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Effects of cattle tick (*Boophilus microplus*) infestation on the bovine immune system

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ABSTRACT

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The immunosuppressive effect of experimental *Boophilus microplus* infestation on bovine peripheral blood lymphocytes (PBL) and on host antibody production to a protein antigen (ovalbumin) was examined. *Boophilus microplus* infestation caused a marginal decrease in the percentage of T lymphocytes in PBL, which was observed in both lightly (5000 larvae) and heavily (40 000 larvae) infested cattle, and began at the second infestation and continued until the end of the fourth infestation. The percentage of B lymphocytes in heavily tick-infested cattle was less than that in non-infested control cattle after the fourth infestation. The response of PBL from tick-infested cattle to phytohemagglutinin (PHA) was always less than that of tick-free cattle after the second infestation. No noteworthy differences were detected between the three stages of tick infestation, that is, 1 week before the peak of adult engorgement, the middle of the peak and 1 week after all ticks had dropped. *Boophilus microplus* saliva (100 μ l ml⁻¹) suppressed 47% of the response of bovine PBL to PHA in vitro. This suppressive effect of saliva may contribute to the lower responsiveness of PBL from tick-infested cattle. Antibody production by tick-infested cattle was examined during the third and fourth heavy tick infestation. Tick-infested cattle showed a diminished response against ovalbumin after the second immunization. The immunosuppressive effects of tick infestation may play an important role in tick survival or in the transmission of tick-borne diseases in the field.

INTRODUCTION

The tropical cattle tick, *Boophilus microplus* is a major economic burden to cattle producers in the tropics. *Boophilus microplus* induces an immune response in the bovine host, which is manifest by mast cell disruption, eosinophil infiltration and concentration at the attachment site (Schleger et al.,

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1976), and humoral antibody production to tick antigens (Roberts and Kerr, 1976; Willadsen et al., 1978).

Parasites can induce host immunosuppression to enhance their chance of survival (Ogilvie and Wilson, 1976). For example, *Demacantor andersoni* caused a reduction in bovine lymphocyte responses to mitogen (Wikel and Osburn, 1982) and *Rhipicephalus appendiculatus* suppressed the humoral immune response in rabbits (Fivaz, 1989). Although Reich and Zorzopulos (1980) suggested that infestation with *B. microplus* caused immunosuppression in the bovine host, there have been few studies of this phenomenon, and the issue is still unclear.

This paper reports studies on the effect of experimental *B. microplus* infestation on the bovine immune system. In particular, we examined the influence of cattle tick infestation on responses by peripheral blood lymphocytes (PBL) and on host antibody production to a protein antigen (ovalbumin).

MATERIALS AND METHODS

Animals and tick infestation

Purebred female Hereford cattle from a tick-free area (New England Tableland, N.S.W., Australia) were used in this study and were infested with the Yeerongpilly strain of *B. microplus*. Six cattle were held in covered pens and infested daily with 1000 larvae for 21 days from Day 0 (first infestation) and 5000 larvae for 1 day on Day 21 (second infestation). They were ranked on resistance to the tick so that two groups (A and B) each containing three cattle had the same average resistance; Group A was then infested with 5000 larvae and Group B infested with 40 000 larvae on Day 70 (third infestation). On Day 112, the animals received a fourth infestation with Group A and Group B receiving 5000 and 40 000 larvae, respectively. The engorged ticks dropping from these cattle were counted and the peak of dropping was on Day 19 after infestation. This day was defined as the peak of the tick infestation.

Separation of lymphocytes from peripheral blood

Lymphocytes were separated from peripheral blood by the method of Outeridge and Dufty (1981). Briefly, 20 ml of heparinized blood were obtained from the jugular vein and the leukocytes concentrated in the buffy coat by centrifugation at $500 \times g$ for 20 min at room temperature. Buffy coat cells were collected and diluted with 2 ml of sterile phosphate buffered saline (PBS, pH 7.2). This cell suspension was layered onto 4 ml of Ficoll-Paque (Pharmacia, Uppsala, Sweden) which was then centrifuged at $800 \times g$ for 40 min at room temperature. The cell layer on top of the Ficoll-Paque was collected

and washed once with sterile PBS at $200 \times g$ for 10 min at room temperature to reduce numbers of platelets in the cell suspension. Contaminating red blood cells were lysed by ammonium chloride Tris buffer (0.017 M Tris, pH 7.2 containing 0.75% NH_4Cl) and the cells were washed twice with sterile PBS. The cells were finally resuspended in complete RPMI 1640 medium (Flow Laboratories, Irvine, UK), which contained 10% heat-inactivated foetal calf serum (Flow Laboratories), 2-mercaptoethanol (5×10^{-5} M), penicillin (100 IU ml^{-1}) and streptomycin ($20 \mu\text{g ml}^{-1}$).

Enumeration of T and B lymphocytes in PBL

Rosette-forming bovine T lymphocytes were detected using 2-aminoethylisothiouonium bromide (AET, Sigma Chemical Company, St. Louis, MO) treated sheep red blood cells (SRBC) (Paul et al., 1979). Packed SRBC (one volume) were incubated with four volumes of 0.1 M AET (pH 9.0) for 20 min at 37°C . The SRBC were washed three times with cold saline followed by two washes with PBS. AET-treated SRBC in PBS ($100 \mu\text{l}$ of 1% solution) were added to the bovine lymphocyte suspension ($100 \mu\text{l}$ of 1×10^7 cells ml^{-1}) in small centrifuge tubes and incubated at 37°C for 10 min. The cells were then centrifuged at $200 \times g$ for 5 min at room temperature. After overnight incubation at 4°C , a drop of 0.5% trypan blue (BDH Chemicals, Poole, UK) was added, and the cells were resuspended by gently rocking the tubes. A drop of the suspension was placed in a hemocytometer and 200 live cells were counted. A lymphocyte with three or more adherent SRBC was counted as a T lymphocyte.

Surface membrane immunoglobulin as a marker for B lymphocytes was detected using indirect immunofluorescence. PBL (5×10^6 cells) were washed twice with PBS containing 0.2% sodium azide, and pelleted by centrifugation at $500 \times g$ for 5 min. The PBL pellets were mixed with $50 \mu\text{l}$ of affinity-purified rabbit anti-bovine immunoglobulin at an optimal dilution and incubated for 30 min at room temperature. Cells were washed twice with PBS containing sodium azide and the pellets were resuspended in $50 \mu\text{l}$ of fluorescein isothiocyanate (FITC) labeled sheep anti-rabbit immunoglobulin and incubated for 30 min at room temperature. The cells were again washed twice with PBS containing sodium azide and then examined using a fluorescence microscope (Zeiss, Germany) with a 485 nm excitation filter and 500 nm barrier filter. The percentage of cells positive for surface membrane immunoglobulin was determined from counting 200 cells.

Lymphocyte response to mitogen

PBL were suspended at 4×10^6 cells ml^{-1} in complete RPMI 1640 medium. $50 \mu\text{l}$ of the PBL suspension were added in triplicate to wells in a 96

well microculture plate (Disposable Products, Adelaide, Australia) along with 50 μl of an optimal concentration of phytohemagglutinin (PHA, Sigma, final concentration 10 $\mu\text{g ml}^{-1}$). The optimal concentration was determined in preliminary experiments with PHA in the range 2.5–40 $\mu\text{g ml}^{-1}$, using cells from three healthy cattle. The cell cultures were incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2 : 95% air. The blastogenic activities of cells in culture were monitored by a colorimetric tetrazolium salt assay (Mosmann, 1983). Briefly, 50 μl of culture supernate were replaced with 10 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma, 5 mg ml^{-1} in PBS) for all wells of an assay and the plates were incubated for 4 h at 37°C. Acid isopropanol (100 μl of 0.04 N HCl in isopropanol) was then added to all wells and mixed thoroughly to dissolve the dark blue crystals. The plates were read using a Titertek Multiscan plus ELISA reader (Flow Laboratories) with a wavelength of 600 nm. Results are recorded as optical density (OD) units which are the OD of control cultures containing no PHA subtracted from the OD of test cultures containing PHA.

Effect of tick saliva and salivary gland extract on PBL mitogen response

Saliva was collected from fully engorged females of *B. microplus* Yeerongpilly strain using the method of Kerlin and Hughes (1992). Briefly, 3 μl of 0.2 M pilocarpine nitrate salt (Sigma) in 1:1 methanol:water was placed on the dorsum of fully engorged ticks to stimulate the production of saliva. Saliva was collected with microcapillary tubes and kept at -20°C until used.

Salivary glands from semi-engorged female *B. microplus* Yeerongpilly strain were homogenized in PBS at 0°C using a glass homogenizer. Homogenized salivary glands were centrifuged at $2000 \times g$ for 15 min to remove large debris. The supernatant was filtered with a 0.22 μm filter and stored frozen at -20°C . The protein concentrations of saliva and salivary gland extract were measured using the BCA Protein Assay (Pierce, Rockford, IL). PBL separated from blood obtained from cattle that had not been exposed to ticks were cultured with PHA and tick saliva or the salivary gland extract. By using reverse phase high-performance liquid chromatography (Kneczke, 1980; Waters Radial-PAK C_{18} column was eluted isocratically with 0.0125 M heptane sulfonic acid, 40% methanol in water, pH 3.0, at 1.0 ml min^{-1} , 213 nm), 1.5×10^{-3} M pilocarpine was found in tick saliva. The effect of pilocarpine on the PBL response to PHA was examined as a control.

Antibody production

Cattle which were infested with 40 000 larvae at the third and fourth infestation were immunized with ovalbumin. Ovalbumin (1 ml of 0.2 mg ml^{-1} , Sigma) was given with 1 ml of QuilA (2 mg ml^{-1} , Superfos Biosector a/s,

Denmark) to animals subcutaneously on the side of the neck at the peak of the third infestation and the peak of the fourth infestation (6 weeks after first immunization). Cattle were bled for serum on Weeks 0, 2, 4, 6, 7, 8 and 9 after the first immunization. Antibody in serum was detected by a kinetic enzyme-linked immunosorbent assay (ELISA, Barlough et al., 1983). Micro-titer plates (Disposable Products) were incubated overnight at 4°C with ovalbumin ($100 \mu\text{g ml}^{-1}$, $150 \mu\text{l}$ per well) in 0.05 M carbonate buffer, pH 9.6. Plates were rinsed twice with ELISA PBS (PBS containing 0.05% Tween 20) and blocked with 1% gelatin in PBS for 2 h at 37°C. The plates were then rinsed three times with ELISA PBS. Sera in serial two-fold dilution from an initial dilution of 1:100 were added to each well ($150 \mu\text{l}$) and incubated for 2 h at 37°C. The plates were then rinsed three times with ELISA PBS, and $150 \mu\text{l}$ horseradish peroxidase conjugated affinity-purified rabbit anti-bovine immunoglobulin diluted 1:1000 in ELISA PBS was added to each well. The plates were incubated for 2 h at 37°C and then rinsed three times with ELISA PBS. Substrate, $150 \mu\text{l}$ of 6.5 mM 5-aminosalicylic acid (Sigma), 10^{-2} mM H_2O_2 , 10^{-2} mM EDTA, 20 mM phosphate buffer (pH 6.0) was added to each well. The optical density (OD) of the developing reaction in each well was read at 2, 4 and 6 min on a Titertek Multiskan plus ELISA reader with a wavelength of 492 nm. The dilution of serum giving an ELISA rate of 0.05 OD units min^{-1} was calculated by regression analysis. The reciprocal of this dilution was used as a measure of serum titer.

RESULTS

Lymphocyte subpopulations

The percentage of T lymphocytes in PBL of tick-infested cattle was always less than in tick-free control animals, but this was only significant ($P < 0.05$) at the end of the second infestation (Fig. 1). The percentage of B lymphocytes in PBL from tick-infested cattle was generally similar to that from tick-free cattle until the fourth infestation, at which time it decreased relative to the controls (Fig. 2).

Lymphocyte response to mitogen

The results of the mitogenic response to PHA of lymphocytes from tick-infested and tick-free cattle are shown in Fig. 3. The response of lymphocytes from tick-infested cattle was consistently less than that of lymphocytes from tick-free cattle from the second infestation onwards. The difference between heavily (40 000 larvae) infested cattle and tick-free control cattle was greater than the difference between the lightly (5000 larvae) infested cattle and control animals (data not shown).

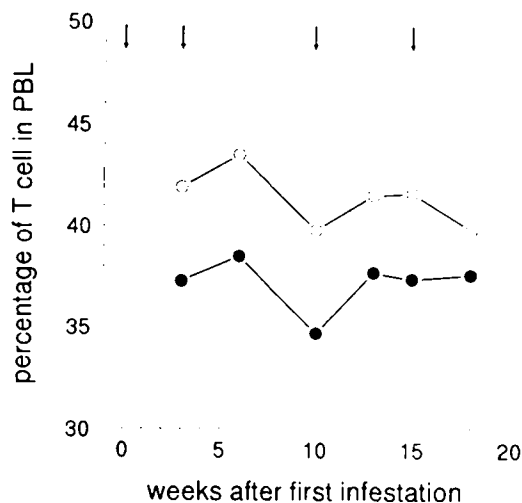


Fig. 1. The mean percentage \pm standard error of T cells in PBL from tick-infested cattle (solid circle, 40 000 larvae at third and fourth infestation) and control cattle (open circle). Arrows represent the time of tick infestation.

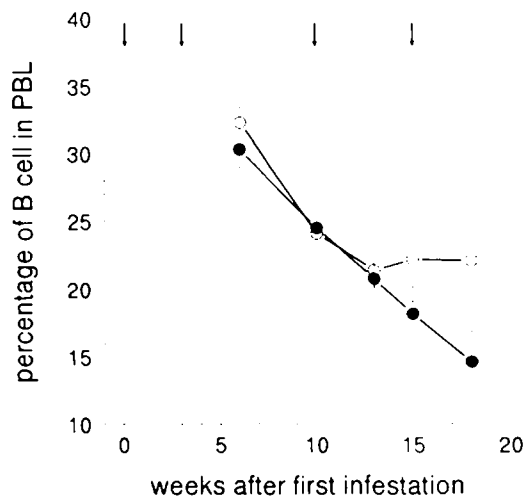


Fig. 2. The mean percentage \pm standard error of B cells in PBL from tick-infested cattle (solid circle, 40 000 larvae at third and fourth infestation) and control cattle (open circle). Arrows represent the time of tick infestation.

Effect of tick saliva and salivary gland extract on PBL mitogen response

Pilocarpine-induced saliva from *B. microplus* suppressed the response of PBL to PHA. In an initial experiment, at a concentration of $100 \mu\text{l ml}^{-1}$ of saliva which contained $73 \mu\text{g ml}^{-1}$ of protein, the mitogenic response was

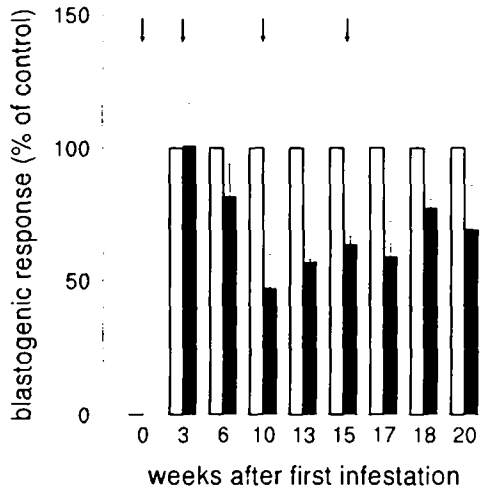


Fig. 3. Response of peripheral blood lymphocytes from tick-infested cattle (black bar) and control cattle (white bar) to PHA. Cells were cultured with optimal concentration of PHA ($10 \mu\text{g ml}^{-1}$) for 48 h. Each bar represents the mean \pm standard error of results from three cattle expressed as a percentage of results from control cultures (non-infested cattle). Arrows represent the time of tick infestation.

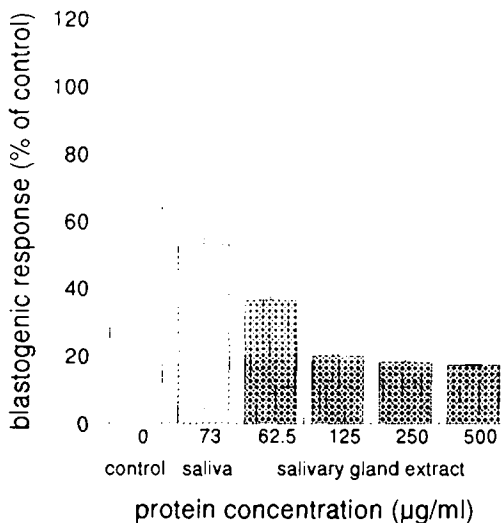


Fig. 4. Effect of saliva and salivary gland extract on response of bovine PBL to PHA. Cells were cultured with optimal concentration of PHA ($10 \mu\text{g ml}^{-1}$) and saliva ($100 \mu\text{l ml}^{-1}$ which contained $73 \mu\text{g ml}^{-1}$ of protein) or various concentrations of salivary gland extract. Each bar represents the mean \pm standard error of the blastogenic response in six replicate treatment wells expressed as a percentage of the response in control wells (containing only PHA).

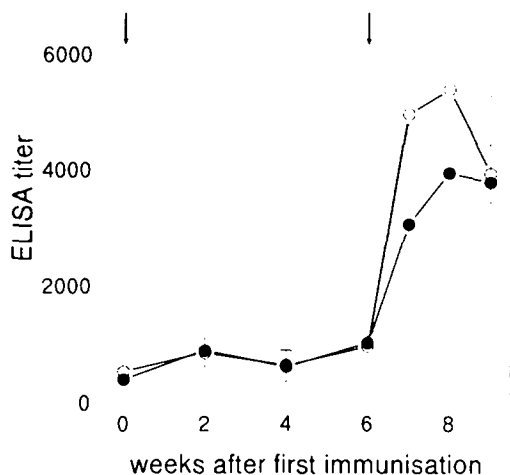


Fig. 5. Antibody production by tick-infested cattle (solid circle) and control cattle (open circle). Ovalbumin was injected with QuilA at the peak of the third and fourth infestation. ELISA titers were measured as described in the text. Arrows represent the time of immunization.

inhibited by 47%. Salivary gland extract showed increasing inhibition up to a concentration of $500 \mu\text{g ml}^{-1}$ (Fig. 4). To confirm this effect, PBL were isolated from three cattle and the effect of saliva at $50 \mu\text{g ml}^{-1}$ and salivary gland extract at 0.5 , 5.0 and $50.0 \mu\text{g ml}^{-1}$ on PHA-induced mitogenesis were measured. Saliva produced a mean inhibition of 46% (range 49–44%) and salivary gland protein at $50 \mu\text{g ml}^{-1}$ induced a mean inhibition of 41% (range 46–32%). The lower concentrations of salivary gland protein did not produce significant inhibition.

It was shown that $100 \mu\text{l ml}^{-1}$ of saliva used as the maximum concentration in the lymphocyte assay contained 1.5×10^{-4} M pilocarpine as a result of the means of isolation. In control experiments it was shown that this concentration of pilocarpine had no detectable effect on lymphocyte mitogenesis.

Antibody production

The kinetics of anti-ovalbumin antibody production in heavily tick-infested and control cattle are shown in Fig. 5. Tick-infested cattle showed diminished antibody production compared with control animals for 2 weeks after the second immunization which was given at the peak of the fourth infestation. However, the group mean values were not statistically different.

DISCUSSION

The present experiments demonstrated that *B. microplus* infestation could reduce the percentage of T and B lymphocytes in PBL, the response of PBL

to a T lymphocyte mitogen and suggested also that antibody production to a protein antigen was diminished. These effects, although some are marginal, may have an influence on the disease status and effects of vaccination in tick-infested cattle.

Boophilus microplus infestation caused a marginal decrease in the percentage of T lymphocytes in PBL. This decrease in the percentage of T lymphocytes was observed in both lightly and heavily infested cattle. The percentage of B lymphocytes in both tick-infested and control cattle dropped at the time of the third infestation, for unexplained reasons. Importantly, there was no difference between the two groups. After the fourth infestation, the percentage of B lymphocytes in heavily tick-infested cattle was less than that from tick-free cattle. Histological studies revealed that lymphocytes could accumulate in skin sites where ticks were feeding (Schleger et al., 1976; Askenase, 1977; Nithiuthai and Allen, 1985). It is possible that tick infestation caused the migration into skin sites of circulating lymphocytes, thus resulting in the slight reduction in mature lymphocytes in peripheral blood.

The response of PBL from tick-infested cattle to PHA, which is a T lymphocyte mitogen, was always less than that of PBL from tick-free cattle after the second infestation, suggesting some immunosuppression in tick-infested animals. The decrease in the percentage of T lymphocytes was too small to explain the reduction of the response of PBL from tick-infested cattle to PHA, suggesting that there may be a direct effect on the capacity of the lymphocytes to respond to stimuli. No noteworthy differences were detected between the three stages of tick infestation, that is, 1 week before the peak engorgement of adults, the middle of the peak and 1 week after all ticks had dropped. This suggests that any suppressive effects of tick infestation are manifested during the entire adult tick infestation period and for some time after ticks have left the host. We did not determine how long the suppressive effect lasted after all the ticks had dropped off the host. It is known that tick infestation causes anemia, loss of appetite, reduced body weight growth, change of blood composition and other deleterious effects on the bovine host (O'Kelly et al., 1971; Seebeck et al., 1971; Springell et al., 1971; Nelson et al., 1977). Consequently, the stress of infestation may also contribute directly to altered lymphocyte function in host animals.

Wikel and Osburn (1982) showed that repeated low-level infestation with *D. andersoni* (ten females and five males) caused a significant reduction of the response of bovine PBL to PHA, while George et al. (1985) found that *Amblyomma americanum* infestation of cattle did not alter the responsiveness of lymphocytes to concanavalin A nor to *Escherichia coli* lipopolysaccharide. Different tick species clearly have different effects on host PBL function. Interestingly, *D. andersoni* produces a strong toxin (Gregson, 1973) which might cause immunosuppression directly or through a general systemic effect such as stress.

The saliva and salivary gland extract from *B. microplus* suppressed the response of bovine PBL to PHA in vitro. This suppressive effect may contribute to the lower responsiveness of PBL from tick-infested cattle. Saliva of *Ixodes dammini* suppressed interleukin-2 (IL-2) secretion by a T lymphocyte hybridoma (Ribeiro et al., 1985) and also inhibited neutrophil function (Ribeiro et al., 1990). In those studies, 0.15–0.57 $\mu\text{l ml}^{-1}$ of saliva suppressed 38–95% of IL-2 production by the T lymphocyte hybridoma. It was proposed that the inhibitory activity of saliva was due to prostaglandin E₂ (PGE₂) and it was estimated that 1 ml of saliva from *I. dammini* contained 94 ng of PGE₂. Higgs et al. (1976) estimated that the saliva of *B. microplus* contained 153 ng ml^{-1} of PGE₂ which is approximately the same concentration as for *I. dammini*. In the present study, 100 $\mu\text{l ml}^{-1}$ of *B. microplus* saliva suppressed 47% of the response of PBL to PHA. Consequently, something other than PGE₂ may cause the strong suppression of lymphocyte function induced by saliva from *I. dammini*, and saliva from *B. microplus* must contain negligible quantities of this substance. Alternatively, the differences may reflect species differences in the lymphocytes or the different sensitivity of IL-2 production and lymphocyte mitogenesis to suppression.

Antibody production by tick-infested cattle was examined during heavy tick infestation. Tick-infested cattle showed a diminished response against ovalbumin for 2 weeks after the second immunization. Although this difference was not significant, the tendency suggested that heavy tick infestation may reduce antibody production. Work with other ticks supports our observation. *Dermacentor andersoni* infestation reduced the antibody response in guinea pigs (Wikel, 1985). *Rhipicephalus appendiculatus* suppressed humoral responses in rabbits during infestation (Fivaz, 1989). This reduction of antibody production may be related to the reduction in the percentage of T and B lymphocytes, and also with reduced mitogen responsiveness of PBL in tick-infested cattle.

As 40 000 larvae yield about 2000 engorged ticks over a period of 1 week and this number of engorged ticks can be observed in the field with uncontrolled tick populations, immunosuppression induced by infestation with *B. microplus* could be occurring under field conditions. The immunosuppressive effects of *B. microplus* infestation may play an important role in tick survival or in the transmission and subsequent pathogenesis of tick-borne diseases.

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