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# Target validation of highly conserved *Amblyomma americanum* tick saliva serine protease inhibitor 19

Tae K. Kim, Zeljko Radulovic, Albert Mulenga*

*Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX, USA*

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ABSTRACT

*Amblyomma americanum* tick serine protease inhibitor (serpin, AAS) 19, is a highly conserved protein that is characterized by its functional domain being 100% conserved across tick species. We also reported that AAS19 was an immunogenic tick saliva protein with anti-haemostatic functions and an inhibitor of trypsin-like proteases including five of the eight serine protease factors in the blood clotting cascade. In this study the goal was to validate the importance of AAS19 in *A. americanum* tick physiology, assess immunogenicity and investigate tick vaccine efficacy of yeast-expressed recombinant (r) AAS19. We confirm that AAS19 is important to *A. americanum* fitness and blood meal feeding. AAS19 mRNA disruption by RNAi silencing caused ticks to obtain blood meals that were 50% smaller than controls, and treated ticks being morphologically deformed with 100% of the deformed ticks dying in incubation. We show that rAAS19 is highly immunogenic in that two 500 μg inoculations mixed with TiterMax Gold adjuvant provoked antibody titers of more than 1:320,000 that specifically reacted with native AAS19 in unfed and partially fed tick tissue. Since AAS19 is injected into animals during tick feeding, we challenge infested immunized rabbits twice to test if tick infestations of immunized rabbits could act as booster. While in the first infestation significantly smaller tick blood meals were observed on one of the two immunized rabbits, smaller blood meals were observed on both rabbits, but 60% of ticks that engorged on immunized rabbits in the second infestation failed to lay eggs. It is notable that ticks fed faster on immunized animals despite obtaining smaller blood meals. We conclude that rAAS19 is a potential component of cocktail tick vaccine.



## 1. Introduction

Ticks and tick-borne diseases (TBD) pause enormous threats to global public and veterinary health. Ticks and important TBDs such as babesiosis, heartwater, and theileriosis are major source of economic loss in the livestock industry (Jongejan and Uilenberg, 2004). Presumably due to improved diagnostics, and climate change that is expanding geographic range of tick vectors, reported human TBDs are on the rise (Brownstein et al., 2005; Kalluri et al., 2007; Walker et al., 2008; Gray et al., 2009). By 2015, the USA Centers for Disease Control listed 14 reportable human TBD agents. *Amblyomma americanum* long considered a nuisance (Childs and Paddock, 2003), is now among medically important tick species. This tick is the principal vector for *Ehrlichia chaffeensis* and *Ehrlichia ewingii*, the causative agents of human monocytic ehrlichiosis (Anderson et al., 1993; Wolf et al., 2000), causative agent of the southern tick-associated rash illness (STARI) (James et al., 2001; Masters et al., 2008), and the heartland virus (Savage et al., 2013). It is also involved in the epidemiology of *Francisella tularensis* (Taylor et al., 1991), and it also transmits *Theileria cervi* (Laird et al., 1988), *E. chaffeensis, E. ewingii*, and *Borrelia lonestari* (Varela-Stokes, 2007) to white tailed deer. Heavy *A. americanum* tick infestation has been reported to reduce productivity in cattle (Barnard et al., 1992; Barnard, 1985; Tolleson et al., 2010, 2012). In absence of tick vaccines against major TBD agents, killing of ticks using acaricides remains the most important tick control method. However serious limitations such as ticks quickly developing resistance to acaricides, environmental and food chain contamination threatens continuity of tick control programs (Graf et al., 2004; George et al., 2004; Ghosh et al., 2007). To solve the problem of acaricide resistance, immunization of animals against tick feeding has been advocated as a sustainable alternative (Opdebeeck et al., 1988; Willadsen, 2004; Sonenshine et al., 2006; de la Fuente and Kocan, 2006; de la Fuente et al., 2007; George, 2000). The rationale is that anti-tick vaccines will be effective against both acaricide susceptible and resistant tick populations (Willadsen, 2004; Merino

* Corresponding author.
*E-mail address:* amulenga@cvm.tamu.edu (A. Mulenga).

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et al., 2013; Mulenga et al., 1999, 2001). Commercialization of the vaccine against *Rhipicephalus* (*Boophilus*) *microplus* validated the feasibility of controlling ticks through immunization (Willadsen et al., 1995). Weaknesses of the *R. microplus* vaccine including effectiveness against one tick species (Rodriguez et al., 1995a, 1995b; Garcia-Garcia et al., 2000), necessitates the search for effective target anti-tick vaccine antigens with potential to control multiple tick species (Mulenga et al., 2013a).

Our goal is to understand tick-feeding physiology as a means of discovering important tick saliva proteins that can be targeted for anti-tick vaccine development. In our laboratory, selection of potential tick vaccine targets is based on high amino acid conservation among ticks and that the candidate antigen is confirmed to be injected into its host. Through this pipeline we identified highly conserved *A. americanum* tick saliva serine protease inhibitor (serpin) (AAS) 19 characterized by 100% conservation of the functional domain reactive center loop in ixodid ticks (Porter et al., 2015). Kim et al. (2015a) recently showed that AAS19 mRNA is expressed in most tick tissues, that the native protein is injected into the host during tick feeding, and that this protein has anti-haemostatic functions with broad inhibitor activity against trypsin, plasmin and five of the eight serine proteases (factors [f] Xa and XIa, strongly, fIIa [thrombin], factors IXa, and XIIa, moderately) in the mammalian blood clotting cascade. In this study the goal was to validate the significance of AAS19 in *A. americanum* tick feeding success and assess its anti-tick vaccine efficacy. We show that AAS19 is important to tick feeding success as revealed by RNAi silencing and that rAAS19 is a potential target in cocktail tick vaccine formulation.

## 2. Materials and methods

### 2.1. Ethics statement

All animal work was conducted and approved according to Texas A&M University Institutional Animal Care and Use Committee (AUP 2011-0207).

### 2.2. Tick feeding, dissection, and protein extractions

*A. americanum* ticks used in this study were purchased from Oklahoma State University tick laboratory (Stillwater, OK, USA). Ticks were maintained in 85% humidity chamber at 25 °C before placing them on rabbits to feed. To feed, *A. americanum* ticks were placed onto the outer portion of the ear of specific pathogen-free (SPF) New Zealand rabbits and restricted in this location using orthopedic stockinettes adhered on the rabbit ear with Kamar Adhesive (Kamar Products Inc., Zionsville, IN, USA). To initiate feeding, 10 male ticks were placed into each ear stocking three days prior to adding 15 female ticks in each ear stocking (total of 30 female ticks per rabbit). To prepare tick protein extracts, five ticks from unfed and manually detached at 24, 72 and 120 h post tick attachment were rinsed in sterile 1X phosphate buffered saline (PBS) pH 7.4 and processed for dissections. Ticks were placed on a sterile glass slide and cut on the extreme anterior, posterior and lateral ends using a sterile razor blade. Tick organs including salivary glands (SG), midguts (MG), synganglion (SYN), Malpighian tubule (MT), ovary (OVR) and the remnants labeled as carcass (CA) were isolated and placed into IP lysis buffer with protease inhibitor cocktail (Thermo Scientific, Waltham, MA, USA). Protein extracts were homogenized and stored in −80 °C.

### 2.3. RNAi silencing of AAS19 mRNA

RNAi-mediated silencing was performed as described (Mulenga et al., 2013b; Kim et al., 2014). Double stranded RNA (dsRNA) was synthesized using the Megascript RNAi kit (Thermo Scientific) targeting position 327–971 of AAS19 nucleotide sequence (NCBI Accession# GAYW01000076). The 644 base pair dsRNA target sequence was searched against tick sequences in GenBank to verify specificity. Using 2 μg of purified PCR product as template, dsRNA was synthesized using primers with added T7 promoter sequence in bold (For: 5′-**TAATACGACTCACTATAGGG**GTACGCCCTGGACGTCGCCAACG-3′ and Rev 5′-**TAATACGACTCACTATAGGG**GAGAGGTCGGCGTCAGCG-GAG-3′). PCR primers for enhanced green fluorescent protein coding cDNA (EGFP; accession number JQ064510.1) with added T7 promoter sequence (Kim et al., 2014) were used to synthesize control EGFP-dsRNA. Two test groups of 15 female *A. americanum* ticks were injected with 0.5–1 μL (~3 μg/μL) EGFP-dsRNA or AAS19-dsRNA in nuclease free water as described (Kim et al., 2014). Injected ticks were kept for 24 h at 25 °C in 85% humidity to recover before being placed on SPF New Zealand rabbits to feed.

The effect of AAS19 mRNA disruption on tick feeding success was investigated by assessing tick attachment and mortality rates, time to feed to repletion, engorgement weight (EW) as an index for amount of blood taken in by tick, and egg mass conversion ratio (EMCR) as measure of utilizing blood meal to produce eggs as described. Tick phenotypes during feeding were documented daily using the Canon EOS Rebel XS camera attached to a Canon Ultrasonic EF 100 mm 1:2:8 USM Macro Lens (Canon USA Inc., Melville, NY, USA).

### 2.4. Validation of RNAi silencing

Disruption of AAS19 mRNA was verified by quantitative RT-PCR as described (Kim et al., 2014). Three ticks each that were injected with EGFP-dsRNA and AAS19-dsRNA were sampled at 48 h post-attachment by manual detachment. Ticks were processed individually. Tick organs (SG, MG, SYN, MT, OVR, CA) were dissected as described above. Extraction of mRNA using the Dynabead mRNA Direct Kit (Thermo Scientific) were performed following the manufacturer's instructions. The extracted mRNA was quantified using the Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland). Template cDNA was synthesized from ~200 ng of mRNA using the Verso cDNA Synthesis Kit following the manufacturer's instructions (Thermo Scientific). Approximately 50 ng cDNA and AAS19 forward (5′-GACAAGACGCACGGCAAAA-3′) and reverse (5′-GAAGTCCGGCGGCTCAT-3′) primers in a final concentration of 900 nM each, were mixed with 2X SYBR Green Master Mix (Thermo Scientific) in triplicates and was subjected to qRT-PCR. For an internal control, *A. americanum* 40S ribosomal protein S4 (accession number GAGD01011247.1) (Koci et al., 2013) was used. Relative quantification (RQ) of AAS19 transcript was determined as described (Kim et al., 2014). AAS19 mRNA suppression was determined using the formula, $S = 100 - (RQ^T/RQ^C \times 100)$ where $S$ = mRNA suppression, $RQ^T$ and $RQ^C$ = RQ of tissues in AAS19-dsRNA injected and EGFP-dsRNA injected ticks, respectively. Data are presented as the mean (M) AAS19 mRNA suppression ± SEM.

### 2.5. Immunization of rabbits with recombinant (r) AAS19 and tick challenge infestation

SPF New Zealand white rabbits of approximately 10–12 weeks of age were immunized with TiterMax Gold adjuvant (Sigma, St. Louis, MO) or 514 μg of rAAS19 antigen in 1X PBS pH 7.4 mixed with an equivalent volume of adjuvant to 1 mL. Expression and purification of the immunizing antigen, rAAS19, in *Pichia pastoris* (X-33) was previously described in Kim et al. (2015a). Two immunizations were administered at days 0 and 30. Rabbits were inoculated subcutaneously and intradermally with ~200 μL of rAAS19: adjuvant or PBS: adjuvant mix into five injection sites. Two weeks after immunization, serum was collected to verify antibody response to rAAS19