ACTA SCIENTIFIC MICROBIOLOGY (ISSN: 2581-3226)

Volume 3 Issue 9 September 2020

Research Article

Assessment of an Immersion Technique for Generating of *Borrelia burgdorferi*-Infected and *Infectious Ixodes* scapularis and *Ixodes ricinus* Ticks

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Received: July 11, 2020

Published: August 31, 2020

Abstract

Experimental infection of ticks with pathogens such as spirochetes of the genus *Borrelia* (*B.*), is a critical step to better understand the mechanisms and the kinetics of infection. At present, four procedures for infection of ticks with *B. burgdorferi* have been described: (i) feeding ticks on infected rodents; (ii) tick immersion in a solution containing the spirochetes; (iii) microinjection of spirochetes directly into the tick gut and (iv) capillary or membrane feeding of ticks with a solution or blood containing *Borrelia* species. organisms. To reduce the use of live animals and for standardization of the conditions of experiments, the three latter procedures are recommended. The present study aimed to investigate the effectiveness of an immersion procedure to generate *B. burgdorferi*-infected ticks and determine whether *Ixodes scapularis* and *I. ricinus* ticks were infective to dogs. Pathogen free, unfed larvae (*I. scapularis* and *I. ricinus*) and nymphs (*I. scapularis*) were immersed in BSK-H medium containing approximately 10^7 *B. burgdorferi* sensu stricto (strain B31) organisms per mL. Immersed ticks were then fed to repletion on rabbits and held under optimum environmental conditions (22°C and 80 ± 10% relative humidity) for moulting. The infection rate in ticks was determined after moulting by qPCR, while their potential infectivity was evaluated on dogs. It was found that immersed larvae and nymphs acquired spirochetes. The spirochetes were detected by qPCR in 18.7% and 37.5% of adult *I. ricinus* and *I. scapularis*, respectively. For nymphs, *B. burgdorferi*-specific DNA was detected in each of three pools of 20 *I. scapularis*. Nevertheless, all infested dogs remained seronegative during the three months after infestation and no clinical signs of borreliosis were detected.

Keywords: *Borrelia burgdorferi; Ixodes scapularis; Ixodes ricinus; Immersion Technique; Transmission, Dogs*

Abbreviations

ss: Sensu Stricto; LB: Lyme Borreliosis; PBS: Phosphate-Buffered Saline; qPCR: Quantitative Polymerase Chain Reaction; Ct: Cycle Threshold

Introduction

Lyme borreliosis (LB) is caused by spirochetes belonging to the complex of *Borrelia burgdorferi* sensu lato. These agents are trans-

mitted to humans, dogs and other animals by infected *Ixodes* species ticks. In Europe, approximately 40 mammal and bird species are reservoirs/hosts of *B. burgdorferi* [1]. Small rodents, especially *Apodemus* sp. and *Clethrionomys* sp., serve as important hosts for the immature stages of *I. ricinus* and as sources of infection with *B. burgdorferi* [2]. In the eastern USA, the white-footed mouse *Peromyscus leucopus* is considered the primary host and a source of infection for *I. scapularis*. Other small mammals such as shrews and chipmunks are also an important reservoirs [3,4]. Although dogs are not reservoirs, they can be infected by these pathogens. Their susceptibility to the infection with *Borrelia* organisms make dogs relevant epidemiological sentinels [5]. In endemic areas LB represents a potential health threat to dogs. LB in dogs is characterized by non-specific clinical signs such as fever, anorexia, lethargy and lymphadenopathy [6]. More severe signs, such as arthritis with lameness, neurologic disorders, and glomerulonephritis can also be observed in dogs [7,8].

Laboratory generation of *B. burgdorferi*-infected ticks used in tick-host-pathogen interaction studies of LB usually requires an animal model. Mice are used most often, because they are natural biological reservoirs. Decreased use of animals in scientific research is desirable for animal well-being. Alternative *in vitro* procedures would allow more effective standardization of techniques and procedures. To date, *I. scapularis* and *I. ricinus* ticks infected with *B. burgdorferi* were generated using *in vitro* methods such as immersion of ticks in solution containing spirochetes [9,10], microinjection of spirochetes directly into the tick gut [11,12], capillary feeding of ticks with solution containing *B. burgdorferi* [13,14] and feeding on artificial membranes [11,12].

In this study, we investigated the effectiveness of an immersion procedure to generate *I. scapularis* and *I. ricinus* ticks infected with *B. burgdorferi.* We also evaluated the capability of ticks that were infected by this technique to transmit spirochetes to dogs.

Materials and Methods

Ethics approval

The protocol was reviewed and approved by the study site Institutional Animal Care and Use Committee (IACUC) prior to implementation (approval no. MDM-001-19).

Ticks and bacteria

Nymphs and larvae of *I. scapularis* and *I. ricinus* were obtained from a laboratory colony initiated in 2012 using adult specimens collected in the field from Georgia (USA) and Utrecht (the Netherlands). Pathogen-free tick are maintained by routine passage on rabbits for larvae and nymphs, and on sheep for adult ticks. Ticks are incubated at 12°C for storage or 22°C for development and at 80 ± 10% relative humidity. To increase their willingness to feed, ticks were transferred under environmental conditions one week before challenge. A low passage (P3) strain of *B. burgdorferi* ss (strain B31, ATCC 35210) was used to infect ticks. *Borrelia* cultures were maintained in complete BSK-H media at 34°C as described previously [15].

Immersion of ticks in suspensions of *Spirochetes*

The immersion protocol was from Policastro and Schwan [9]. About 800 starved larvae or nymphs of each *Ixodes* species (*I. scapularis* or *I. ricinus*) were introduced into 3-mL tubes containing 2 mL of BSK-H medium with 10^7 *Borrelia* cells per mL. Spirochete cell concentrations were determined by counting spirochetes in defined suspension volumes using a dark-field microscope. Tubes containing ticks were incubated for 2 hours at 34°C and vortexed gently every 15 minutes. After immersion, ticks were rinsed twice with PBS, dried on filter paper and then kept in an incubator until they were fed on specific pathogen-free rabbits. Detached and fully engorged ticks were incubated until they molted to the next stage.

Detection of *B. burgdorferi ss* DNA by polymerase chain reaction

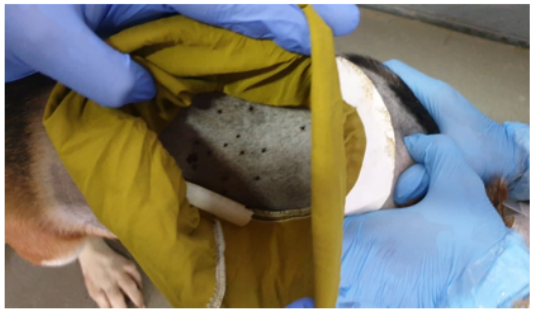
The infection status of *I. scapularis* and *I. ricinus* immersed ticks was determined after moulting by real-time polymerase chain reaction (qPCR) as described previously [16]. DNA was extracted from whole ticks using a NucleoSpin tissue kit according to the manufacturer's recommendations (Macherey-Nagel, Hoerdt, France). Adults were tested individually while nymphs were tested by pooling 20 specimens. DNA-free water and DNA from uninfected ticks were used as negative controls. *Borrelia burgdorferi* DNA were used as positive controls. The samples were considered positive when the threshold cycles (Ct) were inferior to 38.5.

Ability of infected ticks to transmit infections to dogs

Six adult male beagle dogs (aged 2 - 4 years, weighing 10.4 - 13.3 kg) were used: two dogs were infested with nymphal *I. scapularis* (N = 100 per dog), two with 60 (40 females and 20 males) adult *I. scapularis* per dog and two others with 60 (40 females and 20 males) adult *I. ricinus* per dog. Ticks were introduced inside patches previously glued to a dorsolateral area near the ribcage of each dog (Figure 1). Dogs were observed daily for the development of

any signs of pain, lameness, lethargy, or anorexia. Blood was collected from each dog on days 24, 38, 52, 66, 80 and 94 post infestation and tested for *B. burgdorferi*-specific antibodies (SNAP®4DX® Plus, IDEXX Laboratories, Westbrook Maine, USA). The sensitivity and specificity of this assay is 98.8% and 100%, respectively [17].

(a)



(b)

Figure 1: Tick infestation procedure: (a) Patches glued to the dorsolateral region; (b) Infestation with B. burgdorferi-infected I. scapularis/or I. ricinus ticks.

Results

Borrelia burgdorferi-specific DNA was detected successfully in the molted ticks. The infection rate was 18.7% and 37.5% for adult *I. ricinus* and *I. scapularis* ticks, respectively. In the case of *I. scapularis* nymphs, *Borrelia burgdorferi* specific DNA was detected in all three pools of nymphs (Table 1). Of the positive samples, only one pool of *I. scapularis* ticks was moderately loaded with *B. burgdorferi* (Ct value = 26.7 corresponding to 5 x 10^3 spirochetes), while the others were weakly loaded (Ct values ranging between 34.4 and 38.2 (≤ 50 spirochetes).

Tick species	*Ixodes scapularis*		*Ixodes ricinus*
Stage	**Nymphs***	**Adults**	**Adults**
No. tested ticks	3	32	32
No. infected ticks	3	12	6
Infection rate (%)	/	37.5	18.7

***Table 1**:* Borrelia burgdorferi infection of nymphal and adult I. scapularis and I. ricinus ticks by immersion technique (qPCR results).

*: The nymphal stage was tested in pools; each pool contained 20 specimens. So, a total of 60 nymphs were tested.

Adults and nymphal *Borrelia*-exposed ticks attached quickly and fed well on the dogs. The *in-situ* count performed 48 h after tick infestation, demonstrated an attachment rate of 80.8% (97/120) and 64.1% (86/120) for adult *I. scapularis* and *I. ricinus*, respectively. For nymphal *I. scapularis* ticks, the attachment was lower at 44.0% (88/200).

None of the infested dogs seroconverted throughout the three-month follow-up observation period, nor were clinical signs such as lameness, anorexia, weakness, or elevated body temperature detected in these dogs.

Discussion and Conclusion

The development of laboratory methods for infecting ticks with different pathogens of veterinary importance is a key step for further research. The establishment of *in vitro* models would aid in advancing our knowledge of tick-pathogen interactions and would reduce animal use. We chose the immersion technique for infecting ticks with *B. burgdorferi* for several reasons. Firstly, this technique was validated in previous laboratory studies using *I. scapularis* and *I. ricinus* ticks [9,10,18]; secondly, this procedure allows the production of large numbers of infected ticks without the need for infecting host animals; and thirdly, it does not require any special equipment compared to the other more sophisticated procedures (e.g. microinjection).

The infection rate obtained here for *I. ricinus* was lower (18%) compared to those obtained for *I. scapularis* (37%) or to the infection rate of 65% reported by Fiserova., *et al*. [10] using PCR. Even though the detection techniques were different, the infection rate (37.5%) in our study for *I. scapularis*, was similar to those reported (45% - 65%) by Policastro and Schwan [9], using an immunofluorescence assay.

The vector competence of *I. scapularis* and *I. ricinus* for *B. burgdorferi*, strain B31 was investigated previously [19,20]. The susceptibility of dogs to *B. burgdorferi* ss infection using naturally infected ticks was also reported in numerous studies [7,21,22]. In the latter studies, seroconversion was observed from day 10 onward, with maximum titers occurring within 50 to 90 days after infestation. In another published study, the successful transmission of *B. burgdorferi* infection to dogs was documented after ticks were artificially infected with *I. scapularis* by capillary feeding [23]. The authors reported that 100% (5/5) and 20% (1/5) of dogs seroconverted when they were infested with adults and nymphal *I. scapularis*, respectively. Our decision to use dogs to demonstrate transmission by molted ticks was based on these study results.

Ticks in this study failed to induce infections with *B. burgdorferi* ss in dogs, even though we used the same described immersion technique and infection in immature/adult *I. scapularis* and *I. ricinus* was demonstrated. This could be explained by a low spirochete load in immersed ticks after moulting (Ct > 26). It has been reported that dogs exposed to naturally infected, field-collected *I. scapularis* nymphs had lower rates of seroconversion than those infected using adult ticks [21]. This may reflect the smaller number of bacteria transmitted by nymphs during feeding [23]. Interestingly, *I. ricinus* nymphs derived from immersed larvae did not induce seroconversion when they fed on C3H mice [18]. In the present study we did not confirm the viability of *B. burgdorferi* ss bacteria in ticks after their moult to the nymphal/adult stage. Thus, failure to induce infections in dogs might be due to the low bacterial load or to the absence of viable spirochetes in the molted ticks.

Our findings demonstrate that immature/adult *I. scapularis* and *I. ricinus* ticks can acquire *B. burgdorferi* while immersed in a suspension containing spirochetes. Although spirochetes may have survived the moult to subsequent nymphal or adult life stages, molted ticks were unable to transmit the infection to laboratory beagle dogs.

Acknowledgments

The authors extend their appreciation to the personnel at the study site for their assistance in the conduct of this study.

Conflicts of Interest Statement

All the authors declare no conflicts of interest related to this article.

Authors' Contributions

DT and MV conceived the study and designed the experiments. DT, AE and NL performed the experiments. DT drafted the manuscript. All authors read and approved the final version of the manuscript.

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