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Identification of salivary antigenic markers discriminating host exposition between two European ticks: *Rhipicephalus sanguineus* and *Dermacentor reticulatus*

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ABSTRACT

To succeed blood meal, ticks inject salivary proteins to mammalian hosts, eliciting an antibody response against these foreign antigens. Although this immune response has been proposed as a surrogate marker of exposure to tick bites, identification of the corresponding antigens remains elusive. For this aim, a comparison by immunoblots of the kinetic IgG responses to protein salivary gland extracts from two European tick species, *Rhipicephalus sanguineus* or *Dermacentor reticulatus*, in rabbits was performed. A singularity in the immune patterns was observed according to rabbit exposure status and depending on the antigen source. Six and five bands were found specifically associated to *R. sanguineus* and to *D. reticulatus* exposures, respectively. The identity of these salivary antigenic proteins was determined using an original immunoproteomic approach. The utilization of these tick salivary proteins as biomarker candidates to discriminate *R. sanguineus* and/or *D. reticulatus* tick exposure or to develop anti-tick vaccines is discussed.

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1. Introduction

Ticks are currently the most important vectors of human infectious diseases after mosquitoes [1]. Due to the duration of their blood meal, ticks face a strong response from the host defense systems and thus tick saliva has evolved to counter these responses [2]. Recent progress in transcriptome research has uncovered that hard ticks express hundreds of different proteins in their salivary glands (SG) [2,3]. Recently, the elucidation of the

sialotranscriptome from the *R. sanguineus* has been performed opening the opportunity to realize proteomic analysis for identifying for example antigenic salivary proteins reflecting vector–host contacts [4]. Tick saliva components include a cement to anchor the mouthparts to the skin of the host, enzymes, enzyme inhibitors, amine-binding proteins and cytokine homologues that act as anti-hemostatic, anti-inflammatory or immunomodulatory agents [2,3]. This protein cocktail facilitates successful blood feeding and also assists in the transmission of pathogen agents to the host [5]. The immunogenicity of these proteins has been demonstrated in the laboratory and in the field, both in humans and animals [2]. The anti-tick saliva antibody response as a biomarker of tick exposure has been tested using SG extracts or recombinant proteins such as the calreticulin salivary protein [6,7]. Human and animal immune responses against salivary extract or recombinant protein confirmed tick exposure or duration of tick attachment over a span of three days; however, none of these responses was specific to a tick species [8,9].

Prophylaxis of vector-borne diseases, including tick borne diseases, is an important problem both in human and veterinary medicine. One of the perspective approaches to solve this problem is the creation of vaccines against arthropods including pan arthropod vaccines or specific vaccines such as anti-tick vaccines [10,11]. During the last ten years, vaccines developed against *Rhipicephalus* (*Boophilus*) spp. using tick midgut antigens have demonstrated the feasibility of using vaccines for the control of tick infestations and the success of recombinant vaccines [12]. Although this vaccine conferred a level of protection for tick species other than *R. (Boophilus) microplus*, this protection was not optimal against tick species independent of the genus [13,14]. The success of immunological control methods is dependent upon the use of potential key antigens, and there is clearly a need to determine additional tick-protective antigens. Thus, many studies have been conducted to identify other sources of protective antigens in addition to the midgut antigens, including “concealed” antigens (hematophagous arthropod antigens that are not naturally in contact with the host immune response like the midgut antigens are) and “exposed” antigens (antigens secreted during the blood meal) [15,16]. The immunization with “concealed” antigens, such as midgut antigens, testicular proteins, recombinant chitinase proteins and non-secreted salivary proteins, could be efficient; however, the protective immunity that develops is short-lived and repeated vaccination cycles are needed to induce an effective vector-bite protection [17–19]. The use of secreted “exposed” antigens alone or associated with concealed antigens is a method for vaccine design that does not necessitate iterative vaccinations [20]. Many methods have been used to identify protective candidate antigens for the control of ticks, such as the bioinformatic analysis of the amino acid composition of salivary proteins and cDNA expression libraries, use of RNA interference, infections with saliva, salivary extracts, or intestinal extracts or the determination of host immune resistance induced by infestations [21–24]. *R. sanguineus*, commonly known as the brown dog tick, is widely distributed throughout the world and

is a vector of many important pathogens, such as *Rickettsia conorii conorii*, *Rickettsia rickettsii*, and *Rickettsia massiliae* [25,26]. Natural immunization after multiple infestations has been described previously [27]. In some pilot studies, antibody responses against the *R. sanguineus* SG proteins could be used as biomarkers of tick exposure; however, in a recent study conducted in patients affected by Mediterranean spotted fever (MSF), this antibody response was not found in many patients [28–30]. Commonly known as the ornate cow tick, *D. reticulatus* is one of the main vectors of *Rickettsia slovaca* and *Rickettsia raoultii* throughout Europe [31,32]. The involvement of *D. reticulatus* SG extracts in the inhibition of host cytokine production and natural killer cell activity has been well described [33,34]; however, the nature of the antibody responses against *D. reticulatus* SG proteins remains unknown. Therefore, complementary studies are needed to clarify antigenic SG proteins involved in the emergence of host antibody responses following a tick bite.

Thus, the present work proposed to determine the tick SG antigenic proteins that are useful for the determination of distinct exposed vs. un-exposed mammals to tick bites. Furthermore, for the first time, the host exposure according to each tick species is discriminated. A comparative analysis of the kinetic IgG antibody profiles from rabbits exposed to *R. sanguineus* or *D. reticulatus* tick bites against protein salivary gland extracts (pSGE) was performed. The combination of one- and two-dimensional immune-proteomic approaches allowed us to identify shared- and discriminant-*R. sanguineus* and/or *D. reticulatus* salivary antigenic proteins. These exposure biomarker candidates could be helpful in the diagnosis of the diseases transmitted by these vectors, in the assessment of vector control strategies or the development of anti-tick vaccines.

2. Materials and methods

2.1. Tick and sera collection

Pathogen-free *R. sanguineus* ticks, originating from southern France, used in our work have been maintained at the Unité de Rickettsies, the WHO Collaborative Center for Rickettsial and Other Arthropod-Borne Bacterial Diseases, in Marseille, France, since 2003. Pathogen-free *D. reticulatus* ticks have been maintained in this laboratory since 2009.

Three specific-pathogen-free New Zealand white rabbits (Charles River, Lyon, France) were infested successively three times with approximately 20 adult pathogen-free ticks each time. Rabbits were monitored once daily for the engorgement of ticks. The duration of the blood meal varied from 5 to 14 days. Semi-engorged females were collected for SG dissection. Rabbit sera were collected before the manipulation (T0), at the end (T1, T3 and T5) and three weeks (T2, T4 and T6) after the first, second and third infestation, respectively. The detail experimental design was illustrated in Additional file 1. All experimental protocols were approved by the Ethics Committee for Animals of the Medical School of Marseille, France.

2.2. Tick dissection and salivary gland extraction

SG from semi-engorged females were dissected under a stereomicroscope at 1.6× magnification, pooled by species into a microcentrifuge tube on ice in phosphate-buffered saline (PBS) supplemented with protease inhibitor (Sigma, St. Louis, MO, USA), and then disrupted using ultrasonication (Vibracell 72412; Bioblock Scientific, Illkirch, France) for 5 min on ice at maximum amplitude. SG homogenates were then centrifuged for 15 min at 16,100 × g, and the protein concentration of the supernatant was determined in duplicate using the Lowry method (DC Protein assay Kit, Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Each sample was then stored at –20 °C [35].

2.3. CyDye labeling

Tick pSGE were concentrated via precipitation with acetone (Sigma) and were suspended in UTC buffer containing 8 M urea (Sigma), 2 M thiourea (Sigma), 4% (w/v) CHAPS (Sigma) and 30 mM Tris (Sigma), adjusted to pH 8.5, to obtain a protein concentration adjusted to 2.5 µg/µL. pSGE were then minimally labeled with CyDye according to the manufacturer's recommended protocols and as previously described [36,37]. Briefly, proteins were labeled with either cyanine 5 (Cy5) or cyanine 3 (Cy3) (GE Healthcare, Uppsala, Sweden), freshly dissolved in anhydrous DMF (Sigma), and incubated on ice for 30 min in the dark. The reaction was quenched with 1 µL of free lysine (10 nM; Sigma) and incubation for 10 min on ice. Each sample was then immediately used for 1D or 2D experiments.

2.4. One-dimensional (1D) immunoblot

For each tick species, 130 µg of CyDye-labeled tick pSGE was loaded per gel with an equal volume of 2× Tris buffer containing 5% (w/v) SDS (Sigma) [38] and separated using a 12.5% SDS-PAGE in a Mini-protean precast gel (Bio-Rad). For each gel, 10 µL of Precision Plus protein dual-color standard (Bio-Rad) was loaded. After SDS-PAGE, gels were transferred onto a nitrocellulose membrane (0.45-µm, GE Healthcare) with semidry blotting (0.8 mA/cm²) [39]. Blots were incubated with a saturation buffer (5%, w/v, skimmed milk (Becton Dickinson Bioscience, NJ, USA) in PBS containing Tween-20 (0.1%, v/v, Sigma), and each membrane was cut into 15–25 strips, 3–4 mm wide. Immunoblots were performed with rabbit sera diluted 1/100 in PBS supplemented 0.1% (v/v) Tween-20 with 5% (w/v) skimmed milk. After an overnight incubation at 4 °C, blots were incubated with anti-rabbit IgG fluorescein isothiocyanate (FITC) conjugated antibody at 1/1000 (Sigma), and scanned with a TyphoonTM Trio Image scanner (GE Healthcare) at FITC and Cy5 excitation wavelengths (488 nm and 633 nm). The Cy5 labeling of pSGE was used as a control of migration to improve the accuracy of the alignment of antibody reactivity [40]. Pre-scans were performed to adjust the photomultiplier tube voltage to obtain images with a maximum intensity of 60,000–80,000 U. Molecular weights were estimated via comparison with a standard molecular weight marker.

2.5. Two-dimensional (2D) electrophoresis and immunoblot

Reagents that were not purchased from GE Healthcare are indicated. Destreak buffer containing 1% (v/v) IPG buffer 3–10 was used for IPG strips (3–10 linear, 11-cm) overnight rehydration. The CyDye-labeled pSGE for each sample were placed at the acidic end of the IPG strip using a cup-loading technique. For 2D immunoblots and 2D preparative gels, 30 and 60 µg were loaded, respectively. IEF was performed using an Ettan IPGphor II electrophoresis unit at 20 °C, for a total of 30 kVh. Prior to separation in the second dimension, IPG strips were reduced and alkylated in an equilibration buffer containing 50 mM Tris–HCl, pH 8.6 buffer, 6 M urea, 2% SDS and 30% glycerol supplemented with 1% (w/v) DTT or 2.5% (w/v) iodoacetamide instead of DTT for 10 min. IPG strips were then placed at the top of a 12.5% SDS-PAGE Criterion Tris–HCl precast gel (Bio-Rad). For each gel, 10 µg of CyDye-labeled tick pSGE were loaded beside the IGP strip to facilitate the alignment of the 1D antigenic bands with the corresponding 2D antigenic spots. After electrophoresis, gels with CyDye-labeled proteins were scanned with the TyphoonTM Trio Image scanner (GE Healthcare) at Cy3 and Cy5 wavelengths (532 nm and 633 nm, respectively), before performing protein transfer onto nitrocellulose membranes (0.2 µm, GE Healthcare). Immunoblots were performed as described above and analyzed using Decyder v6.5 software (GE Healthcare), as previously described [39]. Briefly, the superimposition of the protein expression profile and the antigenic protein pattern images allowed us to excise accurately the spots of interest on the preparative gel run in parallel. The protein expression profile was used as an “internal standard” to perform a perfect match between the blot and the preparative gel. Antigenic spots were selected for excision and further identification with mass spectrometry (MS).

2.6. Mass spectrometry

Based on the Decyder analysis, spots of interest were excised and digested as described [39,41]. The samples were then analyzed on a NanoLC-LTQ-OrbitrapVelos-ETD (Thermo Scientific, Bremen, Germany). Nano-liquid separation of peptides was performed using a nanoliquid chromatography system (UltiMate[®] 3000 Rapid Separation LC (RSLC), Dionex, Sunnyvale, CA, USA). Five microliters of digested peptide sample was pre-concentrated and washed on a Dionex AcclaimPepMap 100 C18 column (2 cm × 100 µm i.d., 100 Å, 5 µm particle size) and then separated on a Dionex Acclaim PepMap RSLC C18 column (15 cm × 75 µm i.d., 100 Å, 2 mm particle size) (Dionex, Amsterdam, Netherlands) using a linear 30 min gradient (4–40% acetonitrile/H₂O; 0.1% formic acid) at a flow rate of 300 nL/min, under UV detector monitoring (absorption at 214 nm). The nanoLC was coupled to a nanospray source of a linear ion trap Orbitrap mass spectrometer (LTQ OrbitrapVelos, Thermo Electron) at 1.4 kV of LTQ spray voltage and a capillary temperature of 275 °C. All samples were measured in a data-dependent acquisition mode. Each run was preceded by a blank MS run to monitor the system background. A survey full scan (scan range 300–1700

m/z , with 30 K FWHM resolution at $m/z = 400$, target AGC value of 1.00×10^6 and maximum injection time of 500 ms) was performed to measure the peptide masses. The data-dependent CID scans of the 10 most intense precursor ions were fragmented and measured in the linear ion trap (normalized collision energy of 35%, activation time of 10 ms target AGC value of 1.00×10^4 , maximum injection time 100 ms, isolation window 2 Da and wideband activation enabled) and was visualized in parallel to the high-resolution full scan in the Orbitrap. The fragment ion masses were then measured in the linear ion trap to have a maximum sensitivity and the maximum amount of MS/MS data. Dynamic exclusion was implemented with a repeat count of 1 and exclusion duration of 25 s.

2.7. MS data analysis

Data were searched using Mascot software against the *Ixodidae* National Center for Biotechnology Information non-redundant (NCBI nr, NIH, Bethesda, MD, USA) protein database (January 16, 2012), as described [39]. Search parameters were set to allow for one missed tryptic cleavage site, the carbamidomethylation of cysteine, and the possible oxidation of methionine; the precursor and product ion mass error tolerance was <0.2 Da. All identified proteins had a Mascot score greater than 53 (*Ixodidae*, 28587 sequences), corresponding to a statistically significant ($p < 0.05$) confident identification. Moreover, among the positive matches, only protein identifications based on at least two different non-overlapping peptide sequences with a mass tolerance <0.05 Da were accepted. For single peptide-based identification, in addition to Mascot score significance, only peptide sequences with at least six consecutive amino acids detected on MS spectra were considered to limit the number of false positive matches without missing real proteins of interest. Signal peptides were predicted by the submission of the protein sequences to the SignalP server 3.0.

3. Results

3.1. Kinetic analysis of IgG antibody responses from rabbits exposed to tick bites against *R. sanguineus* pSGE

As SG protein composition of ticks could change during the blood feeding [4], a qualitative and quantitative comparison of the SG protein profiles from semi-engorged (SG collected from 4 to 5 days after tick attachment) and engorged ticks (SG collected 10–14 days after tick attachment) was performed. In SG from engorged ticks, protein profiles and protein quantities were found less complex and abundant compared to those obtained from semi-engorged ticks (Additional file 2), reflecting the morphological degradation of SG in completing the engorgement stage [42,43]. Then, SG from semi-engorged ticks were chosen as antigen source for the assessment of rabbit antibody responses.

To determine the characteristic of antibody responses in rabbits exposed to tick bites against *R. sanguineus* pSGE, a comparison of the kinetic IgG rabbit antibody profiles was performed using 1D immunoblot analysis. Although,

non-specific IgG responses against tick pSGE were observed in all naive rabbits at T0, some additional bands were detected from T1 to T6 in sera from rabbits exposed to *R. sanguineus* and *D. reticulatus* (Fig. 1).

Concerning rabbits exposed to *R. sanguineus*, the kinetic analysis of IgG responses indicated that some antigenic bands started to appear at T1 (i.e., bands Rhs4 and Rhs5). In the consecutive rabbit sera collection, these two bands were maintained and their intensity of recognition increased. Moreover, supplementary antigenic bands were revealed following each new infestation (i.e., T3 and T5), and a similar antigenic band detection kinetic was observed in two additional rabbits exposed to *R. sanguineus* bites under similar conditions. These results indicated an important reproducibility of the immune response and suggested that some salivary proteins are more antigenic and that the diversity of this response may be linked to the level of exposure.

Concerning the rabbits exposed to *D. reticulatus*, some antigenic bands appeared at T1 (i.e., two bands at molecular weights of approximately 30–34 kDa) and these bands were maintained in the consecutive rabbit sera collection. However, in contrast to rabbits exposed to *R. sanguineus*, no evolution of the IgG immune profiles was detected despite the following infestations. It is interesting to note that the kinetic antigenic profiles from rabbits exposed to *D. reticulatus* bites, were highly stable and reproducible.

An accurate comparison of these antigenic profiles indicated that some antigenic bands were detected with sera from rabbits exposed to either *R. sanguineus* or *D. reticulatus* (i.e., two bands at molecular weights of approximately 30–34 kDa), suggesting that some salivary antigens could be conserved and then shared between these two tick species.

However, six antigenic bands, arbitrarily named Rhs1 to Rhs6 with a molecular weight range from 72 to 13 kDa, were exclusively recognized with sera from rabbits exposed to *R. sanguineus* bites. A schematic representation of these antigenic bands was depicted on an immunoreactivity diagram (Fig. 1). These six antigenic bands distinguishing rabbit exposures between these two tick species could be considered as relevant antigenic marker candidates of antibody responses following *R. sanguineus* bite exposure. For subsequent antigen identification using a 2D immunoproteomic approach, pooled sera ($n = 3$) from rabbits collected at T6 covering the whole spectrum of these antigenic bands against *R. sanguineus* pSGE were selected.

. Characterization of *R. sanguineus* pSGE antigens recognized by rabbits exposed to tick bites

As each antigenic band could contain numerous proteins, a 2D immunoproteomic approach was then adopted to characterize these tick salivary antigens. To limit the mismatch between antigenic spots detected on 2D immunoblots and their counterparts on the preparative gel, a 2D immunoblot fluorescence-based method was used to increase the confidence in spot matching as previously described [39]. Using *R. sanguineus* pSGE as the antigen source, the pooled sera of rabbits exposed to

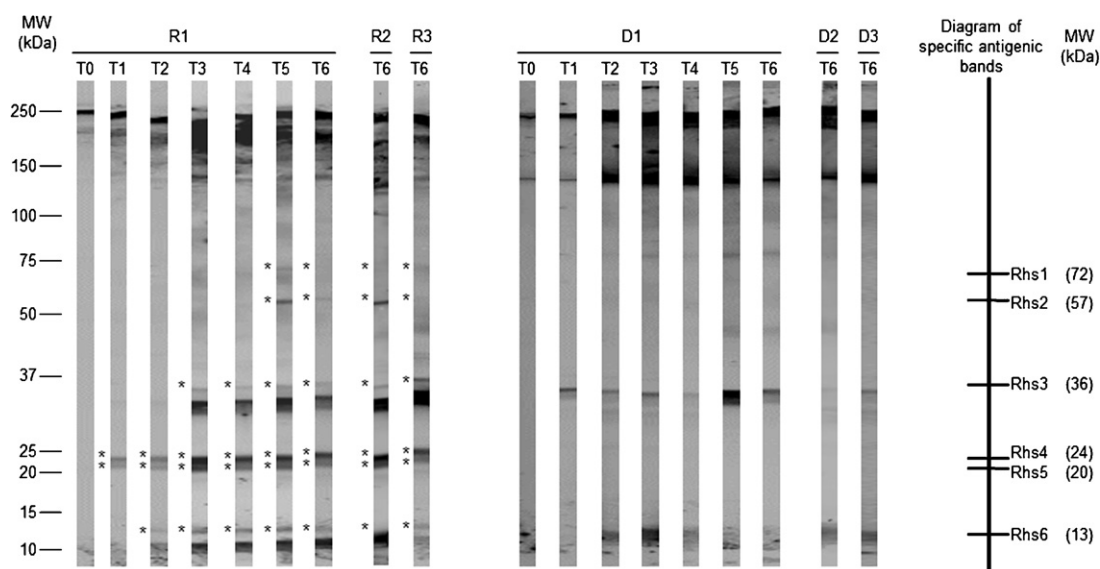


Fig. 1. One-dimensional (1D) *Rhipicephalus sanguineus* salivary gland antigenic profiles from rabbits exposed to different tick species. The evolution of the IgG immune profiles from rabbits exposed to *R. sanguineus* ($n=3$; R1 to R3) or to *D. reticulatus* ($n=3$; D1 to D3) bites against *R. sanguineus* salivary gland protein extracts are presented. Kinetic rabbit sera collection from T0 (naive rabbit) to T6 is indicated at the top (see Section 2 for details). Antigenic bands detected exclusively by rabbits exposed to *R. sanguineus* are indicated on the immune profiles by an asterisk (*) and on the diagram (from Rhs1 to Rhs6). Their corresponding apparent molecular weights are indicated with brackets. MW, molecular weight; kDa, kiloDalton.

R. sanguineus ($n=3$, collected at T6, Fig. 2B), *D. reticulatus* ($n=3$, T6, Fig. 2C) and un-exposed ($n=3$, T0, Fig. 2D) detected 34, 22 and 6 antigenic protein spots on 2D immunoblots, respectively. The six antigenic protein spots detected by naive rabbits were considered as non-specific. They were also detected by pooled sera from rabbits exposed to *R. sanguineus* and *D. reticulatus* bites. Thus, 28 and 16 antigenic protein spots were attributed to the *R. sanguineus* and *D. reticulatus* tick bites, respectively. Interestingly, all of the 16 antigenic protein spots detected by pooled sera from rabbits exposed to *D. reticulatus* were also recognized by pooled sera from rabbits exposed to *R. sanguineus*. These antigenic protein spots were considered as general markers of tick exposure. This phenomenon was attributed to the presence of an ortholog salivary protein from these two distinct species, which could present sequence homologies and then shared-antigens. However, 12 antigenic protein spots were exclusively detected by pooled sera of rabbits exposed to *R. sanguineus* bites that could be considered as discriminant antigens. The loading of *R. sanguineus* pSGE onto a 2D gel next to the IPG strip prior to electrophoresis allowed the alignment of antigenic bands with the corresponding spots between 1D and 2D immunoblots, as described previously [44]. For all of the antigenic bands selected with 1D analysis (Rhs1 to Rhs6), their corresponding spots ($n=6$) were detected on a 2D immunoblot (Fig. 2A and B). The 6 remaining antigenic protein spots detected only by the pooled sera of rabbits exposed to *R. sanguineus* on the 2D immunoblot are presented in Fig. 2E and F. Interestingly, a 2D immunoblot performed with the sera of a single rabbit (R1 collected at T6) exposed to *R. sanguineus* detected the same antigenic protein spots as that observed by the pooled sera ($n=3$, T6) (data not shown). These results support the high

reproducibility of the immune response elicited by these tick bite exposures for each rabbit. A Venn diagram summarizes the distribution of these antigenic protein spots according to rabbit tick species bite status exposure (Fig. 2G).

The 34 antigenic protein spots were then excised and submitted to MS for identification. A total of 23 protein spots (67.6%) were successfully identified, corresponding to 15 distinct proteins according to their NCBI accession number (Table 1). The same protein was detected in more than one spot (e.g., Hsp90 detected in spots numbered 243, 267 and 374), while inversely, several proteins were identified in only one spot (e.g., Vitellogenin-2 and hemelipoglycoprotein precursor detected in spot numbered 293). Among the proteins identified, five proteins detected by naive rabbit sera were considered as non-specific/relevant antigens to tick exposure. Six proteins corresponding to 12 antigenic spots recognized by pooled rabbit sera from both *R. sanguineus* and *D. reticulatus* tick bites reflect an exposure to these two tick species. Five proteins corresponding to five antigenic spots detected only by pooled rabbit sera from *R. sanguineus* tick bites represent particularly interesting antigenic candidates to distinguish these tick expositions. However, among the six *R. sanguineus* antigenic bands (i.e., Rhs1 to Rhs6), corresponding antigenic spots from only four bands were successfully identified (Fig. 2 and Table 1). Despite their abundances, antigenic protein spots corresponding to bands Rhs4 and Rhs5 were not identified. Thus, five *R. sanguineus*-discriminant antigenic proteins, heat shock 70 kDa protein 5 (gi|169809132) (Rhs1), protein disulfide isomerase-2 (gi|148717315) (Rhs2), malate dehydrogenase (gi|241243545) (Rhs3), cement protein (gi|260908636) (Rhs6) and the heme-binding aspartic

Table 1
Rhipicephalus sanguineus antigenic salivary proteins detected by tick-exposed rabbit sera.

Spot number	Band name	Protein name	Accession no. (NCBI)	MW (kDa)	pI value	Signal peptide	Significance (Mascot score)	Peptide number	Tick-exposed rabbit sera		
									R.s.	D.r.	Naive
336 ^a	Rhs1	Heat shock 70 kDa protein 5 [<i>Haemaphysalis longicornis</i>]	gi 169809132	72.5	5.3	Y	936	26	×		
458 ^a	Rhs2	Protein disulfide isomerase-2 [<i>Haemaphysalis longicornis</i>]	gi 148717315	57.3	4.8	Y	592	13	×		
805 ^a	Rhs3	Malate dehydrogenase [<i>Ixodes scapularis</i>]	gi 241243545	36.1	9.2	N	123	3	×		
1141 ^a	Rhs6	Cement protein [<i>Rhipicephalus sanguineus</i>]	gi 260908636	20.6	8.8	Y	53	1	×		
744 ^a		Heme-binding aspartic proteinase [<i>Rhipicephalus microplus</i>]	gi 9858101	38.8	7.2	N	163	3	×		
27		Na ⁺ /K ⁺ ATPase, alpha subunit [<i>Ixodes scapularis</i>]	gi 241124081	114.6	5.5	N	1037	30	×	×	
243							257	9	×	×	
267		Hsp90 protein [<i>Ixodes scapularis</i>]	gi 241830514	84.7	5.0	N	518	19	×	×	
374							1617	37	×	×	
585		Actin [<i>Rhipicephalus microplus</i>]	gi 32330667	42.2	5.3	N	98	7	×	×	
657							6922	32	×	×	
587		Beta tubulin [<i>Ixodes scapularis</i>]	gi 241151391	50.4	4.8	N	1007	20	×	×	
598							867	17	×	×	
609							357	7	×	×	
844		Malate dehydrogenase [<i>Ixodes scapularis</i>]	gi 241243545	36.1	9.2	N	837	11	×	×	
876							810	10	×	×	
877		Mitochondrial voltage dependent anion-selective channel [<i>Rhipicephalus microplus</i>]	gi 315142889	29.7	8.9	N	225	7	×	×	
293		Hemelipoglycoprotein precursor [<i>Dermacentor variabilis</i>]	gi 89954505	178.6	6.3	Y	238	12	×	×	×
		Vitellogenin-2 [<i>Haemaphysalis longicornis</i>]	gi 169159125	177.4	6.1	Y	179	5			
472		D-3-phosphoglycerate dehydrogenase [<i>Ixodes scapularis</i>]	gi 240991833	50.3	6.5	N	940	15	×	×	×
		F0F1-type ATP synthase, alpha subunit [<i>Ixodes scapularis</i>]	gi 241705635	60.0	9.1	N	347	13			
521		F0F1-type ATP synthase, alpha subunit [<i>Ixodes scapularis</i>]	gi 241705635	60.0	9.1	N	1063	19	×	×	×
543		F0F1-type ATP synthase, alpha subunit [<i>Ixodes scapularis</i>]	gi 241705635	60.0	9.1	N	309	11	×	×	×
		Ribosomal DEAD box protein [<i>Ixodes scapularis</i>]	gi 241741609	37.1	8.6	N	415	12			
544		Ribosomal DEAD box protein [<i>Ixodes scapularis</i>]	gi 241741609	37.1	8.6	N	573	15	×	×	×

The proteins were identified using a NanoLC-LTQ-Orbitrap-Velos mass spectrometer, following in-gel digestion with trypsin. Spot numbers and band names correspond to those indicated in Figs. 2A and 1, respectively. The identities of the protein spots, their NCBI accession numbers, the MW and pI values, the presence of a signal peptide and the Mascot score serve as an indicator of confidence of identification (protein scores greater than 53 were considered as significant ($p < 0.05$)). The number of peptide sequences is given. Protein sequences were submitted to the SignalP v.3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) server to predict a signal peptide (Y, presence; N, absence). R.s., *Rhipicephalus sanguineus*; D.r., *Dermacentor reticulatus*; ×, protein spot detected by tick-exposed rabbit sera; MW, molecular weight; kDa, kiloDalton.

^a *Rhipicephalus sanguineus*-specific antigenic proteins.

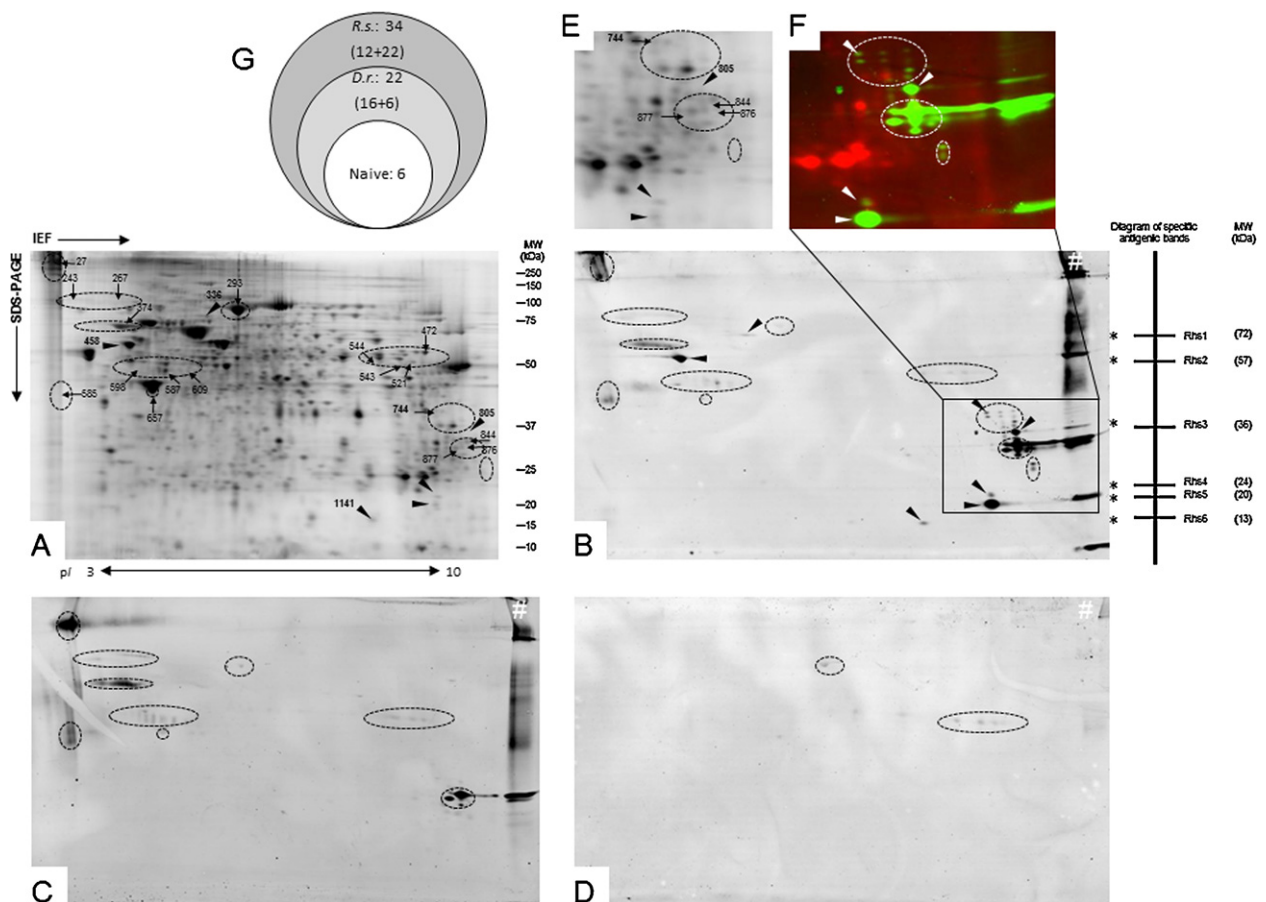


Fig. 2. Two-dimensional *Rhipicephalus sanguineus* salivary gland antigenic profiles from rabbits exposed to different tick species. (A) 2D profile of *R. sanguineus* protein salivary gland extracts (pSGE) labeled with Cy5 CyDye. Circles and arrowheads correspond to antigenic salivary protein spots detected with immunoblot analysis. Antigenic spots identified via mass spectrometry are numbered as indicated in Table 1. IgG immune responses from pooled rabbit sera ($n = 3$) collected at T6 exposed to *R. sanguineus* (B) or *Dermacentor reticulatus* (C) and pooled naive rabbit sera ($n = 3$) collected at T0 (D) against *R. sanguineus* pSGE are presented. Immunoreactive bands on the right part of the immunoblots (#) correspond to *R. sanguineus* pSGE separated into 1D and loaded on the gels beside the IPG strip and used as reference for 1D and 2D profile alignments. Antigenic bands detected exclusively by rabbits exposed to *R. sanguineus* are indicated on the 1D immune profiles with an asterisk (*) and on a diagram (from Rhs1 to Rhs6). Their corresponding molecular weights are indicated with brackets. Enlargement of the gel region (E) and corresponding region from merged images of the electroblot protein profile in red (i.e., "E") and the immunoblot pattern in green (i.e., "B"). A Venn diagram (G) summarizes the number of antigenic salivary protein spots against *R. sanguineus* pSGE detected by sera from rabbits exposed to *R. sanguineus* (R.s.), *D. reticulatus* (D.r.) or unexposed to tick bites (Naive). MW, molecular weight; kDa, kiloDalton.

proteinase (gi|9858101) (protein spot not detected by other pooled rabbit sera), were successfully identified (Table 1). Interestingly, molecular weight isoforms of the malate dehydrogenase protein were detected as both shared- and discriminant antigens. Taking into account the 10 distinct antigenic proteins detected by rabbit sera exposed to both ticks or only to *R. sanguineus*, only three (30%) were predicted to be secreted.

3.3. Kinetic analysis of IgG antibody responses from rabbits exposed to tick bites against *D. reticulatus* pSGE

The qualitative and quantitative alterations of protein samples of SG from tick completing the engorgement, as observed for *R. sanguineus*, lead us to use SG from semi-engorged ticks as antigen source for further analysis of rabbit immune response (Additional file 2). To determine the characteristic of antibody responses against pSGE in

rabbits exposed to *D. reticulatus* tick bites, a comparison of the kinetic IgG rabbit antibody profiles was performed using 1D immunoblots. As observed when *R. sanguineus* pSGE was used as the antigen source, all naive rabbits at T0 presented non-specific IgG responses against *D. reticulatus* pSGE. However, supplementary antigenic bands were revealed with sera from rabbits exposed to *R. sanguineus* and *D. reticulatus* collected from T1 to T6 (Fig. 3).

Concerning rabbits exposed to *D. reticulatus* bites, the kinetic analysis of IgG responses indicated that some antigenic bands started to appear at T1 (i.e., bands Dr1, Dr2 and Dr4). In the following rabbit sera collection, these last bands were maintained and their intensity of recognition increased. Although additional antigenic bands appeared at T2, no new antigenic bands were detectable in the consecutive sera samples collected and similar observations were performed for the kinetic analysis of serological response from the two additional rabbits exposed to *D. reticulatus*

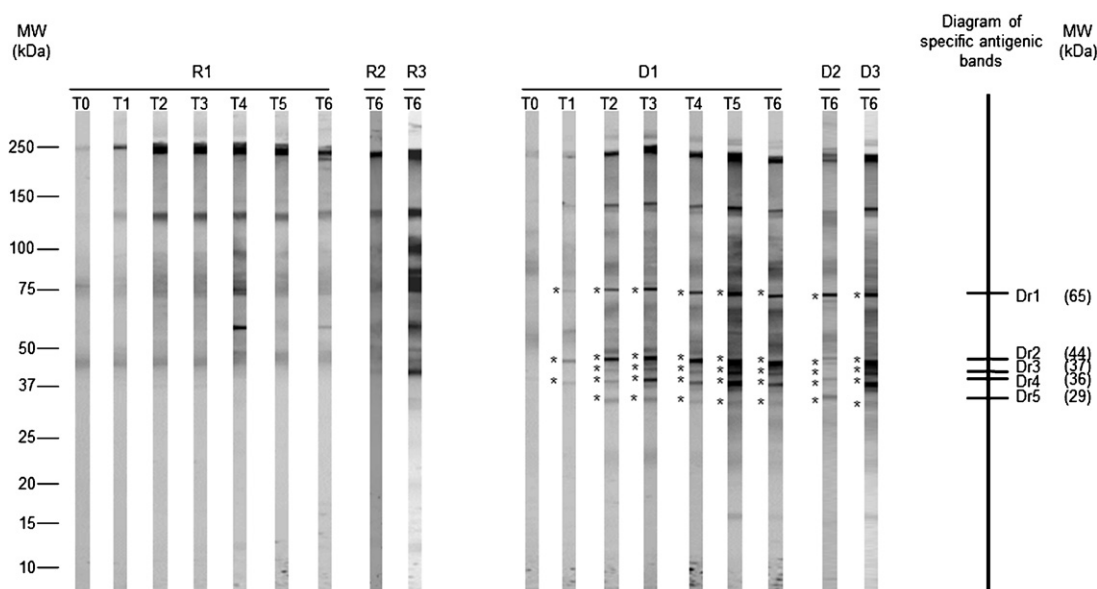


Fig. 3. One-dimensional (1D) *Dermacentor reticulatus* salivary gland antigens from rabbits exposed to different tick species. The evolution of the IgG immune profiles from rabbits exposed to *R. sanguineus* ($n = 3$; R1 to R3) or to *D. reticulatus* ($n = 3$; D1 to D3) bites against *D. reticulatus* salivary gland protein extracts are presented. Kinetic rabbit sera collection from T0 (naive rabbit) to T6 are indicated at the top (see Section 2). Antigenic bands detected exclusively by rabbits exposed to *D. reticulatus* are indicated on the immune profiles with an asterisk (*) and on the diagram (from Dr1 to Dr5). Their corresponding apparent molecular weights are indicated with brackets. MW, molecular weight; kDa, kiloDalton.

bites. These data suggested that IgG antigenic profiles from rabbits exposed to *D. reticulatus* bites seem stable, reproducible and may not be modified by the successive tick infestations (independent from tick infestations). Thus, the antibody response appears quickly and is strong against the different salivary antigenic targets.

Concerning rabbits exposed to *R. sanguineus* bites, some antigenic bands appeared at T1; however, the IgG profiles appeared less marked than those observed for sera from rabbits exposed to *D. reticulatus* bites, and these profiles were not influenced by the time or the level of exposure to tick bites (Fig. 3). Effectively, no major change in the immune responses from the consecutive rabbit sera collection was noted and these IgG responses were found reproducibly between rabbits exposed to *R. sanguineus* bites.

Although distinct antigenic bands were detected using sera from rabbits exposed to either *R. sanguineus* or *D. reticulatus* bites (e.g., one band at a molecular weight of approximately 130 kDa), an accurate comparison of these antigenic profiles indicated that five antigenic bands, arbitrarily named Dr1 to Dr5, with a molecular weight ranging from 65 to 29 kDa, were exclusively recognized by sera from rabbits exposed to *D. reticulatus* bites. A schematic representation of these antigenic bands is depicted on an immunoreactivity diagram (Fig. 3). These five antigenic bands could represent antigenic marker candidates distinguishing exposition to *D. reticulatus* from *R. sanguineus* bites.

Interestingly, IgG immune profiles were not only superimposable according to rabbit tick status exposure (i.e., dependent of the tick bite species), but also according to the antigen source (i.e., the origin of the pSGE used to reveal the

IgG response). The singularity of these immune responses is in favor of a specificity of the antibody response elicited by each tick exposure. The characterization of the corresponding antigens could be useful to determine and to distinguish this host/vector contact. For subsequent antigenic identification using the 2D immunoproteomic approach, pooled sera ($n = 3$) from rabbits collected at T6, covering the whole spectrum of these antigenic bands against *D. reticulatus* pSGE, were selected.

3.4. Characterization of *D. reticulatus* pSGE antigens recognized by rabbits exposed to tick bites

Using *D. reticulatus* pSGE as an antigen source, in the pooled sera of rabbits exposed to *R. sanguineus* ($n = 3$, collected at T6, Fig. 2B), *D. reticulatus* ($n = 3$, T6, Fig. 2C) and un-exposed ($n = 3$, T0, Fig. 2D), 28, 24 and 6 antigenic protein spots, respectively, were detected with a 2D immunoblot. The six antigenic protein spots detected by naive rabbits were considered as non-specific of tick exposure and were also detected by pooled sera from rabbits exposed to *R. sanguineus* and *D. reticulatus* bites. Thus, 22 and 18 antigenic protein spots could be attributed to the *R. sanguineus* and *D. reticulatus* tick bites, respectively. Among them, 13 antigenic protein spots were detected by pooled sera from rabbits exposed either to *R. sanguineus* or to *D. reticulatus* bites and were then considered as general markers of tick exposures. These markers represent potential candidates to measure tick exposure for these two tick species. However, nine and five antigenic protein spots were exclusively detected by the pooled sera of rabbits exposed to *R. sanguineus* and *D. reticulatus* bites, respectively. These last antigenic protein spots

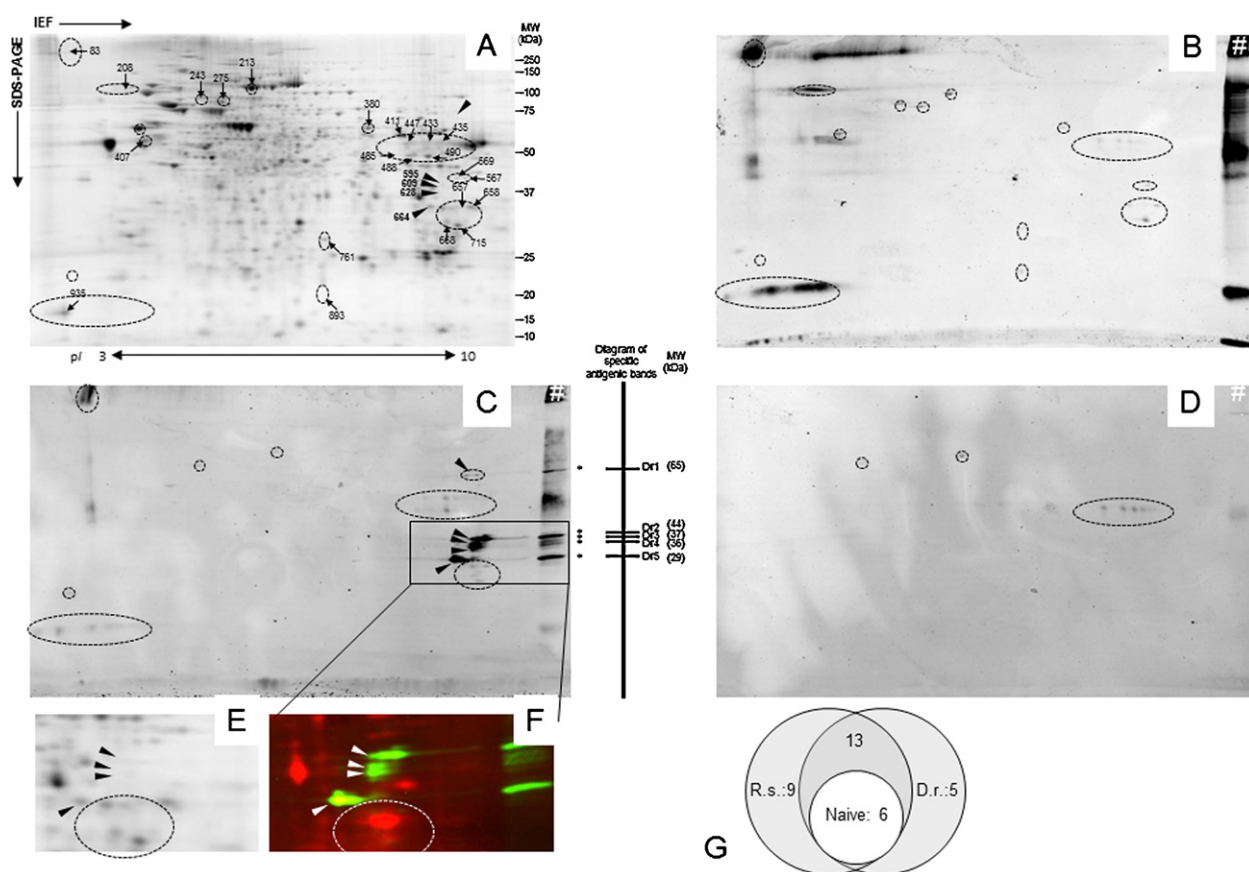


Fig. 4. Two-dimensional *Dermacentor reticulatus* salivary gland antigenic profiles from rabbits exposed to different tick species. (A) 2D profile of *D. reticulatus* protein salivary gland extracts (pSGE) labeled with Cy5 CyDye. Circles and arrowheads correspond to antigenic salivary protein spots detected with an immunoblot. Antigenic spots identified with mass spectrometry are numbered as indicated in Table 2. IgG immune responses from pooled rabbit sera ($n=3$) collected at T6 exposed to *R. sanguineus* (B) or *D. reticulatus* (C) and pooled naive rabbit sera ($n=3$) collected at T0 (D) against *D. reticulatus* pSGE are presented. Immunoreactive bands on the right part of the immunoblots (#) correspond to *D. reticulatus* pSGE separated on 1D and loaded on the gels beside the IPG strip and used as reference for 1D and 2D profile alignments. Antigenic bands detected exclusively by rabbits exposed to *D. reticulatus* are indicated on the 1D immune profiles with an asterisk (*) and on the diagram (from Dr1 to Dr5). Their corresponding molecular weights are indicated with brackets. Enlargement of the gel region (E) and corresponding region from the merged images of the electroblot protein profile in red (i.e., "E") and the immunoblot pattern in green (i.e., "B"). A Venn diagram (G) summarizes the number of antigenic salivary protein spots against *D. reticulatus* pSGE detected by sera from rabbits exposed to *R. sanguineus* (R.s.), *D. reticulatus* (D.r.) or unexposed to tick bites (Naive). MW, molecular weight; kDa, kiloDalton.

could be considered as discriminant antigens. Interestingly, antigenic protein spots presenting the most important recognition at the intensity level correspond to four out of five discriminant antigenic bands detected by rabbit sera exposed to *D. reticulatus* bites (Dr2 to Dr5). A Venn diagram summarizes the distribution of these antigenic protein spots according to rabbit tick species bites exposure status (Fig. 4G).

The 33 antigenic protein spots were then excised and submitted to MS for identification. A total of 27 protein spots (81.8%) were successfully identified, corresponding to 23 distinct proteins according to their NCBI accession number (Table 2). Among the proteins identified, three proteins detected by naive rabbit sera were considered as non-specific/relevant antigens to tick exposure. Eleven proteins corresponding to 13 antigenic spots recognized by the pooled rabbit sera from both *R. sanguineus* and *D. reticulatus* tick bites reflected an exposure to these two

tick species. Six and three proteins corresponding to seven and five antigenic spots were detected only by the pooled rabbit sera from *R. sanguineus* and *D. reticulatus* tick bites, respectively.

Among the six antigenic spots detected exclusively by the pooled rabbit sera from *R. sanguineus* tick bites, a protein from the disulfide isomerase family was also identified suggesting that this last protein could be an interesting antigenic candidate to assess host exposure to *R. sanguineus* tick bites. Concerning the antigenic spots detected only by rabbit sera exposed to *D. reticulatus*, the alignment of the 1D antigenic bands (i.e., Dr1 to Dr5) with 2D antigenic spots with immunoblots permitted the determination of four protein spots that corresponded to the translation elongation factor EF-1 alpha/Tu (gi|325302796) (Dr2), two isoforms of RNA-binding protein musashi (gi|241627626; gi|241710586) (Dr3 and Dr5, respectively) and the malate dehydrogenases protein (gi|325303594) (Dr4). Potentially

Table 2
Dermacentor reticulatus antigenic salivary proteins detected by tick-exposed rabbit sera.

Spot number	Band name	Protein name	Accession no. (NCBI)	MW (kDa)	pI value	Signal peptide	Significance (Mascot score)	Peptide number	Tick-exposed rabbit sera		
									R.s.	D.r.	Naive
595 ^a	Dr2	TPA.exp: translation elongation factor EF-1 alpha/Tu [<i>Amblyomma variegatum</i>]	gi 325302796	43.5	8.5	N	148	9		×	
609 ^a	Dr3	RNA-binding protein musashi [<i>Ixodes scapularis</i>]	gi 241627626	37.4	7.6	N	269	7		×	
628 ^a	Dr4	TPA.exp: malate dehydrogenase [<i>Amblyomma variegatum</i>]	gi 325303594	36.5	7.8	N	427	11		×	
664 ^a	Dr5	RNA-binding protein musashi [<i>Ixodes scapularis</i>]	gi 241710586	28.9	6.9	N	492	15		×	
208		Hsp90 protein [<i>Ixodes scapularis</i>]	gi 241830514	84.7	5.0	N	790	25	×		
275		Hemipoglycoprotein precursor [<i>Dermacentor variabilis</i>]	gi 89954505	178.6	6.3	Y	856	28	×		
380		Protein disulfide isomerase [<i>Ixodes scapularis</i>]	gi 241029459	55.2	6.0	Y	1119	28	×		
567		Conserved hypothetical protein [<i>Ixodes scapularis</i>]	gi 241651548	66.9	8.6	N	673	10	×		
569		TPA.exp: enoyl-CoA hydratase [<i>Amblyomma variegatum</i>]	gi 325303374	21.5	7.7	N	733	8	×		
761		TPA.exp: enoyl-CoA hydratase [<i>Amblyomma variegatum</i>]	gi 325303374	21.5	7.7	N	407	5	×		
893		TPA.exp: emp24/gp25L/p24 family of membrane trafficking protein [<i>Amblyomma variegatum</i>]	gi 325302788	23.2	6.6	Y	116	6	×		
83		Myosin heavy chain, skeletal muscle or cardiac muscle [<i>Ixodes scapularis</i>]	gi 241997622	209.0	6.1	N	1873	57	×	×	
411		FOF1-type ATP synthase, alpha subunit [<i>Ixodes scapularis</i>]	gi 241705635	60.0	9.1	N	1525	22	×	×	
433		Ribosomal DEAD box protein [<i>Ixodes scapularis</i>]	gi 241741609	37.1	8.6	N	414	13	×	×	
435		Calreticulin [<i>Ixodes ricinus</i>]	gi 39726013	48.0	4.6	Y	7759	43	×	×	
447		Ribosomal DEAD box protein [<i>Ixodes scapularis</i>]	gi 241741609	37.1	8.6	N	501	12	×	×	
485		26S protease regulatory subunit [<i>Amblyomma americanum</i>]	gi 11890402	24.3	8.6	N	1073	17	×	×	
488		Glyceraldehyde 3-phosphate dehydrogenase [<i>Dermacentor variabilis</i>]	gi 198443749	36.1	8.6	N	620	14	×	×	
490		Fructose 1,6-bisphosphate aldolase [<i>Ixodes scapularis</i>]	gi 241690479	39.9	8.3	N	220	6	×	×	
657		Malate dehydrogenase [<i>Ixodes scapularis</i>]	gi 241243545	36.1	9.2	N	500	9	×	×	
658		Serpin-2 precursor [<i>Ixodes ricinus</i>]	gi 239530151	44.5	5.8	Y	1105	11	×	×	
668		Mitochondrial voltage dependent anion-selective channel [<i>Rhipicephalus microplus</i>]	gi 315142889	29.7	8.9	N	463	10	×	×	
715		Calmodulin, putative [<i>Ixodes scapularis</i>]	gi 240999683	17.8	4.2	N	750	14	×	×	
935		Calmodulin, putative [<i>Ixodes scapularis</i>]	gi 240999683	17.8	4.2	N	150	4	×	×	
213		Hemipoglycoprotein precursor [<i>Dermacentor variabilis</i>]	gi 89954505	178.6	6.3	Y	1214	29	×	×	×
243		Heme lipoprotein precursor [<i>Ixodes scapularis</i>]	gi 241671868	153.3	6.3	Y	3792	54	×	×	×
407		Beta tubulin [<i>Ixodes scapularis</i>]	gi 241151391	50.4	4.8	N	3715	28	×	×	×

The proteins were identified using a NanoLC-LTQ-Orbitrap-Velos mass spectrometer, following in-gel digestion with trypsin. Spot numbers and band names correspond to those indicated in Figs. 4A and 3, respectively. The identities of the protein spots, their NCBI accession numbers, the MW and pI values, the presence of a signal peptide and the Mascot score serve as an indicator of confidence of identification (protein scores greater than 53 were considered as significant ($p < 0.05$)). The number of peptide sequences is given. Protein sequences were submitted to the SignalP v.3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) server to predict a signal peptide (Y, presence; N, absence). ×, protein spot detected by tick-exposed rabbit sera; R.s., *Rhipicephalus sanguineus*; D.r., *Dermacentor reticulatus*; MW, molecular weight; kDa, kiloDalton.

^a *Dermacentor reticulatus* specific antigenic proteins.

due to its low abundance, MS did not permit us to identify the protein spot corresponding to antigenic band Dr1 (Fig. 4E and F, Table 2).

4. Discussion

Tick-borne diseases are spreading in Europe and, as noted by several studies, understanding their spread is difficult due to changes in epidemiology including distribution, clinical features and severity [26,45,46]. Hence, the characterization of biomarkers of exposure to ticks could be useful for an increased understanding of the epidemiology of tick-borne infections and the immune response to tick bites. Previous studies have been conducted to study the immune responses against tick SG proteins; however, none of these allowed the identification of antigenic biomarkers allowing distinguishing an exposition between *R. sanguineus* and *D. reticulatus* tick species [8,9,30,47].

To determine whether tick bites could elicit in mammalian hosts a reproducible and discriminant antibody response against vector salivary proteins, a comparison of the IgG antibody response against *R. sanguineus* and *D. reticulatus* pSGE using sera from rabbits exposed to these two western European tick species was performed. It was the first study examining the immunological biomarkers of exposure against two main vectors of tick-borne diseases in Europe for humans (MSF, Omsk hemorrhagic fever, tick-borne lymphadenopathy and other rickettsiosis) and animals [1,26,48]. One-dimensional immunoblot analysis of the kinetics of the immune response, the consequences of exposure level and the singularities of the host serological immune responses were performed. For the two tick species, an antibody response was detected immediately after the first exposure at T1, however, the kinetics of the response were not the same for the two species. In the case of *D. reticulatus*, all antibody responses were detected three weeks after the first infestation (T2), and in the consecutive sera samples, only the intensity of the immune responses increased. Conversely, in the case of *R. sanguineus*, although an antibody response occurred at T1, the diversity of these responses occurred after the second (T3) and the third exposures (T5). These results are consistent with the preliminary study conducted before this study showing that a low level infestation of *R. sanguineus* was not sufficient to develop a significant antibody response against these tick pSGE in rabbits (unpublished data). These results suggest that repeated bites of *R. sanguineus* are necessary to elicit a host antibody response against this same tick pSGE. These data could partially explain the discrepancy noted by Nebreda et al. between patient status (MSF or not MSF) and the respective immune responses against SG extracts from distinct tick species [30].

Rabbits exposed to *D. reticulatus* develop antibody responses against *R. sanguineus* pSGE, and rabbits exposed to *R. sanguineus* develop antibody responses against *D. reticulatus* pSGE. Collectively, these results indicated that cross reactivity occurred at the genus level shortly after the first infestation. Thus, determination of these responses to exposure to tick bites could effectively be useful to distinguish exposed vs. unexposed individuals immediately after the first infestation.

Comparative analysis of the IgG immune profiles allowed the detection of 6 *R. sanguineus* antigenic bands. These antigenic bands appear as relevant biomarker candidates for an immunologic assessment of *R. sanguineus* bites. Using this original 2D immunoblot approach, 12 *R. sanguineus* antigenic salivary protein spots were found to be exclusively recognized by sera from rabbits exposed to *R. sanguineus* bites. This highlights that the corresponding salivary proteins could represent the first discriminant salivary antigenic biomarkers of tick exposure. Similarly, using *D. reticulatus* pSGE as an antigen source, a 2D immunoblot defined five discriminant antigenic salivary protein spots from rabbit sera exposed to *D. reticulatus* bites. The reproducibility with the sera of different rabbits exposed to similar conditions enhances the interest of these findings. Moreover, the majority of discriminant antigenic salivary proteins from each tick species elicited the most important rabbit IgG response via 2D immunoblot analysis, underlining the high immunogenicity of their corresponding salivary proteins. Thus, five *R. sanguineus* and four *D. reticulatus* discriminant antigenic proteins were successfully identified using MS. Despite the detection of salivary antigens in rabbits exposed to each of the other tick bite species, which could be attributed to the sequence conservation of some salivary proteins and then shared-antigenic sequences, the independent adaptation of the main tick family to a blood-feeding environment has led to an important protein sequence diversity [49]. Using *R. sanguineus* and *D. reticulatus* pSGE as source antigens, six and 11 proteins, respectively, were recognized by rabbits exposed either to *R. sanguineus* or to *D. reticulatus* bites. Among these 17 antigenic salivary proteins, two corresponded to ortholog salivary proteins and were identified as the malate dehydrogenase and the mitochondrial voltage dependent anion-selective channel. However, two secreted salivary proteins identified in *D. reticulatus* pSGE (calreticulin and serpin) were recognized by rabbits exposed to *D. reticulatus* or *R. sanguineus*.

As host/vector contacts were limited to saliva injected into rabbits in the present study, previously described concealed protective antigens such as glutathione S transferase, myosin alkali light chain-like protein or sialostatin L2 were not identified [50–52]. Exposed antigens previously described as potential protective antigens, such as metalloproteases, were not identified in *R. sanguineus* or in *D. reticulatus* [53]. However, as previously described in other parts of the world, this work confirmed that for European ticks, a determination of an immunogenic response to calreticulin, similar to other common salivary proteins, signals an exposure to ticks but did not allow the determination of the tick species [6,54,55]. Although many studies were conducted to identify the biomarkers of tick exposure [6,9], to our knowledge, this is the first description of tick discriminant antigenic salivary proteins that could be a useful tool to evaluate *R. sanguineus* and/or *D. reticulatus* tick exposure.

The five-discriminant antigenic proteins of *R. sanguineus* correspond to the heat shock (Hsp) 70 kDa protein 5, protein disulfide isomerase-2, malate dehydrogenase, *R. sanguineus* cement protein and heme-binding aspartic proteinase. Hsp70 has been proposed as an

antigenic candidate vaccine against *Haemaphysalis longicornis* bites. Despite the induction of an antibody response in vaccinated rabbits, Hsp70 appears not to confer a protective immunization against further tick infestations [56]. Concerning the cement protein, the use of purified extracts of this protein was shown to be immunogenic and immunoprotective [57]. Recombinant forms of the putative tick cement protein from different tick species were successfully used to immunize and protect animals against tick infestation [58,59]. Interestingly, vaccination of guinea pigs with a secreted cement protein from SG of *R. appendiculatus* provided a cross protection against *R. sanguineus*, and also against *I. ricinus*, *A. variegatum* and *R. microplus* [60]. This protective cross-reactivity suggests that the cement protein could be a good candidate for a global anti-tick vaccine. The tick heme-binding aspartic protease (THAP) is one of the three specific peptidases described in hard ticks [61,62]. The THAP protein was detected in the hemolymph, midgut and fat body and, at a higher quantity, in the ovary of fully engorged *R. microplus* females, and it was present throughout embryo development. Interestingly, the vitelin-degrading cysteine endopeptidase (VTDCE), another peptidase that is present in *R. microplus* embryogenesis, has been tested as a potential vaccine candidate in cattle and presented the best protective capacity among the different assessed antigenic proteins [63]. Similarly, as THAP was identified as a *R. sanguineus* discriminant antigenic protein in this work, it could also be evaluated as a potential vaccine against *R. sanguineus* bites. Lastly, the protein malate dehydrogenase (MDH) is recognized either as common or discriminant antigenic spots by rabbit sera exposed to *R. sanguineus* and *D. reticulatus*. MDH isoenzymes could be used to distinguish cell lines from *I. scapularis* and *R. appendiculatus* [64]. These isoenzymes were also found in *I. ricinus* and *I. persulcatus*, where they could be used as candidates to distinguish *Borrelia*-infected and uninfected ticks [65,66]. Thus, the particular properties of these proteins could explain why some isoforms exhibit common and discriminant immune recognition following exposure to *R. sanguineus* and *D. reticulatus*.

Concerning *D. reticulatus* pSGE as an antigen source, the four-discriminant antigenic proteins were the translation elongation factor EF-1 alpha/Tu (EF-1 α), two isoforms of the RNA-binding protein musashi and malate dehydrogenase. EF-1 α is an intracellular enzyme involved in protein synthesis that has a number of additional functions, including regulation of apoptosis and interaction with the cytoskeleton. It is the first time that this protein was evoked as antigenic. The RNA-binding protein musashi is an ubiquist protein, required intrinsically to maintain stem cell identity in mammals and arthropods [67]. In the present study, this protein appears to be immunogenic exclusively in rabbits infested by *D. reticulatus*. The immune recognition of the RNA-binding protein musashi could reasonably be attributed to SG cell apoptosis, which occurred during the blood meal prior to being injected into their host. SG cell apoptosis was described in ticks feeding on cattle that were vaccinated with tick SG extracts. These detrimental effects could be correlated with a reduced incidence of naturally transmitted tick-borne diseases, as previously described [68].

The mitochondrial voltage dependent anion-selective channel (VDAC), calreticulin and serpin proteins were detected upon exposure to these two main European tick vectors; thus, these proteins are interesting candidates for use in the assessment of the global exposure to ticks at the population or individual level in Europe. Furthermore, through its use as a biomarker, VDAC may be a good candidate to be included in a multi-antigenic anti-tick vaccine that could protect hosts against several tick-borne diseases [12,63,64].

Comparative analyses of tick SG contain between nymphs and adults or between adults at different stages of engorgement have shown that molecule expressions changed according to tick development or behavior [4,69,70]. Then, it is possible that some antigens are injected only during the beginning of the blood meal. Thus, complementary experiments are needed to characterize kinetic host antibody responses against tick pSGE collected from different stage of engorgement. Moreover, this temporal expression variation of SG components could have consequences on their immune recognition by host exposed to tick bites in the field. Therefore, in natural exposition, hosts could be exposed to tick from different stages and duration of the blood feeding or repetition exposition could also be variable between hosts. All these factors could induce heterogeneity of the antibody response between hosts exposed to the same tick species and could limit the detection of representative discriminant salivary antigens. In addition, the exposition to different tick species could also be happened in the field, which could complicate the detection of discriminant tick salivary antigens. Then, our strategy consisting into the identification of discriminant salivary antigenic candidates using artificial exposition, allowed us to characterize potential tick bite markers which should be further validated in their recombinant form using host naturally infested by tick to assess their relevance to determine host exposure to these tick species.

In addition to secreted proteins, tick saliva contains other non-protein salivary molecules participating to the blood feeding success [71]. Among them, the purine nucleoside adenosine and the prostaglandin E2 were shown to interface with the host immune system by altering dendritic cell pro-inflammatory cytokine production. Until now, no host immune response directed against such non-protein salivary molecules has been identified.

New immunogenic proteins have been described both for *R. sanguineus* and *D. reticulatus* that could be used as discriminant biomarkers. As the distribution and spread pattern of these ticks are currently changing in Europe due to human activities (circulation of pets, increase of wild ruminant populations, modifications of agricultural practices, etc.), such discriminant biomarkers could be useful to determine the possible extension areas of the diseases they transmit [72–74]. These discriminant biomarkers should help to determine the risk of tick exposure of individuals or populations in endemic areas of Europe. The development of this serological tool could be useful in epidemiological or entomological surveys for tick-borne diseases in Europe and also in other endemic areas of the world. The protective immunity potentially conferred by these proteins should

be assessed in further studies. This robust and reproducible approach could be applied for the identification of discriminant SG antigens from other ticks, such as *I. ricinus*, the vector of *Lyme borreliosis*, and short-feeder-borne vectors, such as the flea or louse.

Conflict of interest

The authors have declared no conflict of interest.

Authors' contribution

PF, RD, PP, AL, VHV conceived and designed the experiments; VHV, AL, PF, PM, AS performed the experiments; VHV, AL, PF, PP analyzed the data; AS, PM, BN contributed toward reagents/materials/analysis tools; VHV, PF, AL, PP, BN, RD wrote the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cimid.2012.09.003>.

References

- [1] Raoult D, Parola P. Rickettsial diseases. New York: Informa Healthcare USA, Inc.; 2007.
- [2] Steen NA, Barker SC, Alewood PF. Proteins in the saliva of the Ixodida (ticks): pharmacological features and biological significance. *Toxicon* 2006;47(1):1–20.
- [3] Francischetti IM, Sa-Nunes A, Mans BJ, Santos IM, Ribeiro JM. The role of saliva in tick feeding. *Frontiers in Bioscience* 2009;14:2051–88.
- [4] Anatriello E, Ribeiro JM, de Miranda-Santos IK, Brandao LG, Anderson JM, Valenzuela JG, et al. An insight into the sialotranscriptome of the brown dog tick, *Rhipicephalus sanguineus*. *BMC Genomics* 2010;11:450.
- [5] Bowman AS, Nuttall PA. Ticks: biology, disease and control. Cambridge University Press; 2008.
- [6] Alarcon-Chaidez F, Ryan R, Wikel S, Dardick K, Lawler C, Foppa IM, et al. Confirmation of tick bite by detection of antibody to *Ixodes calreticulin* salivary protein. *Clinical and Vaccine Immunology* 2006;13(11):1217–22.
- [7] Schwartz BS, Ford DP, Childs JE, Rothman N, Thomas RJ. Anti-tick saliva antibody: a biologic marker of tick exposure that is a risk factor for Lyme disease seropositivity. *American Journal of Epidemiology* 1991;134(1):86–95.
- [8] Jaworski DC, Rosell R, Coons LB, Needham GR. Tick (Acari: Ixodidae) attachment cement and salivary gland cells contain similar immunoreactive polypeptides. *Journal of Medical Entomology* 1992;29(2):305–9.
- [9] Sanders ML, Glass GE, Scott AL, Schwartz BS. Kinetics and cross-species comparisons of host antibody responses to lone star ticks and American dog ticks (Acari: Ixodidae). *Journal of Medical Entomology* 1998;35(5):849–56.
- [10] Almazan C, Kocan KM, Bergman DK, Garcia-Garcia JC, Blouin EF, de la Fuente J. Identification of protective antigens for the control of *Ixodes scapularis* infestations using cDNA expression library immunization. *Vaccine* 2003;21(13–14):1492–501.
- [11] Carreon D, de la Lastra JM, Almazan C, Canales M, Ruiz-Fons F, Boadella M, et al. Vaccination with BM86, subolesin and akirin protective antigens for the control of tick infestations in white tailed deer and red deer. *Vaccine* 2012;30(2):273–9.
- [12] de la Fuente J, Almazan C, Canales M, Perez de la Lastra JM, Kocan KM, Willadsen P. A ten-year review of commercial vaccine performance for control of tick infestations on cattle. *Animal Health Research Reviews* 2007;8(1):23–8.
- [13] de VS, Zeinstra L, Taoufik O, Willadsen P, Jongejan F. Evidence for the utility of the Bm86 antigen from *Boophilus microplus* in vaccination against other tick species. *Experimental and Applied Acarology* 2001;25(3):245–61.
- [14] Perez-Perez D, Bechara GH, Machado RZ, Andrade GM, Del Vecchio RE, Pedroso MS, et al. Efficacy of the Bm86 antigen against immature instars and adults of the dog tick *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae). *Veterinary Parasitology* 2010;167(2–4):321–6.
- [15] Prevot PP, Couvreur B, Denis V, Brossard M, Vanhamme L, Godfroid E. Protective immunity against *Ixodes ricinus* induced by a salivary serpin. *Vaccine* 2007;25(17):3284–92.
- [16] You MJ. Immunization effect of recombinant P27/30 protein expressed in *Escherichia coli* against the hard tick *Haemaphysalis longicornis* (Acari: Ixodidae) in rabbits. *Korean Journal of Parasitology* 2004;42(4):195–200.
- [17] Imamura S, Namangala B, Tajima T, Tembo ME, Yasuda J, Ohashi K, et al. Two serine protease inhibitors (serpins) that induce a bovine protective immune response against *Rhipicephalus appendiculatus* ticks. *Vaccine* 2006;24(13):2230–7.
- [18] Yamada S, Konnai S, Imamura S, Ito T, Onuma M, Ohashi K. Cloning and characterization of *Rhipicephalus appendiculatus* voraxinalpha and its effect as anti-tick vaccine. *Vaccine* 2009;27(43):5989–97.
- [19] You M, Fujisaki K. Vaccination effects of recombinant chitinase protein from the hard tick *Haemaphysalis longicornis* (Acari: Ixodidae). *Journal of Veterinary Medical Science* 2009;71(6):709–12.
- [20] Nuttall PA, Trimmell AR, Kazimirova M, Labuda M. Exposed and concealed antigens as vaccine targets for controlling ticks and tick-borne diseases. *Parasite Immunology* 2006;28(4):155–63.
- [21] Almazan C, Lagunes R, Villar M, Canales M, Rosario-Cruz R, Jongejan F, et al. Identification and characterization of *Rhipicephalus (Boophilus) microplus* candidate protective antigens for the control of cattle tick infestations. *Parasitology Research* 2010;106(2):471–9.
- [22] Garcia-Varas S, Manzano-Roman R, Fernandez-Soto P, Encinas-Grandes A, Oleaga A, Perez-Sanchez R. Purification and characterisation of a P-selectin-binding molecule from the salivary glands of *Ornithodoros moubata* that induces protective anti-tick immune responses in pigs. *International Journal for Parasitology* 2010;40(3):313–26.
- [23] Sahibi H, Rhalem A, Barriga OO. Comparative immunizing power of infections, salivary extracts, and intestinal extracts of *Hyalomma marginatum marginatum* in cattle. *Veterinary Parasitology* 1997;68(4):359–66.
- [24] Xu Y, Bruno JF, Luft BJ. Identification of novel tick salivary gland proteins for vaccine development. *Biochemical and Biophysical Research Communications* 2005;326(4):901–4.
- [25] Dantas-Torres F. The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae): from taxonomy to control. *Veterinary Parasitology* 2008;152(3–4):173–85.
- [26] Parola P, Paddock CD, Raoult D. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. *Clinical Microbiology Reviews* 2005;18(4):719–56.
- [27] Szabo MP, Bechara GH. Sequential histopathology at the *Rhipicephalus sanguineus* tick feeding site on dogs and guinea pigs. *Experimental and Applied Acarology* 1999;23(11):915–28.
- [28] Inokuma H, Mukai M, Ohno K, Yamamoto Y, Takahashi S, Onishi T. Duration of antibodies against 24 kD protein of *Rhipicephalus sanguineus* extract in dogs infested with the adult ticks. *Journal of Veterinary Medical Science* 1999;61(2):179–81.
- [29] Inokuma H, Ohno K, Onishi T. Is the detection of anti-*Rhipicephalus sanguineus* (Rs24p) antibodies a valuable epidemiological tool of tick infestation in dogs? *Veterinary Research* 2000;31(3):365–9.
- [30] Nebreda MT, Merino FJ, Serrano JL, Fernandez-Soto P, Encinas A, Perez-Sanchez R. Detection of antibodies to tick salivary antigens among patients from a region of Spain. *European Journal of Epidemiology* 2004;19(1):79–83.

- [31] Parola P, Rovey C, Rolain JM, Brouqui P, Davoust B, Raoult D. *Rickettsia slovaca* and *R. raoultii* in tick-borne Rickettsioses. *Emerging Infectious Diseases* 2009;15(7):1105–8.
- [32] Socolovschi C, Mediannikov O, Raoult D, Parola P. Update on tick-borne bacterial diseases in Europe. *Parasite* 2009;16(4):259–73.
- [33] Kubes M, Fuchsberger N, Labuda M, Zuffova E, Nuttall PA. Salivary gland extracts of partially fed *Dermacentor reticulatus* ticks decrease natural killer cell activity in vitro. *Immunology* 1994;82(1):113–6.
- [34] Hajnicka V, Vancova I, Kocakova P, Slovak M, Gasperik J, Slavikova M, et al. Manipulation of host cytokine network by ticks: a potential gateway for pathogen transmission. *Parasitology* 2005;130(3):333–42.
- [35] Almeras L, Fontaine A, Belghazi M, Bourdon S, Boucomont-Chapeaublanc E, Orlandi-Pradines E, et al. Salivary gland protein repertoire from *Aedes aegypti* mosquitoes. *Vector-Borne and Zoonotic Diseases* 2010;10(4):391–402.
- [36] Almeras L, Orlandi-Pradines E, Fontaine A, Villard C, Boucomont E, de Senneville LD, et al. Salivary gland individuality between *Aedes aegypti* colonies. *Vector-Borne and Zoonotic Diseases* 2009;9(5):531–41.
- [37] Fontaine A, Pascual A, Diouf I, Bakkali N, Bourdon S, Fusai T, et al. Mosquito salivary gland protein preservation in the field for immunological and biochemical analysis. *Parasites & Vectors* 2011;4:33.
- [38] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227(5259):680–5.
- [39] Fontaine A, Pophillat M, Bourdon S, Villard C, Belghazi M, Fourquet P, et al. Specific antibody responses against membrane proteins of erythrocytes infected by *Plasmodium falciparum* of individuals briefly exposed to malaria. *Malaria Journal* 2010;9:276.
- [40] Lefranc D, Almeras L, Dubucquoi S, de SJ, Vermersch P, Prin L. Distortion of the self-reactive IgG antibody repertoire in multiple sclerosis as a new diagnostic tool. *Journal of Immunology* 2004;172(1):669–78.
- [41] Torrentino-Madamet M, Almeras L, Desplans J, Le PY, Belghazi M, Pophillat M, et al. Global response of *Plasmodium falciparum* to hyperoxia: a combined transcriptomic and proteomic approach. *Malaria Journal* 2011;10:4.
- [42] Sauer JR, Essenberg RC, Bowman AS. Salivary glands in ixodid ticks: control and mechanism of secretion. *Journal of Insect Physiology* 2000;46(7):1069–78.
- [43] Till WM. A contribution to the anatomy and histology of the brown ear tick *Rhipicephalus appendiculatus* Neumann. *Memoirs of the Entomological Society of Southern Africa* 1961;6:1–124.
- [44] Almeras L, Lefranc D, Drobecq H, de SJ, Dubucquoi S, Vermersch P, et al. New antigenic candidates in multiple sclerosis: identification by serological proteome analysis. *Proteomics* 2004;4(7):2184–94.
- [45] Beugnet F, Marie JL. Emerging arthropod-borne diseases of companion animals in Europe. *Veterinary Parasitology* 2009;163(4):298–305.
- [46] Parola P, Raoult D. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. *Clinical Infectious Diseases* 2001;32(6):897–928.
- [47] Inokuma H, Aita T, Ohno K, Onishi T. Effects of infestation by *Rhipicephalus sanguineus* on lymphocyte blastogenic responses to mitogens in dogs. *Journal of Veterinary Medical Science* 1998;60(9):1013–6.
- [48] Ruzek D, Yakimenko VV, Karan LS, Tkachev SE. Omsk haemorrhagic fever. *Lancet* 2010;376(9758):2104–13.
- [49] Mans BJ, Neitz AW. Adaptation of ticks to a blood-feeding environment: evolution from a functional perspective. *Insect Biochemistry and Molecular Biology* 2004;34(1):1–17.
- [50] Gao J, Luo J, Fan R, Guan G, Ren Q, Ma M, et al. Molecular characterization of a myosin alkali light chain-like protein, a “concealed” antigen from the hard tick *Haemaphysalis qinghaiensis*. *Veterinary Parasitology* 2007;147(1–2):140–9.
- [51] Gao J, Luo J, Fan R, Schulte-Spechtel UC, Fingerle V, Guan G, et al. Characterization of a concealed antigen Hq05 from the hard tick *Haemaphysalis qinghaiensis* and its effect as a vaccine against tick infestation in sheep. *Vaccine* 2009;27(3):483–90.
- [52] Kotsyakis M, Anderson JM, Andersen JF, Calvo E, Francischetti IM, Mather TN, et al. Cutting edge: Immunity against a “silent” salivary antigen of the Lyme vector *Ixodes scapularis* impairs its ability to feed. *Journal of Immunology* 2008;181(8):5209–12.
- [53] Decrem Y, Mariller M, Lahaye K, Blasioli V, Beaufays J, Zouaoui BK, et al. The impact of gene knock-down and vaccination against salivary metalloproteases on blood feeding and egg laying by *Ixodes ricinus*. *International Journal for Parasitology* 2008;38(5):549–60.
- [54] Sanders ML, Jaworski DC, Sanchez JL, DeFraitres RF, Glass GE, Scott AL, et al. Antibody to a cDNA-derived calreticulin protein from *Amblyomma americanum* as a biomarker of tick exposure in humans. *American Journal of Tropical Medicine and Hygiene* 1998;59(2):279–85.
- [55] Sanders ML, Glass GE, Nadelman RB, Wormser GP, Scott AL, Raha S, et al. Antibody levels to recombinant tick calreticulin increase in humans after exposure to *Ixodes scapularis* (Say) and are correlated with tick engorgement indices. *American Journal of Epidemiology* 1999;149(8):777–84.
- [56] Tian Z, Liu G, Zhang L, Yin H, Wang H, Xie J, et al. Identification of the heat shock protein 70 (Hsp70) in *Haemaphysalis longicornis*. *Veterinary Parasitology* 2011;181(2–4):282–90.
- [57] Brown SJ, Shapiro SZ, Askenase PW. Characterization of tick antigens inducing host immune resistance. I. Immunization of guinea pigs with *Amblyomma americanum*-derived salivary gland extracts and identification of an important salivary gland protein antigen with guinea pig anti-tick antibodies. *Journal of Immunology* 1984;133(6):3319–25.
- [58] Hamnoi T, Watchabunsook S, Sakaguchi T, Xuan X, Fujisaki K. Characterization of *Haemaphysalis longicornis* recombinant cement-like antigens and preliminary study of their vaccination effects. *Journal of Veterinary Medical Science* 2006;68(12):1289–95.
- [59] Zhou J, Gong H, Zhou Y, Xuan X, Fujisaki K. Identification of a glycine-rich protein from the tick *Rhipicephalus haemaphysaloides* and evaluation of its vaccine potential against tick feeding. *Parasitology Research* 2006;100(1):77–84.
- [60] Trimmell AR, Davies GM, Lissina O, Hails RS, Nuttall PA. A cross-reactive tick cement antigen is a candidate broad-spectrum tick vaccine. *Vaccine* 2005;23(34):4329–41.
- [61] Pohl PC, Sorgine MH, Leal AT, Logullo C, Oliveira PL, Vaz Ida Jr S, et al. An extraovarian aspartic protease accumulated in tick oocytes with vitellin-degradation activity. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 2008;151(4):392–9.
- [62] Sorgine MH, Logullo C, Zingali RB, Paiva-Silva GO, Juliano L, Oliveira PL. A heme-binding aspartic proteinase from the eggs of the hard tick *Boophilus microplus*. *Journal of Biological Chemistry* 2000;275(37):28659–65.
- [63] Seixas A, Leal AT, Nascimento-Silva MC, Masuda A, Termignoni C, da Silva Jr VI. Vaccine potential of a tick vitellin-degrading enzyme (VTDE). *Veterinary Immunology and Immunopathology* 2008;124(3–4):332–40.
- [64] Munderloh UG, Liu Y, Wang M, Chen C, Kurtti TJ. Establishment, maintenance and description of cell lines from the tick *Ixodes scapularis*. *Journal of Parasitology* 1994;80(4):533–43.
- [65] Radulovic Z, Milutinovic M, Andelkovic M, Vujcic Z, Tomanovic S, Bozic N, et al. Allozyme polymorphism of Mdh and alpha-Gpdh in *Ixodes ricinus* populations: comparison of borreliac-infected and uninfected ticks. *Experimental and Applied Acarology* 2006;40(2):113–21.
- [66] Semenov AV, Alekseev AN, Dubinina EV, Kaufmann U, Jensen RM. Detection of the genotypic heterogeneity of *Ixodes persulcatus* Schulze (Acari: Ixodidae) of the North-West region of Russia and characteristics of distribution of tick-borne pathogens causing Lyme disease and Ehrlichia infections in various genotypes. *Meditinskaja Parazitologija i Parazitarnye Bolezni* 2001;3(3):11–5.
- [67] Siddall NA, McLaughlin EA, Marriner NL, Hime GR. The RNA-binding protein Musashi is required intrinsically to maintain stem cell identity. *Proceedings of the National Academy of Sciences of the United States of America* 2006;103(22):8402–7.
- [68] Jittapalpong S, Jansawan W, Barriga OO, Stich RW. Reduced incidence of *Babesia bigemina* infection in cattle immunized against the cattle tick, *Boophilus microplus*. *Annals of the New York Academy of Sciences* 2004;1026:312–8.
- [69] Ribeiro JM, Alarcon-Chaidez F, Francischetti IM, Mans BJ, Mather TN, Valenzuela JG, et al. An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. *Insect Biochemistry and Molecular Biology* 2006;36(2):111–29.
- [70] McNally KL, Mitzel DN, Anderson JM, Ribeiro JM, Valenzuela JG, Myers TG, et al. Differential salivary gland transcript expression profile in *Ixodes scapularis* nymphs upon feeding or flavivirus infection. *Ticks and Tick-borne Diseases* 2012;3(1):18–26.
- [71] Oliveira CJ, Sa-Nunes A, Francischetti IM, Carregaro V, Anatriello E, Silva JS, et al. Deconstructing tick saliva: non-protein molecules with potent immunomodulatory properties. *Journal of Biological Chemistry* 2011;286(13):10960–9.
- [72] Bartosik K, Sitarz M, Szymanska J, Buczek A. Tick bites on humans in the agricultural and recreational areas in south-eastern

- Poland. *Annals of Agricultural and Environmental Medicine* 2011;18(1):151–7.
- [73] Beugnet F, Kolasinski M, Michelangeli PA, Vienne J, Loukos H. Mathematical modelling of the impact of climatic conditions in France on *Rhipicephalus sanguineus* tick activity and density since 1960. *Geospat Health* 2011;5(2):255–63.
- [74] Cochez C, Lempereur L, Madder M, Claerebout E, Simons L, WN DE, et al. Foci report on indigenous *Dermacentor reticulatus* populations in Belgium and a preliminary study of associated babesiosis pathogens. *Medical and Veterinary Entomology* 2012;26(3): 355–8.