Journal of Visualized Experiments

Disclosing Hemolymph Collection and Inoculation of Metarhizium Blastospores into Rhipicephalus microplus Ticks Towards Invertebrate Pathology Studies

--Manuscript Draft--

Article Type:	Invited Methods Article - Author Produced Video
Manuscript Number:	JoVE59899R1
Full Title:	Disclosing Hemolymph Collection and Inoculation of Metarhizium Blastospores into Rhipicephalus microplus Ticks Towards Invertebrate Pathology Studies
Keywords:	fungal infection; tick hemocytes; immune response; arthropod; model host; immunology
Corresponding Author:	Vânia Rita Elias Pinheiro Bittencourt, PhD Universidade Federal Rural do Rio de Janeiro Seropedica, RJ - Rio de Janeiro BRAZIL
Corresponding Author's Institution:	Universidade Federal Rural do Rio de Janeiro
Corresponding Author E-Mail:	vaniabit@ufrrj.br
Order of Authors:	Jéssica Fiorotti
	Allan Felipe Marciano
	Patrícia Silva Gôlo
	Mariana Guedes Camargo
	Isabele da Costa Angelo
	Vânia Rita Elias Pinheiro Bittencourt, PhD
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)

April 25, 2019

Journal of Video Experiments (JoVE), Editorial Office

Dear Sirs:

Thank you for the thoughtful revise and suggestions for improvement of our manuscript **JoVE59899** "Disclosing hemolymph collection and inoculation in *Rhipicephalus microplus* ticks: towards invertebrate pathology studies" now entitled "Disclosing hemolymph collection and inoculation of *Metarhizium* blastospores into *Rhipicephalus microplus* ticks towards invertebrate pathology studies" Authored by Jéssica Fiorotti, Patrícia S. Gôlo, Allan Felipe Marciano, Mariana G. Camargo, Isabele C. Angelo and Vânia R.E.P. Bittencourt.

We have revised the manuscript in accordance with the wishes of the reviewer. All advices were accepted throughout the manuscript. It is our opinion that these modifications have polished and improved our manuscript, we very much appreciate it.

Please consider our revised manuscript for publication in Journal of Video Experiments (JoVE). We look forward to your response.

Sincerely yours,

Vânia Rita Elias Pinheiro Bittencourt
Full Professor at Federal Rural University of Rio de Janeiro, Department of Animal Parasitology.
BR 465, Km 7, Seropédica, RJ - Brazil
vaniabit@ufrrj.br

TITLE:

Disclosing Hemolymph Collection and Inoculation of *Metarhizium* Blastospores into *Rhipicephalus microplus* Ticks Towards Invertebrate Pathology Studies

AUTHORS AND AFFILIATIONS:

Jéssica Fiorotti[1], Patrícia S. Gôlo[1,2], Allan Felipe Marciano[1], Mariana G. Camargo[1], Isabele C. Angelo[1,3], Vânia R.E.P. Bittencourt[1,2]

[1]Post-Graduate Program in Veterinary Sciences, Federal Rural University of Rio de Janeiro, Seropédica, RJ, Brazil

[2]Department of Animal Parasitology, Federal Rural University of Rio de Janeiro, Seropédica, RJ, Brazil

[3]Department of Epidemiology and Public Health, Federal Rural University of Rio de Janeiro, Seropédica, RJ, Brazil

Corresponding author:

Vânia R.E.P. Bittencourt (vaniabit@ufrrj.br)

Email addresses of co-authors:

Jéssica Fiorotti (jessicafiorot@ufrrj.br)

Patrícia S. Gôlo (patriciagolo@gmail.com)

Allan Felipe Marciano (allanmarc@gmail.com)

Mariana G. Camargo (marigcamargo@gmail.com)

Isabele C. Angelo (isabeleangelo@yahoo.com.br)

KEYWORDS:

fungal infection, tick hemocytes, immune response, arthropod, model host, immunology

SUMMARY:

Analysis of tick hemolymph represents an important source of information on how some pathogens cause disease and how ticks immunologically respond to this infection. The present study demonstrates how to inoculate fungal propagules and collect hemolymph from *Rhipicephalus microplus* engorged females.

ABSTRACT:

Ticks are obligate hematophagous ectoparasites and *Rhipicephalus microplus* has great importance in veterinary medicine because it causes anemia, weight loss, depreciation of the animals' leather and also can act as a vector of several pathogens. Due to the exorbitant costs to control these parasites, damage to the environment caused by the inappropriate use of chemical acaricides, and the increased resistance against traditional parasiticides, alternative control of ticks, by the use of entomopathogenic fungi, for example, has been considered an interesting approach. Nevertheless, few studies have demonstrated how the tick's immune system acts to fight these entomopathogens. Therefore, this protocol demonstrates two methods used for entomopathogen inoculation into engorged females and two techniques

used for hemolymph collection and hemocytes harvesting. Inoculation of pathogens at the leg insertion in the tick female's body allows evaluation of females biologic parameters unlike the inoculation between the scutum and capitulum, which frequently damages Gené's organ. Dorsal hemolymph collection yielded a higher volume recovery than collection through the legs. Some limitations of tick hemolymph collection and processing include i) high rates of hemocytes' disruption, ii) hemolymph contamination with disrupted midgut, and iii) low hemolymph volume recovery. When hemolymph is collected through the leg cutting, the hemolymph takes time to accumulate at the leg opening, favoring the clotting process. In addition, fewer hemocytes are obtained in the collection through the leg compared to the dorsal collection, even though the first method is considered easier to be performed. Understanding the immune response in ticks mediated by entomopathogenic agents helps to unveil their pathogenesis and develop new targets for tick control. The inoculation processes described here require very low technological resources and can be used not only to expose ticks to pathogenic microorganisms. Similarly, the collection of tick hemolymph may represent the first step for many physiological studies.

INTRODUCTION:

The cattle tick, *Rhipicephalus microplus*, is an hematophagus actoparasite with an enormous negative impact on livestock in tropical areas. This tick is the vector of pathogenic agents such as *Babesia bovis*, *Babesia bigemina*, and *Anaplasma marginale* that, combined with the direct hemofeeding damage, can reduce milk and meat production, cause anemia and ultimately death. Losses caused by this ectoparasite were estimated in 3.24 billion dollars annually in Brazil[1]. Sustainable methods are demanded and the use of entomopathogenic agents is considered a promising alternative to reduce the use of chemicals acaricides[2,3,4].

Entomopathogenic fungi, such as *Metarhizium* spp., are natural enemies of arthropods including ticks, and some isolates can be used as biocontrollers. These pathogens actively infect the host through the cuticle and colonize their body[2,5,6]. As the infection develops, cellular and humoral responses are mediated by the tick immune system. Analysis of the tick hemolymph is reported as a useful tool to evaluate the immune responses after the challenging with pathogens[7,8].

Arthropods' immune response is divided into humoral and cellular responses. The humoral response involves hemagglutination processes and production of antimicrobial proteins/peptides, whereas the cellular immune response is performed through the hemocytes. These cells are present in the hemolymph from all arthropods and are reported to develop an expressive role in studies involving innate immune response[9], as it is directly related to the phagocytosis and encapsulation processes. Accordingly, studies about hemocytes can help to investigate death pathway and understand processes such as autophagy, apoptosis, and necrosis. In some invertebrates as bivalves, the hemocytes' collection faces limitations like cell disruption, low hemolymph volume obtained, and low concentration of recovered cells[10]. Very frequently, depending on the methodology applied, reduced concentration of cells is obtained, impacting directly on cells quantification and analysis.

The number of hemocytes circulating in the hemolymph is variable among different arthropods and it can change in the same species due to different physiological stages such as sex, age, and the arthropod's developmental stage[11]. Hemocytes can also be found adhered to some organs and be released into circulation just after the infection process[11]. Nevertheless, most studies reported use insects, while ticks remain less explored regarding their physiology and pathology. Despite pathogen inoculation and hemolymph collection in ticks are less used techniques, establishing standard methods helps the development of more accurate studies.

The aim of the present study was to compare the most used methods for hemolymph collection and inoculation of pathogens into *R. microplus* ticks, evaluating the efficacy in the hemolymph acquisition and hemocytes concentration.

PROTOCOL:

Ticks used in the present study were obtained from an artificial colony, mantained at Federal Rural University of Rio de Janeiro, which methods have been approved by the Committee on Ethics for the Use of Vertebrate Animals (CEUA-IV/UFRRJ #037/2014).

1. Tick engorged females

1.1. After tick gathering, wash engorged females using tap water and immerse them in 0.5% (v/v) sodium hypochlorite solution for 3 min in a 500 mL glass beaker recipient, for cuticle hygiene (**Figure 1**), then dry all females using sterile paper towels (**Figure 2**).

1.2. Divide females in homogeneous weighted groups (with 20 females each): one group without any treatment, one control group inoculated with 5 µL of 0.1% polyoxyethylene sorbitan monooleate aqueous solution (v/v), and one infected-group inoculated with 5 µL at 1.0 x 10^7 blastospores mL^{-1}.

NOTE: Only untreated ticks were used for volume and hemocyte concentration. Three replicates of each group were performed.

2. Pathogens inoculation between the scutum and capitulum

NOTE: In the present study, entomopathogenic fungal suspensions were used as an example.

2.1. Suspend fungal blastospores in 1 mL of 0.1% polyoxyethylene sorbitan monooleate aqueous solution (v/v) and adjust to a final concentration of 1.0 x 10^7 blastospores mL^{-1}. Add an aliquot of 5 µL *Metarhizium* blastospores (**Figure 3**) suspension to the surface of a plastic paraffin film.

NOTE: *Metarhizium* blastospores suspension was adjusted using a Neubauer´s chamber. To speed the inoculation process, multiple bubbles can be added to the plastic paraffin film surface, each bubble corresponding to 5 µL.

2.2. Use a 1 mL ultra-fine insulin syringe and a 0.3 mm needle to pull the suspension and inoculate it into the tick. Remember to take all the air out of the syringe before using it.

2.3. Inoculate 5 µL of fungal suspension into the tick female between the scutum and capitulum. Inoculate females from the control group with 5 µL of 0.1% polyoxyethylene sorbitan monooleate aqueous solution (v/v) with no fungus.

NOTE: A small volume of hemolymph may be present on the foramen after the needle insertion. Be careful to not inoculate air.

3. Inoculation of entomopathogenic fungi between the leg thigh and the tick female's body

3.1. Inoculate females with fungal suspension (5 µL at 1.0 x 10^7 blastospores mL^{-1}) between the leg tigh and the female's body, using a 1 mL ultra-fine insulin syringe coupled to a 0.3 mm needle. Inoculate females from the control group with 5 µL of 0.1% polyoxyethylene sorbitan monooleate aqueous solution (v/v).

NOTE: When *Metarhizium* blastospores inoculation is performed between the leg tigh and the tick female's body, a small volume of hemolymph can be present on the tigh after the needle insertion. Be careful to not inoculate air.

4. Dorsal hemolymph collection

4.1. Use the rubber part of a winged infusion set, a 0.3 mm capillary tube, and a filtered tip to perform the hemolymph collection.

4.2. Disrupt the female dorsal cuticle using a 0.3 mm needle.

4.3. After disruption, apply gentle pressure on the anterior part of the tick body. Observe an almost transparent liquid pulling out of the disruption site. Collect the hemolymph by sucking the liquid through the filter tip coupled to the rubber part of a winged infusion set, and a 0.3 mm capillary tube.

NOTE: Do not press the tick's body hardly during its immobilization because this may disrupt the midgut and contaminate the hemolymph. Wait until gentle pressure can expel the fluid without contamination.

5. Hemolymph collection from the tick leg

5.1. Immobilize the tick, cut a piece of a front leg with a pair of scissors.

NOTE: One or more legs can be cut, as well as, the same leg can be cut more than one time.

5.2. Apply gentle pressure on the tick's posterior body part. Observe a transparent liquid

bubble that shows up on the cut site and collect it with the capillary tube, as described in step 4.3.

NOTE: Do not press the tick's body hardly during its immobilization because this may disrupt the midgut and contaminate the hemolymph. Wait until gentle pressure can expel the fluid without contamination.

6. Hemolymph processing

6.1. After hemolymph collection, deposit it in 1.5 mL microtubes previously filled with 30 µL protease cocktail and 82 µL saline buffer. Keep the microtubes on ice throughout the hemolymph collection.

6.2. Centrifuge samples (500 x *g* for 3 min at 4 °C). A soft pellet of hemocytes will be formed after hemolymph centrifugation.

NOTE: For hemolymph quantification, quantify the hemolymph volume obtained by counting the total volume inside the microtube and discounting the volume of protease cocktail and saline buffer.

6.3. Carefully remove the supernatant (cell-free hemolymph). Gently resuspend the cells in, for example, Leibovitz's L-15 culture adjusted to pH 7.0–7.2. Quantify hemocytes by placing 10 µL of resuspended hemocytes in a Neubauer chamber.

7. Hemocyte sampling slide preparation

7.1. Cut the tick's front leg with a pair of scissors.

7.2. Apply gentle pressure on the tick's posterior body part. Observe a transparent liquid bubble that shows up on the cut site.

7.3. Apply the hemolymph drops directly on clean microscope slides, after that, use appropriated methods to stain the cells.

7.3.1. To stain hemocytes using Giemsa, air-dry the hemolymph on the slide for 30 min, fix it at room temperature with methanol for 3 min, and stain in Giemsa solution (1:9 ratio of Sorensen's buffer solution, pH 7.2) for 30 min at room temperature. Wash the slides with running water to remove the excess of dye, air-dry the slide and evaluate the cells at 400x in an optical microscope.

REPRESENTATIVE RESULTS:

This article approaches inoculation and hemolymph collection methods applied to ticks. After the inoculation between the leg thigh and the tick female's body, some fluid (hemolymph) can be secreted during the process; however, it is important to note that when the inoculation is

finished, no liquid or tissues were present in the needle tip or at the inoculation site, ensuring that the fungal suspension was completely inoculated. When the inoculation process was correctly performed, the needle insertion did not cause tick females' death. On the other hand, ticks died approximately 48 h after inoculation of the entomopathogenic fungus. Inoculation between the leg thigh and tick female's body can damage tick intern organs such as midgut and the Malpighian tubules, while damage to Gene's organ can occur during inoculation between the scutum and capitulum.

When the tick midgut is disrupted by the needle during the dorsal hemolymph collection, the fluid obtained is red, not colorless. This indicates an incorrect hemolymph collection. In these cases, hemolymph shall be discarded since it is contaminated with intestinal content.

The correct hemolymph collection is crucial to properly conduct the experiments and obtain reliable results. When hemolymph was collected from cut tick legs (n = 20 ticks homogenously weighed), the volume obtained was lower than the total hemolymph obtained from the dorsal collection (n = 20 ticks homogenously weighed) (**Figure 4**). It is suggested that, due to the low volume of each drop that pulls out of the cut leg, hemolymph clotting can be frequently present during this process of hemolymph collection. This may negatively interfere in the hemocytes acquisition and classification (**Figure 5** and **Figure 6**). Despite the higher volume achievement of hemolymph, the dorsal collection is considered more difficult to perform.

FIGURE LEGENDS:

Figure 1: Ticks' cuticle hygienization. *Rhipicephalus microplus* fully engorged females were washed using tap water and immersed in 0.5% sodium hypochlorite solution (v/v) for 3 min in a 500 mL glass beaker recipient.

Figure 2: Ticks' cuticle drying. *Rhipicephalus microplus* fully engorged females were dried using sterile paper towels.

Figure 3: *Metarhizium* blastospores. Fungal propaluges used in tick inoculation. Scale bar = 50 µm.

Figure 4: Representative graph demonstrating hemolymph volume obtained with each collecton method. *Rhipicephalus microplus* hemolymph volume obtained after dorsal or leg collection. A pool of 20 homogenously weighed ticks were used for each method. These ticks were not previously inoculated. Mean values ± standard deviation followed for the same letter do not differ statistically after analysis of variance (ANOVA) test *(P* ≥ 0.05).

Figure 5: Hemocytes concentration obtained with each hemolymph collection method. *Rhipicephalus microplus* hemocytes concentration obtained after resuspension in Leibovitz's L-15 culture medium after dorsal or leg collection. Mean values ± standard deviation followed for the same letter do not differ statistically after Kruskal-Wallis test *(P* ≥ 0.05).

Figure 6: Hemocytes found in *Rhipicephalus microplus* hemolymph smear. *R. microplus* hemocytes were stained by Giemsa. Black arrows indicate the different cells in the tick hemolymph. Scale bar = 100 µm.

DISCUSSION:

Inoculation of pathogens is useful when the study aims to investigate the in vivo action of microorganisms in experimental arthropod models because it assures that the pathogen is inside the host. The technique can also be applied to inoculate molecules such as RNA interference (RNAi). Inoculation between the scutum and capitulum is considered easier to perform but frequently damages Gené's organ, impairing the eggs viability[12,13]. Gené's organ is anatomically located close to the anterior part of capitum, and it is an important organ for the tick oviposition[14]. Accordingly, inoculation between the leg tigh and the female's body is more appropriate if the study requires the observation of females' biological parameters since the Gené's organ will not be injured. Despite the inoculation method between the leg thigh and the tick's body is considered more difficult and easily damage or expose the tick internal organs, such as the midgut and the Malpighian tubules, when it is well performed, it will not disrupt these organs.

Hemolymph analysis is essential to understand the arthropod immune system as well as to understand pathogenesis[15,16]. Accordingly tick hemolymph can be used to unveil tick physiology, assure tick infectivity, understand tick-pathogen interactions, and the cellular and humoral immune responses[9,17,18].

Tick hemolymph collection through the leg cutting is reported in several studies[19,20,21]. Despite this method being simple with no or very low hemolymph contamination, it is considered counterproductive for studies that require high concentrations of hemocytes or large volumes of hemolymph. On the other hand, the correct execution of hemolymph collection through the dorsal region of engorged females is not easy to achieve since the gut occupies almost the entire tick body and rupturing it with the needle causes hemolymph contamination. Contamination due to gut disruption can also be observed when the tick is naturally infected with high loads of hemoparasites (viz., *Babesia* spp.), or in the final steps of the death process caused by entomopathogens[8]. In these cases, hemolymph shall be discarded since hemocytes and plasma analyzes can be affected.

The centrifugation speed of hemolymph samples for hemocytes harvesting is also important and directly influence the hemocytes concentration, since high relative centrifugal field (RCF) speeds contribute to: i) cell pellet difficult to resuspend, ii) cells disruption, and iii) hemocytes degranulation. The ideal cell pellet is a soft pellet easy to resuspend. For this reason, the centrifugation at 500 x *g* for three minutes at 4 °C[8] was used. In the present study, hemocytes were resuspended in a culture medium and not in phosphate-buffered saline (PBS) because the culture medium supports better cell viability.

The methods presented here may face limitations when applied to immatures stages (larva and nymph) of ticks or unfed adults. The inoculation method, for example, is only applied for adult

engorged females because the needle size will damage immature stages and possibly unfed adult ticks. To inoculate these stages, a microinjector shall be used. Similarly, hemolymph collection through the dorsal region is more effective when applied to engorged ticks, while hemolymph collection by cutting the legs can be used in studies with unfed adult ticks or immature stages. Despite this, our goal here was to show methods that require very low technological resources. In addition, centrifugation technique has to be performed at low centrifugation force because high force or extended spin time can damage cells.

The methods disclosed here can be used as guidelines for studies involving inoculation of entomopathogens into ticks and hemolymph/hemocytes harvesting. The techniques presented here require very low technological resources and are classical steps for studies about tick physiology, pathology, and tick's immune response.

ACKNOWLEDGMENTS:

This study was financed in part by the Coordenacão de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) from Brazil, finance code 001. CAPES provided Ph.D. scholarship for A.F. Marciano. We thank National Council for Scientific and Technological Development (CNPq) of Brazil for providing Ph.D. scholarship for J. Fiorotti. This research was also supported by grants of Carlos Chagas Filho Foundation for Research of the State of Rio de Janeiro (FAPERJ) and CNPq. V.R.E.P. Bittencourt is a CNPq researcher.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Grisi, L. et al. Reassessment of the potencial economic impact of cattle parasites in Brazil. *Revista Brasileira de Parasitologia Veterinária.* **23** (2), 150−156 (2014).
2. Schrank, A., Vainstein, M.H. *Metarhizium anisopliae* enzymes and toxins. *Toxicon*. **56** (7), 1267−1274 (2010).
3. Camargo, M.G. et al. *Metarhizium anisopliae* for controlling *Rhipicephalus microplus* ticks under field conditions. *Veterinary Parasitology*. **223**, 38−42 (2016).
4. Perinotto, W.M.S. et al. *In vitro* pathogenicity of different *Metarhizium anisopliae* s.l. isolates in oil formulations against *Rhipicephalus microplus*. *Biocontrol Science and Technology*. **27** (3), 338−347 (2017).
5. Pedrini, N., Crespo, R., Juarez, M.P. Biochemistry of insect epicuticle degradation by entomopathogenic fungi. *Comparative Biochemistry and Physiology. Part C: Toxicology and Pharmacolpgy*. **146** (1−2), 124−137 (2007).
6. Ortiz-Urquiza, A., Keyhani, N.O. Action on the surface: Entomopathogenic fungi versus the insect cuticle. *Insects*. **4** (3), 357−374 (2013).
7. Angelo, I. C. et al. Detection of serpins involved in cellular immune response of *Rhipicephalus microplus* challenged with fungi. *Biocontrol Science and Technology.* **24** (3), 351−360, (2014).
8. De Paulo, J.F. et al. *Rhipicephalus microplus* infected by *Metarhizium*: unveiling hemocyte quantification, GFP-fungi virulence, and ovary infection. *Parasitology Research.* **117**, 1847-1856 (2018).

9. Marmaras, V.J., Lampropoulou, M. Regulators and signalling in insect haemocyte immunity. *Cell Signal.* **21**, 186−195 (2009).
10. Hinzmann, M.F., Lopes-Lima, M., Gonçalves, J., Machado, J. Antiaggregant and toxic properties of different solutions on hemocytes of three freshwater bivalves. *Toxicological & Environmental Chemistry*. **95**, 790−805 (2013).
11. Nation, J.L. *Insect Physiology and Biochemistry*. University of Florida. Gainesville, FL (2016).
12. Gene, J. 1848. Mémoires de l'Académie royale des sciences. *Torino*. **9**, 751 (1848).
13. Lees, A.D., Beament, J.W.L. An organ waxing in ticks. *The Quarterly Journal of the Mythic Society*. **7**, 291−332 (1948).
14. Sonenshine, D.E., Roe, R.M. *Biology of ticks*. Oxford University Press. Oxford, UK (2014).
15. Tan, J. et al. Characterization of hemocytes proliferation in larval silkworm *Bombyx mori*. *Journal of Insect Physiology*. **59** (6), 595−603 (2013).
16. Bowden, T.J. The humoral immune systems of the American lobster (*Homarus americanus*) and the European lobster (*Homarus gammarus*). *Fish Research.* **186**, 367−371 (2017).
17. Sonenshine, D.E., Hynes, W.L. Molecular characterization and related aspects of the innate immune response in ticks. *Frontiers in Bioscience*. **13**, 7046−7063 (2008).
18. Tsakas, S., Marmaras, V. Insect immunity and its signaling: an overview. *Invertebrate Survival Journal*. **7**, 228−238 (2010).
19. Burgdorfer, W. Hemolymph Test. A technique for detection of Rickettsiae in ticks. *American Journal of Tropical Medicine and Hygiene*. **19**, 1010−1014 (1970).
20. Dunham-Ems, S.M. et al. Live imaging reveals a biphasic mode of dissemination of Borrelia burgdorferi within ticks. *Journal of Clinical Investigation*. **119**, 3652−3665 (2009).
21. Patton, T.G. et al. Saliva, salivary gland, and hemolymph collection from Ixodes scapularis ticks. *Journal of Visualized Experiments*. **60**, e3894 (2012).

Figure 1

Click here to access/download;Figure;Figure 1.tif

Figure 2

Click here to access/download;Figure;Figure 2.tif

Figure 3

Click here to access/download;Figure;Figure 3.tif

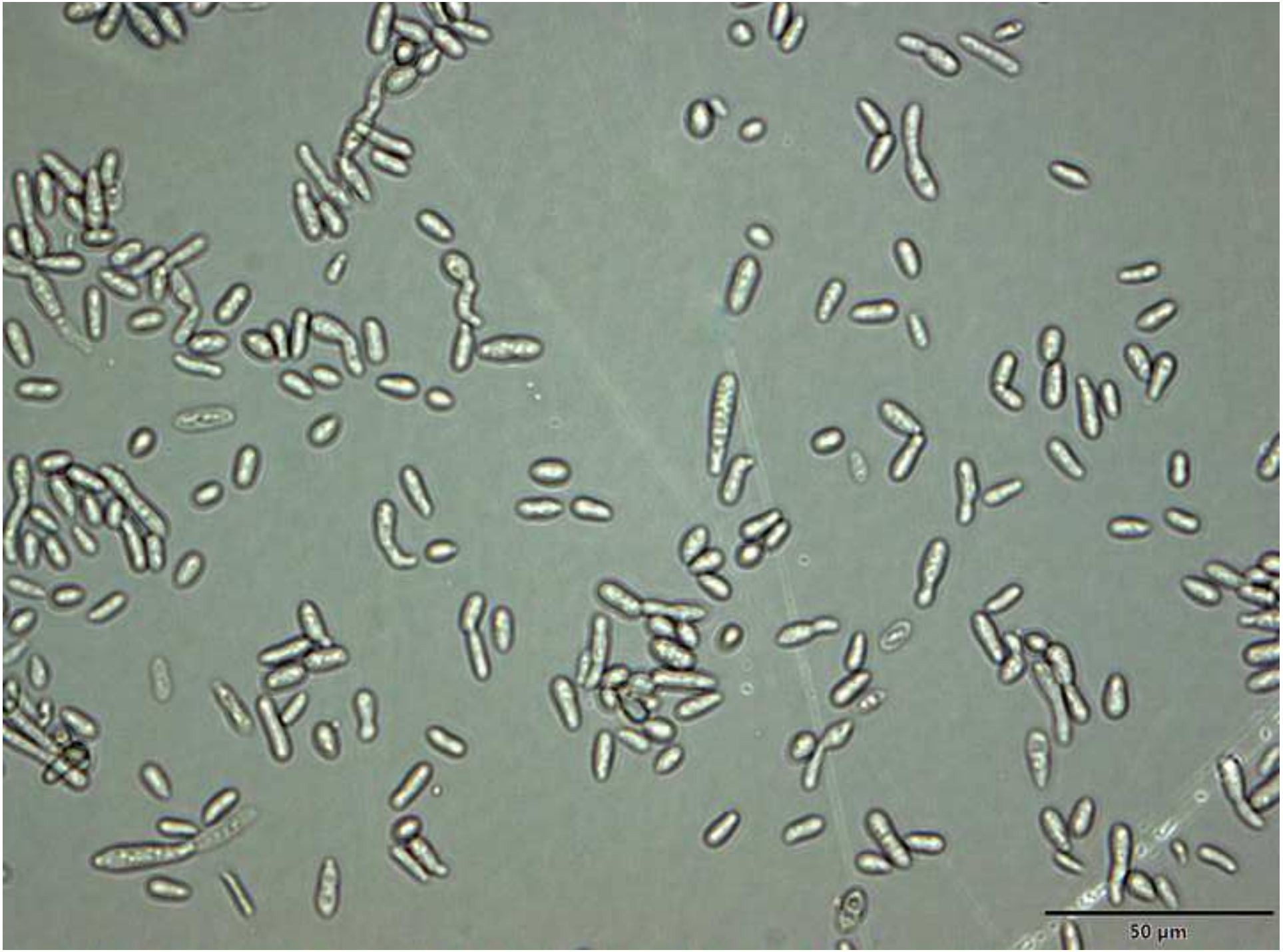


Figure 4

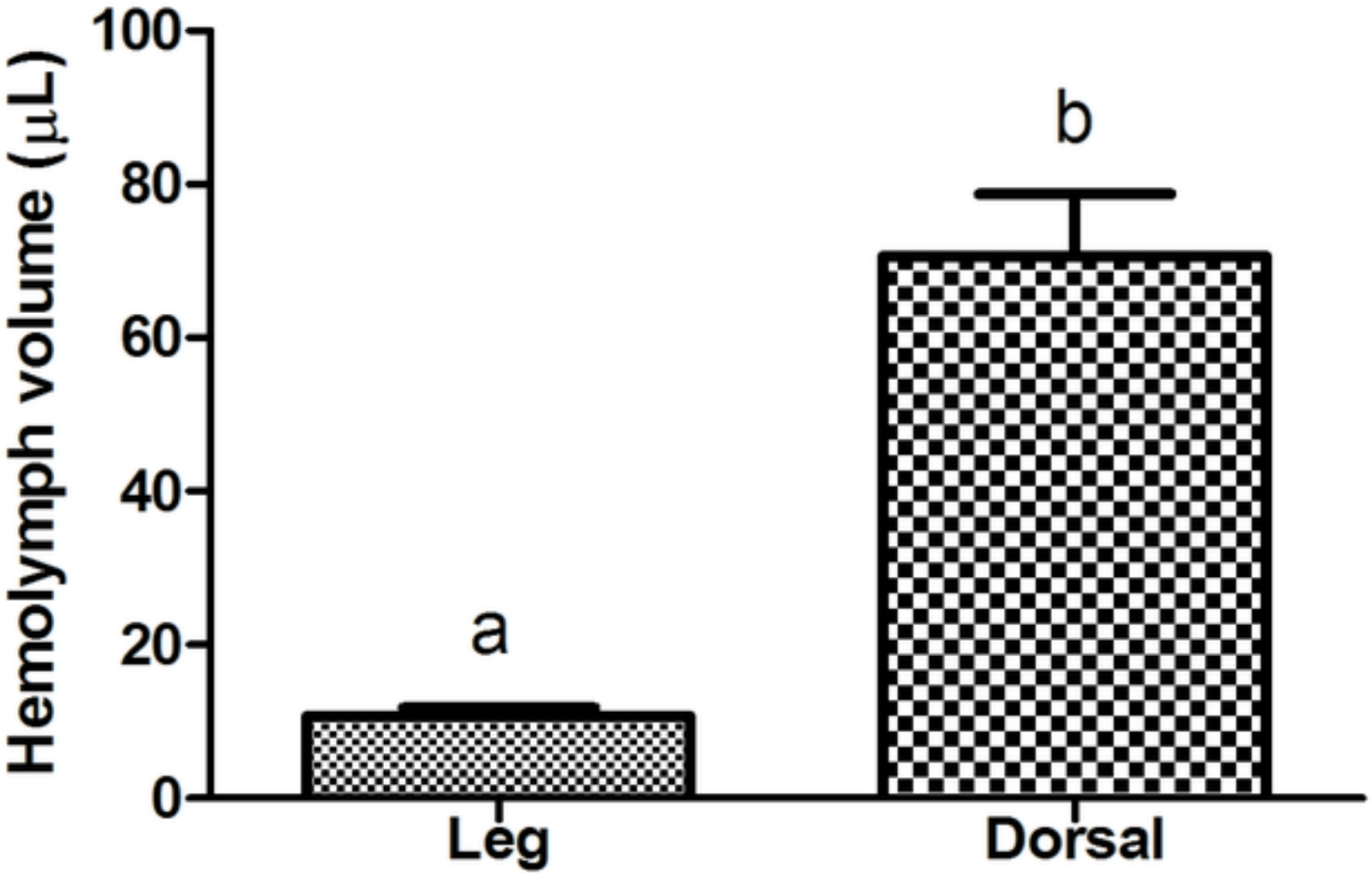


Figure 5

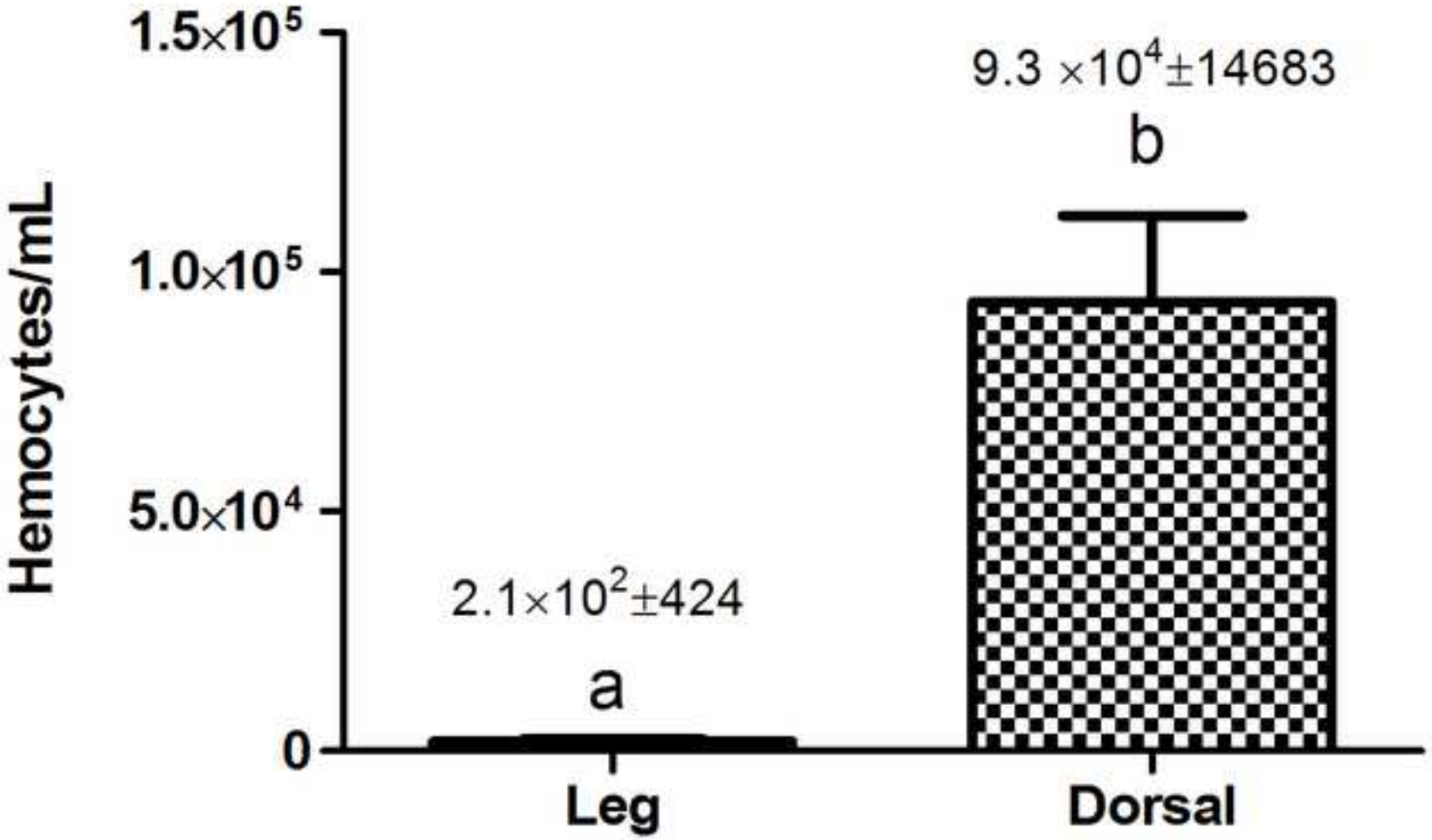
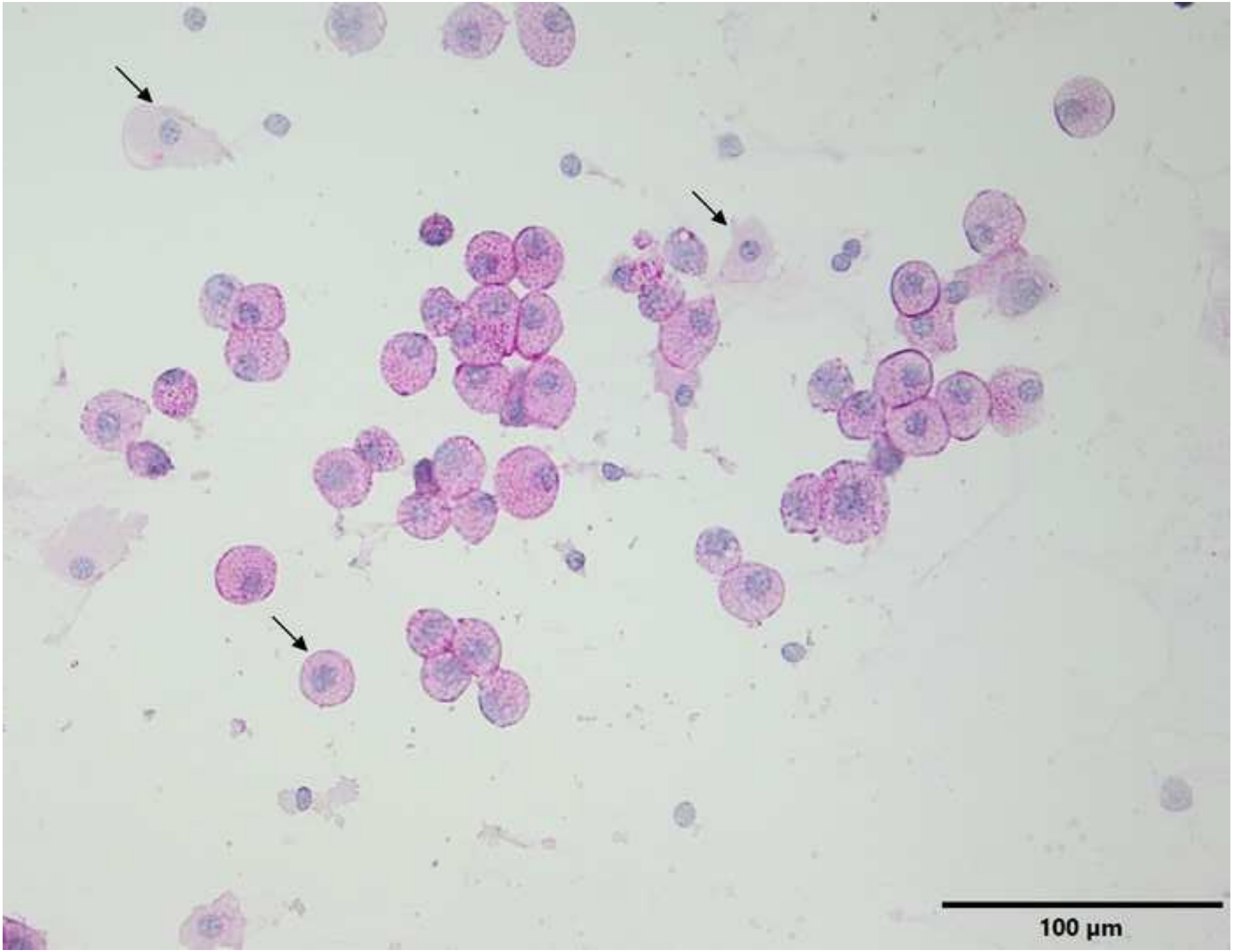
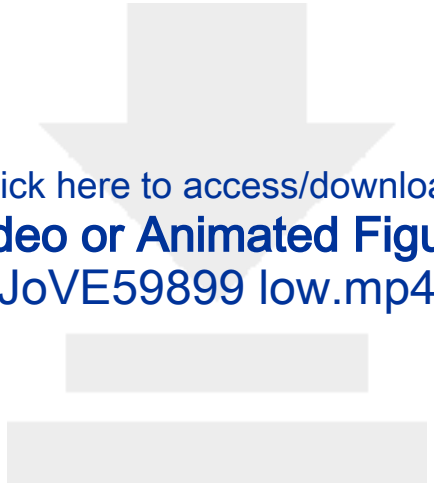


Figure 6

Click here to access/download;Figure;Figure 6.tif



Video



Click here to access/download

Video or Animated Figure

JoVE59899 low.mp4

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Alkaline Hypochlorite solution	Sigma-Aldrich	A1727	
D-(+)-Glucose	Sigma-Aldrich	G8270-1KG	
EDTA	Synth	2706	
Fetal Bovine Serum	Gibco	16000036	
Flexible rubber	BD		
Giemsa stain	Sigma-Aldrich	48900-500ML-F	
Glass capillary	CTechGlass	CT95-02	
Insulin syringe (needle)	BD	SKU: 324910	
KH_2PO_4	Vetec	60REAVET014512	
Leibovitz's L-15 culture medium	Gibco	11415-064	
Methanol	Sigma-Aldrich	34860-1L-R	
Microscope slides	Kasvi	K5-7105	
Microtubes	BRAND	Z336769-1PAK	
Na_2HPO_4	Vetec	60REAVET014593	
NaCl	Sigma-Aldrich	S7653-1KG	
Neubauer chamber	Kasvi	K5-0111	
Penicillin	Gibco	15140163	
Protease inhibitor cocktail	Sigma-Aldrich	P2714	
Tween 80	Vetec	60REAVET003662	

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Disclosing hemolymph collection and inoculation in *Rhipicephalus microplus* ticks: towards invertebrate pathology studies

Author(s): Jéssica Fiorotti, Patrícia S. Gôlo, Allan Felipe Marciano, Mariana G. Camargo, Isabele C. Angelo, Vânia R.E.P. Bittencourt,

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

[X] Standard Access [ ] Open Access

Item 2: Please select one of the following items:

X The Author is **NOT** a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJoVE Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and/or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement their respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **RetentionofRightsinArticle.**Notwithstanding theexclusivelicensegrantedtoJoVEin**Section3**above,the Author shall, with respect to the Article, retain the non-exclusiverighttouseallorpartoftheArticleforthenon-commercialpurposeofgivinglectures,presentationsor teachingclasses,andtopostacopyoftheArticleonthe Institution'swebsiteortheAuthor'spersonalwebsite,in eachcaseprovidedthatalinktotheArticleontheJoVE websiteisprovidedandnoticeofJoVE'scopyrightinthe Articleisincluded.Allnon-copyrightintellectualproperty rights in and to the Article, such as patent rights, shall remain with theAuthor.

5. **GrantofRightsinVideo–StandardAccess.**This **Section5**appliesifthe"StandardAccess"boxhasbeen checkedin**Item1**aboveorifnoboxhasbeencheckedin **Item1**above.InconsiderationofJoVEagreeingtoproduce, display or otherwise assist with the Video, the Author herebyacknowledgesandagreesthat,Subjectto**Section7** below,JoVEisandshallbethesoleandexclusiveownerof all rights of any nature, including, without limitation, all copyrights,inandtotheVideo.Totheextentthat,bylaw, theAuthorisdeemed,noworatanytimeinthefuture,to haveanyrightsofanynatureinortotheVideo,theAuthor herebydisclaimsallsuchrightsandtransfersallsuchrights toJoVE.

6. **GrantofRightsinVideo–OpenAccess.**This **Section6**appliesonlyifthe"OpenAccess"boxhasbeen checkedin**Item1**above.InconsiderationofJoVEagreeing toproduce,displayorotherwiseassistwiththeVideo,the AuthorherebygrantstoJoVE,subjectto**Section7**below, theexclusive,royalty-free,perpetual(forthefulltermof copyrightintheArticle,includinganyextensionsthereto) license(a)topublish,reproduce,distribute,displayand storetheVideoinallforms,formatsandmediawhether now known or hereafter developed (including without limitationinprint,digitalandelectronicform)throughout theworld,(b)totranslatetheVideointootherlanguages, createadaptations,summariesorextractsoftheVideoor otherDerivativeWorksorCollectiveWorksbasedonallor anyportionoftheVideoandexerciseallooftherightsset forth in (a) above in such translations, adaptations, summaries,extracts,DerivativeWorksorCollectiveWorks and(c)tolicenseotherstodoanyorallooftheabove.The foregoingrightsmaybeexercisedinallmediaandformats, whethernowknownorhereafterdevised,andincludethe right to make such modifications as are technically necessarytoexercisetherightsinothermediaandformats. ForanyVideotowhichthis**Section6**isapplicable,JoVEand theAuthorherebygranttothepublicallsuchrightsinthe Video as provided in, but subject to all limitations and requirementssetforthin,theCRCLicense.

7. **GovernmentEmployees.**IftheAuthorisaUnited StatesgovernmentemployeeandtheArticlewasprepared in the course of his or her duties as a United States governmentemployee,asindicatedin**Item2**above,and any of the licenses or grants granted by the Author hereunderexceedthescopeofthe17U.S.C.403,thenthe rightsgrantedhereundershallbelimitedtothemaximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflictwith suchstatuteshallremaininfullforceandeffect,andall provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximumrightspermissiblewithinsuchstatute.

8. **ProtectionoftheWork.**TheAuthor(s)authorize JoVEtotakestepsintheAuthor(s)nameandontheirbehalf if JoVE believes some third party could be infringingor mightinfringethecopyrightofeithertheAuthor'sArticle and/orVideo.

9. **Likeness,Privacy,Personality.**TheAuthorhereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connectionwiththeMaterialsandthesale,promotionand distributionthereof.TheAuthorherebywaivesanyandall rightsheorshemayhave,relatingtohisorherappearance intheVideoorotherwiserelatingtotheMaterials,under allapplicableprivacy,likeness,personalityorsimilarlaws.

10. **Author Warranties.** The Author representsand warrants that the Article is original, that it has notbeen published, that the copyright interest is owned by the Author(or,ifmorethanoneauthorislistedatthebeginning ofthisAgreement,bysuchauthorscollectively)andhasnot beenassigned,licensed,orotherwisetransferredtoany otherparty.TheAuthorrepresentsandwarrantsthatthe author(s)listedatthetopofthisAgreementaretheonly authorsoftheMaterials.Ifmorethanoneauthorislisted atthetopofthisAgreementandifanysuchauthorhasnot entered into a separate Article and Video License AgreementwithJoVErelatingtotheMaterials,theAuthor represents and warrants that the Author has been authorizedbyeachoftheothersuchauthorstoexecutethis Agreementonhisorherbehalfandtobindhimorherwith respecttothetermsofthisAgreementasifeachofthem hadbeenapartyheretoasanAuthor.TheAuthorwarrants thattheuse,reproduction,distribution,publicorprivate performanceordisplay,and/ormodificationofallorany portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectualpropertyorotherrightsofanythirdparty.The Author represents and warrants that it has and will continuetocomplywithallgovernment,institutionaland other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animaltreatment,privacy,andallotherrules,regulations, laws,proceduresorguidelines,applicabletotheMaterials, andthatallresearchinvolvinghumanandanimalsubjects hasbeenapprovedbytheAuthor'srelevantinstitutional reviewboard.

11. **JoVE Discretion.** If the Author requests the assistanceofJoVEinproducingtheVideointheAuthor's facility,theAuthorshallensurethatthepresenceofJoVE employees, agents or independent contractors is in accordancewiththerelevantregulationsoftheAuthor's institution. If more than one author is listed at the beginningofthisAgreement,JoVEmay,initssole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Articleuntilsuchtimeasithasreceivedcomplete,executed Article and Video License Agreements from eachsuch author.JoVEreservestheright,initsabsoluteandsole discretion and without giving any reason therefore, to acceptordeclineanyworksubmittedtoJoVE.JoVEandits employees,agentsandindependentcontractorsshallhave full,unfetteredaccesstothefacilitiesoftheAuthororof theAuthor'sinstitutionasnecessarytomaketheVideo, whetheractuallypublishedornot.JoVEhassolediscretion astothemethodofmakingandpublishingtheMaterials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length,quality,contentandthelike.

12. **Indemnification.** TheAuthoragreestoindemnify JoVEand/oritssuccessorsandassignsfromandagainstany andallclaims,costs,andexpenses,includingattorney's fees,arisingoutofanybreachofanywarrantyorother representations contained herein. The Author further agrees to indemnify and hold harmless JoVE fromand againstanyandallclaims,costs,andexpenses,including attorney'sfees,resultingfromthebreachbytheAuthorof anyrepresentationorwarrantycontainedhereinorfrom allegationsorinstancesofviolationofintellectualproperty rights,damagetotheAuthor'sortheAuthor'sinstitution's facilities,fraud,libel,defamation,research,equipment, experiments, property damage, personal injury,violations of institutional, laboratory, hospital, ethical, humanand animaltreatment,privacyorotherrules,regulations,laws, procedures or guidelines, liabilities and other lossesor damagesrelatedinanywaytothesubmissionofworkto JoVE,makingofvideosbyJoVE,orpublicationinJoVEor elsewherebyJoVE.TheAuthorshallberesponsiblefor,and shallholdJoVEharmlessfrom,damagescausedbylackof sterilization,lackofcleanlinessorbycontaminationdueto themakingofavideobyJoVEitsemployees,agentsor independentcontractors.Allsterilization,cleanlinessor decontamination procedures shall be solely the responsibilityoftheAuthorandshallbeundertakenatthe Author's expense. All indemnifications providedherein shallincludeJoVE'sattorney'sfeesandcostsrelatedtosaid losses or damages. Such indemnification and holding harmlessshallincludesuchlossesordamagesincurredby, or in connection with, acts or omissions of JoVE, its employees,agentsorindependentcontractors.

13. **Fees.** Tocoverthecostincurredforpublication, JoVE must receive payment before production and publicationoftheMaterials.Paymentisduein21daysof invoice.ShouldtheMaterialsnotbepublishedduetoan editorial or production decision, these funds will be returnedtotheAuthor.WithdrawalbytheAuthorofany submittedMaterialsafterfinalpeerreviewapprovalwill resultinaUS$1,200feetocoverpre-productionexpenses incurred by JoVE. If payment is not received by the completion of filming, production and publication ofthe Materialswillbesuspendeduntilpaymentisreceived.

14. **Transfer,GoverningLaw.** ThisAgreementmaybe assignedbyJoVEandshallinuretothebenefitsofanyof JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to anyconflictoflawprovisionthereunder.ThisAgreement maybeexecutedincounterparts,eachofwhichshallbe deemed an original, but all of which together shall be deemedtomeoneandthesameagreement.Asignedcopy ofthisAgreementdeliveredbyfacsimile,e-mailorother meansofelectronictransmissionshallbedeemedtohave *thesamelegaleffectasdeliveryofanoriginalsignedcopy of* thisAgreement.

Asignedcopyofthisdocumentmustbesentwithallnewsubmissions.OnlyoneAgreementisrequiredpersubmission.

CORRESPONDING AUTHOR

Name:	Vânia Rita Elias Pinheiro Bittencourt
Department:	Departmentof Animal Parasitology
Institution:	Federal Rural University of Rio de Janeiro (UFRRJ)
Title:	Full professor, Ph.D.
Signature:	
Date:	26/02/2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. UploadanelectronicversionontheјoVEsubmissionsite
2. Faxthedocumentto+1.866.381.2236
3. MailthedocumenttoJoVE/Attn:JoVEEditorial/1AlewifeCenter#200/Cambridge,MA02140

Manuscript JoVE59899

Formerly "Disclosing hemolymph collection and inoculation *in Rhipicephalus microplus* ticks: towards invertebrate pathology studies" now entitled "Disclosing hemolymph collection and inoculation of *Metarhizium* blastospores into *Rhipicephalus microplus* ticks towards invertebrate pathology studies" Authored by Jéssica Fiorotti, Patrícia S. Gôlo, Allan Felipe Marciano, Mariana G. Camargo, Isabele C. Angelo and Vânia R.E.P. Bittencourt.

Response to Reviewers

We would like to thank the JoVE's reviewers for these very helpful comments on our manuscript. It is our opinion that the incorporation of these revisions into the manuscript has polished and improved it. All changes suggested by the editor are highlighted in yellow in the manuscript text; the changes suggested by reviewer 1 are in blue; pink was used for reviewer 2, and green for reviewer 3.

Editorial and production comments:

***Reviewer's comment*:** *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.*

Authors' response: We appreciate very much your suggestions. The manuscript has been checked for spelling or grammar issues.

***Reviewer's comment*:** *Please revise lines 58-61 to avoid previously published text.*

Authors' response: The text is now re-written. Please refer to lines 59-63.

Reviewer's comment**:** *Title: Please revise to avoid the use of colon or dash.*

Authors' response: The title is now re-written.

Reviewer's comment**:** *Please replace commercial language (Tween®) with a generic term.*

Authors' response: The term was replaced. Please refer to lines 118, 119 and 121.

Reviewer's comment**:** *Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.*

Authors' response: Thank you for your consistent comments. We added more details in our protocol steps. Additionally, we changed the manuscript's protocol text using almost the same content presented in the video's protocol. All information is now included in the protocol and representative results sections.

Reviewer's comment**:** *Figure 1: Please change "µl" to "µL".*

Authors' response: The suggestion was included in Figure 1, now Figure.

Reviewer's comment**:** *Discussion: Please discuss any limitations of the technique.*

Authors' response: Limitations of the techniques were included in the discussion section. Please refer to lines 289, 290, 296 and 297.

Reviewer's comment**:** *Please also include the figure in the written manuscript*.

Authors' response: New figures (Figure 1, 2, 3, and 6) were included in the manuscript text. Please refer to lines 228-233 and 248-250.

Reviewer #1:

Reviewer's comment**:** *Minor Concerns:*

Authors' response: Thank you for all your suggestions. The words are now re-written in the manuscript text.

Reviewer #2:

Reviewer's comment**:** *Nevertheless, described method of injection is questionable as it can potentially cause relatively extensive injury to ticks. We suggest to replace mentioned method by innovative and less invasive inoculation method of microinjection exploiting glass capillaries (e.g. Drummond). Additionally, pathogen inoculation exploiting insulin syringe is limiting as it cannot be applied on other than adult tick stages.*

Authors' response: Thank you for your suggestion. We fully agree with the replacement by microinjection in future studies to allow injection into tick's immature stages. Nevertheless, in the present study, we aimed to use very low technological resources with methods that could be applied to engorged females' ticks, displaying low-cost procedures that when well performed, do not cause any damage to this tick's life stage.

Reviewer's comment**:** *Line 106: How were blastospores of Metarhizium counted? Was Neubauer´s chamber used? If not, please provide details of the calculation method.*

Authors' response: Sorry for the lack of information. *Metarhizium* blastospores were counted in a Neubauer's chamber. We added this information in the manuscript text. Please refer to line 125.

Reviewer's comment**:** *Line 125: For dorsal hemolymph collection 0.03 mm glass capillary was used but in the video protocol the use of 0.3 mm glass capillary is mentioned. Which capillary was actually used?*
Authors' response: Sorry for the misunderstanding. A 0.3mm-glass capillary was used. The manuscript text is now re-written. Please refer to line 147.

Reviewer's comment**:** *Have you detected any tick mortality during the inoculation process? Which of the two described methods seem to be more appropriate?*

Authors' response: After the inoculation process, females were monitored for 72 hours. 72h after the inoculation all females from the control group were alive, and all females inoculated with entomopathogenic fungal propagules were dead. Inoculation between the leg tigh and the female's body is more appropriate to analyse biological parameters because Gene's organ (vital for the eggs' viability) is usually damaged during inoculation through the foramen between the capitulum and the scutum. Despite this, if only mortality will be evaluated, inoculation between capitulum and scutum is more appropriated due to the easiness and speed that this method can be applied.

Reviewer's comment**:** *The author mentioned that during hemolymph collection from the tick leg, cell debris is also present in tick hemolymph (line 181). Are authors able to distinguish cell debris from hemocytes during the cell counting? Is it possible that during hemolymph collection other type of cells are also collected?*

Authors' response: Sorry for the misunderstanding. The term "cell debris" was erroneously used in this context. Actually, hemolymph takes time to pull out from the cut leg and we believe this can facilitate the clotting process, interfering the identification of hemocytes. Regarding the question about the presence of other cell types, if the tick's organs are somehow disrupted, other cell types can be present; similarly, when hard pressure is applied on the tick's body, fatty body cells can detach from this organ and enter the hemolymph. These cells can be erroneously classified as adipohemocytes in some tick species. Sentence was added to the text manuscript. Please refer to lines 220-223.

Reviewer #3:

Reviewer's comment**:** *The title is misleading. At least the object of inoculation should be clarified.*

Authors' response: Sorry for the misunderstanding. The title was reformulated and the object of inoculation was included.

Reviewer's comment**:** *Protocol, 1. 2. Inoculation of pathogens between the scutum and captiulum. Did the authors check the mortality rate after just needle inoculation? It is essential to include such data as a baseline information.*

Authors' response: Thank you for your suggestion. All females did not die after needle insertion, mortality happened just after inoculation of fungal pathogens. We included this information in the manuscript. Please refer to lines 206-208.

Reviewer's comment**:** *Protocol, 2. Inoculation of pathogens between the scutum and capitulum, 2.1, Line 107. The authors used "0.1% Tween 80 aqueous solution" as a solvent. Is there any specific reason why they selected the solvent? Did the authors check the difference between PBS or Milli-Q and the solvent?*

Authors' response: Thank you for your comment. We use 0.1% Tween 80 aqueous solution because to prepare the fungal suspension, blastospores are added to 0.1% Tween 80 sterile aqueous solution to prevent aggregation of fungal propagules. The manuscript text was re-written to clarify this question.

Reviewer's comment**:** *The authors performed the inoculation and hemolymph collection using engorged adult female ticks. Is the method also applicable for unfed adults or nymphal ticks? This information is necessary.*

Authors' response: Thank you for your comment. Our goal was to show methods that require very low technological resources. Unfortunately, the inoculation method is only applied for adult engorged females because the needle size will damage immature stages and unfed adult ticks. To inoculate these stages, a microinjector shall be used. Hemolymph collection through the dorsal region is only applied to engorged ticks, but

hemolymph collection by cutting the legs can be applied to unfed adult ticks and immature stages. We included this information in the manuscript text. Please refer to lines 290-296.

Reviewer's comment**:** *Discussion, Line 219. The reference paper No.21 is not available in the list.*
Authors' response: Sorry for the misunderstanding. This reference was excluded from the manuscript.

Reviewer's comment**:** *Discussion, Line 233. I don't understand what the authors meant by the sentence "Additionally, hemocytes were …*
Authors' response: Sorry for the misunderstanding. Sentence was re-written, please refer to lines 285-287.