich, it was suggested that they could affect vascular biology and adhere to molecules that help tick attachment to host skin [30]. However, the function and expression of CLSP during blood feeding and pathogen transmission is unknown. Here, all the isotigs similar to CLSP were down-regulated in the presence of *B. henselae* and their role in pathogen infection and blood feeding needs to be determined.

Nine isotigs which were down-regulated in response to *B. henselae* infection showed to be involved in stress response biological process. Among them, 8 isotigs were highly similar to *I. scapularis* HSP20 protein (91-95%) and one to *I. scapularis* HSP70 protein (97%). Again, no implication in a potential biology pathway could be identified for any of the isotigs in this group. The heat shock response is a conserved reaction of cells and organisms to high temperatures and other stress conditions and is effected by HSPs [46]. These proteins can protect cells and organisms from damage, allow resumption of normal cellular and physiological activities, and overall provide higher levels tolerance to environmental stress [47]. It has been reported that HSP20 can protect tick cells from stress, impact tick behavior such as questing speed, and can be involved in the *I. scapularis* protective response to *A. phagocytophilum* infection [48]. However, these studies demonstrated that in the natural vector-pathogen relationship, HSPs and other stress response proteins were not strongly activated,

which likely resulted from tick-pathogen co-evolution [48]. The complexity of the tick stress response to infection was also evidenced in the results reported here, suggesting that some pathogens may induce down-regulation of tick heat shock response, likely to increase pathogen survival and multiplication.

Six isotigs showed high similarity to *I. scapularis* microplusin (90-98%), which belong to antimicrobials peptides, and all of them were down-regulated in *B. henselae* infected *I. ricinus* SGs. Functional domains, GO terms or biology pathways were not identified for these isotigs. Microplusins, which also belong to AMPs, were first isolated from the cattle tick *R. (Boophilus) microplus*, as antimicrobial peptides against the Gram-positive bacteria, *M. luteus* and the yeast, *Cryptococcus neoformans* [49,50]. They have been described as members of a family of cysteine-rich AMPs with histidine-rich regions at the N and C termini and have been detected in the hemocytes, ovaries and fat body of *R. microplus* ticks [49]. In *A. americanum*, microplusins are up-regulated before ticks begin to penetrate the host skin for blood feeding [51]. Recently, it was reported that two contigs annotated as Microplusin preprotein-like were down-regulated in Langat virus (LGTV) infected *I. scapularis* ticks [45]. Finding isotigs, with significant similarity to Microplusins, down-regulated after *Bartonella* infection, may suggest a possible co-evolution mechanism similar to that found with defensins.

Twenty-four up-regulated and 32 down regulated isotigs in response to *B. henselae* infection, had a salp15 super-family domain (Acc CDD: c113541). No GO terms or biological pathways could be determined for any of them except for an up-regulated isotig (isotig19777), which harbored metallopeptidase activity but without the associated metalloprotease domain. The salp15 super-family contains 15kDa salivary proteins from Acari that are induced by feeding [52]. Salp15 protein

was first identified as an *I. scapularis* salivary protein with multiple functions such as inhibition of CD4⁺ T cell activation, by specifically binding to the T cells co-receptor CD4 [52-54], and inhibition of cytokine expression by dendritic cells [55]. It has also been implicated in protection of *Borrelia* species, the Lyme disease agent, from complement and antibody-mediated killing by the host as well as allowing the bacteria to remain attached to tick cells [55,56]. During *I. scapularis* blood feeding, it has been shown that salp15 mRNA and protein levels were 13-fold and 1.6-fold higher, respectively, in engorged tick SGs infected with *B. burgdorferi* [56]. In addition, RNA interference-mediated salp15 knockdown in *I. scapularis* drastically reduced the capacity of these ticks to transmit *Borrelia* spirochetes to mice [56]. These findings demonstrated that *Borrelia* sp. exploits salp15 tick protein and is able to induce its expression to facilitate mammalian host infection. An up-regulation of salp15 was also reported in *I. persulcatus* during blood feeding [57]. In our study, 56 genes were identified as belonging to the salp15 family with the CDD domain, 24 of which were up-regulated and 32 down-regulated in response to bacteria infection. Based on the results obtained with *Borrelia* [56,57], it is possible to speculate that *Bartonella* sp. are also capable of increasing the production of some of the salp15 proteins to facilitate their transmission to the vertebrate host.

Forty isotigs were also identified as harboring a tick histamine binding domain (Acc CDD: cl03446): 14 were up-regulated and 26 down-regulated in response to *B. henselae* infection. All of them showed the binding GO molecular function, but any implication in a cellular component or biological process and pathways could be identified. HBPs are lipocalins with two binding sites. Lipocalins are small extracellular proteins that bind to histamine, serotonin and prostaglandin and are implicated in the regulation of cell homeostasis and vertebrate immune response

[58,59]. It has been reported that, out of three closely related HBPs isolated from fed *R. appendiculatus* SGs, two (Ra-HBP1 and Ra-HBP2) are female specific, whereas Ra-HBP3 is exclusively secreted by larvae, nymphs and adult male ticks [60]. It has also been demonstrated that tick female-specific HBPs are found only during the early feeding period, peaking about 48 hours after tick infestation [60]. Such findings showed that HBPs expression is also a dynamic progress during tick feeding and the results reported here with some up- and down-regulated genes after bacteria infection, suggested that HBPs might be also implicated in tick- *B. henselae* interactions.

Two up-regulated and 4 down-regulated isotigs in response to *B. henselae* infection, had a zinc-dependent metalloprotease domain (Acc CDD: c100064). The two up-regulated isotigs (isotig09315 and isotig10110) showed hydrolase and peptidase activity, respectively. All the down-regulated isotigs showed the same metalloprotease molecular function and two of them (isotig03163 and isotig07095) were implicated in proteolysis biological process. No implication in a potential biology pathway could be identified for any isotig in this group. The super-family of metalloproteases contains two major branches, the astacin-like proteases and the adamalysin/reprolysin-like proteases. In tick saliva, metalloproteases were classified as reprolysin-like proteases that contain a zinc-binding motif [61]. Metalloproteases have been described in the SGs of *I. scapularis* [61], *I. ricinus* [62], *Haemaphysalis longicornis* [63] and *R. microplus* [64], but have not been described in other hard tick tissues. The role of SGs metalloproteinases in tick feeding is supposed to be linked to anti-fibrinogen, anti-febrin and anti-hemostatic activities [61]. The hypothesis is that tick salivary metalloproteases, together with other salivary anti-hemostatic proteins, may favor pathogen dissemination through vertebrate host tissues after transmission by ticks [65]. These findings may explain up and down-regulation of metalloproteases

in response to *B. henselae* infection by increasing bacterial dissemination after tick transmission for up-regulated genes and by limiting this process as a host response to infection for down-regulated genes. The balance between these two processes may be essential for both bacteria and tick survival.

Seven up-regulated and seventeen down regulated isotigs in response to *B. henselae* infection have a BPTI/Kunitz domain (Acc CDD: c100101). GO molecular function analysis showed that all isotigs of this group except one (isotig20663, which showed extracellular matrix structural constituent function) have serine-type endopeptidase or peptidase inhibitor activity. No biological pathway was identified for this group of isotigs. BPTI/Kunitz domain is present in an ancient and widespread group of polypeptides containing a disulfide-rich alpha+beta fold that is stabilized by three highly conserved disulfide bridges [66]. With phylogenetic analysis, Schwart *et al.* recently demonstrated that multiple Kunitz domain proteins with more than 3 Kunitz domains appeared widely distributed in different tick species, and, among arthropods, have evolved only in ticks [19]. In hard ticks, BPTI/Kunitz proteins can modulate blood feeding, and disrupt host angiogenesis and wound healing [67]. These proteins are considered vital for hard ticks survival and constitute a potential therapeutic target against ticks and tick-borne pathogens transmission [67]. They belong to the class of protease inhibitors that are the most highly secreted group of proteins represented in the *I. ricinus* SG transcriptome according to Schwarz *et al.* [19]. According to the cysteine patterns of BPTI/Kunitz, Dai *et al* [68] clustered 80 ixodid tick BPTI/Kunitz proteins into three clades (groups I, II and III). In *I. scapularis* and *I. ricinus*, genes from group II are expressed in the middle and late stages of blood feeding, with the exception of *Isc.218* gene that begins to be expressed at 6-12 hours, increases strikingly at 18-24 hours and decreases rapidly at 72 hours

after tick attachment, while genes from group III are only expressed in the late stage of blood feeding [68]. The expression of BPTI/Kunitz proteins is thus a dynamic process during long term blood feeding, a fact that may contribute to the finding of both up and down-regulated BPTI/Kunitz family of serine protease inhibitor genes during *B. henselae* infection.

Silencing *IrSPI*, the most up-regulated gene during bacteria infection that belongs to BPTI/Kunitz family, confirmed the fact that Kunitz proteins contribute to tick blood feeding as tick weight is decreased when the expression of *IrSPI* is impaired. Our results showed also that *IrSPI* has an impact on *B. henselae* development in *I. ricinus* SGs as we demonstrated that silencing *IrSPI* decreases *B. henselae* level in *I. ricinus* SGs, suggesting that *IrSPI* could play a role in SGs invasion by bacteria and/or in bacteria multiplication in SGs during the stimulus of the blood meal [5]. In parallel, *IrSPI* gene expression is induced by *B. henselae* infection in *I. ricinus* SGs at 4 days, that is in accordance with *DvKPI* (*Dermacentor variabilis* kunitz protease inhibitor) expression in *Rickettsia montanensis* infected *D. variabilis* tick midgut [69]. However, silencing *DvKPI* gene enhanced rickettsial colonization of the tick midgut, suggesting that this protein implicated in the defense response, limiting *R. montanensis* invasion [70]. We observed here the opposite result as silencing of *IrSPI* impairs *B. henselae* invasion of SGs. Such a discrepancy is in accordance with the different regulation observed for proteins belonging to BPTI/Kunitz family, which may reflect different functions. In addition, it should be reminded that results obtained with *IrSPI* (this study) and *DvKPI* [70] have been obtained in different tick species, with different pathogens, and concerned different tick organ. It can be hypothesis that, in *I. ricinus* SGs, *IrSPI* is putatively involved with adhesion/invasion/multiplication of *B. henselae*, but also with stress/defense

response as *DvKPI*. Indeed, its over-expression due to infection by foreign bacteria may decrease the amount of other bacteria species in competition with *B. henselae*, allowing its colonization of the SGs. As an example, it has been reported that silencing expression of varisin who belongs to defensin, reduced *A. marginale* infection in *D. variabilis* [71]. Other investigations are then now needed in order to elucidate the role of *IrSPI* and to evaluate the vaccine potential of this molecule in a context of an anti-tick and a transmission-blocking vaccine against *B. henselae* and other tick-borne pathogens.

Conclusion

Results of this study show that the *B. henselae* / *I ricinus* represents a good model for the study of the molecular interactions between ticks and transmitted bacteria. Although the results obtained have to be interpreted carefully because of the use of artificial membrane feeding (avoiding the host responses and using antibiotic and antifungal components), the comparison between infected and non-infected ticks was done in the same conditions validating that differential expression is due to the presence of the bacteria. However, the fact that our study was performed by artificial feeding (because of the difficulties in manipulation of cats, natural hosts of *B. henselae*), implies that expression of selected genes as well as their implication in the bacteria transmission should be confirmed in *in vivo* system. In fact, physiologic changes in SGs are likely to be influenced by host factors that might not be accurately mimicked during artificial feeding. The *B. birtlesii* / laboratory mouse model will then be uses in that way [6].

Our data on differential expression of tick genes during bacteria infection reflect the molecular strategy employed by both tick and bacteria to ensure their survival and development. To analyze in detail the role of genes identified here will lead in the future to a better understanding of the molecular dialogue between the two partners, an essential finding to envisage TBPs transmission blocking strategies.

As a high up-regulated transcript during *B. henselae* infection acting on bacteria development as well as on tick feeding, IrSPI may represent a very interesting candidate to be tested as a vaccine against ticks and bacteria transmission. Indeed, highly effective anti-tick vaccines should reduce both tick burden and vector competence. The deployment of a vaccine designed to reduce transmission of tick-borne pathogens by *I. ricinus* would represent a major improvement over current

control measures as regards to environmental conservation and occupational exposure to tick-borne pathogens.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SB and MVT designed experiments. XYL was responsible for preparing tick and RNA samples, bioinformatics analysis, and performing quantitative real time PCRs and RNAi. MC was responsible for tick feeding. SM participated to qPCR and RNAi experiments. JDLF and RCG were responsible for digital expression analysis. XYL and SB wrote the manuscript. All authors read, corrected and approved the final manuscript.

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Figure Legends

Figure 1: Size description of the transcripts generated by *de novo* assembly of the quality filtered and trimmed 454 pyrosequencing reads using GS *de novo* assembler version 2.5.3 from *B. henselae*-infected and non-infected *I. ricinus* female salivary gland: A) contigs, B) isotigs

Figure 2: Distribution of percentage similarity from the top hit in protein database of transcripts expressed in *B. henselae*-infected and non-infected *I. ricinus* female salivary gland

Figure 3: Gene ontology assignments of transcripts expressed in *B. henselae* infected and non- infected *I. ricinus* female salivary gland: A) Biological Progress, B) Cellular Component, C) Molecular Function

Figure 4: Comparison of the expression profile of 5 *I. ricinus* genes by next generation sequencing data (NGSD) and qRT-PCR analysis in *B. henselae*-infected ticks and non-infected ones

The figure shows differential expression of 5 genes. KF531922 (*IrSPI*) and KF531924 respectively associated with BPTI/Kuntiz family of serine protease inhibitor (*IrSPI*) and Salp15 superfamily protein, which were up-regulated in *B. henselae* infected *I. ricinus* females SGs. KF531923, KF531925, and KF531926 respectively associated with tick salivary peptide group1 protein (20kDa), Salp15 super family protein, and arthropod defensins, which were down-regulated in *B. henselae* infected *I. ricinus* females SGs. The fold changes (FC) were converted into \log_2 values. Error bars of qRT-PCR show the SEM (standard error of the mean). The statistical tests yielded significant values at *** $P \leq 0.0001$.

Figure 5: Influence of *IrSPI* silencing on tick feeding and tick SGs infection by *B. henselae*

IrSPI-siRNA (siRNA) or nuclease free water (control) were microinjected into the body of *B. henselae*-infected *I. ricinus* females before ticks took a non-infected blood meal during 7 days.

A) Quantitative RT-PCR analysis of *IrSPI* gene expression levels in pools of 8 tick SGs from *IrSPI*-siRNA injected ticks and controls. The results are represented as the mean \pm SEM of qRT-PCR performed in triplicated.

B) Weight evaluation of *IrSPI*-siRNA injected ticks body mass compared to controls. The results are represented as the mean \pm SEM of 8 ticks weighted individually.

C) Quantitative PCR analysis of bacteria loaded in pools of 8 tick SGs from *IrSPI*-siRNA injected ticks and controls. The results are represented as the mean \pm SEM of qPCR performed in triplicated.

Figure 1A

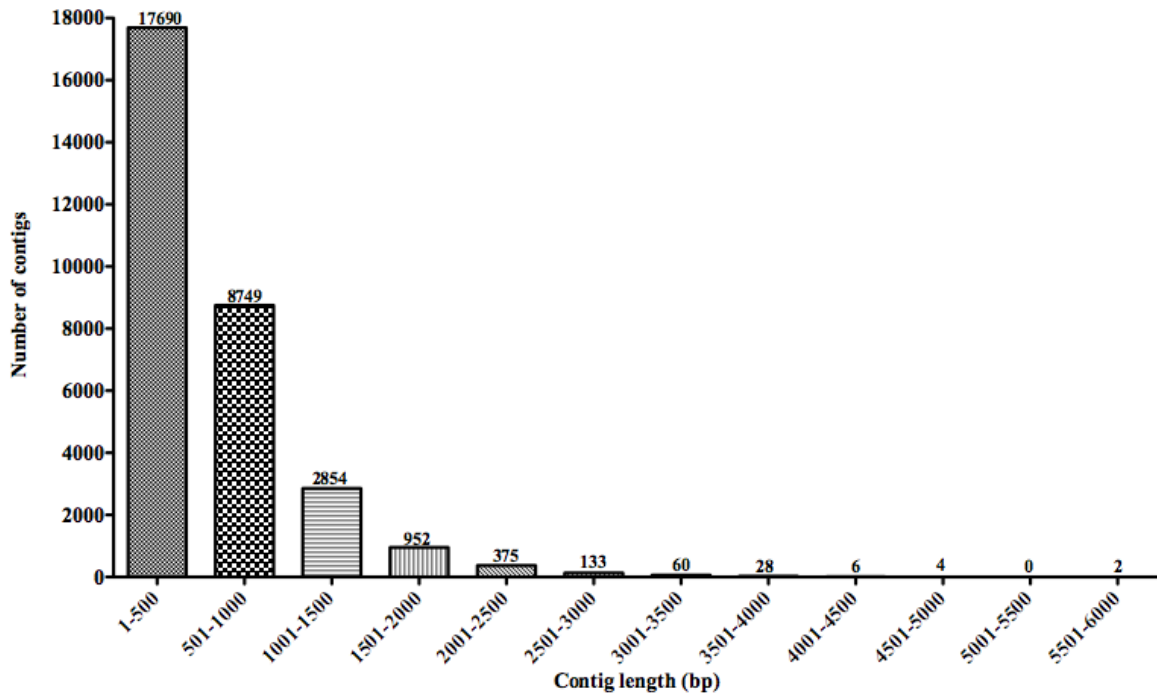


Figure 1B

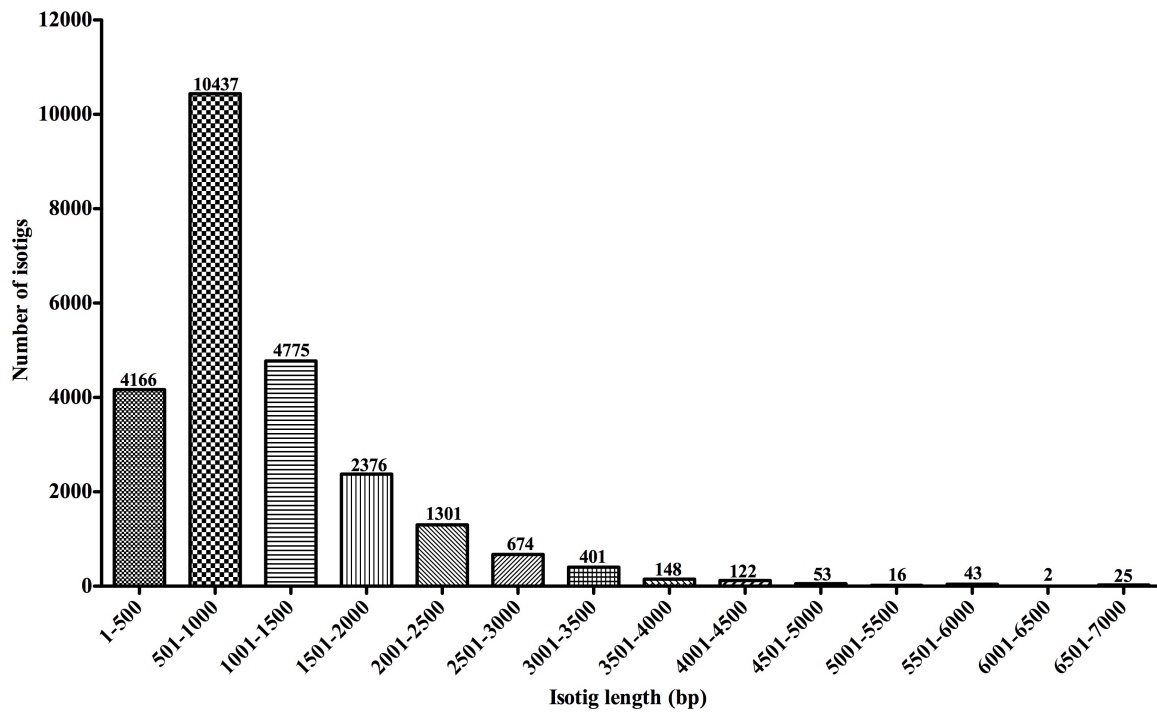


Figure 2

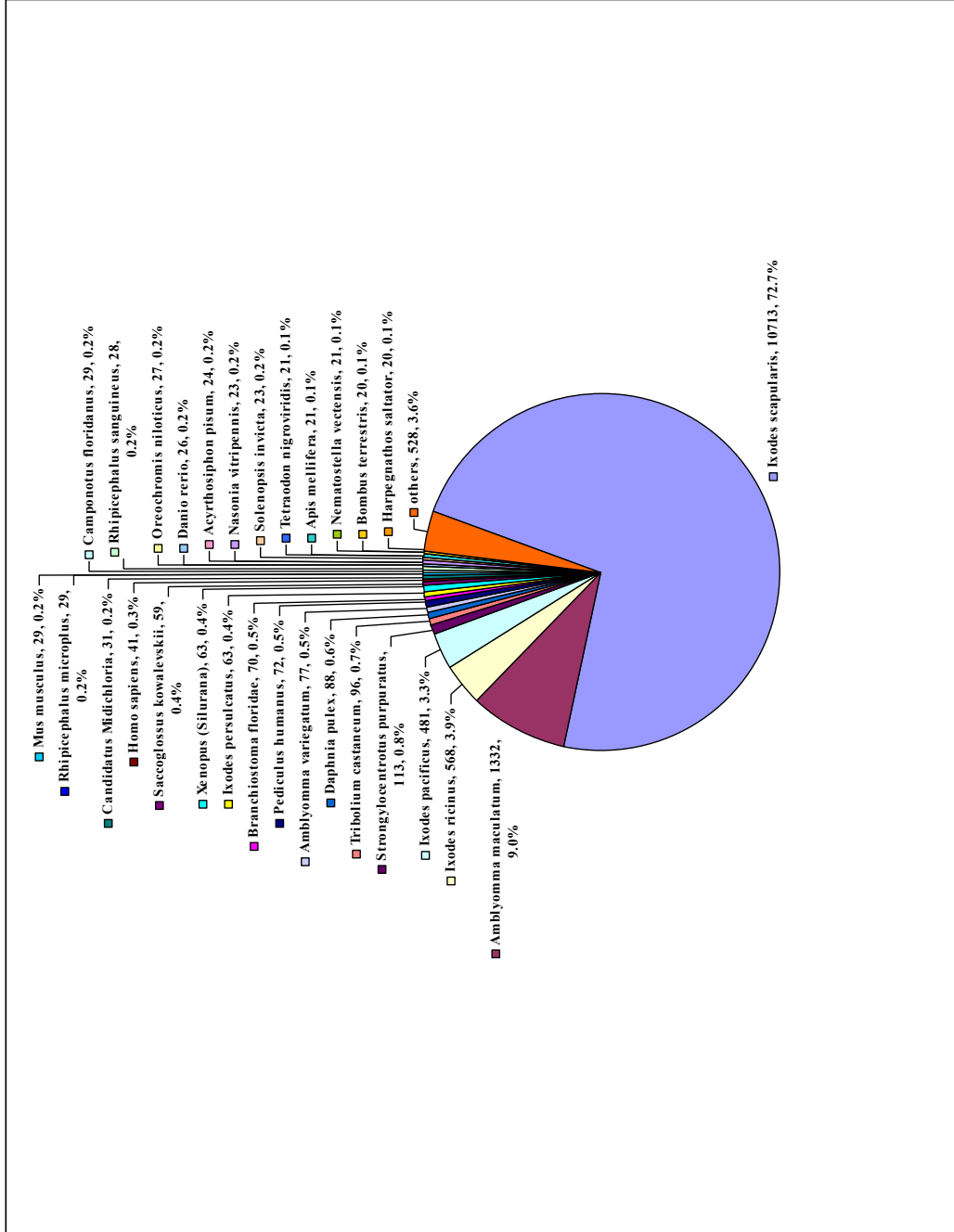


Figure 3A

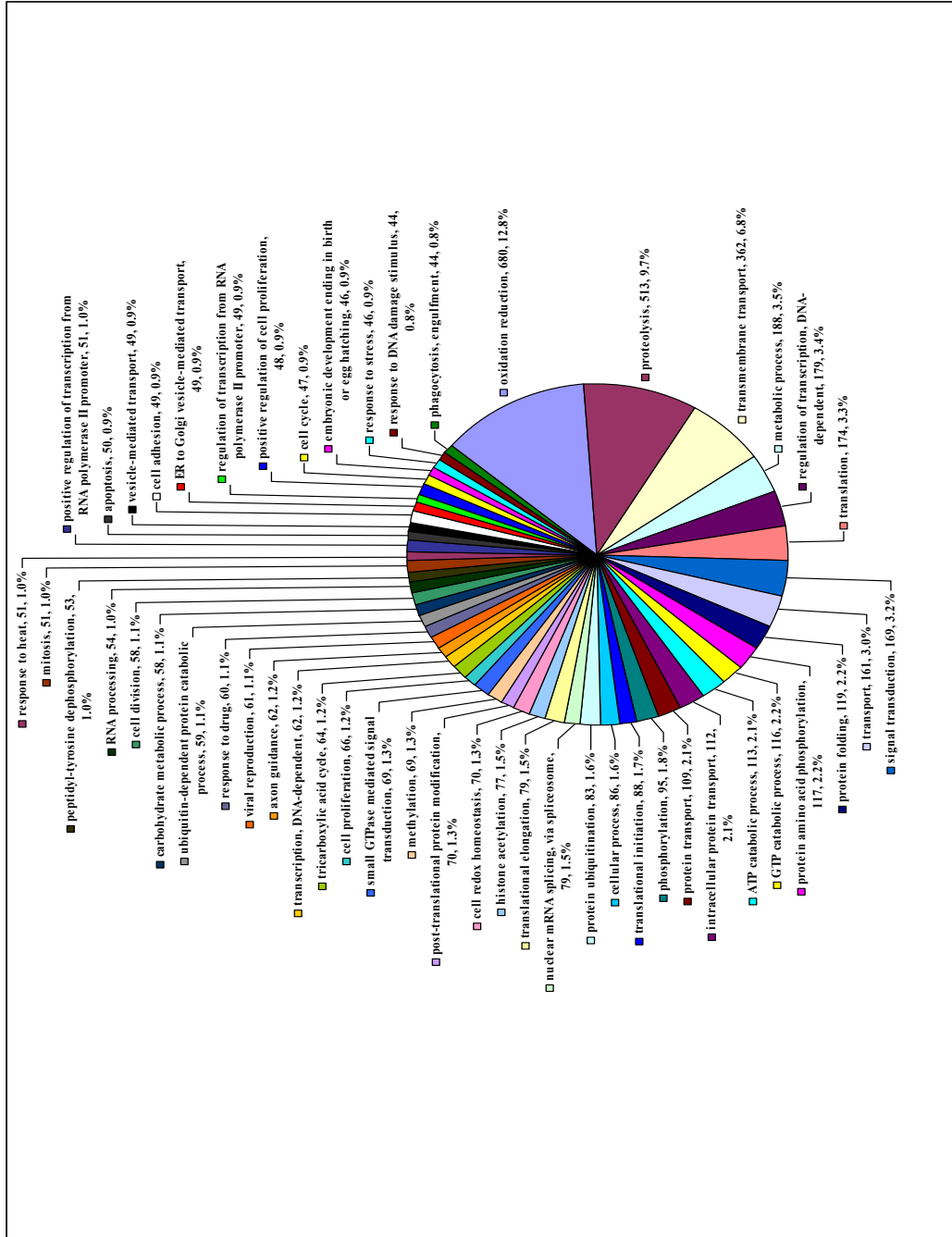


Figure 3B

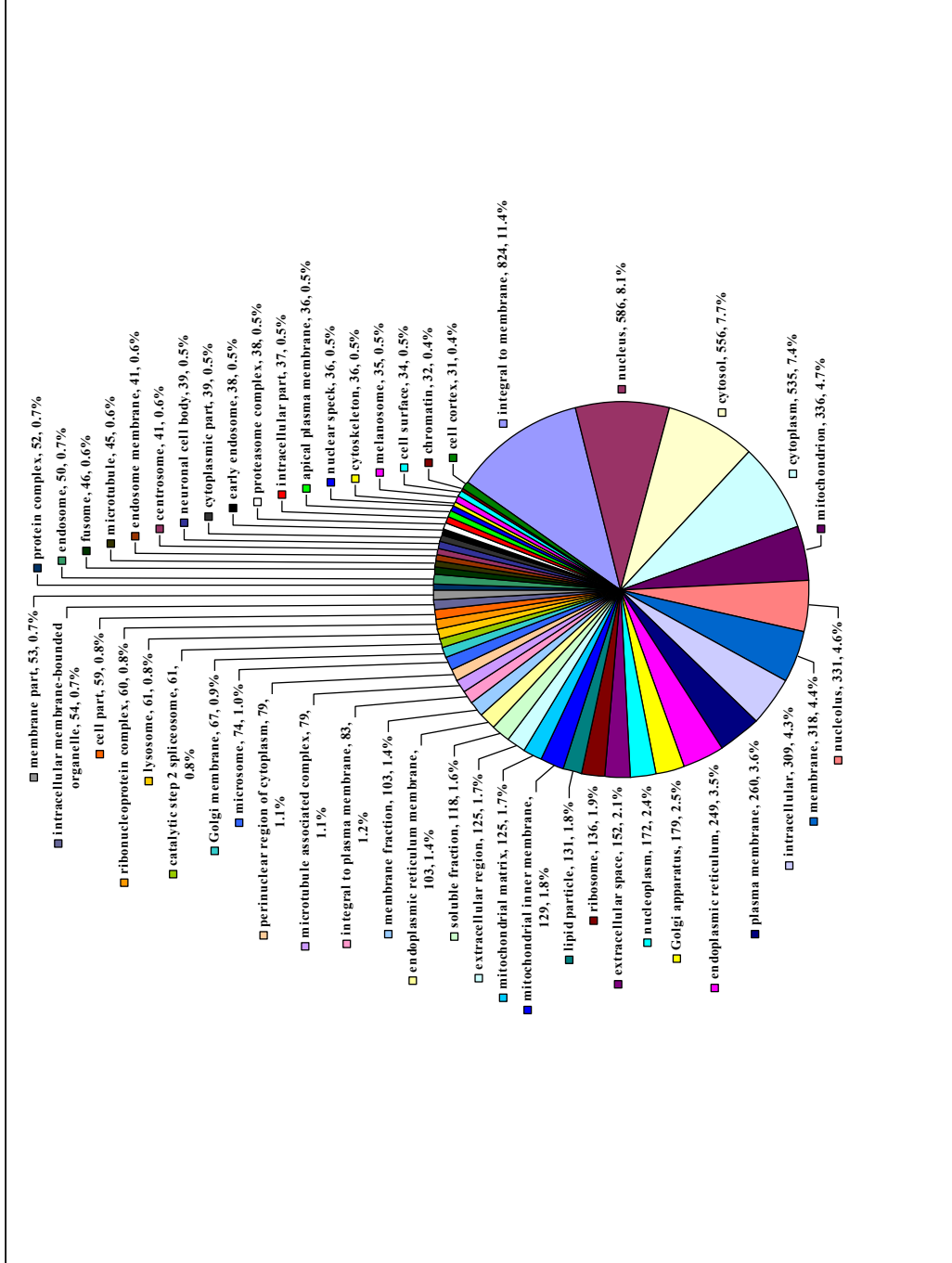


Figure 3C

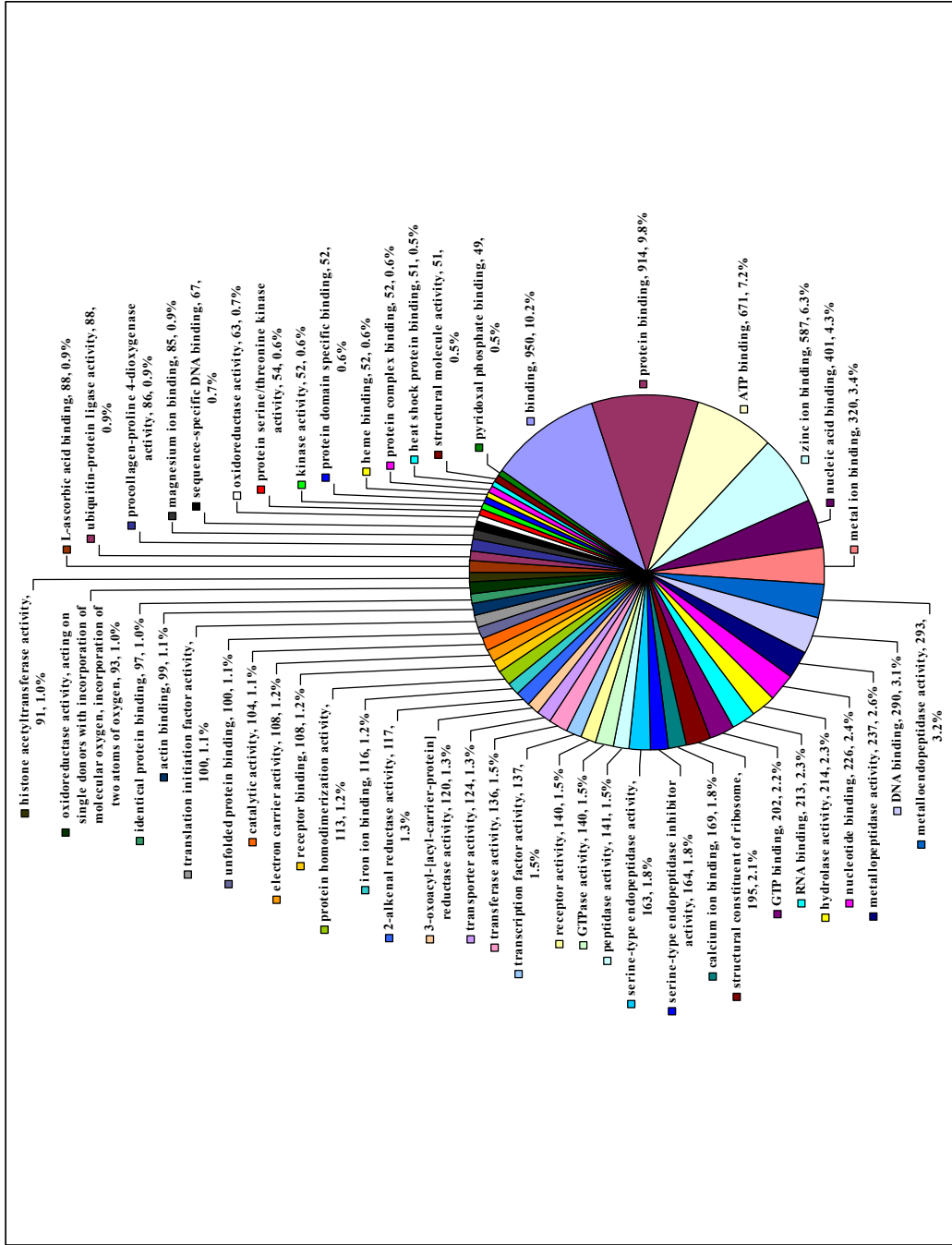


Figure 4

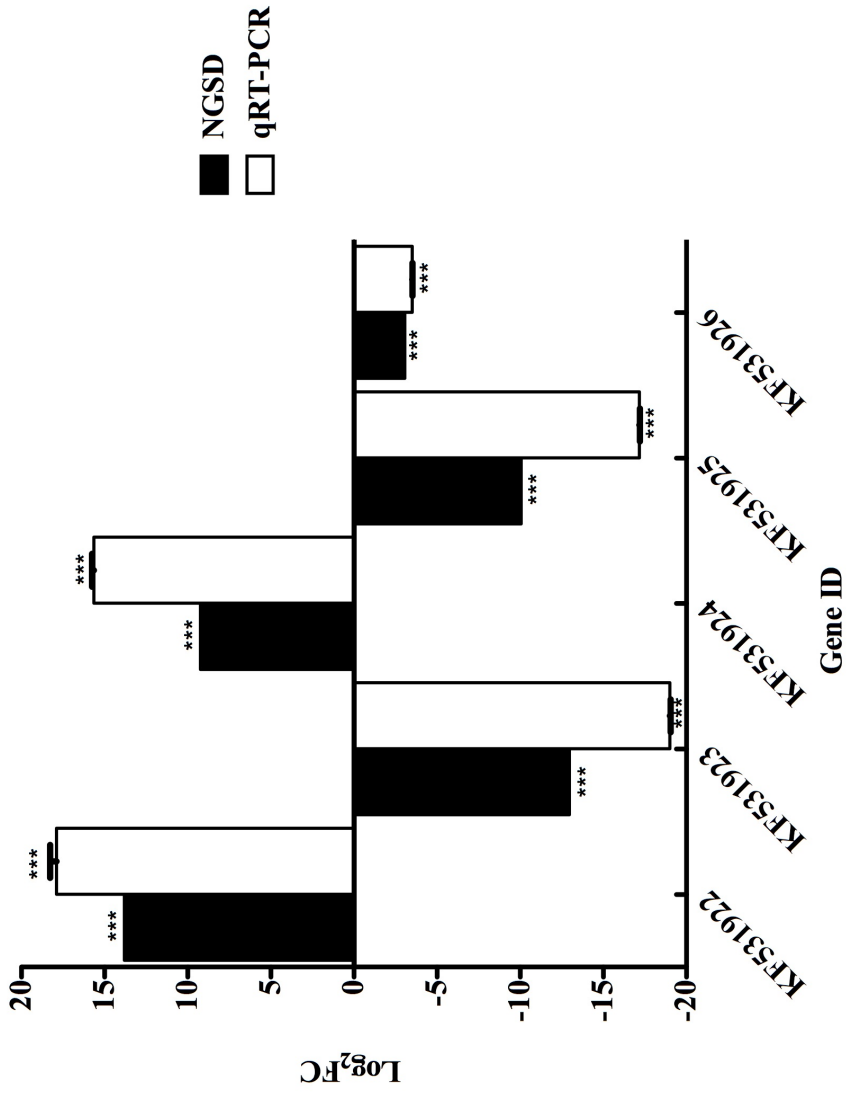


Figure 5

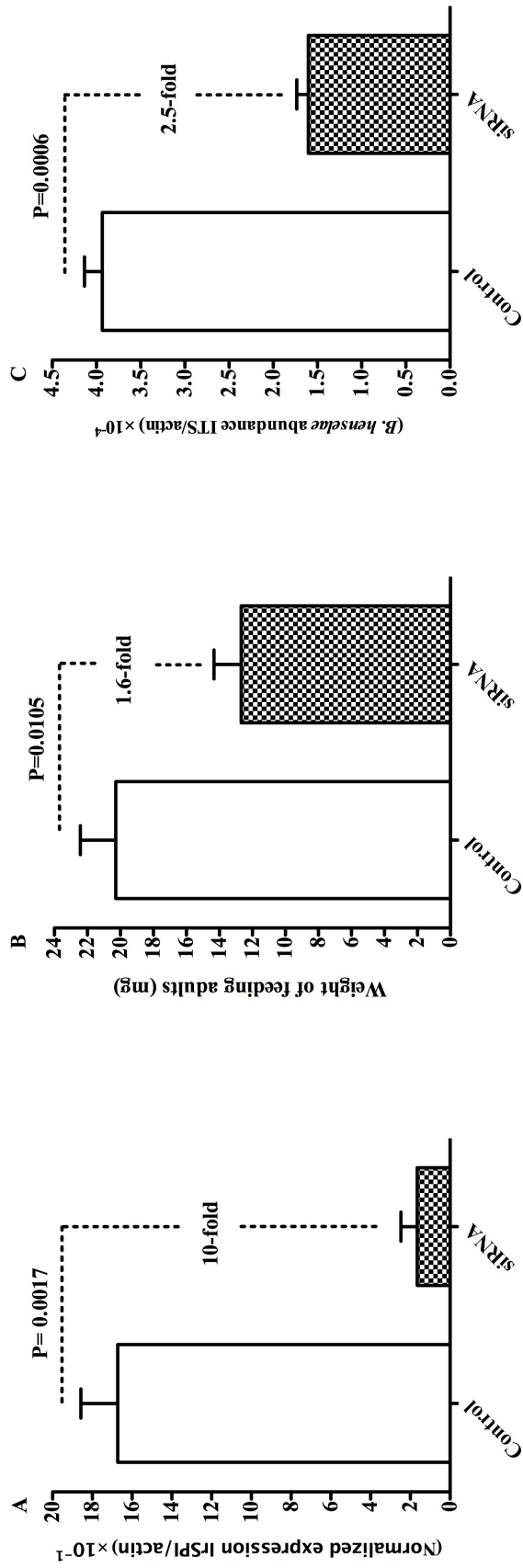


Table 1: List of qPCR primers and siRNA sequences used in this article, and the accession number of the corresponding *I. ricinus* gene

Accession No.	Description	Used/Expected expression	Sense/ anti-sense (5'-3') primers
KF531922	IrSPI BPTI/Kunitz family of serine protease inhibitor	qPCR /Up-regulated	TCTTCGCTGCTGTCTCGTAC CCTTCAAAGGCTCGCATTTGG
KF531923	Tick salivary peptide group 1 protein (20 kDa)	qPCR /Down-regulated	CAGCGACATTTCTCGGTGTAT CCATTTCCAGTTGTGCAATCG
KF531924	Salp15 super-family protein	qPCR /Up-regulated	CAAGACTGATCGTGGCAATGT CTTTTAGGCCACCAAGGGTAT
KF531925	Salp15 super-family protein	qPCR /Down-regulated	GAACTCGTGGACATTTGCCAA GTTTCGGGGCATCTCTAGTG
KF531926	Arthropod defensin	qPCR /Down-regulated	TGAAAATGACGAGGGAGGAGA TGAAACAAGATGCAGGTCCCTTT
AJ889837.1	<i>I. ricinus</i> actin	qPCR internal control	ACGGGTATCGTGTCTCGACT TCAGGTAGTCGGTCAGGTC
AF369529.1	<i>B. henselae</i> ITS	qPCR	AGATGATGATCCCAAGCCTTCTGG GATAAACCCGGAAAACCTTCCC
KF531922	IrSPI	siRNA/ Up-regulated	GCUAAACUUAGAACUCUACUCCU AGGAGUAGACAGUUUCUAAAGUUUAGC

Table 2: Summary and *de novo* assembly of *I. ricinus* salivary gland transcriptome sequenced by 454 Pyrosequencing

	Sequences	Largest length (bp)	Smallest length (bp)	Average length (bp)	N50 (bp)	GC%	Total bases
Sequenced							
1 st	522,670	1,087	34	387	439	44.53	202,288,481
2 nd	778,598	1,185	34	379	436	45.05	295,181,527
Trimmed							
1 st	524,557	1,062	15	360	414	49.9	189,331,404
2 nd	780,228	1,164	15	353	411	44.46	276,127,075
Contigs	30,853	5,647	1	550	1,026		16,970,400
Isotigs	24,539	6,815	52	1,100	1,348		26,884,585

Table 3: Differentially expressed families of transcripts in *B. henselae* infected *I. ricinus* female salivary gland compared to non-infected one

<i>Hypothetical proteins</i>	<i>Number of isotigs</i>	
	<i>Up regulated</i>	<i>Down regulated</i>
Proteins of known function		
Anti-complement proteins	0	10
Arthropod defensins	0	4
BPTI/Kunitz family of serine protease inhibitors	7	17
Collagen-Like Salivary secreted Peptide (CLSP)	0	6
Heat Shock Proteins (HSP)	0	9
Microplusin proteins	0	6
Salp15 super-family proteins	24	32
Tick Histamine Binding Proteins (THBPs)	14	26
Zinc-dependent metalloprotease	2	4
Proteins of unknown function		
<i>Amblyomma maculatum</i> Hypothetical proteins	27	10
<i>Daphnia pulex</i> Hypothetical proteins	4	0
<i>Ixodes scapularis</i> Hypothetical proteins	93	19
Ixodid Secreted salivary gland proteins	55	156
<i>Ixodes scapularis</i> Conserved hypothetical proteins	34	3
Ixodid proteins	164	60
Other species proteins	32	9
Tick Salivary Peptide Group 1 (TSPG-1)	12	30
Zinc finger proteins	14	0
Unknown genes	347	116
Total	829	517

IV.2.3. Conclusion of article 2

In this study, the transcriptome of bacteria-infected *I. ricinus* female SGs was characterized for the first time by using next generation sequencing techniques, leading to a very important source of new data on this medically important vector and its molecular relationships with TBPs. The comparison between *B. henselae* infected and non-infected *I. ricinus* female SGs resulted in the identification of several transcripts that were either up or down-regulated in response to pathogen infection. In the near future, the potential implications of these differentially expressed genes in bacterial transmission will be analyzed in detail to provide insights into the mechanisms of bacteria infection and transmission by ticks and on tick-pathogen interactions. In addition, our results showed that protein coding by the most up-regulated gene (*IrSPI*) contributing to tick feeding and tick salivary glands infection by *B. henselae*, and thus represents a promising candidate to be tested as a vaccine against ticks and bacteria transmission.

V. DISCUSSIONS AND CONCLUSIONS

As presented in the introduction of this manuscript and despite the importance of TBDs, the molecular interactions between ticks and TBPs are poorly understood. The general objective of this PhD was then to investigate *I. ricinus* tick SGs gene expression during *B. henselae* infection in order to improve the understanding of the phenomena that govern the transmission of bacteria by this vector.

In order to infect ticks, we used an artificial membrane feeding system that we have already used for the infection of *I. ricinus* with *Babesia* sp. and *B. henselae* [14,22,38]. This technique, although used by other teams too [70-72], has rarely been evaluated in comparison with direct animal feeding models. Our results demonstrated that even if artificial membrane feeding leads to less engorged ticks than direct feeding on animal, it is a powerful technique to study tick biology and TBP transmission. One of the advantages of this method is that it allows the use of blood of any origin. However, the influence of blood origin on tick feeding behavior has never been evaluated until now. We demonstrated here that there is no influence neither on the proportion and weight of engorged ticks, nor on the duration of tick feeding, whether ticks are fed with sheep blood or chicken blood. By contrast, the analysis of the influence of blood infection, evaluated here for the first time, showed that the proportion and weight of engorged ticks are decreased by *B. henselae* infection, even if the duration of tick feeding is not affected by the infection. This suggests that the presence of a pathogen may directly reduce the motivation to blood feeding, rather than ticks responding indirectly to host cues of infection. Whereas, some vector-borne pathogens (*e.g. Plasmodium* spp.) alter the feeding behavior of their vector (*e.g. Anopheles gambiae*) in order to increase pathogen acquisition and transmission [79-84]. We can assume that these results from the lightly coevolved system represented by the studied model, *i.e. B. henselae* and *I. ricinus*. It also shows that

impact of blood host infection differs according to the pathogen and the vector.

Our results confirmed that this artificial membrane-feeding technique is highly efficient for the infection of *I. ricinus* by *B. henselae*, as an alternative to natural feeding on live animals. However, its use with other models of TBP infection has now to be evaluated. Indeed, it is essential to develop efficient and well-controlled methods for infecting ticks with transmitted pathogens. The development of tick artificial feeding technique provides a more convenient and effective method to obtain as many as possible pathogen-infected ticks at once, especially for the models where the ticks cannot be infected on laboratory animals. In addition, it is also essential to limit the use and suffering of live animals according to the European animal welfare guideline.

To date, studies of tick SGs transcriptome contained a few thousand of expressed sequence tags (ESTs) sequences [85-94], except for the very recent one, which analyzed *I. ricinus* SGs transcriptome using next generation sequencing techniques [95]. In this latest study, all ticks analyzed were collected from nature, fed on various animals, and without any indication of their sanitary status. Four SG samples (*i.e.* early-feeding nymphs, early-feeding adults, late-feeding nymphs, and late-feeding adults) were sequenced and compared after a feeding step on various laboratory animals (*i.e.* rabbit, guinea pig, mice), and generated 272,220 contigs. Finally, a total of 10,796 contigs were classified as secreted proteins that showed significant differences in the transcript representation among the four SG samples, including high numbers of sample-specific transcripts [95]. Despite the high amount of genetic data obtained, results on transcriptome dynamics depending on attachment to the host that are reported in this study should be considered with precaution in the absence of data on the infected status of ticks that were compared. Results obtained in our study confirm higher transcriptome coverage than classical methodologies and generated a reference databank containing 24,539 isotigs, which may be used in several investigations. Indeed, the genome of *I. ricinus* not being sequenced, any contribution

of genetic data represents a major advance for the researches on this vector. Our transcriptome database, representing genes that are expressed in both infected and non-infected *I. ricinus* salivary glands, can provide a valuable reference for *I. ricinus* genome assembling and annotation, as well as serve to genetic studies on both the vector and its interaction with TBPs.

Several investigations performed with different models with varying approaches, report that tick gene expression can be regulated in response to pathogen infection [60-64,66,89], but contained a few differentially expressed tick genes. The comparison between pathogen-infected and non-infected tick SGs gene expression was made here by next generation sequencing techniques for the first time and leads to the identification of 1,346 differentially expressed transcripts when the tick is infected by *B. henselae*. The observed discrepancies between studies may be due to the models but also to the differing sensitivity of techniques used, the new powerful next generation sequencing techniques harboring high sensitivity.

The differentially expressed transcripts identified here may lead to a fundamental contribution toward the future understanding of the mechanisms involved in TBP transmission. Indeed, and as mentioned in the background of this manuscript, various hard tick SG factors, which were identified as being involved in TBP acquisition and/or transmission, are up-regulated in pathogen-infected ticks. All the up-regulated *I. ricinus* SGs transcripts identified here may then be potentially involved in *B. henselae* acquisition and/or transmission; otherwise, they are potentially implicated in tick feeding. Among previous identified tick proteins, some of them are able to enhance pathogen transmission, like those which can specifically bind to pathogen out-surface protein like TROSPA [96], or help pathogens crossing tick intestinal, salivary or ovarian barriers, or invading multiple distinct cell types like salp25D [75]. It has been also reported that silencing expression of defensin like varisin, reduced *A. marginale* infection in *D. variabilis* [97]. On the contrary, some tick proteins are able to inhibit pathogen transmission to control pathogen colonization, presumably to

prevent physiological stress or death and protect ticks, like those belonging to tick anti-complement peptides family as *I. scapularis* 5.3 kDa protein [98], or to tick defensins as longicin [99]. Of course, the down-regulated genes may also have an implication in pathogens' transmission and tick feeding.

Silencing *IrSPI*, the most up-regulated gene during bacteria infection that belongs to BPTI/Kunitz family, confirmed the fact that Kunitz proteins contribute to tick blood feeding as tick weight is decreased when the expression of *IrSPI* is impaired [100]. Our results showed also that product of *IrSPI* has an impact on *B. henselae* development in *I. ricinus* as we demonstrated that silencing *IrSPI* decreases *B. henselae* level in *I. ricinus* SGs, suggesting that *IrSPI* could play a role in SGs invasion by bacteria and/or in bacteria multiplication in SGs during the stimulus of the blood meal [14]. In parallel, *IrSPI* gene expression is induced by *B. henselae* infection in *I. ricinus* SGs, that is in accordance with expression of a protein that belong to the same family: *DvKPI* (*Dermacentor variabilis* Kunitz protease inhibitor) in *Rickettsia montanensis* infected *D. variabilis* tick midgut [101]. However, silencing *DvKPI* gene enhance rickettsial colonization of the tick midgut, suggesting that this protein implicated in defense response, limits *R. montanensis* invasion [102]. We observed here the opposite result as silencing of *IrSPI* impairs *B. henselae* colonization of SGs. Such difference may come from pathogen specificity, *e.g.*, salp16 is able to increase the infection of tick salivary glands by *A. phagocytophilum*, but does not influence *B. burgdorferi* acquisition by tick [103]. Another possibility is that these two proteins, although belonging to the same family may play different roles according to the organ concerned, *e.g.*, silencing *I. scapularis* salivary gland salp25D can impair *B. burgdorferi* acquisition, although silencing midgut Salp25D does not impact on spirochete acquisition [75]. In addition, it should be remembered that results obtained with *IrSPI* (this study) and *DvKPI* [102] have been obtained in different tick species, with different pathogens, and concerned different tick organs. It can be hypothesized that in *I. ricinus* SGs, *IrSPI* is putatively involved with

adhesion/invasion/multiplication of *B. henselae*, but also with stress/defense response as *DvKPI*. Indeed, its over-expression due to infection by a foreign bacterium, may decrease the amount of other bacteria species in competition with *B. henselae*, allowing its colonization of the SGs. As an example, it has been reported that silencing expression of varisin who belongs to defensin, reduced *A. marginale* infection in *D. variabilis* [97]. Other investigations are now needed in order to elucidate the role of *IrSPI* and to evaluate the vaccine potential of this molecule in a context of an anti-tick and a transmission-blocking vaccine against *B. henselae* and other tick-borne pathogens. Additionally, homologous genes belonging to BPTI/Kunitz serine protease inhibitors should also be searched in other tick species and their role in other TBP transmission should be evaluated, in order to know if some common mechanisms exist. Indeed, identification of multiple tick species' molecules with similar structure and/or sequence motifs and role may provide a universal protective antigen for the control of multiple tick infestations and their associated pathogens.

Targeting tick SG antigens that enhance pathogen transmission, such as those interfering with the host response, could potentially reduce transmission of multiple pathogens associated with the targeted tick species. In addition, utilization of so-called “exposed” antigens present in saliva, rather than “concealed” tick antigens to which the host is never naturally exposed, may allow natural boosting of the host response [104]. Moreover, secreted proteins represent good candidates for neutralization by antibodies elicited by anti-tick vaccines. Therefore, genes that are over-expressed in tick salivary glands during a pathogen infection may represent very promising candidates in terms of transmission-blocking vaccine strategy.

In the future, studies will thus focus on the 829 genes that we have been identified here as over-expressed in tick SGs during bacteria infection. First, genes coding secreted proteins that are expected to be secreted in saliva for introduction into the host, will be selected. Annotation and comparison with databank will then permit

to select the best candidates according to their putative function. Proteins belonging to the salp15 super-family for example will be studied with attention because of their recognized functions as inhibition of CD4⁺ T cell activation by specifically binding to the T cells co-receptor CD4 [73,105,106], inhibition of cytokine expression by dendritic cells [107], bacteria protection from antibody-mediated killing, and inhibition of keratinocyte inflammation [108]. Finally, the higher expressed genes will be tested for their implication in pathogen's transmission.

Advances in our understanding of interactions between bacteria and ticks and gene function identification will be facilitated by the introduction of the effective molecular tools for inactivating tick genes, the RNAi approach, that we have adapted to *I. ricinus* ticks. RNAi is now the most widely used gene-silencing technique in ticks where the use of other methods of genetic manipulations has been limited [109], and it has been already successfully used to characterize genes essential for tick survival and feeding as well as for the tick-pathogen interface [109]. In addition, the use of antibodies and host vaccination with tick recombinant proteins is an attractive alternative for the identification of the role of these genes in tick infestations and pathogen infections.

Depending on their role confirmation in bacteria transmission and/or tick survival or development, the selected molecules will be, at last, evaluated as vaccine candidates against tick and bacteria transmission. These candidates should then be tested in various infection models and the underlying mechanisms of host pathogen interaction analyzed in detail. Indeed, mechanisms involved in TBP transmission are multiple and complex [67] and to date, very few antigens appear to be highly effective on their own, suggesting that effective vaccines have probably to integrate several blocking strategies that corresponding to several antigens. This antigen "cocktail" may be present in the differentially expressed transcripts that were identified in this study. Thanks to results obtained here, we effectively expect to have identified one or more vaccine candidates against ticks and transmission of TBPs of very high impact

in domestic animals, livestock and humans in Europe and worldwide. This will contribute to the development of a new generation of pathogen transmission blocking strategies designed to prevent transmission and reduce exposure of vertebrate hosts to TBPs.

Taken together, results obtained during this PhD demonstrated that *B. henselae* infection affect tick blood feeding behavior, and also modulate tick salivary glands genes expression. Understanding all the mechanisms that are involved in bacteria transmission by ticks should provide knowledge to instruct development of next generation vaccine against TBDs. Depending on differentially expressed genes' role confirmation, more and more vaccine candidates for the control of *I. ricinus* and *B. henselae* will be then provided by this work. At the same time, protective antigens that are conserved across tick species should be identified in order to provide a universal vaccine candidate for the control of multiple tick species and their associated pathogens. The strategy of the control of tick and tick-borne diseases will come to a new stage with these 'cocktailed' protective antigens.

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ANNEXES

Prevalence of Tick-Borne Pathogens in Adult *Dermacentor* spp. Ticks from Nine Collection Sites in France

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Abstract

The importance of *Dermacentor* spp. in the transmission of tick-borne pathogens is not well recognized in Europe. To investigate the role of *Dermacentor* spp. in the transmission of tick-borne pathogens, questing ticks were collected in 9 sites from southern to northwestern France (Camargue Delta to Eastern Brittany) where *Dermacentor* spp. exist and tick-borne diseases had occurred previously. Three tick species were collected during the spring and autumn of 2009. Collected ticks (both males and females) included *D. marginatus* ($n=377$), *D. reticulatus* ($n=74$), and *I. ricinus* ($n=45$). All ticks were analyzed by PCR or reverse line blot for the presence of pathogens' DNA. Pathogens analyzed were based on veterinarian reports and included *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Anaplasma marginale*, *Borrelia burgdorferi*, *Bartonella* spp., *Babesia* spp., *Theileria* spp., and *Francisella* sp. *Francisella tularensis* was not detected in any of the analyzed ticks. In *D. marginatus*, infection prevalence for *A. phagocytophilum* (3%) was similar to that found in *I. ricinus* in Europe. Other pathogens present in *D. marginatus* included *A. marginale* (0.5%), *Bartonella* spp. (9%), *C. burnetii* (12%), *F. philomiragia* (1.3%), and *Theileria annulata/Babesia bovis* (0.3%), which were detected for the first time in France. Pathogens detected in *D. reticulatus* included *A. marginale* (1%), *Bartonella* spp. (12%), *C. burnetii* (16%), *Borrelia* spp. (1.5%), and *F. philomiragia* (19%). Pathogens detected in *I. ricinus* included *A. phagocytophilum* (41%), *Bartonella* spp. (9%), *C. burnetii* (18%), *A. marginale* (1%), *Borrelia* spp. (4.5%), and *Babesia* sp. (7%). This study represents the first epidemiological approach to characterize tick-borne pathogens infecting *Dermacentor* spp. in France and that may be transmitted by ticks from this genus. Further experiments using experimental infections and transmission may be now conducted to analyze vector competency of *Dermacentor* spp. for these pathogens and to validate such hypothesis.

Key Words: Tick—Epidemiology—*Dermacentor*—*Ixodes ricinus*—*Anaplasma*—*Coxiella burnetii*—*Bartonella*—*Borrelia burgdorferi*—*Babesia*—*Theileria*—*Francisella tularensis*.

Introduction

TICKS CONSTITUTE THE SECOND VECTOR after mosquitoes in terms of public and veterinary health importance (Toledo et al. 2009a). Ticks transmit the largest variety of pathogens, including parasites, bacteria, and viruses. In addition, most of

the diseases caused by these pathogens are considered emerging or re-emerging diseases (Burri et al. 2011).

The most widespread and abundant tick species in Europe is *Ixodes ricinus*. This ectoparasite is implicated in the transmission of several pathogens including *Borrelia burgdorferi* sensu lato (s.l.) (Smith and Takkinen 2006), *Anaplasma*

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phagocytophilum (Matsumoto et al. 2006, Woldehiwet 2010), *Rickettsia* spp. (Socolovschi et al. 2009), *Babesia* spp. (Chauvin et al. 2009), *Francisella tularensis* (Foley and Nieto 2010), potentially *Bartonella* spp. (Cotté et al. 2008, Reis et al. 2011a), as well as some viruses such as tick-borne encephalitis virus (Kollaritsch et al. 2011).

The second most abundant tick species in Europe belongs to the genus *Dermacentor*, and is also important for public and veterinary health (Pérez-Eid 2007). Compared with *I. ricinus*, little data exist about the role of *Dermacentor* spp. in the transmission of pathogens in Europe. *Dermacentor* spp. are 3-host ticks (larvae, nymphs, and adults feed on different hosts, completing the life cycle in approximately 1 year) feeding on animals and accidentally on humans (Estrada-Peña and Jongejan 1999). Contrary to *I. ricinus*, *Dermacentor* larvae and nymphs are endophilic, *i.e.*, they live in rodents and other micro-mammals burrows, thus limiting the contact with these stages (Pérez-Eid 2007). Also different from *I. ricinus*, *Dermacentor* males are partial bloodsuckers, with implications in the transmission of tick-borne pathogens (Pérez-Eid 2007). In France, spring and autumn are the main periods of activity for *Dermacentor* spp. ticks.

Two *Dermacentor* spp. are present in France—*D. marginatus* and *D. reticulatus*. *D. marginatus* infests ungulates whereas *D. reticulatus* feeds on dogs and horses; both species can bite humans (Estrada-Peña and Jongejan 1999). For example, *Dermacentor* spp. ticks accounted for 10% of the total number of ticks collected on humans in Spain (Estrada-Peña and Jongejan 1999), 0.9% in Italy (Manfredi et al. 1999), 3.25% in Turkey (Bursali et al. 2010), and 3.3% in Romania (Briciu et al. 2011). *Dermacentor* spp. are implicated in the transmission of *Anaplasma ovis* to sheep and goats (Crosbie et al. 1997, Friedhoff 1997), *Babesia caballi* and *Theileria equi* to horses (Kumar et al. 2009), *Babesia canis* to dogs (Cardoso et al. 2010), and *Rickettsia slovaca* to humans (Raoult et al. 2002). In addition, *Dermacentor* spp. are also suspected of transmitting several other pathogens, such as *B. burgdorferi*, *F. tularensis*, *Coxiella burnetii*, *Rickettsia conori*, and some viruses (Pérez-Eid 2007).

Ticks can harbor 2 or more infectious agents and effectively transmit them simultaneously (Swanson et al. 2006). Consequently, it is important to characterize the prevalence of pathogen co-infections in ticks, which is significant for the correct diagnosis and prophylaxis of tick-borne diseases. In Europe, few studies have characterized tick co-infection with several pathogens (Toledo et al. 2009a, Cotté et al. 2010, Halos et al. 2010, Reye et al. 2010, Reis et al. 2011b, Torina et al. 2010, Satta et al. 2011), and there is a need to conduct studies estimating the risk of infection for animal and human populations.

Bovine granulocytic anaplasmosis and tick-borne fever (TBF) of ruminants due to *A. phagocytophilum* has been diagnosed in autumn 2007 in 3 alpine areas where its main vector, *I. ricinus*, is absent or rarely found but *Dermacentor* spp. are abundant (unpublished results). On the basis of these results, we hypothesized that *Dermacentor* spp. are implicated in the transmission of *A. phagocytophilum* and other pathogens. To test this hypothesis, the present study was performed by collecting ticks in different sites across France to characterize the prevalence of tick-borne pathogens in *Dermacentor* spp. and sympatric *I. ricinus* ticks in relation to cattle pathologies reported in the selected areas.

Materials and Methods

Study areas and tick collection

This study was undertaken at locations included in the area known for the presence of *Dermacentor* spp. ticks in France (Pérez-Eid, 2007). According to the indications of veterinary practitioners, sites were selected in pastures where previous TBF, babesiosis, Q fever, Lyme disease, or anaplasmosis outbreaks were diagnosed as acute or subacute diseases identified through clinical signs and/or confirmed by PCR or positive serology. Collection sites were chosen in 9 French departments, corresponding to 11 veterinarian practices, and are presented in Figure 1. Questing adult ticks were collected using the flagging technique (Vassallo et al. 2000) in the spring (April and May) of 2009 for sites 1–3 and 7–9, and in the autumn (September and October) of 2009 for sites 4 and 5. Flagging was conducted from 10:00 AM to 6:00 PM during 2 days. All adult ticks (male and female) were individually preserved in 70% ethanol and identified to the species level using taxonomic keys (Pérez-Eid 2007), categorized by site of collection and sex, and frozen at -20°C until DNA extraction.

DNA extraction

DNA was extracted from individual ticks using the QIAamp® DNA mini kit (QIAGEN, Germany) following manufacturer's instructions. Total DNA was eluted in 100 μL of elution buffer. DNA samples were then distributed at -20°C in 4 96-well plates with 25 μL of DNA per plate to the various laboratories for pathogen DNA characterization.

Pathogen DNA characterization

Pathogens' DNA was characterized by PCR or reverse line blot (RLB) in tick samples using specific primers (Tables 1 and 2). All of the methodologies used here were highly specific for the target pathogen except the PCR performed to detect *Bartonella* sp. and *Borrelia* sp., for which we cannot exclude a cross-reaction with some tick symbiont DNA. In these cases, a sequencing step was performed, when possible, for positive PCR reactions.

Bartonella spp., *Borrelia burgdorferi* s.l., and *Anaplasma marginale*. For *B. burgdorferi* s.l., and *Bartonella* spp., PCR reactions were performed in the MyCycler thermocycler (Bio-Rad, Strasbourg, France). Each reaction was carried out in a 25- μL volume containing 2 μL of tick DNA, 2 μL of 10 $\mu\text{mol/L}$ of each primer, 2 μL of 2.5 mmol/L of each deoxyribonucleotide triphosphate (dNTP), 2.5 μL of 10 \times PCR buffer, and 1 U of *Taq* DNA polymerase (5 U/ μL Takara Biomedical Group, Shiga, Japan). PCR products were sent for sequencing to GATC Biotech Company (Germany). Sequences were compared with known sequences listed in the GenBank nucleotide sequence databases by using the BLAST search option at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST). For *A. marginale*, the major surface protein 4 (*mSP4*) gene was amplified by PCR as reported previously (de la Fuente et al. 2005a). Briefly, 1 μL (1–10 ng) DNA was used with 10 pmol of each primer MSP45 and MSP43 in a 50- μL volume PCR (1.5 mM MgSO_4 , 0.2 mM dNTP, 1 \times AMV/*Tfl* reaction buffer, 5U *Tfl* DNA polymerase) employing the Access RT-PCR system (Promega, Madison, WI). Reactions were performed in an automated DNA thermal cycler (Techne model TC-512, Cambridge, England, UK).

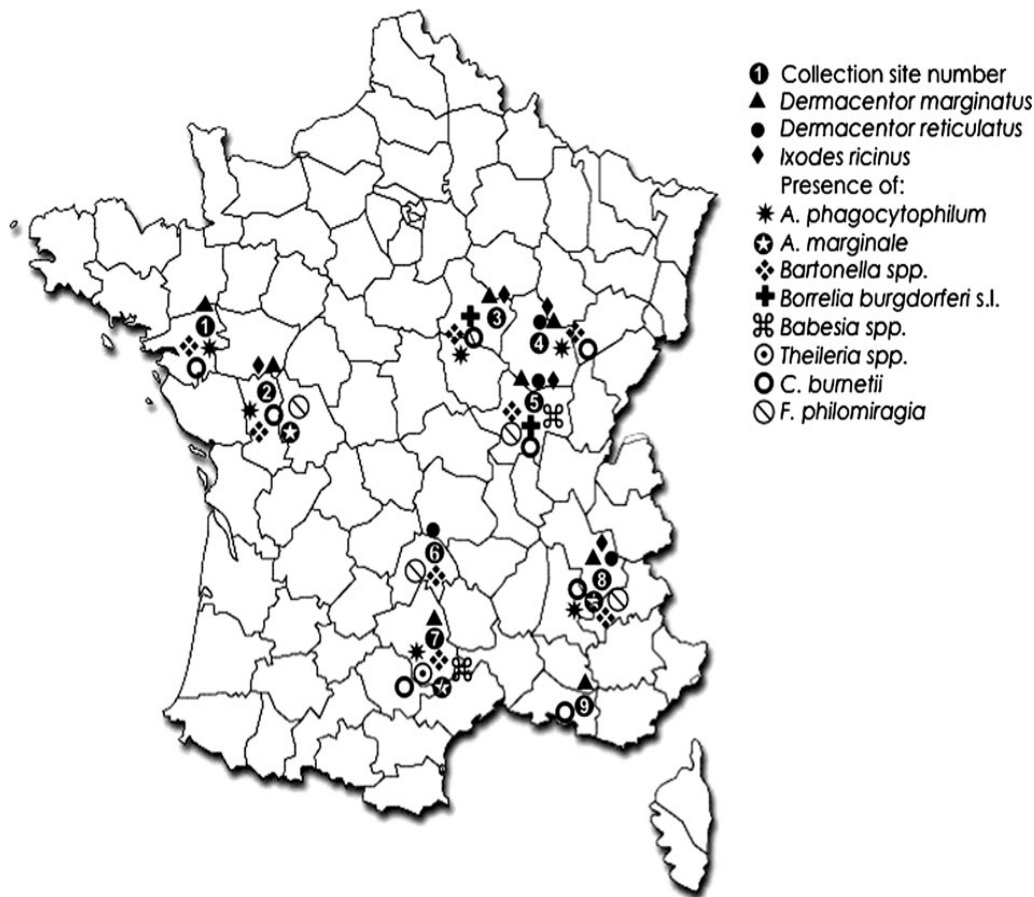


FIG. 1. Tick collection sites in different French departments. Site 1: Loire Atlantique (department no. 44), Chateaubriand (47.24 N, 1.22 W), Soudan (47.44 N, 1.18 W), Louisfer (47.4 N, 1.26 W); site 2: Deux-Sèvres (no. 79), St. Maurice la Fougereuse (47.2 N, 0.3 W), St. Aubain du Plain (46.55 N, 0.28 W), Amailloux (46.44 N, 0.18 W), St. Julien de Vouvante (46.34 N, 0.46 W); site 3: Yonne (No. 89), St. Père (47.27 N, 3.45 E), Etaule (47.31 N, 3.55 E); site 4: Côte d'or (No. 21), Vic de Chassenay (47.28 N, 4.16 E), Chevigny (47.1 N, 5.28 E); site 5: Saône et Loire (no. 71), St Gervais/Couche (46.56 N, 4.56 E), Collonge la M (46.33 N, 4.47 E); site 6: Cantal (no. 15), Villedieu (44.59 N, 3.3 E); site 7: Aveyron (no. 12), Vezouillac (44.12 N, 3.5 E); site 8: Isère (no. 38), Nantes en Rattier (44.56 N, 5.49 E), Notre Dame de Vaux (44.59 N, 5.44 E); site 9: Bouches du Rhône (no. 13), St Martin de Crau (43.38 N, 4.48 E).

A. phagocytophilum. For *A. phagocytophilum*, pathogen detection was conducted by real-time PCR using the commercial kit ADIAVET[®] ANA PHA REALTIME (Adiagène, St. Briec France) targeting the *msp4* gene. PCR amplification was carried out with 2 μ L DNA in a total volume of 25 μ L in a thermocycler CFX 96 (BioRad). Three *A. phagocytophilum* biovars could be detected with this kit, namely biovar *phagocytophilum*, biovar *equi*, and biovar EGH. Negative (DNase- and RNase-free sterile water) and positive controls were included in all experiments. For *Borrelia* spp., positive control DNA was kindly provided by E. Ferquel (CNR *Borrelia*, Institut Pasteur, Paris, France) and for *A. phagocytophilum*, positive control DNA included in the ADIAVET[®] ANA PHA REALTIME kit was used. Amplicons were analyzed by electrophoresis in 1.0% or 1.5% agarose gels containing ethidium bromide and DNA fragments were observed under ultraviolet light.

Francisella sp. Real-time PCR assays were performed using primers and probes that target the *tul4*, *fopA*, and *ISFtu2*

genes of *F. tularensis*, as previously described (Hollis et al. 1989, Versage et al. 2003). The fluorogenic hybridization probes were synthesized by Applied Biosystems (France) with a 6-carboxy-fluorescein reporter molecule (FAM) attached to the 5' end and a quencher (tetramethylrhodamine, TAMRA) attached to the 3' end. Amplification and data analysis were carried out on an ABI PRISM 7000 (Applied Biosystems) thermocycler. Real-time PCR was performed on a 20- μ L final volume using TaqMan Universal PCR Master-Mix, 0.4 μ M forward and reverse primers, 0.1 μ M fluorogenic probe, and 5 μ L of template DNA. For each reaction, both negative (no DNA template and *Escherichia coli* DNA) and positive (2 pg of purified *F. tularensis* subsp. *holarctica* FSC 200 strain) controls were included. To verify if amplified products were the correct size, amplification products were run on 2% agarose gels and visualized by staining with ethidium bromide. This assay is species specific and able to differentiate *F. tularensis* and *F. philomiragia*. Identification of *F. tularensis* occurs when all 3 target sequences (*ISFtu2*, *fopA*, and *tul4*) give a positive result, whereas identification of *F. philomiragia*

TABLE 1. PRIMERS AND PCR CONDITIONS USED FOR DETECTION OF PATHOGENS IN TICKS

Organism detected (laboratory that performed the detection)	Target gene	Primer sequence (5' - 3')	Amplicon size (bp)	PCR conditions	Reference
<i>Borrelia burgdorferi</i> s.l. (1)	16S rRNA	16SLDF: ATGCACACTTGGTGTAACTA 16SLDR: GACTTATCACCGGCAGTCTTA	357	95°C 8 min 35 cycles: 95°C 60s, 53°C 60s, 72°C 60 s 72°C 10 min	Marconi and Garon (1992)
<i>Bartonella</i> spp. (1)	Citrate synthase	bart781: GGGGACCAGCTCATGTGTG bart1137: AATGCAAAAAGAACAGTAAACA	356	95°C 8 min 35 cycles: 95°C 30s, 54°C 30s, 72°C 30 s 72°C 10 min	Norman et al. (1995)
<i>Anaplasma phagocytophilum</i> (2)	16S rRNA	Undisclosed in the kit ADIAVET® ANA PHA REALTIME	130	Undisclosed in the kit	Unpublished
<i>Anaplasma marginale</i> (3)	Major surface protein 4 (MSP4)	MSP45: GGGAGCTCCTATGAATTACAGAGAA TTGTTTAC MSP43: CCGGATCCTTAGCTGTAACAGGAATCTTGC	849	94°C 1 min 35 cycles: 94°C 30s, 60°C 30s, 68°C 1 min 68°C 5min	de La Fuente et al. (2004b)
<i>Coxiella burnetii</i> (2)	IS1111	Undisclosed in the kit ADIAVET® COX REALTIME	90	Undisclosed in the kit	Unpublished
<i>Babesia-Theileria</i> spp. (4)	hypervariable V4 region of 18S rRNA	F2: 5'-GAC ACA GGG AGG TAG TGA CAA G-3' R2: Biotin-5'-CTA AGA AIT TCA CCT CTG ACA GT-3	460-520	94°C 10 min 6 touch down cycles (2 cycles per temp.): 94°C 20s, 67°C, 65°C, 63°C, 61°C, 59°C and 57°C 30s, 72°C 30s 50 cycles: 94°C 20s, 64°C 30s, 72°C 30s	Nagore et al. (2004)
<i>Francisella tularensis</i>	ISFtu2	ISFtu2F: TTGGTAGATCAGTTGGTGGGATAAC ISFtu2R: TGAGTTTTACCTTCGACAAACAATATTC ISFtu2P: AAAATCCATGCTATGACTGATGCTTTAG GTAATCCA	97	50°C 2 min, 95°C 10 min, 50 cycles: 95°C 15 s, 60°C 1 min	Versage et al. (2003)
<i>Francisella philomiragia</i> (5)	Tul4	Tul4F: ATTACAATGGCAGGCTCCAGA Tul4R: TGCCCAAGTTTATCGTCTTCT Tul4P: TTCTAAGTCCCATGATACAAGCTTCCCAA TTACTAAG	91		
	fopA	FopAF : ATCTAGCAGGTCAAGCAACAGGT FopAR : GTCAACACTTGTGAAACATTTCTAGATA FopP : CAAACTTAAGACCACCACCCACATCCCAA	87		

Laboratories: (1) USC INRA Bartonella-Tiques, Maisons-Alfort, France; (2) Laboratoire d'Analyses Sèvres Atlantique, Niort, France; (3) IREC, Ciudad Real, Spain; (4) IZSS, Palermo, Sicily, Italy; (5) Laboratoire National de Référence *Francisella*, ANSES, Maisons-Alfort, France.

TABLE 2. REVERSE LINE BLOT PROBES USED FOR THE DETECTION OF *BABESIA* AND *THEILERIA* SPP. IN TICKS

Genus	Species	Probe sequence
<i>Babesia/Theileria</i>	catch all	Probe 1: TAATGGTTAATAGGAGCAGTTG
<i>Babesia</i>	<i>bigemina</i>	Probe 2: CGTTTTTCCCTTTTGTTGG
<i>Babesia</i>	<i>bovis</i>	Probe 3: CAGGTTTCGCCGTGATAATTGAG
<i>Babesia</i>	<i>divergens</i>	Probe 4: GTTAATATTGACTAATGTCGAG
<i>Babesia</i>	<i>major</i>	Probe 5: TCCGACTTTGGTTGGTGT
<i>Babesia</i>	<i>motasi</i>	Probe 6: GCTTGCTTTTTTGTACTTTG
<i>Babesia</i>	<i>ovis</i>	Probe 7: TGCGCGCGGCCCTTTCGCTT
<i>Babesia</i>	<i>crassa</i>	Probe 8: GTTGGCTTATCTTTTACTTT
<i>Theileria</i>	<i>annulata</i>	Probe 9: CCTCTGGGGTCTGTGCA
<i>Theileria</i>	<i>velifera</i>	Probe 10: CCTATTCTCCTTACGAGT
<i>Theileria</i>	<i>taurotragi</i>	Probe 11: TCTTGGACGTGGCTTTT
<i>Theileria</i>	<i>mutans</i>	Probe 12: CTTGCGTCTCCGAATGTT
<i>Theileria</i>	<i>buffeli/orientalis</i>	Probe 13: GGCTTATTCGGATTGATTTT
<i>Theileria</i>	<i>ovis</i>	Probe 14: TTGCTTTTGTCTCCTTACGAG
<i>Theileria</i>	<i>lestouardi</i>	Probe 15: ATTGCTTGTGTCCCTCCG
<i>Theileria</i>	<i>hiri</i>	Probe 16: CCTCCGGCGTCTGTGCA
<i>Theileria</i>	sp2 (China)	Probe 17: TCCCAAAGTAATGGTTAATAGC
<i>Theileria</i>	sp1 (China)	Probe 18: TACCAAAGTAATGGTTAATAGC
<i>Babesia</i>	sp1 (Turkey)	Probe 19: CCTGGGTAATGGTTAATAGGAA
<i>Babesia</i>	sp2 (Lintan)	Probe 20: CCTTGTAATGGTTAATAGGAA

occurs when the ISFtu2 assay is positive and the *tul4* assay is negative (Versage et al. 2003).

C. burnetii. *C. burnetii* was detected using the ADIA-VEI® COX REALTIME kit (Adiagène, St Brieuc, France). PCR amplification was conducted in a 25-µL volume containing 2 µL of tick DNA using a CFX 96 Thermocycler (BioRad). A control DNA included in the PCR kit was used as positive control, and DNase- and RNase-free water was used as negative control.

Babesia and Theileria spp. PCR amplifications were performed to amplify the hypervariable V4 region of the 18S rRNA gene of *Babesia* and *Theileria* species (Nagore et al. 2004). Reactions were carried out in 50 µL with 5 µL of tick DNA using a thermocycler 2720 (Applied Biosystems). PCR products were then used for RLB hybridization, as previously described (Gubbels et al. 1999, Georges et al. 2001, Schnittger et al. 2004). For each piroplasm, specific oligonucleotide probes were used (Table 2) to detect *Babesia/Theileria* spp., *Babesia bigemina*, *B. bovis*, *B. divergens*, *B. major*, *B. motasi*, *B. ovis*, *B. crassa*, *Theileria annulata*, *T. velifera*, *T. taurotrago*, *T. mutans*, *T. buffeli/orientalis*, *T. ovis*, *T. lestouardi*, *Theileria* all sp2 (China), *Theileria* all sp1 (China), *Babesia* all sp1 (Turkey), and *Babesia* all sp2 (Lintan). After hybridization, the membrane was exposed to a chemiluminescent detection film (Amersham) for 60 min to 24h and then developed on Develop X-ray film (AGFA) and Fixed X-ray film (AGFA). A black spot in the sample-probe cross in the hyperfilm demonstrated a positive signal for that pathogen.

Statistical analysis

A 2x2 chi-squared test or Fisher exact test (when *n* < 10) was performed using the SPSS 11.0 statistical program (SPSS Inc., Chicago, IL) to compare prevalence between tick species for a given pathogen or between pathogens for a given tick species. The differences were considered statistically significant at *p* ≤ 0.05.

Results

Tick collection

A total of 495 adult ticks (60% females and 40% males) were collected (Table 3). Three species of ticks were identified on different collection sites: *D. marginatus* (*n*=377; 76%), *D. reticulatus* (*n*=74; 15%), and *I. ricinus* (*n*=45; 9%) (Fig. 1). Most ticks (57%; *n*=284) were collected in western France in collection site 2 (department no. 79). Despite the importance of this sample, *D. reticulatus* was not collected in this site. A similar result was obtained for site 3 (*n*=11). The second site in terms of the number of ticks collected was site 8 (*n*=62) in southeastern France where the 3 tick species were recovered in sites 4 and 5, in spite of the small number of ticks collected in these 2 sites (*n*=6 and *n*=17 ticks, respectively). In sites 1 (*n*=60), 7 (*n*=38), and 9 (*n*=9), only *D. marginatus* specimens were found, whereas in site 6 (*n*=8) only *D. reticulatus* specimens were found.

Pathogen detection in ticks

Detection of Bartonella spp. Of the 495 tick samples tested, 47 (9.5%) were positive for the 356-bp fragment of the *Bartonella* spp. citrate synthase (*gltA*) gene (Table 4). The presence of *Bartonella* spp. was similarly distributed among the 3 tick species as well as between females and males (Table 4). *Bartonella* spp. were found in almost all collection sites, with the exception of site 9, where only 9 ticks were collected (Fig. 1). Eight sequenced amplicons were homologous to *Bartonella* spp. Four of them showed 100% identity with the uncultured *Bartonella* spp. isolate 10158 BART citrate synthase (*gltA*) gene (GenBank accession no. EF662055) that was isolated from *Ixodes scapularis* in the United States and for which the closest species is *Bartonella rochalimae* (76% identity). The other amplicons showed 97% identity with the uncultured *Bartonella* sp. clone 162 isolated from *Ixodes tasmania* in Australia (accession No. JQ228398), 76% identity with *Bartonella melophagi* strain K-2C (accession No. JQ228399), 77% identity with *Bartonella* sp. pn 1564ga isolated from a rodent in United

TABLE 3. DISTRIBUTION OF TICKS COLLECTED DURING 2009 IN 9 FRENCH COLLECTION SITES

Collection site	Sex	Dermacentor marginatus	Dermacentor reticulatus	Ixodes ricinus	Total
1	Female	42	0	0	42
	Male	18	0	0	18
	Total	60	0	0	60
2	Female	157	0	18	175
	Male	97	0	12	109
	Total	254	0	30	284
3	Female	4	0	3	7
	Male	3	0	1	4
	Total	7	0	4	11
4	Female	1	2	1	4
	Male	1	1	0	2
	Total	2	3	1	6
5	Female	0	5	3	8
	Male	1	8	0	9
	Total	1	13	3	17
6	Female	0	2	0	2
	Male	0	6	0	6
	Total	0	8	0	8
7	Female	24	0	0	24
	Male	14	0	0	14
	Total	38	0	0	38
8	Female	2	24	4	30
	Male	4	26	2	32
	Total	6	50	6	62
9	Female	3	0	0	3
	Male	6	0	0	6
	Total	9	0	0	9
Total	Female	233	33	29	295
	Male	144	41	15	200
	Total	377	74	44	495

States, the closest species being *Bartonella grahamii* (accession no. JQ228400), and 77% identity with *Bartonella* sp. B29044 that was isolated from bats in Guatemala and for which the closest known species is *Bartonella elizabethae* (accession No. JQ228401).

Detection of *B. burgdorferi* s.l. Of the 495 ticks analyzed, 3 (0.6%) were positive for the 357-bp amplified fragment of *B. burgdorferi* s.l. 16S rDNA (Table 4). Two of them corresponded to *I. ricinus* females (collected on sites 3 and 5 in Eastern France) and 1 corresponded to a *D. reticulatus* male collected on site 5 (Fig. 1). Only 1 of the amplified fragments obtained from an *I. ricinus*, was sequenced and showed a 100% identity to *B. burgdorferi* strain Titov gaj 16S rDNA gene that was isolated from *I. ricinus* in Serbia (accession No. JQ228402).

Detection of *A. marginale*. Only 0.6% prevalence was found for *A. marginale* in collected ticks, with 2 *D. marginatus* females collected on sites 2 and 7 and 1 *D. reticulatus* male from site 8 positive for pathogen DNA. *A. marginale* was not detected in *I. ricinus* ticks.

Detection of *A. phagocytophilum*. Of the 495 ticks analyzed, 30 (6%) were positive for the 130-bp fragment of *A. phagocytophilum* *msp4* gene when tested by real-time PCR. *A. phagocytophilum* was found with similar prevalence in all collection sites, with the exception of sites 5, 6, and 9, where

TABLE 4. PREVALENCE OF TICK-BORNE PATHOGENS IN TICKS COLLECTED IN FRANCE

Collection sites	A. phagocytophilum	A. marginale	B. burgdorferi s.l.	Bartonella spp.	C. burnetii	Babesia spp.	Theileria spp.	F. philomiragia
<i>D. marginatus</i>	1,2,3,4,7,8	2,7,8	3,5	1,2,3,4,5,6,7,8	1,2,3,4,5,7,8,9	5	7	2,5,6,8
Females	5/233	2/233	0/233	16/233	25/233	1/233	1/233	2/233
Males	7/144	0/144	0/144	18/144	19/144	0/144	0/144	3/144
% total	3% ^{b, i, iii, vi, vii}	0.5% ^{iv, v, viii}	0% ^{b, iv, v, viii}	9% ^{a, v, vi, viii}	12% ^{vi, vii, viii}	0.3% ^b	0.3% ^b	1.3% ^a
<i>D. reticulatus</i>	0/33	0/33	0/33	5/33	6/33	0/33	0/33	11/33
Females	0/41	1/41	1/41	4/41	6/41	0/41	0/41	3/41
% total	0%	1% ^{iv, v, viii}	1.5% ^{iv, v, viii}	12.2% ^{v, vii, viii}	16% ^{v, vii, viii}	0%	0%	18.9% ^b
<i>I. ricinus</i>	9/29	0/29	2/29	3/29	5/29	3/29	0/29	0/29
Females	9/15	0/15	0/15	1/15	3/15	0/15	0/15	0/15
Males	0/14	0/14	2/14	2/14	2/14	3/14	0/14	0/14
% total	41% ^{v, vi, vii, viii}	0% ⁱ	4.5% ^v	9.1% ^{vii, viii}	18% ^{vii, viii}	6.8% ^{vii, viii}	0%	0%
Total (n=495)	6%	0.6%	0.6%	9.5%	12.9%	0.8%	0.2%	3.8%

The number of positive/tested samples is shown for female and male ticks with percent prevalence shown for all (female + male) ticks. Infection prevalence were compared between host tick species for a given pathogen (significant differences compared against ^a*D. reticulatus* and ⁱ*I. ricinus*; $p < 0.05$) and between pathogens in a given tick species (significant differences are shown with symbols ⁱ to ^{viii} for *A. phagocytophilum*, *A. marginale*, *B. burgdorferi* s.l., *Bartonella* spp., *C. Burnetii*, *Babesia* spp., *Theileria* spp., and *F. philomiragia*).

Collection sites are described in Figure 1.

The laboratories where the experiments were conducted are described in Table 1.

fewer ticks were collected (Table 3 and Fig. 1). None of the *D. reticulatus* collected were positive for *A. phagocytophilum* and only 3% of *D. marginatus* (5% males and 2% females) were positive for *A. phagocytophilum*, whereas 41% of *I. ricinus* collected (60% males and 31% females) were positive for *A. phagocytophilum* (Table 4).

Detection of *F. tularensis* and *F. philomiragia*. All tick samples tested were negative for *tul4* and 19 of them were positive for *fopA* and *ISFtu2*, indicating the absence of *F. tularensis* and a prevalence of 3.8% for *F. philomiragia*. This bacterium was found in 4 different collection sites (Fig. 1). Only *D. marginatus* and *D. reticulatus* showed positive results for *F. philomiragia*, with a higher prevalence of 18.9 % in *D. reticulatus*, especially in females (33 % prevalence) (Table 4).

Detection of *C. burnetii*. *C. burnetii* showed a prevalence of 12.9% in analyzed ticks (Table 4). *C. burnetii* DNA was recovered from ticks at all collection sites, except from site 6 in central France, where only 8 ticks were collected (Fig. 1). The bacterial DNA was found both in males and females of the 3 tick species collected, with a higher prevalence of 18% in *I. ricinus*, followed by *D. reticulatus* (16%) and *D. marginatus* (12%) (Table 4).

Detection of *Babesia/Theileria* spp. Prevalences of 0.8% and 0.2% were found for *Babesia* and *Theileria* spp. parasites, respectively (Table 4). Three *I. ricinus* female ticks collected on site 5 were positive for *Babesia* spp., with 2 of them positive for *B. divergens* and 1 that did not correspond to any of the *Babesia* spp. analyzed (i.e., *B. bovis*, *B. divergens*, *B. major*, *B. motasi*, *B. ovis*, and *B. crassa*). One *D. marginatus* female collected on site 2 was positive for *Babesia* and *Theileria* spp. that did not correspond to any of the *Babesia* spp. and *Theileria* spp. (*T. annulata*, *T. velifera*, *T. taurotragi*, *T. mutans*, *T. hirsii*, *T. buffeli*, *T. ovis*, and *T. lestoquardi*) analyzed. Finally, 1 *D. marginatus* female collected on site 7 was positive for both *B. bovis* and *T. annulata*. None of the samples from *D. reticulatus* were positive for *Babesia* or *Theileria* spp. (Table 4).

Co-infection with different pathogens. Among the 495 ticks tested, 153 (31%) were positive for at least 1 pathogen, 18 (12%) were positive for 2 pathogens, and none of them carried 3 or more pathogens. *C. burnetii* DNA was detected in association with all pathogens tested, except for *B. burgdorferi* s.l. and *Theileria* spp. *Theileria* spp. parasites were found only in a tick also positive for *Babesia* spp. *Bartonella* spp. DNA was found in association with all the pathogens tested with the exception of *A. marginale*, which was detected only together with *C. burnetii* in 1 tick. *B. burgdorferi* s.l. was found in 1 tick in association with *Bartonella* spp. and in another tick together with *Babesia* spp. Finally, *F. philomiragia* was detected in conjunction with *C. burnetii* in 4 ticks and with *Bartonella* spp. in 2 ticks.

Discussion

In this study, we report the results of a survey conducted in 9 study sites in France with the aim of evaluating the prevalence of tick-borne pathogens in *Dermacentor* spp. and sympatric *I. ricinus* ticks. We were interested in *Dermacentor* spp. because these ticks represent the second genus of medical and veterinary importance after *Ixodes* spp. in Europe; however,

few studies have characterized pathogen prevalence in these ticks (Kahl et al. 1992, Sixl et al. 2003, Sting et al. 2004, de la Fuente et al. 2004a, de la Fuente et al. 2005a, Toledo et al. 2009a, Torina et al. 2010, de Carvalho et al. 2011, Satta et al. 2011).

Nine collection sites were chosen from southern to north-western France where *Dermacentor* spp. are abundant and tick-borne diseases have occurred (unpublished results). *D. marginatus* was the most abundant tick species in collected samples, followed by *D. reticulatus* and *I. ricinus*. *Dermacentor* spp. ticks lack host specificity and could infest and transmit different pathogens during their life cycle to several vertebrate hosts, including humans (Estrada-Pena and Jongejan 1999). Therefore, it is important to investigate the prevalence of pathogens of medical and veterinary importance in these ticks. The choice of analyzed pathogens was made according to the pathologies reported by the veterinarians practitioners in the concerned zones and includes *A. phagocytophilum*, *A. marginale*, *B. burgdorferi* s.l., *Bartonella* spp., *C. burnetii*, *Babesia* spp., *Theileria* spp., and *Francisella* spp.

The most prevalent pathogen recovered in *Dermacentor* spp. was *F. philomiragia* in *D. reticulatus*, particularly in female ticks. Vector-borne transmission of *F. philomiragia* has never been suspected, and its detection, for the first time in ticks, was not initially planned in our study. While looking for *F. tularensis*, this bacterium was revealing in the ticks. This bacteria appears to be an opportunistic pathogen, primarily causing serious diseases associated with 2 risk groups of chronic granulomatous disease and immunocompromised patients (Hollis et al. 1989). *F. philomiragia* has been isolated from humans with a febrile syndrome compatible with bacterial infection in Europe, North America, and Australia (Hollis et al. 1989). Knowing whether this bacterium can be transmitted by a vector like a tick must now to be clarified. *F. tularensis* has been suspected to be transmitted by both mosquitoes and ticks (Eliasson et al. 2002, and 3 cases of transmission associated with *Dermacentor* spp. ticks have been described in Spain (Morner 1992, Alkorta et al. 2000, Teijo-Nunez et al. 2006). Furthermore, it was reported that 0.7% of the *D. marginatus* ticks analyzed in another area of Spain carried this pathogen (Toledo et al. 2009a). In Portugal, a *Francisella*-like endosymbiont with significant identity with *F. tularensis* was detected in 39% of the *D. reticulatus* analyzed (de Carvalho et al. 2011). Taken together, these results suggest that *Dermacentor* spp. ticks could play a role in the maintenance and transmission of *Francisella* spp.

C. burnetii was the second most prevalent pathogen recovered from all collection sites, with similar prevalence in all 3 tick species. *C. burnetii* is responsible for Q fever, a zoonotic disease endemic worldwide (Maurin and Raoult 1999). Goats are probably the main reservoir host, and humans become infected mainly by inhalation of contaminated aerosols or dusts containing *C. burnetii* shed by infected animals (Tissot-Dupont et al. 2004). However, although previously considered as negligible, the role of ticks in bacterial transmission to wildlife and pets and in maintaining *C. burnetii* in wild and peridomestic cycles is now clearly recognized (Toledo et al. 2009b). In addition, *C. burnetii* infects several tick species. Other authors have found PCR evidence of *C. burnetii* in *Dermacentor* spp. collected in Spain (Toledo et al. 2009b) and Germany (Beytout et al. 2007) and in *Rhipicephalus* spp. and *Haemaphysalis* spp. collected in Sardinia, Italy (Satta et al.

2011). On the other hand, *C. burnetii* was not detected in *I. ricinus* collected in The Netherlands (Sprong et al. 2012) nor in *D. marginatus* collected in Sardinia, Italy (Satta et al. 2011). These results suggested that several tick species might vector *C. burnetii* in different regions to wild and domestic animals and eventually humans.

Bartonella spp. DNA was detected with similar prevalence in the 3 tick species analyzed and in all collection sites except 1. Sequence results suggested the existence of new *Bartonella* spp. or strains and/or the amplification of DNA from an unknown endosymbiont as was previously reported (Tijssen-Klasen et al. 2011). The presence of *Bartonella* spp. has been reported in ticks from all over the world, including Europe (Angelakis et al. 2010a). However, the tick role in the transmission of *Bartonella* spp. has been debated for many years, despite several reports of indirect evidence (Billeter et al. 2008, Angelakis et al. 2010a, Telford and Wormser 2010). Recent studies demonstrated the transmission of *Bartonella* spp. by *I. ricinus* both *in vitro* (Cotté et al. 2008) and *in vivo* (Reis et al. 2011a). In Italy, *Bartonella* spp. were not detected in *D. marginatus*, whereas pathogen DNA was detected in *Rhipicephalus* spp. (Satta et al. 2011). Recently, a study reported the detection of *B. henselae* infection in a patient following a bite by a *Dermacentor* spp. tick that was infected with the same bacteria (Angelakis et al. 2010b). *Bartonella* spp. prevalence reported here in *I. ricinus* was similar to that reported in ticks collected from northern France (Halos et al. 2005) and higher than that reported in western France (0.2%) (Cotté et al. 2010) and near Paris (0.1%) (Reis et al. 2011b).

A high prevalence of *A. phagocytophilum* was found in *I. ricinus* ticks (41% by real-time PCR), whereas 3% prevalence was found in *D. marginatus*. This obligate intracellular bacterium is the causative agent of granulocytic anaplasmosis in several hosts, including humans, horses, dogs, and ruminants (Woldehiwet 2006). This pathogen is widely distributed in France, where it has been identified in 84 Departments (Matsumoto et al. 2006; unpublished results), beyond the limits of the presence of its main vector *I. ricinus*. This fact suggests the implication of other tick species such as *Dermacentor* spp. in the transmission of *A. phagocytophilum*. Some studies conducted in Spain reported the detection of *A. phagocytophilum* DNA in *D. marginatus* questing ticks (Toledo et al. 2009a) and in ticks feeding on deer and wild boar (de la Fuente et al. 2005a, de la Fuente et al. 2004a), when none was detected in studies conducted in Italy (Satta et al. 2011). In our study, *D. reticulatus* was not infected with *A. phagocytophilum* as previously reported in Austria (Sixl et al. 2003). As expected, *I. ricinus* was confirmed infected as the principal vector of *A. phagocytophilum* with prevalences that were higher than those reported from other studies conducted in different French regions (0.35–10.7%) (Parola et al. 1998, Cotté et al. 2010, Halos et al. 2010, Reis et al. 2011b). However, these results may be affected by differences in the experimental methods used to determine infection prevalence. In fact, the comparison of 2 detection methods used in our study to detect *A. phagocytophilum* has shown that real-time PCR had a better sensitivity than PCR (data not shown).

Some *A. marginale*-positive samples were detected in both *D. marginatus* and *D. reticulatus*, but not in *I. ricinus*, suggesting that *Dermacentor* spp. may be vectors of *A. marginale* in

France. This bacterium, responsible for bovine anaplasmosis, is suspected to be transmitted by several hard tick species in subtropical regions (de la Fuente et al. 2005a) and also mechanically by certain hematophagous dipterans such as tabanid horse flies (de la Fuente et al. 2005b). Although *Dermacentor* spp. ticks are the biological vectors of *A. marginale* in North America, the main tick vector in Europe seems to vary depending on the region (Kocan et al. 2010). The results of a study conducted in 2005 in Sicily showed that among 8 collected tick species, including *D. marginatus* and *I. ricinus*, only *Rhipicephalus turanicus* and *Haemaphysalis punctata* were found to be infected with *A. marginale* (de la Fuente et al. 2005a). In Spain, *H. marginatum* and *Rhipicephalus bursa* were identified as potential biological vectors for *A. marginale* (de la Fuente et al. 2004a). However, a study performed in Hungary in 2008 reported the presence of *A. marginale* in *Tabanus bovis* and not in *D. marginatus*, *D. reticulatus*, *I. ricinus*, and *Haemaphysalis concinna* ticks, suggesting that mechanical transmission by tabanids may be more important than the biological vector role of hard ticks in this region (Hornok et al. 2008).

Theileria spp. were not identified in this study, except for 1 *D. marginatus* female that was found positive for *T. annulata* with a possible co-infection with *B. bovis*. This protozoan parasite is implicated in tropical theileriosis and is transmitted by ticks of the genus *Hyalomma* (Jongejan et al. 1983). Tropical cattle theileriosis is distributed in the Mediterranean and Middle East regions from Morocco to western parts of India and China. This geographical distribution may explain the fact that *T. annulata* was recovered here in southern France only. To our knowledge, this is the first report of *T. annulata* in France and suggested that *D. marginatus* ticks are susceptible to infection with this parasite.

Babesiosis is a worldwide tick-borne hemoprotozoosis affecting many mammalian species (Chauvin et al. 2009). In France, the most prevalent *Babesia* species corresponds to *B. divergens*, a bovine parasite that may infect humans and is transmitted by *I. ricinus* (L'Hostis and Chauvin 1999). In this study, *B. divergens* was detected in *I. ricinus* ticks collected from 2 study sites with a prevalence lower than that previously reported in northern France (20.6%; Halos et al. 2005) but similar to that found in western France (9.8%; Cotté et al. 2010). Although *D. marginatus* is considered a potential vector of *B. divergens* (Estrada-Peña and Jongejan 1999), this parasite was not recovered from *Dermacentor* spp. ticks in France. The fact that our study was performed in bovine pastures may explain why *Babesia* sp. EU1 was not identified in collected ticks. This *Babesia* species was recovered with high prevalence from ticks collected in French forests, where roe deer and not cattle are suspected as the main reservoir host (Duh et al. 2005, Bonnet et al. 2007, Reis et al. 2011b). *B. bovis* was identified in a *D. marginatus* female tick collected in southern France. *B. bovis* is a tick-borne protozoan parasite transmitted by *Rhipicephalus* spp. ticks that infects cattle in tropical and subtropical regions (Bock et al. 2004). As previously discussed, *B. bovis* was identified in the same tick infected with *T. annulata*, a pathogen also found in more tropical regions (Genis et al. 2008). This result suggested the introduction of cattle persistently infected with these pathogens in the study site where these parasites were recovered for the first time in France. However, as in previous cases with other ticks/pathogens, the finding of a *D. marginatus* infected with these 2 parasites does not imply that they are transmitted by this tick but may be simply that the tick acquired

infection after immatures feed on an imported and infected animal.

In this study, 7% of collected *I. ricinus* females were found infected with *B. burgdorferi* s.l. *Borrelia* prevalence in this tick species range from 0% to 36% in France (Randolph 2001, Halos et al. 2005, Ferquel et al. 2006, Beytout et al. 2007, Cotté et al. 2010, Reis et al. 2011b), demonstrating a high variation in pathogen prevalence between different regions. Ticks from the *I. ricinus* complex are considered the main vectors of *B. burgdorferi* s.l. (Nadelman and Wormser 1998), the causative agent of Lyme borreliosis, which is the most significant human vector-borne disease in Europe (Smith and Takkinen 2006). However, other tick species have been suspected to transmit these bacteria, and among them are *Dermacentor* spp. such as *D. marginatus* (Angelov et al. 1996). Furthermore, studies performed in Germany detected viable *Borrelia* spp. in *D. reticulatus* questing ticks with a 11.3% prevalence (Kahl et al. 1992). These results agreed with the finding of *B. burgdorferi* s.l. DNA in 1 *D. reticulatus* male analyzed in our study. As previously reported in Spain (Toledo et al. 2009a), *Borrelia* spp. DNA was not found in *D. marginatus*. Again, the possible role of *D. reticulatus* and *D. marginatus* in the transmission of *B. burgdorferi* s.l. needs to be demonstrated because other *Dermacentor* spp. ticks such as *D. silvaris* (Sun and Xu 2003), *D. andersoni* (Dolan et al. 1997), *D. variabilis* (Dolan et al. 1997), and *D. occidentalis* (Lane et al. 1994) are not vectors of *Borrelia* spp.

The results presented here corresponded to the first systematic study of tick-borne pathogens in *Dermacentor* spp. ticks in France. These results suggest a role for *Dermacentor* spp. as vectors of tick-borne pathogens that affect human and animal health. Several pathogens including *A. phagocytophilum*, *A. marginale*, *B. burgdorferi*, *Bartonella* sp., *C. burnetii*, *B. bovis*, *T. annulata*, and *F. philominagia*, were detected in *D. marginatus* and/or *D. reticulatus*, suggesting a possible role of these tick species in the life cycle and transmission of these pathogens in France. However, without experiments demonstrating the vector competence of these tick species, the epidemiological significance of these findings must be taken with caution, because the presence of a pathogen in ticks does not necessarily mean that they are capable of transmitting it to susceptible hosts. Nevertheless, this information is important for epidemiological studies of tick-borne pathogens in France and to prevent the risks associated with pathogen transmission by *Dermacentor* spp. ticks to humans and animals. Last, the list of pathogens studied here is not exhaustive, and other microorganisms like *Rickettsia* spp. or other species of *Babesia* sp. and *Theileria* sp. that could be carried and possibly transmitted by *Dermacentor* spp. should be studied in the future.

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Abstract

Ticks are obligate blood-feeding ectoparasites of many hosts including mammals, birds and reptiles. After mosquitoes, they are the most important vectors worldwide, and are able to transmit the highest variety of pathogens including virus, bacteria and parasites. *Ixodes ricinus* (Acari: Ixodidae), the most common tick species in Europe, is a three-life stage hard tick. It is frequently associated with bites in humans, and transmits several pathogens, including Tick-Borne Encephalitis, *Babesia* spp., *Borrelia* spp., *Anaplasma* spp., and to a lesser extent *Bartonella* spp. *Bartonella* spp. are facultative intracellular bacteria associated with a number of emerging diseases in humans and animals. It has been demonstrated that *I. ricinus* is a competent vector for *B. henselae* that causes cat scratch disease as well as being increasingly associated with a number of other syndromes, particularly ocular infections and endocarditis.

Recently, emergence or re-emergence of tick-borne diseases (TBDs) is increasingly becoming a problem. Indeed, and because of the limited success and disadvantages of controlling TBDs via acaricides, new approaches are urgently needed. Therefore, vaccine strategies that target conserved components of ticks that play roles in vector infestation and vector capacity have become particularly attractive. Accordingly, the identification of suitable antigenic targets is a major challenge for the implementation of tick and TBDs control strategies.

In the present work, the main objective is to elucidate molecular interactions between *I. ricinus* and *B. henselae* in order to identify some targets that may be used as vaccines against ticks and tick-borne pathogens. Two principal points are focused on: primarily, to identify *I. ricinus* salivary gland differentially expressed transcripts in response to *B. henselae* infection with next generation sequencing techniques (454 pyrosequencing and HiSeq 2000); secondly, to validate the implication of one of these transcripts in the transmission of *B. henselae*. For that purpose, and at first, we validated artificial membrane feeding technique for ticks infection by *B. henselae* and evaluated the impact of several parameters on tick feeding.

Results showed that membrane feeding technique is a suitable method to infect *I. ricinus* with *B. henselae* and that the proportion and weight of engorged ticks are decreased by *B. henselae* infection of the blood meal. Transcriptional analysis of the tick salivary glands generated a reference databank containing 24,539 transcripts, and the comparison of *B. henselae*-infected and non-infected *I. ricinus* female salivary glands showed that 829 and 517 transcripts were significantly up- and down-regulated in response to bacteria infection, respectively. Among them, 161 transcripts corresponded to 9 groups of ticks salivary gland gene families already described, when the other ones corresponded to genes of unknown function. Silencing the most up-regulated gene *IrSPI*, which belongs to BPTI/Kunitz family of serine protease inhibitor, resulted in reduction of tick feeding and bacteria load in tick salivary gland.

In conclusion, this work demonstrated that artificial-membrane feeding technique is a powerful tool for investigating the interactions between tick and tick-borne pathogens as *B. henselae*. It also increases the available genomic information for *I. ricinus* and the knowledge to improve our understanding of the molecular interaction between tick and tick-borne pathogens. At last, it provides a potential vaccine candidate to control tick-borne diseases. In the future, and depending of differentially expressed genes' role confirmation, more and more vaccine candidate will be provided by this work, and the strategy of controlling tick and tick-borne disease will come to a new stage.

Résumé

Les tiques sont des arthropodes hématophages qui parasitent de nombreux hôtes, dont des mammifères, des oiseaux et des reptiles. Après les moustiques, elles représentent les vecteurs de maladies les plus importants au monde et sont à même de transmettre la plus grande variété de microorganismes incluant des virus, des bactéries, et des parasites. Parmi les tiques, *Ixodes ricinus* est l'espèce la plus largement répandue en Europe. Elle est responsable de la transmission de beaucoup d'agents pathogènes importants en santé humaine et vétérinaire comme *Babesia* spp., *Borrelia* spp., *Anaplasma* spp., et à un moindre degré, *Bartonella* spp. Les bartonelles sont de petits coccobactéries Gram-négatif de la classe des alpha-protéobactéries qui sont associés à de nombreuses maladies chez l'homme et l'animal. Il a été démontré que *I. ricinus* est un vecteur compétent pour *B. henselae* qui est à l'origine de la maladie des griffes du chat et de nombreux autres syndromes chez l'Homme.

Aujourd'hui, l'émergence ou la réémergence de maladies transmises par les tiques (TBDs) devient un problème majeur. En raison des problèmes générés par l'utilisation des acaricides (pollution, résistance), il est donc urgent d'identifier de nouvelles approches pour contrôler les populations de tiques. Parmi ces stratégies, la vaccination visant des molécules conservées chez les tiques et impliquées dans leur capacité vectorielle, sont devenues particulièrement attractives. En conséquence, l'identification de cibles antigéniques appropriées est un défi majeur pour la mise en œuvre de ces stratégies de contrôle des tiques et des TBDs.

Dans le présent travail, l'objectif principal est d'élucider les interactions moléculaires entre *I. ricinus* et *B. henselae*, afin d'identifier des molécules qui pourraient représenter des cibles vaccinales contre les tiques et les agents pathogènes qu'elles transmettent. Dans ce but, nous avons identifié, par séquençage à haut débit, des transcrits d'*Ixodes ricinus* différenciellement exprimés au niveau des glandes salivaires de la tique en réponse à une infection par *B. henselae*. Dans un second temps, l'implication d'un de ces transcrits surexprimés lors de l'infection dans la transmission de *B. henselae*, a été évaluée. Enfin, et en premier lieu, nous avons validé l'utilisation de la technique de gorgement artificiel sur membrane pour infecter *I. ricinus* par *B. henselae* et évalué l'impact de différents paramètres sur le gorgement des tiques.

Les résultats ont montré que la technique de gorgement sur membrane est bien adaptée à l'infection d'*I. ricinus* par *B. henselae* en laboratoire, et que la proportion et le poids des tiques gorgées sont diminués lors de l'infection du sang par la bactérie. Le séquençage en 454 des glandes salivaires de tiques a généré une banque de référence contenant 24, 539 transcrits, et la comparaison des glandes salivaires d'*I. ricinus* infectés et non-infectés par *B. henselae* a montré que 829 et 517 transcrits étaient respectivement significativement surexprimés et sous-exprimés en réponse à l'infection par des bactéries. Parmi les gènes de fonction connue, 161 transcrits correspondent à 9 familles déjà identifiées, quand les autres correspondent à des gènes de fonction inconnue. L'extinction par RNA interférence du gène le plus surexprimé, IrSPI qui appartient à la famille des inhibiteurs de sérine protéase BPTI/Kunitz, a entraîné une réduction de la taille du repas sanguin prit par les tiques (et donc sa descendance) ainsi que du niveau d'infection au niveau des glandes salivaires.

En conclusion, cette étude a démontré que la technique de gorgement artificiel des tiques sur membrane est un outil puissant pour étudier les interactions entre les tiques et les agents pathogènes qu'elles transmettent comme *B. henselae*. Ce travail apporte aussi une nette avancée en termes de données génétiques sur *I. ricinus* (dont le génome n'est pas séquencé) et sur les interactions moléculaires entre une bactérie et son vecteur. Enfin, ce travail a permis la mise en évidence d'une molécule représentant un candidat vaccinal très prometteur à la fois pour diminuer la population de tiques et lutter contre les agents pathogènes qu'elles transmettent. Dans le futur, et en fonction de la confirmation du rôle des gènes identifiés ici dans la transmission bactérienne, de nombreux candidats vaccins pourront ainsi être évalués, ouvrant alors de nouvelles perspectives dans la lutte contre les tiques et les maladies dues aux agents qu'elles transmettent.