JOURNAL OF PROTEOMICS 74 (2011) 2892–2908

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An insight into the sialotranscriptome and proteome of the coarse bontlegged tick, *Hyalomma marginatum rufipes*

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ARTICLE INFO

Article history:
Received 2 May 2011
Accepted 13 July 2011
Available online 7 August 2011

Keywords:
Tick
Hematophagy
Salivary glands
Sialome

ABSTRACT

Ticks are mites specialized in acquiring blood from vertebrates as their sole source of food and are important disease vectors to humans and animals. Among the specializations required for this peculiar diet, ticks evolved a sophisticated salivary potion that can disarm their host's hemostasis, inflammation, and immune reactions. Previous transcriptome analysis of tick salivary proteins has revealed many new protein families indicative of fast evolution, possibly due to host immune pressure. The hard ticks (family Ixodidae) are further divided into two basal groups, of which the Metastriata have 11 genera. While salivary transcriptomes and proteomes have been described for some of these genera, no tick of the genus *Hyalomma* has been studied so far. The analysis of 2084 expressed sequence tags (EST) from a salivary gland cDNA library allowed an exploration of the proteome of this tick species by matching peptide ions derived from MS/MS experiments to this data set. We additionally compared these MS/MS derived peptide sequences against the proteins from the bovine host, finding many host proteins in the salivary glands of this tick. This annotated data set can assist the discovery of new targets for anti-tick vaccines as well as help to identify pharmacologically active proteins.

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1. Introduction

Ticks are specialized mites, divided into two large families, the Argasidae (soft ticks) and Ixodidae (hard ticks), and the monotypic Nuttalliellidae [1]. Soft ticks take relatively fast meals on their hosts, usually lasting less than 1 h, while hard ticks stay attached for days or weeks to their hosts. The Ixodidae are further subdivided into the basal Prostriata group, with the single genus *Ixodes*, and the Metastriata, with 11 recognized genera organized into 4 subfamilies [2].

Among the several adaptations to blood feeding, ticks evolved a complex saliva consisting of a mixture of pharmacologically active components that affects their host's hemostasis, inflammation, and immunity and also contains antimicrobial factors [3–8]. Perhaps due to their host's immune response, which could neutralize such activities,

Abbreviations: AMP, antimicrobial peptide; CDS, coding sequence; EST, expressed sequence tag; H class, housekeeping; NR, non-redundant; NCBI, National Center of Biological Information; S class, secreted; SG, salivary gland; TIL, trypsin inhibitor-like; TE, transposable element; U class, unknown function.

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1874-3919/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jprot.2011.07.015

these salivary proteins appear to evolve quickly, as indicated by the discovery of unique protein families among different tick genera and large sequence diversity within protein families that are common to all ticks, such as the lipocalin or Kunitz superfamilies [3]. Gene duplications are also common, leading to the existence of many multigene families within individual tick species, as exemplified by the Kunitz, lipocalin, basic tail, and ixodegrin families [3,9].

In the past 8 years, salivary transcriptomes, or sialomes (from the Greek *sialo*=saliva), have been described from several tick species, including the soft ticks *Argas monolakensis*[10,11], *Ornithodoros parkeri*[12] and *Ornithodoros coriaceus*[13]; the prostriates *Ixodes scapularis*[14,15], *Ixodes pacificus*[16], and *Ixodes ricinus* [17]; the metastriates *Amblyomma americanum*[18], *Amblyomma cajennense*[19], and *Amblyomma variegatum*[20] belonging to the metastriate Amblyomminae subfamily; and *Dermacentor andersoni*[21] and *Rhipicephalus appendiculattus*[22], members of the larger Rhipicephalinae subfamily. Within this last subfamily, the genera *Anomalohimalayia, Cosmiomma, Hyalomma, Margaropus, Nosomma*, and *Rhipicentor* remain unexplored.

To investigate the diversity of the sialome of a member of the *Hyalomma* genus, we analyzed the sialotranscriptome and sialoproteome of adult female *Hyalomma marginatum rufipes*, which is a common three-host tick found in Africa and Europe, and also a competent vector of Crimean Congo fever [23–27]. Immature stages *H. m. rufipes* feed on small vertebrates including mammals but mostly birds, while adults feed on large mammals, including cattle, from where our samples were obtained [28–32].

2. Material and methods

2.1. Ticks and SG preparation

Ticks were removed from zebu cows located on Point G in Bamako, Mali, in December 2008. The SGs were dissected by one of us (JMA) and transferred to RNAlater (Ambion, Austin, Texas, USA). The vials were kept at 4 °C for 24 h then stored at −30 °C until use. Tick carcasses were saved and analyzed by Dr. Dmitry A. Apanaskevich (Assistant Curator, U.S. National Tick Collection, Institute of Arthropodology and Parasitology, Georgia Southern University, Statesboro, Georgia, USA). They were all identified to be adult female specimens of *H. m.rufipes* Koch, 1844.

2.2. cDNA library construction and sequencing

H. m rufipes mRNA from one pair of SGs was isolated using the Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego, California, USA). The PCR-based cDNA library was made following the instructions for the SMART cDNA library construction kit (Clontech, Palo Alto, California, USA). This system utilizes oligoribonucleotide (SMART IV) to attach an identical sequence at the 5′ end of each reverse-transcribed cDNA strand. This sequence is then utilized in subsequent PCR reactions and restriction digests.

First-strand synthesis was carried out using PowerScript reverse transcriptase at 42 °C for 1 h in the presence of the SMART IV and CDS III (3′) primers. Second-strand synthesis was performed using a long distance (LD) PCR-based protocol, using Advantage™ Taq polymerase (Clontech) mix in the presence of the 5′ PCR primer and the CDS III (3′) primer. The cDNA synthesis procedure resulted in the creation of *SfiI A* and *B* restriction enzyme sites at the ends of the PCR products that are used for cloning into the phage vector. PCR conditions were as follows: 95 °C for 20 s; 24 cycles of 95 °C for 5 s, 68 °C for 6 min. A small portion of the cDNA obtained by PCR was analyzed on a 1.1% agarose gel to check quality and range of cDNA synthesized. Double-stranded cDNA was immediately treated with proteinase K (0.8 μg/ml) at 45 °C for 20 min, and the enzyme was removed by ultrafiltration though a Microcon YM-100 centrifugal filter device (Amicon Inc., Beverly, California, USA). The cleaned, double-stranded cDNA was then digested with *SfiI* at 50 °C for 2 h, followed by size fractionation on a ChromaSpin-400 column (Clontech). The profile of the fractions was checked on a 1.1% agarose gel, and fractions containing cDNAs of more than 400 bp were pooled and concentrated using a Microcon YM-100.

The cDNA mixture was ligated into the λ TriplEx2 vector (Clontech), and the resulting ligation mixture was packaged using the GigaPack® III Plus packaging extract (Stratagene, La Jolla, California, USA) according to the manufacturer's instructions. The packaged library was plated by infecting log-phase XL1-Blue *Escherichia coli* cells (Clontech). The percentage of recombinant clones was determined by blue-white selection screening on LB/$MgSO_4$ plates containing X-gal/IPTG. Recombinants were also determined by PCR, using vector primers (5′ λ TriplEx2 sequencing primer and 3′ λ TriplEx2 sequencing) flanking the inserted cDNA, with subsequent visualization of the products on a 1.1% agarose/EtBr gel.

The *H. m. rufipes* SG cDNA library was plated on LB/$MgSO_4$ plates containing X-gal/IPTG to an average of 250 plaques per 150-mm Petri plate. Recombinant (white) plaques were randomly selected and transferred to 96-well MICROTEST™ U-bottom plates (BD BioSciences, Franklin Lakes, New Jersey, USA) containing 100 μl of SM buffer [0.1 M NaCl; 0.01 M $MgSO_4$; 7 H_2O; 0.035 M Tris–HCl (pH 7.5); 0.01% gelatin] per well. The plates were covered and placed on a gyrating shaker for 30 min at room temperature. The phage suspension was either immediately used for PCR or stored at 4 °C for future use.

To amplify the cDNA using a PCR reaction, 4 μl of the phage sample was used as a template. The primers were sequences from the λ TriplEx2 vector and named pTEx2 5seq (5′-TCC GAG ATC TGG ACG AGC-3′) and pTEx2 3LD (5′-ATA CGA CTC ACT ATA GGG CGA ATT GGC-3′), positioned at the 5′ and the 3′ end of the cDNA insert, respectively. The reaction was carried out in 96-well flexible PCR plates (Fisher Scientific, Pittsburgh, Pennsylvania, USA) using the TaKaRa EX Taq polymerase (TAKARA Mirus Bio, Madison, Wisconsin, USA), on a Perkin Elmer GeneAmp® PCR system 9700 (Perkin Elmer Corp., Foster City, California, USA). The PCR conditions were: one hold of 95 °C for 3 min; 25 cycles of 95 °C for 1 min, 61 °C for 30 s; 72 °C for 6 min. Approximately 200–250 ng of each PCR product was transferred to Thermo-Fast 96-well PCR plates (ABgene Corp., Epsom, Surrey, UK) and frozen at −20 °C. Samples were shipped on dry ice to the Rocky Mountain Laboratories Genomics Unit with primer and template combined together in an ABI 96-well Optical Reaction Plate (P/N 4306737)

following the manufacturer's recommended concentrations. Sequencing reactions were set up as recommended by Applied Biosystems BigDye® Terminator v3.1 cycle sequencing kit by adding 1 μl ABI BigDye® Terminator ready reaction mix v3.1 (P/N 4336921), 3 μl 5× ABI sequencing buffer (P/N 4336699), and 2 μl of water for a final volume of 10 μl. Cycle sequencing was performed at 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min for 27 cycles on either a Bio-Rad Tetrad 2 (Bio-Rad Laboratories, Hercules, California, USA) or ABI 9700 (Applied Biosystems, Inc., Foster City, California, USA) thermal cycler. Fluorescently labeled extension products were purified following Applied Biosystems BigDye® XTerminator™ purification protocol and subsequently processed on an ABI 3730xL DNA Analyzer (Applied Biosystems, Inc.). The AB1 file generated for each sample from the 3730xL DNA analyzer was provided to researchers in Rockville, Maryland, USA, through a secure network drive for all subsequent downstream sequencing analysis. In addition to the sequencing of the cDNA clones, primer extension experiments were performed in selected clones to further extend sequence coverage.

2.3. *Bioinformatics tools used*

ESTs were trimmed of primer and vector sequences. The BLAST suite of programs [33], CAP3 assembler [34], and ClustalW [35] software were used to compare, assemble, and align sequences, respectively. For functional annotation of the transcripts, we used blastx [33] to compare the nucleotide sequences with the NR protein database of the NCBI and to the Gene Ontology database [36]. The program reverse position-specific BLAST (RPS-BLAST) [33] was used to search for conserved protein domains in the Pfam [37], SMART [38], Kog [39], and conserved domains databases [40]. We have also compared the transcripts with other subsets of mitochondrial and rRNA nucleotide sequences downloaded from NCBI and to several organism proteomes downloaded from NCBI, ENSEMBL, or VectorBase and to the assembled EST salivary database described before [3], and found in http://exon.niaid.nih.gov/transcriptome/tickreview/Sup-Table-1.xls from where the fasta set can also be recovered at http://exon.niaid.nih.gov/transcriptome/tick_review/tick_proteins_fasta.zip. Segments of the three-frame translations of the EST (because the libraries were unidirectional, six-frame translations were not used) starting with a methionine found in the first 300 predicted amino acids, or the predicted protein translation in the case of complete coding sequences, were submitted to the SignalP server [41] to help identify translation products that could be secreted. O-glycosylation sites on the proteins were predicted with the program NetOGlyc [42]. Functional annotation of the transcripts was based on all the comparisons above.

For sequence comparisons and phylogenetic analysis, we retrieved tick sequences from GenBank, and we have also deducted protein sequences from ESTs deposited in DBEST, as described and made accessible in a previous review article [3]. Phylogenetic analysis and statistical neighbor-joining bootstrap tests of the phylogenies were done with the Mega package [43] after sequence alignment performed by Clustal [44]. Codon volatility was calculated as previously described [45].

2.4. *Proteomic characterization using one-dimensional gel electrophoresis and tandem mass spectrometry (MS)*

The soluble protein fraction from salivary gland homogenates from *H. marginatum* corresponding to approximately 200 μg of protein was brought up in reducing Laemmli gel-loading buffer. The sample was boiled for 10 min and applied to two lanes (~50 and ~150 μg in each lane) and resolved on a NuPAGE 4–12% Bis–Tris precast gel. The separated proteins were visualized by staining with SimplyBlue (Invitrogen). The gel was sliced into 20 individual sections that were destained and digested overnight with trypsin at 37 °C. Peptides were extracted and desalted using ZipTips (Millipore, Bedford, MA) and resuspended in 0.1% TFA prior to S analysis.

Nanoflow reversed-phase liquid chromatography tandem MS (RPLS-MS/MS) was performed using an Agilent 1100 nanoflow LC system (Agilent technologies, Palo Alto, CA) coupled online with a linear ion-trap (LIT) mass spectrometer (LTQ, ThermoElectron, San José, CA). NanoRPLC columns were slurry-packed in-house with 5 μm, 300-Å pore size C-18 phage (Jupiter, Phenomenex, CA) in a 75-μm i.d.×10-cm fused silica capillary (Polymicro Technologies, Phoenix, AZ) with a flame-pulled tip. After sample injection, the column was washed for 30 min with 98% mobile phase A (0.1% formic acid in water) at 0.5 μL/min, and peptides were eluted using a linear gradient of 2% mobile phase B (0.1% formic acid in acetonitrile) to 42% mobile phase B in 40 min at 0.25 μL/min, then to 98% B for an additional 10 min. The LIT-mass spectrometer was operated in a data-dependent MS/MS mode in which each full MS scan was followed by seven MS/MS scans where the seven most abundant molecular ions were dynamically selected for collision-induced dissociation (CID) using a normalized collision energy of 35%. Dynamic exclusion was applied to minimize repeated selection of peptides previously selected for CID.

Tandem mass spectra were searched using SEQUEST on a 20-node Beowulf cluster against the *H. marginatum rufipes* described in this paper and the *Bos taurus* proteome (downloaded from ftp://ftp.ncbi.nih.gov/genomes/Bos_taurus/protein/) with methionine oxidation included as dynamic modification. Only tryptic peptides with up to two missed cleavage sites meeting a specific SEQUEST scoring criteria [delta correlation (ΔC_n)≥0.08 and charge-state-dependent cross correlation (X_{corr})≥1.9 for $[M+H]^{1+}$, ≥2.2 for $[M+2H]^{2+}$, and ≥3.5 for $[M+3H]^{3+}$] were considered as legitimate identifications. The peptides identified by MS were converted to Prosite block format [46] by a program written in Visual Basic. This database was used to search matches in the Fasta-formatted database of salivary proteins, using the program Seedtop, which is part of the Blast package. The result of the Seedtop search is piped into the hyperlinked spreadsheet to produce a text file as shown in supplemental Table S2. Notice that the ID lines indicate, for example, 18_73, which means that one match was found for fragment number 73 from gel band 18. Because the same tryptic fragment can be found in many gel bands, another program was written to count the number of fragments for each gel band, displaying a summarized result in an Excel table. The summary in this form of 11→18| 12→18| 13→2| indicates that 18 fragments were found in Fraction 11, while 18 and 2 peptides were found in

fractions 12 and 13, respectively. Furthermore, this summary included protein identification only when two or more peptide matches to the protein were obtained from the same gel slice.

3. Results and discussion

3.1. *Overview of the assembled salivary expressed sequence tag (EST) set*

A total of 2084 cDNA clones were used to assemble a database (Additional file 1 [Supplemental Table S1]) to yield 1167 clusters of related sequences, 993 of which contained only one EST. The 1167 clusters were compared, using the programs blastx, blastn, or RPS-BLAST [33], to the nonredundant (NR) protein database of the National Center of Biological Information (NCBI), National Library of Medicine, NIH, to a gene ontology database [36], to the conserved domains database of the NCBI [40], and to a custom-prepared subset of the NCBI nucleotide database containing either mitochondrial or rRNA sequences.

Manual annotation of the contigs resulted in four broad categories of expressed genes (Table 1 and Fig. 1). The putatively housekeeping (H) category contained 29% of the sequences and had on average 1.59 sequences per cluster, and the secreted (S) category had 42% of the ESTs with an average of 3.51 ESTs/clusters, while 28% of the ESTs, mostly singletons, were not classifiable, constituting the Unknown (U) group. The transcripts of the U class could represent novel proteins or derive from the less conserved 3′ or 5′ untranslated regions of genes, as was indicated for the sialotranscriptome of *Anopheles gambiae*[47]. Sequences deriving from transposable elements (TE) accounted for the remaining sequences, mostly singletons. TE-related sequences may indicate either the presence of active transposition in the tick, or more likely, the expression of sequences suppressing transposition. Low-level expression of TE sequences have been a relatively common finding in previous sialotranscriptomes.

3.1.1. *Housekeeping (H) genes*

The 594 ESTs attributed to H-class genes expressed in the salivary glands (SGs) of *H. m. rufipes* were further characterized into 20 subgroups according to function (Table 2 and Additional file 1 [Supplemental Table S1]). Transcripts associated with the protein synthesis machinery represented 44% of all transcripts associated within the H class, an expected result given the secretory nature of the organ. Energy metabolism accounted for the second most abundant H class, with 10.8% of the transcripts. Another 10.3% of the transcripts were classified as either "hypothetical conserved" or "conserved secreted" proteins. These represent highly conserved proteins of unknown function, presumably associated with cellular function but still uncharacterized. This functional distribution is typical of previous sialotranscriptomes.

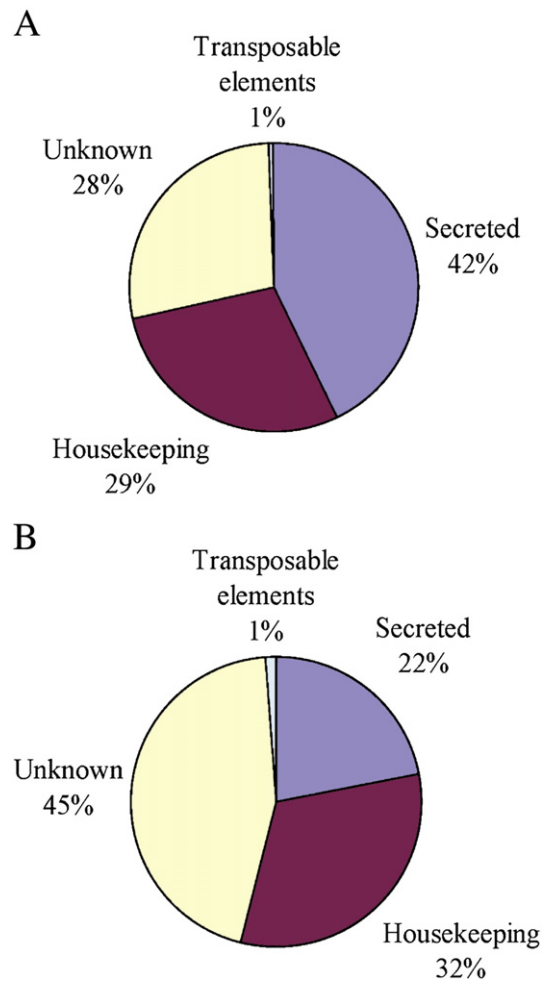

Fig. 1 – Distribution of expressed sequence tags (ESTs) and assembled contigs. Distribution of ESTs (A) and assembled contigs (B) obtained from a cDNA library from the salivary glands of adult female *Hyalomma marginatum rufipes* ticks.

3.1.2. *Possibly secreted (S) class of expressed genes*

A total of 894 ESTs contributed to 255 contigs and singletons associated with putative *H. m. rufipes* salivary-secreted components (Table 3 and Additional file 1). These include previously known gene families [48] such as metalloproteases, lipocalins, protease inhibitor domain-containing peptides,

Table 1 – Main classes of transcripts found in the cDNA library of *Hyalomma marginatum rufipes*.

Class	Number of contigs	Number of ESTs	ESTs/contigs
Secreted	255	894	3.51
Housekeeping	374	594	1.59
Unknown	526	583	1.11
Transposable elements	12	13	1.08
Total	1167	2084	

Table 2 – Housekeeping classes of transcripts found in the cDNA library of *Hyalomma marginatum rufipes*.

Class	Number of contigs	Number of ESTs	ESTs/Contig	Percent
Protein synthesis machinery	123	262	2.13	44.11
Metabolism, energy	50	64	1.28	10.77
Unknown, conserved	48	61	1.27	10.27
Signal transduction	16	44	2.75	7.41
Protein modification machinery	32	40	1.25	6.73
Transcription machinery	29	36	1.24	6.06
Protein export machinery	17	17	1.00	2.86
Cytoskeletal	10	17	1.70	2.86
Nuclear regulation	10	11	1.10	1.85
Proteasome machinery	9	10	1.11	1.68
Oxidant metabolism/detoxification	5	5	1.00	0.84
Metabolism, amino acid	5	5	1.00	0.84
Transporters/storage	4	4	1.00	0.67
Metabolism, carbohydrate	4	4	1.00	0.67
Metabolism, nucleotide	3	4	1.33	0.67
Metabolism, lipid	4	4	1.00	0.67
Transcription factor	1	2	2.00	0.34
Metabolism, intermediate	2	2	1.00	0.34
Extracellular matrix/cell adhesion	1	1	1.00	0.17
Immunity	1	1	1.00	0.17
Total	374	594		

immunomodulators, antimicrobial peptides, basic tail, and glycine-rich peptides. Several other deducted sequences code for proteins that have some sequence similarity to other known proteins or to proteins not previously described in tick sialotranscriptomes.

3.2. *Detailed analysis of the sialome of H. m. rufipes*

From the sequenced cDNAs, a total of 249 protein sequences were derived, 101 of which code for putative secreted products (Additional file 2 [Supplemental Table S2]). This set of 101

Table 3 – Secreted classes of transcripts found in the cDNA library of *Hyalomma marginatum rufipes*.

Subclass	Number of ESTs
Protease inhibitors	
Kazal domain	2
Kunitz domain	15
Madainin family	32
TIL domain	2
Basic tail family found in argasids and ixodids	11
Lipocalins	18
RGD domain family (may not contain RGD)	1
Glycine-rich proteins	
Large GGY protein	73
Cuticle Ala-rich family	1
Metastriate spider silk-like	56
Glycine-rich protein group GYG	3
Glycine-rich similar to RNA binding protein	3
Other glycine-rich proteins	81
Mucins	12
Putative proline-rich polypeptides	4
28-kDa metastriate family	1
8.9-kDa family	6
Immunity-related products	27
DAP-36 fragment	1
Dermacentor 9-kDa family	400
Enzymes	
Metalloproteases	3
Ribonucleases	2
Metastriate insulin growth factor binding protein	1
Conserved secreted protein	1
Similar to previously orphan proteins	23
Other possibly secreted	115
Total	894

proteins includes 74 that are presumably full length, while the remaining 27 are truncated. With this transcriptome-derived protein database, we characterized the tick salivary proteome via analysis of salivary gland homogenates fractionated by electrophoresis on SDS-polyacrylamide gels, bands of which were tryptic digested, fractionated by reversed phase chromatography, followed by in line electro spray into a mass spectrometer for tandem mass spectrometry analysis (Fig. 2). Follows a description of the protein families deducted from the transcriptome analysis, with information of the proteomic experiment as summarized in Table 4 and supplemental files 1 and 2.

3.3. *Putative secreted proteins with presumed or experimentally validated function*

3.3.1. *Metalloprotease domains*

Transcripts coding for metalloproteases have been commonly found in tick sialotranscriptomes [3,10,49], and these may be associated with fibrinogenolytic activity as previously found in *I. scapularis*[50]. HEX-267 is a truncated sequence coding for the carboxy terminal region of a metalloprotease sequence from *Haemaphysalis* found in GenBank, with only 25% similarity over 228 residues but with 68% identity to a homologue deducted from ESTs from *Rhipicephalus microplus*. It also displays the CDD domain for arthropod metalloproteases.

HEX-920 codes for a 5′ truncated endonuclease that may or may not be secreted in saliva. Although not found in *Ixodes* sialotranscriptomes, these types of transcripts have been found in transcriptomes of *Rhipicephalus* and *Amblyomma*, all with a signal peptide indicative of secretion [3]. DNAse activity has not been described in tick saliva but is present in saliva of mosquitoes of the *Culex* genus [51], where it may work in concert with hyaluronidases to decrease the viscosity of the extracellular matrix and help the formation of the feeding lesion. Endonuclease transcripts are also commonly found in sand fly and tsetse sialotranscriptomes [52,53].

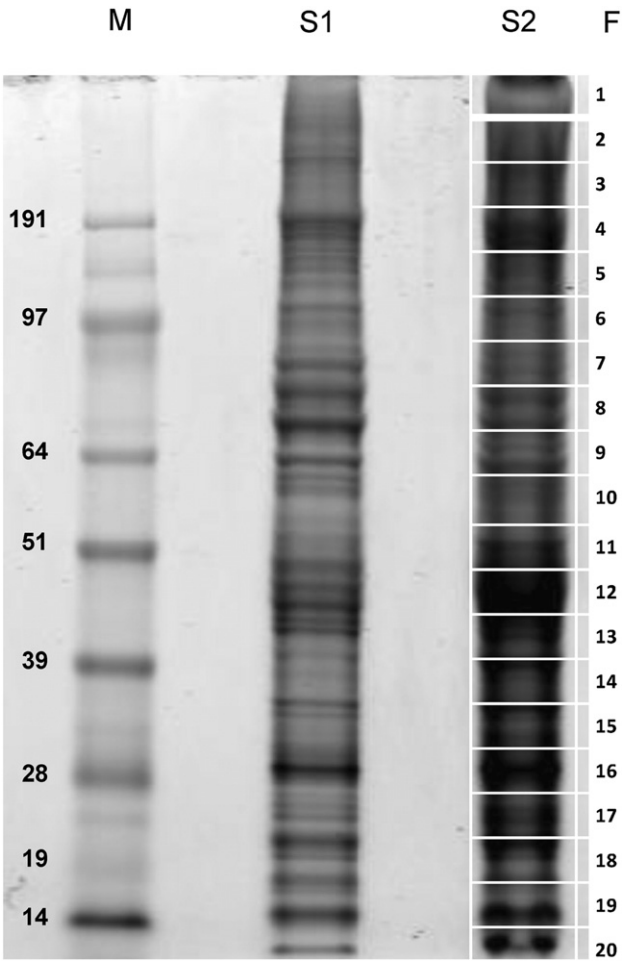

Fig. 2 – 1D gel electrophoresis of *Hyalomma marginatum rufipes* salivary gland homogenates. The numbers at the left indicate the MW in kDA of the protein standards shown in the left lane labeled M. The right gel lane (S2) shows the separation of ~150 μg salivary gland proteins. The grid at the right (1–20) represents the gel slices submitted for tryptic digest and MS/MS identification. S1 depicts a lane with less protein (50 μg) than S2.

3.3.2. *Protease inhibitor-containing domains*

A total of 62 ESTs from the sialotranscriptome of *H. m. rufipes* code for proteins containing signatures of proteins previously associated with a protease inhibitory function, which are either ubiquitous or particular to ticks. A more detailed analysis of these transcripts follows.

3.3.2.1. Kunitz domain containing proteins. The Kunitz domain acquired its name from the Kunitz pancreatic trypsin inhibitor, also known as aprotinin, and later found to be ubiquitous [54]. Tick sialotranscriptomes [3], as well as those of the hematophagous flies of the genera *Culicoides*[55,56] and *Simulium*[57], abound with transcripts coding for members of this family. Proteins containing single or multiple Kunitz domain were described in ticks, where Ixolaris, a double Kunitz protein, and Pentalaris, containing five domains, have been functionally characterized [58–62]. The Kunitz fold can also perform functions beyond protease inhibition, such as ion channel inhibition [63–66]; indeed, recently a modified Kunitz domain peptide from *R. appendiculatus*[67] was shown to activate maxiK channels in an *in vitro* system, suggesting a vasodilator function. Fifteen ESTs were found in the *H. m. rufipes* sialotranscriptome coding for members of the Kunitz family, allowing the deduction of two full-length coding sequences (CDS), one (Hex-1093) from a single and the other (Hex-13) from a double Kunitz family. Both polypeptides have less than 50% identity to their closest matches to the NR and to the assembled dataset described in Francischetti, *et al.* [3].

3.3.2.2. TIL domain containing proteins. The TIL (for trypsin inhibitor-like) domain typically contain ten cysteines forming five disulphide bonds and is found in many protease inhibitors. It belongs to the family I8 of the MEROPS database [68]. These polypeptides may also exert antimicrobial function [69]. Members of this family have been found ubiquitously in blood-feeding insect and tick sialomes, but very few have been characterized. A tick hemolymph anti microbial peptide (AMP) was previously reported to be a member of this family [70]. More recently, tick proteins containing TIL domains were characterized from *R. microplus* as subtilisin inhibitors with antimicrobial activity and expressed in various tick organs,

Table 4 – Tick proteins identified in the polyacrylamide gel shown in Fig. 2.

Sequence name	Description	Fraction number→number of ions	Coverage in aa residues [a]	Percent coverage [a]
HEX-706	Ubiquitin	1→12–2→12–3→12|	144	52.4
HEX-879	40S Ribosomal protein S27	1→4–2→4|	38	45.2
HEX-1118	Ubiquitin/ribosomal protein S27a fusion protein	1→6–2→6–10→6–17→6|	54	37.5
HEX-1165	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 1	6→18|	292	109.0
HEX-1043	Putative cement protein RIM36	8→10–6→8–18→8|	248	306.2
HEX-1057	Hypothetical glycine rich secreted cement protein	8→26–9→20–17→16|	510	314.8
HEX-235	Putative cement protein	8→32–9→24–10→16|	750	230.8
HEX-1097	Apoptosis-promoting RNA-binding protein TIA-1/TIAR	9→18–8→10|	258	80.9
HEX-1107	Mitochondrial chaperonin, Cpn60/Hsp60p	10→8–11→4|	104	46.2
HEX-153	Protein disulfide isomerase	12→10–13→4|	154	46.2
HEX-18	Aldehyde dehydrogenase, putative	12→18–11→4|	220	80.9
HEX-779	Alpha tubulin	12→52–13→26–11→16|	776	236.6
HEX-911	Putative mitochondrial processing peptidase beta-subunit	13→14–12→8–16→4|	182	152.9
HEX-296	40S Ribosomal protein SA (P40)/Laminin receptor 1	13→14–15→6–14→4|	206	86.2
HEX-1089	Ribosomal protein L3, putative	13→8–15→4|	150	56.8
HEX-750	Hypothetical glycine rich secreted protein	15→2|	42	33.6
HEX-1117	Hypothetical glycine rich secreted protein	15→2|	42	22.0
HEX-11	Mitochondrial malate dehydrogenase	15→20–16→8–17→4|	264	201.5
HEX-910	60S Ribosomal protein L6	15→24–16→8|	318	152.9
HEX-350	Guanine nucleotide-binding protein	15→32–16→14–17→4|	448	182.9
HEX-702	60S acidic ribosomal protein P0	15→6|	144	133.3
HEX-115	RNA-binding protein musashi/mRNA cleavage and polyadenylation factor I complex, subunit HRP1	15→8–14→6|	102	49.0
HEX-497	Tick lipocalin superfamily member — Subgroup B	16→14|	172	98.9
HEX-180	Ribosomal protein L8	16→14–17→2|	202	120.2
HEX-186	60S ribosomal protein L23	16→18–17→8|	280	142.9
HEX-1021	ADP/ATP translocase, putative	16→18–2→14–3→12|	296	216.1
HEX-215	40S ribosomal protein S2/30S ribosomal protein S5	16→20–7→2|	342	133.6
HEX-743	40S ribosomal protein S6	16→22|	250	151.5
HEX-549	40S ribosomal protein S3	16→26–15→6–17→6|	324	180.0
HEX-550	Hypothetical secreted protein with basic head	16→3–1→2|	36	40.9
HEX-1143	Putative cement protein	16→6|	48	15.4
HEX-986	Cathepsin L-like cysteine proteinase B	16→6|	86	56.6
HEX-240	Putative cement protein	16→7|	105	52.2
HEX-737	Eukaryotic translation initiation factor 3 subunit 5 epsilon-like	16→8|	128	77.6
HEX-1155	Ribosomal protein L7-like	16→8|	96	90.6
HEX-225	Multifunctional chaperone (14-3-3 family)	17→14–16→10–18→4|	140	69.0
HEX-866	60S Ribosomal protein L13	17→14–19→4|	150	76.5
HEX-619	60S Ribosomal protein L10	17→16–18→6|	166	102.5
HEX-870	40S Ribosomal protein S8	17→20–18→4–19→4|	264	126.9
HEX-892	Glutathione S-transferase	17→67–18→54–19→6|	886	579.1
HEX-423	RNA binding motif-containing protein, putative	17→8|	118	100.0
HEX-975	Mitochondrial F1F0-ATP synthase, subunit OSCP/ATP5	18→14|	204	167.2
HEX-710	60S Ribosomal protein L14	18→16|	162	114.1
HEX-1007	Hypothetical secreted protein with 3 TIL domains	18→2|	20	7.2
HEX-739	NADH-ubiquinone oxidoreductase, NUFS7/PSST/20 kDa subunit	18→24|	372	204.4
HEX-1158	40S Ribosomal protein S7	18→26–17→12–19→4|	385	233.3
HEX-455	Ribosomal protein S4	18→27–19→22–17→18|	265	175.5
HEX-980	Histamine release factor	18→4|	40	26.5
HEX-366	Proteasome (prosome, macropain) subunit, beta type 2	18→6|	94	62.3
HEX-1069	Gly-Tyr rich salivary protein	18→6|	82	38.5
HEX-1036	Ribosomal protein L35a	18→8–19→8|	64	49.6
HEX-896	Lysozyme	19→10–20→5|	144	142.6
HEX-766	Ribosomal protein S19, putative	19→10–20→6|	104	86.0
HEX-68	Ribosomal protein L15	19→12|	122	62.2
HEX-881	40S Ribosomal protein S14, putative	19→12–20→8|	176	144.3
HEX-1090	Ribosomal protein S17	19→14|	264	266.7
HEX-608	Ribosomal protein L28, putative	19→14–18→6|	142	103.6
HEX-751	60S Ribosomal protein L27, putative	19→16–18→4|	176	128.5
HEX-473	Ribosomal protein L31	19→16–20→8|	306	248.8
HEX-909	Translation initiation factor 5A	19→18|	212	146.2
HEX-356	60S Ribosomal protein L32	19→34–18→4|	408	304.5
HEX-1038	Hypothetical secreted peptide precursor	19→4–20→4|	42	49.4

Table 4 *(continued)*

Sequence name	Description	Fraction number→number of ions	Coverage in aa residues [a]	Percent coverage [a]
HEX-244	Ribosomal protein L27A, putative	19→6|	62	52.1
HEX-1139	Translation machinery-associated protein, putative	19→6–18→4|	78	42.9
HEX-392	Ribosomal protein S16	19→8–20→8|	78	70.9
HEX-122	Histone H4, putative	20→31–19→4|	322	322.0
HEX-182	Ribosomal protein S12	20→4|	60	48.4
HEX-247	60S Acidic ribosomal protein P1	20→4|	48	39.3
HEX-600	Cytochrome B5, putative	20→4|	50	58.8
HEX-867	Cytochrome C oxidase subunit VIc	20→4|	56	77.8
HEX-44	Putative superoxide dismutase Cu–Zn	20→6–19→4|	84	54.5
HEX-650	Cytochrome c oxidase polypeptide IV	20→8–19→6|	98	55.1

[a] On fraction with higher number of ions only. Includes redundant ions. Coverage may be greater than 100%.

including the SGs [71]. Hex-1007 is an interesting member of this family, as having 3 TIL domains in tandem starting at positions 91, 147, and 209. It has over 58% identity to proteins deducted from tick sialome ESTs and proteins from *Amblyomma* deposited on GenBank. Two ions matching the Hex-1007 sequence were obtained by MS/MS from gel fraction 18 (Fig. 2, Table 4). This region of the gel is near the 19 kDa marker, a smaller MW than the predicted 28 kDa for this protein. However, it is common for proteins containing many disulfide bonds to appear more compact and thus move faster when submitted to electrophoresis. Alternatively, this protein may be processed into shorter peptides.

Hex-55 is a shorter peptide that actually does not show a typical TIL domain, yet produces weak matches to proteins that are typical members of the family.

3.3.2.3. Basic tail protein family. This protein family was so named due to a stretch of lysine residues in the carboxy terminus region of an expanded family of salivary proteins found in the *I. scapularis* sialome [14,15]. They are unique to ticks [3] and can be identified by the PFAM domain PF07771, although many members are so divergent that they do not register it. Many members also have the conserved block C-x (13,21)-Y-Y-C-x(16,19)-C. Some members of this family have been characterized in *I. scapularis* as anti-clotting [72], thus their inclusion in this section. Hex-449 is a typical member of the family, having the characteristic PFAM domain and the YF-YF block. Its closest known relative is a protein reconstructed from *D. andersoni* ESTs, to which it has 37% identity and 50% similarity. Hex-390 presents 72% identity to a salivary protein from *Hyalomma asiaticum* named P18, and to basic tail proteins from *Ixodes* and *Ornithodoros*. It does not have the characteristic PFAM domain, but has the YF-YF signature, as well as a poly lysine tail. Hex-238 appears to be a very divergent member, presenting weak similarity to the P18 protein when compared to the NR database. Alignment of the *H. m. rufipes* sequences with those of other ticks suggests that Hex-238 may be a truncated member of the family or a protein resulting from a missing exon (Fig. 3A). The phylogenetic tree shows that HEX-449 is a canonical basic tail protein within clade I as shown in Fig. 3B. This clade has strong bootstrap support, indicating a common origin for this protein family in metastriates and prostriates. Clade II, however, does not group with the remaining proteins and does not have strong bootstrap support, suggesting it may derive from a different ancestor, or more likely have evolved beyond recognition of a common ancestor. Future inclusion of novel members of this family may lead to the merging of these clades.

3.3.2.4. Madanin thrombin inhibitor family. Madanins was the name given for related small polypeptides (~6 kDa) isolated from the tick *Haemaphysalis longicornis* that possess anti-thrombin activity [73]. Later another peptide, named chimadanin, was isolated from the same tick [74]. Additionally, variegin[75] was isolated from *A. variegatum* as a novel anti-thrombin peptide. Recently, these peptides were suggested to be part of a exclusive metastriate superfamily [3]. The transcriptome of *H. m. rufipes* indicates the presence of at least four genes that are possibly polymorphic. Alignment of the *Ha. longicornis* sequences with those of *H. m. rufipes* and deducted sequences from *Dermacentor* indicates three regions on these peptides: the signal peptide region, a second region with predominantly negatively charged peptides, and a third proline/serine/threonine-enriched region (marked as 1, 2 and 3 on Fig. 4A). The phylogram provides strong bootstrap support for a common origin between *H. m. rufipes* and *D. andersoni* sequences, both members of the Rhipicephalinae subfamily (marked with I in Fig. 4A), while the *Haemaphysalis* madanins are so divergent as to constitute a separate clade (marked II in Fig. 4B), with the chimadanin (HAELO 67968373) possibly being a link between the two clades. Additional sequencing within the Rhipicephalinae and Haemaphysaline subfamilies may uncover more detailed phylogenetic relationships of these proteins. Notice that these mature peptides are small, with near 60 amino acids, and contain no cysteines, making them relatively straightforward for direct chemical synthesis. The anti-thrombin function of these peptides in *Hyalomma* and *Dermacentor* remains to be confirmed.

3.3.3. *Lipocalin family*

The lipocalin family is extremely diverse in ticks, where it serves multiple functions, as chelators of agonists (kratagonists) of hemostasis and inflammation, and other unrelated functions, such as anti-complement [3]. A previous review

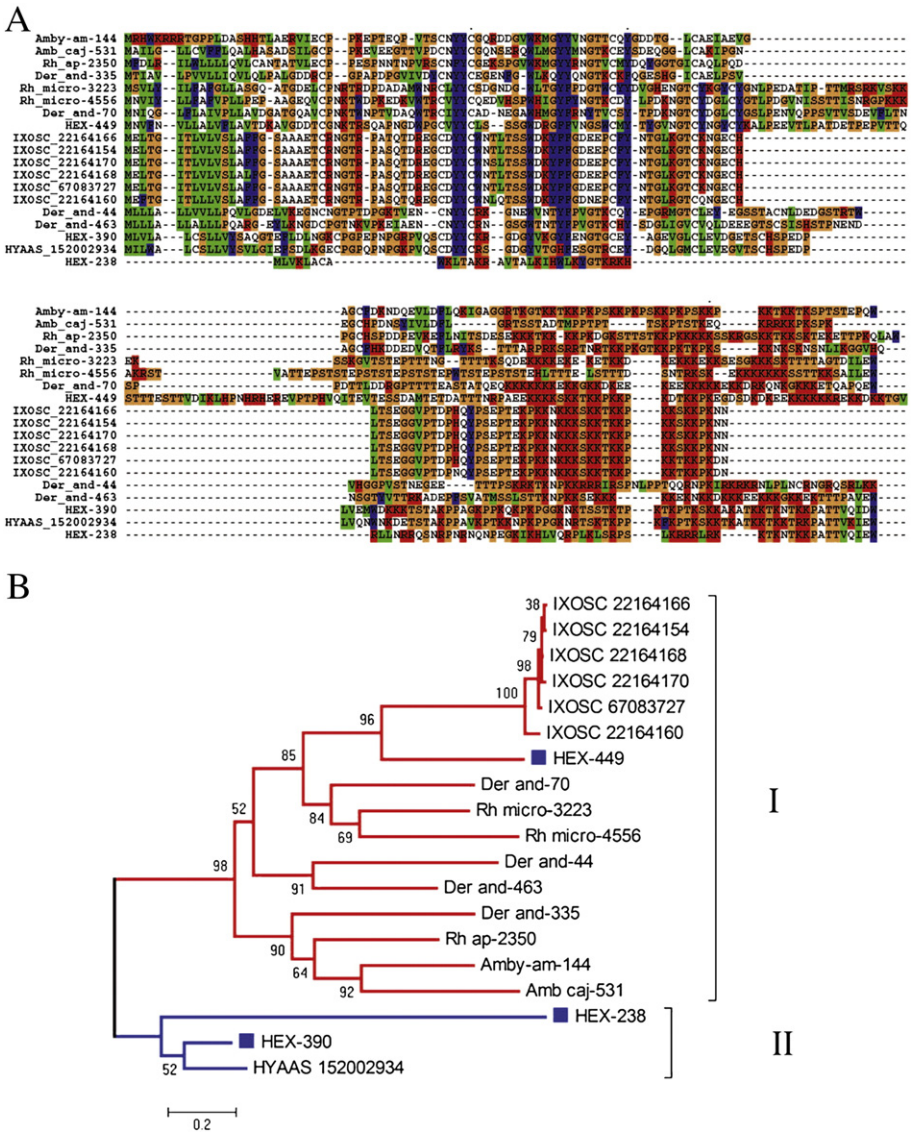

Fig. 3 – The basic tail proteins of *Hyalomma marginatum rufipes* and other ticks. A Clustal alignment. B Phylogenetic tree deducted from the alignment in A after 10,000 bootstraps. The number on the branches indicates percentage bootstrap support. The *Hyalomma marginatum rufipes* sequences are indicated by HEX and a square mark. The names initiating by IXOSC are from *Ixodes scapularis*, and the numbers are NCBI accession codes. HYAAS indicate a protein from *H. asiaticum* followed by its NCBI accession code. The remaining proteins derive from deducted expressed sequence tags available at DBEST and were described in a previous review [3]. The bar at the bottom indicates 20% amino acid divergence. For more detail, see text.

characterized 301 tick salivary lipocalins into 10 major groups [3]. The sialotranscriptome of *H. m. rufipes* yielded 18 ESTs that are similar to previously described tick lipocalins (Table 3). From these ESTs, five lipocalins can be derived. Two of these lipocalins, HEX-614, which is 22% identical and 41% similar to an *A. americanum* salivary protein, and less so to other tick lipocalins and HEX-938, which is most probably a splice variant of the same gene coding for HEX-614, are very divergent, forming a clade of their own with the *Amblyomma* proteins. HEX-133 produces a best match to another *Amblyomma* protein, previously classified as the Metastriate specific group III [3]. HEX-497 matches an *R. microplus* lipocalin with only 34% identity and belongs to Group I, subgroup B of lipocalins. HEX-497 appears abundantly expressed as indicated by the finding of 14 MS/MS ions producing a coverage of 99% of the protein found in gel band 16, near the 28 kDa marker. Finally, HEX-43 matches a *Ha. longicornis* protein at 29% identity, belonging to the Group IIa of lipocalins. The specific functions of any of these lipocalins remain to be identified.

3.3.4. *Glycine-rich family*

Glycine-rich protein is a generic name encompassing a diverse group of proteins, including short and long proteins. Some of these have many GY repeats that are found in small antimicrobial peptides [76] but may be also found in cuticle proteins, where the tyrosine residue may be involved in crosslinking

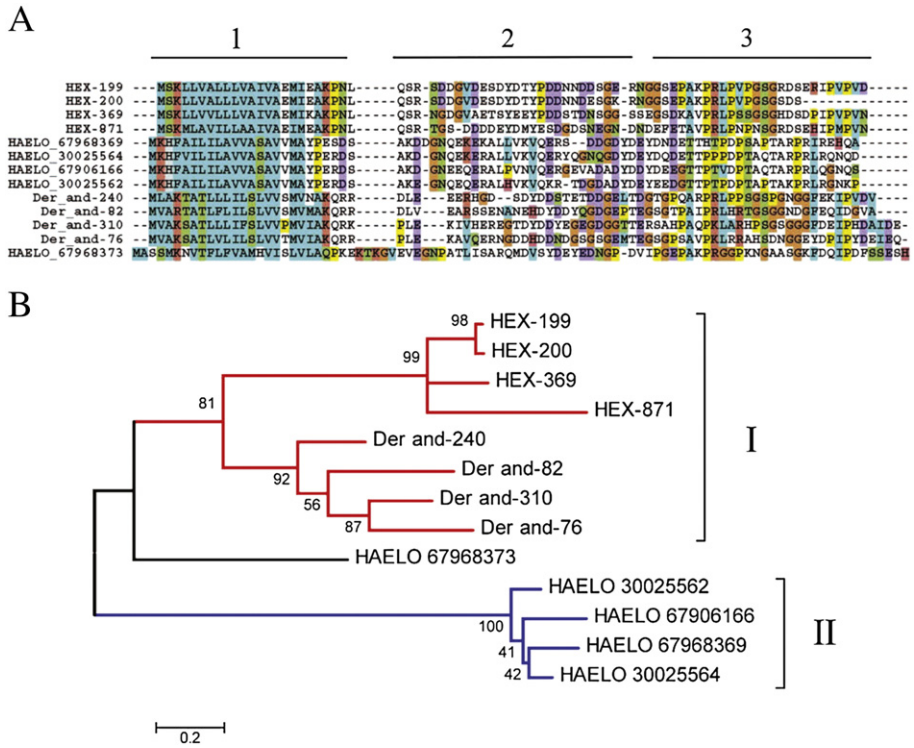

Fig. 4 – The Madanin family of metastriate ticks. *A* Clustal alignment. *B* Phylogenetic tree deducted from the alignment in *A* after 10,000 bootstraps. The number on the branches indicates percentage bootstrap support. The *Hyalomma marginatum rufipes* sequences are indicated by HEX. The names initiating by HAELO are from *Haemaphysalis longicornis*, and the numbers are NCBI accession codes. The remaining proteins derive from deducted expressed sequence tags from *Dermacentor andersoni* available at DBEST and were described in a previous review [3]. The bar at the bottom indicates 20% amino acid divergence. For more detail, see text.

reactions. Very long glycine-rich proteins are found in metastriate ticks, are similar to spider-silk proteins, and may function as cement proteins to attach the tick mouthparts to their hosts [77,78]. A total of 217 ESTs from the *H. m. rufipes* sialotranscriptome was classified as possibly coding for glycine-rich proteins (Table 3), from which 21 CDS were derived. Some of these coding sequences derive from abundantly expressed transcripts such as HEX-1069, a protein containing GY repeats deriving from 30 ESTs, and a homologue of the protein annotated as cement protein 64P-BA1 from *R. appendiculatus*. HEX-1069, as well as HEX-1143 and HEX-20 were identified by MS/MS in the gel shown in Fig. 2 with 6 to 7 ions each at gel fractions 18 (HEX-1069) and 16 (HEX-1143 and HEX-20). HEX-235 is also abundantly expressed, with 42 ESTs, and is similar to the *R. appendiculatus* protein annotated as putative cement protein RIM36 and found to produce strong antibody response in cattle [79]. It was identified in the proteome experiment (Fig. 2 and Table 4) at fraction 8, a region of the gel between the markers for 97 and 64 kDa. The glycine rich proteins HEX-1057 and HEX-1043 were also identified in the same gel fraction, and those coded by HEX-750 and HEX-1117 were found in band 15, between the 28 and 39 kDa markers.

3.3.5. *Mucins*

Mucins are serine- and/or threonine-rich proteins, usually of low complexity, and having the motifs for being linked to N-acetyl-galactosamine residues [42]. Some of these invertebrate mucins also contain chitin-binding domains, suggesting they may coat the feeding channels of blood-sucking arthropods. Transcripts coding for mucins are commonly found in sialotranscriptomes of blood-sucking arthropods. HEX-930 and HEX-264 are probable alleles, deriving from four and three ESTs, respectively. They have a chitin-binding domain and are 50% identical to a *Haemaphysalis* protein annotated as a mucin.

HEX-826 represents the sequence of a threonine-rich protein with seven predicted galactosylation sites, a mature MW of 7.8 kDa, and is similar to a *D. andersoni* protein deducted from salivary ESTs but not to other protein in the NR database.

3.3.6. *Immunity-related products*

Twenty-seven ESTs in the *H. m. rufipes* sialotranscriptome code for proteins assigned to an immunity function (Table 3). Coding sequence for a peptidoglycan recognition protein, an ixoderin/ficolin, also involved in microbial pattern recognition and possibly associated with the activation of the invertebrate complement system [80], and a typical lysozyme were deduced from these ESTs. This lysozyme (coded by HEX-896) was identified in Fig. 2 gel fraction 19 in a region of the gel consistent with its expected MW.

3.4. *Putative secreted proteins with unknown function*

3.4.1. *8.9-kDa family*

We previously characterized 60 tick salivary proteins as members of the uniquely Ixodidae protein family named

8.9-kDa family, of unknown function. The *H. m. rufipes* sialotranscriptome provides evidence for five members of this family. Alignment of these proteins with their relatives (Fig. 5A) allows for detection of a conserved framework of cysteines, including a double Cys–Cys in their carboxy terminals and a few other conserved residues (Fig. 5B and 5C) indicative of a fast divergence of these proteins from a common ancestor. The 8.9 kDa protein coded by HEX-1038 was identified in gel fractions 18, 19 and 20 (Fig. 2 and Table 4).

3.4.2. *Dermacentor-Hyalomma specific 9-kDa family*

We have previously characterized a *D. andersoni*-specific family, based on five protein sequences, named *Dermacentor*-specific 9-kDa expansion, due to their inability to significantly match any protein in the NR database, but being related among themselves, indicating gene duplications in *D. andersoni* followed by fast divergence. Somewhat surprisingly, the sialotranscriptome of *H. m. rufipes* produced 400 ESTs coding for members of this unique family, from which 14 coding sequences were derived. This is the most abundantly expressed family in *H. m. rufipes*. The coding sequences match various *D. andersoni* proteins from 36 to 49% identity, over nearly 100% of their lengths. These 14 proteins are possibly the product of at least 4 genes, two of which may be polymorphic, one producing the proteins HEX-902, HEX-272, HEX-275, HEX-277, HEX-274, and HEX-273, and the other producing proteins HEX-874, HEX-353, HEX-300, HEX-303, and HEX-176, as these proteins are within 10% identity from each other. The remaining proteins, HEX-1077 and HEX-1038, appear to derive from different genes. Alignment of the *Hyalomma* with the *Dermacentor* proteins (Fig. 6A) shows a framework of conserved cysteine residues (Fig. 6B) as well as two leucines and a serine residue. Phylogenetic analysis reveals two main mono-specific clades (marked I and II on Fig. 6C) consisting of *Hyalomma* and *Dermacentor* proteins.

3.5. *Hyalomma proteins similar to previously described orphan tick proteins*

We have previously cataloged 917 tick proteins within 19 protein families as orphans, because they did not produce significant matches to proteins outside their own original genus [3]. The sialotranscriptome of *H. m. rufipes* contains transcripts that allow us to "de-orphanize" a few of these families, as follows: HEX-434 is similar to a *R. microplus* protein, while HEX-421 is similar to a monospecific family within *A. americanum*, within which they share a common framework of six cysteines, three glycines, and two additional sites with hydrophobic amino acids (Fig. 7). Finally, a group of *Hyalomma* proteins with a common polylysine stretch in their mid-region, thus named "basic belly" proteins, matches an *Ornithodoros* protein. The basic belly protein coded by HEX-550 was identified in the gel band 16 (Fig. 2 and Table 4), near the 28 kDa marker. However, HEX-550 has a predicted MW of 7.8 kDa. Although many of the basic belly proteins have a signal peptide, the deducted polylysine stretch is coded by a polyA region, suggesting that these CDS could be artifacts derived from a 3′ untranslated region.

3.6. *Orphan Hyalomma proteins*

Additional file 2 presents 22 protein sequences coding for secreted products without any significant similarities to known proteins. Most of these putative polypeptides are small, and their CDS could derive from the 3′ region of truncated transporters that produce intramembrane helices that are interpreted as signal peptides.

3.7. *Housekeeping proteins and transposable elements*

The EST set acquired in this study allowed for the description of 144 coding sequences associated with housekeeping

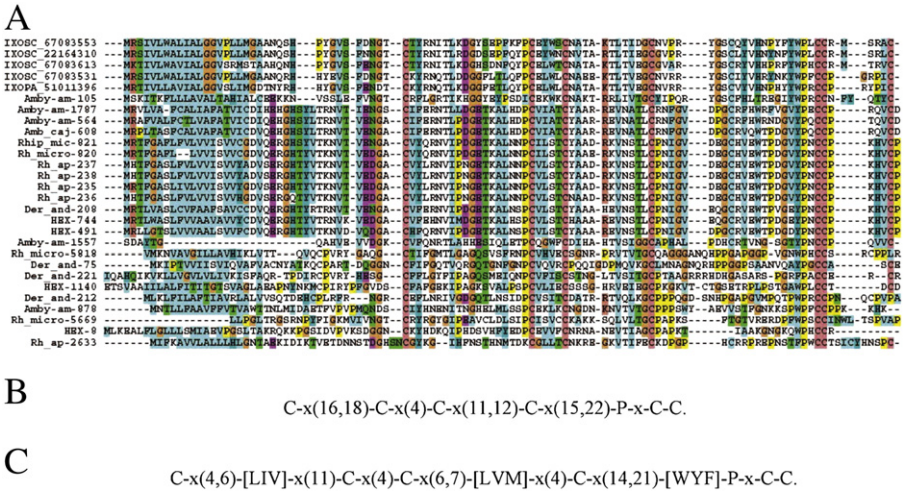

Fig. 5 – The 8.9-kDa family of Ixodidae. A Clustal alignment. B Conserved cysteine and proline framework. C Conserved framework including conserved amino acid substitutions. The *Hyalomma marginatum rufipes* sequences are indicated by HEX. The names initiating by IXOSC are from *Ixodes scapularis*, and the numbers are NCBI accession codes. The remaining proteins derive from deducted expressed sequence tags available at DBEST and were described in a previous review [3]. For more detail, see text.

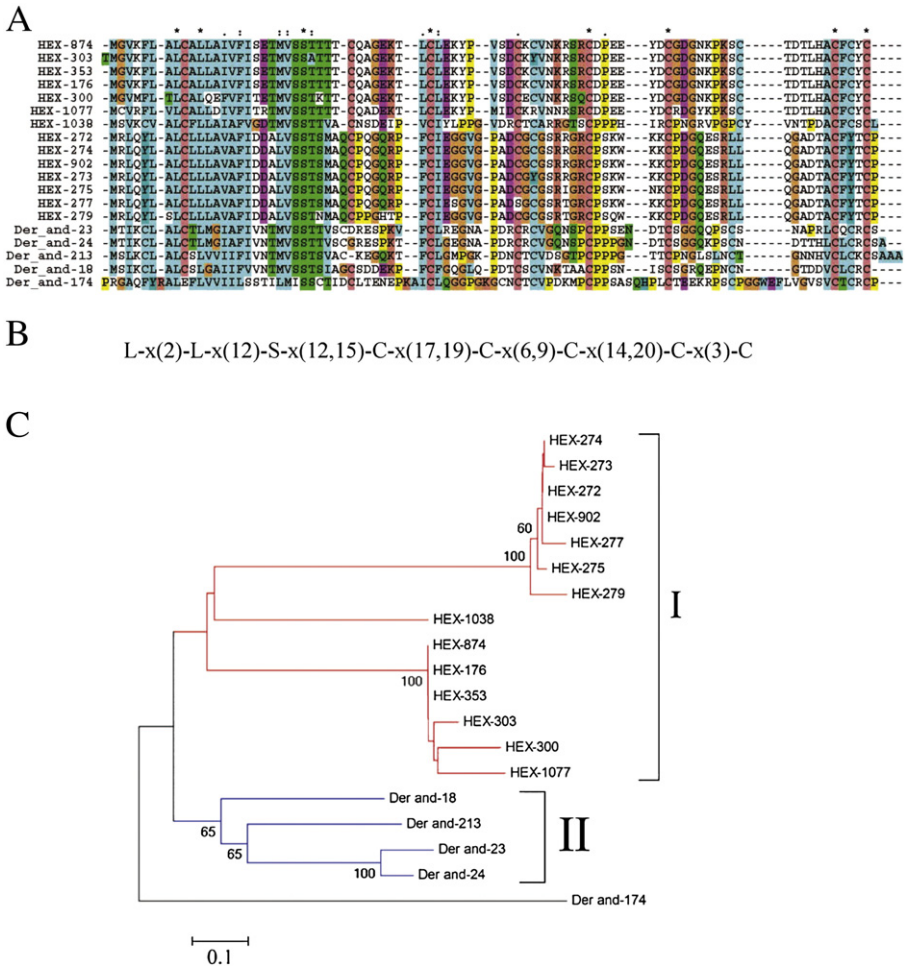

Fig. 6 – The 9-kDa family of *Dermacentor* and *Hyalomma*. *A* Clustal alignment. *B* Conserved cysteine, leucine, and serine framework. *C* Phylogram derived from the alignment in *A*. The numbers on the branches represent percent bootstrap support (less than 50% are omitted). The *Dermacentor* sequences derive from deducted expressed sequence tags available at DBEST and were described in a previous review [3]. The bar at the bottom indicates 20% amino acid divergence. For more detail, see text.

functions, including a set of conserved hypothetical proteins that might be related to protein synthesis or protein modification. Many of these products were identified in various electrophoresis gel bands (Fig. 2 and Table 4), including various ribosomal proteins, products associated with protein modification such as glutathione S-transferases, and proteins associated with energy metabolism. Strong signal for tubulin was also found in fraction 12 and neighboring fractions. Two class I transposon sequence fragments were also extracted from the dataset (Additional file 2).

3.8. *Bovine proteins identified in salivary gland homogenates*

We and others have previously reported that host proteins appear in tick saliva [15,81] . Indeed, there are tick lipocalins postulated to be carriers of host immunoglobulins from the tick hemolymph to tick saliva [81–84], with a possible role of detoxifying host proteins that may cross from the midgut to the hemolymph. We have previously identified host albumin, hemoglobin and immunoglobulin chains in the saliva of *Ixodes scapularis*. This study was done near 10 years ago, with no host mammalian proteomes available, and using low sensitivity Edman degradation of proteins [15]. Presently we searched for host proteins in the salivary gland homogenates of *H. marginatum rufipes*, by supplying the predicted proteome of *Bos taurus* to the Sequest program that searches the MS/MS generated ions against a target database (Table 5 and Supplemental file S3). To properly analyze the bovine proteome, we organized the proteins in a hyperlinked spreadsheet which was blasted against the available predicted proteins of the tick *Ixodes scapularis* (downloaded from http://iscapularis.vectorbase.org/Ixodes_scapularis/Info/Index), the only tick genome known, and, to facilitate protein annotation, against the SWISSPROT protein database and the Gene Ontology database. MS/MS derived peptides originating from the study reported in Fig. 2 were mapped to this spreadsheet as indicated in the methods section. We thus obtained matches to 425 bovine proteins (supplemental file S3, worksheet named Bos matches). However, many of these matches are to very conserved proteins, such as histones, tubulins or ribosomal proteins, that are 100% or nearly so conserved with tick proteins. These matches could derive from tick as well as bovine proteins. We conservatively excluded from the bovine set those proteins producing more than 50% identity to tick proteins, as well as

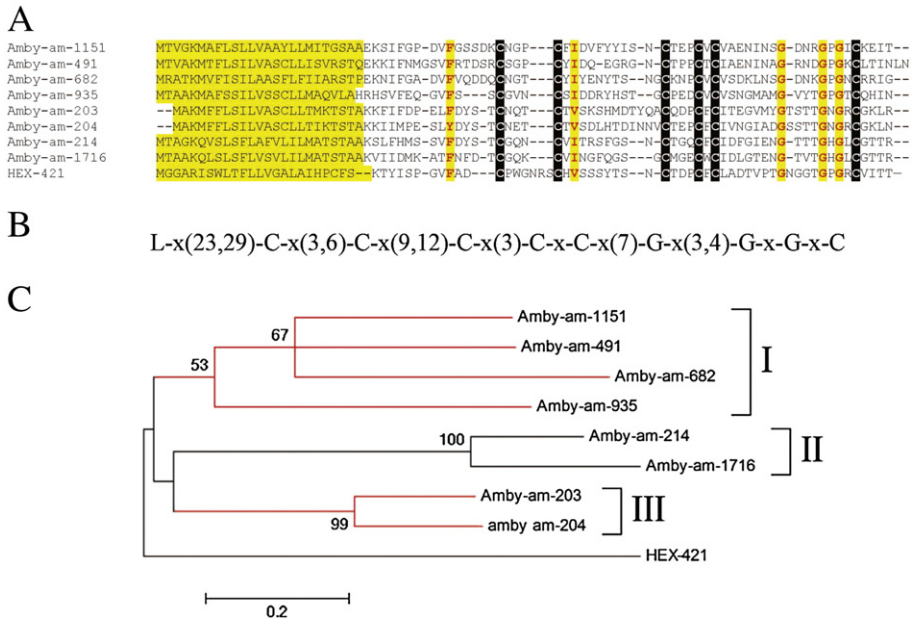

Fig. 7 – The AmbHy9 kDa family of *Amblyomma* and *Hyalomma*. *A* Clustal alignment showing the signal peptide region and a conserved amino acid framework. *B* Conserved cysteine, glycine, and hydrophobic amino acids framework. *C* Phylogram derived from the alignment in *A* showing three *Amblyomma americanum* clades (I–III). The numbers on the branches represent percent bootstrap support (less than 50% are omitted). The *Amblyomma* sequences derive from deducted expressed sequence tags from available at DBEST and were described in a previous review [3]. The bar at the bottom indicates 20% amino acid divergence. For more detail, see text.

all myosins, obtaining a list of 77 bovine proteins (Supplemental file S3, worksheet 2). Several of these 77 proteins were related proteins by being either splice variants, or closely related gene families, such as hemoglobin. We thus removed these redundancies to produce Table 4, with 22 bovine proteins that appear in the tick salivary gland proteome. The table is ordered by the fraction number shown in Fig. 2, from higher to lower MW. The predicted mature masses of the proteins (Table 4) are in accordance with the gel order, except for complement C3 and fibrinogen; C3 appears on fraction 10, between the markers for 64 and 51 kDa, and incompatible with the C3 predicted mass of 185 kDa, indicating C3 cleavage; further C3 fragments appear on fractions 14 and 17. Fibrinogen appears most abundantly covered on fraction 14, under the 39 kDa marker, while the mature protein has a predicted mass of 53 kDa, indicating fibrinogen cleavage. Notice that the list of bovine proteins includes abundant ions for serum albumin, hemoglobin and immunoglobulin chains, as well as for alpha-2-macroglobulin. Proteins abundant in red cells such as band 3 anion transport protein and carbonic anhydrase were also found, as well as leukocyte-derived products azurocidin and the antimicrobial cathelicidin.

The appearance of host proteins on tick salivary gland homogenates could be considered an artifact of contamination during dissection, possibly from the tick gut. However, our samples were carefully collected and no EST produced matches to bovine sequences, as could happen in the case the SG were contaminated with bovine blood. Host Ig in tick hemolymph and saliva were also previously characterized in detailed studies [81]. As indicated before [15], it is interesting to speculate whether these host proteins, while passing through the tick salivary glands, may be submitted to the tick protein glycosylation machinery, although no significant increase in mass for any product was found. Incorporation of these tick epitopes into self molecules may be a strategy for tick suppression of host immunity against carbohydrate antigens.

3.9. *Overview of proteomics results*

One hundred and fifteen contigs were identified by the proteomic data. The distribution of the matched proteins among functional classes, considering only those that obtained at least two ion matches in one gel slice (supplemental file S1, worksheet named “proteome analysis” and Table 6) shows members of the protein synthesis machinery as the most abundantly detected, followed by secreted proteins, protein modification machinery and energy metabolism; these classes account for over 90% of the identified proteins. No correlation was found between the transcript abundance (measured by their number of EST’s) and the number of matching MS/MS ions for each contig (R=0.13) (Supplemental data S1, worksheet “proteome analysis”).

4. Conclusions

Several protein families previously found in tick salivary transcriptomes were identified in *H. m. rufipes*, such as the Kunitz, basic tail, madanin, lipocalin, glycine-rich, mucins, immunity-related, and 8.9-kDa family, as well as protein families previously found only in the metastriate *Dermacentor* genus, such as the 9-kDa family. Most of these proteins have no

Table 5 – Bovine proteins identified in the polyacrylamide gel shown in Fig. 2.

NCBI accession	Mature MW	Description	Fraction→number of ions	Coverage in aa residues (1)	Percent protein coverage (1)
gi|157954061	165	Alpha-2-macroglobulin	Fr-2→16| Fr-3→15| Fr-7→14|	279	18.5
gi|30794360	104	Band 3 anion transport protein	Fr-2→4| Fr-3→4| Fr-5→2|	47	5.1
gi|297462813	119	Ceruloplasmin	Fr-6→2|	33	3.1
gi|76677897	138	Complement factor H precursor	Fr-6→2|	33	2.7
gi|76627249	103	Glucosidase, alpha; neutral C	Fr-7→2|	24	2.6
gi|297462666	80	Primary amine oxidase	Fr-8→3|	48	6.4
gi|27806815	88	Plasminogen precursor	Fr-9→4|	55	6.8
gi|114326282	75	Serotransferrin precursor	Fr-9→8| Fr-10→5|	124	17.6
gi|99028969	185	Complement C3	Fr-10→9| Fr-14→6| Fr-17→3|	155	9.3
gi|30794280	67	Serum albumin precursor	Fr-11→16| Fr-10→14| Fr-12→3|	219	36.1
gi|119914040	31	Serpin A3-3	Fr-11→2|	31	10.4
gi|218931172	53	Fibrinogen beta chain	Fr-14→10| Fr-13→5| Fr-12→4|	138	27.9
gi|297464089	44	Serpin peptidase inhibitor, clade B like	Fr-14→2|	23	5.9
gi|297477165	24	Azurocidin 1 preproprotein-like	Fr-16→2|	32	13.0
gi|30466252	29	Carbonic anhydrase 2	Fr-16→5|	75	28.8
gi|139948632	22	Immunoglobulin lambda-like	Fr-17→16| Fr-18→8| Fr-16→5|	254	108.1
gi|297460000	23	Ig kappa chain	Fr-17→5|	97	41.3
gi|297461641	22	Immunoglobulin lambda-like	Fr-17→8| Fr-18→2| Fr-16→2|	131	55.5
gi|76677514	18	Complement component 4 binding protein	Fr-18→3|	42	21.5
gi|27808640	18	Peptidoglycan recognition protein	Fr-19→4|	57	30.0
gi|27819608	16	Hemoglobin subunit beta	Fr-20→37| Fr-19→7| Fr-18→5|	500	344.8
gi|27807341	14	Cathelicidin-1 precursor	Fr-20→4| Fr-19→2|	64	41.3

(1) On fraction with higher number of ions only; redundant ions are considered; coverage may be higher than 100%.

known function. Many orphan proteins were found that do not match known proteins, but have signal peptides indicative of secretion, suggesting these are *Hyalomma*-specific proteins. This annotated dataset can assist in the discovery of new targets for anti-tick vaccines, as well as help to identify pharmacologically active proteins. In this current study, this annotated transcript data was used to identify salivary protein expression in a proteomic experiment. We additionally identified bovine host proteins in salivary homogenates reinforcing the idea that host proteins are recycled back to the host after ingestion.

Supplementary materials related to this article can be found online at doi:10.1016/j.jprot.2011.07.015.

Acknowledgments

This work was supported by the Intramural Research Program of the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health. We thank NIAID intramural editor Brenda Rae Marshall for assistance. We are grateful for the support of the Research Technologies Section, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840, USA, for DNA sequencing.

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Authors' contributions: IMBF and JMA helped with data analysis, sample preparation, and manuscript editing. NM helped with tick collection and manuscript editing. VMP helped with DNA sequencing. JMCR did most of the data analysis and wrote the bulk of the manuscript. All authors read and approved the final manuscript.

Table 6 – Functional classification of tick transcripts identified by MS/MS.

Class	Number of contigs	Number of ions	Ions/contig
Protein synthesis machinery	61	835	13.69
Secreted	23	182	8.00
Protein modification machinery	12	289	24.08
Metabolism, energy	10	126	12.60
Proteasome machinery	3	46	15.33
Transcription machinery	3	34	11.33
Nuclear regulation	2	62	31.00
Cytoskeletal	2	56	28.00
Oxidant metabolism/detoxification	2	48	24.00
Signal transduction	1	32	32.00
Metabolism, carbohydrate	1	4	4.00
Total	115	1676	

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