



A survey of proteins in midgut contents of the tick, *Haemaphysalis flava*, by proteome and transcriptome analysis

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Abstract

Tick blood meals are stored and digested in their midguts. Blood digestion is complex, and many proteins are involved. Study of the tick-derived proteins in the midgut content may aid in the discovery of active molecules that would be useful for anti-tick vaccines. We analyzed the midgut content proteomes of partially engorged female *Haemaphysalis flava*, fully engorged female *H. flava*, and hedgehog serum using liquid chromatography tandem-mass spectrometry and label-free quantitation. In this study, high-confidence protein profiling of tick midgut content was determined. Based on the search against our in-house transcriptome database, the 28 high-confidence proteins were identified. Of these, 17 were identified as tick-derived, and the rest were of unspecified origin (proteins that could not be differentiated as host-derived or tick-derived proteins). The function of these midgut content proteins identified here may involve nutrient transportation, anti-coagulation, erythrocyte lysis, detoxification, lipid metabolism, and immunization. The presence of hemoglobin suggested that the red blood cells were lysed in the gut lumen. The midgut contents contain a large amount of fibrinogen and it has the ability to clot immediately. The midgut contained mostly host-derived proteins, and these host proteins provide rich nutrients for tick development and reproduction. However, some intracellular proteins were also identified, suggesting the possibility of shedding of the midgut epithelium and ingestion of saliva during feeding. This finding advances our understanding of the digestive mechanism and will be useful in the screening of vaccine antigens.

Keywords Proteome · Midgut content · Blood meal · High-confidence proteins

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Introduction

Haemaphysalis flava (Ixodidae) is a public health pest that feeds on cattle, horses, pigs, dogs, cats, hedgehogs, pandas, wild boars, birds, and humans (Chae et al. 2017; Choi et al. 2014; Yoon et al. 1996). It carries many pathogens including thrombocytopenia syndrome virus (Jo et al. 2019), Kabuto Mountain virus (Ejiri et al. 2018), *Rickettsia* species (Zhang et al. 2018), tick-borne encephalitis virus, *Borrelia* spp., *Cercopithifilaria* spp. (Nematoda: Onchocercidae), *Anaplasma* and *Bartonella* spp. (Yun et al. 2016; Ishiguro et al. 2000; Uni et al. 2013; Kang et al. 2016). It has a geographic distribution in Asia that includes China, Japan, and Sri Lanka (Doi et al. 2018; Ejiri et al. 2018; Jo et al. 2019; Kang et al. 2018; Zheng et al. 2019; Chen et al. 2010).

To screen for anti-tick antigens that may help control *H. flava* and reduce the diseases it transmits, Xu et al. (2015, 2016a) characterized its midgut and salivary gland transcriptome next-generation sequencing technology. Liu et al. (2018a, b) studied the protein components in the midgut contents and feces of *H. flava* by liquid chromatography tandem-mass spectrometry (LC–MS/MS) and label-free quantitation (LFQ). Additionally, some full-length cDNAs were cloned and gene expression levels were observed (Xu et al. 2016b, 2019; Liu et al. 2016). For instance, the recombinant heat shock protein 70 (HSP70) and enolase were expressed in vitro, and their anti-coagulant activities were studied (Liu et al. 2017; He et al. 2019).

In a previous study, the peptides were used to search the public database so that some proteins may be missed and only 131 unique peptides and 102 proteins were identified, fifteen of these proteins were high-confidence proteins (unique peptides ≥ 2). Here, it is the first time that the peptides were used to search the in-house protein libraries, the midgut and salivary gland transcriptome databases of *H. flava* published previously by Xu et al. (2015, 2016a). The proteome of host serum was not performed in the study of Liu et al. (2018a), so that the host-derived proteins could not be excluded. The in-house transcriptome databases were utilized and the LC–MS/MS of host serum was performed to clarify their presence in the midgut contents.

Materials and methods

Ethical approval

All animal experiments were approved and overseen by Institutional Animal Care and Use Committee at HUNAU (No. 43321503). Animals were protected from unnecessary suffering throughout the experiments.

Host and tick

Six hedgehogs (*Erinaceus europaeus*) (600 ± 50 g) were purchased from Xinyang, Henan, China ($32^{\circ} 13' N$, $114^{\circ} 08' E$) and kept in our laboratory at the Hunan Agricultural University (Changsha, Hunan province, China). Three hedgehogs were placed into the experiment group and three into the control group. Thirty unfed adult *H. flava* were

placed on hedgehogs in the experimental group. Hedgehogs in the control group were not infected with ticks.

Ticks were harvested from the hedgehogs when they were partially or fully engorged according to visual inspection. We randomly chose three female ticks with similar weights (180 ± 20 mg) as the partially engorged female *H. flava* (PEF) group and three female ticks (350 ± 20 mg) as the fully engorged female *H. flava* (FEF) group.

Collection of midgut contents and the host blood sample

The PEF and FEF were dissected immediately after harvest and the midgut contents from three PEF and three FEF (about 30 μ L) were carefully squeezed into three clean tubes with 500 μ L 4% sodium citrate-physiological saline, respectively. Three biological replications were performed in the following experiment. The tubes were centrifuged at 5000 rpm for 15 min to remove the blood cells. A 50 μ L sample of the supernatant was transferred into clean tubes. Hedgehog blood from the control group of hedgehogs was also collected, and the serum was separated by centrifugation.

Samples (50 μ L) were mixed with 30 μ L SDT buffer (4% sodium dodecyl sulfate, 1 mM DL-Dithiothreitol (DTT), 150 mM Tris-HCl pH 8.0) and boiled for 5 min. The undissolved debris was removed after centrifugation at $14,000 \times g$ for 15 min. Supernatant samples were quantified with a bicinchoninic acid (BCA) protein assay kit (Bio-Rad, Berkeley, CA, USA) and were visualized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Protein digestion

Protein digestion was carried out according to the filter-aided sample preparation (FASP) procedure described by Wiśniewski et al. (2009). DL-Dithiothreitol was added to samples (100 μ g) up to the final concentration at 100 mM, boiled for 5 min, and then cooled to room temperature. Thereafter, to remove detergent, DL-Dithiothreitol and other low-molecular-weight components, the 200 μ L UA buffer (8 M urea, 150 mM Tris-HCl pH 8.0) was added, and then centrifuged with 10 kDa ultrafiltration centrifuge tubes at 14,000 rpm for 15 min. The filtrate was then discarded and the process was repeated. Then 100 μ L UA buffer containing 50 mM iodoacetamide was added to block reduced cysteine residues. The samples were then incubated for 30 min in darkness, and then centrifuged at 14,000 rpm for 10 min. The filtrates were washed three times with 100 μ L UA buffer and then three times with 100 μ L 100 mM NH_4HCO_3 . Eventually, the resultant samples were digested in trypsin buffer (4 μ L Trypsin in 40 μ L NH_4HCO_3) with incubation for 16 h at 37 °C. The peptide filtrates were collected and the peptide content was estimated crudely by UV light at 280 nm before mass spectrometry.

Mass spectrometry

Each product of enzymatic hydrolysis was desalted on C18 Cartridges (Empore™ SPE Cartridges C18; Sigma, Santa Clara, CA, USA) and concentrated using a vacuum centrifuge concentrator. Then 40 μ L of 0.1% (v/v) trifluoroacetic acid was added for acidification or protonation. A total of 2 μ g of concentrated peptide products were loaded onto a C18-reversed phase column (Agilent Technologies, Wilmington, DE, USA), which were

balanced by the buffer A (2% acetonitrile and 0.1% formic acid). Peptides were separated on the EASY nLC100 HPLC system (Proxeon Biosystems; Thermo Fisher Scientific, Waltham, MA, USA) at a flow rate of 300 nL/min with a linear gradient of buffer B (80% acetonitrile and 0.1% formic acid) from 0 to 45% for 55 min, 45 to 100% for 2 min, and 100% for 3 min.

After separation by HPLC, a data-dependent top-10 method that used the most abundant precursor ions from the survey scan (300–1800 m/z) was dynamically chosen for HCD fragmentation and used to acquire MS data. The MS and MS/MS scan at 200 m/z was set as 17,500 and 70,000 for the survey scans acquisition, respectively. The maximum ion injection times were 10 ms for MS and 60 ms for MS/MS. Normalized collision energy for dissociating ions was 30 eV, and the isolation window was 2 m/z. Precursor ions were chosen after a dynamic exclusion duration of 40 s, and the under fill ratio was defined as 0.1%.

MS data analysis and label-free quantitation (LFQ) with MaxQuant

MaxQuant software (v.1.3.0.5) was used for the analysis and the detection of peptides. The MS data of midgut contents from PEF and FEF were searched against the protein libraries deduced from Unigenes of the midgut transcriptomes and salivary gland transcriptome of *H. flava* (GSE67247 and GSE69721 in NCBI GEO) (Xu et al. 2015, 2016a, b), respectively, and the MS data of host serum were searched against the NCBI database (<https://www.ncbi.nlm.nih.gov/taxonomy/?term=Erinaceinae>). The main search parameters included: a mass deviation filter of 6 ppm; enzyme set to trypsin; a maximum missed cleavage set at 2; and a mass tolerance of 20 ppm. The fixed modification was set as carbamidomethyl (C), and the variable modification was oxidation (M), acetyl (Protein N-term). Peptide and protein false discovery rate (FDR) was set at 0.01.

Label-free quantification was carried out in MaxQuant as the description by Lubner et al. (2010). Label-free quantification intensity was obtained based on the normalized spectral protein intensity. The label-free quantity of each protein of PEF and FEF were compared, significantly different expressed proteins in abundance were observed if the ratio between PEF and FEF intensity was > 1.5 or < 0.67 , and the *P* value was < 0.05 .

Annotation of proteins and analysis of protein origin

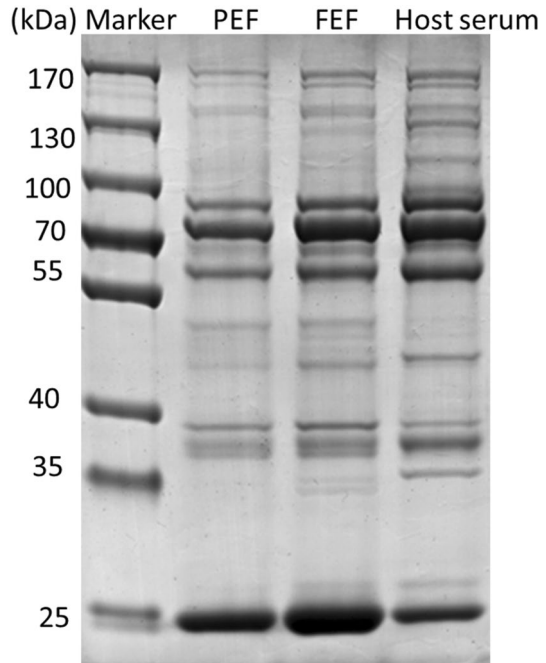
The high-confidence peptide sequences (unique peptides ≥ 2) were searched against the Ixodoidea dataset downloaded from UniProt (190,995 protein sequences downloaded on 12/07/2018, <https://www.UniProt.org>), and then the same peptide sequences were grouped based on sequence alignment. The coverage of unique peptides was the method used to exclude hedgehog-derived proteins and to identify tick-derived proteins.

Results and discussion

Protein profiling with SDS-PAGE

Protein concentrations of midgut contents from PEF, FEF, and the hedgehog serum were determined by the BCA method and were, respectively, 54.79 ± 4.68 , 19.90 ± 1.47 , and 23.56 ± 2.06 $\mu\text{g}/\mu\text{L}$. The protein profiling of samples was determined by SDS-PAGE

Fig. 1 Comparison between *Haemaphysalis flava* midgut contents and hedgehog serum. Marker: 10 μ L protein marker; partially engorged female *H. flava*: 10 μ L midgut content extract from partially engorged female *H. flava* ($54.79 \pm 4.68 \mu\text{g}/\mu\text{L}$); fully engorged female *H. flava*: 10 μ L midgut content extract from partially engorged female *H. flava* with concentration ($19.90 \pm 1.47 \mu\text{g}/\mu\text{L}$); and host serum: 10 μ L hedgehog serum ($23.56 \pm 2.06 \mu\text{g}/\mu\text{L}$)



(Fig. 1). Bands at 25, 38, 63, 80, 90, 140, and 170 kDa were intense and common between tick midgut contents and hedgehog serum. The band at 50 kDa was visible in PEF and FEF but not in host serum samples.

Annotation and the assembly of peptide sequences

The 174 unique peptides and 107 peptide sequences were identified by searching against the midgut transcriptomic library of *H. flava* (NCBI GEO: GSE69721), and 17 peptide sequences were confirmed as high-confidence proteins (Table 1). The 191 peptides and 84 peptide sequences were identified by a search of the salivary gland transcriptomic library of *H. flava* (NCBI GEO: GSE67247), and 21 peptide sequences were confirmed as high-confidence proteins (Table 1). These results showed that the corresponding mRNA of midgut content proteins present in the midgut or the salivary gland because the same gene may be expressed in the different tissues. However, it is unknown that the midgut content proteins may be derived from the midgut or salivary gland. Due to the variation among different species, more proteins may be identified by searching against the libraries from the *H. flava* transcriptomes than universal databases such as NCBI GenBank and the UniProt database.

The high-confidence peptide sequences were annotated and the peptide sequences deduced from the same transcript were merged (Table 2). A total of 28 high-confidence proteins were identified in the tick midgut contents. Only one (peptide sequence MG-2799 (SG-5002)) was present at the phase of fully engorged female and two (peptide sequence MG-631 (SG-4096) and peptide sequence MG-9922 (SG-5824)) present in the partially engorged phase. The rest were found in both. Most of peptide sequences had a high

Table 1 Identification of high-confidence peptide sequences from tick midgut contents of partially and fully engorged female *Haemaphysalis flava*

No	Unique peptides	Peptide sequence/protein	NL	Cover (%)	Origin (T/H)
1	DCSEHQHFLJLGTK, DEAFYADIAPSVR, DYEEAYVSVR, EILLPIYR, EPTTEILDFEYR, IANLDSLVSDDDNAISTNFK, IELLPVTDSPAIAR, SEYPCHFELSQHRLR DYEEAYVSVR, EILLPIYR, SEYPCHFELSQHRLR	SG-12613 MG-1058 MG-13914 SG-195	1564 374 189 1075	6.71 27.8 17.99 22.13	Tick Tick
2	EFFHEMTWR, GHLDHGLEDSLRL, MYLLPAAEDATK AAGNLATR, ATLSLLEGG, DIFVQGLDK, EDGLHGVCDTLYIVHEEDHDYMEVTK, EFFHEMTWR, EHSLVQEFATSEHLQNPPELTK, EIEDALPVADR, FICPTWHA NEHQRL, GHLDHGLEDSLRL, LAASAVASGVDCR, LINGLVGPQPGSTK, MYLLPAAEDATK, NHVVNAPEDIK, NNELSALDADR, SHDSFLPR, SNEINLVGALEHPFAGK, SSLSGSIDQR, TLELEAEHDAPTSET-TLPEDAVK				
3	IINEPTAAALAYGLDK, STAGDTHLGGEDFDNR, VEIANDQQGNR	SG-8235 MG-26577 MG-41014	673 502 485	6.39 8.56 14.43	Tick Tick
4	IINEPTAAALAYGLDK, SQIHDIVLVGGSTR, STAGDTHLGGEDFDNR, TTPSYVAFIDTER, VEIANDQQGNR				
5	AYTESGASLPVNLK, EGGSPVTLTLQEVTK, SVILLPMGASDDGAHSQNEK	MG-896 SG-3575	454 392	11.20 13.01	Tick
6	DTCDNPNQYQCR, HNLYVGGRR, NDLIIJTAACHCVAGK	MG-3859 SG-13671	348 477	9.77 7.12	Tick
7	NVQAEEMVEFSSGLK, TGAIVDVPVGPPELLGR	MG-631 SG-4096	415 297	7.47 10.43	Tick
8	AGFAGDDAPR, AVFPSIVGR, CPEALFQPSFLGMEACGIIHETTYSIMIK, DLITDYLMIK, DLYANT-VLSGGTTMYPGIADR, DSYVGDQEAQSK, HQGVMVGMGQK, GYSFTTTAER, SYELPDGQVIT-IGNER, VAPEEHPVLLTEAPLNPK	MG-240 SG-217	349 329	40.68 43.16	Tick
9	AAAAFMGK, LSESENFDEFK	MG-879	132	15.15	Tick
10	FDLTMSPPPPVAAK, GQDAAGEFFCFR, TDACNFMGVSCPLEAGK	MG-5701	156	28.21	Tick
11	AVEEFLAEVAEATK, LGDPLNPNSTLCGPLHTER	MG-9922 SG-5824	337 579	9.50 5.53	Tick

Table 1 (continued)

No	Unique peptides	Peptide sequence/protein	NL	Cover (%)	Origin (T/H)
12	CSGALIDDQHVLTAAK, GYQGNHVNNAIILK, LTVLAGAHSR, SADVEEACVYK, SVFCTVY-DQGSPCMHDVGGPVMISK, TVANNQCYDENDETEVPK, VDGAWTVHGVVSGGAK	MG-291	131	43.51	Tick
		SG-20125	289	37.37	
13	VPLPNP*, TLTVTGIEFR	MG-627	155	10.32	Tick
	QDANNYIDLVLRL, TLTVTGIEFR	SG-27392	227	9.69	
14	LLQGNDVSTK, SLETLGR	MG-990	99	17.17	Tick
		SG-8698	128	13.28	
15	FECENMSCVQDK, EKFECEMNSCVQDK	SG-228	105	13.33	Tick
16	AAFPGNDVTVQLFTK, PVHSETHPLNAQDSDYR	SG-9354	153	21.59	Tick
17	ALTGALNYYR, ASAGNQPCR	SG-10717	215	9.30	Tick
18	DSTLIMQLLR, NLLSVAYK	MG-1059	245	7.20	Tick
		SG-2838	278	6.47	
		A0A1S3ABS6	429	4.20	Hedgehog
19	EGIPPDQQR, ESTLHLVLR, TITLVEPSDTIENVK	MG-5055	128	29.70	Tick
		SG-123	337	10.08	
		A0A1S3ADB0	128	26.56	Hedgehog
20	EHALLAYTLGVK, IGGIGTVPVGR, STTTGHLIYK	MG-390	462	6.93	Tick
		SG-6	481	6.86	
		A0A1S2Z998	462	7.14	Hedgehog
21	ADLNLNLTIAK, GVVVSEDLPLNISR	MG-1572	388	6.70	Tick
		SG-2705	751	3.46	
		A0A1S3AM25	724	3.59	Hedgehog
22	VGINYQPPTVPPGGDLAK, QLFHPEQLITGK, TIGGGDDSFNTFFSETGAGK	MG-34	416	12.01	Tick
		A0A1S3WID6	417	11.99	Hedgehog
	QLFHPEQLITGK, TIGGGDDSFNTFFSETGAGK	SG-1206	126	25.40	Tick

Table 1 (continued)

No	Unique peptides	Peptide sequence/protein	NL	Cover (%)	Origin (T/H)
23	ALVPELTQQMFDK, LAVNMVPPR, GHYTEGAELVDSVLDVVR	MG-2799 SG-5002	374 411	8.82 10.46	Tick
24	NLQYYDISAK, VCENIPIVLCGNK	A0A1S3W9D3 MG-352	401 221	10.72 10.41	Hedgehog Tick
25	HAVSEGTK, LLLPGELAK	SG-968 A0A1S3A4Q1	221 216	10.41 10.65	Hedgehog
26	EMVELPLR, LAGESENLR	MG-1248 SG-27398	124 124	13.71 13.71	Tick
27	DAGTIAGLNVLR, TTPSYVAFDTER	A0A1S3WR78 MG-2872	125 304	13.60 5.92	Hedgehog Tick
28	GAAQNIIPASTGAAK, VIPELNGK	SG-942 A0A1S3WAZ7	816 789	3.19 2.28	Hedgehog
		MG-1176	615	6.67	Tick
		SG-949	620	6.66	
		A0A1S3A423	646	3.87	Hedgehog
		MG-1624	271	8.49	Tick
		SG-1683	353	6.52	
		A0A1S3A1J4	333	6.91	Hedgehog

Peptide sequence/protein: Peptide sequences in the salivary gland (SG) and midgut (MG) transcriptome datasets or protein in UniProt dataset that peptides matched at least two; NL nucleic acid length; Cover (%) Coverage (%); Origin (T/H) Origin (Tick/Hedgehog)

Table 2 Annotation results of high-confidence peptide sequences blasting against the salivary gland transcriptome and midgut transcriptome of *Haemaphysalis flava*

No	Unigene ID	Protein, gene, organism, protein accession number	E-value	Score	Identity (%)
1	MG-1058/SG-12613	Vitellogenin-1, Vg-1, <i>Haemaphysalis flava</i> , A0A346JM05	0.00	8294	100.0
2	MG-13914/SG-195	Vitellogenin-2, Vg-2, <i>Haemaphysalis flava</i> , A0A346JM06	0.00	5619	100.0
3	MG-26577/SG-8235	Heat shock protein 70-8235, N/A, <i>Haemaphysalis flava</i> , A0A1L5L8R3	0.00	2575	100.0
4	MG-41014	Heat shock 70 kDa protein 1/8, <i>Rhipicephalus appendiculatus</i> , A0A131YYR4	0.0	2420	97.7
5	MG-896/SG-3575	Metalloexopeptidase, N/A, <i>Amblyomma cajennense</i> , A0A023FKE7	0.00	1744	83.5
6	MG-3859/SG-13671	Serine protease, HISP, <i>Haemaphysalis longicornis</i> , Q6L7Z5	0.00	1366	73.5
7	MG-631/SG-4096	ATP synthase subunit alpha, N/A, <i>Rhipicephalus appendiculatus</i> , A0A131Z614	0.00	2088	79.8
8	MG-240/SG-217	Actin, <i>Amblyomma triste</i> , A0A023GHC4	0.00	1967	100.0
9	MG-879	Fatty acid-binding protein, N/A, <i>Amblyomma triste</i> , A0A023GHZ0	2e-80	616	91.5
10	MG-5701	M1 domain-containing protein, N/A, <i>Amblyomma aureolatum</i> , A0A1E1WZY1	7.8e-85	649	76.8
11	MG-9922/SG-5824	Aldehyde dehydrogenase, N/A, <i>Amblyomma aureolatum</i> , A0A1E1X7D6	0.00	2714	95.7
12	MG-291/SG-20125	Serine protease-like protein, N/A, <i>Amblyomma sculptum</i> , A0A1E1XU45	1.1e-176	1279	81.1
13	MG-627/SG-27392	Sulfotransferase, N/A, <i>Rhipicephalus zambeziensis</i> , A0A224Z9G4	1.6e-121	904	79.0
14	MG-990/SG-8698	Secreted protein, N/A, <i>Amblyomma triste</i> , A0A023G1Z2	4.1e-46	389	69.2
15	SG-228	Microplasin-like antibacterial peptide, <i>Amblyomma aureolatum</i> , A0A1E1WWG1	4.3e-8	134	33.8
16	SG-9354	Reeler, N/A, <i>Amblyomma sculptum</i> , A0A1E1XTJ8	6.2e-36	325	47.7
17	SG-10717	Soluble epoxide hydrolase, N/A, <i>Amblyomma triste</i> , A0A023G8Q4	2.2e-77	629	57.1
18	MG-1059/SG-2838	14-3-3 domain-containing protein, <i>Amblyomma triste</i> , A0A023GGR3	1.9e-171	1241	100.0
19	MG-5055/SG-123	Polyubiquitin, N/A, <i>Amblyomma americanum</i> , A0A0C9SDC7	2.2e-83	641	100.0
20	MG-390/SG-6	Elongation factor 1-alpha, N/A, <i>Amblyomma aureolatum</i> , A0A1E1XAX2	0.00	2409	99.4
21	MG-1572/SG-2705	Heat shock protein 90, N/A, <i>Haemaphysalis flava</i> , A0A2D1C0A7	0.00	3719	100.0
22	MG-34/SG-1206	Tubulin alpha chain, <i>Rhipicephalus appendiculatus</i> , A0A131YP40	0.00	2186	99.0
23	MG-2799/SG-5002	Tubulin beta chain, <i>Rhipicephalus appendiculatus</i> , A0A131YRB1	0.00	2340	99.1
24	MG-352/SG-968	GTP-binding nuclear protein, <i>Ornithodoros moubata</i> , A0A1Z5KX72	5.4e-164	1173	98.2
25	MG-1248/SG-27398	Histone H2-B, N/A, <i>Hyalomma excavatum</i> , A0A131XLQ7	3.2e-80	612	100.0

Table 2 (continued)

No	Unigene ID	Protein, gene, organism, protein accession number	E-value	Score	Identity (%)
26	MG-2872/SG-942	Valosin containing protein, <i>Haemaphysalis longicornis</i> , A7BF19	0.00	4044	98.3
27	MG-1176/SG-949	Heat shock cognate 70 kDa protein, N/A, <i>Haemaphysalis flava</i> , A0A097A1J8	0.00	3102	100
28	MG-1624/SG-1683	Glyceraldehyde-3-phosphate dehydrogenase, <i>Haemaphysalis flava</i> , A0A2P1DPZ4	0.00	1730	100.0

Unigene ID: Unique gene ID in the salivary gland (SG) and midgut (MG) transcriptome datasets

similarity to known proteins such as peptide sequence SG-12613, which shared 100% identity with Vitellogenin 1 (Vg 1), and peptide sequence MG-41014, which was similar (97.7%) to HSP70 protein (Table 2). However, the peptide sequences of SG-228, SG-9354 and SG-10717 had low similarity with the proteins of microplusin-like anti-bacterial peptide, reeler and soluble epoxide hydrolase.

Characterization of tick-derived protein and function analysis

The comparison of peptide sequences identified in *H. flava* and proteins identified in hedgehogs showed that 17 proteins were tick-derived proteins such as Vgs and HSP70. However, the others like polyubiquitin and heat shock protein 90 (HSP90) could not be determined to tick-derived proteins, because these proteins are highly conserved and the identified peptides were shared by tick and host protein sequences. Some of the 17 tick-derived proteins play important roles in tick development, and they are discussed here.

Vitellogenin (Vg)

Based on the sequence alignment, there was 100% identity between the peptide sequence MG-1058 and peptide sequence SG-12613, and these two were considered to be the same protein. Likewise, the peptide sequence MG-13914 and peptide sequence SG-195 were also considered to the same protein. These two proteins were annotated to the *H. flava* Vg 1 (GenBank number: MH178299) and Vg 2 (GenBank number: MH178300) (100% identity) (Table 2). Vgs were expressed in the tick midgut, fat body, and ovary (Boldbaatar et al. 2010; Raikhel and Dhadialla, 1992; Khalil et al. 2011) and were then transferred into the oocytes by receptor-mediated endocytosis with the hemolymph and finally converted to Vn. Vn could sequester, transport, and store heme for embryo development (Logullo et al. 2002). However, Vgs have also played a role in heme sequestration (Thompson et al. 2007; Boldbaatar et al. 2010) and transportation of carbohydrates, lipids and other nutrients (Kunke and Nordin 1985; Dhadialla and Raikhel 1990). Vgs were classed as transport proteins in a similar proteomic study by Tirloni et al. (2014), suggesting that Vgs in this experiment may function in nutrients transportation. The Vgs identified in the midgut content did not change in abundance significantly between the two fully engorged and partially engorged phages.

Heat shock protein 70 (HSP70)

HSP70 is a family of proteins with at least eleven members found in humans (Shrestha and Young 2016). It is not known how many HSP70 members occur in ticks, but six HSP70 cDNA sequences were found in *H. flava*, and five of these were released by NCBI GenBank. Here, three of these were identified in the midgut contents, namely: peptide sequence MG-26577 (SG-8235), peptide sequence MG-41014 and peptide sequence MG-1176 (SG-949). Two of these HSP70 identified here were considered to be tick-derived proteins, but the derivation of the other (peptide sequence MG-1176 (SG-949)) is difficult to resolve.

The basic functions of HSP70 are involved in protein folding and refolding, preventing protein aggregation, and solubilizing proteins (Hartl and Hayer-Hartl 2002). HSP70 also has anticoagulation and immunological properties (Bolhassani and Rafati 2008). HSP70 can inhibit thrombosis without bleeding risk in mice (Allende et al. 2016, 2017). Tick HSP70 elicited fibrinolytic activities (Vora et al. 2017). He et al. (2019) showed

that *H. flava* recombinant HSC70 could delay prothrombin time and thromboplastin time in vitro. Silencing of RH-Hsc70 inhibited tick blood feeding and significantly decreased the tick engorgement ratio (Wang et al. 2019). These data suggested that HSP70 may have potential value for the development of drugs or vaccines to control tick and tick-borne diseases (Vora et al. 2017; Bolhassani and Rafati 2008).

Serine protease

Red blood cells are lysed in the gut lumen, and serine protease plays an important role in this process (Sojka et al. 2013; Miyoshi et al. 2007). The recombinant serine protease from *Haemaphysalis longicornis* was potently able to hydrolyze the substrates Bz-(DL)-Arg-pNA and Z-Ala-Ala-Leu-pNA (Miyoshi et al. 2004a, 2007). The serine proteases mRNAs of *Rhipicephalus appendiculatus* were expressed in tick midgut (Mulenga et al. 2003). The peptide sequence MG-3859 (SG-13671) shared 73.5% similarity to the *H. longicornis* serine protease published by Miyoshi et al. (2004b), suggesting that the serine protease of *H. flava* midgut content may be involved in the degradation of host erythrocyte membranes.

Aldehyde dehydrogenase

The release of heme, free iron radicals, H_2O_2 , and other stress-inducing molecules results in significant oxidative stress during blood digestion. These molecules are scavenged by enzymes, such as aldehyde dehydrogenase. Anderson et al. (2008) studied the mialome in *D. variabilis* midguts and speculated that aldehyde dehydrogenase might function in the detoxification of aldehydes and be involved in oxidoreductase activity.

Fatty acid-binding protein

Fatty acid-binding proteins belongs to the calycons superfamily. Fatty acid-binding protein can bind hydrophobic ligands, such as fatty acids and pigments (Bernier and Jollès, 1987; Ockner, 1990). Some members of this family are secreted and are involved in the metabolism and transportation of lipids (Bernier and Jollès, 1987; Ockner, 1990; Glatz and van der Vusse 1990). The difference of fatty acid-binding protein content between PEF and FEF was small. However, there are no studies on tick fatty acid-binding proteins and their function remains unknown.

ML domain-containing protein

Horácková et al. (2010) reported that a member of ML (MD-2-related lipid-recognition) domain protein family, which contain an MD-2-related lipid-recognition (ML) domain, was strongly induced by a blood meal. It appeared to involve a tick's immune response because it had the ability to interact with immunoglobulin E. Herein, the peptide sequence MG-5701 annotated to the ML domain-containing protein also contained an ML domain, implied that the protein from *H. flava* may be related to tick immunization. The peptide sequence MG-5701 showed a significant increase (PEF: FEF = 3.38, Table 3) in abundance in the FEF compared to the PEF.

Table 3 Label free quantitative results of high-confidence peptide sequences in tick midgut contents of partially and fully engorged female *Haemaphysalis flava*

No	Peptide sequences	Quantitative value (x10E6)			PEF/FEF		
		LFQ (PEF)	LFQ (FEF)	Host serum	Ratio	P	Conclusion
1	MG-1058/SG-12613	131.0±12.0	127.0±3.8	0±0	0.97	0.68	
2	MG-13914/SG-195	1188.3±587.6	659.4±40.1	0±0	0.56	0.27	
3	MG-26577/SG-8235	215.3±9.7	183.7±10.1	73.9±52.4	0.85	0.033	
4	MG-41014	608.6±55.7	906.0±47.4	595.1±107.0	1.49	0.004	
5	MG-896/SG-3575	62.2±2.9	64.1±8.5	0±0	1.03	0.78	
6	MG-3859/SG-13671	140.8±21.9	190.0±49.7	0±0	1.35	0.27	
7	MG-631/SG-4096	31.5±3.7	0±0	0±0	–	–	Down
8	MG-240/SG-679	476.3±114.2	1148.6±40.4	0±0	2.41	0.001	Up
9	MG-879	202.7±65.5	244.1±6.9	0±0	1.20	0.43	
10	MG-5701	105.0±5.1	354.9±36.9	0±0	3.38	<0.001	Up
11	MG-9922/SG-5824	26.4±4.7	0±0	0±0	–	–	Down
12	MG-291/SG-20125	2087.7±203.2	3267.0±162.8	0±0	1.56	0.003	Up
13	MG-627/SG-27392	415.0±4.2	367.1±33.4	0±0	0.88	0.12	
14	MG-990/SG-8698	196.8±5.7	160.1±281	0±0	0.81	0.15	
15	SG-228	56.7±6.5	182.5±12.2	0±0			
16	SG-9354	205.7±5.2	262.4±15.6	0±0	1.28	0.008	
17	SG-10717	39.7±5.1	34.0±1.4	0±0	0.86	0.20	
18	MG-1059/SG-2838	1.1±0.1	0.9±0.1	0.0±0.0			
19	MG5055/SG-123	3553.7±251.9	2989.0±222.6	1843.0±380.0	0.84	0.076	
20	MG-390/SG-6	289.1±30.8	229.6±45.6	527.2±113.8	0.80	0.20	
21	MG-1572/SG-2705	173.7±27.3	114.8±2.6	83.5±4.5	0.66	0.038	Down
22	MG-34/SG-1206	98.6±6.2	353.2±93.2	131.2±45.5	3.58	0.018	Up
23	MG-2799/SG-5002	0±0	51.5±15.2	47.1±7.8	0	0.009	Up
24	MG-352/SG-968	125.3±12.0	253.2±23.6	142.1±64.0	2.02	0.002	Up
25	MG-1248/SG-27392	204.5±21.9	95.5±7.0	0±0	0.47	0.003	Down
26	MG-2872/SG-942	183.5±28.0	157.6±3.0	127.0±19.1	0.86	0.26	
27	MG-1176/SG-949	594.7±28.8	690.6±5.8	166.0±66.9	1.16	0.010	
28	MG-1624/SG-1683	458.1±57.2	543.7±42.3	234.7±67.6	1.19	0.16	

Serine protease-like, ATP synthase, actin and metalloexopeptidase

The peptide sequence MG-291 (SG-20125) was annotated to the serine protease-like protein. However, it showed a low similarity with serine protein in amino acid levels. In addition, it is probable that the midgut content samples contained the secretome with intracellular proteins such as actin. The peptide sequence MG-896 (SG-3575) shared 80% similarity with the putative metalloexopeptidase based on the alignment results online. The information related to the metalloexopeptidase was from the transcriptome (de Castro et al. 2016; Garcia et al. 2014). There is no published information on the function of ATP synthase (subunit alpha), serine protease-like protein, metalloexopeptidase.

These proteins, including heat shock protein 70, aldehyde dehydrogenase, ATP synthase and actin, are intracellular proteins. It is unknown whether these proteins were secreted into the midgut lumen or originated from the breakdown of epithelium cells.

Host proteins in *Haemaphysalis flava* midgut contents

The identified peptides were used to blast against the NCBI database (NCBI, Erinaceinae 29978, 13/06/2018). The 2805 peptides and 372 proteins were obtained, and 299 proteins of those were considered as high-confidence proteins. The tick midgut stores the blood meal so that the 10 proteins with the highest content in the tick midgut were host-derived, and they are listed in the Table 4.

In these host-derived proteins, the hemoglobin subunit beta, hemoglobin subunit alpha and carbonic anhydrase 2 were the main components of red blood cells, and the serum albumin, serotransferrin, apolipoprotein A-I, complement C3, alpha-2-macroglobulin-like, haptoglobin and fibrinogen alpha chain were important components of the host serum.

Hemoglobin and serum albumin

During tick feeding, the host blood was imbibed to the midgut lumen. The midgut will greatly enlarge, meanwhile, the proliferation of undifferentiated reserve cells to digestive and secretory cells. The host blood will be lysed in the midgut lumen and the components of host red blood cells will be released from host erythrocytes. Therefore, a large amount of hemoglobin, a major component of tick blood meals (Graça-Souza et al. 2006), was detected in the tick midgut contents. After the hemoglobin was released, the hemoglobin was phagocytosed by digestive cells and digested in acidic vesicles of the tick gut cells.

During tick development and reproduction, the ultimate source of amino acids used for the de novo protein synthesis is hemoglobin and serum albumin (Sojka et al. 2016). The hemoglobin and serum albumin rank highest in the midgut content proteins (Table 4).

Table 4 Label free quantitative results of hedgehog's serum proteins in tick midgut contents of partially and fully engorged female *Haemaphysalis flava*

No	Accession number in UniProt, protein	Quantitative values (x10E9)			PEF/FEF		Conclusion
		LFQ (PEF)	LFQ (FEF)	Host serum	Ratio	P	
1	A0A1S3WPY1, Hemoglobin subunit β	718.0 \pm 79.7	725.5 \pm 16.6	247.3 \pm 5.7	1.01	0.90	
2	A0A1S2ZRW6, Serum albumin	455.3 \pm 37.9	592.3 \pm 14.3	401.5 \pm 7.3	1.31	<0.01	
3	A0A1K0GV58, Hemoglobin subunit α	353.6 \pm 33.9	401.2 \pm 31.6	159.3 \pm 7.01	1.24	0.08	
4	A0A1S3W2S7, Serotransferrin	193.2 \pm 9.9	184.0 \pm 3.3	216.0 \pm 3.0	0.95	0.28	
5	A0A1S2ZQM1, Apolipoprotein A-I	73.4 \pm 6.0	99.3 \pm 5.1	99.2 \pm 3.3	1.39	<0.01	
6	A0A1S3AIG2, Carbonic anhydrase 2	37.9 \pm 4.9	57.1 \pm 1.9	17.5 \pm 0.6	1.51	<0.01	Up
7	A0A1S3WE67, Complement C3	51.8 \pm 1.3	51.6 \pm 2.4	73.9 \pm 0.5	0.97	0.93	
8	A0A1S3W2D8, α 2-macroglobulin-like	60.5 \pm 1.7	71.6 \pm 2.5	87.9 \pm 1.6	1.18	<0.01	
9	A0A1S3WFY0, Haptoglobin	27.7 \pm 0.4	48.7 \pm 1.8	74.7 \pm 0.8	1.75	8.53	Up
10	A0A1S3A559, Fibrinogen chain α	29.7 \pm 1.8	22.9 \pm 0.45	0.11 \pm 0.03	0.77	<0.01	

Fibrinogen

The high level of the fibrinogen in tick midgut contents suggests that the midgut contents have the ability to clot immediately.

Serotransferrin

Laskay et al. (2013) monitored the mammalian proteins present in nymphal ticks and detected transferrin on the 29th day. Mori et al. (2014) reported that host-derived transferrin occurred in all developmental stages. Tick-derived transferrin was detected in the midgut and the ovary of adult females, but not in the eggs and the larvae, indicating that host-derived transferrin was not transferred to offspring. In addition, host-derived transferrin was located in the midgut digestive cells and the oocytes of engorged adult female ovaries, Mori et al. (2014) suggested that host-derived transferrin may be the main iron source in the ovary.

Alpha-2-macroglobulin and the complement C3

The alpha-2-macroglobulin and the complement C3 evolved from a common ancestor (Samonte et al. 2002). Both play an important role in innate immunity. The rest of the proteins including carbonic anhydrase 2, apolipoprotein A-I and haptoglobin were the serum components. It is unknown whether these proteins be digested and utilized by ticks.

Unspecified proteins (proteins that could not be differentiated as host-derived or tick-derived proteins)

The origin of polyubiquitin, EF- α , HSP90, tubulin alpha chain, tubulin beta chain, GTP-binding nuclear protein, H2B, valosin-containing protein, and GAPDH has not been determined because their peptides shared by the host and ticks. Intracellular proteins, such as polyubiquitin, EF- α , HSP90, tubulin alpha chain, tubulin beta chain, were detected in tick midgut contents, suggesting a breakdown of the midgut epithelium or the release of host red blood cells.

Compared to PEF, the content of tubulin alpha chain, tubulin beta chain, and GTP-binding nuclear protein was up-regulated and the histone H2B was down-regulated in FEF.

Quantitation of HSP70 and H2B

The HSP70 (Peptide sequence MG-26577 (SG-8235), peptide sequence MG-41014) identified as a tick-derived protein was not only detected in tick midgut contents but also in hedgehog serum. This may be because HSP70 is highly conserved. The peptide sequence MG-26577 (SG-8235) and peptide sequence MG-41014 were similar to hedgehog HSP70 (A0A1S3AMB4) and shared 75.2 and 78.1% identity. Based on the detected peptides used to identify HSP70, four (STAGDTHLGGEDFDNR, TTPSY-VAFTDTER, SQIHDIIVLVGGSTR, and VEIANDQGNR) were peptides common to ticks and hedgehogs. Only IINEPTAAALAYGLDK of *H. flava* is different with

IINEPTAAAAYGLDK of hedgehogs by a single amino acid. As the leucine and the isoleucine had the same molecular weight, the peptides matched to HSP70 were not specific. Therefore, the HSP70 was detected in host serum (Table 3).

Although the quantitative results showed that histone H2B was detected only in tick midgut contents. However, the evidence that the HAVSEGTK and LLLPGELAK identified in histone H2B were in the sequence of both the *H. flava* H2B protein and also in hedgehogs (UniProt accession number: A0A1S3WR78) cannot determine whether histone H2B was a tick-derived protein. Histone H2B was detected in tick midgut contents but not in host serum, probably because H2B is released extracellularly when blood cells are broken, or neutrophil extracellular traps are formed (Yang et al. 2016).

Conclusion

In-house protein libraries, inferred from the midgut and salivary gland transcriptomes of *H. flava*, were used in conjunction with the proteome to identify the midgut content proteins and to observe mRNA expression of midgut content proteins in midgut tissue and salivary gland tissue. The host serum was used as control group for excluding host protein interference with identification of the midgut content proteins. The profiles of high-confidence proteins of the midgut content were discovered. However, the midgut contents of *H. flava* were more complex than anticipated, and had a high percentage of host-derived proteins. These proteins present in the midgut content, such as Vgs, HSP70, serine protease, are associated with diverse biological functions in ticks including the transportation, anticoagulation, degradation of host erythrocyte membranes, detoxification, lipid metabolism and immunization. For example, the HSP70 involved as an anticoagulant. Vgs may play a role in transportation and the serine protease might be related to erythrolysis. These proteins play an important role in the digestion of tick blood meals.

Hemoglobin and carbonic anhydrase 2 released from the red blood cells lysed in gut lumen are basal elements of red blood cells (Sojka et al. 2013). A high content of host-derived proteins was detected in the midgut contents and these host proteins were mainly used as nutrients for tick development and oviposition. The study of tick midgut contents may help illuminate the mechanism of tick digestion and to aid in the screening for anti-tick antigens.

In the midgut contents, many highly conserved proteins were identified and the origin of these proteins could not be differentiated. These proteins can be intracellular and even nuclear and the shedding of the midgut epithelium and the ingestion of saliva may contribute to the midgut contents. Surprisingly, some proteins involved in the anti-haemostatic, anti-inflammatory and immunomodulatory functions such as serpin and lipocalin (Chmelar et al. 2011; Prevot et al. 2006; Sangamnatdej et al. 2002), which play an important role in tick saliva anti-haemostatic and anti-inflammatory activity (Radulović et al. 2014; Kazimířová and Štibrániová 2013), were not found in this study.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the national research committee and Hunan Agricultural University Institutional Animal Care and Use Committee.

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