

Video Article

Feeding of Ticks on Animals for Transmission and Xenodiagnosis in Lyme Disease Research

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Abstract

Transmission of the etiologic agent of Lyme disease, *Borrelia burgdorferi*, occurs by the attachment and blood feeding of *Ixodes* species ticks on mammalian hosts. In nature, this zoonotic bacterial pathogen may use a variety of reservoir hosts, but the white-footed mouse (*Peromyscus leucopus*) is the primary reservoir for larval and nymphal ticks in North America. Humans are incidental hosts most frequently infected with *B. burgdorferi* by the bite of ticks in the nymphal stage. *B. burgdorferi* adapts to its hosts throughout the enzootic cycle, so the ability to explore the functions of these spirochetes and their effects on mammalian hosts requires the use of tick feeding. In addition, the technique of xenodiagnosis (using the natural vector for detection and recovery of an infectious agent) has been useful in studies of cryptic infection. In order to obtain nymphal ticks that harbor *B. burgdorferi*, ticks are fed live spirochetes in culture through capillary tubes. Two animal models, mice and nonhuman primates, are most commonly used for Lyme disease studies involving tick feeding. We demonstrate the methods by which these ticks can be fed upon, and recovered from animals for either infection or xenodiagnosis.

Video Link

The video component of this article can be found at <https://www.jove.com/video/50617/>

Introduction

In 2011, Lyme disease was the 6th most common Nationally Notifiable disease in North America (<http://www.cdc.gov/lyme/stats/index.html>). *B. burgdorferi* is a versatile microbe, both genetically and antigenically (reviewed in ¹). Its genetic constitution includes a large (>900 kB) chromosome and up to 21 plasmids (12 linear, 9 circular), with plasmid content varying among isolates. Much is to be learned about this spirochete, as over 90% of the plasmid open reading frames are unrelated to any known bacterial sequences ^{2,3}. *B. burgdorferi* presents a wide variety of antigens as potential targets of host immunity. However, an untreated infection often persists. The interaction of spirochetes with the tick milieu and the vertebrate host environment necessitates adaptation by *B. burgdorferi* throughout the infection process. Several plasmid-encoded genes are known to be differentially expressed in response to changes in temperature, pH, cell density and even stage of the tick life cycle ⁴⁻⁸.

The study of *B. burgdorferi* adaptation throughout its enzootic cycle, and host responses following infection by the natural route relies on the ability to feed ticks on appropriate animal models. Such studies are met with the technical challenges of generating ticks that harbor *B. burgdorferi*, and ensuring the efficient transmission and/or feeding of ticks on the model host. In addition, the containment and recovery of infected ticks is essential. Among the models used are mice and nonhuman primates, each of which serves as a valuable tool in Lyme disease research. As with the white-footed mouse, which is a natural reservoir host for *B. burgdorferi*, the laboratory mouse is a highly susceptible host that supports persistent infection by *B. burgdorferi* ⁹. Following infection of disease-susceptible mice, such as the C3H strain, the spirochetes disseminate to multiple tissues, including the skin, bladder, muscles, joints and heart. Inflammatory responses to the infection lead to diseased heart and joint tissue. While the spirochetes persist in this host and remain infectious, inflammatory lesions may become intermittent, not unlike the process in humans. The mouse model has thus provided much information on *B. burgdorferi*-induced pathology, including arthritis and carditis and host immune responses ¹⁰⁻¹². From the perspective of the pathogen, certain genes differentially expressed during mammalian infection have been characterized, as have some necessary for transmission from the tick vector ¹³⁻²¹.

Though several animal species have been used to study Lyme disease ²², rhesus macaques most closely mimic the multi-organ character of human disease ²³. Unlike other animal models, the breadth of disease manifestations such as erythema migrans, carditis, arthritis, and neuropathy of the peripheral and central nervous systems are observed in macaques. In mice, the reservoir host for *B. burgdorferi*, disease varies by mouse strain and age ²⁴, while the early and late-disseminated manifestations are uncommon ⁹. In addition, other rodents, lagomorphs, and canines all fail to exhibit neurological disease from *B. burgdorferi* infection ²⁵. Importantly, macaques exhibit signs that are characteristic of all three phases of Lyme borreliosis, namely, early-localized, early-disseminated, and late-stage Lyme disease ²⁶⁻²⁸. Erythema migrans (EM) is thought to occur in 70-80% of human cases ²⁹, and is also seen in rhesus macaques ^{28,30}. Following infection, the spirochetes disseminate from

the site of inoculation to multiple organs. Spirochetal DNA has been detected in skeletal muscles, heart, bladder, peripheral nerve and plexus, as well as in the central nervous system (cerebrum, brainstem and cerebellum, spinal cord, and dura mater)³¹.

Tick feeding on mice has been utilized by us and other research teams for propagation of tick colonies, in reservoir competence studies³²⁻³⁶ and in studies of *B. burgdorferi* pathogenesis³⁷⁻⁴⁰. This technique has also been used for xenodiagnosis and testing of vaccine efficacy in mice⁴¹⁻⁴⁴. We have fed *Ixodes* ticks on nonhuman primates for model development²⁸, a study of vaccine efficacy⁴⁵, and for xenodiagnosis in the assessment of persistence post-antibiotic treatment⁴⁶. Ticks that harbor *B. burgdorferi* can be maintained in a natural enzootic cycle by feeding larvae on infected mice and using the nymphs for studies, as the spirochetes are transmitted through the life stages. In this report, we instruct on how to generate ticks infected with wild type or mutant *B. burgdorferi*, using capillary tube-feeding. This can also be accomplished by microinjection⁴⁷ and by immersion⁴⁸. The purpose of artificial introduction of *B. burgdorferi* into ticks can be to study mutant strains whose transmissibility is unknown, to generate a group of ticks with a high infection rate, and to reduce potential for error by maintaining a clean and otherwise uninfected tick colony. In addition, we demonstrate tick feeding on mice and nonhuman primates, so as to assure containment and recovery of replete ticks. The use of tick feeding is essential for future studies of immune responses to *B. burgdorferi* infection, potential Lyme vaccine efficacy, and xenodiagnosis for detection of occult infections.

Protocol

An experimental outline of tick inoculation and feeding upon animals for Lyme disease research is depicted in **Figure 1**.

1. Inoculating Nymphal *Ixodes* Ticks with *B. burgdorferi* Using Capillary Tube-feeding

When performing manipulations with ticks, white lab coats with elastic sleeves, gloves, and disposable bouffant caps are worn.

1. Our technique is a modified version of that reported by Broadwater *et al.*⁴⁹. Prepare capillary tubes by heating and pulling Pasteur pipets to breaking thinness using a pipet puller. Using forceps and dissecting scope, break the tips to the optimal diameter (about 0.2 mm). A tube standardized to the tick mouthpart size is used as a sizing guide. A face shield should be worn when preparing the pipets.
2. Grow *B. burgdorferi* to between $2-8 \times 10^7$ /ml (mid-log phase) in BSK-H medium (Sigma) containing 6% rabbit serum.
3. Use nymphal ticks that have been stored at 23 °C for 4-6 weeks post-larval molt. Place ticks onto a small 60 x 15 mm petri dish with double-sided tape on the outer bottom surface of the dish. Place the ticks ventral-side facing up.
4. Dip the capillary tube tip into the *B. burgdorferi* culture tube after mixing. Place the capillary tube over the hypostome of the tick mouth parts using a dissecting scope. Use molding clay to fix the tube in place, as shown in **Figure 2A**.
5. Place the petri dishes with affixed ticks inside a large clear plastic tub for an added level of containment. Wet paper towels are added to provide moisture. Place the ticks in a 37 °C thermal incubator for 30 min-2 hr until defecation is apparent. This indicates that the media containing spirochetes has passed through the tick.
6. Rest the ticks for 2-4 weeks at 23 °C to allow adaptation to the tick environment before feeding them on animals.

2. Infecting Mice with *B. burgdorferi* by Tick

1. Dilute ketamine stock 1:10 in sterile water. Anesthetize each mouse with 100 mg/kg of ketamine by intraperitoneal injection with a tuberculin syringe.
2. Once the mouse is fully anesthetized, shave the mouse from the ears to the middle back using a fine (Remington smooth & silky) electric trimmer.
3. In a white pan with no other objects nearby, transfer the nymphal ticks (that harbor *B. burgdorferi*) by a moistened paintbrush to the hairless area of the mouse. Alternatively, uninfected ticks can be placed on mice for xenodiagnosis of mice with suspect infection. The use of a clean, white surface for tick placement helps to ensure that any unattached ticks will be readily seen.
4. Place the mouse in specialized caging (Allentown Caging, Allentown, PA). The caging consists of a stainless steel grill elevated from the bottom of the cage. The cage top was modified by our in-house machine shop to elevate the water bottle holder enough to allow free movement of the mouse underneath. The pan is filled with about ½ inch of water to trap any ticks that fall off mice (**Figure 3A**). To minimize the risk of hypothermia, reusable heating pads, microwaved prior to use, are placed underneath the cages until mice wake completely from anesthesia. Animals are often ataxic as they recover from anesthesia and rub against food and water trays, so these must be removed. The water level is low enough to prevent limbs of mice from submersion.
5. Place the cage inside a tray that has been lined with Tangle trap paste (Contech, Victoria, BC, Canada) and tape to ensure the entrapment of arthropods. Mice are caged singly and observed continuously during the period of anesthesia.
6. Within 2 hr, when mice are completely awake from the anesthesia, the food tray and water bottle are replaced to the cage. After 24 hr, the housing enrichment consisting of a plastic hut and nylabone is replaced.
7. After 3, 4, and 5 days, check the mouse, cage and cage water for fed ticks. The cage water is sifted through a white metal pan (*i.e.* "panning for gold"). Rinse fed ticks in clean water and store in plastic jars (**Figure 3B**). On days 3 and 4, replace water in cage with clean water. On day 5, check not only the cage, but the mouse thoroughly for ticks. Usually by this point all ticks have fed and the mice can be returned to regular caging.
8. Place all waste from the mouse cages, including liquids, in biohazard containers for autoclaving and disposal. Keep a log of the number of ticks placed on mice and those recovered at all times.

3. Feeding Ticks on Nonhuman Primates for Infection with *B. burgdorferi* or xenodiagnosis

1. Prepare the tick containment device: Cut a 1 ¾ inch diameter circle in the 3 inch x 3 inch LeFlap (flap) using a clean scalpel and the measurement guide. Use the cut-out as a template to cut circles of identical size in the Biatane foam and Duoderm. The foam is used to

- elevate flap over the surface of the skin and prevent possible crushing of tick. The Duoderm adds another layer of cushioning and overlays the edges of the containment device for added security from tick escape. A diagram of the containment device is depicted in **Figure 4**.
2. Veterinary staff will anesthetize the animal with 5-8 mg/kg Telazol by intramuscular injection.
3. Clip the hair of the animal using electric trimmer (Oster) equipped with size 40 blades. All areas that will be covered by the jacket are clipped: back, front, upper arms. Using shaving cream and dual-blade disposable razors, closely shave an area of approximately 25 cm vertical x 20 cm horizontal. Wipe clean with moist paper towels and blow dry with low heat to dry the skin.
4. Place the flap on the animal's dorsum, just below the scapula, on either side of the spine. Use a marker to trace the circle in that spot. Prepare the area of skin around the circle by wiping it with SkinPrep. This removes oil in skin that could affect adhesion of glue and containment devices. Leaving about a 1 cm circumference of space around the circle, apply a layer of skin glue (SkinBond) with a width of ~4 cm.
5. Remove the adhesive backing from the Biatane foam and affix to the skin in the appropriate spot. Animals are again anesthetized by veterinary staff with 5 mg/kg Telazol. Seal the edges with skin glue and Hypafix tape. Remove the adhesive backing from the flap and affix on top of the Biatane. Place Hypafix tape around the edges of the LeFlap, then tape down the mesh flap of the flap and place the jacket on the animal. Tape and Tangle Trap paste is applied to the floor in a perimeter surrounding the nonhuman primate caging for added security.
6. To minimize the effects of chemicals used in Step 3.4 on tick feeding, the ticks are added 24 hr after the containment device is in place. At this point, the security of the device is also inspected and reinforced if needed. Typically, 20 unfed nymphs (4-8 weeks post-larval molt) are added to the skin within the device using a paintbrush.
7. Remove the adhesive backing from the mesh of the flap and seal it in place. Lastly, remove the Duoderm backing to expose adhesive, and place on top of the containment device. Add a piece of Hypafix tape across the open mesh circle, and replace the jacket. The completed containment device is shown in **Figure 5A**.
8. After 5 days, anesthetize the animals as above and the jackets are removed. Remove the tape first to inspect tick feeding through the mesh (**Figure 5B**). Carefully peel the Duoderm away from the flap.
9. Pull back the mesh portion at the edges to provide access to the ticks. Fed ticks are frequently found near or under the foam circle (**Figure 5C**) and are removed and placed in clean water with a paintbrush. Remove the device once all visible fed ticks are collected (**Figure 5D**).

Note: Oftentimes, the containment device can simply be peeled away from the skin. If adhesion is strong and could potentially damage the skin, Unisolve solvent is applied to the area for gentle removal. The skin is wiped with isopropanol and ticks are stored at 23 °C. If used for infection, the ticks can be crushed to confirm the number that contained *B. burgdorferi*. If used for xenodiagnosis, the ticks are kept for 1-3 weeks prior to analysis of midgut contents.

Representative Results

Following the completion of capillary feeding, the ticks are typically rested at 23 °C for 2-3 weeks before they are fed on animals for transmission. Using the capillary-feeding technique, we have found that over 90% of the fed ticks harbor *B. burgdorferi*. The percentage of positive ticks is determined by washing ticks in peroxide and ethanol, then crushing them in sterile PBS with a microfuge tube-shaped pestle. The midgut contents spilled into the PBS are fixed on slides and stained with an anti-*Borrelia* species antibody that is FITC-conjugated. Representative tick midgut smears viewed by fluorescent microscopy are depicted in **Figure 2B-C**.

Mouse infection rates with low passage strain B31 wild type *B. burgdorferi* are near 100%. A combination of serology and culture of *B. burgdorferi* from mouse tissues is used to determine if each mouse has become infected. A western blot showing serum antibody responses from mice infected with *B. burgdorferi* by tick is shown in **Figure 3C**. This technique has been used to examine the transmissibility and infectivity of *B. burgdorferi* mutant strains³⁷⁻³⁹.

We have used tick feeding on nonhuman primates for infection and for xenodiagnosis. Efforts have been made to improve tick feeding and recovery of fully fed ticks by implementing the flap containment device. The flap product is used for application of medicinal maggots in humans, but we have modified it for tick feeding on primates. In previous studies, we utilized a hard capsule for tick containment^{27,28,45,46} and obtained an average feeding rate (# fed ticks/# ticks added to capsules) of 35.2%, ranging between 23.5-52.5%. In infection studies, rates of transmission (# animals infected/# fed upon) averaged 86.5%. In more recent experiments, the feeding rates using LeFlap have been between 50-90%. On rare occasions, using the previous method, ticks have crawled under the capsule and into the adhesive tape, where they desiccate and die. Using the flap the improved feeding and multiple layers of adhesive have kept the ticks contained.

In addition to direct fluorescent staining of tick midgut preparation (**Figure 2B-C**), more sensitive methods can be used to detect *B. burgdorferi* within ticks. Molecular detection can, and has been used to detect *B. burgdorferi*-specific DNA^{42,50,51} with either standard or quantitative PCR. Common targets for detection are the *flaB*^{46,50}, *OspC*⁴⁶ and *OspA*^{42,51} genes. The viability of recovered spirochetes has also been examined by culture of midgut preparations and feeding xenodiagnostic ticks on naïve mice⁴².

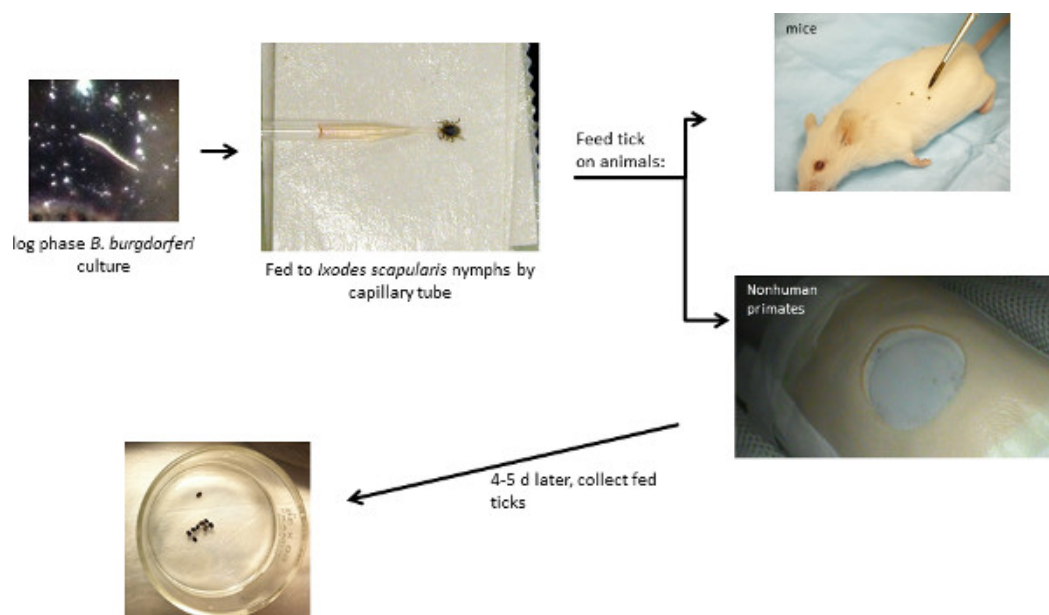


Figure 1. Feeding Ticks on Animals for Transmission of *Borrelia burgdorferi*. Overall scheme of the techniques involved in feeding ticks on animals for Lyme disease studies. Ticks are capillary tube-fed cultures of *B. burgdorferi* and can be fed upon animal models of Lyme disease, such as mice and nonhuman primates (rhesus macaques). [Click here to view larger figure.](#)

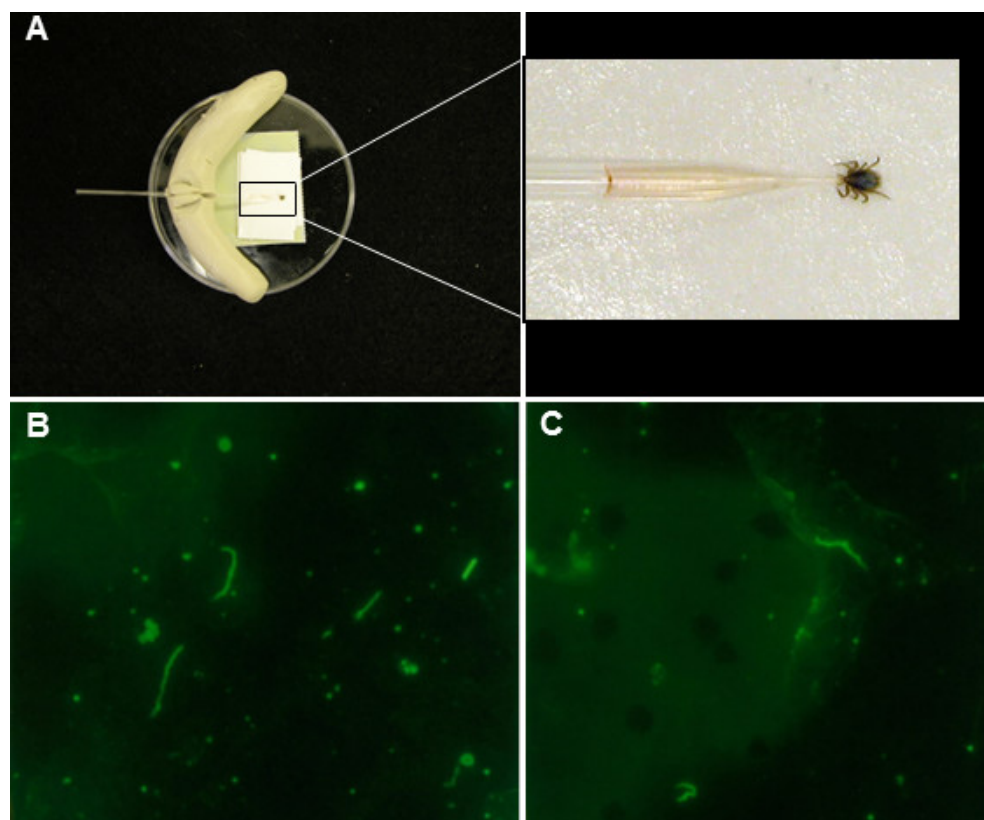


Figure 2. The capillary tube-feeding method and results. **A)** The apparatus used to feed ticks is shown, with a magnified view of the tick and capillary tube on the right. **B-C)** representative images from the midguts of ticks fed *B. burgdorferi*. The midgut smears were stained with anti-*Borrelia* species-FITC polyclonal antibodies (Kirkegaard & Perry Labs) and viewed under fluorescent microscope.

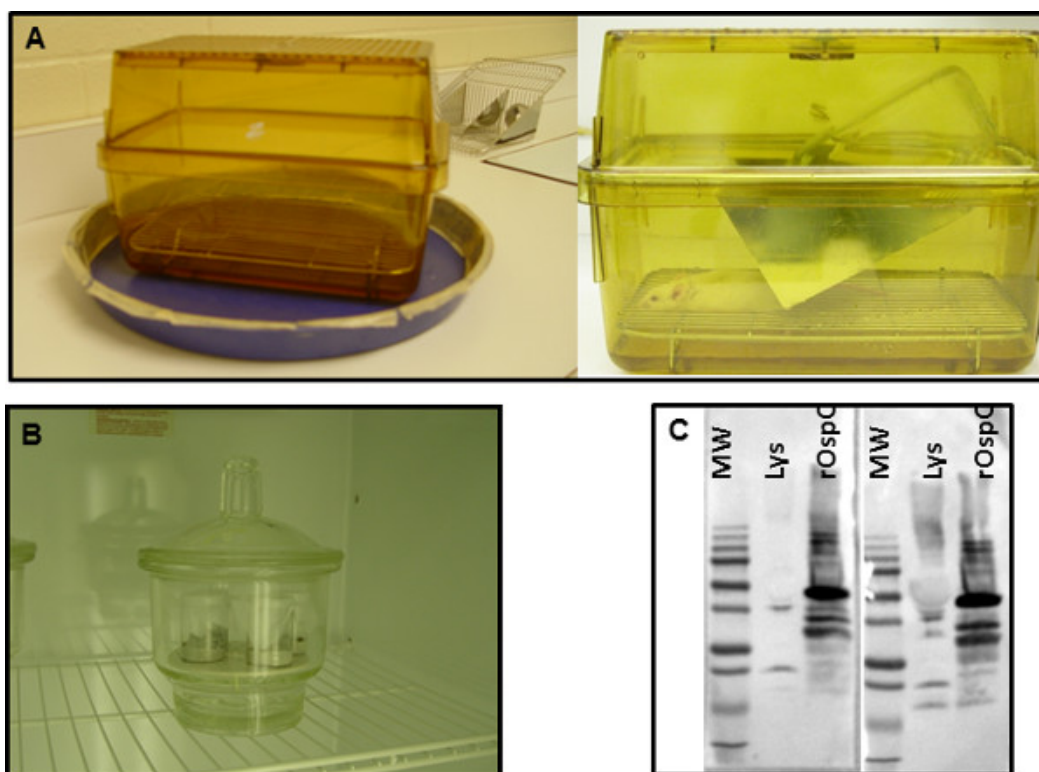


Figure 3. *Ixodes scapularis* tick feeding on laboratory mice. **A)** The specialized caging for mice when used for tick feeding. The wire floor is elevated above a pan of water to collect ticks. An anesthetized mouse is shown in the image on the right. **B)** The storage containers used for ticks. **C)** Representative immunoblots from tick-infected mice. Serum from day 21 post-infection was used to probe blots containing *B. burgdorferi* lysates and recombinant OspC protein, an immunodominant antigen.

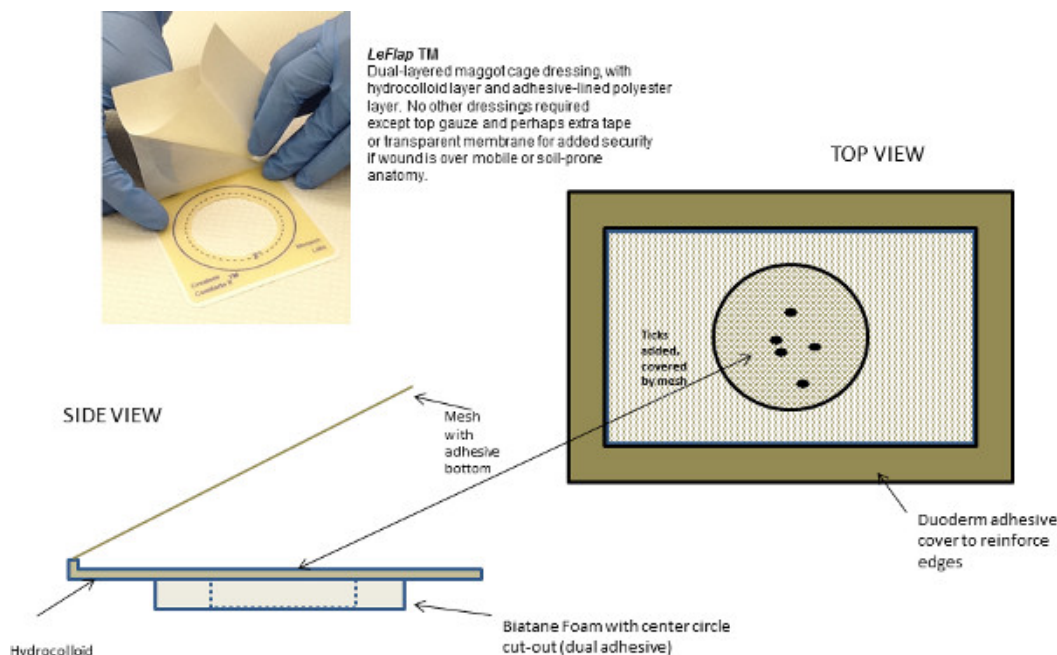


Figure 4. Diagram of the tick containment device used to feed ticks on rhesus macaques. The first layer consists of Biatane foam. The flap is placed on top of the foam and the Duoderm is the third layer. [Click here to view larger figure.](#)

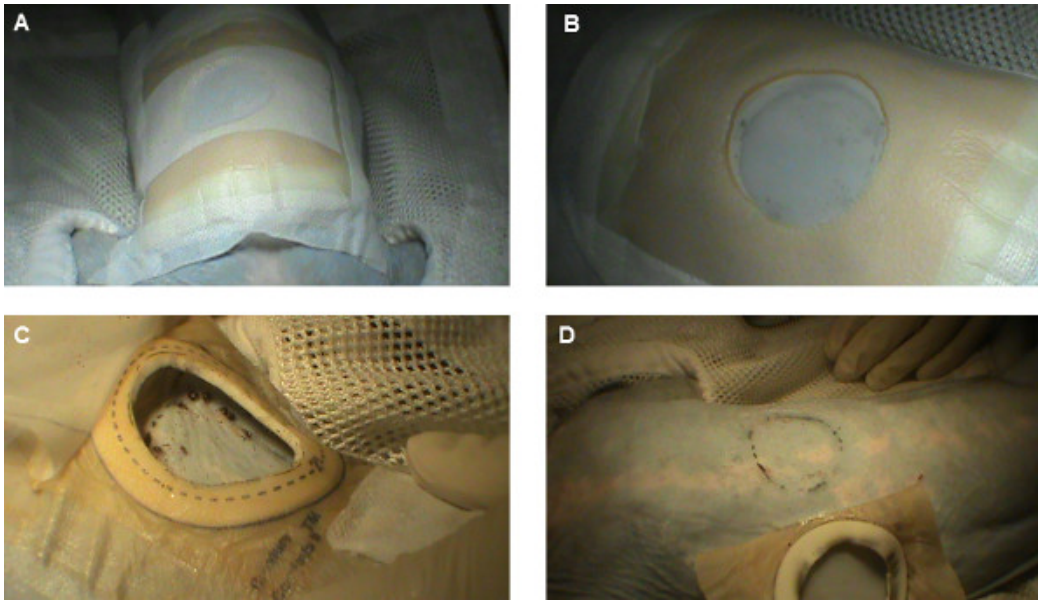


Figure 5. *Ixodes scapularis* tick-feeding on rhesus macaques. **A)** The complete containment device. **B,C)** Views of ticks feeding through the device, and following removal of the flap. **D)** The site of tick feeding following complete removal of the device.

Discussion

In order to obtain ticks that harbor *B. burgdorferi* for downstream studies, the ticks can be: (1) fed on infected mice at the larval stage; (2) immersed in *B. burgdorferi* cultures at either the larval or nymphal stage⁴⁸; (3) microinjected with *B. burgdorferi*⁴⁷; or (4) capillary tube-fed *B. burgdorferi*⁴⁹. While each of these methods has its purpose, for ensuring that a large portion of the ticks to be used for infection harbor *B. burgdorferi*, we favor capillary tube feeding. If inoculation with known quantities of spirochetes is not required, the capillary-feeding methods may have less potential to damage the ticks. This is of importance if they are to be fed on animals. This method may also be preferred if the investigators are testing mutants for transmissibility/infectivity. It is important to recognize that growth in culture can result in plasmid loss⁵², so the use of low passage *B. burgdorferi* is essential. Also, the medium and density of spirochetes is artificial upon introduction, so the ticks should not be used immediately post-capillary feeding. Instead, a period of not less than 2 weeks is allowed for the spirochetes to adapt to the tick microenvironment before use in experiments.

When feeding ticks on mice, it is not necessary to shave the mice beforehand. In a previous study (unpublished) in which we sought to examine the effects of tick saliva on skin, shaving was necessary. In doing so, and counter intuitively, we discovered that the ticks: a) attach readily to hairless skin; b) have a high feeding rate; and c) are easily visible. The feeding rates vary depending on whether larvae or nymphs are used but are consistently above 50% when nymphs are used. As such, shaving mice prior to feeding has become common practice in our laboratory, not only for experiments, but also for propagation of the colony.

Previously, in our Division at the Tulane National Primate Research Center, 34 monkeys have been fed upon by 770 *Ixodes* nymphs in 5 different studies. Tick feeding rates (# fed/# added to capsules) average 35.2%, ranging between 23.5-52.5%. In infection studies, rates of transmission average 86.5%. In a recent pilot study (unpublished), tick feeding rates varied from 5-75% and no resistance to subsequent feeding was apparent. However, the success rates between attempts 2 and 3 varied significantly, where feeding rates were much higher for the 3rd attempt than for the 2nd. The "attempt 2" ticks were housed at ambient temperature longer than the "attempt 3" ticks. The most important factor we have found that affects tick feeding is the tick age and environment pre-experiment. Those kept at 4 °C post-molt until shortly before use generally feed better. As such, we recommend continuously propagating ticks, storing them accordingly and having two separate lots of ticks available when performing feeding on animals.

In our most recent study (unpublished) we compared feeding ticks on macaques using the hard capsule to feeding with the flap device. Ten monkeys were fed upon once with capsules and twice with LeFlap. In this set of experiments, we observed an average feeding rate of 17% (range 5-25%) with capsules and an average rate of 54.75% (range 35-90%) with the flap. We surmise that the broader surface area for tick feeding and the reduced use of harsh adhesives improves feeding. Use of the flap containment also allows the investigators to either let ticks feed longer or add more ticks, as the tick can be removed without removal of the entire device. Lastly, though the adhesives may cause mild skin irritation in some animals (which could affect or induce cutaneous immune responses) the flap device itself may have limited, if any, discomfort for the animals.

Disclosures

The authors declare that they have no competing financial interests.

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