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Expression pattern of subA in different tissues and blood-feeding status in *Haemaphysalis flava*

Lei Liu[1] · Tian-yin Cheng[1] · Fen Yan[1]

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Abstract Tick-borne-diseases (TBD) pose a huge threat to the health of both humans and animals worldwide. Tick vaccines constitute an attractive alternative for tick control, due to their cost-efficiency and environmental-friendliness. Subolesin, a protective antigen against ticks, is reported to be a promising candidate for the development of broad-spectrum vaccines. However, the entire length of its gene, subA, and its gene expression pattern in different tissues and blood-feeding status (or different levels of engorgement) have not been studied extensively. In our study, the full-length of subA in *Haemaphysalis flava, Rhipicephalus haemaphysaloides*, *Rhipicephalus microplus,* and *Dermacentor sinicus* was cloned by RACE–PCR. The subA expression pattern was analyzed further in *H. flava* in different tissues and blood-feeding status by RT-PCR. We found that the full-length of subA in *H. flava*, *R. haemaphysaloides, R. microplus,* and *D. sinicus* was 1318, 1498, 1316, and 1769 bp, respectively, with encoded proteins at 180, 162, 162, and 166 aa in length, respectively. The primary structure of subolesin in *H. flava* included three conserved regions and two hypervariable regions, with no signal peptide. SubA expression in female *H. flava* of different blood-feeding status was in the order of the fasted $<$ the 1/4-engorged $<$ the half-engorged $<$ the fully-engorged ($p < 0.01$). Tissue expression of subA was in the order of salivary gland $>$ midgut $>$ integument ($p < 0.01$), but its expression in salivary glands was not statistically different from that in ovaries. We concluded that subolesin was a conserved antigen and that subA was expressed differentially in *H. flava* in different tissues and blood-feeding status. Those features made subolesin feasible as a potential target antigen for development of a universal vaccine for the control of tick infestations and a reduction in TBD.

Keywords Tick · Subolesin · SubA · RACE · Vaccine

✉ Tian-yin Cheng
hn5368@163.com

[1] College of Veterinary Medicine, Hunan Agricultural University, Changsha, Hunan 410128, China

Introduction

Ticks are hematophagous ectoparasites of multiple classes of arthropods, which can cause irritation, emaciation, anemia, and paralysis in host animals. Ticks are known to be competent vectors that transmit a variety of pathogens, such as viruses, bacteria, protozoa, fungi, and helminths. TBD have become a great threat to the health of both animals and humans throughout the world (e.g., tick-borne-encephalitis (TBE), spotted fever, babesiosis, Lyme disease, and anaplasmosis; Baneth 2014). Recently, novel TBD have been discovered. Since 2007, more than 279 cases of the severe fever known as thrombocytopenia syndrome have been reported in Henan, Zhejiang, and Shandong, China (Wang et al. 2016; Ye et al. 2015), which indicates that the disease now has a wide distribution; bunyavirus, which was isolated from ticks, was shown to contribute to the disease (Xu et al. 2011).

Thus far, tick control strategies have been implemented by applying acaricides. However, application of acaricides tends to cause selection of acaricide-resistant ticks, environmental pollution, and drug residues in milk and meat products (Graf et al. 2004). Thus, it is urgent to seek safer, more effective, and environmentally-friendly alternatives (Liu et al. 2014); vaccination has emerged as one of the viable alternatives (de la Fuente et al. 2013). The efficiency of vaccines that use antigens Bm86 and Bm91 from *R. microplus* (Rand et al. 1989; Willadsen et al. 1996) have provided a promising way to develop vaccines against ticks. However, vaccine trials showed various levels of efficacy among different strains of *R. microplus* and among closely related tick species, which suggested that ticks exhibit genetic differences in the susceptibility to Bm86 vaccination (Canales et al. 2009). Lacking broad-spectrum vaccinations against ticks awaits screening of possible antigen candidates that would be expressed in all classes of ticks.

Subolesin (previous known as 4D8) was recognized and cloned originally from a cDNA library of *Ixodes scapularis* (Almazan et al. 2003). It was reported to be effective in reducing vectorial capacity and fertility of *I. scapularis*, *Dermacentor variabilis*, *Dermacentor marginatus*, *Amblyomma americanum,* and *Rhipicephalus sanguineus* (de la Fuente et al. 2006b) and, hence, subolesin was considered to be a tick protective antigen that might be of significance in developing universal vaccines for the control of ticks (de la Fuente et al. 2013).

To obtain more information on subolesin, we cloned the full-length of the subolesin gene, subA, in *H. flava*, *R. haemaphysaloides*, *R. microplus,* and *D. sinicus* by RACE-PCR. In particular, we analyzed the subA expression pattern further in different tissues and feeding status of *H. flava* using RT-PCR.

Materials and methods

Tick collection

Haemaphysalis flava, *R. haemaphysaloides*, *R. microplus,* and *D. sinicus* were collected from hedgehogs, goats, or dogs in Xinyang, Henan province (E114°06′, N31°125′), Hengyang, Hunan province (E110°32′, N26°07′), Tongren, Guizhou province (E107°45′, N27°07′), and Taigu, Shanxi province (E111°23′, N36°39′).

Total RNA extraction

RNA was extracted by EasyPure RNA Kit (Transgen Biotech, Beijing, China) from whole ticks or dissected tick organs in the four species of ticks listed above. The quality of total RNA was assessed by an A260 to A280 ratio by a NanoDrop ND-2000 (Thermo Scientific, Waltham, MA, USA) and the quantity of RNA was evaluated by an Agilent Bioanalyzer 2100 (Agilent, CA, USA). cDNA was synthetized by following the manual of TransScript All-in-One First-Strand cDNA Synthesis SuperMix for PCR (Transgen Biotech).

Cloning the open reading frame (ORF) of subA

ORF-F (5′-ATGGCTTGTGCGACATTAAAGC-3′) and ORF-R (5′-GCTACGCCCAGC-TATTTGTCGT-3) were used as primers. PCR was performed at 94 °C for 5 min followed by 30 cycles that included denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s, and an extension at 72 °C for 30 s for each cycle. A final extension at 72 °C for 7 min was included also.

RCR products were electrophoresed in 1.5 % agarose. Target bands were recovered by a gel extraction kit (Takara, Dalian). Ligation and transformation of PCR products were performed according to the manual for the kit. Positive clones were selected and sequenced by Sangon (Shanghai, China).

Rapid amplification of cDNA ends (RACE) for subA

3′ and 5′ RACE were employed to clone full-length of subA. Primers were designed based on conserved nucleic acid sequences that were revealed by a multiple sequence alignment of annotated subolesin cDNA sequences (EU301808, EU326280, DQ159968, JX856138) from closely related tick species. Gene-specific primers (GSP) for 3′-RACE and 5′-RACE were designed according to the amplified ORF of subA (Table 1).

A 1.5 µl sample of RNA from fully-engorged female ticks was used and first-strand cDNA was synthesized by 3′-Full RACE Core Set with PrimeScript™ Rtase (Takara, Dalian). The first round of amplification used 3′ RACE-GSP1 and 3′ RACE-outerP as primers (Table 1) and first-strand cDNA was used as templates through 20 cycles. Conditions were as follows: initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 50 s, and a final extension at 72 °C for 10 min. The second round of amplification was completed with 3′ RACE-GSP2 and 3′ RACE-innerP as primers. Conditions were as follows: 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 64.5 °C for 30 s, 70 °C for 50 s, and a final extension at 72 °C for 10 min.

Dephosphorylation, decap-reaction, addition of 5′-RACE adaptor, and reverse transcription were done according to the manual of the SMARTer™ RACE cDNA Amplification Kit (Takara, Dalian). 5′ RACE-GSP1 and 5′ RACE-outerP were used as primers (Table 1). Conditions were as follows: 94 °C for 3 min, 20 cycles at 94 °C for 30 s, 64 °C for 30 s, 72 °C for 40 s, and 1 cycle at 72 °C for 10 min. The second round of amplification was performed with 5′ RACE-GSP2 and 5′ RACE-innerP as primers. Conditions were as follows: 94 °C for 3 min, 26 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and 1 cycle at 72 °C for 10 min.

A phylogenetic tree of subA was also plotted using software MEGA 5.0 based on available mRNA sequences of multiple tick species in Genbank. The signal peptide of subolesin was analyzed in http://www.cbs.dtu.dk/services/SignalP/.

Table 1 The design of RACE Primers

Primer	Sequence (5′ → 3′)	T_m (°C)	Ticks
3′ RACE-GSP1 (Bm)	CGTGCCACCAAAGTTGACT	55.5	*R. microplus*
3′ RACE-GSP2 (Bm)	CGAACGAATGATGAAGGAGCGAG	60.0	
3′ RACE-GSP1 (Rh)	CGTGCCACCAAAGTTGACT	55.5	*R. haemaphysaloides*
3′ RACE-GSP2 (Rh)	AGGAGCGAGAGAGCAAGATACG	60.0	
3′ RACE-GSP1 (Hf)	CGAAGATGTATGCCTTTGTCG	56.0	*H. flava*
3′ RACE-GSP2 (Hf)	AAGCTGGCCGAGCAGTACGAC	64.0	
3′ RACE-GSP1 (Ds)	CGTGCCACCAAAGTTGACT	55.5	*D. sinicus*
3′ RACE-GSP2 (Ds)	GCTCGCAGAACAATACGACACATTTG	60.0	
3′ RACE-outerP	TACCGTCGTTCCACTAGTGATTT	–	All four ticks
3′ RACE-innerP	CGCGGATCCTCCACTAGTGATTTCAC TATAGG	–	
5′ RACE-GSP1 (Bm)	CCCGTATCTTGCTCTCTCGCTCCTTC	65.0	*R. microplus*
5′ RACE-GSP2 (Bm)	TCGCAAATGAGCCCAACCT	58.0	
5′ RACE-GSP1 (Rh)	GCTCTCTCGCTCCTTCATCATCCGT	65.0	*R. haemaphysaloides*
5′ RACE-GSP2 (Rh)	TCGCAAATGAGCCCAACCT	58.0	
5′ RACE-GSP1 (Hf)	GCTCCTTCATCATCCGCTCGCAG	64.0	*H. flava*
5′ RACE-GSP2 (Hf)	AGGCATACATCTTCGTCGTTTCGG	60.0	
5′ RACE-GSP1 (Ds)	GCGTAGCACCCTCAAACCGCTTCT	64.0	*D. sinicus*
5′ RACE-GSP2 (Ds)	AAGCAGTGGTATCAACGCAGAGT	58.0	
5′ RACE-outerP	CATGGCTACATGCTGACAGCCTA	–	All four ticks
5′ RACE-innerP	CGCGGATCCACAGCCTACTGATG ATCAGTCGATG	–	

SubA expression in *H. flava* in different tissues and blood-feeding status

Fully-engorged and 1/2-engorged ticks were dissected, and three replicates each of salivary glands, ovaries, midguts, and integument were isolated carefully. Total RNA was extracted from whole ticks and also from different organs. The purity and density of total RNA were tested by UV-spectrophotometry. cDNA was synthesized according to the manual of the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian) with total RNA at 0.70 μg. Standard curves were plotted based on serial dilution of cDNA from female adult *H. flava*. Dissociation curves for subA and house-keeping gene β-actin were plotted also in a regular way. RT-PCR was manipulated using SYBR® Premix Ex Taq™ (Takara, Dalian), and an ABI7300 (Applied Biosystems, Waltham, MA, USA) was employed to do quantitative analysis. The conditions of RT-PCR were as follows: initial denaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, annealing at 60 °C for 60 s, and amplification for 40 cycles.

The $2^{-\triangle\triangle Ct}$ method was used for assessment and comparison of subolesin expression in different tissues and blood-feeding status. Normalized expression of subA was compared among different tissues and different feeding status using Student's test.

Results

RNA extraction

A260/A280 of RNA extracts were 1.84, 1.98, 1.98, and 2.00 in *H. flava*, *R. haemaphysaloides*, *R. microplus,* and *D. sinicus,* respectively. RNA extracts were analyzed further using an Agilent Bioanalyzer 2100 (Fig. 1). There were two major bands at 2000 and 25 nt, respectively.

Cloning the ORF of subA

The ORF of subA were 540, 486, 486, and 498 bp in *H. flava*, *R. haemaphysaloides*, *R. microplus,* and *D. sinicus,* respectively (Fig. 2).

Cloning the full-length of subA

The 3′ of subA were 614, 975, 971 and 1216 bp (Fig. 3) and the 5′ of subA were 457, 553, 464 and 313 bp in *H. flava*, *R. haemaphysaloides*, *R. microplus*, and *D. sinicus* (Fig. 4), respectively. After reassembly of the complete gene sequence, the full-length of subA were 1318 bp (KJ829652), 1498 bp (KM115650), 1316 bp (KM115649), and 1769 bp (KM115651) in *H.*

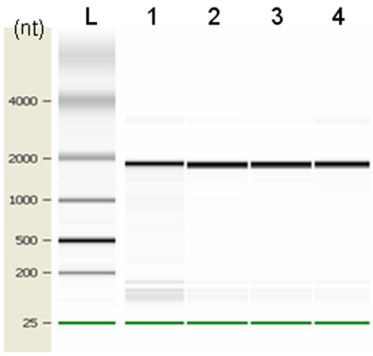

Fig. 1 Electrophoresis of total RNA. (1— *H. flava*; 2— *R. haemaphysaloides*; 3— *R. microplus*; 4— *D. sinicus*; L. Ladder)

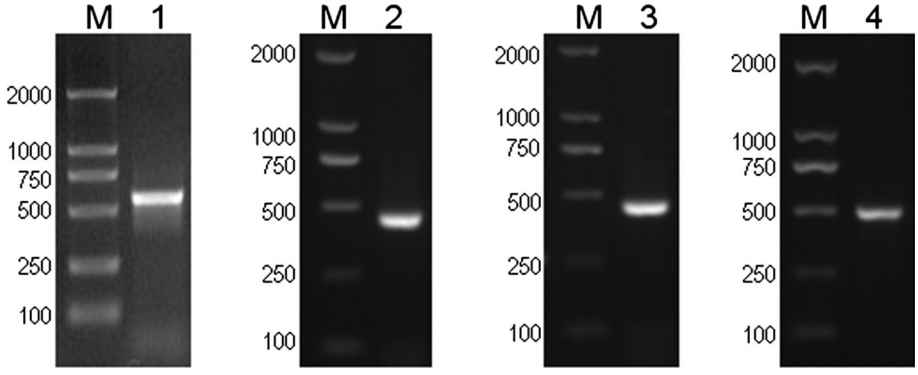

Fig. 2 Electrophoresis for amplified products of ORF of subA. (1— *H. flava*; 2— *R. haemaphysaloides*; 3— *R. microplus*; 4— *D. sinicus*; M. Marker)

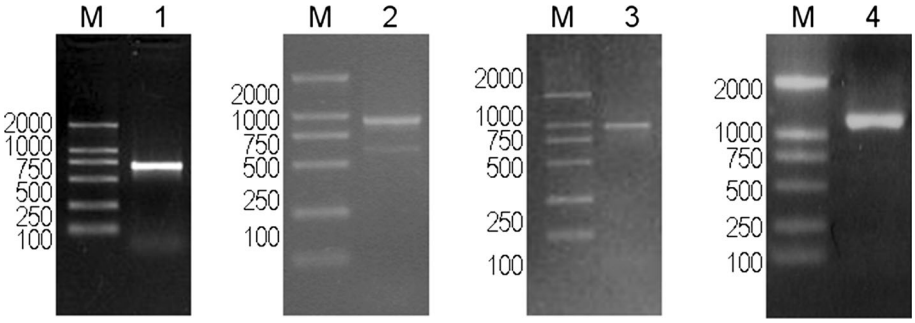

Fig. 3 Electrophoresis for amplified products of 3′ RACE. (1— *H. flava*; 2— *R. haemaphysaloides*; 3— *R. microplus*; 4— *D.sinicus*; M. Marker)

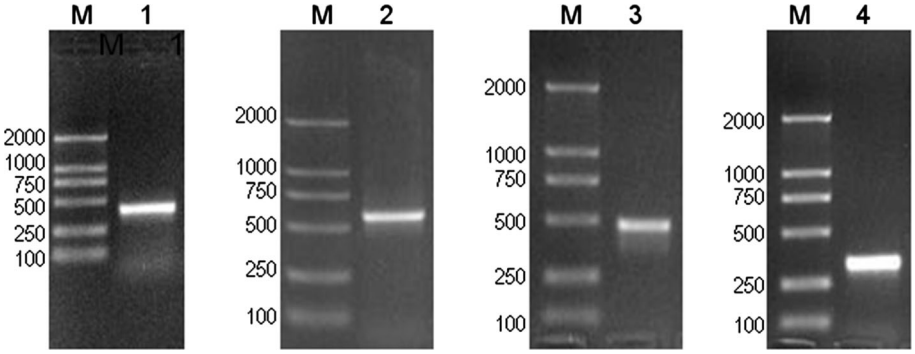

Fig. 4 Electrophoresis for amplified products of 5′ RACE. (1— *H. flava*; 2— *R. haemaphysaloides*; 3— *R. microplus*; 4— *D.sinicus*; M. Marker)

flava, *R. haemaphysaloides*, *R. microplus*, and *D. sinicus*, respectively. Hence the subolesin encoded were 180, 162, 162, and 166 aa accordingly.

Analysis of ORF of subA and subolesin encoded

The DNA sequence of four species of ticks were compared with that of the other 25 species of ticks in Genbank. The ORF of subA were highly conservative, especially at the 5′ end and the 3′ end. The ORF of subA included three conservative regions and two hypervariable regions. In addition, the conservative regions showed more similarity within the same genus.

The phylogenetic tree showed species of *Dermacentor* had an individual monophyletic clade and the other classes of ticks shared a monophyletic clade, including species of *Rhipicephalus*, *Amblyomma*, *Haemaphysalis*, *Ixodes*, and *Hyalomma* (Fig. 5).

Based on the sequence of the known 29 species of ticks, we concluded that the primary structure of the protein encoded was also highly conservative (Fig. 6). The 30 amino acid residues in the amino terminal, the 43 amino acid residues in the middle, and the 57 amino acid residues in the carboxyl terminal were identical. Furthermore, the first 17 amino acid residues were identical, all in the form of MACATLKRTHDWDPLHSP. There were two classes of amino acid sequences in the carboxyl terminal; one sequence ended with

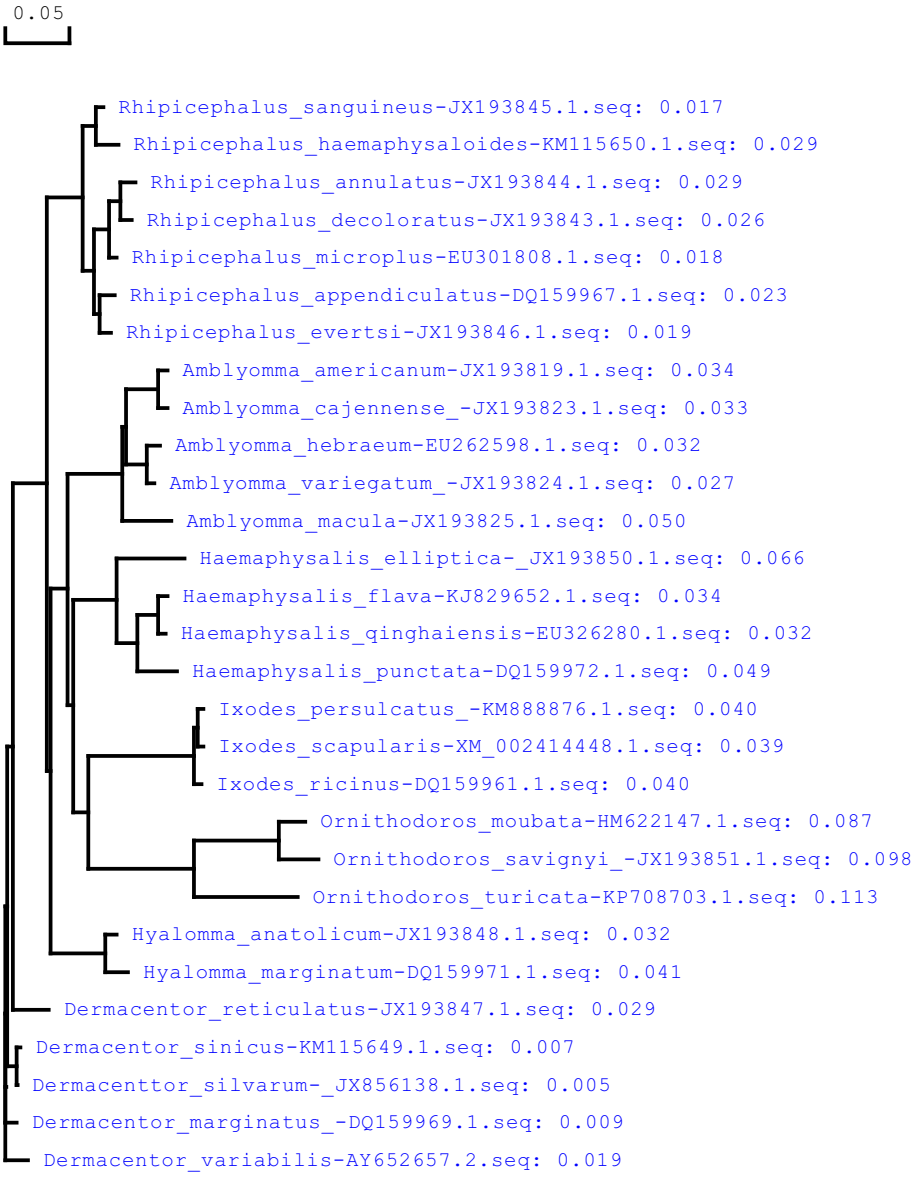

Fig. 5 Phylogenetic tree of subA for various tick species based on nucleotide sequences. A phylogenetic tree of subA was constructed by software MEGA 5.0 based on available mRNA sequences of multiple tick species in Genbank. The accession numbers of subA sequences from multiple species of ticks are shown after their names

KLAEQYDTFVKFTYDQ, and the other sequence ended with DQIQKRFEGATPSYLS following KLAEQYDTFVKFTYDQ. Accordingly, amino acid sequences in hypervariable regions differed greatly.

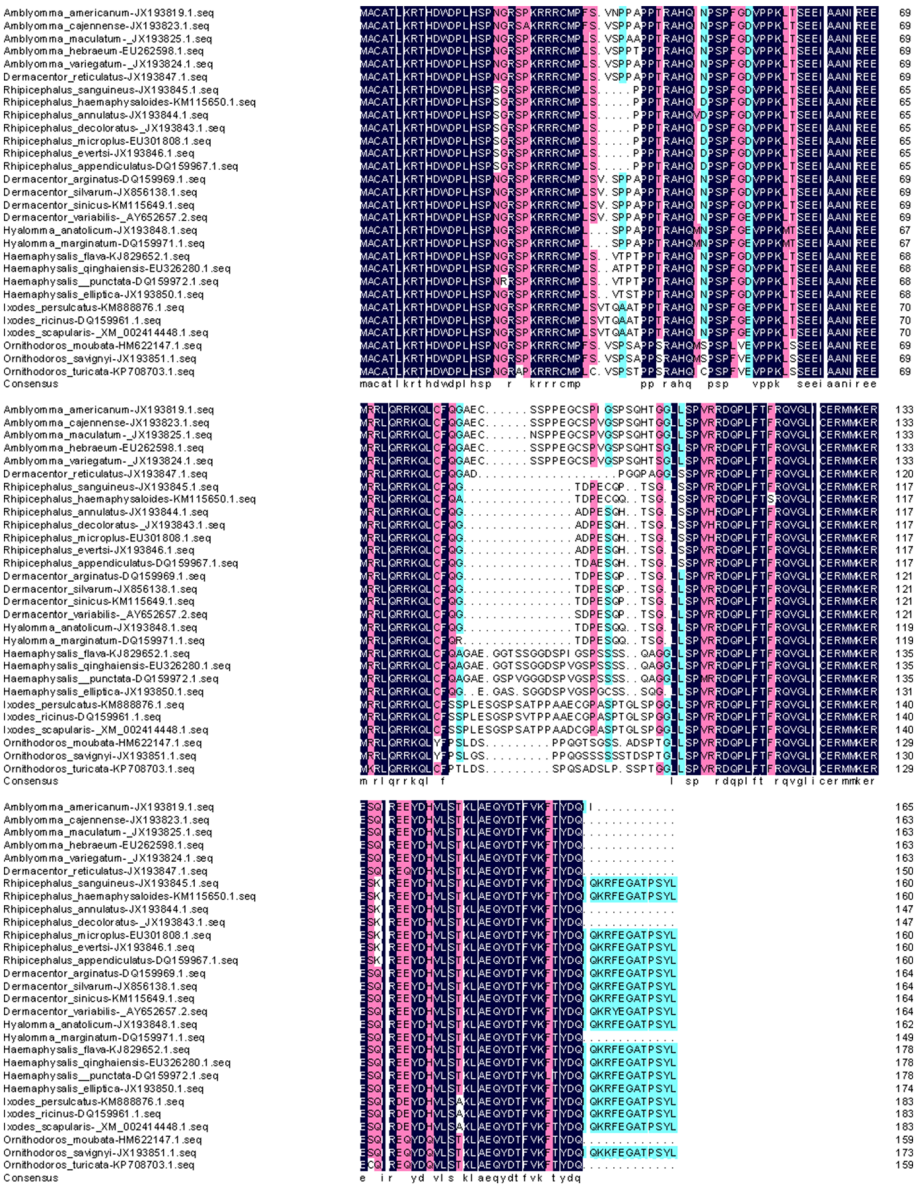

Fig. 6 Multiple alignment of subA amino acids sequences in ticks. The predicted amino acid sequences of four species of ticks were compared with that of an additional 25 species of ticks in Genbank. The accession numbers of subA sequences from multiple species of ticks are shown after their names

SubA expression in *H. flava* in different tissues and blood-feeding status

A standard curve was made based on a fivefold serial dilution of cDNA templates. Standard slopes of the target gene and β-actin were −3.39 and −3.34, and correlation coefficients were 0.999 and 0.998, respectively (Fig. 7). Amplification efficiency of the target

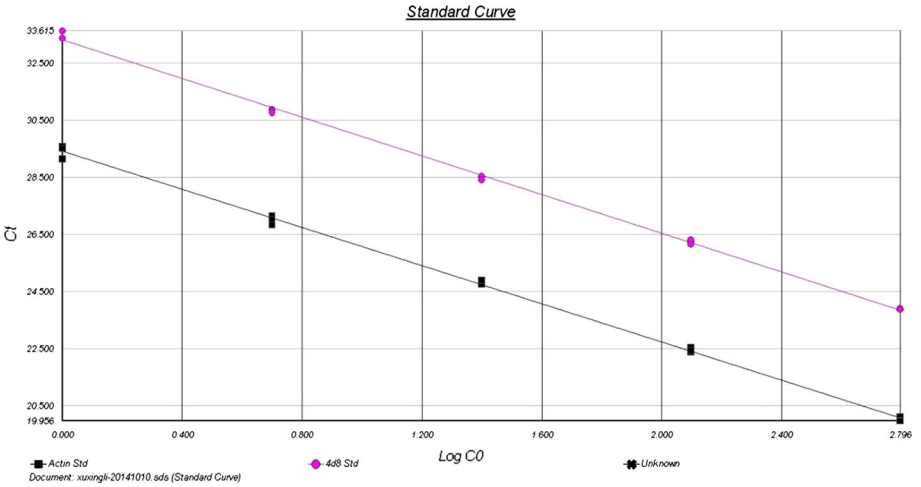

Fig. 7 The standard curves of subA and β-actin. Standard curves were made based on a fivefold serial dilution of cDNA templates. Standard slopes of the target gene and β-actin were −3.39 and −3.34, and correlation coefficients were 0.999 and 0.998, respectively

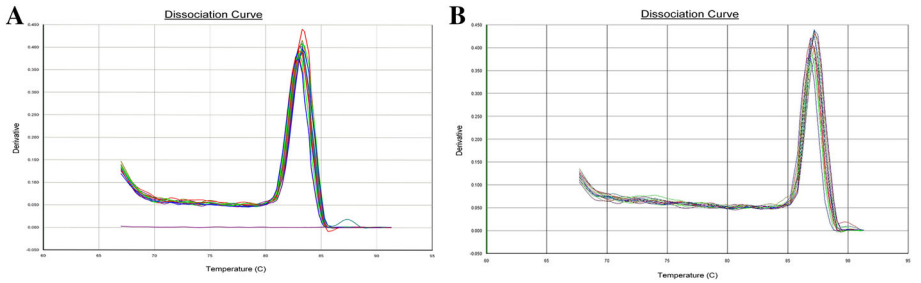

Fig. 8 The dissociation curves of subA (**a**) and β-actin (**b**). T_m of the target gene and β-actin were 83.2 and 87.2 °C, respectively

gene and β-actin were estimated to be 97.3 and 99.4 %, respectively. T_m of the target gene and β-actin were 83.2 and 87.2 °C, respectively, based on the dissociation curves (Fig. 8).

Expression of subA was associated positively with the amount of blood consumed by ticks, because fully-engorged ticks had the highest expression ($p < 0.01$) (Fig. 9a). Tissue expression of subA was in the order of salivary gland > midgut > integument ($p < 0.01$), but its expression in the salivary gland was not statistically different from that in the ovary (Fig. 9b).

Discussion

Almazánet al. (2003) screened subolesin initially from a cDNA library of *I. scapularis* and the author obtained its partial gene sequence. Further studies showed that subolesin was a conservative antigen with a mRNA full-length of 2693 bp, an ORF length of 555 bp, and a coded protein length of 184 aa (mw 20.7 kDa); in addition, the animals that were

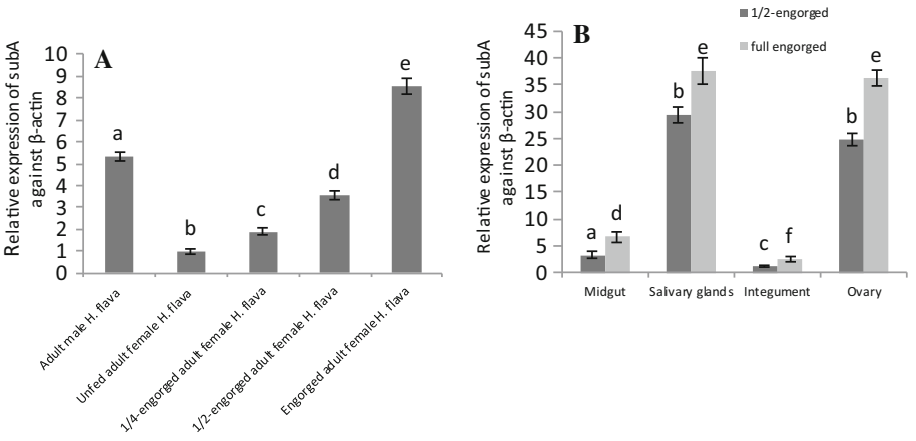

Fig. 9 The expression of subA in *H. flava* of different blood-feeding status (**a**) and tissues (**b**) by RT-PCR. SubA expression in different feeding status and tissues was compared by Student's *t* test. Data not sharing a common letter in (**a**) indicated there was a significant difference regarding total subA expression in *H. flava* of different blood-feeding status ($p < 0.01$). Data within the same tissue not sharing a common letter in (**b**) indicated there was a significant difference ($p < 0.01$). Data not sharing a common letter among different tissues indicated there was a significant difference ($p < 0.01$)

immunized with recombinant protein of subolesin showed reduced tick infections, tick egg production, and tick vitalities (Almazan et al. 2005; de la Fuente et al. 2006a, b, c). However, the full-length and expression pattern of subolesin in other ticks has not been studied so far. Thus, our study aimed to clone the full-length of subA of the primary species of ticks found near/in Hunan Province, China and to investigate its expression in different blood-feeding status and tissues of *H. flava*.

Only 18 s rRNA band was found after total RNA extraction. However, based on the OD260/OD280 and our years of experience, it was unlikely due to any error during RNA extraction. It was deduced that there was also a "hidden break" in 28 s RNA of ticks as well as in other animals like protozoans, coelenterates, platyhelminthes, nemathelminthes, and some arthropods (Ishikawa 1977). At 40–60 °C, the hydrogen bond that links two fractions of 28 s rRNA break into two fractions. In bees, those two fractions were 1900 and 2000 nt, which was similar to the length of 18 s rRNA (Winnebeck et al. 2010).

The present study succeeded in cloning the full-length of subA in *H. flava*, *R. haemaphysaloides*, *R. microplus*, and *D. sinicus*, which were 1318, 1498, 1316 and 1769 bp, respectively. As far as we know, there was only one full-length of subA mRNA of *I. scapularis* in GenBank before our study. After conducting a similarity comparison of the ORF of subA among 29 species of ticks, we found that ORF of subA was highly conservative, especially at the 5′ end and the 3′ end, which made cloning of subA from other ticks possible. However, we also found that certain regions in ORF of sub A were hypervariable. Those sequences could be used to identify different genera of ticks.

Compared to the nucleic acid sequence, the primary structure of the subolesin protein showed greater similarity, especially within the same genus. The putative protein consisted of three conservative regions and two hypervariable regions, without any known signal peptide as revealed by bioinformatics methods. Also, conservative regions were much wider than hypervariable regions. Secondary structure of the putative protein showed major irregular coils and a-helixes. Our findings were thus consistent with studies by Fuente and other researchers (de la Fuente et al. 2006a, b, 2013; Goto et al. 2008), who also

reported that the subolesin protein was highly conservative. However, given the existence of hypervariable regions, the conserved sequence of subolesin should be used to develop a universal vaccine.

The total expression of subA was associated positively with the amount of blood volume in the tick; fully-engorged ticks showed the highest expression. Compared with half-engorged ticks, the subA expression in salivary glands, midguts, ovaries, and integument were higher in fully-engorged ticks ($p < 0.01$). Yu (2015) reported that in adult *R. haemaphysaloides*, expression of subA was higher in half-engorged ticks than in ticks that had fasted, which was also consistent with our results. Our results were also similar to studies that showed a reduced blood uptake in subolesin-challenged ticks, which indicated that subolesin might be related closely to blood feeding (Bensaci et al. 2012; de la Fuente et al. 2006a; Lu et al. 2016).

As we showed, subA expression in the salivary glands and ovaries were higher than in midguts and integument of both engorged and half-engorged ticks ($p < 0.01$). Luet al. (2016) also reported a high level of subA transcription in salivary glands and ovaries in *R. haemaphysaloides*, which was similar to our study, but its transcriptional level in the midgut and integument was consistently high and not different from that in the salivary gland and ovary (Lu et al. 2016). It was possible that subolesin expression varied between tick species (Zivkovic et al. 2010). The subA expression pattern in the ovary might explain why ticks that sucked blood from recombinant, subolesin immunized hosts showed reduced fertility: perhaps, antibodies that bound with subolesin blocked its specific physiological function in the ovary. In fact, one of the criteria for an effective vector-protective antigen was that the formation of the antibody-antigen complex should disrupt the normal function of the vector protein (Elvin and Kemp 1994). Because salivary glands and their secretions played a critical role in pathogen transmission (Liu et al. 2014), related proteins in salivary glands were considered to be potential target antigens for vaccine development. Previous studies revealed that ticks submitted to RNA interference of subA had a lower intake of blood (de la Fuente et al. 2006a). Together with our data, it seems possible that subolesin, in addition to its function in the regulation of innate immunity and gene expression in vectors, was involved in the regulation of feeding of ticks directly or indirectly. However, those assumptions remain to be confirmed by trials in vivo.

Our results showed that subolesin, as well as its gene subA, were both conservative among tick species that we tested. SubA expression in *H. flava* was higher after a blood meal. In addition, subA expression was higher in salivary glands and ovaries than in any other tissues that we tested. In conclusion, our data further indicated that subolesin is an ideal candidate as an antigen target for development of a universal vaccine for the control of ticks.

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