The Prevalence of *Babesia* sp., *Rickettsia* sp., and *Ehrlichia* sp. in the Upper Midwestern United States

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Abstract

Rickettsia spp, *Babesia* spp, and *Ehrlichia* spp. are all responsible for emerging tick-borne diseases within the United States. The intention of this study was to complete a survey of American dog ticks and rodent hosts for the presence of all three parasites in order to determine which animals acted as hosts for each parasite in the midwestern United States, and how common each parasite was within each host. American dog tick (*Dermacentor variabilis*), white-footed mouse (*Peromyscous leucopus*), deer mouse (*Peromyscus maniculatus*), masked shrew (*Sorex cinereus*), and northern short-tailed shrew (*Blarina brevicauda*) samples were collected at the University of Notre Dame Environmental Research Center (UNDERC) located in Wisconsin and Michigan in the summer of 2012. The samples were surveyed for all three parasites primarily using PCR and gel electrophoresis. All three parasites were discovered in the dog ticks, and the rodent hosts contained a variation of parasites. *Babesia* spp. was the most common of the three parasites. Given that all three of these parasites were discovered in dog ticks, it is suggested that patients in this region should be screened for all three diseases plus Lyme Disease following a tick bite and subsequent signs of illness.

Keywords

Dermacentor variabilis, *Rickettsia*, *Babesia*, *Ehrlichia*, tick-borne disease, midwestern United States

Introduction

Lyme disease is currently the most common reported tick-borne illness in the United States. However, three more tick-borne diseases, Rocky Mountain Spotted Fever, Human Monocytotropic Ehrlichiosis, and Babesiosis are becoming more common. In fact, Human Monocytotropic Ehrlichiosis only became a reportable disease within the past twenty years, and Babesiosis just became reportable in 2011 (Herwaldt et. al 2012). Currently, patients are usually only tested for Lyme disease following a tick bite, thus allowing the symptoms of other potential tick-borne diseases to progress if a Lyme disease test comes back negative (Daley 2013).

Rocky Mountain Spotted Fever (RMSF) is the most severe rickettsial infection in the United States with a 23.4% rate of hospitalization of between 2000 and 2007 (Openshaw et al. 2010). The bacteria *Rickettsia rickettsii* is the causative organism of RMSF, and is most often found in the American dog tick *Dermacentor variabilis*. *R. rickettsii* are a small, aerobic, Gram-negative coccobacilli that may infect humans after an infected tick has been feeding on a host for six to ten hours. Rocky Mountain Spotted Fever has been documented throughout the United States. Between the years 2000 and 2007, it was reported in 46 states, including Wisconsin and Michigan where our research facilities are located. However, compared to states like Oklahoma, the incidence rate was quite low in Michigan and Wisconsin, though it is increasing. To illustrate, in Wisconsin, there were no cases between 2000 and 2003, but between 2004 and 2007 there were seven per every one million persons. There was also an increase in the number of cases in Michigan (Openshaw et al. 2010). These are only human cases however, and it is generally accepted that other mammals including small rodents play a role in the zoonotic life cycles of rickettsias (Milagres et al. 2013). It is also important to note that *R. rickettsii* is not the only

species that is capable of causing human infection; recently, at least 11 other rickettsial agents have been discovered as pathogenic in humans (Parola et al. 2005).

Human Monocytotropic Ehrlichiosis (HME) is also considered an emerging tick-borne disease. HME is caused by the bacteria *Ehrlichia chaffeensis*, which is most often transferred by the Lone Star tick, *Amblyomma americanum*. *E. chaffeensis* is a zoonotic organism, meaning that part of its life cycle occurs within a mammalian reservoir. The most common reservoir is the white-tailed deer (*Odocoileus virginianus*) (Ewing et al. 1995), while dogs have also been observed as a notable host (Cheng et al. 2009) In 2010, cases were observed within humans in 36 states, including both Wisconsin and Michigan[1]. Wisconsin had as many as 550 cases of some form of Ehrlichiosis or Anaplasmosis (formerly known as Human Granulocytic Ehrlichiosis) reported in 2010 (Johnson 2012). HME did not become a reportable disease until 1999, but since then there as mostly been a steady increase in reported cases. For example, in 2000, there were only 200 reported cases, and in 2008 there were 961 reported.

Because Babesiosis became classified as a reportable disease in 2011, there is very little epidemiological data available. In 2011, only 18 states were designated for the surveillance of Babesiosis. During that year, 1124 confirmed cases were reported with 97% (1092) occurring within seven states, one of which was Wisconsin. In the 689 cases for which the data is available, 46% of patients were hospitalized (Herwaldt et. al 2012). *Babesia microti* or *Babesia divergens* are usually the species of *Babesia* responsible for human infection, though there are similar species of *Babesia* that cause a similar range of symptoms. *B. microti* is the most common cause of Human Babesiosis in the United States. Like *Ehrlichia* and *Rickettsia*, *Babesia* spp. are zoonotic. Small rodents including mice and shrews (Tadin et al. 2012), and cattle (Holman et al.

2005) are the most common reservoirs, and deer have also been identified as potential hosts (Gray 2006). *Peromyscus leucopus*, the white-footed mouse, is one of the most important reservoirs for *B. microti* in the United States (Gray 2006).

The midwestern United States has been an area where all three of these diseases have been reported and are currently increasing in reported cases, indicating these are potentially 'emerging diseases.' Our intent was to screen for all three of these diseases in both ticks and potential reservoirs that are native to the University of Notre Dame Environmental Research Center (UNDERC), which lies on the border of Wisconsin and Michigan. The habitats within UNDERC include northern hardwood forests, lakes, and wetlands. Wildlife samples were taken from UNDERC. The samples include several species of shrews, mice, and *D. variabilis*. *Dermancentor variablis* is really only accepted as a common vector for *R. rickettsii*, but because knowledge of these diseases is limited, screening them for all three of the diseases could be informative. We were also interested in seeing how many, if any, ticks were co-infected because those ticks could be more threatening to human health if they were able to infect a human host with multiple diseases.

Materials and Methods

Sample Collection

All of the samples were collected at UNDERC, which is located at 46' 13' North by 89' 32' West during the spring to fall months in 2012, specifically when night lows were above 40ºF. For the rodents, traps were laid out in a grid like pattern in 10 different locations. The trapping grids contained 25 traps in a 5x5 configuration with 15 meter spacing between consecutive traps. All

mice were live-trapped using Sherman traps baited with rolled oats, black oil sunflower seeds, and peanut butter. Upon initial capture, all animals were identified to species, sexed, weighed and marked with ear tags. The rodents captured in this study included *Peromyscus leucopus* (white-footed mouse), *Peromyscus maniculatus* (deer mouse), *Blarina brevicauda* (Northern short-tailed shrew), and *Sorex cinereus* (masked shrew). Ear punches were taken from all of the rodents, from which DNA was later extracted. Ticks were collected using the common practice of flagging, in which a 1 m^2 white flag is dragged along the forest floor and then examined for ticks approximately every 10 meters. In addition to performing drags, ticks were removed from small mammals captured in our traps. All ticks and ear punches were placed into individual tubes containing 70% ethanol for storage until further analysis could be conducted.

DNA Extraction

DNA extraction for both the ear tissue from rodents and ticks was done using a QIAGEN blood and tissue kit using the standard protocol for this kit. Samples were lysed in a solution of 180 µl ATL buffer and 20 µl proteinase K while being heated for an hour at 56°C in a digital dry bath (USA Scientific). Samples were then divided into 100 µl aliquots and stored at -20°C until PCR reactions were performed. All samples were tested using PCR for the presence of *Rickettsia*, *Babesia*, and *Ehrlichia*.

Identification of *Rickettsia* spp.

10 µl reactions containing the primers, NEB Taq Mastermix, and water were used to amplify the rickettsial outer membrane protein A (r*ompA*) gene. The primers Rr.190 70 P 5'-ATGGCGAATATTTCTCCAAAA-3' and Rr.190 602N 5'-AGTGCAGCATTCGCTCCCCCT-3'

were used to amplify the gene (Williamson et al. 2010). The samples were run in a Bio-Rad thermocycler initially at 95°C for 5 minutes and then for 40 cycles with a denaturation step at 95°C for 1 minute, an annealing step at 72°C for 30 seconds, and an extension step 72°C for 30 seconds. These steps were followed by a final extension at 72°C for 5 minutes.

IDENTIFICATION OF *EHRLICHIA* SPP.

10 µl reactions containing the primers, NEB Taq Mastermix, and water were used to amplify the 16s rRNA gene. The primers HE1 5'-CAATTGCTTATAACCTTTTGGTTATAAAT-3' and HE3 5'-ATAGGGAAGATAATGACGGTACCTATA-3' were used to identify the gene (Anderson et al. 1992). Samples were run in a Bio-Rad thermocycler for 3 cycles initially with the denaturation step at 94°C for 1 minute, the annealing step at 55°C for 2 minutes, and the extension step at 70°C for 1 minute at 30 seconds. These initial 3 cycles were then followed by 37 cycles with the denaturation step at 88°C for 1 minute, the annealing step at 55°C for 2 minutes, and 70°C for 1 minute and 30 seconds.

IDENTIFICATION OF *BABESIA* SPP.

10 µl reactions containing the primers, NEB Taq Mastermix, and water were used to amplify the 18s rRNA gene. The primers BJ1 5'-GTCTTGTAATTGGAATGATGG-3' and BN2 5'-TAGTTTATGGTTAGGACTACG-3' were used to identify the gene (Schorn 2011). Samples were run in a Bio-Rad thermocylcer for 40 cycles with the denaturation step at 94°C for 30 seconds, the annealing step at 55°C for 30 seconds, and the extension step for 72°C for 40 seconds.

Visualization and Confirmation

All of the PCR products were detected by electrophoresis of 2 μL of product with 2 μL of loading buffer on a 1% agarose gel containing GelRed. Each gel was run for approximately 70 minutes. Once a potential positive sample was determined via gel electrophoresis, the product was cleaned with Exo-SAP for subsequent cycle-sequencing. In order to confirm there was still a potential positive after the Exo-SAP procedure, 2 μL of the product from the cleaned PCR-product was rerun through electrophoresis on a 1% agarose gel. Once confirmed, the remaining product was run through a BigDye reaction and sequenced by the Genomics Core Facility at the University of Notre Dame using an ABI 3730xl 96-capillary sequencer. The sample was run through the BLAST program to confirm a positive.

Once the potential positive was confirmed for each disease, the confirmed positive was used for comparison to the remainder of the samples that had yet to be tested. Therefore, all other samples were tested with both a positive and a negative control, and the future positives were determined based upon the results of the gel electrophoresis. Figure 1 shows a typical electrophoresis result for all three pathogens.

Results

Over the course of the summer months, 76 ticks were collected, all *Dermacentor variabilis.* There were also 259 *Peromyscus maniculatus*, 101 *Peromyscus leucopus*, 30 *Sorex cinereus,* and 46 *Blarina brevicauda* collected over the same period. There were also 73 samples of *Peromyscus* blood samples collected. *Babesia, Rickettsia,* and *Ehrlichia* were found in almost every sample set. Following the sequencing of the potential *Rickettsia* positive, the sample was

identified as *Rickettsia cooleyi*, the *Babesia* sample was identified as *Babesia bigemina* and it also matched the 18s rRNA gene for general *Babesia* spp, and the sample of *Ehrlichia* was identified as *Ehrlichia chaffeenis*. Table 1 shows the percent of positives broken down by species.

There were also only 3 sets of coinfections taken from all of the samples. 2 of the coinfections occurred within ticks. The first one was infected with a combination of *Rickettsia* and *Babesia,* and the second was infected with a combination of *Babesia* and *Ehrlichia.* The 3rd set of coinfections came from one of the *Peromyscus* blood samples and was also a combination of *Babesia* and *Ehrlichia.*

Chi-squared tests were performed to determine the significance of several factors. A chi-squared test was done to compare the infection rates (pooled across host species) for all three pathogens. The test was statistically significant with a χ^2-value of 39.3 (p = 2.873e-9, df =2). There were significantly more *Babesia* sp. positives than either of the other two pathogens. Chi squared tests were done to compare the positives in the ticks to the positives in the rodent hosts for each pathogen. The only pathogen for which there was a statistical difference was *Babesia* ($\chi^2 = 4.05$, df = 1, p = 0.044). There were significantly more ticks carrying the pathogen when compared to rodent hosts. The chi-squared test was statistically insignificant for the other two pathogens. (*Rickettsia* sp. $\chi^2 = 0.60$, df = 1, p = 0.44 and *Ehrlichia* sp. $\chi^2 = 0.013$, df = 1, p = 0.91). Chi-squared tests were also performed to determine if there was a significant difference between the infection rates in the shrews, *Sorex cinereus* and *Blarina brevicauda*, and the mice, *Peromyscus maniculatus* and *Peromyscus leucopus.* There was no significant difference for

Babesia sp. ($\chi^2 = 0.223$, df = 1, p = 0.64) or *Ehrilichia* sp. ($\chi^2 = 0.71$, df = 1, p = 0.40). Contrarily, *Rickettsia* sp. were significantly more likely to be in shrews when compared to mice. ($\chi^2 = 163$, df = 1, p = 2.2e-16) Finally, chi squared tests were performed to determine if there was a statistically significant difference between all 4 rodent hosts for each pathogen. Once again, there was no significant difference between the rodent *Babesia* sp. ($\chi^2 = 1.88$, df = 3, p = 0.60) and *Ehrlichia* sp. ($\chi^2 = 5.57$, df = 3, p = 0.13) The incidence rates in the hosts for *Rickettsia* sp. were significantly different. There were significantly more positives in *Blarina brevicauda* ($\chi^2 = 208$, df = 3, p < 2.2e-16) when compared to *Sorex cinereus, Peromyscus maniculatus,* and *Peromyscus leucopus*.

Discussion

The top hit for the *Rickettsia* sample was a 91% identification (461/501 identities, bit-score = 730) to the *Rickettsia* sp. ompA gene (GenBank: KF702334). The first species specific match was to *R. cooleyi,* which is a strain of *Rickettsia* that was discovered in 1998 (Billings et al. 1998). The match was to the 190 kDa antigen gene (GenBank: AF031535.1) with a 91% identification (452/494 identities, bit-score = 725). *Rickettsia cooleyi* is highly divergent in three conserved genes compared to other Rickettsial species. Since *R. cooleyi* is a newly identified strain of Rickettsia, the exact method of pathogenesis and the clinical presentation has still not been identified. The sample positive was also quite similar to *Rickettsia monacensis* (88% identification, bit-score = 669 bits), which is known to infect humans, especially in Europe (Oteo and Portillo 2012). When the search with the positive is narrowed for *Rickettsia rickettsii,* the top

hit shares 79% identification (bit-score = 377) with the complete genome (GenBank: CP00318). We speculate the rickettsial species discovered in the *Dermacentor variabilis* and the rodents found at UNDERC are most likely capable of infecting humans given the high identity with *R. rickettsii* and *R. monacensis* though proper testing would need to be done to confirm this. There were only 3 positive ticks out of the 76 tested, or 3.9%. This is a significantly low percentage of ticks infected when compared to the amount of *Sorex cinereus* and *Blarina brevicauda*, which were 20% and 58.7% respectively. These shrews appear to be better hosts for Rickettsial infections.

There are several possible explanations for this. First of all, rickettsial infections may be easily transmitted by other ticks, such as *Ixodes scapularis* (deer tick), which are present in Wisconsin and Michigan. If these ticks may be more likely to come in contact with shrews than mice, this could explain why mice were not positive for any form of rickettsial disease. However, it may be more likely that *B. brevicauda* and *S. cinereus* are better hosts than *P. maniculatus* and *P. leucopus* for *Rickettsia* spp, and that rickettsial diseases survive more readily in the shrews. The fact that *Rickettsia* spp. were found in *Dermacentor variabilis* is not surprising because American Dog Ticks are recognized as one of the most common vectors for rickettsial diseases in the United States (Rolland et al. 1998).

The positive sample of *Ehrlichia* that was sequenced had greater than 99% identification with *E. chaffeensis*, which was the specific species of *Ehrlichia* that was being searched for. Specifically, the sample had the greatest match with a 16S ribosomal RNA sequence of *E. chaffeensis* from Arkansas (29/29 identities, bit-score =54.7). *E. chaffeensis* is responsible for HME. There were 5 ticks infected with *Ehrichia* spp. which is consistent with previous results

(Roland et al. 1998). However, this is not what was expected because *D. variablis* are not usually considered a common vector for *Ehrlichia* spp. (Williamson et al. 2010). There were no positives found in *P. leucopus, S. cinereus,* and *B. brevicauda.* There was also a low percentage (3.1%) of positives found in *P. maniculatus*, but a significantly higher percentage of positives found in the *Peromyscus* blood samples in comparison with the rest of the *Peromyscus* spp. samples. This could potentially be attributed to the effectiveness of the HE1 and HE3 primers used. The primers were found to be effective in detecting *E. chaffeensis* in *D. variablis*, but were more successful in identifying infections in blood samples. This indicates that *Peromyscus* spp. may actually be natural reservoirs for *Ehrlichia* spp. but the HE1 and HE3 primers may be useful for detecting the pathogen within blood samples. If this is the case, then the prevalence rate in the *D. variablis* ticks may in fact be higher. It is also possible, and we believe more likely, that *E. chaffeensis* does not reside in the ear tissue of rodents. Both *S. cinereus* and *B. brevicauda* do not appear to be natural reservoirs for *Ehrlichia* spp. It is also worth noting that the bands produced in the gel were the weakest of the 3 pathogens (Figure 1) which may be an indication that the primers are not very effective. This may also indicate that the PCR conditions used for the primers were not ideal.

Babesia spp. had the highest and most consistent prevalence rates among all of the samples. Upon sequencing, the positive sample had strong identification with *B. crassa, B. bigemina,* and the 18s rRNA gene for general *Babesia* spp. The highest identification, at 84% (194/231 identities, bit-score = 219), was with a sequence of *Babesia* sp. 18s ribosomal RNA gene (GenBank: KF841442) that lacked species identification. Other top identifications were with the *Babesia crassa* isolate hlj143 18s ribosomal RNA gene (84% identification, bit-score =

219); (GenBank: JX542614) and with the *Babesia bigemina* strain 563 18s ribosomal RNA gene (83% identification, bit-score = 207) ; (GenBank: HQ840960). *B. crassa* is a strain of Babesia that infects sheep and is common in Asia (Hashemi-Fesharki and Uilenberg 1981). There are no reports of the existence of *B. crassa* in the United States, so it is unlikely that the sample was positive for *Babesia crassa*. It is also unlikely that the sample was positive for *B. bigemina* (though more likely than *B. crassa*). *B. bigemina* is one of several species that are responsible for bovine Babesiosis. It has been documented in North America, but only southern parts of the United States and in Mexico (Holman et al. 2005). There were even reports that *B. bigemina* has been eradicated from the United States (Cantu et al. 2007). This means that the *Babesia* detected could be another form of *Babesia* spp. such as *Babesia microti* which has been documented in Wisconsin and Michigan. There was a strong match (188/230 (82%) identities, bit-score = 191) when the search was narrowed to *Babesia microti* (GenBank: AY144698). Despite a slightly lower matching score, we hypothesize it is more likely that the positive is *Babesia microti*, given it's documented existence in the UNDERC region.

It is also worth noting that a screening for a fourth pathogen, *Anaplasma phagocytophilum* was attempted, but proved unsuccessful. The protocol for the *Anaplasma* spp. was different because it was a nested PCR. The primers ge3a 5'-CACATGCAAGTCGAACGGATTATTC-3' and ge10r 5'-TTCCGTTAAGAAGGATCTAATCTCC-3' were used in the first PCR cycle and the primers ge9F 5'-AACGGATTATTCTTTATAGCTTGCT-3' and ge2 5'-GGCAGTATTAAAAGCAGCTCCAGG-3' (Massung et al. 1998). There were several times unidentified DNA bands were observed via gel electrophoresis, but no positive BLAST matches

were found for the sequenced bands. There are several possible explanations for this. It is definitely possible that none of the samples were infected with *Anaplasma phagocytophilum*, and some random DNA strands may have been accidentally amplified. It is also possible that the primers or the protocol is ineffective, in which case there may have been positive *Anaplasma* spp. samples that were not detected. This is definitely a possibility considering *Anaplasma* spp. has been repeatedly documented in Wisconsin and Michigan. We find it surprising that *Anaplasma* spp. would be missing from all of the sample sets, particularly given the prevalence of disease within our tested samples and their geographic locale..

The fact that there were three sets of coinfections is also interesting. This demonstrates that *D. variabilis* is capable of surviving and carrying multiple infections, which means that it may also be capable of transmitting both pathogens with a single bite. There was also a single *Peromyscus* blood sample containing a coinfection, which suggests that some species of mice are also capable of carrying multiple tick-borne pathogens. As mentioned previously, further research is needed to determine if *D. variabilis* would be a competent vector for the detected pathogens (versus being a "dead-end" host) and to determine how multiple infections could affect the fitness of mammalian hosts.

Finding *Ehrlichia* spp., *Babesia* spp., and *Rickettsia* spp. has public health relevance for the midwestern United States. *Babesia* spp. was the most common, but all three pathogens may exist in *D. variabilis*, which is the most common type of tick in the midwestern United States. Therefore, it would be worth ill screening patients following a tick bite for all three diseases, plus Lyme Disease. Such tests could be highly beneficial because all three diseases are easily treatable once they are detected, and the most severe symptoms only occur when these diseases

go undiagnosed for too long. Of course, the best way of preventing the diseases is achieved by avoiding tick bites; thus methods of preventing ticks from biting humans (such as wearing appropriate clothes while outdoors) or removal of ticks, for example by the application of acaricides, would be ideal in controlling the spread and burden of these three emerging diseases in the Upper Midwest.

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Table 1. Surveillance results across pathogens and hosts that were surveyed. The percentages represented the amount of percent positive for each parasite discovered in the host samples (± SE). The P-value is for each percentage with a null hypothesis of 0 positive samples for each host.

Disease	Host	Positives/Total	Percentage	P (H_o: p = 0)
Babesia	*Peromyscus maniculatus*	36/259	13.9 ± 2.1%	1.47E-17
	Peroomyscus leucopus	17/101	16.8 ± 3.7%	1.47E-17
	Dermacentor variabilis	17/76	22.4 ± 4.8%	8.24E-09
	Sorex cinereus	5/30	16.7 ± 6.8%	4.39E-09
	Blarina brevicauda	4/46	8.7 ± 4.2%	0.015
	Peromyscus (Blood)	4/73	5.5 ± 2.7%	0.016
Rickettsia	*Permoyscus maniculatus*	0/259	NA	NA
	Permoyscus leucopus	0/101	NA	NA
	Dermacentor variabilis	3/76	3.9 ± 2.2%	0.047
	Sorex cinereus	6/30	20 ± 7.3%	0.001
	Blarina brevicauda	27/46	58.7 ± 7.3%	2.17E-18
	Permoyscus (Blood)	0/73	NA	NA
Ehrlichia	*Permoyscus maniculatus*	8/259	3.1 ± 1.1%	0.00029
	Peromyscus leucopus	0/101	NA	NA
	Dermacentor variabilis	5/76	6.6 ± 2.8%	0.0057
	Sorex cinereus	0/30	NA	NA
	Blarina brevicauda	0/46	NA	NA
	*Peromyscus (*Blood)	15/73	20.5 ± 4.7%	5.10E-08

Figure 1. Top left. An image of a gel containing 5 *Rickettsia* positives in lanes 2,3,6,7,8. Column 5 was not counted due to the double band that was produced. The positives were about 550 base pairs each. **Top Right**. An image of a gel containing 1 *Ehrlichia* positive in lane 2. The positive is about 400 base pairs. **Bottom.** An image of a gel containing 3 *Babesia* positives in lanes 1, 3, and 4 along with a known *Babesia* positive on the far right. The positives ranged in size between 290 and 320 base pairs.