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A newly optimized protocol to extract high-quality hemolymph from the cattle tick *Rhipicephalus microplus*: Improving the old conditions

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ABSTRACT

Ticks are hematophagous ectoparasites with importance to animal and human health. In recent years, the study of ticks has had significant development, including immune response, vector-host interactions, physiological and multi-omics approaches. However, one of the main impediments is obtaining a significant amount of high-quality hemolymph. For this reason, we developed a protocol that allows obtaining up to 100 μl of hemolymph free of host blood per engorged tick. The technique consists of continuous hipocuticular punctures of the tick dorsum and an anticoagulant buffer that impedes hemolymph coagulation, allowing constant extravasation and ensuring high yields. Additionally, the hemocytes recovered with this protocol are intact and can be used for further analysis. The high-quality hemolymph obtained using this protocol and its applications will help to better understand the processes involving the hemolymph and its components. Although there are other hemolymph extraction protocols, the method developed here is very well suited for *Rhipicephalus microplus*, and in our experience, results in better yields and high-quality samples.

1. Introduction

Ticks are arthropod vectors capable of transmitting many pathogens that affect animal and human health. *Rhipicephalus microplus* is the vector of *Anaplasma* spp. and *Babesia* spp., the causative agents of bovine anaplasmosis and babesiosis, respectively. Studying tick tissues and organs is essential due to pathogens carrying out part of their life-cycle inside the vector. In this regard, many reports focus on studying the midgut, salivary glands and hemolymph, as approaches to identify the components participating in the transmission process and the pathogen-vector interaction (Patton et al., 2012; Narasimhan et al., 2014; Aounallah et al., 2020). Hemolymph plays a significant role in tick physiology and vectorial capacity and is the major source of nutrients, osmoregulation, and transport of molecules and hormones. Additionally, the hemolymph consists of protein-rich plasma and different types of cells (hemocytes) that play a significant role in immune response (Grubhoffer et al., 2013). On the other hand, hemolymph also protects the tick when superficial injuries occur by activating different coagulation mechanisms that prevent microbes internalization.

Although hemocytes control microorganisms within the tick, many pathogens have developed strategies to evade the immune response and are transported though the hemolymph from the midgut to the salivary glands (Sonenshine & Macaluso, 2017). Due to the importance of the hemolymph in pathogen transmission process, several collection and extraction protocols exist for some tick species, including *Ixodes scapularis* (Patton et al., 2012), *Rhipicephalus sanguineus* (Velásquez-Serra, 2016) and *R. microplus* (Angelo et al., 2010; Fiorotti et al., 2018, 2019).

The hemolymph extraction protocols reported for *I. scapularis* and *R. sanguineus* usually imply cutting the tick coxae at the distal joint with a scalpel and then gently applying pressure to the tick dorsum for the hemolymph to secrete out of the leg (Patton et al., 2012; Velásquez-Serra, 2016). However, the yield of hemolymph extracted per tick is very low.

On the other hand, in the protocol for hemolymph extraction in *R. microplus*, the dorsal tick surface is perforated with a glass microtube and then gently pressed to allow hemolymph extravasation. This technique requires constant pressure on the tick body, resulting in the rupture of internal tissues, including the midgut, thus leading to contamination of the hemolymph with blood ingested from the host (Sousa et al., 2013).

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Additionally, one of the main impediments to obtaining hemolymph is the rapidity with which clotting occurs because when the cuticle is damaged, the molecules involved in the clotting block the incision site, and coagulation occurs in the extravasated hemolymph (Blisnick et al., 2017). So far, the importance of hemolymph in various physiological processes of ticks is known, so the study of resistance to acaricides, physiology, immune response, and performing multi-omics approaches requires obtaining uncontaminated and high-quality hemolymph.

Undoubtedly, the constant improvement of protocols leads to obtaining samples with higher purity and high yields that allow the development of techniques with more accurate results, contributing to a better understanding of the pathogen-vector interaction. Here, we report a newly optimized method for hemolymph extraction for *R. microplus*, with a broad specter of applications. It is an easy to perform, fast, and high yield technique (up to 100 μl) for obtaining high-quality hemolymph free of host blood.

2. Materials and methods

2.1. Protocol for tick preparation

(i) Rinse engorged and semi-engorged ticks with distilled water to remove remnants and excess skin or bovine hair.
(ii) Wash 3 times by immersion in 10% benzalkonium chloride (BZK) for 10 min at room temperature (25 °C). Discard excess BZK and rinse 3 times with sterile distilled water, covering the entire surface of the ticks. Optional: Using cold water and solutions (4 °C) during tick preparation can optimize hemolymph integrity.
(iii) Immediately perform a final wash with sterile distilled water and an antibiotic-antimycotic 100× mixture (10,000 units/ml penicillin, 10,000 μg/ml streptomycin and 25 μg/ml amphotericin B) (Thermo Fisher Scientific, Waltham, USA) at a ratio of 1:100 for 10 min at room temperature (25 °C), followed by two rinses with sterile distilled water. Dry ticks on sterile gauze.

Recommendation: store ticks at 4 °C if the extraction is to be performed at a later stage. Prior incubations at 4 °C overnight may increase the extraction yield of hemocyte cells by 30–40%, possibly due to detachment of circulating cells in the hemolymph. Additionally, keeping ticks at 4 °C for up to 9 days has been reported to prevent oviposition in *R. microplus* (Oshiro et al., 2021).

After tick preparation, proceed directly to hemolymph extraction.

2.2. Protocol for hemolymph extraction

(i) Wash ticks with 70% ethanol for 5 min at room temperature (25 °C) and leave to dry on sterile gauze (Fig. 1A). Note: For a sterile environment, perform the extraction in a laminar flow hood or in the presence of Fisher burners.
(ii) Place 20–100 μl (according to the tick size) of cold (4 °C) sterile anticoagulant citrate buffer in a 0.5 ml microtube. Note: According to the tick size, an average of 50,000 hemocytes/female can be obtained, as we observed from the quantification of the hemolymph collected from 100 engorged females (approximately 400 mg) (5 replicates). Anticoagulant citrate buffer preparation: 20.8 g/l anhydrous α-D-glucose (Sigma-Aldrich, Darmstadt, Germany); 8 g/l sodium citrate (Sigma-Aldrich); 3.36 g/l EDTA-dehydrate (Merck Millipore), 23 g/l sodium chloride (Sigma-Aldrich), pH 7.4. Sterilize by filtration and keep on ice.
(iii) Under the stereoscopic microscope, hold the tick at the lateral sides with a sterile dissection forceps (preferably curved or flat-tip forceps) and place 10–15 μl of cold sterile anticoagulant citrate buffer on the tick dorsal region (anterior-posterior direction) (Fig. 1B and C).
(iv) Critical step: perform one to seven continuous hipocuticular punctures with a 25 × 16 mm sterile needle over the dorsal region (anterior-posterior direction) where the anticoagulant citrate buffer drop was placed (Fig. 1D). Recommendation: hipocuticular punctures must be performed in the hemocele at an angle of about 45° to the tick's dorsum avoiding damage to the tick organs. The quality and quantity of the sample obtained depends on the accuracy of the puncture.
(v) After each hipocuticular puncture, press the lateral tick sides gently with the forceps to induce the extravasation of the hemolymph through the cuticle. Press gently as many times as necessary to recover the hemolymph (Fig. 1E) or make new punctures in different areas of the dorsal region (anterior-posterior direction) (Fig. 2), and repeat the extraction as described above.
(vi) Collect the uncontaminated hemolymph with a micropipette (Fig. 1F) and transfer it to 1.5 ml microtubes with 20–100 μl (according to the tick size) of cold anticoagulant citrate buffer and keep on ice. Mix gently by pipetting (Video S1). Note: The volume of the anticoagulant citrate buffer used depends on the tick size and the amount of the extracted hemolymph. Optional: We suggest immediate proceeding to hemolymph separation (pellet and plasma) or freezing at −20 °C until use, depending on the final applications.

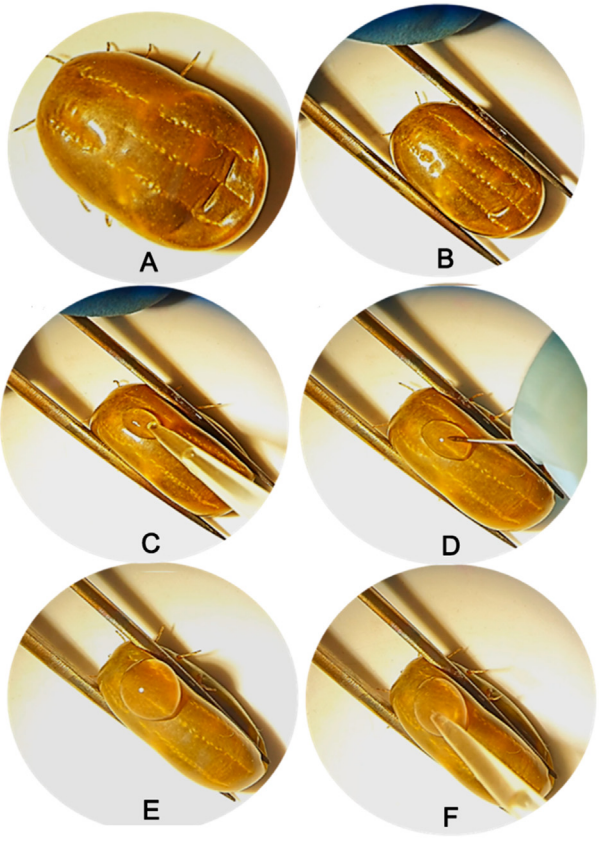

Fig. 1. High-quality hemolymph extraction with the newly optimized protocol. **A** Engorged tick previously washed with 10% BZK, 70% ethanol and dried on sterile gauze. **B** Manipulation of the tick must be carried out gently by the lateral sides with sterile dissection flat-tip forceps to avoid damage to inner tissues. **C** To avoid extravasated hemolymph coagulation, place a drop of citrate buffer on the dorsal tick region where the punctures will be made. **D** Punctures must be performed continuously with a sterile needle over the tick dorsal region at 45°. **E** Hemolymph extravasation is induced by gentle pressure on the lateral sides of the tick. **F** Collection of the extravasated hemolymph with a micropipette. *Abbreviation*: BZK, benzalkonium chloride.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.crpvbd.2021.100066.

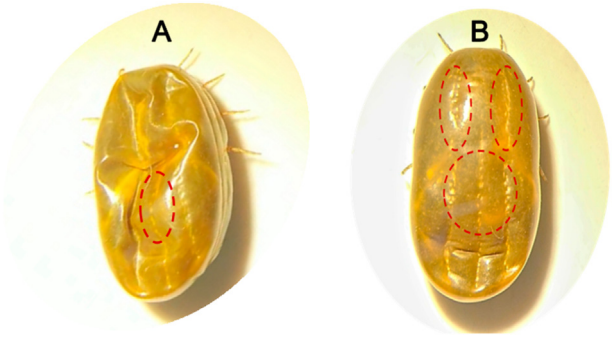

Fig. 2. Schematic representation of the recommended regions (dotted red circles) to perform the hipocuticular punctures based on the tick dorsal plane and amount of blood ingested. **A** Semi-engorged tick. **B** Engorged tick.

2.3. Protocol for hemolymph separation

(i) Centrifuge the collected hemolymph at 500× *g* for 3 min at 4 °C (Fiorotti et al., 2018). The supernatant (plasma) is recovered in a sterile 1.5 ml microtube and stored at −20 °C. Optional: Before freezing, adding protease inhibitors (Complete, Roche) may help preserve the supernatant components (plasma).

(ii) Resuspend the obtained pellet (hemocytes) in 100 µl of anticoagulant citrate buffer and wash twice by centrifugation at 500× *g* for 1 min at room temperature. Optional: The obtained hemocytes can be maintained for up to one month at 30 °C in 37% MEM (Eagle's Minimum Essential Medium, Sigma-Aldrich), 37% L15 (Leibovitz's Medium, Thermo Fisher Scientific), 10% tryptose phosphate broth (Thermo Fisher Scientific), supplemented with 15% fetal bovine serum (Thermo Fisher Scientific), and 1% antibiotic-antimycotic mixture (100×, Thermo Fisher Scientific) (Cobaxin-Cardenas et al., 2019).

2.4. Quantification and staining of hemocytes

(i) Use a clean pipette tip to transfer 10 µl of hemocytes on the hemocytometer.

(ii) Using a compound microscope, count the four corner squares and the central square of the hemocytometer, record the number of hemocytes and calculate concentrations as follows: Cell concentration = Total no. of cells counted/No. of squares × 10,000 (Bastidas, 2015). Optional: Perform a vital staining assay with conventional Trypan Blue 1:1. Optional: Perform a vital qualitative assay with CFDA (5(6)-carboxyfluorescein diacetate, Thermo Fisher Scientific). Briefly, resuspend 1 × 10^6 hemocytes in 50 µl of anticoagulant citrate buffer and add 1 µg/µl of CFDA solution. Resuspend and incubate at 37 °C for 15 min at room temperature with gentle agitation. (Stock solution: 10 µM CFDA in DMSO). Wash twice by centrifugation with anticoagulant citrate buffer. Observe under a wide-field fluorescence microscope with a FITC filter (Fig. 3).

3. Results and discussion

Tick-borne diseases are an object of study due to the harm caused to humans and animals. In cattle, *R. microplus* causes severe infestations with significant economic losses worldwide, increasing the risk of selecting acaricide-resistant strains. So far, the information about the transmission, vectorial capacity, tick immune response, and chemical resistance, among other processes, is still scarce, and the unveiled components and molecules that participate represent a significant field to explore (Aguilar-Díaz et al., 2018). In this regard, hemolymph plays a central role in several biological processes related to tick-borne pathogen transmission and tick survival. Although hemolymph extraction methods exist, most of them do not show significant yields, and contamination of the sample is recurrent (Angelo et al., 2010; Patton et al., 2012; Velásquez-Serra, 2016; Fiorotti et al., 2018).

The present protocol is fast and with a broad application to fully engorged and partially engorged ticks. With this protocol, we have obtained up to 100 µl of hemolymph per engorged female tick with a weight of up to 400 mg, representing approximately 50,000 hemocytes per individual (see *Section* 2.2, (ii) of the protocol). On the other hand, based on tick weight, we suggest that the yield of extracted hemolymph in partially engorged ticks is slightly lower than in engorged ticks. We must highlight that in our experience, the engorged ticks obtained from bovines under controlled conditions have a higher weight than ticks from naturally infested bovines. In this regard, based on our observations, the extractions from naturally obtained ticks require a greater number of individuals to obtain up to 100 µl of hemolymph.

The samples of hemolymph extracted with our protocol are high-quality, i.e. bovine blood-free hemolymph, ready to be used for several purposes such as the study of tick physiology, multi-omic approaches, cellular and humoral immune responses, among others (Kopácek et al., 2010; Stopforth et al., 2010; Aguilar-Díaz et al., 2018). The results obtained with our protocol show it is an efficient method for extracting hemolymph free of host blood components, with higher yields than reported with other methods (Angelo et al., 2010; Patton et al., 2012; Velásquez-Serra, 2016; Fiorotti et al., 2018).

The present technique consists of a series of continuous hipocuticular punctures on the dorsal region of the tick with a needle and involves pressure with forceps to induce hemolymph extravasation. Additionally, we know that ticks use clotting hemolymph to reduce tissue damage (Blisnick et al., 2017). Therefore, it is essential to consider using the anticoagulant buffer that prevents immediate

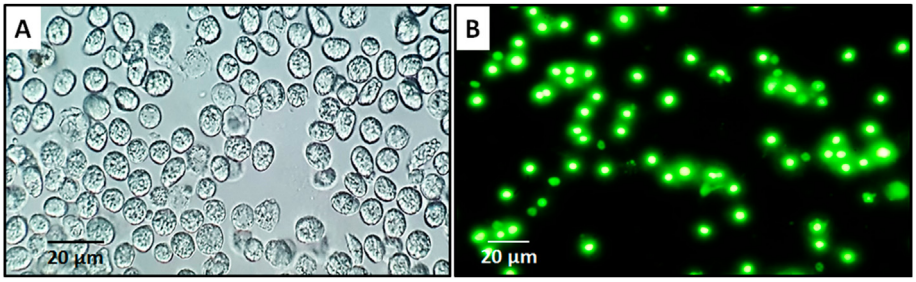

Fig. 3. Assessment of the quality and viability of two different hemocyte samples obtained from extravasated hemolymph. **A** Sample 1, bright field micrograph (light microscopy) of intact and viable hemocytes (40×). **B** Sample 2, confocal micrograph (fluorescence microscopy) of hemocytes stained with CFDA. Fluorescent hemocytes indicate viable cells (40×).

coagulation of the hemolymph, maintaining its continuous extravasation and promoting a higher yield. The use of an anticoagulant in the extraction buffer immediately after the puncture favors a higher quality and volume of extracted hemolymph. This extra-component is not used in the same way in other reported techniques for hemolymph extraction (Sousa et al., 2013).

An essential advantage of the method described here is that several punctures can be made at different dorsal regions to achieve a more significant collection (Fig. 2). It is important to note that punctures should be made at an angle of about 45° to the tick dorsum since vertical punctures increase the risk of passing through the cuticle and hemocele and damaging internal organs thus contaminating the sample. Using this improved protocol, we obtained up to 100 μl of hemolymph. Additionally, this technique provides whole hemocytes that can be maintained in culture for cellular and molecular studies. In our experience, we have maintained viable hemocytes for up to one month under the conditions reported in the protocol. On the other hand, we point out that tick longevity may affect the extraction and the quality of the hemolymph. A long-lived tick has a solid and rigid cuticle that increases the risk of perforating the inner organs; additionally, we have obtained a smaller amount of hemolymph from longer-lived ticks.

The study of ticks is limited to obtaining biological samples; in this regard, obtaining high-quality hemolymph is essential for developing molecular techniques that require samples of bovine blood-free hemolymph. Although buffer components such as EDTA do not interfere in some molecular approaches, EDTA can be eliminated from the sample during extraction if there is a particular interest in the nucleic acids from hemocytes.

4. Conclusion

The development of new technologies that allow obtaining hemolymph free of host blood components opens the possibility of optimizing molecular and immunological protocols for studying ticks. In addition to the feasibility and simplicity of the method we present, this protocol allows obtaining higher yields and high-quality samples than previously reported methods suited for *R. microplus*.

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Ethical approval

Not applicable.

CRediT author statement

Hugo Aguilar-Díaz: conceptualization, project administration, funding acquisition, writing - original draft, writing - review & editing. Rosa Estela Quiroz-Castañeda: validation, writing - original draft, writing - review & editing. Karina Salazar-Morales: methodology, visualization, writing - review & editing. Estefan Miranda-Miranda: design of the study, writing - review & editing. All authors read and approved the final manuscript.

Data availability

Raw data are available from the corresponding author upon reasonable request.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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