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TROPSA and TRE31 Gene Knockouts to Prevent the Transmission of Lyme Disease from Tick to Host: A Research Protocol

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Abstract

Introduction: *Ixodes scapularis*, the blacklegged tick, is responsible for the transmission of Lyme disease. Rising temperatures and shorter winter seasons, due to climate change, is resulting in the Northward expansion of tick range. This is correlated with the increasing prevalence of Lyme disease in Canada. This research protocol aims to address this issue by genetically mutating the blacklegged tick which is primarily responsible for the transmission of *Borrelia burgdorferi*, the bacterium that causes Lyme disease, in North America. The proposed mutation involves two gene knockouts: TROSPA and TRE31. The blacklegged tick mutant is predicted to be unable to transmit Lyme disease to the white-footed mouse, *Peromyscus leucopus*.

Methods: Mutated ticks will feed on the blood of Lyme positive mice and later naïve mice. The rate of Lyme disease transmission from mutated ticks will be compared to transmission rates in positive and negative wild type control groups. The statistical significance of the difference between these groups' transmission rates will be evaluated by Student's t-test with Fisher's protected least significant difference test.

Results: Based on the results from literature testing each mutation independently, we predict our *I. scapularis* mutant, having both TROSPA and TRE31 gene knockouts, will be unable to transmit Lyme disease to the white-footed mouse.

Discussion: Unsuccessful transmission of Lyme disease from mutated ticks indicates that the TROSPA and TRE31 knockouts are effective in preventing *B. burgdorferi* from completing its lifecycle within the tick. Based on the expected results, the combined gene-knockout model presents a novel method to hinder the transmission of Lyme disease more effectively than previously investigated single gene knockouts.

Conclusion: This research protocol suggests a strategy to decrease the rate of Lyme disease amongst ticks, and thus humans. Future research could explore efficacies of knocking out other genes in combination with TROSPA or TRE31.

Keywords: lyme disease; *Ixodes scapularis*; *Borrelia burgdorferi*; gene knockout; vector borne diseases

Introduction

Ticks are parasitic haematophagous arthropods and important vectors for diseases such as Rocky Mountain spotted fever, Lyme disease, ehrlichiosis, and tularemia [1,2]. In North America, the blacklegged tick, Ixodes scapularis, is responsible for the transmission of Lyme disease [3]. Lyme disease, which can be transmitted from ticks to humans, can cause flu-like symptoms and serious effects such as arthritis, cranial neuropathies, and radiculoneuritis [4]. Lyme disease is caused by the spirochete bacterium *Borrelia burgdorferi* [5]. *I. scapularis*, has three life stages (larva, nymph, and adult) and during each stage the tick will commonly feed on mammalian blood, including that of mice, voles, deer, and sometimes humans [3]. During feeding, *I. scapularis* can transmit B. burgdorferi to and from hosts, potentially spreading Lyme disease [6]. Increasing temperatures due to climate change is resulting in spatial and temporal shifts in the distribution of many species, blacklegged ticks included [7]. In fact, the northward expansion for these ticks is occurring at an exceptional rate of ~46 km/year [8]. Increasing northern boundaries of these ticks is also increasing the ranges of tick-borne diseases. One study concluded that in Ontario, Lyme positive ticks have increased from 8.4% in 2008 to 19.1% in 2012 [9]. The rapid increase in the prevalence of Lyme disease can be halted by inhibiting *B. burgdorferi* transmission from ticks to their mammalian hosts.

In unfed ticks, *B. burgdorferi* colonization is isolated to the gut. During feeding, the bacterial population increases and mobilizes allowing migration to the haemolymph, then salivary glands, enabling transmission to the host [10]. *I.*

scapularis gut colonization is facilitated by an interaction between a tick gut receptor, TROSPA, and a bacterial outer-surface protein, OspA [10]. Previous research has demonstrated TROSPA deficient *I. scapularis* were unable to support *B. burgdorferi* attachment resulting in failed colonization and death [11]. Despite this result, a small number of ticks became *B. burgdorferi* positive indicating the presence of multiple tick gut receptors involved in *B. burgdorferi* colonization [11]. To address this concern, we propose a mutant *I. scapularis* species with two knockouts: TROSPA and TRE31. TRE31 is another *I. scapularis* gut protein that when interacting with BBE31, an outer surface bacterial lipoprotein, facilitates the dissemination of *B. burgdorferi* from the tick gut [12]. This has been supported by findings of preferential expression of BBE31 in fed nymphs and decreased Lyme disease transmission when ticks were given a BBE31 antiserum [10]. Our proposed *I. scapularis* mutant, with both TROSPA and TRE31 gene knockouts, will be unable to transmit Lyme disease to its host, the white-footed mouse, *Peromyscus leucopus*. *B. burgdorferi* will be unable to colonize the tick gut because the TROSPA knockout will prevent binding. The small proportion of *B. burgdorferi* that does manage gut colonization will still fail to transmit Lyme disease due to a lack of BBE31-TRE31 binding.

We hypothesize that *I. scapularis* with the combination of a TROSPA gene and TRE31 gene knockout will be immune to *B. burgdorferi* colonization in the midgut (TROSPA) and transmission from midgut to salivary glands (TRE31) preventing Lyme disease transmission from parasite to host. Our proposed research protocol aims to test the efficacy of these specified mutations to reduce the transmission of Lyme disease.

Methods

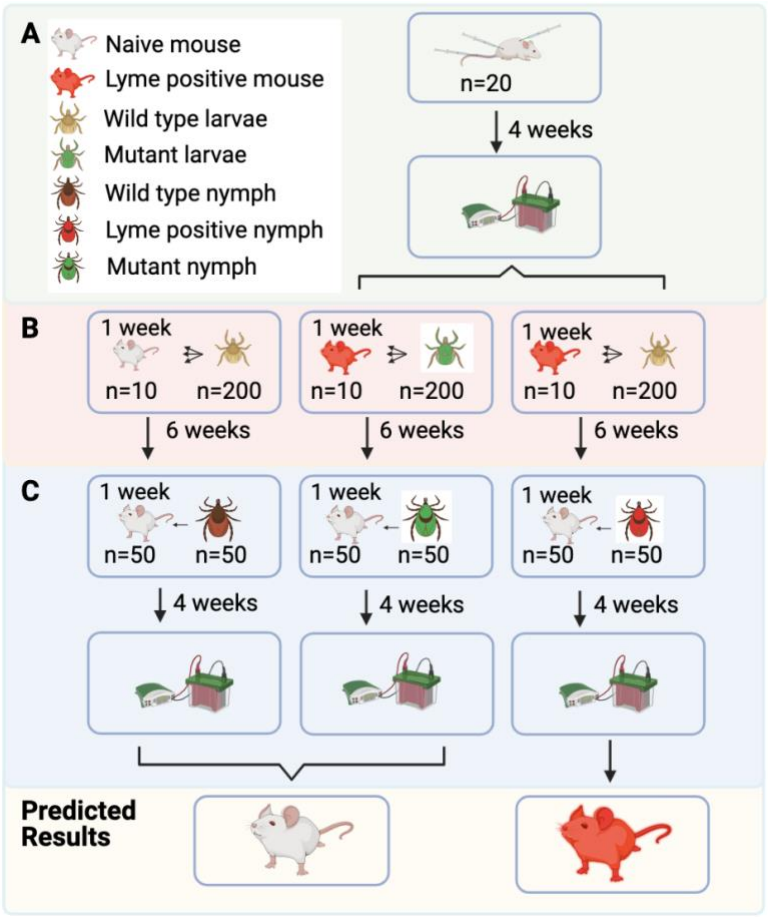

Figure 1. Workflow for the inoculation and detection of Lyme disease in white-footed mice. (A) Inoculation and confirmation of *B. burgdorferi* in mice, (B) mutant and wild type larvae treatment groups feeding on Lyme (+) and Lyme (-) mice (C) wild type, mutant and Lyme (+) nymph feeding on naive mice. Created with BioRender.com.

Study System

Our experiment aims to demonstrate our mutant *I. scapularis'* inability to transmit Lyme disease to its host, the white-footed mouse. White-footed mice are one of the primary hosts of blacklegged ticks in North America and are therefore an excellent model to study transmission of Lyme disease due their accessibility and relevance [13].

Male mice will be individually housed in cages in a climate-controlled room at 21 ℃ and maintained on a 12-hour light-dark cycle. Cages will be wrapped in a fine mesh. The mice will have continuous access to food (Lab Rodent Diet 5001) and water.

Prior to and following feeding, ticks will be housed in sterile clear plastic vials sealed with piece of fine mesh [14]. During feeding, ticks will be placed on mice in mesh wrapped cages and handling of ticks will be done with fine forceps when unfed and blunt-end forceps when fed [14].

Sourcing Tick Models

Prior to commencing this protocol, TROSPA and TRE31 knockout ticks will be generated using CRISPR-Cas-9 according to Sharma et al.'s *I. scapularis* embryo injection protocol [15].

Infection of White-Footed Mice

In the first stage (Figure 1A) of our protocol, 20 naive white-footed mice will be inoculated with *B. burgdorferi* via injection (10 [5] spirochetes/mouse) [11]. After four weeks, each mouse will be tested for Lyme disease indicated by *B. burgdorferi* antigen biomarkers that can be detected using western blot assays. GAPDH, a housekeeping gene, will be used as a loading control. Any mice that do not test positive for Lyme disease will be excluded from the experiment.

Tick Exposure to Lyme Disease

The second stage (Figure 1B) exposes the mutated ticks to *B. burgdorferi* through feeding on infected mice. This stage will consist of mutant, negative, and positive control *I. scapularis* treatment groups. The mutant treatment will consist of 200 *I. scapularis* larvae with TROSPA and TRE31 gene knockouts. The negative and positive control treatments will each contain 200 wild type *I. scapularis* larvae. Mutant treatment larvae and positive control treatment larvae will feed on Lyme (+) mice from stage (A) at a ratio of 20 ticks per mouse. Meanwhile, the negative control treatment larvae will feed on naive Lyme (-) mice. For each treatment group, ticks will be transferred from their vials and placed onto the mice in their cages. *I. scapularis* will be given a one week feeding period (in contact with white-footed mice) to ensure sufficient time for *B. burgdorferi* transmission [11]. After this time, ticks will be removed from the mice and placed back into their vials. Six weeks after feeding, *I. scapularis* will molt and enter the nymph stage of their life cycle [11].

Tick Transmission of Lyme Disease

In the final experimental stage (Figure 1C), *I. scapularis* nymphs from each treatment group will feed on naive white-footed mice at a ratio of 50 ticks per mouse (n=150). Again, ticks are removed from their vials and placed on mice in their cages. The purpose of this step is to determine if *B. burgdorferi* is able to be transmitted from the mutant tick to the naive mouse.

Verifying Lyme Disease Transmission Status

Following a one week tick feeding period and a four-week bacterial colonization period, the ticks will be removed from their hosts and tested for the presence of *B. burgdorferi* DNA by polymerase chain reaction (PCR) of the flagellin gene [16]. Additionally, blood samples will be collected from each mouse. Western blot assays will be conducted on each blood sample to confirm the presence, or absence, of *B. burgdorferi* antigens indicating Lyme disease. Once again, GAPDH, a housekeeping gene, will be used as a loading control.

Results will be expressed as the proportion of *B. burgdorferi* infected mice. The statistical significance of the difference between treatment groups will be evaluated by Student's t-test with Fisher's protected least significant difference test using RStudio software [17].

Results

Based on our hypothesis, we predict naive white-footed mice to be Lyme (-) when interacting with the negative control and mutant blacklegged tick treatment groups and naive white-footed mice to be Lyme (+) when interacting with the positive blacklegged tick control group.

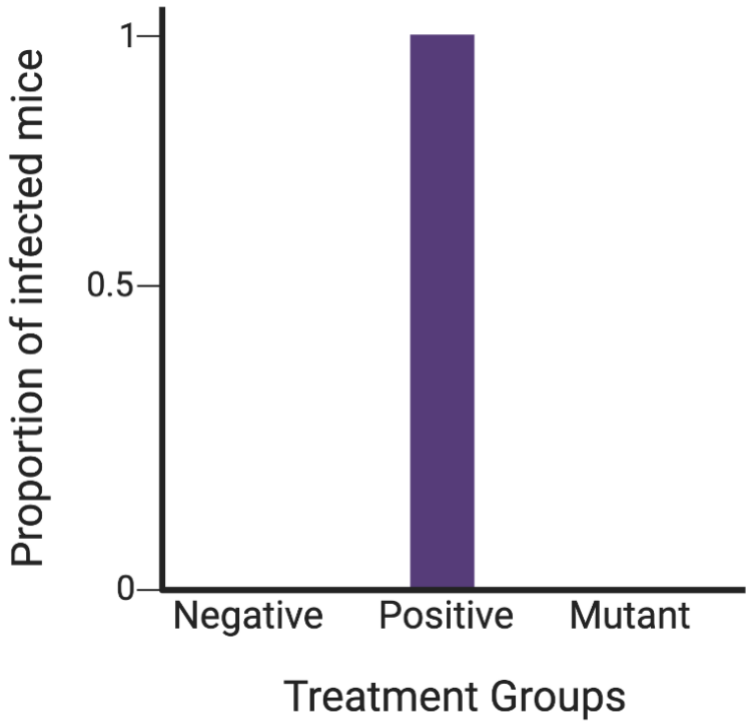

Figure 2. Predicted results for the proportion of infected mice with *B. burgdorferi* amongst three treatment groups. Created in BioRender.com.

Discussion

Here, we present a novel protocol to test the efficacy of a combined gene-knockout to combat the spread of Lyme disease. Previous research has demonstrated the down regulation of either TRE31 or TROPSA both results in the decreased transmission of *B. burgdorferi* from tick to host [10,11]. Additionally, successful protocols for CRISPR/Cas9-mediated gene editing in the black-legged ticks are available [15]. Our protocol would thereby serve as the second step to determine the combined efficacy of TROPSA and TRE31 knockouts in eliminating *B. burgdorferi* transmission between tick and host. Results that corroborate the predicted statistically significant difference between mutant and positive treatment groups, would establish this combined gene-knockout method as an effective technique to eliminate transmission. Future steps would involve ensuring that the TROSPA and TRE31 knockout mutations are heritable and do not reduce fitness of ticks.

Outcomes without a significant difference between positive and mutant treatments would refute our hypothesis. The successful transmission of Lyme disease from tick to host, in TROSPA and TRE31 knockout ticks, would indicate that the novel combination of mutations were not effective in preventing *B. burgdorferi* from colonizing the gut and migrating to the salivary glands of the tick.

Possible limitations to our protocol include the unconfirmed heritability of the mutated ticks' TROSPA and TRE31 knockout mutations, and the viability of these mutated ticks. As with any attempt to limit bacterial infection, resistance is of concern. Over time, the selective pressures created by our proposed mutations may favour *B. burgdorferi* binding to other tick receptors allowing gut colonization and dissemination to salivary glands.

After ensuring heritability, a future application could involve releasing mutated ticks into areas where Lyme disease is of concern. These ticks may breed with wild blacklegged ticks to decrease the wild population's ability to transmit Lyme disease. Furthermore, future studies could involve knocking out other genes, in combination with TROSPA or TRE31, involved in *B. burgdorferi* and blacklegged tick interactions. A candidate is Salp25D, a salivary protein that is secreted during tick feeding [18]. Salp25D combats host neutrophils at the bite site therefore aiding *B. burgdorferi* survival [18]. Previous research found a Salp25D knockdown model of *I. scapularis* demonstrated impaired *B. burgdorferi* acquisition when compared to the wildtype control [18].

Conclusions

While climate change increases the unpredictability of the natural world, certain trends such as the northward expansion of the tick vector have been clearly documented. To ensure the health of the Canadian population, it is essential to take immediate action to counter the propagation of Lyme disease in Canada. The results of this proposed research protocol could play an essential role in stopping the spread of Lyme disease, particularly in these unnatural northern ranges.

List of Abbreviations Used

TROSPA: tick receptor outer surface protein A
OspA: outer surface protein A
PCR: polymerase chain reaction

Conflicts of Interest

The authors declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent

No Research Ethics Board (REB) review was required as this paper is a proposal, not primary research.

Authors' Contributions

ADWA: made substantial contributions to the design of the study, revised the manuscript critically, and gave final approval of the version to be published.
ERD: made substantial contributions to the design of the study, revised the manuscript critically, and gave final approval of the version to be published.
SAL: made substantial contributions to the design of the study, revised the manuscript critically, and gave final approval of the version to be published.
HSP: made substantial contributions to the design of the study, revised the manuscript critically, and gave final approval of the version to be published.

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