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Host Blood Proteins and Peptides in the Midgut of the Tick *Dermacentor variabilis* (Say) Contribute to Bacterial Control

by

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ABSTRACT

Antimcrobial midgut proteins and peptides that result from blood digestion in feeding American dog ticks *Dermacentor variabilis* (Say) were identified. Midgut extracts from these ticks showed antimicrobial activity against *Micrococcus luteus,* regardless of whether they were challenged with bacteria (*Bacillus subtilis, Escherischia coli* and *Borrelia burgdorferi),* purified peptidoglycan, blood meal components and/or whole blood (rabbit). However, no peptide band co-migrating with defensin was found in midgut extracts from ticks challenged with these microbes or blood meal components. Partial purification of the midgut extracts using C_{18} Sep Paks and gel electrophoresis showed the presence of 4 distinct bands with rMW 4.1, 5.3, 5.7 and 8.0 kDa identified by tryptic digestion-mass fingerprinting as digestive fragments of rabbit α-, ß- γ-chain hemoglobin, and rabbit ubiquitin. No evidence of varisin, a defensin previously identified in the hemolymph of *D. variabilis,* was found in the tryptic digest, although varisin was found in a hemocyte lysate isolated and analyzed by the same methods. However, varisin transcript was detected in midgut cell lysates. Also present in all midgut samples was a cluster of 3 overlapping bands with rMW 13.0, 14.1 and 14.7 kDa which were identified by tryptic-digestion LC-MS and MALDI-TOF as rabbit α- and ß-chain hemoglobin (undigested) and transtherytin. Lysozyme transcript was detected in midgut cell extracts but the peptide was not. Studies done on the midguts of other tick species demonstrated that hemoglobin digestion resulted in fragments that were antimicrobial. Our findings for *D. variabilis* confirmed the presence of these hemoglobin digestive fragments as well as an additional peptide from rabbit blood, ubiquitin, a peptide known to occur as part of an antimicrobial complex in vertebrate leukocytes. In addition, we show antimicrobial activity due to larger hemoglobin fragments

than those reported previously. In this respect, the midgut's response to microbial challenge appears different than the hemolymph. The midgut's antimicrobial activity appears to be primarily a byproduct of hemoglobin digestion rather than expression of immune peptides and proteins. In addition to the absence of direct evidence of defensin and lysozyme peptides, we also noted that *Borrelia burgdorferi* spirochetes were not lysed in the midgut lumen which would be expected if defensin was active in this location. In view of these findings, no evidence was found that defensin and lysozyme, despite the presence of transcript, contributes to the midgut's immune defense.

Keywords: Midgut, antimicrobial peptides, hemoglobin fragments.

Running head: Tick midgut response to blood feeding and bacterial challenge.

INTRODUCTION

Ticks are notorious as vectors of the agents of infectious disease which they transmit when feeding. As blood feeders, the midguts of these parasites are exposed to microbes, both pathogenic and non-pathogenic, in addition to the nutritive components of their food source. How some microbes survive in the tick's midgut and internal tissues while other microorganisms are destroyed is a question that has baffled investigators for many years.

In insects and other invertebrates, ingestion of various microbes provokes an effective defense by upregulating the innate immune system (Gillespie et al. 1997, Beerntsen et al. 2000). Foreign molecules, e.g., LPS, lipoteichoic acid, etc., on the surfaces of many prokaryotes and some eukaryotic parasites trigger receptor responses by pattern recognition molecules on the luminal surfaces of the midgut cells. The response induces or enhances expression of an array of antimicrobial peptides such as lectins, lysozyme and defensins. In mosquitoes, defensin expressed following challenge by motile malaria ookinetes effectively destroys most of the cell penetrating parasites (Richman et al. 1997; Vizioli et al. 2001; Lowenberger et al. 999). As many as three different antimicrobial peptides, a cercropin, an attacin and a defensin, are expressed in tsetse flies, when challenged with tsetse-specific *Trypanosoma* organisms. Even non-pathogenic microbes such as *Micrococcus luteus* and *Escherichia coli* can induce expression of these potent defense compounds (Boulanger et al. 2002). In addition, heme and microbe-inhibiting peptidic fragments that result from hemoglobin digestion contributes to the rapid elimination of most invading microorganisms.

In contrast to blood feeding insects, much less is known about the ability of ticks to recognize and destroy invading organisms. The tick midgut has long been regarded as a favorable environment for survival of ingested microorganisms because of the presumed

absence of intraluminal proteolytic enzymes. Digestion in ticks is almost entirely intracellular. However, antimicrobial peptides have been found in ticks. A defensin, varisin was purified from the hemolymph of American dog ticks, *Dermacentor variabilis* (Johns et al. 2001). More recent evidence indicates the hemocytes as an important site of defensin synthesis and storage (Ceraul et al. 2003). However, no unequivocal evidence of its expression in the midgut of these ticks has been found (Ceraul et al. unpublished). Defensins have also been reported in both the hemolymph and digestive tract of the soft tick, *Ornithodoros moubata* (Nakajima et al. 2002). Other antimicrobial peptides, including lysozyme isolated from the midgut (Kopacek et al. 1999; Grunclova et al. 2003) and a lectin from the hemolymph (Kovar et al. 2000) have been in found in soft ticks. Evidence of lectin-like activity was also reported in *D. variabilis* hemolymph when challenged with *E. coli* (Ceraul et al. 2002). Aside from these few reports, little information is available regarding the occurrence of antimicrobial peptides in the midgut or whether they are expressed in response to microbial challenge.

In addition to the antimicrobial peptides, tick digestion of blood meal hemoglobin may result in peptides that can destroy invading microbes. Fragments of hemoglobin digestion in the lumen of the midgut of a hard tick, *Boophilus microplus* (Fogaca et al.1999) and the soft tick *O. moubata* (Nakajima et al.2003) have been reported to have antimicrobial activity against non-pathogenic, non-invasive bacteria. Whether such peptidic fragments would kill pathogenic microbes has not been reported.

The use of capillary oral feeding has made it possible to introduce specific microbes or compounds into the tick's digestive tract (Macaluso et al. 2001, 2002) and to observe the response. Using this method, we sought to determine whether challenge with selected

microbes, e.g., *Borrelia burgdorferi,* bacterial components or blood products would induce expression or secretion of antimicrobial peptides by the tick's midgut. Knowledge of how the tick's digestive tract responds to microbes and molecules in its blood meal may provide new insights into how pathogenic microbes are able to survive and colonize their tick vectors and be transmitted to cause disease.

MATERIALS AND METHODS

Ticks. *D. variabilis* was colonized and maintained as described previously (Sonenshine, 1993). Blood-fed females were detached from New Zealand White Rabbits (*Oryctolagus cunniculus)* following 4 days feeding on these animals. All use of animals for this research was done in accordance with protocols approved by the Old Dominion University Institutional Animal Care and Use Committee (IACUC). The approved protocols are on file in the Old Dominion University Animal Care Facility Office.

Bacteria and Bacterial Components. *Bacillus subtilis (*ATCC strain 6051) and *Escherischia coli* (ATCC # 25922) were obtained from the American Type Culture Collection. *B. subtilis* were grown in Tryptic Soy Broth (TSB) (Difco, Detroit, MI). *E. coli* were maintained on TSA agar plates. *Borrelia burgdorferi* strain B-31 was obtained from the Centers for Disease Control, Fort Collins, CO and cultured in BSK II (Sigma, St. Louis, MO) at 33°C in a 5% CO_2 incubator. Bacterial suspensions were prepared by centrifugation (3000 g for 10 min) and washing the pellet with 10 mM Phosphate-buffered saline (PBS) pH 7.2 to remove the growth medium. Cells were re-suspended in the same buffer. *B. burgdorferi* suspensions were adjusted with a Hausser Brightline hemocytometer (Hausser, Horsham, PA) using a Nikon Optiphot phase contrast compound microscopy to approximately 50,000 cells per microliter. *B. subtilis* and *E. coli* were re-suspended in 10

mM PBS and adjusted to 135,000 colony forming units (CFU) per microliter for *B. subtilis* and 155,000 CFU per microliter for *E. coli*.

Oral Feeding. Artificial capillary feeding (oral feeding) was done as described by Macaluso et al. (2002). Ticks fed on rabbits for 4 days were immobilized on glass slides and allowed to imbibe medium in glass microcapillaries positioned over their mouthparts. Ticks were incubated at 27 ± 1^0 C and 90 ± 1% RH during the procedure. At first, oral feeding was done for 3 – 4 h; subsequently, oral feeding was increased to periods of 6 – 15 h. ATP (1 mM) was added to 0.1 M PBS (pH 7.2) buffer as a feeding stimulant. Medium was replenished 1X or 2 X to compensate for any evaporation or leakage. Ticks were challenged with the 3 different bacterial genera in buffer as described below. In addition to bacteria, ticks also were challenged with peptidoglycan (Sigma Chemical Co., St. Louis, MO) at a concentration of 1 μg/μl in buffer. Other ticks were challenged with 2% rabbit whole blood, 2% rabbit serum, 2% hemoglobin (Sigma), hematin (Sigma) and heme, all diluted in buffer. Heme was prepared by digesting 5% hemoglobin with protease, then precipitating the protein and collecting the heme-containing supernatant; the presence of heme was confirmed by spectrophotometry (Shimadzu UV160U, Shimadzu Instrument Co., Columbia, MD). Controls consisted of ticks allowed to feed only on buffer (including ATP) and blood fed ticks without challenge.

To determine whether ticks consumed fluid from the glass capillaries, 20μl of 1 μm FITC-labeled fluorescent microspheres (Molecular Probes, Inc., Eugene, OR) per mL of blood was included in the medium. To confirm fluid uptake, the ticks were washed 3 X in buffer, agitated (Vortex) and the capitulum (containing the mouthparts) excised to remove surface contamination. Samples of midgut tissue collected during tick dissections (10

ticks/sample) were smeared onto glass slides, mounted in Slo-Fade mounting medium (Molecular Probes) and examined by epifluorescence microscopy.

Tissue Collections and Protein Assays. Following host or capillary feeding, ticks were surface washed with 70% ethanol: 3% H_2O_2 to remove contaminants. The midguts were removed, washed in PBS buffer and homogenized in cold (4^0 C) protein collection buffer consisting of 100 mM PBS supplemented with 0.1 – 0.2 mM PMSF and a 200-fold dilution of protease inhibitor cocktail (Sigma, St. Louis, MO, cat.no. P8340). Samples (N = 45) were sonicated, then frozen (-20^0 C) until needed. One sample of midguts from 6-day blood-fed ticks was collected in lysis buffer (Ceraul et al. 2003), homogenized, sonicated and filtered using 3.5 MWCO Microcon filters (Millipore Corp., Bedford, MA) prior to freezing. Bradford protein assays were performed as described by the manufacturer (BioRAD, Richmond, CA) using immunoglobulin G as the standard.

Gel Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 2-mercaptoethanol was done using Tris-Bis 4 -12% gradient NuPage minigels gels, 10 cm x 10 cm x 1 mm thick (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's recommendations. Midgut samples collected as described above were adjusted to similar protein content (10 μg) and loaded into each lane. Gels were stained with silver (Silver Express, Invitrogen) or Commassie Brilliant Blue (R), photographed with a Kodak Gel Logic 100 Imaging System (Kodak, Rochester, NY) and relative molecular weights (rMW) assigned using the Kodak ID Software.™ Controls included 1) midguts from blood-fed ticks without bacteria; 2) extracts of sonicates made from cultures of the three different bacterial genera; 3) serum from a tick-infested rabbit; 4) hemoglobin only (Sigma); 5) heme (Sigma) and 6) lysate of *D. variabilis* hemocytes.

To detect basic proteins (pH > 7.0), samples of midgut lysate were assayed on a 1 mm thick native 15% acidic gel as described by Lebendiker (2004) and run using reversed polarity. Gels were stained with Coomassie Brilliant Blue (R). Bands that migrated into the gel were excised, eluted, dialyzed overnight, concentrated and stored (-20 ° C) for protein identification.

Western Blot. This was done as described by Ceraul et al. (2003). Antiserum was prepared in a rabbit (*Orytolagus cunniculus*) immunized with synthetic defensin conjugated to keyhole limpet hemocyanin (KLH) and affinity purified using a 4% agarose Aminolink Plus Immobilization affinity column (Pierce Biotechnology Inc., Rockford, IL) prior to use. Defensin controls were synthetic defensin and *D. variabilis* hemocyte lysate as described by Ceraul et al. (2003). Trials were repeated twice with midguts collected in normal buffer and with midguts collected in lysis buffer.

Protein Purification and Identification. Crude extracts of midguts collected were fractionated by loading them onto C_{18} Sep Paks (Waters, Milford, MA) and eluting with mixtures of 0.1% trifluoroacetic acid (TFA): acetonitrile (ACN) ranging from 100% TFA to 100% ACN. Fractions were concentrated by lyophilization (LabConco, Lyphlock Lyophilizer, Kansas City, MO) and reconstituted in 50 mM PBS to the desired concentration for further analysis. Aliquots of fractions were assayed by SDS-PAGE as described above, silver-stained (Silver Quest, Invitrogen) or Coomassie Blue (R) stained and bands of interest excised, eluted, filtered to remove buffer salts using 3.5 MWCO Microcon filters, concentrated and frozen for further analysis.

Gel slices excised from the protein gels were analyzed in accordance with standard protocols at the University of Virginia's W.M. Keck Biomolecular Research Facility,

Charlottesville, VA (www.healthsystem.virginia.edu/internet/biomolec/) as described by previously (Cohen and Chait, 1997; Rao et al. 2003). After digestion with trypsin, peptides were introduced into a Thermo-Finnigan LCQ DecaXP mass spectrometer and the resulting spectra were searched against the NCBI (BLAST P) non-redundant database. The sensitivity of detection would detect proteins or peptides present in low concentrations, even as low as 1% of the sample.

Assay for Anti-microbial Activity. Aliquots of midgut protein extracts and fractions eluted with C_{18} SepPaks were adjusted to equivalent concentrations (approximately 15 μg/μl) and pipetted into 8 mm wells cut into agar plates as described previously (Johns et al. 2000). Subsequently, the agar plates were seeded with a 3 h log phase culture of *Micrococcus luteus* to create a bacterial lawn. Samples included crude and fractionated extracts of midguts from female ticks forcibly detached after 3 – 4 days of feeding as well as those oral fed on 1) 2% hemoglobin, 2) 5% heme; 3) 5% hematin; 4) 2% globin (from protease digestion of hemoglobin); 5) *B. burgdorferi;* 6) *B. subtilis* and 8) *E. coli*. Controls included 1) ticks fed 4 days, no other treatment and 2) ticks fed with PBS + ATP buffer. Antimicrobial activity, as detected by a zone of growth inhibition around each well, was measured after 24 h.

Electron Microscopy. Midgut samples were collected after capillary feeding for 3 h and prepared for TEM by conventional methods. Tissues were fixed in cold (4^0 C) 4% glutaraldehyde buffered in 0.1M S-collidine, washed, postfixed, dehydrated and embedded (EM 812) as described previously (Sonenshine et al. 1981). Thin sections in the gray-silver spectrum were cut on an RMC Ultramicrotome MT2C using a Dupont Diatome 45^o diamond knife. The sections were positively stained using uranyl acetate and lead citrate. Thick

sections were examined with the Nikon Optiphot microscope at 400 and 1000X and photographed with the Spot digital camera (Diagnostic, Inc., Milwaukee, WI). Thin sections were viewed and photographed using a JOEL 100 CX II transmission electron microscope at an accelerating voltage of 60 kV.

Molecular Analysis. mRNA was isolated from bacteria-challenged or blood-fed midguts using QuickPrep™ Micro mRNA Purification Kit (Amersham Biosciences, Piscataway, NJ) in accordance with the manufacturer's recommendations. Reverse transcription (RT) and PCR amplification was done as described by Ceraul et al (2003) except as noted below. Primers used for detection of defensin (varisin) and lysozyme transcript in tick midguts are given in table 1. To determine the presence of defensin or lysozyme transcript, RT was done using the ImProm-II Reverse Transcription System (Promega, Madison, WI) with the specific primer VsnR for varisin and the specific primer LysR for lysozyme. PCR was carried out using PCR Supermix High Fidelity (Invitrogen) with 200 nM of the defensin primers VsnF and VsnR (Ceraul et al. 2003) and 200 nM of the lysozyme primers LysF and LysR designed from the tick lysozyme sequence (GenBank AY183671.1). RT negative controls were included to monitor for DNA contamination. For both defensin and lysozyme, PCR cycling was as follows: 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 58°C for 1min, 68°C for 1 min followed by a final extension at 68°C for 10 min. The resultant amplicons were visualized on a 2% agarose gel. Following PCR, the ~ 225 (putative defensin) and ~400 bands (putative lysozyme) were extracted from the agarose gel using a Qiagen gel extraction kit (Qiagen) and cloned into pCR4 TOPO (Invitrogen, Carlsbad, CA). DNA sequencing was carried out using the ABI Bigdye Terminator V3.1 Ready Reaction Kit and run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Identification of the DNA sequences was by BLAST analysis from the National Center for Biotechnology Information (NCBI), National Library of Medicine, NIH, Bethesda, MD.

RESULTS

Smears of midguts from samples of female ticks (10 females/sample) challenged by capillary oral feeding showed numerous fluorescent microspheres in all specimens examined with the fluorescent microscope, indicating that the ticks imbibed at least some of the contents from the capillaries (figure not shown).

Agar plates containing the midgut extracts showed zones of anti-bacterial activity in the *M. luteus* lawn surrounding wells containing midgut extracts from females challenged with *B. burgdorferi*, 2% hemoglobin and unchallenged (fed on rabbits only) (**Fig. 1 A**). Similar results were obtained with samples challenged with the other bacteria and heme. No inhibition zones were observed surrounding the wells left untreated, those treated with buffer, or midgut extracts from ticks challenged with hematin (**Fig. 1 A**). Similarly, no growth inhibition was observed with samples challenged with peptidoglygcan or rabbit serum. When the midgut was extracted with lysis buffer, a distinct clear zone of growth inhibition was observed surrounding the crude extract; much larger zones of growth inhibition were observed around the wells treated with fractions partially purified by SepPak elution of the midgut lysate with increasing strength acetonitrile, especially around the wells with the 40% and 80% eluates. No growth inhibition was found around the well with buffer only (**Fig. 1 B**). When the 40% SepPak eluate was electrophoresed (see below), strongest growth inhibition was observed around the 8 kDa band but weaker inhibition around the 4-5 kDa bands excised from the gel (**Fig. 1 C**).

Transmission electron micrographs (TEM) showed that spirochetes, *B. burgdorferi,* remained intact in the lumen of the midgut as long as 3 h after oral feeding **(Fig. 2).** Numerous specimens were observed in the lumen; however, none had penetrated the midgut epithelium or migrated to the midgut's outer lining.

When the SDS-PAGE profiles of the midguts of ticks fed naturally or challenged with *B. B. burgdorferi,* were examined, a small protein band with rMW of 6.2 kDa was evident; in some specimens, a smaller band at 5.30 kDa was barely detectable. However, neither band co-migrated with the rMW 4.95 kDa band in the hemocyte lysate subsequently identified by tryptic digestion as tick defensin. Neither band was evident in the samples from ticks challenged with 2% hemoglobin solution or 30 % rabbit serum while only the upper band was faintly evident in the sample from ticks challenged with peptidoglycan (Fig. 3 A). These bands were subsequently identified as fragments of α and β-chain hemoglobin (see below). No difference was found in the presence or intensity of these peptide bands when capillary feeding was done for 3 h or 6 –15 h. Midguts from ticks challenged with *B. subtilis, E. coli,* the heme moiety of hemoglobin or hematin showed a similar response; no evidence of a band that co-migrated with defensin was seen (data not shown).

Gel electrophoresis of the 40% SepPak purified eluate showed the presence of 3 distinct bands with rMW 4.1, 5.3, 5.7 kDa. Also present was a band at rMW 8.0 kDa, and a cluster of up to 3 bands, rMW 13.0, 14.1 and 14.7 kDa. The latter 3 peptides migrated as a broad band that was evident in all blood fed specimens and in the rabbit hemoglobin control. Arrows indicate the peptides or peptide clusters submitted for protein identification (Fig. 3 B).

Western blots done on the protein gels did not detect varisin in the midgut samples (data not shown). Acidic gels, which separated basic proteins such as defensin, showed a distinct band in the hemocytes lysate that co-migrated with the defensin standard, but did not reveal any peptides from the midgut lysate that co-migrated with defensin (**Fig. 3 C**).

Tryptic digestion –mass spectrometry of the gel slice from the hemocyte lysate containing the 4.95 kDa band revealed a fragment with the sequence of varisin, the *D. variabilis* defensin. In contrast, tryptic digestion –mass spectrometry of the 4.1, 5.3 and 5.6 kDa bands from the midguts revealed digestive fragments of rabbit α-chain and ß-chain hemoglobin and fragments of rabbit ubiquitin, but no evidence of defensin. Similarly, tryptic digestion of the 13.0 – 14.7 kDa band showed rabbit α-chain and ß-chain hemoglobin and rabbit transtherytin, but no evidence of lysozyme. Tryptic digestion of the antimicrobial 8.0 kDa band also showed fragments of rabbit α-chain and ß-chain hemoglobin, indicating larger fragments of α-and ß-chain hemoglobin than those noted above. Representative fragments of the tryptic digests are shown in Table 2. MALDI-TOF of the 8 kDa band (from the SDS-PAGE gel) showed one predominant peak at 11,293 daltons constituting 65.4% of the total peaks present, and several smaller ones (**Fig. 4 A**). Clearly, one (the largest) or several of the α-and ß-chain hemoglobin fragments is responsible for the antimicrobial activity found in this midgut sample. MALDI-TOF analysis of the 13.0 – 14.7 kDa band showed peaks consistent with the undigested rabbit α-chain and ß-chain moieties of hemoglobin, but no evidence of lysozyme (**Fig. 4 B**).

RT-PCR using varisin primers showed a band (~ 225 bp) in the sample from tick midguts, which was identified by sequencing as varisin, the *D. variabilis* defensin (GenBank AY181027). RT-PCR using lysozyme primers showed a band (~ 400 bp) in samples from

tick midguts. Sequencing of this amplicon gave a sequence with similarity with the *D. variabilis* tick lysozyme sequence (GenBank AY183671.1).

DISCUSSION

The results of these studies showed the presence of fragments of rabbit α- ß-and γ-chain hemoglobin, resulting from breakdown of these proteins, as well as rabbit ubiquitin and rabbit transthyretin in the midguts of *D. variabilis* females. However, there was no evidence of tick defensin (varisin) peptide, even though varisin transcript was amplified from tick midgut tissue. Western blots, which previously showed defensin in hemocyte lysates (Ceraul et al. 2003) did not detect this peptide in midgut extracts. Acidic gels, which separate basic proteins such as defensin based on their polarity, showed a band in the hemocyte lysate, subsequently identified as varisin by tryptic digestion/mass spectrometry, but no comparable peptide band in the midgut lysates. On SDS protein gels, bands that migrated at or close to the relative mobility of defensin were found instead to be fragments of the rabbit proteins, α- and ß-chain hemoglobin and ubiquitin. The importance of the finding of these digestive fragments of rabbit proteins is related to their antimicrobial activity reported in previous studies. Digestion of rabbit α- chain hemoglobin by the soft tick, *O. moubata* (Nakajima et al. 2003) and bovine hemoglobin by the cattle tick, *Boophilus microplus* (Fogaca et al. 1999) led to the production of small fragments that were antimicrobial against gram positive bacteria. In this study, even larger fragments of α- and ß-chain hemoglobin digestion, including one as large as 11.29 kDa, were found to have antimicrobial activity. This has not been reported elsewhere.

In contrast to blood feeding insects, where defensin is expressed in response to microbial challenge, no evidence of tick defensin was found in samples from tick challenged

with different bacteria, components of bacterial cell wall such as peptidoglycan or increased concentration of hemoglobin.

Erythrocytes and other blood cells are lysed in the midgut lumen, but there is no evidence that the hemolysins of ticks are also anti-microbial (Ribeiro, 1988). Hemoglobin released by hemolysis of erythrocytes is digested intracellularly. There, the hemoglobin tetramer is disrupted, releasing heme (hemin) and the α-, ß-, γ- and δ-chain hemoglobin moieties. The α- and ß-chain hemoglobin moieties are known to be active against a variety of gram-positive and gram-negative bacteria (Parish et al. 2001). Hemin also has significant antibacterial activity (Stojilkovic et al. 2001). Escape of these moieties into the midgut lumen is expected to occur after break up of the midgut digestive cells, a normal facet of the digestive process in ticks (Sonenshine, 1991).

This study is the first to report evidence of host ubiquitin in the tick midgut. Members of the ubiquitin protein family found in the cytosol of leukocytes form a complex with ribosomal S30 that exhibits strong antimicrobial activity against gram-negative as well as gram-positive bacteria (Hiemstra et al. 1999). Since ticks are known to secrete hemolysins into the lumen that lyse leukocytes as well as erythrocytes (Sonenshine, 1991) this would allow release of the ubiquitin-S30 complex, which may inhibit any microbes present.

It is not clear whether tick lysozyme contributes to the inhibition of ingested bacteria in the tick's midgut. No evidence of this protein was found in the tryptic digests of the midgut extracts. Although it is possible that lysozyme was not extracted from the midgut tissues, this seems unlikely in view of the tissue lysis procedure used. However, lysozyme transcript was found in the midgut tissue lysate and its identity confirmed by DNA sequencing. In *O. moubata,* Grunclova et al. (2003) reported that tick gut lysozyme is upregulated in response

to blood feeding (measured by semi-quantitative PCR), but lysozyme in hemocytes remained unchanged. These authors found no evidence of an increase in lysozyme transcript in response to bacterial infection. Other digestive enzymes that might inhibit or kill bacteria have been found in tick midgut cells, e.g, acid phosphatase (Agyei et al. 1992), alkaline phosphatase (Gough and Kemp, 1995) and serine proteases (Mulenga et al. 2003), but were localized to intracellular vesicles within the midgut epithelia and would not normally be available in the lumen.

In contrast to the hemolymph, exposing the tick's midgut to challenge with different bacteria or peptidoglycan, a bacterial wall component, did not lead to defensin secretion previously shown to occur in the hemolymph (Johns et al. 2000; Johns et al. 2001b). No band consistent with the rMW for defensin was found in response to these different types of challenge. Challenging the midgut with hemoglobin, heme or serum also did not result in production of defensin, although blood feeding was reported to induce defensin expression in soft ticks (Nakajima et al. 2002). One possibility is that the number of microbes or peptidoglycan molecules ingested during the oral feeding was insufficient to induce such a response. Alternatively, direct contact with the midgut epithelium may be blocked by the developing peritrophic membrane, in those species where it occurs, but this probably occurs too late be effective (Vaughn and Azad, 1993). Blood feeding insects, however, showed expression of specific antimicrobial peptides in response to similar challenges with infectious microbes (Boulanger et al. 2002; Beernsten et al. 2000; Lowenberger et al. 1999; Richman et al. 1997; Vizioli et al. 2001).

Previous studies (Sonenshine et al. 2002) showed that, contrary to earlier beliefs, the midgut lumen may be an unsuitable environment for survival of bacteria. Attempts to

culture *B. subtilis, E. coli,* or *B. burgdorferi* from the midguts of ticks that had ingested these bacteria were unsuccessful. The mode of inhibition is unknown, although the survival of intact *B. burgdorferi* spirochetes as late as 3 hours is not surprising in view of the apparent absence of varisin and lysozyme in the midgut lumen.

In summary, our evidence suggests that the antimicrobial activity observed in the midguts of *D. variabilis* is related primarily to host-derived blood meal proteins, namely, digestive fragments of α- and ß-chain hemoglobin and, possibly, the ubiquitin ribosomal S-30 complex, rather than expression of defensin, lysozyme and/or other tick specific antimicrobial peptides.

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Table 1. Primers used for determination of antimicrobial peptides in tick midgut.

Name	Sequence
VsnF	5'GACTGCGCTTTGAGACGACAAA 3'
VsnR	5'AGAAAGCATAACCATTTTTAATATGCATTT 3'
LysF	5' ATGCAGCTGCACCTGCCGCTCGCG-3'
LysR	5' ATATCGGCACCCCTTGACGTAGGA-3'

Table 2. Results of tryptic digestion/mass spectrometry analysis for identification of antimicrobial peptides recovered from gel slices of protein gels.

rMW band	Representative partial amino acid sequence found	Mass (D)	Identification[1]	Native MW (D)
A. Hemocytes				
4.95 kDa band	GFGCPLNQGACHNHCRS[a]	1884.8	Tick defensin (varisin)	4229.0
B. Midgut				
4.1,5.3,5.7 kDa bands	AVGHLDDLPGALSTLSDHAHK[a]	2267.2	α -chain hemoglobin	15482.5
"	VLAAFSEGLSHLDNLK[a]	1713.9	β-chain hemoglobin	16141.5
"	TITLEVEPSDTIENVK[a]	1787.9	Ubiquitin	17959.7
8 kDa band	AVGHLDDLPGALSTLSDHAHK[a]	2267.2	α -chain hemoglobin	15482.5
"	KVLAAFSEGLNHLDNLK[a]	1869.0	β-chain hemoglobin	16141.5
13.0 – 14.7 kDa bands	FLANVSTVLTSK[a]	1279.7	α -chain hemoglobin	15482.5
"	VVAGVANALAHK[a]	1149.7	β-chain hemoglobin	16141.5
"	AADETWEPFASGK[a]	1408.6	Transthyretin	

[1] Determined by tryptic digestion/mass spectrometry. See Materials and Methods for details.

[a] Selected sequences from among numerous others from the tryptic digest.

Abbreviations: D = Daltons; kDa = kilodaltons.

Description of Figures

Fig. 1. Photograph of an anti-bacterial assay done on an agar plate. The plate was seeded with a lawn of *Micrococcus luteus*. Extracts of *D. variablis* midguts with or without challenge with bacteria or nutrient solutions were deposited in the 8 mm diameter wells. **A.** Midguts extracted in protease inhibitor buffer. 1 = blank; 2 = buffer solution only; 3 = midguts challenged with 5% hematin solution; 4 = midguts challenged *B. burgdorferi*; 5 = midguts challenged with 2% hemoglobin solution; 6 = midguts from fed ticks, no challenge. Protein content of midgut samples adjusted to an average of 154 μg protein per well. **B.** Midguts extracted in lysis buffer. 1 = Midgut, crude extract; 2 = TFA only; 3 = 30% acetonitrile eluate; 4 = 40% acetonitrile eluate; 5 = 80% acetonitrile eluate; 6 = 100% acetonitrile eluate; 7 = buffer only. Protein content was adjusted to approximately 150 μg/well. **C.** Protein bands from 30% acetonitrile fraction excised from an SDS reducing gel. 1 = buffer only; 2 = α, β-chain moieties of hemoglobin; 3 = 3 kDa; 4 = 4 – 5 kDa; 5 = 8 kDa; 6 = 14 kDa; 7 = midgut lysates (40% acetonitrile eluate).

Fig. 2. Transmission electron microscopy profile showing persistence of *B. burgdorferi* in *D. variabilis* midgut collected after 3 hours of capillary oral feeding with these bacteria. Arrows (white) indicate *B. burgdorferi* spirochetes. Bar = 0.66 μm.

Fig. 3. Protein profiles (gel electrophoresis) of *D. variabilis* midgut extracts from partially fed females following challenge with different treatments versus a hemocyte lysate (known to contain defensin). **A.** Representative gel of midguts extracts collected 6 - 15 h after capillary oral feeding 1 = hemocyte lysate; 2 = midgut, no challenge; 3 = midgut challenged with *B. burgdorferi;* 4 = molecular weight markers; 5 = midgut challenged with peptidoglycan; 6 = midgut challenged with 2% hemoglobin; 7 = midgut challenged with

30% rabbit serum. Sample loading 10 μg total protein per lane. Arrow in lane 1 indicates band identified as tick defensin (varisin). **B.** Proteins in 40% SepPak eluate of midgut extract showing low molecular proteins tested for antimicrobial activity and identification by mass fingerprinting. 1 = midgut extract; 2 = molecular weight markers. **C.** Midgut and hemocyte proteins separated in relation to polarity on an acidic gel. Arrow indicates defensin (varisin). 1 = defensin synthetic peptide, 5 μg; 2 = band in hemocyte lysate subsequently confirmed as defensin by tryptic digestion/mass spectrometry, 60 μg; 3 = midgut lysate, 60 μg. Note absence of defensin (varisin) in the lane for the midgut lysate.

Fig. 4. MALDI-TOF of extracts from protein bands at rMW 8.0 kDa and rMW 13.0 –14.65 kDa showing peaks for rabbit α-and ß-chain hemoglobin. **A.** 8.0 kDa band showing 7 peaks, all fragments of α- and ß-chain hemoglobin. Arrow indicates predominant peak at 11,279 daltons. **B.** 13 – 14.65 kDa band. Left arrow indicates α-chain hemoglobin (141 amino acids, mass weight 15,482); right arrow indicates ß-chain hemoglobin (147 amino acids, mass weight 16,022).

30% rabbit serum. Sample loading 10 μg total protein per lane. Arrow in lane 1 indicates band identified as tick defensin (varisin). **B.** Proteins in 40% SepPak eluate of midgut extract showing low molecular proteins tested for antimicrobial activity and identification by mass fingerprinting. 1 = midgut extract; 2 = molecular weight markers. **C.** Midgut and hemocyte proteins separated in relation to polarity on an acidic gel. Arrow indicates defensin (varisin). 1 = defensin synthetic peptide, 5 μg; 2 = band in hemocyte lysate subsequently confirmed as defensin by tryptic digestion/mass spectrometry, 60 μg; 3 = midgut lysate, 60 μg. Note absence of defensin (varisin) in the lane for the midgut lysate.

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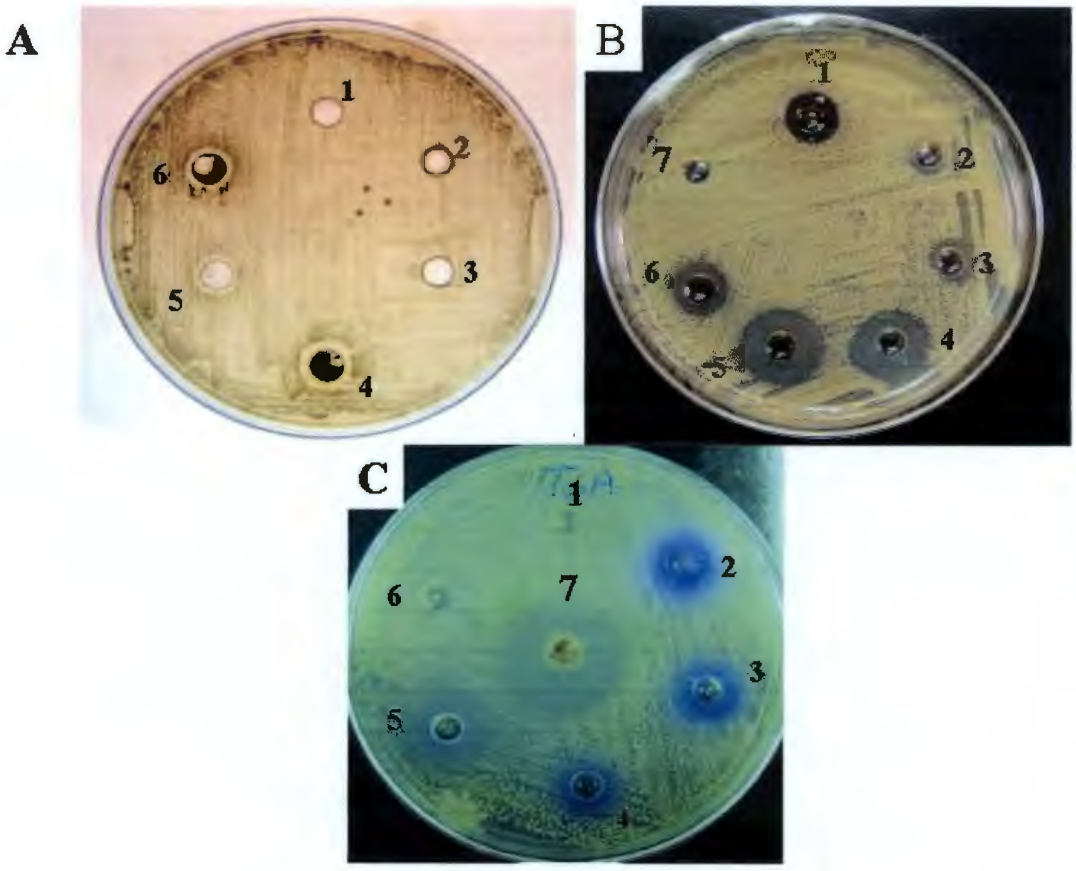
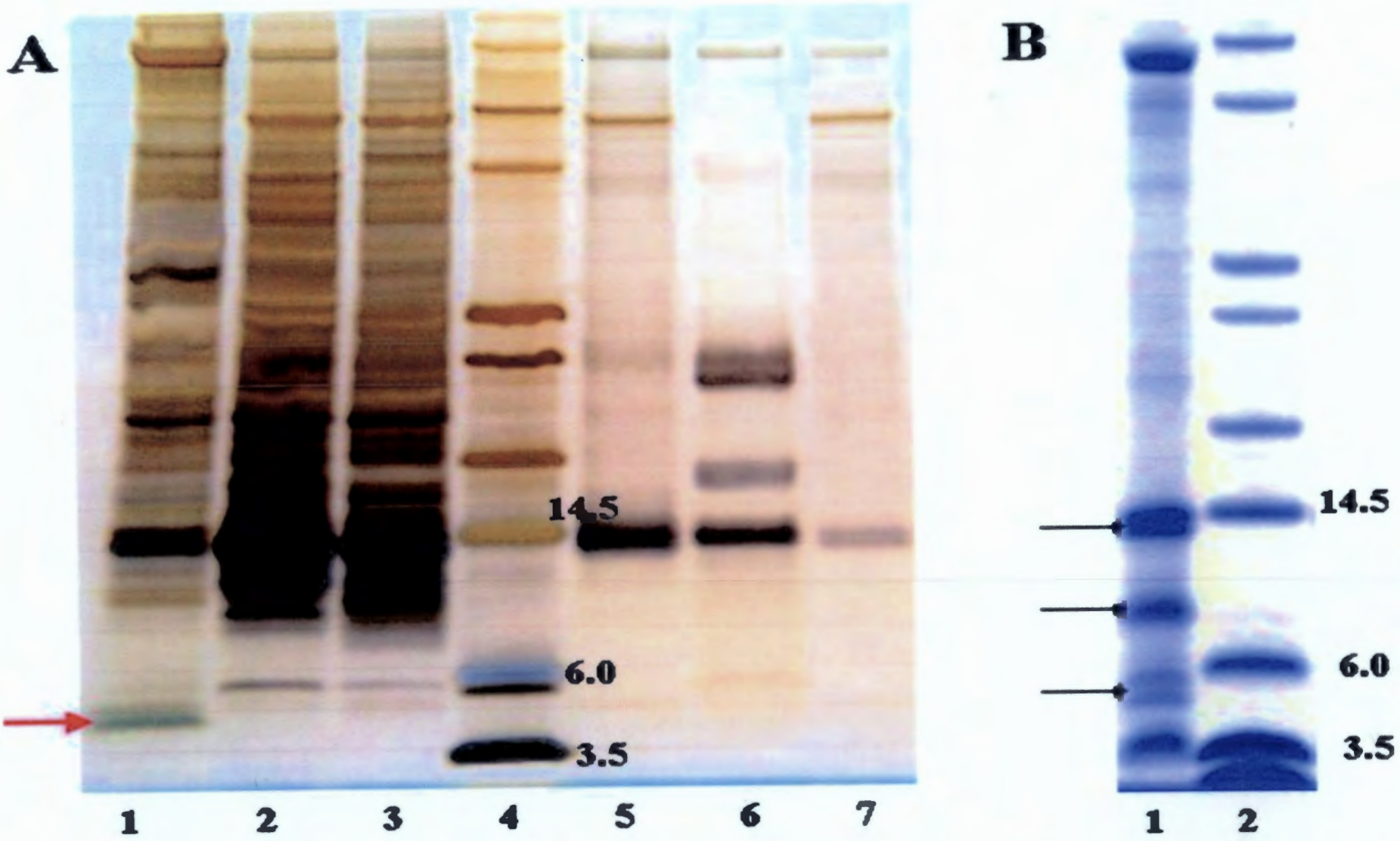
Fig. 1




A
14.5
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