

Expressed sequence tags (ESTs) from the salivary glands of the tick *Amblyomma cajennense* (Acari: Ixodidae)[☆]

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Abstract

The neotropical tick *Amblyomma cajennense* is a significant pest to domestic animals, the most frequently human-biting tick in South America and the main vector of Brazilian spotted fever (caused by *Rickettsia rickettsii*), a deadly human disease. The purpose of this study is to characterize the adult *A. cajennense* salivary gland transcriptome by expressed sequence tags (ESTs). We report the analysis of 1754 clones obtained from a cDNA library, which reveal mainly transcripts related to proteins involved in the hemostatic processes, especially proteases and their inhibitors. Remarkably, five types of possible serine protease inhibitors were found, including a molecule with a distinguished structure that contains repeats of the active motif of hirudin inhibitors. Besides, other components that may be active over the host immune system or acting as defensins against infecting microorganisms were also described, including a molecule similar to insect venom allergens. The conjunction of components from this transcriptome suggests a diverse strategy of *A. cajennense* tick during feeding, but emphasized in the coagulation system.

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1. Introduction

Ticks (class Arachnida, subclass Acari, suborder Ixodida) are large (2–30 mm in length) mites that are obligatory hematophagous ectoparasites in postembryonic stages. They may injure their vertebrate hosts by causing dermatoses (inflammation, itching, swelling and ulceration), envenomization (from tick saliva), exsanguinations or otoacariasis (infestation of the ear canal), and they can transmit

[☆] **Ethical statement:** We declare that the experiments were done according to the ethical principles of experimentation from our institution.

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toxins causing tick paralysis. However, ticks are chiefly important as a vector of viruses, bacteria, protozoa and nematodes, which may result in severe illness or the death of their hosts (Guglielmone et al., 2003).

Currently, there are around 870 tick species described in the World (Wheller and Hayashi, 1998; Horak et al., 2002), divided into three families: Ixodidae (683 species), Argasidae (183 species) and Nuttalliellidae (1 species). General tick life cycle is comprised of egg, larval, nymphal and adult (male and female) stages. For most of the species, each one of these stages (except for the egg) needs a blood meal in order to develop to the next stage. In the case of females, large amounts of blood are usually needed for oviposition of hundreds to thousand of eggs.

Tick salivary glands are vital structures responsible for their biological success, in order to survive under off-host conditions, and chiefly during blood feeding, when a significant obstacle in obtaining a blood meal is counteracting the hemostatic system of the host. In fact, the crude saliva is a mixture of innumerable substances that are directly involved in blood coagulation, platelet aggregation, vascular contraction, host immunity and inflammation (Ribeiro and Francischetti, 2003), besides a possible active role in the transmission of several tick-borne diseases (Nuttall and Labuda, 2004).

Screening and characterization of the compounds present in salivary secretions have been performed by different ways, and these studies are generally known as sialomics (Champagne, 2004). They have been done by mass spectrometry, transcriptomic and proteomic methods, and are useful analytical strategies to improve the knowledge of new bioactive molecules (Ribeiro et al., 2006; Alarcon-Chaidez et al., 2007; Faria et al., 2005; Junqueira-de-Azevedo et al., 2006). Besides the existence of almost 900 tick species in the world, tick sialomics have been limited to very few species. Since tick sialomics have been restricted to species of the Ixodidae family, including *Boophilus* (Rhipicephalinae subfamily), *Ixodes* (Ixodinae), *Haemaphysalis* (Haemaphysalinae), their profiles have revealed various transcripts similar to different species. On the other hand, several other transcripts have been found to be restricted in each of the studied species (Ribeiro et al., 2006; Alarcon-Chaidez et al., 2007). This diversity is not a surprise, since each tick species has its ecological niche, where it is exposed to specific environmental conditions and usually feed on a specific group of host species.

Among the Brazilian tick fauna, the Ixodidae species *Amblyomma cajennense* (Fabricius), Amblyomminae subfamily, bears the greatest medical and veterinary importance, since it is a significant pest to horses, cattle and dogs, and it is the most frequently human-biting tick and the main vector of Brazilian spotted fever (caused by *Rickettsia rickettsii*), a re-emerging deadly disease that occurred in Southeastern Brazil (Horta et al., 2004). In addition, *A. cajennense* has been incriminated as a possible vector of equine babesiosis (Heuchert et al., 1999) and an uncharacterized spirochetosis, called Lyme-like disease (Yoshinari et al., 2000).

Herein, there is a report of the analysis of 1754 clones obtained from the cDNA library of the *A. cajennense* salivary glands. The profile of transcripts is presented focusing those related to proteins involved on the hemostatic process, especially the proteases and their inhibitors.

2. Materials and methods

2.1. Salivary gland extractions

Adult females of *A. cajennense* were obtained from a laboratory colony that originated from field-collected ticks in Pirassununga, SP, Brazil. They were maintained in total darkness at 27 °C and 90% relative humidity in an incubation oven, and then allowed to feed on the shaved skin of *Oryctolagus cuniculus* rabbit backs (Rechav et al., 1997). After 5 days, partially fed females were removed from the rabbits, and the salivary glands were separated from the rest of the organs. The extracted salivary glands were transferred into cryotubes and stored at –80 °C until use.

2.2. cDNA library construction and DNA sequencing

The generation of expressed sequence tags (ESTs) was performed as described previously (Junqueira-de-Azevedo et al., 2006), as follows. The salivary glands were mixed to the Trizol reagent and total RNA was purified according to the manufacturer's protocol (Invitrogen). Poly A + enriched RNAs were isolated on an oligo (dT)-cellulose column (GE Healthcare). The cDNAs were synthesized from 5 µg of Poly A + RNA using the Superscript Plasmid System (Invitrogen). Random colonies were selected and the DNA was isolated by alkaline lysis. The DNA was sequenced on an ABI 3100 sequencer using BigDye2 dideoxy-terminators (Applied Biosystems) and M13 forward primer.

2.3. Cluster assembly and identification

After sequencing, the electropherogram files were analyzed in a semi-automatic way, as described in Junqueira-de-Azevedo et al. (2006). The Phred program was used (www.phrap.com) to remove poor-quality sequences (window length of 75 bases with 75% of standard quality of 25). Then, adapter and vector sequences were removed using the CrossMatch Program. A manual examination was carried out and sequences below 150 bp were discarded. ESTs were then assembled in clusters of contiguous sequences using CAP3 program (Huang and Madan, 1999), with 98% or more of base identity in a high-quality region. The cluster sequences were searched against GenBank NCBI database using BLASTX and BLASTN algorithms. The clusters were screened for the presence of potentially full-length open reading frames (ORFs). The possible signal peptide (www.cbs.dtu.dk/services/SignalP) as well as the conserved domains was predicted by SMART (Schultz et al., 2000).

3. Results and discussion

3.1. EST sequencing and cluster identification

The diversity of components from the saliva of *A. cajennense* was explored here through the generation and analysis of an EST database. A total of 1920 clones were sequenced, yielding 1754 readable sequences (92% efficacy), with an average

size of 472 bp. All sequenced clones were deposited in the GenBank (dbEST division) under accession numbers from EC778445 to EC780213. These ESTs were assembled in 1234 clusters (256 clusters composed of two or more ESTs, and 985 singlets).

After database comparisons, 69% were associated with previously described sequences that were basically divided into those with putative roles in the several levels of the feeding process (feeding proteins) and those that are not involved in feeding (non-feeding proteins) (Fig. 1-center). These non-feeding proteins enroll molecules with different cell functions (Fig. 1-left) and most were similar to insect proteins. The number of products with unknown function (17%) is also remarkable. Besides, the remaining 31% did not show significant hits in databases, suggesting novel arthropod transcripts. The complete lists of all putatively identified gene products are provided in Supplementary tables 1 and 2, and the sequences of clusters cited in the text are in Supplementary table 3.

3.2. Proteins involved in the blood-feeding process

By “feeding”, all the processes that would be important to ticks in maintaining their interaction with the host, for example, helping to prevent blood from coagulating or impairing the host immune and inflammatory systems to attack, were considered (Steen et al., 2006). Abundance is another indication of how important the proteins can be to the main fate of some systems, such as the case of toxins to

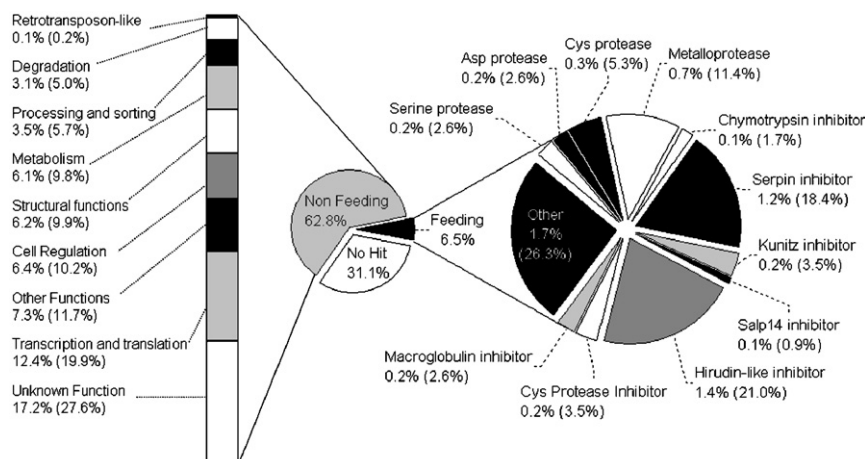


Fig. 1. Relative proportion of each category of product found. Feeding and non-feeding categories are assigned through GenBank comparisons with known sequences with an e -value $< 10e-05$ on Blastx search. Non-feeding was categorized by their cellular function, whereas feeding by their structural nature. Number of clusters and percentages of total ESTs and of ESTs in the category (in brackets) are presented.

the snake venom glands (Junqueira-de-Azevedo et al., 2006). In fact, among the 10 most abundant transcripts (Table 1) found here, half are involved in blood feeding. Thus, a combined criterion was adopted to compose a very general category of proteins that may be enrolled in the blood feeding process (Supplementary table 1).

This group is mainly composed of proteases and protease inhibitors, representing around 6% of the total ESTs from *A. cajennense* (Fig. 1—right). This exacerbation of the protease/protease-inhibitor system is probably related to the main tick's needs. To have a blood meal, ticks penetrate their hypostomes into the host skin to achieve the blood vessels. This procedure damages (with aid of proteases) many capillaries and small vessels, and the cell disruption causes a hemorrhage, which will increase the blood volume for feeding. Then, perhaps the most essential action that any hematophagous organism should take care is to keep the blood in a liquid form, flowing to its oral cavity. Thus, they directly interfere in the blood clotting and in the platelet aggregation systems of their hosts by the action of proteases and their inhibitors (Champagne, 2004; Ribeiro and Francischetti, 2003; Steen et al., 2006; Prevot et al., 2006).

The process of hemostasis (Fig. 2) occurs in four distinct phases. In the primary phase, the injured blood vessel promotes vasoconstriction and exposure of endothelial cells. Platelets participate in hemostasis through a sequence of events beginning with adherence at the site of injury. Platelets then release contents from internal granules, aggregate to form the platelet plug and promote coagulation by providing a procoagulant surface on their phospholipid membranes. In the secondary phase, initiation of the classic coagulation cascade begins with the interaction of tissue factor and factor VII, present in

its activated form in plasma (factor VIIa). At the end of a series of enzymatic steps involving other coagulation factors, prothrombin is converted to thrombin, thrombin converts fibrinogen to fibrin, and then fibrin and platelets form a hemostatic thrombus. Organization of the formed clot provides cessation of bleeding. The tertiary phase employs endogenous anticoagulants that control and slow the procoagulant processes. Finally, during the quaternary phase, fibrinolytic proteins are activated and result in clot lysis; thus, the thrombus does not continue to expand. Primary hemostasis refers to the cellular response at the site of injury, whereas secondary hemostasis refers to the circulating clotting factors that work through enzymatic processes to generate the end product of fibrin to form the platelet plug (Zimmerman, 2007).

A brief description of such components found in *A. cajennense* transcriptome is presented below.

3.3. Proteases in blood-feeding organisms

The proteolytic enzymes, widely distributed in all organisms, operate in every biological phenomenon and are frequently involved in cascades or networks. In the saliva of hematophagous organisms there are several enzymes that probably are responsible to promote a pre-oral or even an oral digestion, acting also in the maintenance of blood in its liquid form (Cohen, 1995). This phenomenon contributes to the efficiency of the tick feeding process that will be completed by the gut enzymes. In this context, metalloproteases are emerging as substances with toxic effects that disturb the hemostatic system and degrade the cellular matrix proteins (Steen et al., 2006).

Our cDNA library has seven clusters that code for metalloproteases (Supplementary Table 1),

Table 1
The most abundant ESTs from *Amblyomma cajennense* salivary glands

#	Cluster	No. of clones	Best match to NR protein database (<i>Organism</i>)	Accession no.	e-Value
1	ACAJ0236C	12	ADP/ATP translocase [<i>Ixodes scapularis</i>]	gb AAAY66852.1	100E–165
2	ACAJ0147C	12	Serpin [<i>Ixodes scapularis</i>]	gb AAV80788.1	100E–81
3	ACAJ0069C	12	Ferritin [<i>Amblyomma americanum</i>]	gb AAQ54708.1	100E–94
4	ACAJ0137C	11	60S Ribosomal protein L15 [<i>Ixodes scapularis</i>]	gb AAAY66921.1	400E–95
5	ACAJ0177C	11	B-cell translocation gene 1 [<i>Gallus gallus</i>]	sp P34743	400E–36
6	ACAJ0085C	11	Hirudin-like protein [<i>Amblyomma variegatum</i>]	dbj BAD29729.1	400E–33
7	ACAJ0207C	10	Heat shock-related protein [<i>Dermacentor variabilis</i>]	gb AAO92281.1	700E–74
8	ACAJ0059C	10	Cyclin B4 [<i>Xenopus laevis</i>]	emb CAC24492.1	200E–08
9	ACAJ0222C	9	Hirudin-like protein [<i>Amblyomma variegatum</i>]	dbj BAD29729.1	300E–32
10	ACAJ0158C	9	Prohibitin protein Wph [<i>Bombyx mori</i>]	gi 114053221	500E–75

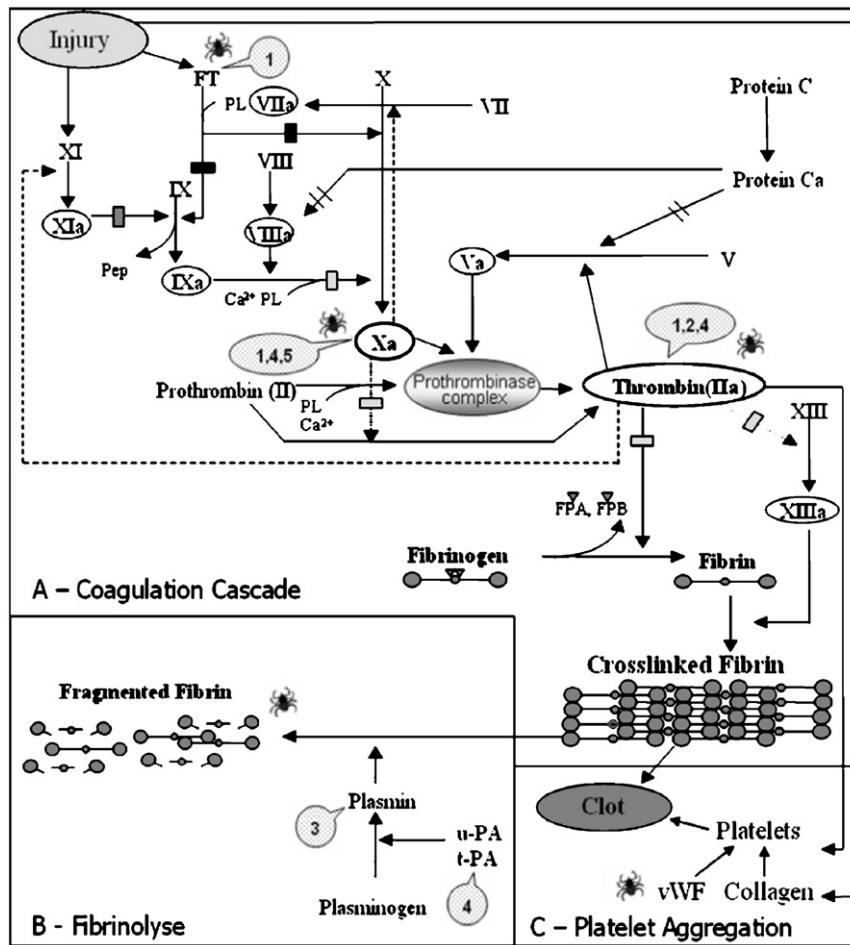


Fig. 2. Representation of the hemostasis process pointing out the potential inhibition sites by *Amblyomma cajennense* transcript products. The blood coagulation cascade (A) consists of the proteolytic activation of several serine proteases leading to the thrombin formation. Thrombin is the protease responsible for the conversion of fibrinogen (soluble protein) into fibrin (insoluble protein). After the coagulation, the clot must be degraded by the fibrinolytic process (B), which is initiated by the activation of plasminogen into plasmin, the enzyme that is able to lyse the cross-linked fibrin. Another important event in the hemostasis process is the platelet aggregation (C), which is initiated by platelet activation and adhesion of platelet membrane receptors to vWF and collagen. In this scheme, the ticks represent previously described proteins that interfere in the hemostatic system (Maritz-Olivier et al., 2007) and the numbers represent the potential action of the feeding proteins described in this paper. (1) (ACAJ0015C, ACAJ1187S, ACAJ1219S), (2) (ACAJ0085C, ACAJ0222C, ACAJ0247C, ACAJ0788S, ACAJ0914S), (3) (ACAJ0514S, ACAJ0917S, ACAJ1035S), (4) (ACAJ0069C), (5) (ACAJ0950S).

which are similar to members of the reprotolysin family, including the hemorrhagic snake venom metalloproteases and enzymes recently described in other ticks, such as *Ixodes scapularis* Say (Francischetti et al., 2003). Interestingly, the tick enzymes present a Cys-rich domain that is not homologous to P-III class of snake metalloproteases or ADAMs (data not shown). Although these clones were only partially sequenced, the region “HEVAHVLCGMH”, which is highly homologous to the consensus Zn²⁺-binding motif, could be recognized (Fig. 3B). A phylogenetic analysis of the

catalytic portion compared with matrix metalloproteases, ADAMs proteases and snake venom proteases (the last two, reprotolysins) placed the tick molecules close to the reprotolysin group (Fig. 3C) rather than to matrix metalloproteases, suggesting them as new members of the reprotolysin family that lack the disintegrin domain or that are related to their ancestor. The signal peptide and prodomains of the three longest 5' sequences are conserved with other tick metalloproteases, suggesting that they undergo the same processing pathways probably adopted by the *Haemaphysalis longicornis*

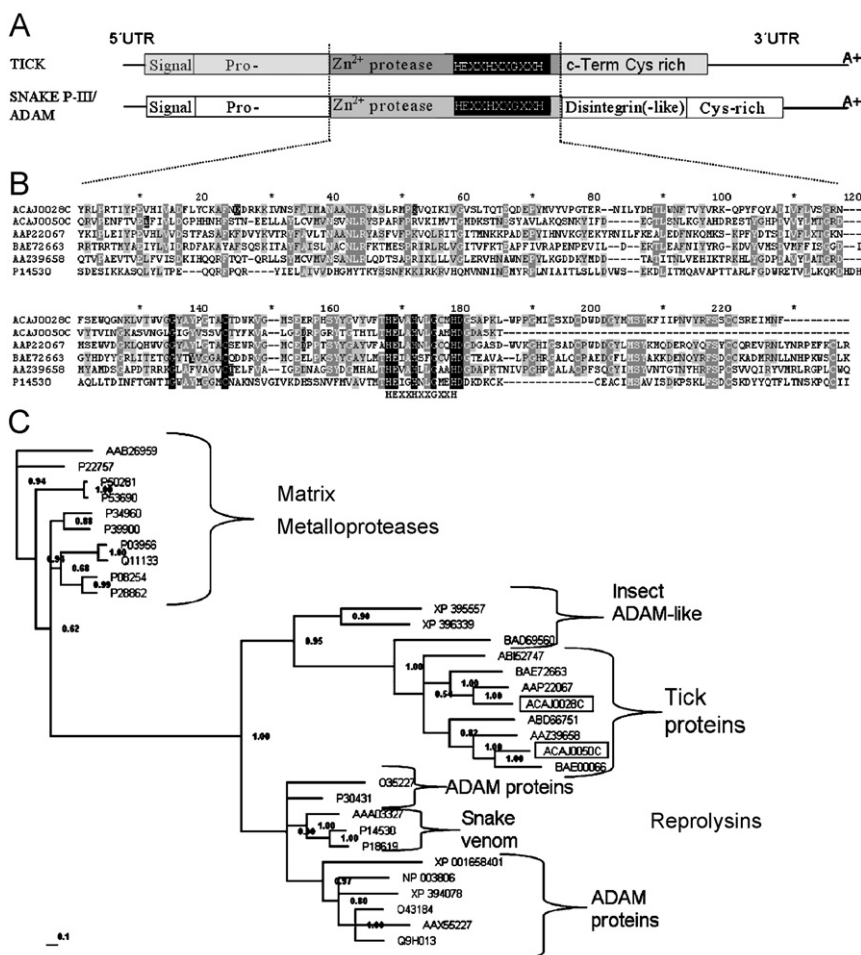


Fig. 3. The metalloproteases from *Amblyomma cajennense*. (A) Schematic view of the ADAM/SVMP precursor and that of tick metalloproteases. (B) Alignment of the translated amino acid sequence of the mature proteolytic domain, with the catalytic motif remarked above. ACAJs are sequences from this work, AAP22067 is from *Ixodes scapularis*, BAE72663 from *Haemaphysalis longicornis*, AAZ39658 from *Boophilus microplus* P14530 from snake *Trimeresurus flavoviridis*. (C) Bayesian inference of the phylogeny based on the posterior probability distribution of the trees. MrBayes software version 3.1.1-p1 (Ronquist and Huelsenbeck, 2003), with 10⁶ number of cycles for the Markov chain Monte Carlo algorithm, was used for the analysis of the proteins indicated by their accession numbers on the tree.

Neumann: the protein is intracellularly processed and released as a soluble mature enzyme into the acini lumen (Harnnoi et al., 2007). Interestingly, angiotensin-converting enzyme-like metalloproteases were not detected here, suggesting that *A. cajennense* metalloproteases may not be associated with anti-inflammatory mediation as in other cases (Steen et al., 2006) but rather to the anti-hemostatic activity.

The cysteine and aspartic proteases play important roles in the biology of parasitic organisms, especially because they are used to be involved from embryogenesis to hemoglobin degradation during blood feeding (Renard et al., 2000; Delcroix et al.,

2006). Three sequenced clusters code for cysteine proteases, similar to cathepsin-B or L-like enzymes, and the other three clusters were similar to asparaginyl proteases of the legumain-like family (Supplementary table 1). These enzymes may be directly involved in blood degradation.

Vertebrate blood coagulation is mainly modulated by the action of serine proteases, enzymes that are generally produced as inactive zymogens, which are activated by peptide cleavage. Although these enzymes are also found in arthropods, they are not very well characterized in them (Miyoshi et al., 2004). Three clusters code for serine proteases previously reported in ticks: two of them

(ACAJ0368S and ACAJ0968S) are trypsin-like serine proteases characterized from *Rhipicephalus appendiculatus* Neumann and the last one (ACAJ0802S) was described in *I. scapularis*. The serine proteases may be involved in the feeding process since it was demonstrated that some of them are able to degrade serum albumin (Miyoshi et al., 2004). Although they are mostly found in the gut, the hypotheses of pre-oral digestion performed by the saliva enzymes could not be discarded.

3.4. Protease inhibitors

Many important physiological mechanisms are mediated by proteolytic enzymes and their control can be achieved by the action of specific inhibitors. In our cDNA library, 26 clusters (59 clones) code for possible serine and cysteine protease inhibitors (Supplementary table 1) that may act in different parts of the coagulation cascade (Fig. 2).

The most abundant type of molecule among the putative feeding protein transcripts (almost 23%) corresponds to a hirudin-like protein (BAD29729), which is supposed to be a thrombin inhibitor. In fact, these proteins are called “hirudin-like” probably because they present a C-terminal sequence (~12 amino acids) that resembles the hirudin C-terminal. Hirudin is an exogenous anticoagulant peptide isolated from the leech *Hirudo medicinalis* Linnaeus. It is perhaps, the most studied natural thrombin inhibitor, which gave rise to several analogs, including a 20 amino acid-long synthetic peptide, named Hirulog (Weitz, 2003). The Hirulog C-terminus is the region responsible for thrombin binding.

This protein coded by cluster ACAJ0085C is similar to the hirudin-like protein identified in the tick *Amblyomma variegatum* (Fabricius) (BAD29729), but both of them are distinct from whole hirudin, although they share a related peptide resembling Hirulog C-terminus (Fig. 4). Moreover, the *Amblyomma* spp. proteins present a highly repetitive structure, composed by repeats of 34 residues (four repeats for *A. cajennense* and five for *A. variegatum*). Each segment repeat has around 97% identity to each other and the Hirulog C-terminal peptide could be aligned to every segment (Fig. 4).

Whether these novel molecules in fact exhibit a thrombin (or other serine protease) inhibitory activity, may be potentiated by the several copies. Probably, this multimeric structure could be cleaved

at one of the four basic residues outside the blocking motif, giving rise to several processed active peptides. Besides, the molecules found in this transcriptome indicate that the hirudin inhibitory active motif is more spread than previously thought and not restricted to leeches.

The Serpin (SERine Protease INhibitors) is a ubiquitous superfamily of the serine protease inhibitors, basically composed of 350–400 amino acids that have a typical reactive center loop (RCL) located near the C-terminus. The RCL is responsible for the interaction with target proteases, causing irreversible losses in the enzymatic activity and distortion in the structures (Prevot et al., 2006). Nine clusters (21 clones) coding for proteins with sequences similar to several Serpins isolated in the last years from tick cDNA libraries were identified. One of them (ACAJ0069C) is highly expressed (12 clones), with a signal peptide that could be observed on the assembled sequence and is similar to the *Ixodes* Serpins (data not shown).

The role of these tick Serpins is not clear, as they are found either in the hemolymph or in the saliva, thus possibly they act on parasite/host clotting systems or as a defense molecule against host proteases. Better evidences came from a recombinant protein derived from the salivary glands of *Ixodes ricinus* Linnaeus, named Iris, which was first described as an immunosuppressor but then as an inhibitor of blood proteases (Prevot et al., 2006). Considering this and the multiplicity of serine proteases in the coagulation system, it is conceivable that the Serpins expressed in salivary glands may act on the host blood.

A more orthodox example of the arthropod inhibitor acting on the blood host coagulation enzymes is the Kunitz family of serine protease inhibitors. These proteins have one or more domains of the bovine pancreatic trypsin inhibitor (BPTI), which is a characteristic of Kunitz-like structure (pfam00014.13). Amblin is a 151 amino acid-long thrombin inhibitor from the hemolymph of the *Amblyomma hebraeum* Koch tick (Lai et al., 2004). Other examples are the Ixolaris, a Factor Xa inhibitor with two Kunitz-like domains (Francischetti et al., 2002), and Boophilin, a thrombin inhibitor from the *Boophilus microplus* (Canestrini) tick (CAC82583).

The sequences of these Kunitz serine protease inhibitors were compared with the three deduced proteins found in our library (Fig. 5). ACAJ0015C is very similar to Boophilin and should represent its

Signal Peptide		
ACAJ0085C	(1)	MEKNIIVLLAVALTGPAVLAKPQGHP-----
BAD29729	(1)	MLKNIVLLLAAGLSGPAVLAKPKWSPLFEEA
Hirudin		-----
Repeat 1: present in both tick proteins.		
ACAJ0085C	(27)	HDALEARSDAVHTAVPKMPK-GGHGGFEPIPID-YD
BAD29729	(32)	TNALEARSGGHQTAVPKVSKQGLGGDFEEIPSDIIE
Hirudin		-----
Hirulog		GDFEEIP-EEYL
Repeat 2: present only in <i>A. variegatum</i> protein.		
ACAJ0085C		-----
BAD29729	(68)	KRALED RSGGHQTAVPKISKQGLGGDFEEIPSDIIE
Hirudin		-----
Hirulog		GDFEEIP-EEYL
Repeat 3: Both ticks; not well aligned with Hirudin.		
ACAJ0085C	(61)	ERALEARSDAVHTAVPKMPK-GGLGGFEPIPID-YD
BAD29729	(104)	ERALEARSDGHQTAVPKMSKQGLGGDIEEIPSDIIE
Hirudin	(1)	VYYTDC TESGQNLCLCEGSNVCGQGNKCILGSD---
Hirulog		GDFEEIP-EEYL
Repeat 4: Both ticks; best match to Hirudin C-term.		
ACAJ0085C	(95)	ERALEARSDAVHTAVPKMPK-GGHGGFEPIPID-YD
BAD29729	(140)	ERALEARSGGHQTAVPKMSKQGLGGDSEIIPSDIIE
Hirudin	(34)	GEKNQCVTG-EGTPKPQSHN---D GDFEEIP-EEYL
Hirulog		GDFEEIP-EEYL
Repeat 5: Both ticks; not well aligned with Hirudin.		
ACAJ0085C	(129)	ERALEARSDAVHTAVPKMPK-GGHGGFEPIPID-YD
BAD29729	(176)	ERALEARSGGHE TAVPKMSKQGLGGDFEEIPSDIIE
Hirudin	(65)	Q-----
Hirulog		GDFEEIP-EEYL
ACAJ0085C	(163)	ERALHA--
BAD29729	(212)	ERALQARG

Fig. 4. Alignment of the hirudin-like proteins from ticks *Amblyomma cajennense* (cluster ACAJ0085C—this work) and *Amblyomma variegatum* (GenBank BAD29729) with hirudin from the leech *Hirudo medicinalis* (GenBank 1002207A). The alignment was wrapped to fit the five repeats found. The C-terminal of hirulog was manually aligned to the C-terminal of the repeats. Dark background denotes better conservation and box indicates the predicted signal peptide.

A. cajennense version. The two others have more similarity to vertebrate inhibitors than to known hematophagous ones. ACAJ1219S codes for a 109 amino acid polypeptide corresponding to a single copy of the Kunitz domain that is similar to the tissue factor pathway inhibitor precursor (TFPI). ACAJ1187 has also a single Kunitz domain in the N-terminal half of the molecule that is similar to some trophoblast Kunitz domain protein precursor. However, its C-terminal has no similarity to inhibitors or to any other sequences.

Another kind of serine protease inhibitor found here is similar to the trypsin inhibitor like cysteine-rich domain (pfam01826.13). These inhibitors have been recently characterized in ticks and other hematophagous animals, and cluster ACAJ0463S is very similar to Ixodidin from *B. microplus* (Fig. 6), the best characterized of such peptides that was described functionally as a defensin (against several microbes), and biochemically as a protease inhibitor (chymotrypsin and elastase inhibitor) (Fogaca et al., 2006). Another cluster,

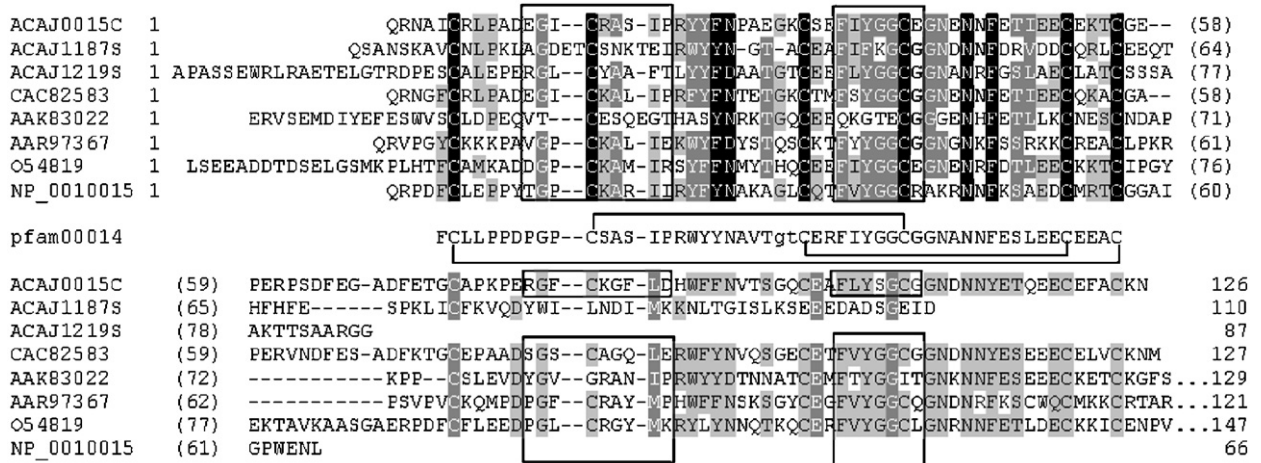


Fig. 5. Alignment of kunitz domain containing inhibitors. ACAJs: clusters from this work; CAC82583: boophilin from *B. microphilus* (tick); AAK83022: Ixolaris from *I. scapularis* (tick); AAR97367: thrombin inhibitor from *A. hebraeum* (tick); O54819: TFPI from *Mus musculus* and NP_0010015: BPTI from *Bos taurus*. The pfam00014 domain consensus is aligned between the two blocks of the alignments, with the disulfide arrangement indicated. Boxes indicate the protease recognition regions.

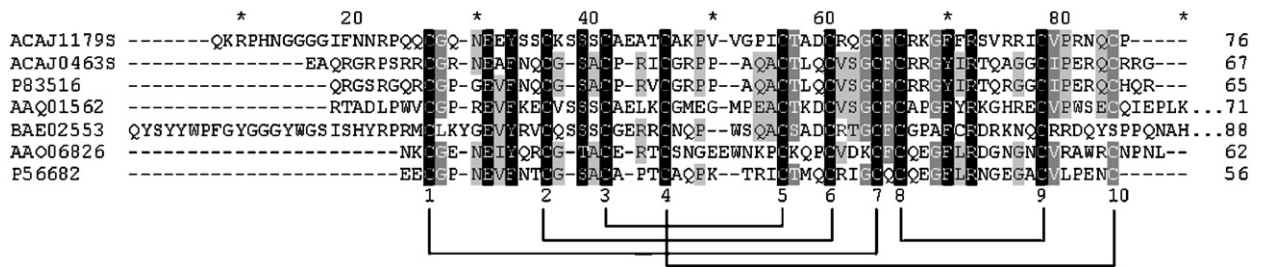


Fig. 6. Alignment of the predicted mature chymotrypsin/elastic inhibitors. ACAJs: clusters from this work; P83516: ixodidin from *B. microphilus* (tick); AAQ01562: von Willebrand factor from *I. ricinus* (tick); BAE02553: hypothetical ORF from *H. longicornis* (tick); AAQ06826: serine protease inhibitor from *Anopheles stephensi* (bug); P56682: chymotrypsin inhibitor from *Apis mellifera* (bee). The disulfide arrangement is indicated above the alignment.

ACAJ1179S, also shows this domain, but it is much less similar to the Ixodidin.

The cysteine protease inhibitors, known as cystatins, were isolated from a variety of microbes and tissues of animals and plants. In our cDNA library, there is a cluster (ACAJ0181C) that codes for a protein that is homologous to secreted cystatin 2 from the salivary glands of *I. scapularis* (data not shown). A recombinant protein from *H. longicornis* was shown to be active against papain and cathepsins L and B, suggesting its involvement in tick innate immunity (Zhou et al., 2006). Curiously, cathepsin L and B were also found in the library, as shown before.

Among the other families of protease inhibitors, three singlet clusters (ACAJ0514S; ACAJ0917S; ACAJ1035S) found in our cDNA library have

similarity (only around 30%) with the alpha-2-macroglobulin-like proteins from the soft tick *Ornithodoros moubata* (Murray) (Saravanan et al., 2003).

3.5. Other proteins possibly involved in tick feeding

Besides impairing blood coagulation and the proteolytic attack from host immune system, other mechanisms are required by hematophagous animals to deal with the host during feeding. One of them is the ability to manage host residues that can be toxic. Iron, a fundamental molecule from blood, would accumulate in tick digestive system due to the great amounts of hemoglobin they stock intracellularly in the gut, without excretion (Sonenshine, 1991). So, ferritin is an intracellular iron-carrying

molecule that may be supposed to sequester this metal in ticks (Kopacek et al., 2003). Cluster with 12 clones (ACAJ0147C) was identified as coding for a full-length ferritin, highly conserved to that from other ticks, including the absence of signal peptide.

Regarding microbial infections, ticks protect themselves through several defensive molecules. Defensins are small polypeptides (4 kDa) present in most invertebrates, generally in their hemolymph but also in the midgut, in response to several bacteria or after a blood meal (Fogaca et al., 2004; Nakajima et al., 2002). Two different defensins were detected, one (ACAJ0367S) is more similar to defensins from *A. hebraeum*, and the other (ACAJ0330S) is related to varisin A1 from *Dermacentor variabilis* (Say) (AAO24323) (data not shown). Also a singlet sequence (ACAJ0534S) similar to molecules such as microplusin and hebraein (AAR97292), a new class of antimicrobial peptides recently described, was found in this cDNA library (Fogaca et al., 2004).

Finally, other molecules that may regulate the host immune system were found. A tick IgE-dependent histamine releasing factor-like protein, possessing the TCTP domain, was found in three *A. cajennense* ESTs (ACAJ0078C). These proteins were shown to cause a release of histamine (Mulenga and Azad, 2005). Paradoxically, ticks are known to have other proteins that capture histamine, but they were not detected here. Another potent allergen, the venom allergen 3 or 5 from wasps and ants, was also found matching a singlet cluster (ACAJ0502S) showing around 35% identity over half of the molecule. These allergens are among the main toxins of insects (Hoffman, 1993) and were also correlated to a very extensive family of proteins, the Crisps, which includes snake venom toxins, but was not yet described in ticks. And at last, a tumor necrosis factor (TNF)-receptor-associated factor (TRAF) 6 was found also for the first time in ticks, in six of our clusters (e.g. ACAJ0136C and ACAJ0205C). TRAF is a broad action immunological regulator that acts as an adapter for the downstream signaling of TNF receptor, and the interleukin-1 receptor/Toll-like receptor superfamily (Wu and Arron, 2003).

4. Conclusion

In this work, we investigated the transcriptome of *A. cajennense* salivary glands, a common tick in Brazil with medical and veterinary importance due to its bite and pathogen transmission. From the

sample of 1754 clones, we could associate almost 70% with related sequences in databases. Proteases and protease inhibitors dominate the scene in *A. cajennense* salivary glands. Most of these components show features of endogenous or exogenous molecules active in blood coagulation cascade. Remarkably, five different types of possible serine protease inhibitors were found, including a molecule with a distinguished structure that contains repeats of the active motif of hirudin inhibitors. Besides, other components that may be active over the host immune system or act as defensins against infecting microorganisms were also described. Surprisingly, some expected transcripts coding, for instance, histamine-binding proteins, cement proteins, angiotensin-converting enzyme, besides others found in some tick transcriptomes (Ribeiro et al., 2006; Alarcon-Chaidez et al., 2007) were not observed among the ESTs obtained here.

Although many more studies on the activities of most of the molecules pointed here have to be done prior to the final assignment of their role, the real aim of this report is to provide a primary dataset of expressed sequences that may help to guide such investigations.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.toxicon.2007.12.011](https://doi.org/10.1016/j.toxicon.2007.12.011).

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