

## **Tick Defensins: Why is *Ixodes scapularis* (Acari: Ixodidae) a great vector for diseases?**

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### **Abstract**

Defensins are small proteins involved in the innate immunity of an organism. In this study, scapularisin, a defensin found in the tick *Ixodes scapularis*, and varisin, a defensin found in the tick *Dermacentor variabilis*, were successfully translated using an *in vitro* eukaryotic translation system. Proteins translated *in vitro* were analyzed on SDS-PAGE gel. Both scapularisin and varisin are shown to form dimers.

### **Introduction**

Lyme disease is the most prevalent vector-borne disease in the United States, infecting more than 20,000 people each year (CDC 2010). The blacklegged tick (*Ixodes scapularis*) is the primary vector of the spirochete *Borrelia burgdorferi sensu stricto*, the causative agent of Lyme disease, as well as two emerging pathogens, *Anaplasma phagocytophilum* and *Babesia microti*, the causative agents of Human Granulocytic Anaplasmosis and Babesiosis, respectively (Holman et. al. 2004, Coleman et. al. 2005). By studying the antimicrobial peptides found in the tick, it becomes possible to draw conclusions on the innate immunity of the tick and the role innate immunity plays in vector competence. Therefore, much knowledge can be gained by studying any antimicrobial peptides found in *I. scapularis*.

Recently, defensins have been isolated in several species of ticks (Hynes et. al. 2005, Todd et. al. 2007, Zhou et. al. 2007). Defensins are small arginine rich proteins, usually containing six cysteine residues that form disulfide bridges (Hynes

et. al. 2005). They have been found in both invertebrates and vertebrates, functioning as host defense proteins (Hoffmann and Hetru 1992, Ganz 2003). These peptides kill invading microorganisms by forming membrane-penetrating channels in the cells of the microorganisms (Gillespie et. al. 1997, Ganz 2003). Defensins are highly effective against gram-positive bacteria, but have been shown to inhibit gram-negative bacteria, fungi, and protozoan parasites (Gillespie et. al. 1997, Richman et. al. 1997, Shahabuddin et. al. 1998). Recently, a defensin, called scapularisin, was found in *I. scapularis*, but its role as an antimicrobial peptide remains unclear (Hynes et. al. 2005).

The American dog tick, *Dermacentor variabilis*, has a defensin, varisin, that is similar to the defensins found in insects (Johns et. al. 2001). Varisin has been shown to have antimicrobial activity against gram-positive bacteria and *B. burgdorferi* (Ceraul et. al. 2003). The amino acid sequences of varisin and scapularisin are 78.9%, and the six cysteine residues are located in that same relative positions (Hynes et. al. 2005). Therefore, it is unclear why scapularisin is not effective against *B. burgdorferi*.

In this study scapularisin and varisin genes were isolated using RT-PCR from *I. scapularis* and *D. variabilis* ticks. The cDNA was then amplified and sent out for sequencing. Once the genes were confirmed, the cDNA was placed in a eukaryotic in-vitro transcription/translation system to see if both sequences could be translated. The structure of the translated proteins were also studied.

## **Methods**

### *Tick Collection and Handling*

Adult *I. scapularis* were collected at The Louis Calder Center, the biological station of Fordham University, located 45 km north of New York City in Armonk, New York (41° 8' N, 73° 48' W) during March 2012 by drag sampling (Milne 1943). Adult *Dermacentor variabilis* ticks were donated by Dr. Daniel Sonenshine from Old Dominion University in Norfolk, Virginia. Ticks were shipped overnight in a cooler with dry-ice, around 4°C, to prevent tick death. Ticks were stored in vials with mesh tops and stored in a plastic bag with a damp paper towel, and the vials were placed in a refrigerator at a temperature of 4°C. This temperature allows tick metabolism to slow until placement in survival cages (Ogden et. al. 2004). Ticks were placed in 100% ethanol twenty-four hours prior to RNA extraction.

### *RNA Extraction*

Ticks were removed from the ethanol and allowed to air dry briefly to rid the excess ethanol. Ticks were then placed in Lysing Matrix A tubes (MPBio) with a sterile garnet matrix and one ¼ inch ceramic sphere. The tube containing adult *I. scapularis* ticks had 5 ticks and the tube containing adult *D. variabilis* ticks had 7 ticks. The ticks were homogenized using the Fastprep 24 (MPBio) set at a speed level of 4.0 m/s for 40 seconds. Immediately following homogenization, total RNA extraction was started with the addition of 600 µL of Buffer RLT Plus from the RNeasy® Plus Minikit (QIAGEN). Manufacturer's instructions were followed with minor modifications. The RNA was eluted once with 50 µL of dH<sub>2</sub>O.

### *Primers*

Primers specific for the reported mRNA sequences of scapularisin (GenBank Accession Number AY660970.1) and varisin (GenBank Accession Number AY181027.1) were designed. The sequence for a T7 promoter and a kozak sequence were added to the forward primers. The primer pair for scapularisin (F 5'-GGATCCTAATACGACTCACTATAGGGAACAGCCACCATGAGGGTCATTGCTGTTAC-3', R 5'-TGGTTTCTTCTAGTTGTGGTAGCATGTGC-3') amplified from just upstream from the start codon to after the stop codon. The primer pair for varisin (F 5'-GGATCCTAATACGACTCACTATAGGGAACAGCCACCATGCGCGGACTTTGCATCTG-3', R 5'-GGAGGCATTAATTCCTGTAGCAGGTGCAG-3') amplified from just upstream from the start codon to after the stop codon.

### *RT-PCR*

RT-PCR was performed using QIAGEN® One-Step RT-PCR Kit following the kit instructions. Twenty nanograms of RNA were amplified in 20 µL RT-PCR reactions (4.0 µL 5X RT-PCR buffer, 0.8 µL 10mM dNTPs, 0.8 µL enzyme mix, 0.8 µL 10pmol/µL forward primer, 0.8 µL 10pmol/µL reverse primer, x µL 10ng/µL RNA, and dH<sub>2</sub>O to final volume of 20.0 µL). There was one tube for each primer pair that served as a negative control in which no RNA was added. Temperature cycles for both scapularisin and varisin were: one cycle each of 50°C for 30 minutes and 95°C for 15 minutes; 50 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; followed by a final extension at 72°C for 10 minutes; and a final hold at 4°C.

### *Agarose Gel Electrophoresis*

2.5  $\mu$ L of loading dye was added to the PCR products. 5  $\mu$ L of each reaction was then added to 1.2% agarose gels containing ethidium bromide (EtBr), and electrophoresis was performed at 170 V for 1 hour. Bands were then visualized in a UV trans-illuminator (BioRad).

### *Gel Extraction, Ligation, Transformation, Miniprep, and Sequencing*

Bands representative of scapularisin and varisin from the RT-PCR products were cut out and purified using the QIAquick Gel Extraction Kit (QIAGEN). Following gel extraction, ligation and transformation were performed using pGEM-T and pGEM-T Easy Vector Systems (Promega) with minor modifications. Following transformation, the plasmid DNA was purified using QIAprep<sup>®</sup> Miniprep Kit (QIAGEN) with minor modifications. The samples were then sent out for sequencing by Genewiz Inc. The results were compared by BLAST to confirm product identities.

### *In-vitro Translation*

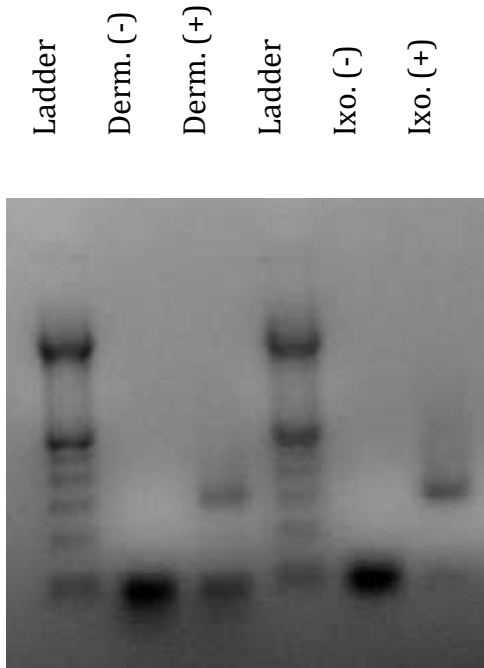
*In-vitro* transcription/translation was performed using TnT<sup>®</sup> Quick Coupled Transcription/Translation Systems Kit following the kit instructions. 200 nanograms of DNA were transcribed/translated in a 50  $\mu$ L reaction (40  $\mu$ L of TnT Quick Master Mix, 2  $\mu$ L [<sup>35</sup>S]methionine, 1  $\mu$ L T7 TnT<sup>®</sup> PCR Enhancer, 200 ng DNA, and dH<sub>2</sub>O to a final reaction volume of 50  $\mu$ L). The reaction was incubated at 30°C

for 90 minutes. A positive control with the luciferase gel, provided in the kit, was run to determine if translation was successful. The results were analyzed on a 10% Bis-Tris SDS polyacrylamide gel. Both scapularisin and varisin were run with and without the presence of  $\beta$ -mercaptoethanol.

## Results

The RT-PCR successfully amplified both scapularisin and varisin (Fig. 1). Each defensin was expected to be around 225 base pairs and this was confirmed when the agarose gel was visualized using a UV trans-illuminator. Both RT-PCR products for the defensins showed the presence of primer-dimer, therefore the bands representing scapularisin and varisin had to be cut out of the gel and purified. Following gel extraction, the products were placed into a plasmid vector for amplification. Both scapularisin and varisin produced numerous white colonies. Sequencing confirmed the presence of both scapularisin and varisin (Figs. 2 and 3). There were a few point mutations in the sequence of scapularisin from the reported sequence for scapularisin; however, there were no premature stop codons because of the mutations, and all six cysteine residues were in the same relative position. After confirming the presence of both scapularisin and varisin, *in-vitro* transcription/translation was performed. When the products were run on an SDS polyacrylamide gel in the presence of  $\beta$ -mercaptoethanol, both scapularisin and varisin produced one band just about 10 kDa (Fig. 4). However, when the same products were run on an SDS polyacrylamide gel without  $\beta$ -mercaptoethanol, both

scapularisin and varisin produced one band just above 20 kDa, suggesting that both defensins form dimers.



**Figure 1.** RT-PCR products from total RNA extracted from *Dermacentor variabilis* (Derm.) and *Ixodes scapularis* (Ixo.) showing the presence of varisin and scapularisin (both are ~225 bp).

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>gb|AY181027.1| Dermacentor variabilis varisin A1 (vsnA1) mRNA, complete cds
Length=624

Score = 416 bits (225), Expect = 3e-113
Identities = 225/225 (100%), Gaps = 0/225 (0%)
Strand=Plus/Plus

Query 1   ATGCGCGGACTTTGCATCTGCCTTGTCTTTCTCCTTGTGTTGGTCTTGTATCCGCCAGG 60
          |||
Sbjct 80   ATGCGCGGACTTTGCATCTGCCTTGTCTTTCTCCTTGTGTTGGTCTTGTATCCGCCACG 139

Query 61   GCTGCCGCCCCGGCTGAAAGCGAGGTGGCTCACCTCCGTGTCGTCGAGGCTTCGGATGC 120
          |||
Sbjct 140  GCTGCCGCCCCGGCTGAAAGCGAGGTGGCTCACCTCCGTGTCGTCGAGGCTTCGGATGC 199

Query 121  CCACTGAACCAAGGCGCTGTCAACCACTGCCGCAGCATTGGGCGCCGAGGCGGCTAC 180
          |||
Sbjct 200  CCACTGAACCAAGGCGCTGTCAACCACTGCCGCAGCATTGGGCGCCGAGGCGGCTAC 259

Query 181  TGCTCTGGCATCATCAAGCAGACCTGCACCTGTACAGGAATTAA 225
          |||
Sbjct 260  TGCTCTGGCATCATCAAGCAGACCTGCACCTGTACAGGAATTAA 304

```

**Figure 2.** Sequencing of the RT-PCR product from the Derm. product resulted in a 100% match to varisin, the defensin found in *D. variabilis*.

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>lcl|38917
Length=225

Score = 383 bits (207), Expect = 3e-111
Identities = 219/225 (97%), Gaps = 0/225 (0%)
Strand=Plus/Plus

Query 74   ATGAGGGTCATTGCTGTTACCTTGATCGCCCTTCTGGTTGCTGGAGCGTTTATGACTTCC 133
          |||
Sbjct 1     ATGAGGGTCATTGCTGTTACCTTGATCGCCCTTCTCGTTGCTGGAGCGTTTATGACTACC 60

Query 134  AGCGCACAAAGAGGAAGAAAACCAAGTGGCTCACGTTTCGAGTTCGACGTGGTTTTGGATGT 193
          |||
Sbjct 61   AGCGCACAAAGAGGAAGAAAACGAAGTGGCTCACGTTTCGAGTTCGACGTGGTTTTGGATGT 120

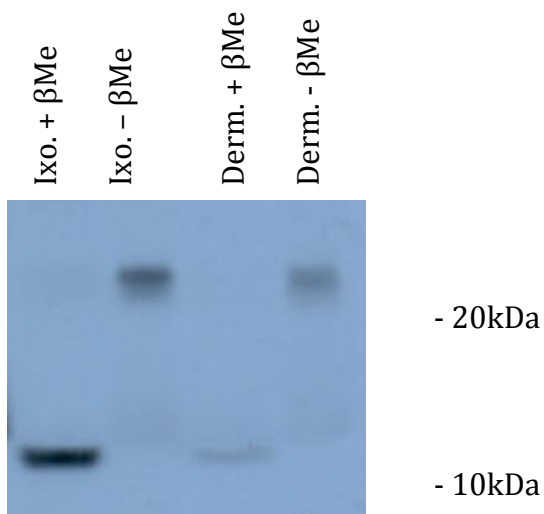
Query 194  CCCTTCGACCAAGGGGCGTGTACAGGCACTGCCAGAGCATCGGACGTCGCGGAGGTTAC 253
          |||
Sbjct 121  CCCTTCAACCAAGGGGCGTGTACAGACACTGCCAGAGCATCGGACGACGCGGAGGTTAC 180

Query 254  TGCGCGGGATTATCAAGCAGACGTGCACATGTACCACAACCTAG 298
          |||
Sbjct 181  TGCGCGGGATTATCAAGCAGACGTGCACATGTACCACAACCTAG 225

```

**Figure 3.** Sequencing of the RT-PCR product from the Ixo. product resulted in a 97% match to scapularisin, the defensin found in *I. scapularis*. There were a few point mutations in the sequence.





**Figure 4.** Both scapularisin and varisin appear to form dimers in the absence of  $\beta$ -mercaptoethanol.

## Discussion

Defensins have been studied in various insects and select species of ticks (Hoffmann and Hetru 1992, Hynes et. al. 2005, Wang and Zhu 2011). However, little knowledge exists regarding the functioning of the defensins in *I. scapularis*. The results discovered in this study show that scapularisin and varisin can successfully be extracted from ticks as RNA, amplified using RT-PCR, and then transcribed and translated in a eukaryotic *in-vitro* system. Now that it is possible to produce these defensins in the laboratory, it becomes possible to study their antimicrobial properties.

Scapularisin and varisin were also shown to form dimers when analyzed using SDS-PAGE without the presence of  $\beta$ -mercaptoethanol. Dimers and multimers

have been reported in the defensins of other species (Ganz 2003). This is the first time dimerization has been shown in a tick defensin. All defensins act by similar mechanisms, however it is possible that defensins that form dimers and multimers have evolved to target different types of bacteria (Ganz 2003).

In the future, it would be beneficial to study the stability of scapularisin and varisin to monitor the decay rates of these peptides. One hypothesis as to why scapularisin does not defend the tick against *B. burgdorferi* is the peptide immediately decays upon synthesis. The antimicrobial activity of scapularisin also needs to be clarified to determine whether or not this peptide can be effective against vector-borne diseases.

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