Published in final edited form as:

Ticks Tick Borne Dis. 2018 March ; 9(3): 506–518. doi:10.1016/j.ttbdis.2018.01.001.

Disruption of blood meal-responsive serpins prevents *Ixodes scapularis* from feeding to repletion

Mariam Bakshi, Tae Kwon Kim, and Albert Mulenga*

Department of Veterinary Pathobiology, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, 422 Raymond Stotzer, TAMU 4467, College Station, TX 77843, USA

Abstract

Serine protease inhibitors (serpins) are thought to mediate the tick's evasion of the host's serine protease-mediated defense pathways such as inflammation and blood clotting. This study describes characterization and target validation of 11 blood meal-responsive serpins that are associated with nymph and adult *Ixodes scapularis* tick feeding as revealed by quantitative (q)RT-PCR and RNAi silencing analyses. Given the high number of targets, we used combinatorial (co) RNAi silencing to disrupt candidate serpins in two groups (G): seven highly identical and four non-identical serpins based on amino acid identities, here after called GI and GII respectively. We show that injection of both GI and GII co-dsRNA into unfed nymph and adult *I. scapularis* ticks triggered suppression of cognate serpin mRNA. We show that disruption of GII, but not GI serpins significantly reduced feeding efficiency of both nymph and adult *I. scapularis* ticks. Knockdown of GII serpin transcripts caused significant respective mortalities of 40 and 71% of nymphal and adult ticks that occurred within 24–48 h of attachment. This is significant, as the observed lethality preceded the tick feeding period when transmission of tick borne pathogens is predominant. We suspect that some of the GII serpins (S9, S17, S19 and S32) play roles in the tick detachment process in that upon detachment, mouthparts of GII co-dsRNA injected were covered with a whitish gel-like tissue that could be the tick cement cone. Normally, ticks do not retain tissue on their mouthparts upon detachment. Furthermore, disruption of GII serpins reduced tick blood meal sizes and the adult tick's ability to convert the blood meal to eggs. We discuss our data with reference to tick feeding physiology and conclude that some of the GII serpins are potential targets for anti-tick vaccine development.

Keywords

Ixodes scapularis; tick serpins; target validation; tick feeding physiology; combinatorial RNAi silencing

*Author for correspondence: amulenga@cvm.tamu.edu, Phone: 979 458 4300.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Ticks are among the most important ecto-parasites with global public health and veterinary health impact. In terms of the diversity of transmitted animal and human disease pathogens, ticks are the second most important vectors after mosquitoes (Jongejan and Uilenberg, 2004). In public health, the impact of tick-borne diseases (TBD) has been on the rise since the description of *Borrelia burgdorferi* as the causative agent of Lyme borreliosis vectored by *I. scapularis* (Burgdorfer et al., 1982; 1989; Burgdorfer, 1984). *I. scapularis* and its sister species, *I. ricinus*, *I. pacificus*, and *I. holocyclus* are major vectors of human TBD agents in North America, Europe and Australia (Davis et al., 2015; Piesman and Stone, 1991; Rizzoli et al., 2014). Currently, the US Centers for Disease Control (<http://www.cdc.gov/ticks/diseases/>) has listed 16 reportable human TBDs agents, six of which are transmitted by *I. scapularis*. Other *I. scapularis* vectored agents include the newly described Lyme disease agent, *B. mayonii* (Dolan et al., 2015, 2016; 2017; Pritt et al., 2016; Telford et al., 2015), *B. miyamotoi*, closely related to tick relapsing fever causative agents (Breuner et al., 2017), *Anaplasma phagocytophilum* (animal and human anaplasmosis, Levin and Ross, 2004), *Babesia microti* (Prusinski et al., 2014), Powassan encephalitis virus (Dupuis II et al., 2013), and *B. odocoilei* (cervid babesiosis) (Steiner et al., 2006). On the west coast of the United States, *I. pacificus* is the primary vector for Lyme disease and human anaplasmosis agents (Burgdorfer et al., 1985; Dahlgren et al., 2015). The importance of *I. scapularis* in public health justified sequencing of the full genome (Gulia-Nuss et al., 2016; Hill and Wikel, 2005). Availability of the *I. scapularis* genome sequence data provided for opportunities for in depth studies to understand tick feeding physiology as means through which to find out ways to design better tick control strategies.

In absence of effective vaccines against TBD agents, killing ticks by chemical acaricides is currently the only reliable alternative method to protect against animal and humans TBD infections (Cisak et al., 2012; Drummond, 1976). The limitations of the current acaricide-based tick control methods including development of resistance against these chemicals and environmental contaminations are known (Abbas et al., 2014; Coles and Dryden, 2014; George et al., 2004; Graf et al., 2004). Immunization against tick feeding is a validated sustainable alternative tick control method (de la Fuente et al., 2015; Mulenga et al., 2001; Willadsen et al., 1995; Willadsen, 2004). The major limitation toward the global adoption of anti-tick immunization as an alternative tick control method is the lack of effective tick vaccine antigens. Effective vaccine antigens could confer better anti-tick protection levels that are comparable to acaricide-based tick control, and this would result to broader acceptance of tick vaccines as a tick control strategy. As transmission of animal and human TBD infections primarily occur via tick feeding (Liu and Bonnet, 2014), understanding the molecular basis of tick feeding physiology is likely to lead to discovery of effective tick vaccine target antigens. We are interested in understanding the role(s) of tick serine protease inhibitors (serpins) in tick feeding physiology.

The tick feeding style of lacerating host tissue and sucking up blood that bleeds into the wounded area is expected to provoke host defenses such as coagulation, inflammation, complement activation, all of which are serine protease mediated pathways that are under tight control of serpins (Gettins, 2002; Huntington, 2011; Olson and Gettins, 2011). Serpin

malfunctions that lead to human diseases, emphysema, thrombosis, and angioedema (Carrell and Lomas, 2002; Carrell and Travis, 1985) attest to the importance of serpins. It is possible that, similar to other organisms, serpins play important role(s) in tick feeding physiology and if disrupted could reduce tick-feeding efficiency. On this basis, ticks were suspected to inject serpins into the host to evade host defense to tick feeding and thus this family of proteins represent important candidate tick vaccine antigens (Mulenga et al., 2001). Several serpin encoding cDNAs have been cloned and characterized in several tick species (Imamura et al., 2005; Kaewhom et al., 2007; Lebouille et al., 2002; Mulenga et al., 2003; Tirloni et al., 2016; Toyomane et al., 2016; Yu et al., 2013) including at least 45 in *I. scapularis* (Mulenga et al., 2009). Some of the tick serpins are functional inhibitors of protease mediators of host defense pathways including blood clotting, inflammation, and complement activation (Chmelar et al., 2011; Kim et al., 2015b; Ibelli et al., 2014; Mulenga et al., 2013a; Radulovi and Mulenga, 2017; Prevot et al., 2006; Rodriguez-Valle et al., 2015). In other studies, immunization of animals with recombinant tick serpins reduced tick-feeding efficiency (Imamura et al., 2005; Imamura et al., 2006; Imamura et al., 2008; Jittapalapong et al., 2010; Kim et al., 2016; Prevot et al., 2007; Sugino et al., 2003)

The goal of this study was to characterize the roles of 11 blood meal-responsive serpins in *I. scapularis* tick feeding. These serpins were first described among 45 *I. scapularis* serpin sequences (Mulenga et al., 2009). Semi-quantitative RT-PCR expression analysis showed that some of the 11 candidate serpins were apparently up regulated during adult *I. scapularis* tick feeding, suggesting a role in regulating tick feeding. In this study, we have used quantitative RT-PCR expression analysis to relate the transcriptional profiles of candidate serpins to different stages of nymph and adult *I. scapularis* tick feeding. Target validation using the combinatorial RNAi silencing approach was successfully used to validate that four of the 11 *I. scapularis* serpins are important to nymph and adult *I. scapularis* tick feeding and thus represent potential target antigens for anti-tick vaccine development.

2. Materials and Methods

2.1 Ethics statement

All experiments were done according to the animal use protocol approved by Texas A&M University Institutional Animal Care and Use Committee (IACUC) (AUP 2011–207 and 2011–189, 2014-0310) that meets all federal requirements, as defined in the Animal Welfare Act (AWA), the Public Health Service Policy (PHS), and the Humane Care and Use of Laboratory Animals.

2.2 Phylogeny and Comparative sequence analyses

The Neighbor joining method in MacVector (MacVector Inc., Cary, North Carolina) was used to construct the phylogeny tree of the 11 candidate *I. scapularis* serpins (Mulenga et al., 2009) and 15 other tick serpin amino acid sequences that were downloaded from GenBank and Uniprot databases (Table 1). Specifications were set to the Poisson correction of amino acid substitution model and calculation of bootstrap values was set at a 1000 replications. Human alpha-anti trypsin (Accession number: AAB59495) was used as outgroup in the phylogeny tree. Pairwise sequence alignments to determine amino acid identity levels

between sequences that segregated together in the phylogeny tree was done using ClustalW in MacVector software (MacVector Inc., Cary, North Carolina).

2.3 Tick feeding, dissections, total RNA extraction, and cDNA synthesis

I. scapularis nymph and adult ticks used in this study were obtained from BEI resources or purchased from the tick laboratory at Oklahoma State University (Stillwater, OK). Ticks were fed on New Zealand white rabbits. Ticks were restricted to feed on top of the rabbit ear using the two-inch cotton stockinet tick containment cells that were glued onto rabbit ears using the Kamar adhesive (Kamar Products Inc., Zionsville, IN) as described (Kim et al., 2016). To feed nymphs, the inside of the stockinet was lined with pantyhose material to prevent nymphal ticks from escaping. For adult ticks, females were pre-mated prior to feeding. To mate, ticks were placed into a petridish on ice and we visually monitored for paired male and female ticks, which were subsequently placed into a separate container to complete mating.

Dissections of adult tick salivary glands (SG), midgut (MG) and carcass (tick remnant after removal of SG and MG) of unfed and ticks that were partially fed for 24, 48, 72, 96 and 120 h was done as described (Kim et al., 2015b). Both unfed and partially fed ticks were washed in diethylpyrocarbonate water prior to dissection. Total RNA extraction was done using the TRIzol total RNA extraction reagent according to manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Approximately 200 μ L Chloroform was added per 1 mL of TRIzol and aqueous phase was separated by centrifugation at 12000 x g at 4°C. Total RNA was precipitated from aqueous phase using isopropanol, washed with 70% ethanol and total RNA was solubilized in diethylpyrocarbonate water. Adult tick total RNA was extracted from organs pooled of five ticks in three replicates. Tick organs were lysed in TRIzol by pipetting up and down followed by sonication using tissue dismembrator (Thermo Fisher Scientific, Waltham, MA). For nymphs, total RNA was extracted from pools of ten unfed nymphs and those that were fed for 24, 48, and 72 h. Nymphs were minced using a pair of soft tissue scissors directly in TRIzol. Total RNA concentration was determined using NanoDrop spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) and stored at -80°C.

Template cDNA was routinely synthesized from 500 ng of total RNA using the Verso cDNA synthesis kit according to instructions by the manufacturer (Thermo Fisher Scientific, Waltham, MA, USA). The Verso cDNA synthesis kit contains a reverse transcriptase enhancer reagent, which prevents genomic (g) DNA carryover into the synthesized cDNA. This eliminates the need for separate DNase treatment of total RNA. This allowed us not to conduct a separate "no reverse transcriptase control reaction to test for gDNA contamination in total RNA extracts(below). The reverse transcription step included cDNA synthesis steps at 42°C for 30 min and 95°C for 2 min. After cDNA synthesis, the working stock for each sample was diluted 1:10 with DEPC treated water.

2.4 Temporal and spatial transcription analysis during adult and nymph *I. scapularis* tick feeding

Temporal and spatial reverse transcriptase (RT) quantitative (q) PCR expression analysis of candidate *I. scapularis* serpins (Table 2) in cDNA of unfed and 24–72 h partially fed nymphs, and dissected adult tick organs SG, MG and carcass of unfed and 24–120 h fed adult ticks was done on the Biorad® CFX96 Touch Real-Time PCR Detection System according to the manufacturer's instructions (Biorad® Hercules, California, USA). Specific primers (Table 2) were designed using the MacVector software. To increase specificity of reactions, all reverse qRT-PCR primers were based on encoding sequences of the functional reactive center loops (RCL) domains of candidate serpins. The RCL region tends to be unique to each serpin. Prior to qRT-PCR expression analysis, cDNA dilutions, primer concentrations, and specificity of qRT-PCR primers were optimized. Optimization of qRT-PCR primers and the ribosomal protein S4 internal reference gene control (F: 5'-GACTCGCAGGGACACTCGTTTCG-3'; R: 5'-TGCTCTTGCCGATCACAAAAT-3') was tested at 50, 300, and 900 nM in triplicate reactions. The ribosomal protein S4 used for internal reference gene is stably expressed in both nymph and adult mRNA samples. To check for primer dimer formation, a no cDNA template control was included. The specificity of the RT-qPCR reaction was monitored by melting curve analysis in the Biorad® software (Biorad® Hercules, California, USA). Detection of a single curve in melt curve analysis confirmed that a single specific product was amplified. Primer concentrations that met the above parameters were used in the reaction.

Routinely qRT-PCR was done with three biological replicates with reactions of each biological sample done in three technical replicates. The qRT-PCR reaction was performed in triplicate samples of 50 µL final reaction containing 25 µL 2X SYBR green PCR master mix (Thermo Fisher Scientific, Waltham, MA), 5 µL of 1:10 diluted cDNA template (synthesized as above), 3 µL each of optimized (300 nM for each primer) forward and reverse primers, and 14 µL nuclease free water. The following cycling conditions were used: 50°C for 2 minutes for one cycle followed by 95°C for 10 min, 95°C for 40 cycles of 15 seconds each, and 60°C for 1 min. For reproducibility, data was accepted when the threshold cycle (Ct) values for the internal reference gene were 1 or 2 cycle differences in all three biological replicates. The mRNA expression levels were determined using the comparative delta delta Ct method (Livak and Schmittgen, 2001). For each biological replicate, we did qRT-PCR in three technical triplicates and data reported as Mean ± standard errors of mean.

2.5 Target validation of serpins using combinatorial RNAi silencing

Due to the high number of targets, we used the combinatorial RNAi silencing approach (Grimm and Kay, 2007) to streamline work. To forestall off target effects of RNAi silencing, sequence targets that were used to construct dsRNA did not show any matches to other *I. scapularis* sequences in GenBank. This was accomplished by searching dsRNA target sequence regions against other *I. scapularis* sequences in GenBank. Two combinatorial (co) double stranded (ds) RNA were synthesized. The first template, hereafter referred to as Group I (GI), targeted seven serpins (S) that segregated together in cluster D (CD) on the phylogeny tree (Fig. 1). The seven GI serpin (S) included S12 (Accession number [acc#] EW892008), S14 (acc# B7QNY6), S18 (acc# ISCW024013), S21 (acc#: EW881762), S22

(acc#: EW949864), S10 (acc# ISCW023617), and S25 (acc# ISCW014257). We selected ~200 base pairs (bp) region for S10 (nucleotide position [NP]: 1-202), ~200 bp for S14 and S25 (NP: 1-199), and ~200 bp region that was conserved in S12, S18, S22 (NP: 302-501) and S21 (NP: 272-471) to make ~800 bp DNA for GI co-dsRNA synthesis template (Supplemental Fig. 1). The second template DNA hereafter referred to as Group II (GII), targeted four serpins, S9 (acc# B7QL31), S17 (acc# B78JF1), S19 (acc# B7PH24) and S32 (acc# B7Q0E8), that segregated separately in CA, CB, CC and CD on the phylogeny tree (Fig. 1). Likewise, we spliced ~200 bp for S17 (NP: 239-438), S9 (NP: 298-497), S32 (NP: 409-608), and S19 (NP: 849-1173) to make an ~800 bp GII co-dsRNA synthesis template (Supplemental Fig. 1). These sequences were submitted for custom DNA synthesis (Genescript® Piscataway, NJ, USA). Synthesized DNA was cloned into the pGEM®-T vector system (Promega Corp., Madison, WI, USA).

To synthesize co-dsRNA, the cloned template cDNA was amplified using specific primers with added T7 promoter primer sequences (underlined) GI (Forward: 5' - TAATACGACTCACTATAGGGATGAAGCTCGGCCGTGCCCA-3'; Reverse: 5' - TAATACGACTCACTATAGGGTTTCCTCGGGTCTTCTGGCTC-3') and GII (F: 5' - TAATACGACTCACTATAGGGCGGTTACGCTGCGGATGAACT-3'; R: 5' - TAATACGACTCACATAGGGGTGGACGCGCTCGCCTGCGTGC-3'). For control dsRNA, Green Fluorescent Protein (GFP) cDNA was amplified as described (Kim et al., 2014b). Approximately 2 µg of purified template cDNA was used to synthesize co-dsRNA (five reactions for each target) using the Megascript RNAi Kit according to manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). We have previously determined that five dsRNA synthesis reactions yields sufficient amount of dsRNA, which allow us to inject high amount of dsRNA in a low volume (Kim et al., 2014b). Synthesized co-dsRNA from five reactions was purified and concentrated using ethanol precipitation as adopted by us (Kim et al., 2014b). The concentration was determined using Nanodrop spectrophotometer (BioTeK Instruments, Inc., Highland Park, Winooski, Vermont, USA).

Unfed nymphs (n = 50) and adult (n = 25) ticks were respectively microinjected with ~0.5 µL and 0.5–1 µL of dsRNA (1.7 µg/µL) via the fourth leg coxa using pulled glass capillaries for nymphs. For adults, we injected with custom-made 45°C angled, 0.5-inches, 33 gauge Hamilton needles and 10 µL syringes (Hamilton Company, Reno, NV, USA). Injected ticks were incubated at 22–24°C for one week with relative humidity set to ~90% and 12h/12h of day/night cycle before assessing the effects of RNAi silencing on tick feeding. The one week incubation period was empirically pre-determined during preliminary studies as the length of time that was needed to substantially deplete mRNA of GI and GII serpins that predated the injection of GI and GII co-dsRNA in unfed nymph or adult *I. scapularis* ticks. The goal was to attempt at preventing the tick from making the cognate protein before RNAi silencing was induced. To determine the time line to depletion of cognate mRNA that predated the injection of GI and GII co-dsRNA in unfed tick, we determined suppression levels of cognate mRNA in co-dsRNA injected ticks at days 3, 7, 14, 21, and 28 post co-dsRNA injection as detailed in section 2.6 below.

Glass capillary needles were prepared using the Narishige needle puller (Narishige International Inc, East Meadow, NY, USA) and Kwik-Fil Borosilicate Glass Capillaries

(World Precision Instruments, Sarasota, FL, USA). A glass capillary (0.8–1.10 × 100 mm) was inserted under the metal heat strip dome and clamped onto metal arms on each ends. Heat was applied to the glass capillaries by setting the heat knob to 6 where the glass softened in ~2–5 seconds and quickly pulled. To load dsRNA into pulled capillaries, a bevel was prepared on the pointed end and the plunger of 10 µL Hamilton syringe was fit into the blunt side of the capillary and an appropriate dsRNA volume was aspirated and subsequently injected into each tick

Following incubation, co-dsRNA injected ticks were placed on naive rabbit ears to feed as described above. The effects of RNAi silencing on tick feeding success was investigated by assessing the following tick feeding parameters: tick attachment rate (subtract non-attached ticks at 24 h after placing ticks on the animal), time to complete feeding, tick mortality, engorgement weight (EW) as index for amount of host blood the tick takes in, egg laying capacity (adults), and molting for nymphs. For nymphs, in addition to determining the EW, we measured hemoglobin (Hb) content per tick unit mass using the Hb assay kit (Abnova, Taipei City, Taiwan) according to instructions by the manufacturer. Hb content represented the amount of blood taken in by the tick. Mortality of ticks was determined during feeding by counting and removing dead ticks every 24 h. For adult ticks, numbers of the ticks that laid eggs and the ability of ticks to convert the blood meal into eggs [egg mass conversion ratios (EMCR)] were also determined. EMCR was calculated by dividing the weight of the egg mass over the EW. Tick phenotypes during feeding were documented daily using the Canon Rebel VS camera (Canon USA Inc., Melville, NY, USA). The RNAi silencing for the GII cluster was repeated twice to verify first round data.

2.6. Validation of RNAi silencing

To validate RNAi silencing, we sampled three ticks at 24 h (adults and nymphs) post-attachment. The rationale to sample at 24 h is to confirm if cognate mRNA was suppressed at start of feeding. These ticks were individually processed for validation of target mRNA disruption by qRT-PCR using primers in Table 2. For nymphs, validation of RNAi silencing was done in cDNA of whole animals and for adult ticks, in cDNA of dissected SG, MG, and CA. Relative mRNA suppression was determined using the formula $S = 100 - (RQ^T / RQ^C \times 100)$ where S is equal to percent mRNA suppression of serpin, RQ^T and $RQ^C = RQ$ of tissues of GI or GII dsRNA injected ticks and EGFP-dsRNA injected ticks, respectively (Kim et al., 2016). For each biological replicate, three mRNA suppression values were generated by independently comparing the RQ value of each treatment to the RQ value of each of the three independent GFP-dsRNA-injected controls. The mRNA suppression level was presented as mean (M) serpin mRNA suppression ± SE of as described.

2.7. Statistical analysis

The statistical t-tests and the fisher's exact test in Prism software 7 (GraphPad Software, San Diego, CA, USA) were used to determine statistical significance between controls and treatments for effects of RNAi on amount of blood taken in by the tick (engorgement weight, EW) and tick mortality respectively. The statistical significance of mRNA transcript abundance between different tick feeding stages was determined for each replicate in each tissue using the formula 2^{-CT} as described (Livak and Schmittgen, 2001).

3. Results

3.1. Comparative sequence and phylogeny analyses

Blastp scanning of the 11 selected serpins in this study against entries in GenBank and Uniprot retrieved 15 other tick serpin sequences that showed more than 75 % amino acid identity to one of the sequences in this study (Table 1). When subjected to phylogeny analysis (Fig. 1), the 11 blood-meal-responsive *I. scapularis* serpins and 15 other tick serpin (S) sequences downloaded from public databases segregated into five clusters (C): A–E. Seven of the 11 serpin sequences in this study (S10, S12, S14, S18, S21, S22, and S25) segregated together with *Ixodes ricinus* serpin (Uniprot acc# V5HBT7) in CD. Of the remaining four sequences, S32 segregate with *I. ricinus* serpin (Uniprot acc# A0A131XZ77) in CA, S17 cluster together with nine serpins from nine tick species: *I. ricinus* (Uniprot acc# Q06B72), *Amblyomma maculatum* (Uniprot acc# G3ML50), *A. triste* (Uniprot acc# A0A023GPF9), *A. cajennense* (Uniprot acc# A0A023FM57), *A. americanum* (Uniprot acc# A0A0E9Y2F), *Rhipicephalus pulchellus* (Uniprot acc# L7LRY7), *R. appendiculatus* (Uniprot acc# A0A131YYG3), *R. microplus* (Uniprot acc# A0A0R6CDI0), *Hyalomma excavatum* (Uniprot acc# A0A131XKU1) in CB, S9 with *I. ricinus* (Uniprot acc# Q06B75) in CC, and S19 with *I. ricinus* (Uniprot acc# A0A147BTY8), *A. cajennense* (Uniprot acc# A0A023FFQ10) and *A. americanum* (Uniprot acc# A0A0E9Y314) in CE. Pairwise amino acid sequence alignment show that S32 and *I. ricinus* serpin (A0A131XZ77) in CA, S9 and *I. ricinus* serpin (Q06B75) in CC are 95 % and 96 % identical respectively, with 100% bootstrap values. In CB, S17 amino acid sequence is 99 % identical to *I. ricinus* serpin (Q06B72) and 77 – 80 % to other metastrongylid tick serpin sequences. As previously reported (Porter et al., 2015), the functional reactive center loop (RCL) domain is 100% conserved among serpin sequences in CB (not shown). Amino acid identity levels among sequences in CD and CE range between ~14 and 95 % and ~72– 99 % respectively. Amino acid identity levels among *I. scapularis* serpins in CD, range between 85– 96 % for S12, S18, S21 and S22, ~14–39 % for S10, S14, and S25. It is notable that among S12, S18, S21 and S22 sequences, differences are mostly confined within the RCL (not shown). In subsequent sections, we refer to the seven *I. scapularis* serpins that segregated together in CD (S10, S12, S14, S18, S21, S22, and S25) as Group (G)I and the remaining four sequences in CA, CB, CC, and CE (S9, S17, S19, and S32) as GII serpins.

3.2. GI and GII serpins are associated with the nymph and adult *I. scapularis* tick feeding process

We previously used semi-quantitative RT-PCR analysis to identify blood meal-responsive *I. scapularis* serpins (Mulenga et al., 2009). In this study, we have used qRT-PCR to relate transcription patterns of four of the seven GI serpins S12, S14, S18, and S21 (Fig. 2A) and GII serpins, S9, S17, S19, and S32 (Fig. 2B) to the feeding process of nymph and adult *I. scapularis* ticks. For GI serpins, transcript abundance of the four tested GI serpins increased with feeding in both nymph (Fig. 2A–B) and adult ticks (Fig. 2C–D). Please note that transcription patterns for three (S10, S22, and S25) of the seven GI serpins were not determined in this study. Limited sequence information at the time of designing this experiment affected our ability to design optimum RT-qPCR primers for S10, S22, and S25.

Fig. 2A shows that the four tested GI serpins were in general up regulated in response to nymph tick feeding. As shown in Figs. 2A1 and 2A3, GI serpins, S12 and S18 continued to increase with feeding with highest mRNA levels observed at the 72 h feeding time point. For S14 and S21, mRNA was respectively highly expressed at 48 and 24 h before decreasing (Figs. 2A2 and 2A4). Similarly, except for mRNA of S19, which was down regulated (Fig. 2A7), mRNA of other GII serpins, S9 (Fig. 2A5), S17 (Fig. 2A6), and S32 (Fig. 2A8) were up regulated in response to nymph feeding with highest transcript abundance respectively observed at 24, 24–48, and 72 h.

Figs. 2B and 2C respectively summarizes transcription profiling of GI and GII serpins in dissected salivary glands (SG), midgut (MG), and carcass (tick remnant after removal of SG and MG). Similar to transcription profiles of GI serpins in whole nymphs (Fig. 2A), mRNA expression profiles of majority of GI serpins in dissected adult tick organs increased with feeding (Figs. 2B) with exception of S18 and S21 that was respectively down regulated in response to feeding in SG (Fig. 2B7) and MG S21 (Fig. 2B11). As shown in Fig. 2B, mRNA expression levels for all GI serpins was highest at start of feeding (unfed or 24–72 h feeding time points) and dropped to low levels from the 96 and 120 h feeding time points. Similarly, except for S19, which was downregulated in response to feeding in all tissues (Figs. 2. C7–C9), mRNA of GII serpins, S9, S17, and S32 was up regulated in response to feeding in all tissues (Fig. 2C). Except for S9 in the SG (Fig. 2C1) for which highest mRNA levels were observed at the 120 h time point, S17 in SG (Fig. 2C4) and S32 (Fig. 2C12) for which transcript abundance oscillated every 24 h, mRNA of majority of GII serpins was highest in organs of unfed ticks or those that fed for 24 through 72 h and low at the 96 and 120 h feeding time points (Fig. 2C).

3.3. Disruption of GII, but not GI serpins affected both nymph and adult *I. scapularis* tick feeding

We successfully used the combinatorial (co) RNAi silencing approach to validate the importance of the 11 candidate serpins in nymph and adult *I. scapularis* tick feeding success. We used phylogeny clustering patterns (Fig. 1), to assemble and custom synthesize DNA templates for synthesis of GI and GII co-dsRNA (Supplemental Fig. 1). Prior to assessing the effects of RNAi silencing, we used qRT-PCR to validate RNAi silencing in nymphs and adult ticks that were attached for 24 h as summarized in Fig. 3. In nymphs, for GI serpins, except for S12 mRNA for which we confirmed 85% suppression in one of the three animals (A1), mRNA of other GI serpins, S14, S18 and S21 was suppressed in all three animals between 48–93, 43–98, and 42–95% respectively (Fig. 3A2–3A4). Likewise, we confirmed that injection of co-dsRNA into nymphs suppressed S9, S17, S19, and S32 mRNA in all three animals between 66–87%, 58–67%, 53–72%, and 68–75% respectively (Fig. 3A5–3A8).

Figs. 3B and 3C respectively summarizes validation of RNAi silencing of GI and GII serpins in dissected organs of adult ticks that were partially fed for 24 h. For GI serpins, mRNA of S12 was suppressed between 43–94% in salivary gland (SG) of all three animals, 79% and 95% in midgut (MG) of two of animals, and by 78% in carcass of one of the three animals (Figs. 3B1–B3). For S18, its mRNA was suppressed in SG, MG, and carcass of two of the

three animals by 90 and 91%, 42 and 92%, and 44 and 60% respectively (Fig. 3B4–B6). In the case of S21, its mRNA was suppressed by 76% in SG of one of the three animals, by 55% and 74%, 54% and 66% in MG and carcass of two of three animals respectively (Figs. 3B7–B9). We would like to note that suppression of S14 mRNA could not be validated in three tested animals (not shown), while validation of RNAi silencing of three (S10, S22, and S25) of the seven GI serpins was not done in that we could not design optimal qRT-PCR primers.

Fig. 3C1–C11 summarizes validation of RNAi silencing of GII serpin mRNA in adult tick organs. As shown in Fig. 3C1–C3, transcript of S9 was respectively suppressed in SG and MG of all three animals by 51–80% and 43–62%, and in carcass of two of the three animals by 15 and 23%. Similarly, mRNA of S17 was respectively suppressed in SG and MG of two of the three animals by 41 and 67% and 50 and 67%, and by 34–60% in carcass of all three animals (Fig. C4–C6). In the case of S19, its mRNA was suppressed in SG (Fig. 3C7) of all three animals between 54–61%, 61–73% in MG (Fig. 3C8) and by 29% in carcass (Fig. 3C9) of one of three animals and.. For S32, its mRNA was suppressed in SG and carcass of all three animals by 54–83 and 55–85% (Fig. 3C10–3C11) but not in MG (not shown).

Following validation of RNAi silencing, ticks were allowed to feed to repletion to assess the effects of RNAi silencing. Comparatively, RNAi silencing of GI serpins did not dramatically impact both nymph and adult tick feeding. Knockdown of GI serpins did not affect feeding parameters of nymphs (not shown). In adult ticks, 38% (5/13) of GI co-dsRNA-injected compared to 12% (2/16) for GFP-dsRNA injected adult ticks died during feeding ($p = 0.1917$, Two-tailed Fisher exact test), while the remainder (62%, 8/13) of treated ticks fed and laid eggs similar to controls (not shown). Likewise, injection of GI co-dsRNA did not affect nymph tick feeding (not shown). In contrast, 71% (12/17) of GII co-dsRNA injected adult ticks but not GFP-dsRNA injected ticks ($n=18$) died within 24 h of attachment ($P < 0.0001$, Two-tailed Fisher exact test) (not shown). The engorgement weight of one GII dsRNA treated tick (107 mg) that fed to repletion was 14% lower than the EW of GFP dsRNA injected ticks (124 ± 40.08 mg) (not shown) and this tick failed to lay eggs (not shown). Similarly, in the first round of RNAi silencing, disruption of GII serpins in nymph caused 40% (4/10, $p = 0.0103$, Two-tailed Fisher exact test) mortality within 24 h of attachment as opposed to no mortality in GFP dsRNA injected ticks ($n=18$). Engorgement weight of GII dsRNA injected nymphs (3.81 ± 0.16 mg) was apparently smaller than, but not significantly different from engorgement weight of GFP dsRNA injected nymphs (4.11 ± 0.166 mg) (not shown).

Given the significant impact of RNAi silencing of GII serpins in the first round, we sought to repeat this experiment to validate data. Comparable to first round, 38% (8/21, $p = 0.0109$, Two-tailed Fisher exact test) of GII co-dsRNA injected adult ticks died overall with majority of mortality observed within 24–48 h of attachment. The remaining ticks were allowed to feed to repletion. After detachment from the host, the ticks were photographed to document physical phenotypes (Fig. 4A), In Fig. 4A, five of ticks that detached from the host retained whitish gel like material on their mouthparts (arrowhead marked, the asterisks marked tick died on the host), which we suspect could be the tick cement cone. The mean engorgement weight of GII co-dsRNA injected ticks (28.56 ± 10.57 mg) was significantly smaller than

GFP-dsRNA (89.69 ± 23.36 mg) injected ticks (Fig. 4B, $p < 0.05$, unpaired student t-test). When incubated for egg laying, 80% (8/10, $p = 0.1968$, Two-tailed Fisher exact test) of GII co-dsRNA injected as opposed to 46% (6/13) of GFP-dsRNA injected ticks failed to lay eggs (not shown). Likewise, the egg mass conversion ratio (ability to utilize blood meal to produce eggs) of GII co-dsRNA injected ticks (18.40 ± 8.8 mg) was lower than GFP-dsRNA injected control ticks (38.92 ± 14.38 mg) (not shown).

Although not statistically significant, second round RNAi silencing of GII serpins reduced *I. scapularis* nymph feeding efficiency as revealed by mortality of 21% (14/68) in GII co-dsRNA injected as opposed to 16% (11/68) mortality in GFP dsRNA injected. Visually we could see that GII co-dsRNA injected nymphs were smaller, however engorgement weight of GII co-dsRNA nymphs from rabbit#1 (3.95 ± 0.26 mg) and rabbit#2 (3.80 ± 0.23 mg) though apparently smaller, was not significantly different from engorgement weight of control ticks rabbit1 (4.21 ± 0.21 mg) and rabbit2 (4.11 ± 0.166 mg) (not shown). To further get a sense of the amount of blood that was taken in by nymphs, we determined the hemoglobin content per tick unit mass as summarized in Fig. 4C. Similar to nymph engorgement weight (not shown), hemoglobin (μ M) per unit weight of GII co-dsRNA injected nymphs was apparently lower, but not significantly different from GFP dsRNA injected ticks (Fig. 4C). It is notable that, of the six nymphs hemoglobin content values, three were substantially smaller than GFP dsRNA injected, which was not very variable.

4. Discussion

Tick serpins are among proteins that are thought to facilitate tick feeding and thus represent potential target antigens for anti-tick vaccine development (Mulenga et al., 2001; 2003; 2007). The medically important *I. scapularis* tick species encodes for at least 45 serpins (Mulenga et al., 2009; Porter et al., 2015; 2017). In a previous study, we used semi-quantitative RT-PCR to identify a group of blood meal induced tick serpins in SG and MG of adult *I. scapularis* ticks (Mulenga et al., 2009). This study describes molecular and biological characterization of the 11 blood meal-responsive *I. scapularis* tick serpins (S) (S9, S10, S12, S14, S17, S18, S19, S21, S22, S25, and S32, number IDs as in (Mulenga et al., 2009). The observation that all 11 serpin sequences were conserved in other tick species suggested that they are involved in regulating functions that are important to all ticks. Our spatial and temporal quantitative RT-PCR expression analyses data indicated that tick serpins investigated here are associated with nymph and adult *I. scapularis* tick feeding. Results show that transcript abundance of more than half of the candidate serpins was highest in unfed ticks or those that fed for 24 h, suggesting that some of the candidate serpins could be involved with regulation of early tick feeding events such as tick attachment on host skin, creation of feeding lesion, and preparation for tick transmission of tick borne disease (TBD) agents.

An important goal of this study was to determine the importance of the 11 serpins in tick feeding using RNAi silencing. The RNAi silencing approach is widely used to establish functions of tick genes in tick feeding physiology (Aljamali et al., 2003; Gao et al., 2011; Karim et al., 2005; Kim et al., 2014b; 2015a; 2015b; Mulenga and Khumthong, 2010a; 2010b; Mulenga et al., 2013b; Mulenga et al., 2010a; Mulenga et al., 2010b; Pedra et al.,

2006; Radulović et al., 2015) and transmission of TBD agents (Adamson et al., 2013; Antunes et al., 2012; Coumou et al., 2016; Ferrolho et al., 2017; Kocan et al., 2009; Merino et al., 2011). Similar to other organisms (Garrett et al., 2009; Gulley et al., 2013; Reichhart et al., 2011; Tong et al., 2005), the tick serpin family is large with high potential for redundancy (Mulenga et al., 2009; Porter et al., 2015). From the perspective of target validation studies to identify effective anti-tick serpins, the large tick serpin family presents a challenge: how to narrow down the list of potential effective anti-tick serpin targets? In RNAi silencing approaches for which a single target is evaluated per treatment, it will be a laborious task to screen the 45 *I. scapularis* serpins (Mulenga et al., 2009). Additionally, given the apparent redundancy in the tick serpin family (Porter et al., 2015), it is also possible that if one tick serpin is blocked, the tick could utilize an alternate serpin, the consequence of which would be failure to identify key serpin(s) that are potential candidate anti-tick vaccine antigens. To circumvent this limitation, we have successfully adopted the combinatorial RNAi silencing approach that was described by others (Barnard et al., 2012; Tzin et al., 2015; Yang et al., 2009), and disrupted the 11 candidate serpins in two clusters: group of seven highly identical serpins (S10, S12, S14, S18, S21, S22, and S25) here after referred to as group I (GI) serpins, and four non-identical serpins (S9, S17, S19, and S32) that we have referred to as GII serpins. At a glance, one would expect that disrupting seven GI serpins would be more effective against tick feeding than disrupting the four GII serpins. Thus, it was interesting to note that disruption of the four GII serpins, and not the seven GI serpins caused significant lethality that affected both adult and nymph tick feeding success. Our RNAi silencing data here could be explained by the possibility that disruption of GI serpin transcripts was not significant enough to affect tick feeding. However, we believe that this is not likely the case in that validation of RNAi silencing confirmed that, similar to GII serpins, transcripts of GI serpin were depleted by up to 90% during feeding. We speculate that biological functions that are regulated by GI serpins may not be critical to tick feeding success and thus GI serpins may not represent good targets for anti-tick vaccine development. On the other hand, the observed significant anti-tick lethality in RNAi silencing of GII serpins strongly suggest that GII serpins could be potential effective target antigens for development of a vaccine against both nymph and adult *I. scapularis* ticks. The observation that significant mortality of ticks that were injected with GII co-dsRNA occurred within 24–48 h of attachment is significant as this effect precedes the tick feeding time points when majority of TBD agents are transmitted. With exception of the Powassan virus, which might be transmitted within minutes of attachment (Ebel and Kramer, 2004), other *I. scapularis* transmitted TBD agents including Lyme disease spirochetes, *B. burgdorferi* (Peavey and Lane, 1995; des Vignes et al., 2001; Piesman et al., 2001) and *B. mayonii* (Dolan et al., 2016), *Babesia microti* (Richards et al., 2017), and *A. phagocytophilum* (Hodzic et al., 1998; Katavolos et al., 1998) are predominantly transmitted after the *I. scapularis* tick has attached for more than 48 h. We speculate that immunization against tick feeding with GII serpins will disrupt tick feeding before transmission of major TBD agents starts, and thus could protect against TBD infections.

When ticks complete feeding, they cleanly disengage from their feeding site with their mouthparts intact. Thus, it was notable that some of the RNAi co-dsRNA injected retained a whitish gel-like tissue on their mouthparts, which we suspect is the tick cement cone. We

speculate that, some of the GII serpins might play roles in maintenance of the tick-feeding site. Whether or not the observed effects of RNAi silencing of GII serpins are attributable to all four serpins or to an individual serpin remains to be validated. Interestingly, one of the four GII serpins, S17 is the homolog to *A. americanum* serpin (AAS) 19 (Porter et al., 2015), a broad-spectrum inhibitor of trypsin-like proteases, anti-coagulant, and inhibitor of platelet aggregation (Kim et al., 2015b), which when disrupted by RNAi silencing caused tick deformities and significant mortality in adult *A. americanum* ticks (Kim et al., 2016). Additionally, ticks that fed on rabbits immunized with yeast expressed recombinant AAS19 obtained significantly smaller blood meals (Kim et al., 2016). Whether or not the significant effect observed in RNAi silencing of GII serpin was attributed to S17 is currently being investigated. It will be interesting to characterize functions of S17, which like it's *A. americanum* homolog has its functional reactive center loop domain 100% conserved in all ticks for which sequence data is available (Porter et al., 2015).

The application of the RNAi silencing technology in target validation studies to identify effective anti-tick druggable or vaccine antigens has inherent limitations. One of the limitations is that, dsRNA is delivered by manual injections, which may result in delivery of different amounts of dsRNA. We believe that this could explain the discrepancy in tick mortality rates that we observed between first and second round of RNAi silencing of GII serpins. Another limitation is that the RNAi silencing approach that requires incubating ticks that are injected with dsRNA for 24 h (Chalaire et al., 2011; Kim et al., 2014b; Mulenga and Khumthong, 2010) before starting to evaluate the effects of RNAi silencing may not provide for sufficient time to suppress mRNA that predated triggering of the RNAi silencing pathway (Chalaire et al., 2011). The consequence of this could be that cognate proteins that are translated before the RNAi silencing pathway is induced could mask the effects of RNAi silencing. Here we successfully circumvented this limitation, we included the step to deplete cognate mRNA in unfed ticks before starting to evaluate the effects of RNAi silencing. We believe that this could explain the fact that mortality of ticks that were injected with GII serpin co-dsRNA predominantly occurred within 24–48 h of attachment.

We would like to note here that approach used here was previously reported. Conserved regions were targeted to knock down *Aam*-IGFBP-rP6S and *Aam*-IGFBP-rP6L in *A. americanum* (Mulenga and Khumthong, 2010a) and five metalloproteases in *A. maculatum* (Bullard et al., 2016). In a recent study, our group has utilized combinatorial RNAi silencing as used here to characterize tick cement glycine rich proteins (Hollmann et al., 2017). In conclusion, this study makes a contribution toward discovery of serpins that are important to the feeding success of *I. scapularis* nymph and adult ticks. The protocol described in this study could be applied to target validation of other large tick protein families. The strategy is to use sequence analysis to identify potentially redundant groups of tick genes and then knock them down as a group. Altogether, this is a powerful way to validate the importance in tick feeding of groups of functionally redundant genes. In conclusion, our data here suggest that some of the GII serpins regulate important *I. scapularis* tick feeding functions and thus represent potential targets for anti-tick vaccine development.

Acknowledgments

Authors would like to thank BEI resources for providing *Ixodes scapularis* adult and nymph ticks that were used in this study. We also would like to thank Oklahoma State University tick lab for prompt delivery of our tick orders. This research was supported by National Institutes of Health grants [AI081093, AI093858, AI074789] to AM.

References

- Abbas RZ, Zaman MA, Colwell DD, Gilleard J, Iqbal Z. Acaricide resistance in cattle ticks and approaches to its management: the state of play. *Vet Parasitol.* 2014; 203:6–20. [PubMed: 24709006]
- Adamson SW, Browning RE, Budachetri K, Ribeiro JM, Karim S. Knockdown of selenocysteine-specific elongation factor in *Amblyomma maculatum* alters the pathogen burden of *Rickettsia parkeri* with epigenetic control by the Sin3 histone deacetylase corepressor complex. *PLoS One.* 2013; 8:e82012. [PubMed: 24282621]
- Aljamali M, Bior A, Sauer J, Essenberg R. RNA interference in ticks: a study using histamine binding protein dsRNA in the female tick *Amblyomma americanum*. *Insect Mol Biol.* 2003; 12:299–305. [PubMed: 12752664]
- Antunes S, Galindo RC, Almazán C, Rudenko N, Golovchenko M, Grubhoffer L, Shkap V, do Rosário V, de la Fuente J, Domingos A. Functional genomics studies of *Rhipicephalus (Boophilus) annulatus* ticks in response to infection with the cattle protozoan parasite, *Babesia bigemina*. *Int J Parasitol.* 2012; 42:187–195. [PubMed: 22265898]
- Barnard A, Nijhof AM, Gaspar AR, Neitz AW, Jongejan F, Maritz-Olivier C. Expression profiling, gene silencing and transcriptional networking of metzincin metalloproteases in the cattle tick, *Rhipicephalus (Boophilus) microplus*. *Vet Parasitol.* 2012; 186:403–414. [PubMed: 22142943]
- Breuner NE, Dolan MC, Replogle AJ, Sexton C, Hojgaard A, Boegler KA, Clark RJ, Eisen L. Transmission of *Borrelia miyamotoi* sensu lato relapsing fever group spirochetes in relation to duration of attachment by *Ixodes scapularis* nymphs. *Ticks and Tick-borne Dis.* 2017; 8:677–681.
- Bullard RL, Williams J, Karim S. Temporal gene expression analysis and RNA silencing of single and multiple members of gene family in the lone star tick *Amblyomma americanum*. *PloS one.* 2016; 11:e0147966. [PubMed: 26872360]
- Burgdorfer W, Hayes SF, Corwin D. Pathophysiology of the Lyme disease spirochete, *Borrelia burgdorferi*, in ixodid ticks. *Rev Infect Dis.* 1989; 11:S1442–S1450. [PubMed: 2682956]
- Burgdorfer W. Discovery of the Lyme disease spirochete and its relation to tick vectors. *Yale J Biol Med.* 1984; 57:515–520. [PubMed: 6516454]
- Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP. Lyme disease—a tick-borne spirochetosis? *Science.* 1982; 216:1317–1319. [PubMed: 7043737]
- Burgdorfer W, Lane RS, Barbour AG, Gresbrink RA, Anderson JR. The western black-legged tick, *Ixodes pacificus*: a vector of *Borrelia burgdorferi*. *Am J Trop Med Hyg.* 1985; 34:925–930. [PubMed: 3898886]
- Carrell RW, Lomas DA. Alpha1-antitrypsin deficiency—a model for conformational diseases. *N Engl J Med.* 2002; 346:45–53. [PubMed: 11778003]
- Carrell R, Travis J. α 1-Antitrypsin and the serpins: variation and countervariation. *Trends Biochem Sci.* 1985; 10:20–24.
- Chalautre KC, Kim TK, Garcia-Rodriguez H, Mulenga A. *Amblyomma americanum* (L.) (Acari: Ixodidae) tick salivary gland serine protease inhibitor (serpin) 6 is secreted into tick saliva during tick feeding. *J Exp Biol.* 2011; 214:665–673. [PubMed: 21270316]
- Chmelar J, Oliveira CJ, Rezacova P, Francischetti IM, Kovarova Z, Pejler G, Kopacek P, Ribeiro JM, Mares M, Kopecky J, Kotsyfakis M. A tick salivary protein targets cathepsin G and chymase and inhibits host inflammation and platelet aggregation. *Blood.* 2011; 117:736–744. [PubMed: 20940421]
- Cisak E, Wójcik-Fatla A, Zajac V, Dutkiewicz J. Repellents and acaricides as personal protection measures in the prevention of tick-borne diseases. *Annals of Agricultural and Environmental Medicine.* 2012; 19:625–630. [PubMed: 23311778]

- Coles TB, Dryden MW. Insecticide/acaricide resistance in fleas and ticks infesting dogs and cats. *Parasit Vectors*. 2014; 7:8. [PubMed: 24393426]
- Coumou J, Narasimhan S, Trentelman JJ, Wagemakers A, Koetsveld J, Ersoz JI, Oei A, Fikrig E, Hovius JW. *Ixodes scapularis* dystroglycan-like protein promotes *Borrelia burgdorferi* migration from the gut. *Journal of Molecular Medicine*. 2016; 94:361–370. [PubMed: 26594018]
- Dahlgren FS, Heitman KN, Drexler NA, Massung RF, Behravesh CB. Human granulocytic anaplasmosis in the United States from 2008 to 2012: a summary of national surveillance data. *Am J Trop Med Hyg*. 2015; 93:66–72. [PubMed: 25870428]
- Davis RS, Ramirez RA, Anderson JL, Bernhardt SA. Distribution and Habitat of *Ixodes pacificus* (Acari: Ixodidae) and Prevalence of *Borrelia burgdorferi* in Utah. *J Med Entomol*. 2015; 52:1361–1367. [PubMed: 26336263]
- de la Fuente J, Estrada-Peña A, Cabezas-Cruz A, Kocan KM. *Anaplasma phagocytophilum* uses common strategies for infection of ticks and vertebrate Hosts. *Trends Microbiol*. 2015; 24:173–180. [PubMed: 26718986]
- des Vignes F, Piesman J, Heffernan R, Schulze TL, Stafford KC 3rd, Fish D. Effect of tick removal on transmission of *Borrelia burgdorferi* and *Ehrlichia phagocytophila* by *Ixodes scapularis* nymphs. *J Infect Dis*. 2001; 183:773–778. [PubMed: 11181154]
- Dolan MC, Hojgaard A, Hoxmeier JC, Replogle AJ, Respicio-Kingry LB, Sexton C, Williams MA, Pritt BS, Schrieffer ME, Eisen L. Vector competence of the blacklegged tick, *Ixodes scapularis*, for the recently recognized Lyme borreliosis spirochete Candidatus *Borrelia mayonii*. *Ticks Tick Borne Dis*. 2016; 7:665–669. [PubMed: 26922324]
- Dolan MC, Breuner NE, Hojgaard A, Boegler KA, Hoxmeier JC, Replogle AJ, Eisen L. Transmission of the Lyme disease spirochete *Borrelia mayonii* in relation to duration of attachment by nymphal *Ixodes scapularis* (Acari: Ixodidae). *J Med Entomol*. 2017; 54:1360–1364. [PubMed: 28874016]
- Dolan MC, Breuner NE, Hojgaard A, Hoxmeier JC, Pilgard MA, Replogle AJ, Eisen L. Duration of *Borrelia mayonii* infectivity in an experimental mouse model for feeding *Ixodes scapularis* larvae. *Ticks Tick-Borne Dis*. 2017; 8:196–200. [PubMed: 27838290]
- Drummond R. Chemical control of ticks. *World Animal Review (Italia)*. 1976; 4(19):28–33.
- Dupuis AP II, Peters RJ, Prusinski MA, Falco RC, Ostfeld RS, Kramer LD. Isolation of deer tick virus (Powassan virus, lineage II) from *Ixodes scapularis* and detection of antibody in vertebrate hosts sampled in the Hudson Valley, New York State. *Parasites & vectors*. 2013; 6:1. [PubMed: 23281838]
- Ebel GD, Kramer LD. Short report: duration of tick attachment required for transmission of powassan virus by deer ticks. *Am J Trop Med Hyg*. 2004; 71:268–271.
- Ferrolho J, Antunes S, Sanches GS, Couto J, Évora PM, Rosa C, André MR, Machado RZ, Bechara GH, Domingos A. Ferritin 1 silencing effect in *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae) during experimental infection with *Ehrlichia canis*. *Ticks Tick-Borne Dis*. 2017; 8:174–184. [PubMed: 27825659]
- Gao X, Shi L, Zhou Y, Cao J, Zhang H, Zhou J. Characterization of the anticoagulant protein Rhipilin-1 from the *Rhipicephalus haemaphysaloides* tick. *J Insect Physiol*. 2011; 57:339–343. [PubMed: 21147114]
- Garrett M, Fullaondo A, Troxler L, Micklem G, Gubb D. Identification and analysis of serpin-family genes by homology and synteny across the 12 sequenced Drosophilid genomes. *BMC Genomics*. 2009; 10:1. [PubMed: 19121221]
- George J, Pound J, Davey R. Chemical control of ticks on cattle and the resistance of these parasites to acaricides. *Parasitology*. 2004; 129:S353–S366. [PubMed: 15938518]
- Gettins PG. Serpin structure, mechanism, and function. *Chem Rev*. 2002; 102:4751–4804. [PubMed: 12475206]
- Graf J, Gogolewski R, Leach-Bing N, Sabatini G, Molento M, Bordin E, Arantes G. Tick control: an industry point of view. *Parasitology*. 2004; 129:S427–S442. [PubMed: 15938522]
- Grimm D, Kay MA. Combinatorial RNAi: a winning strategy for the race against evolving targets? *Molecular Therapy*. 2007; 15:878–888. [PubMed: 17311009]

- Gulia-Nuss M, Nuss AB, Meyer JM, Sonenshine DE, Roe RM, Waterhouse RM, Sattelle DB, de la Fuente J, Ribeiro JM, Megy K. Genomic insights into the *Ixodes scapularis* tick vector of Lyme disease. *Nature communications*. 2016; 7:10507.
- Gulley MM, Zhang X, Michel K. The roles of serpins in mosquito immunology and physiology. *J Insect Physiol*. 2013; 59:138–147. [PubMed: 22960307]
- Hill CA, Wikel SK. The *Ixodes scapularis* Genome Project: an opportunity for advancing tick research. *Trends Parasitol*. 2005; 21:151–153. [PubMed: 15780833]
- Hodzic E, Fish D, Maretzki CM, De Silva AM, Feng S, Barthold SW. Acquisition and transmission of the agent of human granulocytic ehrlichiosis by *Ixodes scapularis* ticks. *J Clin Microbiol*. 1998; 36:3574–3578. [PubMed: 9817875]
- Hollmann T, Kim TK, Tirloni L, Radulovic ŽM, Pinto AF, Diedrich JK, Yates JR, da Silva Vaz I, Mulenga A. Identification and characterization of proteins in the *Amblyomma americanum* tick cement cone. *Int J Parasitol*. 2017
- Huntington J. Serpin structure, function and dysfunction. *Journal of thrombosis and haemostasis*. 2011; 9:26–34. [PubMed: 21781239]
- Ibelli AM, Kim TK, Hill CC, Lewis LA, Bakshi M, Miller S, Porter L, Mulenga A. A blood meal-induced *Ixodes scapularis* tick saliva serpin inhibits trypsin and thrombin, and interferes with platelet aggregation and blood clotting. *Int J Parasitol*. 2014; 44:369–379. [PubMed: 24583183]
- Imamura S, da Silva Vaz Itabajara Junior, Sugino M, Ohashi K, Onuma M. A serine protease inhibitor (serpin) from *Haemaphysalis longicornis* as an anti-tick vaccine. *Vaccine*. 2005b; 23:1301–1311. [PubMed: 15652673]
- Imamura S, Konnai S, da Silva Vaz, Itabajara, Yamada S, Nakajima C, Ito Y, Tajima T, Yasuda J, Simuunza M, Onuma M. Effects of anti-tick cocktail vaccine against *Rhipicephalus appendiculatus*. *Jpn J Vet Res*. 2008; 56:85–98. [PubMed: 18828446]
- Imamura S, Namangala B, Tajima T, Tembo ME, Yasuda J, Ohashi K, Onuma M. Two serine protease inhibitors (serpins) that induce a bovine protective immune response against *Rhipicephalus appendiculatus* ticks. *Vaccine*. 2006; 24:2230–2237. [PubMed: 16314008]
- Jittapalapong S, Kaewhom P, Pumhom P, Canales M, De La Fuente J, Stich R. Immunization of rabbits with recombinant serine protease inhibitor reduces the performance of adult female *Rhipicephalus microplus*. *Transboundary and emerging diseases*. 2010; 57:103–106. [PubMed: 20537119]
- Jongejan F, Uilenberg G. The global importance of ticks. *Parasitology*. 2004; 129:S3–S14. [PubMed: 15938502]
- Kaewhom P, Sirinarumitr T, Chantakru S, Jittapalapong S. Cloning and characterization of cDNA encoding a serine protease inhibitor from salivary glands of Thai cattle tick (*Boophilus microplus*). *Kasetsart J (Nat Sci)*. 2007; 41:74–80.
- Karim S, Miller NJ, Valenzuela J, Sauer JR, Mather TN. RNAi-mediated gene silencing to assess the role of synaptobrevin and cystatin in tick blood feeding. *Biochem Biophys Res Commun*. 2005; 334:1336–1342. [PubMed: 16040003]
- Katavolos P, Armstrong PM, Dawson JE, Telford SR III. Duration of tick attachment required for transmission of granulocytic ehrlichiosis. *J Infect Dis*. 1998; 177:1422–1425. [PubMed: 9593039]
- Kim TK, Porter LM, Sze S, Lewis L, Mulenga A. A 24–48 h fed *Amblyomma americanum* tick saliva immuno-proteome. *BMC Genomics*. 2014a; 15:518. [PubMed: 24962723]
- Kim TK, Radulovic Z, Mulenga A. Target validation of highly conserved *Amblyomma americanum* tick saliva serine protease inhibitor 19. *Ticks Tick-Borne Dis*. 2016; 7:205–414.
- Kim TK, Ibelli AMG, Mulenga A. *Amblyomma americanum* tick calreticulin binds C1q but does not inhibit activation of the classical complement cascade. *Ticks Tick-Borne Dis*. 2015a; 6:91–101. [PubMed: 25454607]
- Kim TK, Tirloni L, Radulovic Z, Lewis L, Bakshi M, Hill C, da Silva Vaz I, Logullo C, Termignoni C, Mulenga A. Conserved *Amblyomma americanum* tick Serpin19, an inhibitor of blood clotting factors Xa and XIa, trypsin and plasmin, has anti-haemostatic functions. *Int J Parasitol*. 2015b; 45:613–627. [PubMed: 25957161]
- Kim TK, Curran J, Mulenga A. Dual silencing of long and short *Amblyomma americanum* acidic chitinase forms weakens the tick cement cone stability. *J Exp Biol*. 2014b; 217:3493–3503. [PubMed: 25189365]

- Kocan KM, Zivkovic Z, Blouin EF, Naranjo V, Almazán C, Mitra R, de la Fuente J. Silencing of genes involved in *Anaplasma marginale*-tick interactions affects the pathogen developmental cycle in *Dermacentor variabilis*. BMC developmental biology. 2009; 9:42. [PubMed: 19607704]
- Koci J, Simo L, Park Y. Validation of internal reference genes for real-time quantitative polymerase chain reaction studies in the tick, *Ixodes scapularis* (Acari: Ixodidae). J Med Entomol. 2013; 50:79–84. [PubMed: 23427655]
- Leboulle G, Crippa M, Decrem Y, Mejri N, Brossard M, Bollen A, Godfroid E. Characterization of a novel salivary immunosuppressive protein from *Ixodes ricinus* ticks. J Biol Chem. 2002; 277:10083–10089. [PubMed: 11792703]
- Levin ML, Ross DE. Acquisition of different isolates of *Anaplasma phagocytophilum* by *Ixodes scapularis* from a model animal. Vector-Borne and Zoonotic Diseases. 2004; 4:53–59. [PubMed: 15018773]
- Liu XY, Bonnet SI. Hard tick factors implicated in pathogen transmission. PLoS Negl Trop Dis. 2014; 8:e2566. [PubMed: 24498444]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻CT method. Methods. 2001; 25:402–408. [PubMed: 11846609]
- Merino O, Almazán C, Canales M, Villar M, Moreno-Cid JA, Galindo RC, De la Fuente J. Targeting the tick protective antigen subolesin reduces vector infestations and pathogen infection by *Anaplasma marginale* and *Babesia bigemina*. Vaccine. 2011; 29:8575–8579. [PubMed: 21951878]
- Mulenga A, Kim T, Ibelli A. *Amblyomma americanum* tick saliva serine protease inhibitor 6 is a cross - class inhibitor of serine proteases and papain - like cysteine proteases that delays plasma clotting and inhibits platelet aggregation. Insect Mol Biol. 2013a; 22:306–319. [PubMed: 23521000]
- Mulenga A, Tsuda A, Onuma M, Sugimoto C. Four serine proteinase inhibitors (serpin) from the brown ear tick, *Rhipicephalus appendiculatus*; cDNA cloning and preliminary characterization. Insect Biochem Mol Biol. 2003; 33:267–276. [PubMed: 12535684]
- Mulenga A, Khumthong R. Disrupting the *Amblyomma americanum* (L.) CD147 receptor homolog prevents ticks from feeding to repletion and blocks spontaneous detachment of ticks from their host. Insect Biochem Mol Biol. 2010a; 40:524–532. [PubMed: 20451606]
- Mulenga A, Khumthong R, Chalaire KC. *Ixodes scapularis* tick serine proteinase inhibitor (serpin) gene family; annotation and transcriptional analysis. BMC Genomics. 2009; 10:217. [PubMed: 19435496]
- Mulenga A, Kim TK, Ibelli AMG. Deorphanization and target validation of cross-tick species conserved novel *Amblyomma americanum* tick saliva protein. Int J Parasitol. 2013b; 43:439–451. [PubMed: 23428900]
- Mulenga A, Sugino M, Nakajimo M, Sugimoto C, Onuma M. Tick-Encoded serine proteinase inhibitors (serpins); potential target antigens for tick vaccine development. Journal of Veterinary Medical Science. 2001; 63:1063–1069. [PubMed: 11714020]
- Mulenga A, Khumthong R. Silencing of three *Amblyomma americanum* (L.) insulin-like growth factor binding protein-related proteins prevents ticks from feeding to repletion. J Exp Biol. 2010b; 213:1153–1161. [PubMed: 20228352]
- Mulenga A, Khumthong R, Blandon MA. Molecular and expression analysis of a family of the *Amblyomma americanum* tick Lospins. J Exp Biol. 2007; 210:3188–3198. [PubMed: 17766296]
- Mulenga A, Khumthong R, Chalaire KC. *Ixodes scapularis* tick serine proteinase inhibitor (serpin) gene family; annotation and transcriptional analysis. BMC Genomics. 2009; 10:217. [PubMed: 19435496]
- Olson ST, Gettins PG. Regulation of proteases by protein inhibitors of the serpin superfamily. Prog Mol Biol Transl Sci. 2011; 99:185–240. [PubMed: 21238937]
- Peavey CA, Lane RS. Transmission of *Borrelia burgdorferi* by *Ixodes pacificus* nymphs and reservoir competence of deer mice (*Peromyscus maniculatus*) infected by tick-bite. J Parasitol. 1995:175–178. [PubMed: 7707191]
- Pedra JH, Narasimhan S, Deponte K, Marcantonio N, Kantor FS, Fikrig E. Disruption of the salivary protein 14 in *Ixodes scapularis* nymphs and impact on pathogen acquisition. Am J Trop Med Hyg. 2006; 75:677–682. [PubMed: 17038693]

- Piesman J, Stone BF. Vector competence of the Australian paralysis tick, *Ixodes holocyclus*, for the Lyme disease spirochete *Borrelia burgdorferi*. *Int J Parasitol*. 1991; 21:109–111. [PubMed: 2040556]
- Piesman J, Schneider BS, Zeidner NS. Use of quantitative PCR to measure density of *Borrelia burgdorferi* in the midgut and salivary glands of feeding tick vectors. *J Clin Microbiol*. 2001; 39:4145–4148. [PubMed: 11682544]
- Porter LM, Radulovi ŽM, Mulenga A. A repertoire of protease inhibitor families in *Amblyomma americanum* and other tick species: inter-species comparative analyses. *Parasites & vectors*. 2017; 10:152. [PubMed: 28330502]
- Porter L, Radulovi Ž, Kim T, Braz GR, Vaz IDS, Mulenga A. Bioinformatic analyses of male and female *Amblyomma americanum* tick expressed serine protease inhibitors (serpins). *Ticks Tick-borne Dis*. 2015; 6:16–30. [PubMed: 25238688]
- Prevot P, Couvreur B, Denis V, Brossard M, Vanhamme L, Godfroid E. Protective immunity against *Ixodes ricinus* induced by a salivary serpin. *Vaccine*. 2007; 25:3284–3292. [PubMed: 17270322]
- Prevot PP, Adam B, Boudjeltia KZ, Brossard M, Lins L, Cauchie P, Brasseur R, Vanhaeverbeek M, Vanhamme L, Godfroid E. Anti-hemostatic effects of a serpin from the saliva of the tick *Ixodes ricinus*. *J Biol Chem*. 2006; 281:26361–26369. [PubMed: 16672226]
- Pritt BS, Mead PS, Johnson DKH, Neitzel DF, Respicio-Kingry LB, Davis JP, Schiffman E, Sloan LM, Schrieffer ME, Replogle AJ. Identification of a novel pathogenic *Borrelia* species causing Lyme borreliosis with unusually high spirochaetemia: a descriptive study. *The Lancet Infectious Diseases*. 2016; 16:556–564. [PubMed: 26856777]
- Prusinski M, Kokas J, Hukey K, Kogut S, Lee J, Backenson P. Prevalence of *Borrelia burgdorferi* (Spirochaetales: Spirochaetaceae), *Anaplasma phagocytophilum* (Rickettsiales: Anaplasmataceae), and *Babesia microti* (Piroplasmida: Babesiidae) in *Ixodes scapularis* (Acari: Ixodidae) collected from recreational lands in the Hudson Valley Region, New York State. *J Med Entomol*. 2014; 51:226–236. [PubMed: 24605473]
- Radulovi ŽM, Mulenga A. Heparan sulfate/heparin glycosaminoglycan binding alters inhibitory profile and enhances anticoagulant function of conserved *Amblyomma americanum* tick saliva serpin 19. *Insect Biochem Mol Biol*. 2017; 80:1–10. [PubMed: 27845251]
- Radulovi Ž, Porter L, Kim T, Bakshi M, Mulenga A. *Amblyomma americanum* tick saliva insulin-like growth factor binding protein-related protein 1 binds insulin but not insulin-like growth factors. *Insect Mol Biol*. 2015; 24:539–550. [PubMed: 26108887]
- Reichhart JM, Gubb D, Leclerc V. The *Drosophila* serpins: multiple functions in immunity and morphogenesis. *Methods Enzymol*. 2011; 499:205–225. [PubMed: 21683256]
- Richards SL, Langley R, Apperson CS, Watson E. Do Tick Attachment Times Vary between Different Tick-Pathogen Systems? *Environments*. 2017; 4:37.
- Rizzoli A, Silaghi C, Obiegala A, Rudolf I, Hubalek Z, Foldvari G, Plantard O, Vayssier-Taussat M, Bonnet S, Spitalska E, Kazimirova M. *Ixodes ricinus* and Its Transmitted Pathogens in Urban and Peri-Urban Areas in Europe: New Hazards and Relevance for Public Health. *Front Public Health*. 2014; 2:251. [PubMed: 25520947]
- Rodriguez-Valle M, Xu T, Kurscheid S, Lew-Tabor AE. *Rhipicephalus microplus* serine protease inhibitor family: annotation, expression and functional characterisation assessment. *Parasites & vectors*. 2015; 8:7. [PubMed: 25564202]
- Steiner FE, Pinger RR, Vann CN, Abley MJ, Sullivan B, Grindle N, Clay K, Fuqua C. Detection of *Anaplasma phagocytophilum* and *Babesia odocoilei* DNA in *Ixodes scapularis* (Acari: Ixodidae) collected in Indiana. *J Med Entomol*. 2006; 43:437–442. [PubMed: 16619631]
- Sugino M, Imamura S, Mulenga A, Nakajima M, Tsuda A, Ohashi K, Onuma M. A serine proteinase inhibitor (serpin) from ixodid tick *Haemaphysalis longicornis*; cloning and preliminary assessment of its suitability as a candidate for a tick vaccine. *Vaccine*. 2003; 21:2844–2851. [PubMed: 12798626]
- Telford SR, Goethert HK, Molloy PJ, Berardi VP, Chowdri HR, Gugliotta JL, Lepore TJ. *Borrelia miyamotoi* disease: neither Lyme disease nor relapsing fever. *Clin Lab Med*. 2015; 35:867–882. [PubMed: 26593262]

- Tirloni L, Kim TK, Coutinho ML, Ali A, Seixas A, Termignoni C, Mulenga A, da Silva Vaz I. The putative role of *Rhipicephalus microplus* salivary serpins in the tick-host relationship. *Insect Biochem Mol Biol*. 2016; 71:12–28. [PubMed: 26844868]
- Tong Y, Jiang H, Kanost MR. Identification of plasma proteases inhibited by *Manduca sexta* serpin-4 and serpin-5 and their association with components of the prophenol oxidase activation pathway. *J Biol Chem*. 2005; 280:14932–14942. [PubMed: 15695806]
- Toyomane K, Konnai S, Niwa A, Githaka N, Isezaki M, Yamada S, Ito T, Takano A, Ando S, Kawabata H. Identification and the preliminary in vitro characterization of IRIS homologue from salivary glands of *Ixodes persulcatus* Schulze. *Ticks Tick-borne Dis*. 2016; 7:119–125. [PubMed: 26460162]
- Tzin V, Yang X, Jing X, Zhang K, Jander G, Douglas AE. RNA interference against gut osmoregulatory genes in phloem-feeding insects. *J Insect Physiol*. 2015; 79:105–112. [PubMed: 26071792]
- Willadsen P. Anti-tick vaccines. *Parasitol*. 2004; 129(Suppl):S367–S387.
- Willadsen P, Bird P, Cobon G, Hungerford J. Commercialisation of a recombinant vaccine against *Boophilus microplus*. *Parasitology*. 1995; 110:S43–S50. [PubMed: 7784128]
- Yang X, Weber M, ZarinKamar N, Posnien N, Friedrich F, Wigand B, Beutel R, Damen WG, Bucher G, Klingler M. Probing the Drosophila retinal determination gene network in Tribolium (II): The Pax6 genes eyeless and twin of eyeless. *Dev Biol*. 2009; 333:215–227. [PubMed: 19527703]
- Yu Y, Cao J, Zhou Y, Zhang H, Zhou J. Isolation and characterization of two novel serpins from the tick *Rhipicephalus haemaphysaloides*. *Ticks Tick-borne Dis*. 2013; 4:297–303. [PubMed: 23601911]

Figure 1. Blood meal-responsive *Ixodes scapularis* serpins in this study are conserved in other ticks

A guide phylogeny tree of *I. scapularis* serpin protein sequences with other tick serpins that were downloaded from GenBank and Uniprot was constructed using the Neighbor-joining method as described. Number IDs of *I. scapularis* serpins (asterisks marked) were previously described (Mulenga et al., 2009). Phylogeny clusters (C) are indicated as CA, CB, CC, CD and CE. Amino acid identity levels within each cluster are noted.

Bakshi et al., Figure 2A

Bakshi et al., Figure 2B

Bakshi et al., Figure 2C

Figure 2. Spatiotemporal expression profiles of group (G) I and GII serpins during nymphs (fig. 2A) and adult (figs. 2B and 2C) *Ixodes scapularis* ticks feeding

Quantitative RT-PCR analysis was done in cDNA of unfed nymphs and those that were partially fed 24–72 h and dissected salivary glands (SG), midgut (MG), and carcass (tick remnant after removal of SG and MG) of unfed and adult ticks that were partially fed for 24–120 h as described under section 2.4. Relative abundance of serpin transcripts was normalized against transcript levels of ribosomal protein S4 (RPS4) using comparative Ct (Ct) method as described. Data is reported as Mean (M ± SEM).

Bakshi et al., Figure 3A

Bakshi et al., Figure 3B

Bakshi et al., Figure 3C

Figure 3. Injection of combinatorial (co) dsRNA induced suppression of cognate mRNA of GI and GII serpins in nymph (fig. 3A) and adult ticks (figure 3B and 3C)

Whole nymph and dissected organs (salivary glands, midgut, and carcass) adult *I. scapularis* ticks that were injected with GI and GII co-dsRNA, or GFP (control) dsRNA were incubated for one week before placing them onto the rabbit ear to feed. At 24 h post attachment, three ticks each: nymph and adult were sampled and individually processed for validation of RNAi silencing as described under section 2.6. Relative mRNA suppression was determined using the formula $S = 100 - (RQ^T / RQ^C \times 100)$ where S is equal to percent mRNA suppression of serpin, RQ^T and RQ^C = RQ of tissues of GI or GII dsRNA injected ticks and EGFP-dsRNA injected ticks. Data is reported as Mean ($M \pm SEM$) of duplicate readings in adults and duplicates readings in nymphs.

Figure 4. RNAi silencing of group (G) II serpins (nomenclature for this study) reduced tick feeding efficiency

Adult ticks that completed feeding and spontaneously detached from the host were photographed (Fig. 4A). Asterisk shows the dead tick found on host during feeding and arrowheads shows the ticks detached from the host retaining a whitish gel like material on their mouthparts. Ticks were individually weighed as an index for amount of blood taken in by the tick as engorgement mass (Fig. 4B). Likewise, hemoglobin content of detached nymphs was individually determined as described under section (Fig. 4C) under section 2.5. The unpaired student t-test was used to determine statistical significance. Data is reported as $M \pm \text{SEM}$, p-values are indicated in the figures.

Table 1

Tick serpin sequences used for construction of phylogenetic tree

Source sequence	Accession numbers in public databases
<i>Ixodes scapularis</i> Serpin 12	EW892008
<i>Ixodes scapularis</i> Serpin 14	ISCW015204
<i>Ixodes scapularis</i> Serpin 18	ISCW024013
<i>Ixodes scapularis</i> Serpin 21	EW881762
<i>Ixodes scapularis</i> Serpin 22	EW949864
<i>Ixodes scapularis</i> Serpin 10	ISCW023617
<i>Ixodes scapularis</i> Serpin 25	ISCW014257
<i>Ixodes scapularis</i> Serpin 9	B7QL31
<i>Ixodes scapularis</i> Serpin 17	B78JF1
<i>Ixodes scapularis</i> Serpin 19	B7PH24
<i>Ixodes scapularis</i> Serpin 32	B7Q0E8
<i>Ixodes ricinus</i> Serpin	AOA131XZ77
<i>Ixodes ricinus</i> Serpin	Q06B72
<i>Amblyomma maculatum</i> Serpin	G3ML50
<i>Amblyomma triste</i> Serpin	A0A023GPF9
<i>Amblyomma cajennense</i> Serpin	A0A023FM57
<i>Amblyomma americanum</i> Serpin	A0A0E9Y2F
<i>Rhiphicephalus pulchellus</i> Serpin	L7LRY7
<i>Rhiphicephalus appendiculatus</i> Serpin	A0A131YYG3
<i>Rhiphicephalus microplus</i> Serpin	A0A0R6CDI0
<i>Hyalomma excavatum</i> Serpin	A0A131XKU1
<i>Ixodes ricinus</i> Serpin	Q06B75
<i>Ixodes ricinus</i> Serpin	V5HBT7
<i>Amblyomma cajennense</i> Serpin	A0A023FQ10
<i>Amblyomma americanum</i> Serpin	A0A0E9Y314
<i>Ixodes ricinus</i> Serpin	A0A147BTY8

Table 2

Quantitative PCR primers for group I and group II serpins

Serpin sequences (Accession number)	Primer sequences (5'-3')	Amplicon Size (bp)	Concentration (nM)
Group 1	Serpin 12 (EW892008)	F: GCAGTGACGTCAATTTCAT R: TGATGCACCTCTCCATAAA	F: 300 nM R: 300 nM
	Serpin 18 (ISCW024013)	F: TATGGTCGAGGTGAACGAAA R: TGTAGAACAGGAACGGGTGA	F: 300 nM R: 300 nM
	Serpin 21 (EW881762)	F: CAAGGCTATGGTTGAGGTGA R: GACGTGGAAGTGCACAATTT	F: 300 nM R: 300 nM
	Serpin 14 (B7QNY6)	F: GTTAGGGCTGAAGTCGGTGT R: CGGCAGTACCTTCTTCGTTC	F: 300 nM R: 300 nM
	Serpin 17 (B7QJF1)	F: GCTGTGCTGGAGGTCAATGAA R: TTGGCAACGGGGCGGCGTCA	F: 300 nM R: 300 nM
	Serpin 9 (B7QL31)	F: AAGGATTGGCACGCAGGCATT R: CTTGTCCCGCAAACAGGATGTCA	F: 300 nM R: 300 nM
	Serpin 19 (B7PH24)	F: TGCTCGAGCTGCCTTACA R: AGGTCCTGCAGGTCTCTCTG	F: 300 nM R: 300 nM
	Serpin 32 (B7Q0E8)	F: AAGCCTTTGTGGAGGTGAAC R: ACACCGAGCATTTACTGCAA	F: 300 nM R: 300 nM