Video Article

Saliva, Salivary Gland, and Hemolymph Collection from *Ixodes scapularis* Ticks

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Abstract

Ticks are found worldwide and afflict humans with many tick-borne illnesses. Ticks are vectors for pathogens that cause Lyme disease and tick-borne relapsing fever (*Borrelia* spp.), Rocky Mountain Spotted fever (*Rickettsia rickettsii*), ehrlichiosis (*Ehrlichia chaffeensis* and *E. equi*), anaplasmosis (*Anaplasma phagocytophilum*), encephalitis (tick-borne encephalitis virus), babesiosis (*Babesia* spp.), Colorado tick fever (Coltivirus), and tularemia (*Francisella tularensis*) [1-8]. To be properly transmitted into the host these infectious agents differentially regulate gene expression, interact with tick proteins, and migrate through the tick [3,9-13]. For example, the Lyme disease agent, *Borrelia burgdorferi*, adapts through differential gene expression to the feast and famine stages of the tick's enzootic cycle [14,15]. Furthermore, as an *Ixodes* tick consumes a bloodmeal *Borrelia* replicate and migrate from the midgut into the hemocoel, where they travel to the salivary glands and are transmitted into the host with the expelled saliva [9,16-19].

As a tick feeds the host typically responds with a strong hemostatic and innate immune response [11,13,20-22]. Despite these host responses, *I. scapularis* can feed for several days because tick saliva contains proteins that are immunomodulatory, lytic agents, anticoagulants, and fibrinolysins to aid the tick feeding [3,11,20,21,23]. The immunomodulatory activities possessed by tick saliva or salivary gland extract (SGE) facilitate transmission, proliferation, and dissemination of numerous tick-borne pathogens [3,20,24-27]. To further understand how tick-borne infectious agents cause disease it is essential to dissect actively feeding ticks and collect tick saliva. This video protocol demonstrates dissection techniques for the collection of hemolymph and the removal of salivary glands from actively feeding *I. scapularis* nymphs after 48 and 72 hours post mouse placement. We also demonstrate saliva collection from an adult female *I. scapularis* tick.

Video Link

The video component of this article can be found at http://www.jove.com/video/3894/

Protocol

1. Hemolymph collection for slide preparation

(Movie 1)

1. Gently remove actively feeding ticks from an animal and place in 3% topical hydrogen peroxide for 5 minutes and then in 70% ethanol for 10 minutes to surface sterilize.
2. With a pap pen draw a circle onto a silane coated microscope slide and place the tick within the pap pen circle.
    1. Silane coated slides are used for best adherence of the hemolymph to the microscope slide.
3. View the tick under a dissecting microscope (1X objective, 10X eyepiece, 3.5X magnification).
4. Gently push down on the tick's dorsum with forceps to splay the tick's legs and immobilize the tick.

Note: When immobilizing the tick do not press too hard because this may disrupt the midgut or puncture the tick and contaminate the hemolymph.

5. Amputate the tick's leg or legs at the distal joint with a fine point disposable scalpel. To determine infectivity via hemolymph only 1 leg needs to be amputated. For collection of hemolymph on a slide several legs may be amputated.

Note: Do not cut the leg too close to the body because this may cause midgut contamination of the hemolymph.

6. After the leg or legs are cut continue to gently apply pressure to the tick's dorsum for the hemolymph to secrete out of the legs onto the slide. Gently move the tick around on the slide to spread the hemolymph.

2. Salivary gland removal

(Movies 2 & 3)

1. Spot several 25 µl pools of phosphate buffered saline (PBS) onto a microscope slide and place a tick into one of the PBS pools.
2. View the tick under a dissecting microscope (1X objective, 10X eyepiece, 3.5X magnification).
3. Stabilize the tick with fine tipped forceps by holding the basis capitulum (mouth parts) or the rear of the tick.
4. Insert the fine tipped forceps into the rear of the tick and slice up the tick's dorsum to expose the organs. If desired the midgut can be removed at this time and transferred to a fresh pool of PBS on a microscope slide or to a microfuge tube containing PBS.
5. Find the pair of salivary glands (grape-like clusters) located bilaterally alongside the legs of the tick. If the salivary glands are not visible amongst the tick debris move the main tick portion, still containing the salivary glands, to a fresh pool of PBS to reduce the disruption and loss of the salivary glands.

Note: Earlier in a feeding, the salivary glands are harder to locate because they are not as developed as compared to later on in the feeding.

6. Remove the salivary glands from the tick with fine tipped forceps and place in a fresh pool of PBS.
7. With fine tipped forceps transfer the salivary glands to another clean 25 µl PBS pool, repeat this wash step 3-4 more times to remove any external microorganisms and tick debris.

Note: Wash the salivary gland clusters gently to reduce the loss and disruption of individual salivary glands.

8. Place the glands into a clean pool of PBS on a silane coated slide or in a microfuge tube containing PBS.

3. Saliva collection

(Movie 4)

1. Gently remove adult female ticks from the rabbit or other host using fine tipped forceps just before they drop off fully engorged, approximately 5-7 days post-attachment.
2. Adhere the nearly engorged tick onto one end of a microscope slide with scotch tape. The tape should be placed approximately ¾ of the way up the tick's dorsum toward the head, leaving the tick's basis capitulum (mouth parts) exposed.
3. Where the tape meets the anterior edge of the ticks dorsal surface pipet 5 µl of 5% pilocarpine solution (in methanol). Allow the tape to wick the pilocarpine over the tick's dorsum, without allowing the pilocarpine to come in contact with the tick's basis capitulum.
4. Mount a piece of non-toxic modeling clay onto the microscope slide approximately one inch from the tick's mouthparts.
5. Using fine tipped forceps break off the tip of a pulled capillary tube[28] to the desired diameter.
6. View the tick's basis capitulum under a dissecting microscope.
7. Gently fit the tick's hypostome into the pulled capillary tube allowing the maxillary palps to reside on the outside of the capillary tube.
8. Press the opposite end of the capillary tube into the modeling clay to hold the capillary tube in place.
9. Place the mounted salivating tick inside a dark chamber with high humidity (e.g., a lidded styrofoam box lined with wet paper towels). Tilt the slide so the hypostome points to the bottom of the container, allowing gravity to aid in saliva collection.
10. Place the container at room temperature.
11. Closely monitor the salivating ticks for the first hour, and collect saliva as it is generated by expelling it out of the capillary tubes with a Pasteur pipet bulb. After the first hour, check accumulation every hour for at least 4 hours. Continue to collect the saliva as it is generated.

Note: Saliva acquisition can be stopped once enough saliva is collected for the study being performed.

12. If the ticks are not salivating or if more saliva is required, salivation can occasionally be induced by using the capillary tube to massage the hypostome.
13. Add 0.1 volumes of protease inhibitor cocktail to the saliva and store at -80°C until needed.

4. Representative Results

Movie 1 demonstrates how to hold a partially fed *I. scapularis* nymph and amputate the legs to collect hemolymph onto a microscope slide. Once the leg or legs are amputated a clear fluid is secreted (figure 1A and 1B). If the midgut is ruptured the hemolymph appears cloudy as it is comes out of the amputated leg(s) (figure 1C and 1D).

The extraction of salivary glands after the nymph has been feeding for 48 or 72 hours is demonstrated in movies 2 and 3. After the tick is punctured there is generally a lot of debris (consisting of trachea, malpighian tubules, blood, connective tissue etc.), to prevent the loss or disruption of the salivary glands move the tick to a fresh pool of PBS. Figures 2A and 2B show where the salivary glands are located after the nymph has been cut open and figure 2C shows removed salivary gland clusters in a pool of PBS.

Saliva collection set up from *I. scapularis* adult females is shown in movie 4 and figure 3. A tick salivating into a capillary tube is observed in movie 5. This method of saliva collection used pilocarpine to stimulate salivation and can yield over 20 µl of saliva per adult female tick.

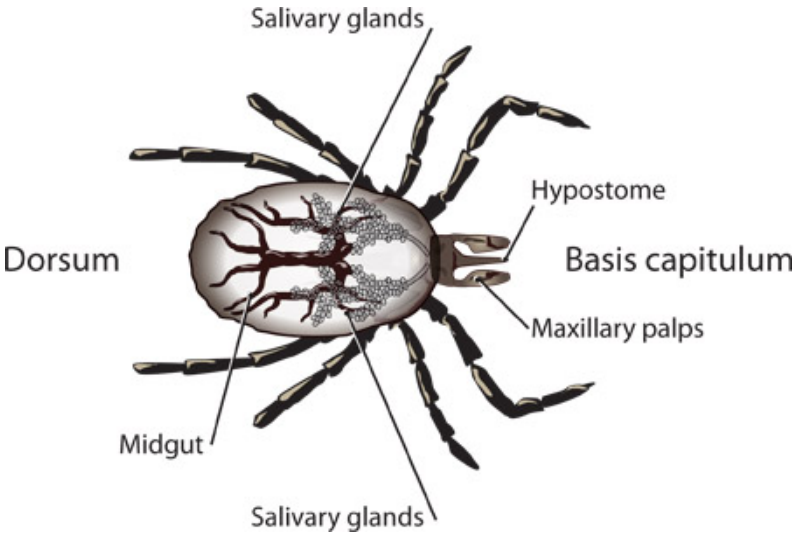

Figure 1. Labeled structures of an I. scapularis nymph.

Movie 1. *Ixodes scapularis* hemolymph collection. Click here to watch movie.

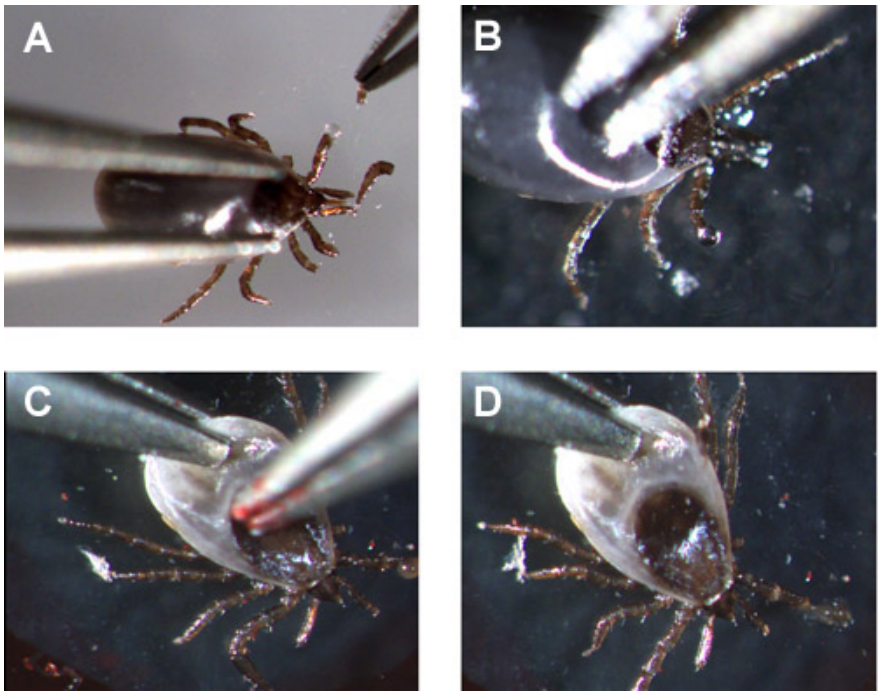

Figure 2. Uncontaminated (A & B) and contaminated (C & D) hemolymph exuding from the nymph's leg.

Movie 2. Salivary gland extraction from a 48 hour fed *I. scapularis* nymph.Click here to watch movie.

Movie 3. Salivary gland extraction from a 72 hour fed *I. scapularis* nymph. Click here to watch movie.

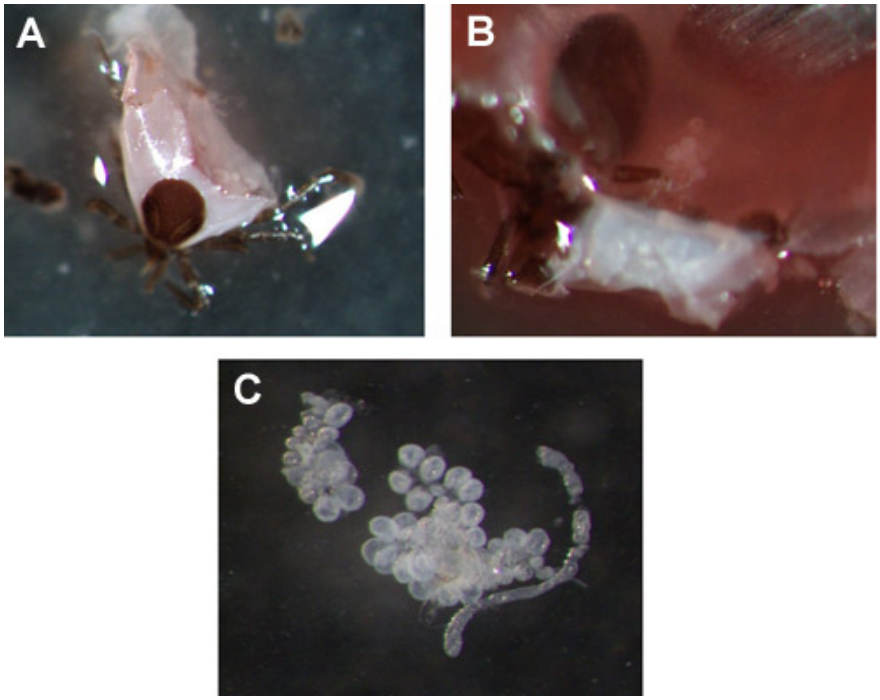

Figure 3. *Ixodes scapularis* nymph salivary glands. (A & B) Examples of salivary glands in a 72 hour fed nymph, prior to extraction. (C) Removed salivary gland cluster.

Movie 4. Saliva collection set up from an adult *I. scapularis* female tick. Click here to watch movie.

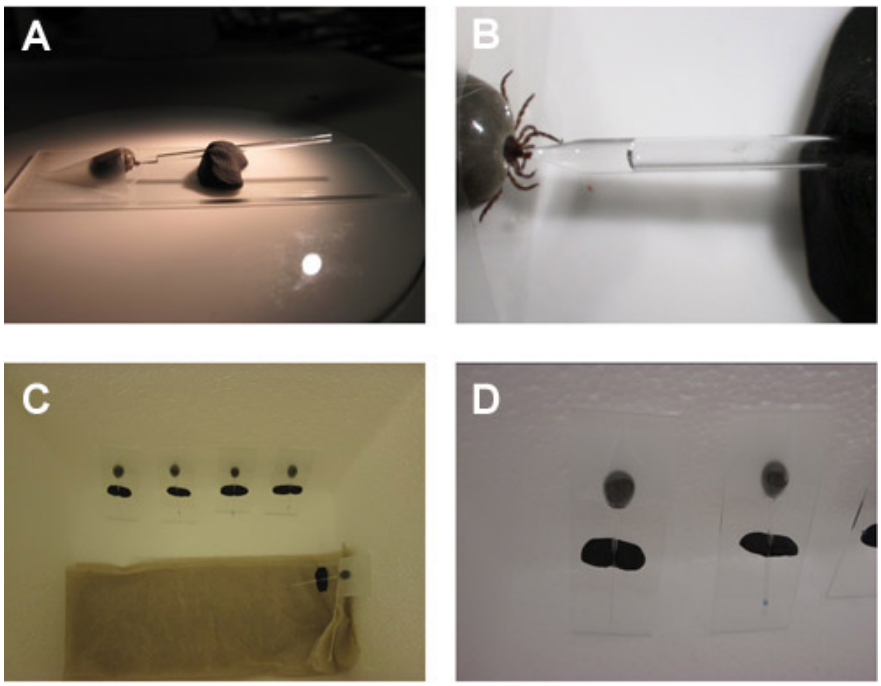

Figure 4. Saliva collection from adult *I. scapularis* female ticks. (A & B) Tick mounted on a slide with its hypostome in the pulled end of the capillary tube with the unpulled end of the capillary tube held by modeling clay. (C & D) Humidified chamber containing salivating adult *I. scapularis* female ticks.

Movie 5. *Ixodes scapularis* female tick salivating into a capillary tube. Click here to watch movie.

Discussion

The collection of tick hemolymph, salivary glands, and saliva is important in the study of tick-borne pathogen transmission, prevalence, dissemination, proliferation, and persistence in both the tick and the host [6,11-13,20,23,29]. There are several ways to dissect a tick[30,31]. However, when collecting salivary glands it is critical to dissect the tick properly so the salivary glands are not ruptured or lost in the tick's remains. Once the salivary glands are removed from the tick they need to be washed several times to remove midgut contamination and then can be fixed onto a slide for staining or ground in PBS to obtain salivary gland extract (SGE). SGE is easier to collect than saliva and possesses attributes similar to saliva; therefore, it can be used as a saliva alternative. However, SGE contains additional proteins originating from the salivary gland cells that are not present in tick saliva. The addition of extra proteins in SGE can be an advantage or a disadvantage depending on the study being performed, but is something a researcher needs to be aware of when working with SGE. SGE does have the advantage that pilocarpine is not used during the collection of salivary glands. Pilocarpine, a muscarinic cholinomimetic agent that acts as an agonist of salvation, has been shown to have a cytotoxic effect on *B. burgdorferi* during saliva collection [32,33]. Dopamine is another agonist of salivation but is rapidly destroyed by the hemolymph and other tick fluids, as compared to pilocarpine that has a sustained effect[33]. Other stimulatory techniques have been explored for saliva collection and all were shown to affect the salivary composition [34].

Hemolymph collection can be difficult because the ticks are moving and it can be problematic to immobilize them without rupturing the midgut. Immobilizing the tick with double sided tape is an option, but the hemolymph can be lost onto the tape if the tick is not removed. Other studies have collected hemolymph from several ticks by cutting the tick's legs at the distal joints and using centrifugation to collect the hemolymph [16,35]. Once hemolymph collection is mastered the hemolymph can be used to determine tick infectivity, tick to pathogen interactions, and the migration of pathogens from the midgut to the salivary glands.

Although our laboratory's focus is on *B. burgdorferi* and *I. scapularis* ticks, the techniques mentioned in this protocol can be used to study other tick-borne infectious agents and tick species. Furthermore, these techniques can also be used on nymphs or adults with relative ease. Using these techniques with larva could be challenging due to their size. Until the dissection technique is mastered it is suggested to use uninfected ticks. It is also important to exercise the proper biosafety measures when dissecting infected or field collected ticks. The methods in this video protocol can be used as guidelines on how to carry out tick dissections and what to look for when performing these techniques.

Disclosures

We have nothing to disclose.

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