Establishing a Protocol for the Long-term Sampling and Analysis of Tick-borne Pathogens in the Lehigh Valley

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Abstract
The blackedlegged tick, *Ixodes scapularis,* transmits several human pathogens including the spirochete that causes Lyme disease (*Borellia burgdorferi),* which is endemic to the Lehigh Valley region of eastern Pennsylvania. Additional tick-borne diseases have also been emerging in the region including tick-borne relapsing fever (caused by *Borrelia miyamotoi*), human granulocytic anaplasmosis (caused by *Anaplasma phagocytophilum*), and human babesiosis (caused by *Babesia microti*). The dog tick, *Dermacentor variabilis*, is capable of transmitting Rocky Mountain Spotted Fever (caused by *Rickettsia rickettsii).* To determine the entomological risk of infection by a tick-borne pathogen, a protocol was established to gather data for a long-term study. Eleven collection sites were screened and selected for analysis throughout the three-year duration of the study, and the iPhone application TRAILS was selected to digitally document collection paths. A sample size of 50 ticks per site was determined to create a total sample size of 550 ticks for the Lehigh Valley region. A total of 1596 ticks were collected during a one-month period, with an average of 136 deer tick nymphs, 2.7 deer tick adults, 6 dog ticks and 0 lone star ticks per site. Molecular studies will be conducted to determine the prevalence of pathogens carried by the ticks and used in conjunction with the tick abundance data to produce a risk assessment of tick-borne illnesses in the Lehigh Valley.

Introduction
Ticks transmit many infectious diseases that have become of increasing concern throughout the Lehigh Valley. The region is composed mainly of suburban environments in Berks, Lehigh and Northampton counties and houses approximately 650,000 residents. This region provides suitable environments for several enzootic pathogen cycles; especially those that involve tick interactions with the white-footed mouse, *Peromyscus leucopus* as well as other small mammals and birds. Blacklegged ticks (*Ixodes scapularis*), also known as deer ticks, are endemic to this mid-Atlantic region with a humid continental climate and plenty of undisturbed forests.

Deer ticks are known vectors of several human pathogens including *Borrelia burgdorferi*, *Anaplasma phagocytophilum* (ha strain), *Babesia microti*, and *Borrelia miyamotoi*. Dog ticks (*Dermacentor variabilis)* are capable of transmitting the bacteria *Rickettsia rickettsii*. Finally, lone star ticks (*Amblyomma americanum*) may induce meat allergies by transferring an alpha-gal sugar into the blood stream of the host, eliciting an immune response. Ticks may be infected with a single pathogen or co-infected with multiple infections, which may be the result of contracting different pathogens from different hosts or by taking a blood meal from a single co-infected host (Hersh et al. 2014). Ticks in the nymph life stage have taken a single blood meal, and thus the ticks that we will test have had only one possible source of contraction.

Lyme disease is the result of infection with the spirochete B. *burgdorferi* (senso lato). In 2013 Pennsylvania accounted for approximately 18% of the confirmed national cases of Lyme Disease (CDC, 2015). Human granulocytic anaplasmosis (HGA) is caused by a strain of the intracellular bacterium, *Anaplasma phagocytophilum* that infects neutrophils. *Babesia microti*, a protozoan parasite of red blood cells, causes human babesiosis. Babesiosis is emerging in the Lehigh Valley where three cases were reported in 2013. *B. miyamotoi* is the causative agent of tick-borne relapsing fever (Scoles et al., 2001). Although no human cases have been reported in Pennsylvania, one case of infection with B. *miyamotoi* was reported in New Jersey in 2012 (Gugliotta et al., 2013). *Rickettsia rickettsii* is the causative agent of Rocky Mountain Spotted Fever and is transmitted by Dog ticks. This infection is currently rare in Pennsylvania, and in 2010, 0.2-1.5 cases per 1 million persons were reported in the state according to the CDC (2013).

The objective of this study is to establish a series of methods for determining the infection rates of *B. burgdorferi*, *A. phagocytophilum*, *B. microti*, and *B. miyamotoi* in deer ticks in the Lehigh Valley of Pennsylvania. We established a series of methods to locate sampling sites, collect samples and determine a sample size for analysis. We also established a baseline of the current tick populations so that we will be able to accurately describe changes over time regarding species distributions and the pathogens that are carry. This information will allow us to construct a risk assessment of contracting tick-borne illnesses in this region. Assessing the prevalence of these human pathogens in ticks, and understanding the likelihood of humans to contract tick-borne illnesses in these regions, can help to guide health care providers in time-sensitive clinical decisions regarding prophylaxis and treatments.

Methods

Collection sites were found using the satellite images of Google Maps to locate closed-canopy, forested areas in the Lehigh Valley. Each location was then placed on a map to ensure that we were representing a broad range of the area of interest. Each location was screened to determine if it would qualify to be included. For each site we asked a series of five questions to ensure the compatibility of the site with our study. The criteria were: 1. Can a sufficient number of ticks be collected to provide a minimally representative sample? 2. Is the understory possible to navigate? 3. Will the environment remain relatively unchanged for the next 20 years? 4. Can we obtain permission to collect from the site? 5. Where is the site located in relation to the other sites in the study? All locations that conformed to the first four questions and provided additional geographic distribution were included (Figure 1).

Ticks were collected in June and July of 2015 by dragging a 1-m^2 white corduroy cloth in forested areas throughout Lehigh, Berks, and Northampton Counties. We conducted 30-minute drag "transects" in which we walked through the forest towing the cloths over leaf litter and low vegetation. We attempted to drag over logs and rocks preferentially, and took care to ensure that we did not drag over the same area twice. Drag cloths were checked for the presence of ticks approximately every 20 meters to avoid previously bound ticks falling off. We recorded the number of ticks found during each 30-minute transect and used this data to determine the number of ticks found per distance and per time.

The iPhone TRAILS Application (Iosphere GmbH) was used to map our paths for all transects. Topographic maps were available through this application from the work of the OpenStreetMap Community. TRAILS provided JPEX images of our locations on a topographical map of the area that we traveled that could then be layered on various different maps, including satellite images. By recording the precise locations of where ticks were collected, it will be possible to repeat the collections at the same sites in future years. Ticks were collected into vials containing 70% ethanol and stored in a -20°C freezer until DNA extraction. We recorded the temperature, humidity, dew point, weather conditions and vegetation characteristics at the start of each collection to determine the environment of each site. We then classified each tick according to species and life stage, and adults were identified as male or female.

We determined a sample size that balanced our need for an adequate number of ticks to be tested as well as our ability to test large numbers of samples by determining the 95% confidence intervals for different sample sizes based on a 20% Lyme disease infection rate. All calculations were conducted according to the Clopper-Pearson method. We determined that a sample size of 50 ticks per site would constitute a total sample of 550 ticks from the Lehigh Valley, thus producing a 95% confidence interval between 16.7%- 23.6% infection (Table 1).

We extracted DNA from 52 ticks from each site to allow for a 2 tick buffer in the event of a failed extraction. The number of ticks to be tested from each vial was determined by finding the proportion of the content of each vial to the total sample size for each location according to the following equation:

$$\frac{\#\ nymphs\ in\ vial}{Total\ \#\ numphs\ at\ site} * (52) = \#\ nymphs\ for\ extraction\ from\ the\ vial$$

The appropriate number of nymphs were randomly selected from each vial for extraction and analysis to create a final sample size of 50 ticks per site.

Tick DNA was extracted using a Qiagen QIAamp Mini Kit (Qiagen, Valencia, CA). A no-template control was also prepared to ensure that the reagents used in the extraction process were not cross-contaminated with tick DNA. The extracted DNA will be tested for the presence of various tick-borne pathogens. All procedures for extraction and analysis are conducted according to the methods of Edwards et al. (2015).

Results

A total of 1596 ticks were collected over a one month period between June-July 2015. Deer tick nymphs were found to be the most abundant with an average of 136 deer tick nymphs collected per site and individual site collections ranging from 65-276 ticks (Table 2). Adult deer ticks were less abundant, with an average of 2.7 adult ticks per site, and a range between 0-15 for individual sites. An average of 6 dog ticks per site were collected, with a range of individual site counts between 0-59. Neither dog tick nymphs, nor any lone star ticks were found. We collected an average of 25.7 deer ticks per hour throughout the Lehigh Valley. Using the TRAILS software, we also determined that we collected an average of 17.2 deer ticks per kilometer. We found that the standard error of the mean for the number of ticks per hour was 9.00 and the standard error of the mean for the number of

ticks per kilometer was 9.21. Graphing the deer tick prevalence for each site allowed us to observe the relative differences between sites that we will be able to compare to future collection years (Figure 2).

Discussion

Determining a specific method for collecting ticks, in which we track our paths is important because it allows us to account for environmental differences that may impact tick abundance. In addition, it was important that we recorded the time of year in which the collections took place as well as the weather conditions during the drags, which may also affect the life stage and density of ticks searching for blood meals. In order to accurately describe the current prevalence of tick-borne pathogens and predict future changes, we needed to first establish a baseline of both the tick and bacteria populations. The TRAILS application recorded the precise locations of where ticks were collected, which makes it possible to repeat the collections in the same areas of each site in future years.

Establishing the optimum sample size for our study allowed us to test a reasonable number of samples considering the resources available to us, while still producing significant results. The sample size for each site was determined by the need for a sufficient number of samples as well as our time available to extract and test the DNA for each site. We based our calculations upon a 20% infection rate, which is the infection prevalence for an area in which doctors prescribe antibiotics as a preventative measure for tick bites.

With this data we will be able to establish a risk assessment for the tested area. We based our inquiry off of the determination for entomological risk, which takes into account the size of the vector population as well as the likelihood that a given vector will be carrying, and thus able to pass along, a pathogen. Our assessment will combine the likelihood of acquiring a tick bite in the Lehigh Valley as well as the likelihood that the tick is infected, and thus able to transmit, one or more tick-borne pathogens. This assessment will be conducted on deer ticks by extracting DNA and analyzing it using qPCR for the presence of *B. burgdorferi, B.miyamotoi, A. phagocytophilum,* and *B. microti*. Gathering data regarding the presence of deer ticks, dog ticks and lone star ticks in the Lehigh Valley will allow us to understand the changes in these populations over time and how they will affect the health of human populations living in the region.

Acknowledgements

I would like to thank Dr. Luther Rhodes and the Luther Rhodes Endowment Fund of the Lehigh Valley Health Network and the Lehigh Valley Scholars Program for providing the funding to conduct this study. I would also like to thank Dr. Marten Edwards for his mentorship and assistance throughout this project. Finally, I would like to thank Bess Fleishman and Amulya Makkapatti for their participation in the collection of ticks and extraction of DNA.

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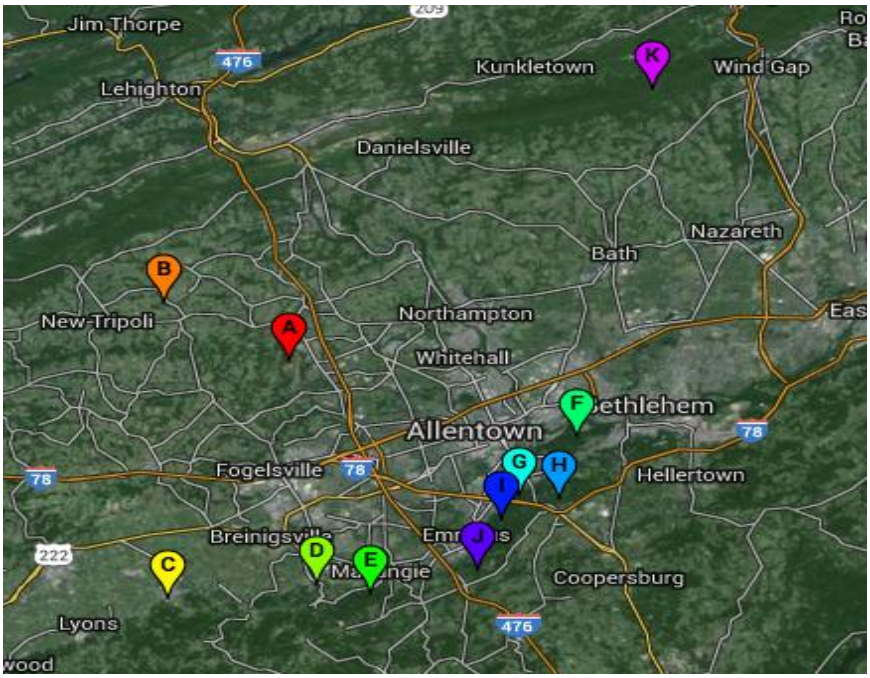
Appendix

Figure 1. Tick collection sites.



(A. Trexler Nature Preserve, B. Raker Wildlife Preserve, C. Park in Topton, D. Aburtis Mountain Road Tract, E. Reimert Memorial Bird Haven, F. Lehigh Uplands Preserve. G. South Mountain Preserve, H. Scholl Woodlands Preserve, I. Robert Rodale Reserve, J. Burkhart Preserve, K. Graver Arboretum.)

Table 1. Determination of an appropriate sample size.

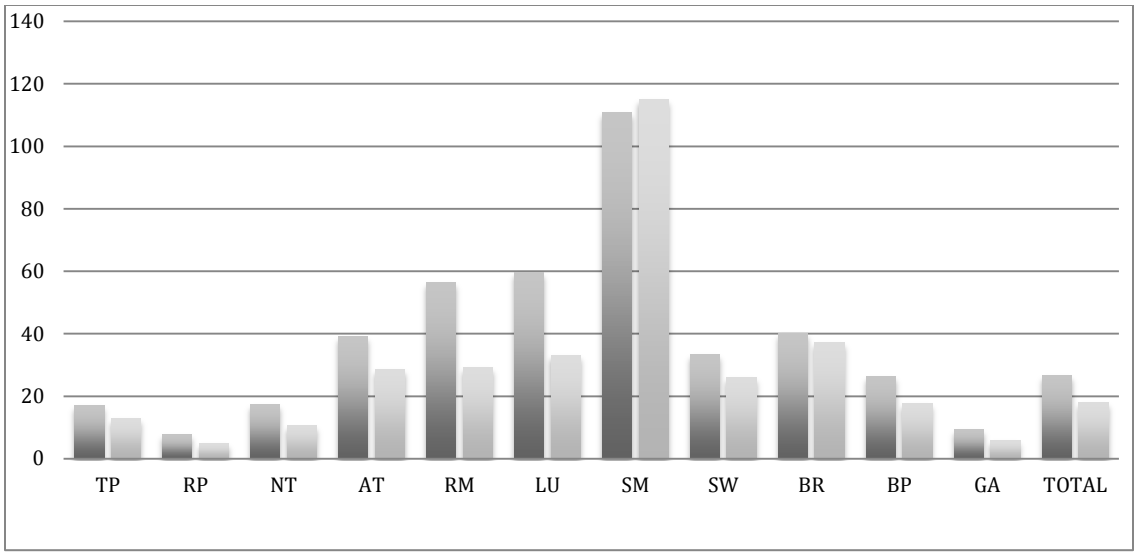
Sample Size	N Positive	Percent Positive	Lower 0.95 Confidence Limit	Upper 0.95 Confidence Limit
5	1	20%	0.5%	71.6%
10	2	20%	2.5%	55.6%
25	5	20%	6.8%	40.7%
50	10	20%	10.0%	33.7%
100	20	20%	12.7%	29.2%
500	100	20%	16.6%	23.8%
1000	200	20%	17.6%	22.6%

(All confidence interval values were calculated according to the Clopper-Pearson method.)

Table 2. Total tick counts by site.

Site Name	Deer Tick Adults	Deer Tick Nymphs	Dog Tick Adults	TOTAL
Trexler Preserve	4	131	59	**194**
Raker Wildlife Preserve	0	95	5	**100**
Park in Topton	3	136	3	**142**
Alburtis Mt. Rd. Track	1	116	1	**118**
Reimert Memorial	1	168	1	**170**
Lehigh Uplands Preserve	15	209	0	**224**
S. Mountain Preserve	1	276	0	**277**
Scholl Woodlands	0	113	0	**113**
Robert Rodale Reserve	2	119	0	**121**
Burkhart Preserve	1	65	0	**66**
Graver Arboretum	2	68	1	**71**
TOTAL	**30**	**1496**	**70**	**1596**

Figure 2. Deer tick abundance by site.



(A. Deer ticks per hour, B. Deer ticks per kilometer).