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Original article

Identification of 24 h *Ixodes scapularis* immunogenic tick saliva proteins

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ABSTRACT

Ixodes scapularis is arguably the most medically important tick species in the United States. This tick transmits 5 of the 14 human tick-borne disease (TBD) agents in the USA: *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *B. miyamotoi*, *Babesia microti*, and Powassan virus disease. Except for the Powassan virus disease, *I. scapularis*-vectored TBD agents require more than 24 h post attachment to be transmitted. This study describes identification of 24 h immunogenic *I. scapularis* tick saliva proteins, which could provide opportunities to develop strategies to stop tick feeding before transmission of the majority of pathogens. A 24 h fed female *I. scapularis* phage display cDNA expression library was biopanned using rabbit antibodies to 24 h fed *I. scapularis* female tick saliva proteins, subjected to next generation sequencing, *de novo* assembly, and bioinformatic analyses. A total of 182 contigs were assembled, of which ~19% (35/182) are novel and did not show identity to any known proteins in GenBank. The remaining ~81% (147/182) of contigs were provisionally identified based on matches in GenBank including ~18% (27/147) that matched protein sequences previously annotated as hypothetical and putative tick saliva proteins. Others include proteases and protease inhibitors (~3%, 5/147), transporters and/or ligand binding proteins (~6%, 9/147), immunogenic tick saliva housekeeping enzyme-like (17%, 25/147), ribosomal protein-like (~31%, 46/147), and those classified as miscellaneous (~24%, 35/147). Notable among the miscellaneous class include antimicrobial peptides (microplusin and ricinusin), myosin-like proteins that have been previously found in tick saliva, and heat shock tick saliva protein. Data in this study provides the foundation for in-depth analysis of *I. scapularis* feeding during the first 24 h, before the majority of TBD agents can be transmitted.

Introduction

Ticks are obligate blood-feeding ectoparasites that vector viruses, bacteria, and protozoa. Inflicting many negative consequences on their hosts, ticks are considered to be one of the most medically and veterinary important arthropods and rank second only to mosquitoes in terms of disease transmission (Sonenshine, 1993). There are at least 53 tick species frequently found to parasitize animals and humans (Dantas-Torres et al., 2012). It is estimated that ticks cause between 13.9 and 18.7 billion US dollars in damages worldwide annually (de Castro, 1997). With the habitat ranges of ticks quickly expanding due to the movement of humans and migration of animals as well as an increase in suitable environments due to climate changing, it is plausible to suggest that total cost of damages will only increase (Madder et al., 2012).

In the past, ticks and tick-borne diseases were solely associated with veterinary medicine, however from the discovery of the causative agent of Lyme disease in the 1980s, the importance of ticks to human health was further emphasized (Benach et al., 1983; Burgdorfer et al., 1982, 1983). The Centers for Disease Control and Prevention lists 14 human disease agents transmitted by ticks in the United States. Of those 14, the deer tick, *Ixodes scapularis* transmits causative agents for four diseases: human granulocytic anaplasmosis, human babesiosis, Lyme disease and Powassan virus disease. Specifically, *I. scapularis* transmits lineage II of the Powassan virus disease, the deer tick virus (Dupuis II et al., 2013). Lyme borreliosis, the most prevalent vector-borne disease in the northern hemisphere, was originally estimated to affect 30,000 however now it is thought to affect 300,000 people per year in the United States (CDC, 2013). Recently it was discovered that *Borrelia miyamotoi* is transmitted by *I. scapularis* and linked to human illness as seen in Russia and the U.S (Gugliotta et al., 2013; Krause et al., 2013;

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Platonov et al., 2011; Scoles et al., 2001). Another concerning factor to human public health, *I. scapularis* has demonstrated the ability to co-transmit pathogens and co-infect hosts with *Borrelia burgdorferi* and *Anaplasma phagocytophilum* (Levin and Fish, 2000).

Large-scale tick control by acaricide treatment represents the main way ticks are eliminated from hosts. However, this method is only a short-term solution as tick resistance to these chemicals is quickly emerging. The development of new acaricides is time consuming and expensive. Acaricides also contaminate the environment and animal feed as well as posing as health risks to humans (Graf et al., 2004). The concept of immunizations against ticks appears to be a practical solution and has been demonstrated with anti-tick vaccines against *Rhipicephalus microplus* (de la Fuente et al., 2007; Olds et al., 2013; Willadsen, 2004). However, this vaccine remains the only commercially available vaccine against ticks. With new molecular biology technologies and bioinformatic analyses advances, the process of identifying suitable universal tick antigenic targets has become more promising.

Tick vaccine development is centered on two main strategies including targeting "exposed" or "concealed" antigens. "Exposed" antigens are secreted in tick saliva and are exposed to the host at the feeding site whereas concealed antigens are hidden from the host immune system therefore failing to trigger an immune response. We propose that immunizing hosts with exposed antigens would eliminate the need for booster vaccinations because the host's immune system would be naturally primed by repetitive tick feeding. "Concealed" antigens would require repeated immunizations to maintain elevated antibody titers, making their use impractical. Except for viruses such as the Powassan virus disease that are transmitted within minutes of tick attaching onto host skin, the majority of TBD need more than 24 h after attachment to be successfully transmitted to host (Ebel and Kramer, 2004; McQuiston et al., 2000). For instance, *B. burgdorferi*, the causative agent of Lyme disease, invades the tick's salivary glands to enter the host 48 h post-attachment (De Silva & Fikrig, 1995). Likewise, studies have also shown that *A. phagocytophilum* transmission occurs between 24 and 48 h after tick attachment, while *Babesia microti* migrate to the salivary glands after 2–3 days of tick attachment and multiply to 10,000 sporozoites (des Vignes et al., 2001; Hodzic et al., 1998; Katavolos et al., 1998; Kjemtrup and Conrad, 2000). The purpose of this study was to identify *I. scapularis* immunogenic tick saliva proteins that are secreted into the host during the first 24 h after tick attachment onto host skin. Immunization against 24 h *I. scapularis* tick saliva proteins could impede the tick feeding process before the majority of pathogens would be transmitted to the host.

Materials and methods

Ticks

For this study, unfed *I. scapularis* ticks were purchased from the tick laboratory at Oklahoma State University. In our lab, ticks were maintained in tick chambers at room temperature, >85% relative humidity and fed on New Zealand white rabbits according to the animal use protocol approved by Texas A&M University IACUC. Female *I. scapularis* ticks were mated prior to feeding. Female ticks were presumed mated when found paired up with male ticks. Ticks were restricted to feed on the tops of rabbits' ears using an orthopedic stockinet tick containment device adhered onto rabbit skin with Kamar Adhesive (Kamar Products Inc., Zionsville, IN). Female ticks were manually detached with forceps at the 24 h time point. Ticks were then washed in dietylpyrocarbonate (DEPC) treated water to remove pieces of rabbit skin and hair. The tick mouthparts were also inspected for any remaining rabbit skin tissue. Following cleaning, ticks were air dried on paper towels, pooled in groups of 8–10, chopped up using scissors and subsequently disrupted using a sonic dismembranator model 100 (Fischer Scientific, Itasca, IL) in 1 mL of TRIzol (Life Technologies, Carlsbad, CA, USA). Finally, ticks were stored at −80 °C until total RNA extraction. Whole tick RNA was used in this experiment to ensure that all salivary proteins were identified, including those translated elsewhere and subsequently transported to the salivary gland for tick feeding.

Preparing of 24 h Ixodes scapularis cDNA expression phage display library

Tick messenger RNA extraction, cDNA synthesis, phage display libraries preparation, and phage selection through biopanning protocol were performed as previously described (Radulović et al., 2014). Total RNA was extracted by using TRIzol reagent according to manufacturer's protocol and mRNA was isolated from total RNA with Straight A's™ mRNA isolation system (Novagen, Madison, WI, USA). cDNA synthesis was performed using OrientExpress™ Oligo(dT) cDNA Synthesis Kit (Novagen, Madison, WI, USA) starting from a total of 4 μg of mRNA. The quality of cDNA was checked by amplification of actin gene sequence as previously described, followed by preparation of 24 h fed female *I. scapularis* parent phage display expression cDNA library using T7 Select System (Novagen, Madison, WI, USA) (Radulović et al., 2014). In order to verify the quality of the phage display library, the individual lengths of the cloned cDNA sequences were checked by PCR amplifications using T7 "up" and "down" primers provided in T7 Select System kit. *Escherichia coli* BLT5403 strain grown in M9TB liquid and LB solid media with carbenicillin (final concentration 50 μg/mL) was used as host cells for T7 library phages. Plate lysate amplification protocol was used for amplification of parent library. Selection of phages containing cloned cDNA sequence encoding antigenic tick proteins secreted through saliva during first 24 h post attachment was performed with the biopanning protocol using antibodies to 24 h fed *I. scapularis* females (Radulović et al., 2014).

Production of rabbit antibodies to tick saliva proteins was previously described (Mulenga et al., 2013). Briefly, pre-mated female ticks were placed on rabbit ears to feed for 24 h. A total of 15 specimens were fed per ear. After 24 h ticks were manually removed and rabbits were re-infested with unfed pre-mated females. A 24 h feeding cycle was repeated 4 times within one week, followed by a one-week break. The entire cycle was repeated four times before rabbit exsanguination and serum collection. The antibody response to tick saliva protein was verified by western blot analysis using proteins extracted from unfed ticks as well as from ticks at different stages of engorgement. As a negative control we used serum obtained from rabbits before tick feeding. The biopanned library was established after four rounds of biopanning. As a control for nonspecific binding of phages to produced antibodies, a control biopanned library was established using rabbit serum obtained prior to tick feeding as a source of antibodies.

Next generation sequencing, de novo assembly, and sequence analysis

24 h fed female and control libraries were subjected to next generation sequencing as previously described (Radulović et al., 2014). Illumina HiSeq2000 system with the following parameters was used: paired-end sequencing, read length of 100 bp, and 800,000 reads per sample. Sequence reads trimmed at the default 0.05 limit value were *de novo* assembled using CLC Genomics Workbench software version 6.0.2 (CLC Bio-Qiagen, Cambridge, MA, USA). All other parameters were set to the default settings. Contigs that were found in the control biopanned library were eliminated from further analysis due to non-specificity. Annotation of assembled contigs was performed by using BlastX