ENZOOTIC TRANSMISSION OF BABESIA DIVERGENS AMONG COTTONTAIL RABBITS ON NANTUCKET ISLAND, MASSACHUSETTS

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INTRODUCTION

In the last 30 years, diverse tick-borne zoonoses have emerged as public health problems. In northeastern North America, a guild of pathogens is maintained by the northern deer tick, *Ixodes dammini*. *Borrelia burgdorferi* (agent of Lyme disease), *Babesia microti* (agent of human babesiosis), *Anaplasma (Cytoecetes) phagocytophilum* (agent of human granulocytic ehrlichiosis), and *B. divergens* (agent of human granulocytic ehrlichiosis) are intensely transmitted between cottontail rabbits. To test the hypothesis that a piroplasm may also be maintained in rabbits, we sampled these hosts from Nantucket Island, Massachusetts and tested their blood and tissues by a polymerase chain reaction for evidence of infection. Surprisingly, the agent of bovine redwater and of European human babesiosis, *Babesia divergens*, was detected in 16% of the rabbits sampled during 1998–2002 (>99% sequence similarity in the 18S ribosomal DNA). The vector of *B. divergens* on Nantucket appears to be *Ixodes denitatus*, a rabbit- and bird-feeding tick that may feed on humans. Although the risk of human infection appears to be minimal, an autochthonously acquired Kentucky case due to this rabbit agent was recently reported. Physicians should entertain the diagnosis of babesiosis due to *B. divergens* for severe hemolytic febrile syndromes in American patients exposed to sites where rabbits are common.

Abstract. Specific ticks seem to locally serve as vector for characteristic microbial assemblages (guilds) comprising spirochetes, piroplasms, ehrlichiae, and arboviruses. *Borrelia andersoni* and *Anaplasma phagocytophilum* are intensely transmitted between cottontail rabbits. To test the hypothesis that a piroplasm may also be maintained in rabbits, we sampled these hosts from Nantucket Island, Massachusetts and tested their blood and tissues by a polymerase chain reaction for evidence of infection. Surprisingly, the agent of bovine redwater and of European human babesiosis, *Babesia divergens*, was detected in 16% of the rabbits sampled during 1998–2002 (>99% sequence similarity in the 18S ribosomal DNA). The vector of *B. divergens* on Nantucket appears to be *Ixodes denitatus*, a rabbit- and bird-feeding tick that may feed on humans. Although the risk of human infection appears to be minimal, an autochthonously acquired Kentucky case due to this rabbit agent was recently reported. Physicians should entertain the diagnosis of babesiosis due to *B. divergens* for severe hemolytic febrile syndromes in American patients exposed to sites where rabbits are common.

MATERIALS AND METHODS:

Sample collection. Cottontail rabbits, *Sylvilagus floridanus*, were collected by live trap (Tomahawk Live Traps, Tomahawk, WI) or shot on Nantucket Island, Massachusetts from 1998 to 2002. Collections occurred during the spring and fall of 1998 and then monthly (April to October) from April 1999 to August 2002. All work was performed under a scientific collecting permit issued by the Massachusetts Division of Fisheries and Wildlife. Anticoagulated blood (acid citrate dextrose) was collected from all animals. Spleens were collected from dead animals; a small piece was sliced off for DNA extraction and the rest was frozen at −70°C. All rabbits were visually inspected for the presence of ticks, which were removed by gentle traction with forceps. Most rabbits were caged over water overnight to allow replete ticks to drop off. Live-trapped animals were marked with an ear tag and released at the point of capture.

Ticks from rabbits were collected, sorted by species and stage, and held at 24°C and a relative humidity of 90%, and allowed to molt. Replete females were allowed to oviposit. Partially engorged ticks were retained for polymerase chain reaction (PCR) analysis.

Polymerase chain reaction. DNA was extracted from blood and spleens using the Isoquick Blood Kit (Orca Research, Bothell, WA) or the DNEasy Tissue kit (Qiagen, Inc., Valencia, CA). Ticks were individually homogenized in 50 μL of phosphate-buffered saline. An aliquot was then pooled with those from five other ticks and extracted with phenol/chloroform. The PCR was performed initially using the genus-wide primers Pir0A/Pir0B as described previously. Briefly, 25-μL reactions containing 12.5 pmol of each primer, 0.2 mM dNTPs, 0.03 mM dUTP, 1× Qiagen reaction buffer, 0.125 units of *Taq* polymerase (Qiagen, Inc.), and 2.5 μL of template DNA were used. Subsequently, specific *B. divergens* primers (Div818f: GAACCTTAGTAATGGTTAATAG/H9262 and Div1347r: GGACGAACCTTTTTACGGAG/H9262) were designed to amplify a 560-bpepair piece of the 18S ribosomal DNA (rDNA) gene. Amplification proceeded as previously described, except that a lower annealing temperature (55°C) was used. The β-tubulin gene was amplified using...
Babtub1 and Babtub3 as described elsewhere. Care was taken to minimize the possibility of PCR contamination. Safe PCR methods were practiced, including designated pre- and post-PCR areas, aerosol barrier tips, and negative controls for every reaction. Due to the possibility of false-positive results from blood meal contamination, pools of ticks from PCR-positive rabbits were excluded from the analysis. Amplicons were visualized by agarose gel electrophoresis. A random sample of positive amplicons was excised from the gel, purified by a spin column (Qiagen, Inc.), and sent to the University of Maine sequencing facility (Orono, ME) for sequence analysis. Prevalence estimates are expressed as the percent positive, and 95% confidence intervals (CIs) were calculated by the exact binomial using STATA (Stata Corp., College Station, TX) software.

Phylogenetic analysis. Phylogenetic analysis of the *Babesia* sp. in Nantucket rabbits was done with two different genes: 18S rDNA and β-tubulin. To obtain a large piece of the 18S rDNA gene for phylogenetic analysis, PiroA was combined with primer B to yield a 1300-basepair piece. Neighboring and maximum parsimony analysis was performed. Results were compared for Jukes Cantor as well as HKY models. The robustness of the tree was assessed using 500 bootstrap replicates. *Toxoplasma gondii* and *Cryptococcidium cohnii* were used as outgroups.

GenBank accession numbers. The GenBank accession numbers were AY144688 Nantucket Rabbit 1, Z4875 Babesia 1, U16370 Babesia 2, U07885 Babesia 3, U16369 Babesia 4, L19079 Babesia 5, AF158702 Babesia 6, AF175300 Babesia 7, AF049999 Babesia 8, AB050732 Babesia 9, Japan, AF231348 Babesia 10, GI strain, AF158700 WA1, X68523 T. gondii, and M64245 C. cohnii for 18S rDNA, and AY144704 Nantucket Rabbit 1, AY144705 Babesia, AY144706 Babesia Texas, AY144703 Babesia, and M20025 T. gondii for β-tubulin DNA.

Blood and spleen smears. Blood smears and spleen impression smears were taken at the time of sample collection and stained with Giemsa. Slides from PCR-positive rabbits were re-examined by oil immersion microscopy for parasites.

Rabbit serology. Sera collected from rabbits in 2001 and 2002 were tested for evidence of exposure to Babesia and Babesia using a routine immunofluorescence assay (IFA) as described previously for Babesia. Babesia antigens were obtained by primary culture of deer blood obtained from hunter-killed deer from Massachusetts. Babesia Babesia Babesia IFA slides were made from the Purrnell strain maintained in bovine red blood cell culture and were a kind gift of Dr. Patricia Holman (Texas A&M University, College Station, TX). The IFA slides were fixed with acetone before use. A screening dilution of 1:64 was used for the rabbit sera.

Minimum infection rate of molted ticks. Fully fed ticks removed from rabbits were held in 3-dram vials at 24°C and allowed to molt. Molted ticks were then pooled in groups of five, had their DNA extracted, and were tested for evidence of infection by PCR as described in this report.

**RESULTS**

Prevalence of *Babesia* in rabbits. *Babesia* was frequently detected in rabbits from our study site. A total of 203 cottontail rabbits were examined over five transmission seasons; the prevalence of Babesia infection ranged from 11% to 29%, with an overall estimate of 16% (Table 1). The DNA of this *Babesia* was successfully amplified from cottontail rabbits every year. All 95% CIs for our yearly prevalence estimates overlap, suggesting that transmission did not differ significantly between years. Parasites morphologically consistent with Babesia were identified in blood and spleen impression smears from PCR-positive rabbits (Figure 1). Parasitemias were typically extremely low; often only a single parasite could be identified from an entire slide. *Babesia microti* was only rarely detected (3 of 79 rabbits tested). Rabbits from 2001 and 2002 showed seroreactivity to both Babesia (50%) and Babesia (26%). However, 62% of those seroreactive to Babesia showed a four-fold greater titer than they did to Babesia, while only 6% of Babesia reactive rabbits had a four-fold greater titer than to Babesia. Titers ranged from 64 to 8,192 for Babesia (geometric mean titer [GMT] = 385) and from 64 to 1,024 for Babesia (GMT = 136).

Phylogenetic analysis. Sequence analysis of 18S rDNA and β-tubulin gene show that the rabbit piroplasm is most closely related to Babesia, and was virtually identical to a Babesia isolate (Purrnell strain) from Europe (GenBank U16370) with a 99.8% sequence similarity in the 18S rDNA gene or only three unique nucleotides of 1300 basepairs sequenced. Phylogenetic analysis of this gene shows that the Nantucket rabbit piroplasm clusters together with Babesia with a bootstrap value of 100% (Figure 2). This was true for both methods of analysis: neighbor-joining or maximum parsimony. The DNA sequence obtained from Nantucket rabbits is also identical to a sequence obtained from a human babesiosis case from Kentucky in 2000. The β-tubulin gene also groups the rabbit Babesia together with Babesia.

This gene, however, has a greater amount of sequence diversity. The rabbit agent is 97.8% similar to Babesia divergens from Europe (a difference in 24 of 1080 basepairs analyzed).

Analysis of ticks. Three species of ticks were collected from cottontail rabbits: *I. dentatus, Haemaphysalis leporispalustris,* and *I. dammini.* All stages of *I. dentatus* and *H. leporispalustris* were found on rabbits. Although larval and nymphal stages of *I. dammini* were routinely identified on rabbits, adults (males and females) were rarely found and are not included in this analysis. All three species yielded Babesia PCR-positive ticks. Two percent of both male and female (95% CI = 0–5 and 0–6, respectively), but none of the nymphal, *I. dentatus* tested positive (Table 2). Similarly, 2% (95% CI = 0–9) of *I. dammini* nymphs tested positive. Positive *H. leporispalustris* were rare; only one pool tested positive (0.4%, 95% CI = 0–2).

Replete larval ticks collected from rabbits were held at

<table>
<thead>
<tr>
<th>Year</th>
<th>Percent infected (95% confidence interval)</th>
<th>No. tested</th>
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<tbody>
<tr>
<td>1998</td>
<td>29% (10–56)</td>
<td>17</td>
</tr>
<tr>
<td>1999</td>
<td>28% (12–49)</td>
<td>24</td>
</tr>
<tr>
<td>2000</td>
<td>11% (3–25)</td>
<td>38</td>
</tr>
<tr>
<td>2001</td>
<td>16% (7–29)</td>
<td>51</td>
</tr>
<tr>
<td>2002</td>
<td>11% (5–20)</td>
<td>73</td>
</tr>
<tr>
<td>Overall</td>
<td>16% (11–22)</td>
<td>203</td>
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24°C, allowed to molt, and then tested as nymphs for evidence of infection by PCR. Approximately 5% of the I. dentatus nymphs, but none of the H. leporispalustris, were found to have retained infection through the molt (Table 3). Initial experiments were conducted at 18°C with no resulting infected nymphs, suggesting an extrinsic incubation temperature greater than 18°C. Unfortunately, most of the I. dammini larvae held at 24°C were killed by mold and could not be included in the analysis. These results support the conclusion that I. dentatus, but not H. leporispalustris, is a competent vector for B. divergens.

To incriminate the stage of I. dentatus that may be responsible for transmission of B. divergens, we attempted to correlate the infection status of the rabbits with the stage feeding on them (Figure 3). The mean number of ticks per rabbit for each stage each month was plotted on the same graph as the prevalence of active B. divergens infection. The two peaks of infection correspond well with the peaks of larvae suggesting that larval ticks are responsible for perpetuation.

**DISCUSSION**

Although B. divergens is currently considered to be solely endemic in Eurasia (and indeed, is legally excluded from importation into the United States in any form by the U. S. Department of Agriculture [USDA]), it appears to be enzootic in cottontail rabbits on Nantucket Island, Massachusetts. We sequenced large pieces of two genes, 1300 basepairs of the 18S rDNA and 1040 basepairs of the /H9252-tubulin gene, and found that the rabbit agent is most closely related to B. divergens. Although the 18S rDNA sequence is not identical to that of B. divergens from Europe, the small differences between the two fall well within sequence heterogeneity of ribosomal genes noted for other Babesia and Theileria species.22−25 Given the degree of 18S rDNA divergence among the three European cattle-derived strains that have been sequenced and whose sequences have been deposited in GenBank (Figure 2), we conservatively conclude that the few nucleotide differences that are apparent between the Nantucket parasites and the European B. divergens represent that which might be expected from allopatric populations. Re-
Our results suggest that the mode of perpetuation among rabbits may be similar and that *I. dentatus* may be the vector. Although all three species of ticks collected from rabbits yielded amplicons by the PCR, only *I. dentatus* was shown to maintain infection through the molt. Interestingly, there was no correlation between infection in nymphal *I. dentatus* (collected as replete larvae) and the infection status of the rabbit from which they derived, implying that the larvae were already infected before attachment. The monthly prevalence of *B. divergens* correlated well with the appearance of larval *I. dentatus* on rabbits (Figure 3). We have failed, to date, to document TOT because none of the *I. dentatus* larvae derived from females that had fed on PCR-positive rabbits have proven to be infected; however, our sample size was limited and tissues from newly eclosed larvae appeared to inhibit the PCR. An unfortunate episode of mold contamination prevented us from testing sufficient numbers of nymphal *I. dammini* derived from larvae engorging on rabbits. Further observations under controlled conditions, using laboratory-infected animals and specific pathogen-free colony-derived *I. dentatus* and *I. dammini* seem required for proof that perpetuation depends on TOT.

Although a few (about a dozen) head of cattle are present on a couple of Nantucket farms, these dairy animals are carefully maintained and are unlikely to represent the source of the rabbit *B. divergens*. Five years of sampling have shown that cottontail rabbits are consistently infected with *B. divergens* at a prevalence ranging from 11% to 23% each year, thereby excluding the possibility of an improbable, transient importation by ticks on birds. Although *B. divergens* has not been reported to infect European rabbits, the ecology of this parasite, particularly with respect to alternative reservoir hosts, remains poorly explored. We note that *B. leporis* was described from European hares, and a small *Babesia* was identified in rabbits from California. It may be that our findings represent a rediscovery of these agents, but for now a tentative identification can only be based on our phylogenetic analyses, which demonstrate that the Nantucket rabbit parasite is virtually identical to European *B. divergens*.

The public health significance of our finding remains unclear. *Babesia divergens* is the main source of human babesiosis in Europe. Although cases are extremely rare (22 since human babesiosis was first described in Yugoslavia in 1957), occurring only in splenectomized people, the course of disease is severe. Infection with *B. divergens* is considered a medical emergency, and a 38% case fatality rate has been observed. To date, there have been two cases in the United States consistent with the diagnosis of *B. divergens*. The first was a fatal case in a splenectomized man from Missouri. Parasites identified on the blood smear were indistinguishable from *B. divergens*, and the 200-basepair piece of 18S rDNA was also identical to that of *B. divergens*. Although referred to by the investigators as a new parasite, MO1, the evidence is problematic inasmuch as the long-held dogma of *Babesia* host specificity now seems untenable, particularly given the prevalence and diversity of human babesiosis.

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obtained from the blood of this patient is 100% identical to our sequence from rabbits, confirming the identity of this infection. Interestingly, this patient reported hunting rabbits prior to his illness. To date, only cases of babesiosis due to *B. microti* have been identified on Nantucket Island. Physicians and laboratory staff there are vastly experienced in the clinical presentation and diagnosis of babesiosis, and it seems unlikely that morphologically atypical parasites would be ignored. However, determination of the potential public health burden awaits serologic analysis with specific antigens, and a careful reanalysis of archived blood smears or frozen blood samples from babesiosis cases.

Intense enzootic transmission of *B. divergens* and other members of the microbial guild among rabbits is possible because all three stages of *I. dentatus* tend to concentrate their bites on rabbits, although birds are infested by subadult ticks. Because of this host specificity, it is often assumed that humans will not be exposed to *I. dentatus*-maintained agents. This assumption has not been rigorously examined. As part of a study of Lyme disease in a Maryland community, participants collected and retained for identification ticks that they found on their bodies. Surprisingly, there was one *I. dentatus* for every four *I. dammini* identified. Perhaps these ticks attack people more often than appreciated. Unlike deer ticks that quest on the vegetation in an edge area, rabbit ticks quest in the low grass, and people may encounter them on their well-manicured lawns. Furthermore, subadult *I. dammini* feed on rabbits and could serve as a bridge vector. We failed to amplify *B. divergens* DNA from questing nymphal ticks, either *I. dentatus* or *I. dammini*, but the sensitivity of the PCR in detecting *Babesia* within unfed ticks is limited (Telford SR, Persing DH, unpublished data). Thus, the risk of *B. divergens* transmission to humans remains to be defined.

How *B. divergens* became enzootic in Massachusetts, as well as in Missouri and Kentucky, is a matter of speculation. Cattle are not native to the Americas, and it may be that *B. divergens* was brought to the United States with the introduction of cattle. Beginning in the 1600s, cattle were imported into the United States, first from Mexico (where they had been introduced by the Conquistadors) and later from Europe and elsewhere. Once in America, the cattle were extensively cross-bred and shipped throughout the country. Most of the popular beef cattle bred in the United States today trace their roots to European stocks. These importation practices would have allowed for the efficient introduction and distribution of a cattle pathogen, particularly one that may cause chronic infection, throughout the country. There is no historical or other evidence, however, that cattle in the United States have ever sustained episodes of disease caused by *B. divergens*. The USDA has maintained intensive surveillance for outbreaks of bovine babesiosis since the description of the natural history of *B. bigemina* in 1893 by Smith and Kilbourne, and it is unlikely that outbreaks of redwater disease would be missed. Chronically infected, subclinical cattle might have remained unidentified frequently enough to allow the parasite to adapt to rabbits and their ticks. Among other possible hypotheses for the presence of *B. divergens* in American rabbits include introduction from Europe via the transport of infected *I. ricinus* by errant migratory birds; or the Arctic migration of infected Eurasian caribou. However, these hypotheses seem less probable than introduction within imported cattle.

There is great potential for wide distribution of *B. divergens* and the rabbit microbial guild in the United States. Subadult *I. dentatus* occasionally feed on migratory birds, allowing for wide dissemination of infected ticks. The range of cottontail rabbits or related species extends over most of the continental United States, providing susceptible hosts wherever the ticks are dropped. Alternatively, the rabbit-rabbit tick microbe guild could have dispersed to new areas with the game stocking programs of the early 1900s. During the 1920s and 1930s, 20,000 cottontail rabbits were shipped from Kansas and Missouri to Massachusetts to stock lands for sporting purposes, eventually competitively displacing the native New England cottontail, *Sylvilagus transitionalis*. This act introduced tularemia to Massachusetts.5,36 We surmise that the rabbit tick-maintained microbial guild was introduced as well.