Published in final edited form as:

Ticks Tick Borne Dis. 2013 December ; 4(6): 469–477. doi:10.1016/j.ttbdis.2013.05.001.

Proteome of *Rhipicephalus sanguineus* tick saliva induced by the secretagogues pilocarpine and dopamine

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Abstract

One dimensional gel electrophoresis was used to separate proteins from the saliva of *Rhipicephalus sanguineus* female ticks fed on rabbits. Gel slices were subjected to tryptic digestion and analyzed by reversed-phase HPLC followed by MS/MS analysis. The data were compared to a database of salivary proteins of the same tick and to the predicted proteins of the host. Saliva was obtained by either pilocarpine or dopamine stimulation of partially-fed ticks. Electrophoretic separations of both yielded products that were identified by mass spectrometry, although the pilocarpine-derived sample was of much better quality. The majority of identified proteins were of rabbit origin, indicating the recycling of the host proteins in the tick saliva, including hemoglobin, albumin, haptoglobin, transferrin, and a plasma serpin. The few proteins found that were previously associated with parasitism and blood feeding include 2 glycine-rich, cement-like proteins, 2 lipocalins, and a thyroprotease inhibitor. Among other of the 19 tick proteins identified, albeit with undefined roles, were SPARC and cyclophilin A. This catalog provides a resource that can be mined for secreted molecules that play a role in tick-host interactions.

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Conflict of interest: None declared.

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Keywords

Proteome; Tick saliva; *Rhipicephalus sanguineus*; Dopamine; Pilocarpine

Introduction

Rhipicephalus sanguineus, the brown dog tick, is an ubiquitous ectoparasite associated with pet dogs, cats, rabbits, rodents, pigeons, and occasionally found infesting humans. It is also a vector of canine babesiosis and Rocky Mountain spotted fever, among other diseases (Beugnet and Marie, 2009; Dantas-Torres, 2008; Dantas-Torres and Figueredo, 2006; Demma et al., 2005, 2006; Mariotte et al., 1944).

In order to suck blood, ticks use their chelicerae to lacerate the skin and then insert hypostome and chelicerae into the host's epidermis. Concomitantly, a salivary secretion builds a cement cone composed of many proteins, some of which are rich in the amino acid glycine. This structure maintains the tick mouthparts firmly attached, and acarologists believe that it also creates a gasket that facilitates blood sucking (Parola and Raoult, 2001; Sonenshine, 1992).

Tick saliva also assists blood feeding by means of a complex mixture of compounds. It contains vasodilators, inhibitors of blood clotting and platelet aggregation, and modulators of immunity and inflammation (Francischetti et al., 2009a; Hovius et al., 2008; Sá-Nunes and Oliveira, 2010).

Many salivary components are genus-specific proteins indicating a fast evolution of these proteins, perhaps due to different homeostatic pressures posed by the hosts (Francischetti et al., 2009b). Recently, the sialotranscriptome (from the Greek sialo=saliva) of females of *R. sanguineus* was described (Anatriello et al., 2010). This allows for identification of salivary proteins from tick saliva, which can be obtained by stimulation of partially-fed ticks with the secretagogues dopamine (DA) or pilocarpine (PC) (Kaufman, 1976; Sauer et al., 1995). Tick saliva also contains host proteins (Valenzuela et al., 2002), and the predicted proteomes of several mammals are also available in public databases. Such databases have now made it feasible to identify in greater detail the proteins secreted in tick saliva. In the present work, we describe the proteome of saliva from female *R. sanguineus* ticks partially fed on rabbits and obtained by stimulating the ticks with DA and PC.

Material and methods

Saliva collection

R. sanguineus ticks were laboratory-reared, as previously described (Ferreira and Silva, 1998). To obtain engorged ticks for saliva collection, rabbits (n=4) were infested with 70 pairs of adult *R. sanguineus* ticks restricted by plastic feeding chambers fixed to their backs (Ferreira et al., 1998). The experiments with rabbits are in agreement with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (COBEA) in line with the Guidelines for Animal Users as issued by the National Institute of Health, and the animal protocol was approved by the School of Medicine of Ribeirão Preto of the University of São Paulo, Institutional Animal Care and Use Committee (IACUC) under protocol number 144/2010. The procedure for collection of saliva was performed on partially-engorged (after 5–7 days of feeding) female ticks rinsed in distilled water and dried with filter paper. A solution of DA (10 µL at 0.2% in phosphate-buffered saline, pH 7.4) was inoculated into each tick's hemocoel using a micro-fine 29 Gauge (12.7×0.33 mm) needle (BD Biosciences, San Diego, CA). Alternatively, 2 µL of PC hydrochloride (5%

solution in 0.7 M NaCl) was inoculated near the border of the dorsal scutum together with a topical application of 5 μ L of PC hydrochloride (5% solution in methanol) to their dorsal scutum. Tick saliva was harvested from tick mouthparts using a micropipette, kept on ice, pooled, filtered through a 0.22- μ m pore filter (Costar-Corning Inc., Cambridge, MA), and stored at -70°C until further use.

Gel electrophoresis studies

Tick saliva samples (50 μ L) collected with DA or PC (0.97 mg/mL and 1.21 mg/mL of protein, respectively) were resolved by one-dimensional (1D) sodium dodecylsulfate polyacrylamide gel electrophoresis (4–12% gradient gels) and visualized with Coomassie blue staining (Pierce, Rockford, IL). Excised gel bands were destained using 50% acetonitrile in 25 mM NH_4HCO_3 , pH 8.4, and vacuum dried. Trypsin (20 μ g/mL in 25 mM NH_4HCO_3 , pH 8.4) was added, and the mixture was incubated on ice for one hour. The supernatant was removed, and the gel bands were covered with 25 mM NH_4HCO_3 , pH 8.4. After overnight incubation at 37°C , the tryptic peptides were extracted using 70% acetonitrile, 5% formic acid, and the peptide solution was lyophilized and desalted using ZipTips (Millipore, Bedford, MA).

Nanoflow reversed-phase liquid chromatography tandem mass spectrometry (nanoRPLC - MS/MS)

Tryptic peptides were analyzed using nanoRPLC-MS/MS. A 75- μ m i.d. $\times$ 360 μ m o.d. $\times$ 10 cm long fused silica capillary column (Polymicro Technologies, Phoenix, AZ) was packed with 3 μ m, 300 Å pore size C-18 silica-bonded stationary RP particles (Vydac/Grace, Deerfield, IL). The column was connected to an Agilent 1100 nanoLC system (Agilent Technologies, Santa Clara, CA) that was coupled online with a linear ion-trap mass spectrometer (LTQ; ThermoElectron, Waltham, MA). Peptides were separated using a gradient consisting of mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The peptide samples were injected, and gradient elution was performed under the following conditions: 2% B at 500 nL/min for 30 min; a linear increase of 2–42% B at 250 nL/min for 110 min; 42–98% for 30 min including the first 15 min at 250 nL/min and then 15 min at 500 nL/min; 98% at 500 nL/min for 10 min. The linear ion-trap mass spectrometer was operated in a data-dependent tandem MS (MS/MS) mode in which the 5 most abundant peptide molecular ions in every MS scan were selected for collision-induced dissociation using a normalized collision energy of 35%. Dynamic exclusion was applied to minimize repeated selection of previously analyzed peptides. The capillary temperature and electrospray voltage were set to 160°C and 1.5 kV, respectively. Tandem MS spectra from the nanoRPLC-MS/MS analyses were searched against a protein FASTA database derived from the tick transcriptome previously reported (Anatriello et al., 2010), or from *Ixodes scapularis*-predicted proteins (obtained from www.vectorbase.org/), or from human and rabbit proteins (obtained from the NCBI sites ftp://ftp.ncbi.nih.gov/genomes/Oryctolagus_cuniculus/protein/ and ftp://ftp.ncbi.nih.gov/genomes/H_sapiens/protein/). These searches used the SEQUEST software operating on an 18-node Beowulf cluster. For a peptide to be considered legitimately identified, it had to achieve stringent charge state and proteolytic cleavage-dependent cross correlation (X_{corr}) and a minimum correlation (ΔC_n) score of 0.08.

MS results from legitimately identified peptides as described above were mapped to the Excel spreadsheets using a homemade program. The following example illustrates the convention for interpreting the data: The hit F₂ $\rightarrow$ 6 indicates that a particular protein had 6 MS/MS peptide hits in gel fraction 2.

Results

To obtain the salivary proteome of *R. sanguineus* females, we induced tick salivation by 2 different methods, using either DA or PC. Although DA is a commonly used and efficient secretagogue for obtaining tick saliva (Fezza et al., 2003; Kaufman, 1978; Ribeiro et al., 1992; Zhu et al., 1998), we observed that DA-induced saliva was brown in color and produced a precipitate following centrifugation at 14,000 g for 5 min. On the other hand, PC-induced saliva was clear and did not produce a precipitate. Comparison of the 1D gels for saliva induced by these 2 secretagogues showed that the DA-derived saliva had diffuse bands while the sample stimulated by PC had better defined bands (Figs. 1 and 2, respectively). The diffuse bands could derive from protein aggregation/polymerization due to presence of DA or due to the prophenoloxidase-activating system which participates in immunity of invertebrates causing melanization reactions and clotting of fluid proteins (Söderhäll and Cerenius, 1998). Important to highlight that we cannot exclude other forms of protein degradation, as it is caused by free radicals (Dean et al., 1997) during oxidation of DA (Chen et al., 2008).

After tryptic digestion of gel plugs from saliva induced by DA, only 2 lipocalins (Lip-629 and Lip-5) were identified in a region of the gel corresponding to the 55 kDa marker, even though these proteins have a predicted mass of 22–23 kDa (Fig. 1 and Supplemental Table S1). More proteins were identified in gel plugs deriving from saliva induced by PC, although far less than predicted from the transcriptome (Anatriello et al., 2010): Lipocalin RS-368 was found in several gel fractions, including at fraction 15 near the 17-kDa marker, compatible with its expected mature molecular mass of 19 kDa, but also in fractions 4, 5, and 6, which span from below the 188-kDa marker to below the 98-kDa marker. Thyropin (RS-899), containing 2 thyropin domains, as indicated by its comparison to the Pfam database, with predicted mass of 23 kDa, was also found in bands 4, 5, and 6, far above its predicted molecular mass. Similarly, RS-54, belonging to a protein family exclusive of ticks, named GLC, was found in fraction 6, just below the 98-kDa marker; the mature protein is predicted to have a mass of 25 kDa, suggesting these proteins may have further post-translation modifications. Two members of the “Metastriate one-of-each family”, RS-935 and RS-757, were also identified; RS-935, with predicted molecular mass of ~20 kDa, in fractions 5 and 6, near the 98-kDa marker, while RS-757 was found at fractions 15 and 17, near the markers for 17 and 14 kDa. The glycine-rich proteins RS-24 and RS-25 were found in fraction 18, near the 14-kDa marker. Because we do not have the full-length sequences of these proteins, it is not possible to correlate predicted masses with the gel fraction where they were found. Surprisingly, the lipocalins Lip-629 and Lip-5 found in the DA-induced saliva were not found in the saliva induced by PC. Other proteins matching the *R. sanguineus* tick sialotranscriptome (n=19) are listed in Table 1 and shown in Fig. 2, including ribosomal proteins, cyclophilin A, thioredoxin peroxidase, and a glycosyltransferase. Interestingly, although they are among the most abundant transcripts in the transcriptome of *R. sanguineus* (Anatriello et al., 2010), only 2 glycine-rich proteins were identified in the secreted proteome. This finding is compatible with the possibility that most glycine-rich proteins are indeed fixed in the cement cone and are not secreted into the host via saliva.

Previous work has reported the presence of host blood proteins in the saliva of *I. scapularis* (Valenzuela et al., 2002) and *Hyalomma marginatum rufipes* (Francischetti et al., 2011). In fact; here we are also documenting 56 rabbit proteins in *R. sanguineus* saliva induced by PC. We used the predicted proteins of the rabbit (available from the NCBI) as identification targets for the electrophoresis MS/MS study of PC-induced saliva. This led to the identification of many rabbit proteins, with much larger peptide hit intensity than the identified tick proteins themselves (Table 2 and Fig. 3). The precursor of rabbit serum

albumin (SA), for example, was found with high intensity (measured by the number of peptide ions matching SA) on bands spanning fractions 8 (188 ions detected), 9 (84 ions), 12 (82 ions), 7 (70 ions), 11 (60 ions), and 5 (54 ions), to name only the 6 most intense bands. These MS-MS-derived ions were found to produce over four-fold coverage of the protein on fraction 8, located near the 62-kDa marker, compatible with its predicted MW, and indicative of its large abundance. In comparison, the most abundant tick salivary protein identified, a protein disulfide isomerase, had only 17 ions detected in band 6, and the thyroperin (RS-899) putative cysteine proteinase inhibitor had only 10 ions detected in band 5.

Several other abundant rabbit blood proteins were identified, such as serotransferrin, haptoglobin, alpha-1 proteinase inhibitor, vitamin D-binding protein histidine-rich glycoprotein, erythrocyte band 3, an anion carrier, complement C3, carbonic anhydrase, and hemoglobin. Cytoskeletal proteins such as actin and tubulin were also found with large signals. Both of these latter proteins are largely conserved, and these signals could have been derived from tick proteins instead. Many hits to rabbit ribosomal proteins and enzymes were also found, as indicated in Tables 2 and 3 and in Supplemental Spreadsheet S2 and in Fig. 3. Interestingly, rabbit immunoglobulins were not identified in this study, although they are among the 3 most abundant in mammalian serum (Adkins et al., 2002; Pieper et al., 2003).

Some of the rabbit proteins identified in Tables 2 and 3 and Fig. 3 could actually be artifacts due to conservation of protein sequences among species. Because we do not have the full repertoire of proteins from *R. sanguineus*, these matches could be derived from nearly identical tick proteins and not host proteins. Accordingly, to further control the origin of the putative rabbit proteins identified in the tick salivary sample, in Supplemental Spreadsheet S2 we show the comparisons of these host protein sequences with proteins derived from the tick *I. scapularis* (Ribeiro et al., 2006). Indeed, a great part of the rabbit proteins identified is highly conserved. For example, mammalian tubulin is 97% identical to the *I. scapularis* tubulin while actin is 98% identical to the tick protein. Supplemental Spreadsheet S2 shows several such comparisons, with many proteins having >90% similarity to tick proteins, and thus their origin cannot be determined. They could have originated from *R. sanguineus* and not from host proteins, with several significant exceptions, as the proteins named in the previous paragraph and indicated in Table 3, listing proteins with no closely related tick homologs, and in Supplemental Spreadsheet S2.

Discussion

Apart from leakage of intracellular components from degenerated or damaged salivary gland cells, the presence of 'housekeeping' components in tick saliva might have functions in parasitism and is supported by previous observations of apocrine and merocrine secretion in tick salivary glands (Coons and Roshdy, 1981). In this regard, among the tick salivary proteins identified by this work in saliva induced by PC, only a few have known functions in parasitism by ticks, i.e. glycine-rich, cement-like proteins, lipocalins, and thyroperin protease inhibitor (Anatriello et al., 2010; Francischetti et al., 2009b). On the other hand, the other tick proteins identified in this proteomic study have undefined roles. Two such proteins are SPARC and cyclophilin A; in our previous work describing the transcriptome of female salivary glands, their transcript sequences had in silico evidence of a signal peptide indicating that the tick can secrete these expressed proteins into saliva. In mammals, SPARC functions as a matricellular glycoprotein and modulates cellular interactions within the extracellular matrix, inhibits angiogenesis (Bhoopathi et al., 2010), regulates the expression of plasminogen activator inhibitor-1 by fibrotic fibroblasts (Chang et al., 2010), organizes the assembly of collagen fibrils (Giudici et al., 2008), and participates of Th17 differentiation and germinal centre formation (Piconese et al., 2011). Given the intimate

relationship of ticks with their hosts' extracellular matrix, their SPARC protein may interfere in this structure to the tick's advantage. Tick SPARC must be further studied. In vertebrates, cyclophilin A is a ubiquitous, abundant protein of the cytosol. During inflammatory responses, it is released into extracellular spaces by live and dying cells and binds to cell surface heparans and to its main signaling receptor, CD147, displaying potent chemotactic activity for monocytes, neutrophils, eosinophils, and T cells in the periphery (Xu et al., 1992; Yurchenko et al., 2002). In addition, secretion of cyclophilin A by thymocytes is able to modulate bone marrow cell migration (Khromykh et al., 2007). Through its peptidyl-prolyl cis-trans isomerase activity, it assists the folding of proteins by catalyzing cis-trans isomerization of proline imidic peptide bonds. In bacterial pathogens, cyclophilin A is a virulence factor that promotes colonization of mucosa by *Streptococcus pneumoniae*, although the exact mechanism is still unknown (Hermans et al., 2006). In *Legionella pneumophila*, a surface-exposed peptidyl-prolyl cis-trans isomerase called Mip promotes secretion or activation of *p*-nitrophenol phosphorylcholine hydrolase beyond the outer membrane of the pathogen (Debroy et al., 2006). In *Toxoplasma gondii*, a secreted cyclophilin stimulates interleukin-12 from dendritic cells and has a high affinity for the CCR5 chemokine receptor (Aliberti et al., 2003). Whether the peptidyl-prolyl cis-trans isomerase property of cyclophilin A could affect the activity of parasitic and/or host proteins or the chemotaxis and activation of host leukocytes, remains to be elucidated.

Regarding the large amount of host proteins present in *R. sanguineus* tick saliva, this apparently unusual finding merits some speculation. We suggest that these ectoparasites recycle these molecules from the hemolymph. It is widely demonstrated that ticks as well as other kinds of blood-feeding and non-blood-feeding insects, after their meal, move intact proteins across the digestive system to the hemolymph (reviewed by Jeffers and Roe, 2008). Once in the hemolymph, it is suggested that these proteins may cross the digestive system and are transported to the salivary glands where they are secreted back into the host. This hypothesis has been supported by different groups that demonstrated the presence of host proteins in tick saliva (Valenzuela et al., 2002; Madden et al., 2002; Wang and Nuttall, 1994; Francischetti et al., 2011). Hence, looking at our own results, several data support our suggestion that ticks are able to recycle host protein from the hemolymph: First, prior to collection of saliva, the tick mouthparts were carefully washed, but the amount of host protein in the proteome is too high to explain contamination from such a source; some of the host proteins described in this proteome (e.g. hemoglobin, transferrin, albumin) have been found in the tick hemolymph; host proteins can be found in the salivary glands of *R. microplus* nymphs and adults (I.K. de Miranda Santos, pers. communication); host proteins (e.g. albumin and hemoglobin) were found only selectively in the saliva because IgG, an abundant protein of the blood, was not detected. We also exclude regurgitation because in this case the saliva would be red in color, which was not the case. In addition, the strongly stained bands in the gel contained mostly host proteins. Indeed, this feature was correlated with the abundance of peptide ions for the respective proteins.

The intense bands near the 62-kDa marker (Fig. 3) correspond to the strong recovery of peptide ions for the abundant plasma proteins rabbit SA and serum transferrin, which should appear in this region of the gel (Tirumalai et al., 2003). Hemoglobin, band 3 anion exchanger, and carbonic anhydrase, important red cell constituents were also found. Leukocyte products such as myeloperoxidase and the abundant neutrophil gelatinase-associated lipocalin (NGAL) were found, as were the C3 protein of the complement system, a haptoglobin precursor and a plasma serpin that inhibits elastase and is the most abundant serine protease inhibitor in the circulation of mammals (Ranes and Stoller, 2005).

Why would ticks waste precious host proteins while feeding on them? Perhaps they do not need such proteins. Indeed, tick excretions are very rich in protein, and SDS gel

electrophoresis of *I. scapularis* feces has the same Coomassie-stained bands as a sample of rabbit blood (Ribeiro, unpublished). Over 70 years ago, a classical study showed that the amounts of blood taken by ticks in the first days of feeding is much larger than the tick's increase in weight, leading to exsanguination and death of their rabbit hosts (Jellison and Kohls, 1938). It has been suggested that in the first days of feeding, ticks are skimming the blood for oligonutrients and/or lipids, while excreting the unnecessary or excessive components via saliva or fecal material (Ribeiro, 1988), similar to feeding aphids that excrete a sugary liquid when feeding on sugar-rich but amino acid-poor diet (Klingauf, 1987). Another explanation is that ticks are, instead, recycling pivotal host proteins in order to subvert their role in the host. We have previously shown that tick infestations induce systemic acute-phase reaction proteins, including transferrin and haptoglobin (Carvalho et al., 2010). The presence of a host haptoglobin precursor and transferrin in the tick salivary proteome indicates that it probably is present locally in the blood feeding pool. Among plasmatic proteins, haptoglobin has the highest binding affinity for hemoglobin. It defends the host's vasculature against hemoglobin and heme by removing them with the CD163 scavenger receptor of the monocyte/macrophage system. CD163 is specific for the haptoglobin-hemoglobin complex. Once it binds to this complex, it signals anti-inflammatory responses that culminate in the release of IL-10 and the induction of heme oxygenase-1 (Abraham and Drummond, 2006), an enzyme that generates the immunosuppressive metabolites CO, Fe⁺⁺, and biliverdin from heme. Haptoglobin might thus establish a favorable milieu for the tick, and the possibility that ticks recirculate host haptoglobin through their salivary glands for this exact purpose is intriguing. Transferrin is a negative acute-phase protein and a regulator of levels of free iron. It affects the immune response by regulating the amount of iron available to cells of the immune system and ticks may be recirculating or sequestering host transferrin in order to regulate the host immune and/or the levels of a potentially toxic ion.

We can also speculate that if the host proteins pass through the intracellular tick compartment, these proteins could be subjected to the tick glycosylation machinery and thus immunogenic 'tick' sugar-based antigens would be presented to the host, possibly to function as decoys that divert host immunity away from tick proteins, or the host self epitopes associated with the tick glycosyl-epitopes may lead to immune suppression towards tick epitopes. Indeed, bovine beta chain hemoglobin shown to be present in saliva from females of the cattle tick, *Rhipicephalus microplus*, is recognized by IgG antibodies from tick-infested bovines and, furthermore, the reactive protein is acidic (pI 6.09) in comparison to native bovine hemoglobin, indicating that the native hemoglobin was modified in the tick (I.K. de Miranda Santos, unpublished).

It is also interesting to note that the lipocalin marked as Lip-368 (Fig. 2) is identified in 4 gel fractions where it is expected (15, 16, 17, and 18 in Fig. 2, near the 14-kDa marker), as well as on gel fractions of much higher MW, in particular in fraction 6, by the 98-kDa marker. The tick lipocalin Lip-38 (Fig. 2), although having a MW near 20 kDa as most lipocalins, is found in fractions corresponding to 100 kDa and above (fractions 4 and 5 in Fig. 2). The same occurs with some host proteins. Rabbit hemoglobin appears in fraction 13, above the 17-kDa marker, but also on bands 5 and 6, near the 98-kDa marker (Fig. 3). This increased molecular weight could, again, reflect glycosylation of part of the proteins, other post-translation modifications, or strong binding of tick salivary proteins with host proteins. The possible modification of host proteins by ticks is indeed a subject worth of consideration.

While this putative modification of host proteins by ticks needs further confirmation, there is much evidence showing that the binding of host proteins by tick salivary components is strong enough to survive to SDS denaturation. Sá-Nunes and colleagues (Sa-Nunes et al., 2009) demonstrated that sialostatin L, a tight-binding cysteine protease inhibitor from *I.*

scapularis saliva, binds to cathepsin S and that the complex is stable under SDS conditions. Unbound cathepsin S was detected only upon boiling the sample. In fact, sialostatin L presents a nanomolar affinity to its major targets (Kotsyfakis et al., 2006), and high-affinity binding was also demonstrated for other tick salivary-binding molecules, such as salivary anti-complement proteins (Nunn et al., 2005; Valenzuela et al., 2000) and disintegrins (Mans et al., 2002). Modification of host proteins by ticks can have yet another biological role. It can obviously involve proteolysis, but with consequences far-reaching those that facilitate absorption of amino acids: Hemoglobin degradation leads to formation of hemorphins, opioid peptides active in the immune system and in pain reception and hemoglobin-derived peptides also have antimicrobial activities (Nyberg et al., 1997).

Tick saliva induced by either DA or PC has been employed in different experimental approaches in order to identify its immunopharmacological activities. A few years ago, the concentration of PC present in PC-induced saliva was determined, and it ranged from 3 to 50 mM (Ribeiro et al., 2004). Because pilocarpine presents effects on different cell types, appropriate controls are required when employing tick saliva in biological assays, in order to avoid experimental artifacts. At least for activation assays of dendritic cells in the presence of *I. scapularis* and *R. sanguineus* saliva, the fraction containing PC has not induced significant changes of cytokine production (Sa-Nunes et al., 2007). In the same manner, DA does not affect the biological activities of saliva obtained from *R. microplus* (Carvalho et al., 2010). However, our experience with tick salivation employing DA or PC suggests that researchers using dopamine to obtain tick saliva should take into consideration the possible melanization of their salivary sample via the tick's prophenoloxidase pathway or spontaneous sample oxidation. Perhaps, it should be advisable that the salivary sample be used immediately, that inhibitors for the prophenoloxidase pathway be employed (e.g. EDTA and DTT), or that the proteins be separated from the dopamine as soon as possible to avoid dopamine oxidation and possible protein cross-linking.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP through grant 2009/53645-3 to IKMS, and scholarships to EA (2007/50869-2) and CJFO (2006/54985-4); by the Brazilian National Science Foundation-CNPq through grants 559603/2009-6 and 471946/2010-9 to IKMS, Fundação de Amparo à Pesquisa do Estado de Minas Gerais- FAPEMIG and the Intramural Research Program of the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health. We thank the Research Technology Branch under Dr. Robert Hohman for preparing the gel and mass spectrometric data collection and analysis, and to the NIAID intramural editor Brenda Rae Marshall for assistance.

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Fig. 1. Gel electrophoresis, tryptic digestion, and tick protein identification by MS analysis of digested peptides from *R. sanguineus* saliva induced by DA. Left lane: Molecular weight standards (M). Right lane, DA-induced tick saliva. Lip stands for lipocalin, and the number refers to the protein numbers shown in Supplemental Spreadsheet S2, which is an updated version of a previously published file (Anatriello et al., 2010).

Fig. 2. Gel electrophoresis, tryptic digestion, and tick protein identification by MS analysis of digested peptides from *R. sanguineus* saliva induced by PC. Left lane: Molecular weight standards (M). Right lane, PC-induced tick saliva. The grid numbered 1–23 corresponds to the fractions subjected to tryptic digest and mass spectrometric detection of peptidic fragments matching the tick sialotranscriptome, identified to the right side of the fraction number. For the description of protein symbols see Table 1.

Fig. 3. Gel electrophoresis, tryptic digestion, and rabbit protein identification by MS analysis of digested peptides from *R. sanguineus* saliva induced by PC. Left lane: Molecular weight standards (M). Right lane, PC-induced tick saliva. The grid numbered 1–23 corresponds to the fractions subjected to tryptic digest and MS detection of peptidic fragments matching the tick rabbit proteome, identified to the right side of the fraction number. For the description of protein symbols see Table 2.

Table 1

Tick proteins identified in *R. sanguineus* saliva induced by pilocarpine. See Fig. 2 legend for more details.

Tick protein	MW (kDa)	Description	Symbol in Fig. 2	Fraction → number of ions ^a
RS-899	23.5	Putative thyropin precursor	Thyr-899	F ₅ → 10 F ₄ → 4 F ₆ → 3
RS-126	22.9	Matricellular protein osteonectin/SPARC/BM-40	Sparc-126	F ₅ → 3
RS-54	29.3	GLC family member	GLC-54	F ₆ → 3
RS-935	17.2	One of each family member 1	OOE-935	F ₆ → 6 F ₅ → 4 F ₄ → 2 F ₁₅ → 2 F ₁₆ → 2
RS-757	18.3	One of each family member 2	OOE-757	F ₁₅ → 2 F ₁₇ → 2
RS-671	17.2	One of each family member 3	OOE-671	F ₆ → 4 F ₄ → 2 F ₅ → 2
RS-38	18.3	Lipocalin	Lip-38	F ₅ → 4
RS-368	21.3	Lipocalin	Lip-368	F ₆ → 11 F ₅ → 6 F ₁₆ → 4 F ₄ → 3 F ₁₅ → 3 F ₁₇ → 2 F ₁₈ → 2
RS-25	19.9	Glycine-rich protein	GR-24/25	F ₁₈ → 2
RS-24	32.0	Glycine-rich protein	GR-24/25	F ₁₈ → 2
RS-104	17.3	40S ribosomal protein S13	Rib-S13	F ₆ → 3 F ₁₅ → 2
RS-67	21.8	60S ribosomal protein L9	Rib-L9	F ₆ → 3 F ₁₂ → 2
RS-78	18.8	Ribosomal protein L21	Rib-L21	F ₄ → 4 F ₅ → 4
RS-815	20.9	Eukaryotic translation initiation factor 3 subunit 2 beta	ETI	F ₄ → 2
RS-766	17.1	Ubiquitin/ribosomal protein S27a fusion protein	S27a	F ₄ → 4 F ₅ → 3 F ₆ → 3 F ₁₅ → 2 F ₁₆ → 2 F ₁₇ → 2
RS-279	22.3	Thioredoxin peroxidase - truncated at 5'	TRP	F ₆ → 2
RS-310	17.5	Cyclophilin A	Cyc	F ₅ → 3
RS-878	26.8	Protein disulfide isomerase	PDI	F ₆ → 17 F ₅ → 15
RS-99	17.7	Glycosyltransferase, putative	GT	F ₁₆ → 2 F ₁₇ → 2

^aF₅ → 10 indicates 10 ions were found for the protein in fraction 5.

Table 2

Rabbit proteins identified in *R. sanguineus* saliva induced by pilocarpine. Some of these proteins may have closely related tick protein homologs. See Table 3 for rabbit proteins without known tick homologs. Some proteins are isoforms of the same gene or closely related gene products. See Fig. 3 legend for more details.

Rabbit protein	MW (kDa)	Description	Symbol in Fig. 3	Fraction → number of ions ^a
gi 126723746	69	Serum albumin precursor	SA	F_8 → 188 F_9 → 84 F_12 → 82 F_7 → 70 F_11 → 60 F_5 → 54
gi 156119356	77	Serotransferrin	STF	F_7 → 72 F_12 → 36 F_9 → 36 F_10 → 28 F_8 → 24 F_11 → 22
gi 291409423	50	Tubulin, beta-like	TB	F_9 → 56 F_12 → 4 F_10 → 4
gi 156119364	42	Actin, cytoplasmic 1	ACT	F_10 → 48 F_11 → 36 F_12 → 22 F_13 → 20 F_9 → 15 F_5 → 13
gi 291415548	51	Tubulin, beta 4	TB	F_9 → 44 F_10 → 2
gi 126722957	46	Alpha-1-antiproteinase S-1 precursor	α-1-AT	F_9 → 43 F_8 → 10 F_7 → 6 F_10 → 4
gi 126722876	46	Alpha-1-antiproteinase E	α-1-AT	F_9 → 42 F_8 → 8 F_10 → 4 F_7 → 4
gi 126722912	46	Alpha-1-antiproteinase F precursor	α-1-AT	F_9 → 34 F_8 → 10 F_7 → 6 F_10 → 4
gi 126723457	16	Hemoglobin subunit beta-1/2	Hb	F_12 → 30 F_13 → 30 F_4 → 30 F_10 → 28 F_3 → 28 F_5 → 26
gi 126723668	53	Vitamin D-binding protein precursor	VDdb	F_9 → 28 F_8 → 4
gi 291400273	61	Histidine-rich glycoprotein	HRGP	F_7 → 28 F_12 → 14 F_10 → 12 F_9 → 12 F_6 → 10 F_11 → 8
gi 283806600	46	Alpha-1-antitrypsin	α-1-AT	F_9 → 25 F_8 → 6 F_10 → 4 F_7 → 4
gi 291392526	37	L-lactate dehydrogenase B	LacDeh	F_12 → 25
gi 291388359	71	Poly A-binding protein, cytoplasmic 4	PolA	F_14 → 24 F_17 → 24 F_16 → 20 F_18 → 18 F_15 → 16 F_21 → 4 F_18 → 23 F_16 → 23 F_14 → 23 F_19 → 23 F_17 → 23 F_15 →
gi 291393283	33	Ribosomal protein SA	RibSA	20
gi 291415360	39	Hypothetical protein LOC10009097	HypP	F_2 → 22 F_3 → 20 F_9 → 16 F_11 → 10 F_5 → 10 F_4 → 8 F_18 → 21 F_16 → 21 F_14 → 21 F_19 → 21 F_17 → 21 F_15 →
gi 291402382	33	Ribosomal protein SA-like	RibSA	18
gi 291395394	42	Beta actin	ACT	F_11 → 21 F_10 → 20 F_12 → 9 F_13 → 4 F_17 → 4 F_9 → 3
gi 291406576	70	Heat shock 70 kDa protein 2	HS70	F_7 → 20 F_8 → 10 F_9 → 4
gi 291389089	50	Tubulin, alpha 4a	TB	F_9 → 18 F_11 → 8 F_10 → 6 F_12 → 4
gi 291402475	52	GL12416-like	GL	F_9 → 18 F_11 → 8 F_10 → 6 F_12 → 4
gi 291408513	36	Delta-aminolevulinic acid dehydratase	DALD	F_12 → 18 F_11 → 4
gi 283837861	39	Annexin A1	Annex	F_11 → 18 F_2 → 16
gi 156119416	37	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	PPh	F_12 → 18 F_11 → 8
gi 291400199	71	Heat shock 70 kDa protein 8	HS70	F_7 → 17 F_8 → 8 F_9 → 4 F_18 → 16 F_16 → 16 F_17 → 16 F_15 → 14 F_14 → 14 F_22 →

Rabbit protein	MW (kDa)	Description	Symbol in Fig. 3	Fraction → number of ions ^a
gi 291395508	30	Ribosomal protein S4, X-linked X-like	RibS4	12
gi 291407056	34	Acidic ribosomal phosphoprotein P0	RibP0	F_17 → 16 F_18 → 12 F_16 → 12 F_14 → 12 F_15 → 10
gi 291408341	72	Heat shock 70 kDa protein 5	HS70	F_7 → 16 F_8 → 4 F_9 → 2
gi 291410975	84	Heat shock 90 kDa protein 1, beta	HS90	F_7 → 16 F_6 → 6 F_9 → 4 F_4 → 4
gi 291389085	50	Tubulin, alpha 4a	TB	F_9 → 16 F_11 → 4 F_12 → 2 F_10 → 2
gi 291389091	73	mCG18413-like	mC	F_9 → 16 F_11 → 4 F_12 → 2 F_10 → 2
gi 291411146	50	Tubulin, beta 1-like	TB	F_9 → 15
gi 291391538	42	Beta actin	ACT	F_10 → 15 F_12 → 10 F_13 → 6 F_4 → 6 F_11 → 5 F_9 → 5 F_18 → 14 F_16 → 14 F_17 → 14 F_15 → 12 F_14 → 12 F_22 →
gi 291407691	26	Ribosomal protein S4, X-linked X-like isoform 3	RibSA	10
gi 126723309	82	Complement C3 alpha chain	C3	F_5 → 14 F_4 → 12 F_11 → 8 F_2 → 6 F_10 → 4 F_3 → 4
gi 291403445	32	Nucleoside phosphorylase	NPp	F_13 → 14 F_12 → 8
gi 291413837	34	Serine protease inhibitor A3N-like, partial tyrosine 3/tryptophan 5 -monooxygenase Activation protein,	SPI	F_10 → 14 F_9 → 14
gi 291405401	29	epsilon polypeptide	Tmox	F_13 → 12 F_12 → 10
gi 291388220	33	Carbonic anhydrase I	CA	F_12 → 12 F_13 → 12
gi 156119384	36	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	PPh	F_12 → 12 F_11 → 8
gi 126723706	39	Haptoglobin precursor	Hapto	F_10 → 12 F_11 → 8 F_9 → 6
gi 291400837	30	Ribosomal protein S4, X-linked X-like	RbSA	F_18 → 10 F_16 → 10 F_17 → 10 F_22 → 8 F_15 → 8 F_14 → 8
gi 291398505	34	Ribosomal protein L5	RibL5	F_17 → 10 F_18 → 8 F_15 → 8 F_14 → 8 F_16 → 6 F_20 → 4
gi 291395123	39	G-protein beta subunit-like protein	Gptn	F_18 → 10 F_15 → 10 F_14 → 10 F_17 → 10 F_16 → 8 F_22 → 6
gi 291406239	102	Solute carrier family 4, anion exchanger	SC	F_11 → 10 F_12 → 6 F_2 → 6
gi 291385496	31	Protein phosphatase 1, catalytic subunit, alpha	PPh	F_12 → 10 F_11 → 6
gi 291397052	53	Heat shock 70 kDa protein 8	HS70	F_7 → 9 F_8 → 6 F_9 → 4
gi 291405719	81	Myeloperoxidase-like	Mpox	F_9 → 8
gi 126723487	34	Heterogeneous nuclear ribonucleoprotein C	NRNP	F_18 → 8 F_14 → 8 F_15 → 6 F_16 → 6 F_17 → 6 F_19 → 4
gi 291394305	35	DNA-binding protein B-like	DNABp	F_17 → 8 F_15 → 6 F_16 → 6 F_14 → 6
gi 130488167	36	Nuclease-sensitive element-binding protein 1	NSEBP	F_17 → 8 F_15 → 6 F_16 → 6 F_14 → 6
gi 291383209	29	Ribosomal protein S6-like	RbS6	F_17 → 8 F_18 → 6 F_15 → 6 F_21 → 6 F_16 → 6 F_14 → 6
gi 291386387	34	Ribosomal protein L5-like	RibL5	F_17 → 8 F_18 → 6 F_15 → 6 F_16 → 6 F_14 → 6 F_20 → 4
gi 291410580	32	Ribosomal protein L7a-like	RibL7	F_17 → 8 F_22 → 6 F_21 → 6 F_16 → 6 F_19 → 6 F_18 → 4
gi 291396282	84	Heat shock 90 kDa protein 1, beta	HSP90	F_7 → 8 F_6 → 6 F_4 → 4 F_9 → 2
gi 130500366	52	Hemopexin precursor	HEMOP	F_8 → 6 F_9 → 4

$a_{F_8} \rightarrow 188$ indicates 188 ions were found for the protein in fraction 8.

Table 3

Rabbit proteins without similarity with tick proteins identified in *R. sanguineus* saliva.

Seq name	Description	Symbol in Fig. 3
gi 126723746	Serum albumin precursor	SA
gi 156119356	Serotransferrin	STF
gi 126722957	Alpha-1-antiproteinase	α -1-AT
gi 126723457	Hemoglobin subunit beta-1/2	Hb
gi 126723668	Vitamin D-binding protein precursor	VDb
gi 291400273	Histidine-rich glycoprotein	HRGP
gi 291408513	Delta-aminolevulinic acid dehydratase	DALD
gi 283837861	Annexin A1	Annex
gi 126723309	Complement C3 alpha chain	C3
gi 291403445	Nucleoside phosphorylase	NPp
gi 291388224	Carbonic anhydrase	CA
gi 126723706	Haptoglobin precursor	Hapto
gi 291406239	Band 3 anion exchanger	SC
gi 291405719	Myeloperoxidase-like	Mpox
gi 291413525	Neutrophil gelatinase-associated lipocalin	NGAP
gi 130500366	Hemopexin precursor	HEMOP