

2000

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Johns, Robert; Sonenshine, Daniel E.; and Hynes, Wayne L., "Response of the Tick *Dermacentor variabilis* (Acari: Ixodidae) to Hemocoelic Inoculation of *Borrelia burgdorferi* (Spirochetales)" (2000). *Biological Sciences Faculty Publications*. 119.
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Journal of Medical Entomology

Running Head: Control of *B. burgdorferi* in *D. variabilis*.

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Control of *Borrelia burgdorferi* (Spirochetales) Infection in the Tick *Dermacentor variabilis* (Acari: Ixodidae).

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ABSTRACT. When *Borrelia burgdorferi* B31 low passage strain spirochetes are directly injected into the hemocoel of *Dermacentor variabilis* females, the bacteria are cleared from the hemocoel within less than 24 hours. Viable spirochetes could not be found in either hemolymph, salivary gland or ovary tissues by subculturing or by IFA. The hemocyte population increased ~ 6 times within the first 6 hrs after inoculation compared with the uninoculated controls . In contrast, the soluble total hemolymph protein content decreased inversely with the increase in hemocytes. Borreliacidal activity was demonstrated with cell-free hemolymph from *D. variabilis*. *In vitro* antimicrobial assays using hemolymph from borrelia-challenged and non-challenged ticks resulted in spirochete reductions of 72.0% versus only 11.5%, respectively, within 1 hr. Additional evidence of induced antimicrobial hemolymph protein activity was demonstrated by SDS-PAGE, which revealed upregulation of a lysozyme-like peptide (~15 kDa) (22% increase) and the induction of a ~5.8 kDa peptide in the *B. burgdorferi*-challenged ticks. In contrast with the non-vector borne *Bacillus subtilis*, *D. variabilis* presents a rapid and very robust response to challenge with cultured *B. burgdorferi* spirochetes, leading to their early elimination. The role of the tick immune system, including possible differences between vector and non-vector ticks, in determining the success of invasive bacteria is discussed.

Key words: *Dermacentor variabilis*, *Borrelia burgdorferi*, antimicrobial peptides, phagocytosis.

Ticks are able to transmit a wide variety of pathogenic organisms to vertebrate hosts, such that tick transmitted diseases are the most prevalent vector-borne diseases reported in the United States (CDCP, 1997; Reed, 1993). Of particular importance are Rocky Mountain spotted fever (RMSF), Ehrlichiosis and Lyme disease, which are considered as resurging or emerging zoonotic diseases of major public health importance (Fishbein and Dennis, 1995; Gratz, 1999). The American dog tick, *Dermacentor variabilis*, is the primary vector for the agent of RMSF, *Rickettsia rickettsii* (Burgdorfer, 1989). This tick has also been reported to be one of the several vectors of *Ehrlichia spp.*, the agent of Human Granulocytic Ehrlichiosis (Gratz, 1999). In contrast, *Borrelia burgdorferi*, the causative agent of Lyme disease, is transmitted exclusively by ticks of the genus *Ixodes*, in particular *Ixodes scapularis* and *I. pacificus* (Lane *et al.*, 1991). Although immatures of *D. variabilis* ticks often acquire the spirochetes during feeding on infected vertebrates, there is no transstadial transmission and no spirochetes have been shown to survive in the midgut after the molt (Piesman and Sinsky, 1988; Mather and Mather, 1990). However, the reason for this is unknown. The question of whether *B. burgdorferi* could survive in the tissues of *D. variabilis* should they be able to gain access, has not been addressed. Also the question of whether the immune system of *D. variabilis* can recognize these bacteria and destroy them outside of the gut environment has not been considered. Evidence, although limited, of the ability of ticks to control infection when challenged with various bacteria has been reported by Podboronov (1991) and Grubbhoffer *et al* (1991). *D. variabilis* was shown to mount a vigorous and highly effective immune response to the gram-positive *Bacillus subtilis* efficiently eliminating this non-invasive microbe from the tick haemocoel (Johns *et al*, 1998). However, the question

of whether *D. variabilis* can also control a challenge from a tick-specific tissue invasive bacterium such as *B. burgdorferi* has not been addressed.

In this study, we inoculated cultured *B. burgdorferi* into the hemocoel of *D. variabilis*, bypassing the midgut, and examined the tick's response to this microbial challenge. Hemocyte population increases and induction or enhancement of antimicrobial peptides are described. We also studied the ability of *B. burgdorferi* to survive in tick hemolymph and other tick tissues. This study further illuminates the ability of *D. variabilis* to selectively mount an effective anti-microbial response to an intercellular bacterium, specifically a tick-borne spirochete. This study also serves as a basis for understanding how certain tick species selectively harbor pathogens like *B. burgdorferi* while other species of ticks do not tolerate the same microbes. The significance of these findings in relation to a more general understanding of how pathogenic microbes can colonize their tick hosts is discussed.

Materials and Methods

Ticks. *Dermacentor variabilis* ticks were colonized from wild caught ticks collected near Williamsburg and Chincoteague, Va. Larvae and nymphs were fed on rats (*Rattus norvegicus*): adults were fed on rabbits (*Oryctolagus cuniculus*). All life stages were maintained in an AMINCO-AIRE incubator at 27°C ± 1°C and 92 ± 1% RH. All feeding and rearing of ticks were done as described by Sonenshine (1993).

Bacteria. The *Borrelia burgdorferi* low passage B31 strain used in this study was provided by Martin E. Schriefer, Bacterial Zoonoses Branch, Center for Disease Control, Fort Collins, Co. Stock cultures were stored at -70°C. Aliquots were removed and subcultured in BSKII-H media (Sigma) at 33°C in a 5% CO₂ incubator. To maintain their

low passage state, cultures were subcultured no more than 5 times. Bacterial suspensions for all assays were made by centrifuging cells at 3000 g for 10 minutes, washing the pellet with 8mM Phosphate-buffered saline (PBS) pH 7.4, and resuspending in the same buffer. Bacterial cells were adjusted with a Brightline hemocytometer using a Nikon Optiphot phase contrast compound microscope or by dark field microscopy.

Tick inoculations and hemolymph collection. All tick inoculations with *B. burgdorferi* were done as described by Johns *et al.* (1998). Briefly, 50,000 bacterial cells in a 5ul PBS (pH 7.4) suspension were injected into the hemocoel cavity of partially fed virgin female *D. variabilis* via the foramen between the capitulum and the anterior end of the scutum. A 50 μ l Hamilton syringe (Hamilton, Reno, NV) with a 30-gauge hypodermic needle was used for the bacterial inoculations. Collection of hemolymph was made by severing the forelegs at the coxal-trochanteral joint and drawing hemolymph into a glass micropipette. Hemolymph was diluted 1:1 in Shen's tick saline (Oliver *et al.*, 1974) with 10 mM Phenylmethyl-sulfonyl Fluoride (PMSF) (Sigma), centrifuged at 3000 X g for 10 minutes, and the supernatant (plasma portion) retained and stored at -80°C until needed for assay.

Hemocyt counts. These were done as described previously (Johns *et al.*, 1998) using a Brightline hemocytometer and viewed with the Nikon Optiphot compound microscope. Hemolymph samples were assayed at 1, 6, 18, 24, 48, and 72 hours post-inoculation.

Hemolymph protein concentration. Total soluble protein concentration of tick hemolymph was ascertained with the BioRad Protein assay kit (BioRad, Richmond, Va.)

or the BCA Protein assay Reagent (Pierce, Rockford, IL) according to the manufacturer's instructions.

Bacterial survival within Hemolymph of infected ticks. To assess the ability of *B. burgdorferi* to survive *in vivo* in *D. variabilis*, 5 μ l of hemolymph taken from borrelia-infected ticks at 1, 24, and 48 hours post inoculation was added to 5 mls of BSK media and visually assessed for spirochetes at 7 days. Furthermore, to ascertain if the spirochetes were evading detection and destruction from hemolymph factors by hiding within intracellular niches in other tick tissue, salivary gland and ovary tissues were dissected at 1, 24, and 48 hour intervals, washed 3 x's in PBS buffer pH 7.5, subcultured individually in BSK II and assayed for viable spirochetes at 7 days. Visual detection of *B. burgdorferi* spirochetes was done using dark field microscopy.

Immunofluorescence assay tests (IFA) were also done in accordance with previously published methods (Bissett and Hill, 1987) on tick hemolymph, salivary, and ovary tissue at 1, 24, and 48 hrs for detection of spirochetes. Tick tissue was smeared onto silylated glass slides (CEL Associates, Inc., Houston, Texas), air dried for 20 minutes at 4°C then acetone fixed for 20 minutes at 4°C. Slides were washed 3 times in PBS after each step. Ten μ l of undiluted monoclonal antibody (Mab) H5332 specific for *B. burgdorferi* OspA (from Dr. A Barbour, University of California, Irvine, CA., courtesy of Dr. D. Jaworski) was added to each slide and incubated for 1 hour at 33°C. After incubation, the slides were washed 3 times in PBS, and 10 μ l of goat anti-mouse IgG fluorescein-isothiocyanate conjugate at 1:40 dilution was added and incubated at 33°C. After incubation, the slides were washed 3 x's in PBS and stored (-20°C) until viewed by fluorescence microscopy (Nikon Optiphot with fluorescence attachment). Controls were

whole *B. burgdorferi* spirochetes from the laboratory cultures. As a check on the accuracy of detection by IFA using the monoclonal antibody, a fluorescein-labeled affinity purified polyclonal antibody to *B. burgdorferi* (Kirkegaard & Perry Lab. Inc., Gaithersburg, Md.) at a 1:40 dilution was also used on a duplicate set of samples.

Microtiter inhibition assays. Whole tick hemolymph plasma collected at 1-hour post inoculation from *B. burgdorferi* infected ticks or sham inoculated (non-infected) ticks was assayed for anti-*B. burgdorferi* activity. Antimicrobial activity was assessed using a 96 well microtiter plate (Corning, Corning, NY). Fifty μ l of a 3 - 5 day *B. burgdorferi* culture (adjusted to 4.5×10^3 cells per μ l) was added to wells containing 10 μ l of tick hemolymph plasma sample. Samples were evaluated for bacterial numbers on a hemocytometer by phase contrast microscopy at 5, 15, 30, 45, and 60 min intervals.

Gel electrophoresis. Separation and visualization of proteins within tick hemolymph plasma (from ticks inoculated with *B. burgdorferi* as well as sham inoculated control ticks) and from whole cell lysates of *B. burgdorferi* was done as previously described (Johns *et al.*, 1998). Briefly, electrophoresis was done by SDS-PAGE according to the method of Laemmli (1970) using a Pharmacia Biotech Model SE 260-10A vertical minigel system (Pharmacia, Biotech, Piscataway, NJ). Gels were 10-20% Novex precast Tricine gradient gels with a 4% stacking gel (Novex, San Diego, CA). The protein concentration of hemolymph plasma samples collected at 1, 6, 18, 24, and 48 hours post-inoculation and from a sham inoculated control were determined as previously described. Approximately 785 μ g of protein per sample was loaded onto the gel and electrophoresed according to the manufacturer's instructions. Staining was done by either silver stain or Commassie Brilliant Blue in accordance with the manufacturer's instructions

(BioRad). Gels were digitally photographed with the Kodak DC40 Digital Camera and densitometry was performed with Kodak ID Software™

Results

Hemocyte counts. Changes in hemocyte numbers were assayed at 6 different time intervals following inoculation of part-fed female *D. variabilis* ticks with *B. burgdorferi*. Hemocyte numbers in control ticks remained relatively constant with a mean average of 1182.3 ± 645.4 cells / μl (Fig. 1). In contrast, hemocyte numbers in *B. burgdorferi*-challenged ticks increased significantly at 1 hour post-inoculation to 7608.3 ± 1271.3 cells / μl , approximately 6.4 times the average control numbers ($t = 19.6$, $df = 15$, $P < 0.001$). At 6 hours post-inoculation hemocyte numbers in bacteria challenged ticks, 3552.9 ± 1204.4 cells / μl , were still significantly elevated compared to the controls at 3.0 times the average hemocyte control numbers ($t = 7.6$, $df = 15$, $p < 0.05$). At 18 hours bacteria challenged hemocyte numbers were 1680.8 ± 740.6 cells / μl or approximately 1.4 times the control numbers still showing a significant increase ($t = 2.6$, $df = 15$, $p < 0.05$). However, by 24 hours, hemocyte numbers in *B. burgdorferi*-challenged ticks were the same as in the non-challenged control ticks and this trend remained constant throughout 72 hours.

Protein content. Protein concentration of hemolymph plasma from non- infected control ticks assayed at 6 different time intervals showed a mean average of 109.432 ± 12.1 μg / μl protein (Table 1). Hemolymph protein concentration was significantly reduced at 1 hour in the *B. burgdorferi*-challenged ticks ($t = 3.8$, $df = 2$, $p = 0.007$) compared to the mean average control. However, by 6 hours post inoculation, the *B.*

burgdorferi-challenged tick hemolymph protein concentration was similar to the mean average control concentration. Thereafter, the protein concentration levels remained similar in both the control and challenged tick samples throughout 72 hours.

Survival of *B. burgdorferi* in *D. variabilis* tissue. *B. burgdorferi* is a highly motile intercellular bacterium with the ability to invade salivary glands and other tissues of its natural vector, *Ixodes scapularis* (Johnson *et al.*, 1984; Burgdorfer *et al.*, 1982). In order to assess the ability of *D. variabilis* to effectively eliminate *B. burgdorferi* from infection of the hemocoel it was necessary to assay other tick tissue, in addition to hemolymph. Assays of hemolymph, salivary gland, and ovary tissue to determine the presence of viable spirochetes were done at time intervals of 1, 24, and 48 hours (Table 2). At 1 hour post-inoculation viable spirochetes were present in 50% of samples of whole tick hemolymph tissue, 17% of salivary tissue samples (representing 1 out of 6 ticks sampled), and 17% of ovary tissue (1 out of 6 ticks sampled). No viable spirochetes were detected at 24 or 48 hours in any tissues sampled for any of the replicates.

IFA was performed on all tissue samples that assayed negative for spirochetes in the BSK II subculturing assay shown in Table 2. In all tissue samples collected after 1 hour no spirochetes were detectable by immunofluorescence thus confirming the absence of viable *B. burgdorferi in vivo* following inoculation of spirochetes into these ticks (data not shown).

Microtiter inhibition assays. Hemolymph from *B. burgdorferi* infected *D. variabilis* females effectively destroyed 72% of a *B. burgdorferi* cell population within 1 hour as compared with a control bacterial culture not exposed to tick hemolymph (Fig. 2). By comparison, hemolymph from non-infected ticks effected an 11.5% decrease of

bacterial cells at 1 hour. Antimicrobial activity was first observed at 15 minutes with *B. burgdorferi*-challenged tick hemolymph destroying 41% of borreliae while non-challenged control tick hemolymph had a 16.6% decrease in bacterial cells. Thereafter, the *B. burgdorferi* population treated with hemolymph from challenged ticks showed a steady decrease in numbers through 1 hour while borrelia cells treated with hemolymph from non-infected ticks had a mean average decrease in cells of 14.6% which remained relatively constant through 1 hour.

Gel electrophoresis. SDS PAGE gel analysis showed an increase in a protein band(s) at the molecular weight range of ~15 kDa in borrelia-stimulated tick hemolymph collected at 1 hour (Fig. 3, lane 2) versus non-challenged tick hemolymph (Fig. 3, lane 7). Densitometry analysis showed an increase of 22.5% in the bands in this range. A band of approximately 5.8 kDa appeared in lane 2 that was only faintly visible in the control lane 7. This band was also present in diminishing intensities in *B. burgdorferi*-stimulated tick hemolymph collected at 6 hours (lane 3) and at 18 hours (lane 4).

Discussion

Ticks possess the innate ability to survive bacterial infections in the hemocoel cavity from an array of environmentally ubiquitous gram positive or gram negative bacteria (Johns, *et al.*, 1998; Podboronov, 1991). In the case of the gram positive bacterium *Bacillus subtilis*, *D. variabilis* females clear these bacilli from their body tissues within 48 to 72 hrs. Although some bacterial growth occurs within the first few hours following inoculation, bacteria are rapidly destroyed and few bacteria can be cultured from these ticks after 24 hours. Increases in phagocytic hemocytes peaks within 48 hrs, by which time virtually all viable bacilli have been eliminated (Johns *et al.*, 1998). In

contrast, the response of these same ticks to challenge with the Lyme disease spirochetes, *B. burgdorferi* appears to be more vigorous and rapid. Direct inoculation of cultured *B. burgdorferi* into the hemocoel of *D. variabilis* results in elimination of the spirochetes *in vivo* in less than 24 hours. There is no evidence of borrelia multiplication and most spirochetes are destroyed within 1 hour.

Phagocytosis has been shown to be an important factor in suppressing *B. burgdorferi* migrating from the midgut of the vector ticks into the hemolymph (Munderloh and Kurtii, 1995; Coleman *et al.*, 1997). Phagocytosis by hemocyte cells is believed to be a primary cellular defense response to microbial infection in several tick species (Kühn and Haug, 1994; Zhioua *et al.*, 1997). Encapsulation is another means used by arthropods to clear the hemocoel, particularly for gram negative bacteria or for organisms too large to be engulfed by phagocytes (Gagen and Ratcliff, 1976). Silica bead implants in the haemocoel of *D. variabilis* provoke an encapsulation response by plasmatocytes and granulocytes in a manner similar to the two-phase response of insects (Munderloh and Kurtii, 1995; Eggenberger *et al.*, 1990). Whether the rapid (1 hr) surge in hemocyte numbers in response to *B. burgdorferi* challenge represents a phagocytic or encapsulation process is unknown, although no evidence of encapsulation was detected in the many tissue samples examined. Thus the findings reported in this study are consistent with the hypothesis of phagocytosis by hemocyte cells as a major response to borrelia challenge. This observed difference in hemocyte response time between *B. subtilis* infected ticks and *B. burgdorferi* infected ticks could reflect variation in the ticks' response due to bacterial type specificity such as broad differences of gram positive bacteria versus that of spirochetes (which more closely resembles gram negative bacteria). Also, as *B. subtilis*

has the ability to sporulate, this could explain the gradual rise in hemocyte numbers over a 2-day period post-infection, since sporulating organisms would continue to provoke the tick's immune system. Thus, the tick's immediate control of *B. subtilis* bacterial challenge can be explained primarily by anti-microbial peptides (accounting for an 88% reduction in *B. subtilis* cells within 12 hrs after their peak), with phagocytosis repeatedly activated to control the occasional new cells arising from germinating spores (Johns *et al.*, 1998). However, when provoked by *B. burgdorferi*, the hemocyte response is activated immediately, but in the absence of further challenge, hemocyte numbers decline within less than 24 hrs to normal levels.

In addition to the massive early mobilization of hemocytes, borrelia challenge in *D. variabilis* also stimulates rapid expression of antimicrobial peptides. This includes the upregulation of a putative lysozyme-like peptide as well as the induction of a small ~5.8 kDa peptide similar in size to the cercropins or proline-rich peptides of lepidopteran and dipteran insects. The latter act primarily against gram negative bacteria (Cociancich, *et al.*, 1994). The rapid death of borreliae in the presence of cell-free hemolymph from borrelia-challenged versus non-stimulated ticks (72% versus 11%) strongly supports borreliacidal activity independent of phagocytosis, presumably due to the combination of lysozyme and the induced peptide. While lysozyme is most effective against gram positive bacteria it can be upregulated in response to a gram negative challenge ((for example, lipopolysaccharides (LPS) are known to provoke an increase in lysozyme expression)) (Wittwer *et al.*, 1997). Furthermore, small molecular weight peptides, acting as porins, are known to open channels in the LPS layer of gram negative bacteria, facilitating penetration of lysozyme and subsequent destruction of the organisms in insects (Gillespie

et al., 1997). We previously reported a constitutive anti-gram positive plasma protein of approximately 14.5 kDa in hemolymph of *D. variabilis* females which we believe to be a lysozyme, and which is enhanced upon challenge with *Bacillus subtilis* (Johns *et al.*, 1998).

B. burgdorferi spirochetes in their natural vector penetrate gut epithelial cells during tick host feeding, and adhere to the basal surfaces of the gut epithelium, the basal and lateral surfaces of salivary gland acini, and developing oocytes within the hemocoel (Burgdorfer, 1989; Munderloh and Kurtii, 1995). Motility, as well as tissue tropism, are probably important determinants in *B. burgdorferi* survival and subsequent transmission within *I. scapularis*, and the delay in transmission from the initial time of tick feeding could argue that an infective form of the spirochete must develop either in its passage to, or penetration of the salivary glands (Munderloh and Kurtii, 1995). That *B. burgdorferi* regulates the expression of several outer surface proteins during its migration from the midgut to the salivary glands (most notably Osp A and Osp C) reveals the plasticity of this organism and its ability to respond and adapt in this environment (Schawn *et al.*, 1995). The rapid rise in hemocytes in *D. variabilis* upon infection by *B. burgdorferi*, as well as the suppression of spirochete viability, apparently by soluble hemolymph lytic peptides, could sufficiently eliminate the bacteria before such adaptive changes occur, thereby preventing enough of the bacteria surviving and successfully colonizing other tissues. Whether or not *B. burgdorferi* retains the same tissue tropism for *D. variabilis* as it has for *I. scapularis* has not been determined. Also, caution must be taken in assigning any role to the antigenic changes in *B. burgdorferi* as aiding in its ability to survive in *D. variabilis*, because the natural route of tick infection has not occurred and this may

certainly alter the expression of spirochete outer surface proteins. Finally, it should be noted that the cultured B-31 *B. burgdorferi* used in these studies lacked plasmin (a protease), which was shown by Coleman *et al.* (1997) to be important for survival of spirochetes migrating from the midgut. We interpret Coleman *et al.*'s data to suggest that plasminogen/plasmin binding provides protection against the tick's immune response, allowing at least a few spirochetes to enter the salivary glands. Plasmin binding prior to their inoculation into *D. variabilis* might have conferred sufficient protection to enable some of these organisms to survive in this normally non-vector competent host.

These studies with *D. variabilis* may provide a basis for comparing the response of the immune system of other ticks, especially the natural vector, *I. scapularis*, to spirochete challenge. Differences in the immune system of *I. scapularis*, if any, may allow a more permissive environment in which some hemolymph-invading spirochetes may survive long enough to infect the salivary glands and other internal organs.

In future studies we will investigate and compare antimicrobial mechanisms in the *I. scapularis* hemocoel with that of *D. variabilis* and assess the degree of competency of *I. scapularis* in suppressing *B. burgdorferi* viability. Furthermore, we will investigate how the regulation of borrelia outer surface proteins and plasmin coating may protect the spirochete from antimicrobial proteins in tick hemolymph.

Acknowledgements

We are grateful to Dr. Martin E. Schriefer, Division of Vector-borne Diseases, Centers for Disease Control, Fort Collins, CO for the supply of the low passage B31 strain of *Borrelia burgdorferi*. We also thank Dr. Allan Barbour and Dr. Deborah Jaworski, University of California, Irvine, CA for the supply of the monoclonal antibody H5332

against *Borrelia burgdorferi*. We thank Dr. James V. Koch, President, Old Dominion University, for the Eminent Scholars award used to support this study.

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Figure Legends

Figure 1. Changes in *D. variabilis* hemocyte populations following inoculation of *B. burgdorferi* (10^4 cells/ul) into partially fed virgin females ($n = 30$). Borrelia = *B. burgdorferi*-infected ticks. Control = noninfected ticks.

Figure 2. Mean \pm SD of visible spirochetes as determined by phase contrast microscopy using a Brightline hemocytometer at different time intervals. Values are an average of 3 replications. Dv heme = control hemolymph from noninfected ticks. DVBB 1 hr = hemolymph collected at 1 hour from *b. burgdorferi*-infected ticks

Figure 3. Photograph of SDS-PAGE gel showing upregulation of 15 kDa protein and induction of new 5.8 kDa protein in hemolymph plasma from *D. variabilis* fed females inoculated with *Borrelia burgdorferi* or Shen's buffer (control). Lane 1, Molecular weight (MW) standards; Lane 2, 1-hr post-inoculation; Lane 3, 6-hr post-inoculation; Lane 4, 18-hr post-inoculation; Lane 5, 24-hr post-inoculation; Lane 6, 48-hr post-inoculation; Lane 7, sham-inoculated control.

NOTE:

Graphs (Fig. 1 and 2) were done in Microsoft Excell, format Times New Roman (12 point). Single column display OK.

Photograph (Fig. 3) was done from an original scanned into manuscript and labeled in Times New Roman (12 point). Single column display for the photo OK

Table 1. Total soluble hemolymph protein concentration (ug / ul) of *Dermacentor variabilis* following inoculation of cultured *Borrelia burgdorferi* (B₃₁ strain)*

Status	1 hr	6 hr	18 hr	24 hr	48 hr	72 hr	Mean
Infected	73.8 ±16.1	122.4 ± 28.1	113.5 ± 31.5	113.6 ± 6.2	99.1 ± 8.9	115.9 ± 3.4	106.3 ± 17.7
Infected	95.7 ± 2.1	102.1 ± 5.0	116.5 ± 22.1	127.9 ± 17.9	100.5 ± 7.4	113.8 ± 6.9	109.4 ± 12.1

* Values represent an average of 3 separate trials using six ticks per trial per time period.

Table 2. Presence of viable *Borrelia burgdorferi* in *Dermacentor variabilis* female tick tissues following inoculation of cultured spirochetes (B₃₁ strain)*

Samples positive for viable spirochetes / total replications sampled

Time (hrs) P.I.	Hemolymph	Salivary gland	Ovary
1 hr	3/6	1/6	1/6
24 hr	0/6	0/6	0/6
48 hr	0/6	0/6	0/6

* Six replications were done at each time period. Tissue was extracted and cultured in BSK-H media and incubated at 33° C for 7 days, then inspected for spirochetes using dark field microscopy. Numbers represent number of samples positive for viable spirochetes of the total number of replications. P.I. = post-inoculation.

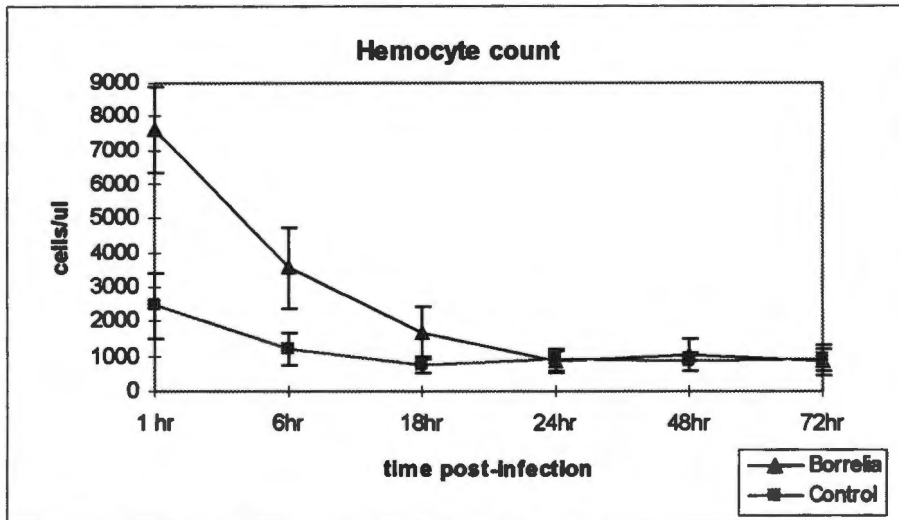


Fig. 1

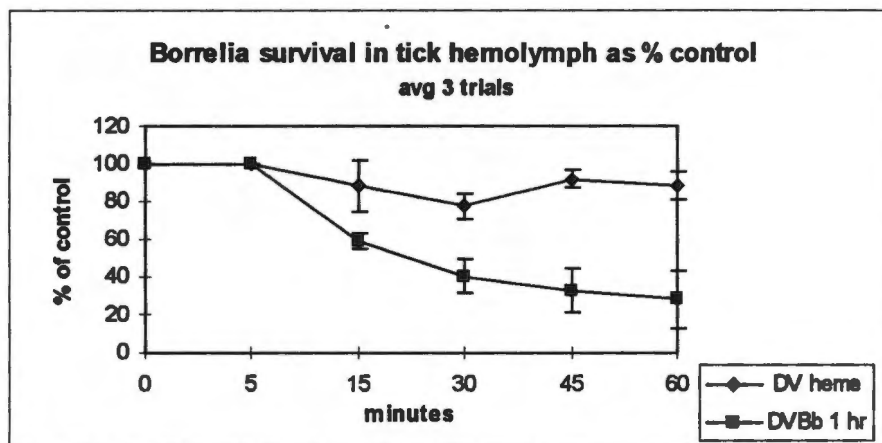


Fig. 2.

