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Research Article

Serosurvey for Select Pathogenic Agents, Particularly Eastern Equine Encephalitis and West Nile Viruses, in Free-Ranging Michigan White-Tailed Deer

Melinda K Cosgrove[1]*, Daniel J O'Brien[1], Ailam L Lim[2,3], Steven R Bolin[3] and Stephen M Schmitt[1]

[1]*Wildlife Disease Laboratory, Michigan Department of Natural Resources, USA*

[2]*Wisconsin Veterinary Diagnostic Laboratory, University of Wisconsin-Madison, USA*

[3]*Veterinary Diagnostic Laboratory, Michigan State University, USA*

***Corresponding Author:** Melinda K Cosgrove, Wildlife Disease Laboratory, Michigan Department of Natural Resources, Lansing, Michigan, USA.

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Abstract

In late summer 2005, an outbreak of Eastern equine encephalitis (EEE) in free-ranging white-tailed deer (WTD; *Odocoileus virginianus*) was described in Michigan, USA. Thereafter, subclinical infections of WTD were hypothesized to be more common than previously realized. Consequently, exposure of deer hunters and processors to EEE virus during butchering was plausible. Because the prevalence of EEE virus infection in Michigan WTD remained undescribed, a serosurvey was conducted in the autumn of 2005. Its principal objective was to assess infection rates of EEE in Michigan WTD in areas where clinical cases had been reported previously in deer, horses or humans. Serum was also opportunistically tested for antibodies to *Anaplasma phagocytophilum* (*A. phagocytophilum*), *Babesia bovis* (*B. bovis*), *Borrelia burgdorferi* (*B. burgdorferi*), bovine viral diarrhea virus (BVDV-1 and 2), *Rickettsia rickettsii* (*R. rickettsii*), and West Nile virus (WNV). One hundred thirty-eight hunter-harvested deer were surveyed in multicounty areas of the southwestern and northeastern Lower Peninsula. Diagnostic tests included plaque reduction neutralization, viral neutralization, and indirect immunofluorescent antibody assays. Overall prevalences for EEE, *A. phagocytophilum*, *B. bovis*, *B. burgdorferi*, BVDV-1 and 2, *R. rickettsia*, and WNV were 4.3, 1.4, 25, 7.6, 25 and 21, 0 and 63%, respectively. Significant regional differences were noted only for *B. bovis* and the BVDVs. In Michigan, the sporadic occurrence of clinical EEE in WTD did not coincide with high seroprevalence. Conversely, clinical cases of WNV have been rare despite the majority of deer having been infected. Hunter exposure to EEE virus in venison appears unlikely even in areas where clinical cases occur.

Keywords: *Babesia; Borrelia burgdorferi; Bovine Viral Diarrhea; Eastern Equine Encephalitis; West Nile Virus; White-Tailed Deer*

Abbreviations

BVDV: Bovine Viral Diarrhea Virus; C.I.: Confidence Interval; EEE: Eastern Equine Encephalitis; IFA: Immunofluorescent Antibody; MH: Mantel-Haenszel; MDNR: Michigan Department of Natural Resources; MSU-VDL: Michigan State University Veterinary Diagnostic Laboratory; NE: Northeast; NVSL: National Veterinary Services Laboratory; PRNT: Plaque Reduction Neutralization Test; SD: Standard Deviation; SW: Southwest; VN: Viral Neutralization; WNV: West Nile Virus; WTD: White-tailed Deer

Introduction

Background

The first clinical case of EEE infection in WTD was diagnosed in Georgia, USA, in 2001 [1] and Wisconsin diagnosed a single deer in 2004. Eastern equine encephalitis was documented for the first time in Michigan WTD in 2005 [2]. In total, seven clinical cases were diagnosed, presenting the first reported outbreak of EEE in WTD.

Significance

Because subclinical EEE infections of free-ranging WTD were hypothesized to be more common than previously realized [1], exposure of deer hunters and processors to EEE virus during butchering was plausible [2] and the prevalence of EEE virus infection in Michigan WTD remains undescribed. In addition, seroprevalence of EEE virus in WTD has been scrutinized for its potential value for monitoring virus activity in relation to public health [3-5]. Quantifying potential human and domestic animal exposure and further exploring the role of WTD in the ecology of all these pathogens remain topics of discussion [6-11].

Objectives of the Study

Consequently, a serosurvey was conducted in the autumn of 2005. The principal objective was to assess infection rates of EEE in Michigan WTD in areas where clinical cases had been reported previously in deer, horses, or humans. Serum was also opportunistically tested for antibodies to *A. phagocytophilum, B. bovis*, *B. burgdorferi*, BVDV-1 and BVDV-2, *R. rickettsii* and WNV.

Materials and Methods

Sample collection

Blood collection kits, containing a uniquely numbered 10 cc Vacutainer serum separator blood collection tube (Becton-Dickinson, Cockeyville, MD) and disposable gloves, were distributed to deer hunters in the southwestern Lower Peninsula of Michigan (hereafter, the southwest (SW) site: Cass, Kalamazoo, Kent, Montcalm, St. Joseph, and Van Buren counties; 41°48' - 43°30'N, 85°18' - 86°21'W). Hunters were asked to collect blood from the jugular vein, heart, or other large blood vessels immediately after harvesting a deer and return the blood sample to a Michigan Department of Natural Resources (MDNR) deer check station. In addition, serum samples collected during a separate study [12] in the northeastern Lower Peninsula of Michigan (Alcona, Alpena, Oscoda, and Montmorency counties: (44°40' - 45°00'N, 83°30' - 84°05'W) were also used as part of this survey. These counties were considered the northeast (NE) study site.

Location of harvest, gender, and age as determined previously [13], was recorded for each sample. Blood was centrifuged for 10 minutes at 1,163 X G and the serum decanted into 5-ml low temperature freezer vials at the MDNR Wildlife Disease Laboratory. Up to 5 mL of serum was sent to the United States Department of Agriculture's National Veterinary Services Laboratory (NVSL) in Ames, Iowa for EEE antibody detection. Any remaining serum was transferred to Michigan State University's Veterinary Diagnostic Laboratory (MSU-VDL) for detection of antibodies to *A. phagocytophilum*, *B. bovis.*, *B. burgdorferi*, BVDV-1, BVDV-2, *R. rickettsii* and WNV.

Serology

Serum samples were tested for viral neutralizing antibodies against EEE using standard plaque reduction neutralization test (PRNT) procedure at NVSL [14]. Serial 10-fold dilutions of each serum sample were assayed for inhibition of the infectivity of the reference strain EEE. Antibody titer was considered to be the highest serum dilution at which the cytopathic effect of the reference strain virus was completely inhibited. Samples were considered positive if any amount of EEE-specific antibody was detected.

At MSU-VDL, serum samples were tested for viral neutralizing antibodies against WNV, BVDV-1 and -2 using standard microtitration assay procedures [15]. The viral neutralization (VN) assay was conducted using Vero cells and a local WNV isolate (isolated from a puppy in Michigan [DCPAH LAR# 2841200]) was used as the reference strain. Serial 2-fold dilutions of each serum sample ranging from 1:8 to

1:8,192 were assayed for inhibition of the infectivity of the reference strain WNV on the Vero cells. Similarly, the VN assays for BVDV were conducted using bovine turbinate cells and cytopathic Singer as the BVDV-1 reference strain and cytopathic 125C as the BVDV-2 reference strain. The bovine turbinate cells and fetal bovine serum supplement used were free of adventitious BVDV and antibody against BVDV. Serial 2-fold dilutions of each serum ranging from 1:4 to 1:4,096 were tested for antibody against BVDV. Antibody titer was considered to be the highest serum dilution at which the cytopathic effect of the reference strain virus was completely inhibited. Serum was considered positive at ≥1:16 for WNV, BVDV-1 and BVDV-2.

Serum samples were tested for antibody against select bacterial pathogens and *Babesia spp.* using indirect fluorescent antibody (IFA) methods as recommend by commercial suppliers of antigen slides (*A. phagocytophilum* and *B. bovis*, Fuller Laboratories Inc., Fullerton, CA; *B. burgdorferi* and *R. rickettsia*, Veterinary Medical Research and Development Inc., Pullman, WA). Briefly, serial 2 fold dilutions of each sample of sera were made in phosphate buffered saline solution (0.01M, pH 7.4) and the 1:80, 1:160, 1:320, 1:640 dilutions were tested for antibody against *A. phagocytophilum*; the 1:40, 1:80, 1:160 and 1:320 dilutions were tested for antibody against *B. bovis* and against *R. rickettsii*; and the 1:160, 1:640, 1:1280 and 1:2560 dilutions were tested for antibody against *B. burgdorferi*. A fluorescein isothiocyanate labeled rabbit anti-deer IgG (Kirkegaard and Perry, Gaithersburg, MD) was used at a 1:50 dilution as the secondary antibody for detection of primary antibody from deer. Sample sera were considered positive at ≥ 1:40 for *R. rickettsii*, ≥ 1:80 *for A. phagocytophilum* and *B. bovis* and ≥ 1:640 for *B. burgdorferi* as specified by the commercial suppliers of the antigen slides.

Statistical analysis

Two-tailed Fisher's Exact Tests were used to test the strength of association between test-positive status and sex or harvest location for each disease agent using freely-available software (EpiInfo 7, Centers for Disease Control and Prevention, Atlanta, Georgia, USA). Samples from deer for which sex was unknown were excluded from gender analyses. Because both the number and size of cattle farms tend to be higher in southern Michigan [16], deer there have greater potential for exposure to cattle viruses. Consequently, a stratified Mantel-Haenszel (MH) chi-square analysis was used to control for the confounding effect of variation in cattle numbers on BVDV seroprevalence in WTD across the study sites. Mean herd sizes by county (< 50 head, ≥ 50 head) within each study site were used to define strata [16]. Ninety-five percent confidence intervals (C.I.) on prevalence were generated using the prop.test function in R (R Core Team, R Foundation for Statistical Computing, Vienna, Austria). For all analyses, significance was considered attained at $p \leq 0.05$.

Results

Serum samples were obtained from 138 deer; 84 (61%) were from the SW site. Age was unavailable for 31 (22%) deer, and gender unknown for 11 (8%). The remaining animals consisted of 7 (7%) fawns, 45 (42%) yearlings, and 55 (51%) adults. Mean (SD) age was 2.2 (1.8) years. Seventy-one (56%) were male.

Antibodies to EEE virus were detected in six (4.3% [95% C.I.:1.8, 9.6]) of 138 white-tailed deer sera. Five (83%) of the six were from the SW study site, although this difference was not statistically significant ($p = 0.4$). There was no significant difference ($p = 0.23$) in EEE seroprevalence by gender.

One hundred thirty-two serum samples were available for the remaining serological tests. No significant differences in positivity by gender were found for any of the remaining disease agents; nor were there significant differences by site for *A. phagocytophilum*, *B. burgdorferi, R. rickettsii* or WNV. Thirty-three (25% [18, 33]) samples were positive for *Babesia bovis*, with seroprevalence being significantly higher ($p = 0.002$) in the SW site. Two (1.5% [0.26, 5.9]) sera were positive for *A. phagocytophilum*, ten (7.6% [3.9, 13.8]) for *B. burgdorferi*, and 83 (63% [54, 71]) for WNV. Antibodies to *R. rickettsii* were not detected in any sample.

Twenty-two (17% [11, 24]) and 20 (15% [9.7, 23]) samples were positive for BVDV-1 and BVDV-2 antibodies, respectively. All seropositive deer were from the SW study site, hence seroprevalence was significantly higher there for both BVDV-1 ($p < 0.00001$) and BVDV-2 ($p = 0.0001$). Even after controlling for cattle numbers, deer harvested in the SW retained a significantly higher risk of being seropositive for both BVDV-1 (Odds Ratio $(OR)_{MH} = 31$ [9.9, 98]; $\chi^2_{MH} = 58.9$, $p < 0.00001$) and BVDV-2 ($OR_{MH} = 109$ [13, 889]; $\chi^2_{MH} = 61$, $p < 0.00001$) than deer from the NE site.

Discussion

Considerable attention has been devoted in the last decade to cervids (particularly WTD) and their role as hosts for arboviruses such as EEE and WNV. Studies have focused principally on vector ecology [17-21], serosurveys as indicators of virus activity and tools for public health surveillance [3-5,8,22-24] and reports of clinical cases and disease outbreaks [1,2,25,26]. Infections with EEE virus in white-tailed deer are not uncommon, with reported seroprevalence ranging between 1 and 14% [3-5], but clinical cases of EEE are rarely reported [1,2]. In Michigan, the sporadic occurrence of clinical disease in WTD and other species has not necessarily correlated with high seroprevalence. Although two counties (Kent, Montcalm) with 7 of the 8 clinical EEE cases in the September 2005 outbreak were within the SW site sampled two months later for this study, EEE virus seroprevalence in that region was only 6% (5/84 [2.2, 14]). Yet this region has been a persistent area of EEE activity for decades, with epizootics in horses reported there since the 1940's [27,28] and the first human case in 1980 [29]. Although NE Michigan is not historically known as an EEE endemic area [27], there was one equine fatality from EEE in that area in 2005 and one deer in this study with antibodies present. Similarly, there was little apparent association between clinical disease due to WNV and seroprevalence. Only one clinical case from Kent County (coinfected with EEE virus; [2]) was diagnosed in 2005 in spite of high WNV seroprevalence both in the SW (46/78 [59%]) and NE (37/54 [69%]) samples taken two months later. West Nile virus seroprevalence in this study was considerably higher than previous reports (0.9 - 13%; [8, 24]). During the intervening years since this study was conducted, our labs have confirmed an additional eight clinical cases of WNV, one each in 2006 and 2014, two in 2016 and four in 2015. One of the two deer in 2016 and three of the four deer in 2015 were coinfected with EEE virus. Six of the eight cases were from southwestern lower Michigan.

Despite expressed concerns regarding the potential for WTD to act as reservoirs of *Babesia spp*. [30], in contrast to EEE, little attention has been devoted to quantifying infection in free-ranging deer. Consequently, there is limited context for our results. Related to ongoing Texas Cattle Fever surveillance, infection rates for *Babesia bovis*, *B. bigemina,* and *B. odocoilei* were determined in 85 WTD in three counties in south and central Texas, where prevalence varied from 4-16%, 4% and 12-30% for the three agents, respectively [31,32]. Ramos., *et al.* [32] reported that 60% of tested deer were positive by screening PCR for all *Babesia spp.* Prevalence of antibody against *B. bovis* in our SW site (27/78; 35% [24, 46]) exceeds all of the Texas sites. However, no positive result was obtained on a screening PCR for all *Babesia spp.* performed on the sero-positive sera (data not shown). Despite the reported seroprevalence to *B. bovis*, the organism has not been detected in this region. In contrast*, B. odocoilei* has frequently been found in ticks collected from the southwest region of Michigan in recent years (Bolin SR personal communication). We speculate that the test subjects in our study may very likely have been infected with *B. odocoilei*, and the antibodies that we detected were simply cross-reactivity to *B. bovis*. Cross- reactivity of antibodies among closely related *Babesia* spp. have been reported [31,32]. Due to lack of knowledge of *Babesia spp.* in Michigan at the time of the study, the sera were not tested for *B. odocoilei.* In contrast to *Babesia spp.,* Stuen., *et al.* [11] documented seroprevalence of *A. phagocytophilum* in WTD ranging from 3 - 47%, easily exceeding our findings.

Reported seroprevalence of *B. burgdorferi* has varied widely [33-36], with the results from the current study falling at the lower end of that range. Our finding of six seropositive deer in our NE site is perhaps notable. While established populations of *Ixodes scapularis* (*I. scapularis,* which is the principal competent vector of *B. burgdorferi*) have yet to be documented in that portion of Michigan, its invasion is ongoing in the state [37]. These seropositive deer may indicate the presence of an undetected population of *I. scapularis*, adventitious ticks dropped from migrating birds [38], or the presence of a cryptic cycle of *B. burgdorferi*. The cryptic cycling of *B. burgdorferi* in the north-eastern Lower Peninsula may hasten the establishment of transmission cycles involving *I. scapularis* once the tick arrives [37]. Thus, these data provide a baseline for future comparison, and indication to public and domestic animal health officials that Lyme Disease should receive consideration in the differential diagnoses for patients in the area with consistent clinical signs.

A pair of recent reviews [7,39] have summarized epidemiological issues related to BVDV infections in free-ranging cervids and noted that wild populations with a high percentage of seropositive animals are generally in close proximity to cattle. This was also the case in our

study, although significant regional differences persisted even after controlling for cattle numbers. Reported seroprevalences in studied populations of WTD have varied widely, ranging from 0% [40] to 64% [41]. Infection rates in our SW site (20/78; 26% [17, 37] and 22/78; 28% [19, 40] for BVDV-2 and BVDV-1, respectively) were most consistent with prevalence found in southern Minnesota [42]. Several other studies that quantified the proportions of WTD persistently infected with BVDV by quantifying viral antigen in tissue samples noted predictably lower prevalence rates of 0 - 0.3% [43-46]. Although transmission of BVDV from cattle to WTD has been demonstrated via a natural challenge model [47], previous spatial analyses have also suggested that BVDV can be maintained independently by cattle and WTD populations [6]. If that situation holds in Michigan as well, it may help explain our findings.

Our study had a number of limitations which must be kept in mind. Our sample size was modest and only covered two regions of the state, so inference to other regions of the state is uncertain. However, as noted, the SW region of Michigan has historically shown the most clinical cases of EEE. Because we were primarily interested in potential recreational exposures to hunters from EEE, we chose to measure seroprevalence, and consequently did not obtain skin samples to determine the percentage of persistently infected BVDV cases. Because persistently infected animals are the epidemiologically critical shedders of the virus, and typically comprise a minority of all infected animals, our prevalence estimates for BVDV may overstate the importance of the disease in WTD. They do however point out their substantial exposure to this economically important cattle pathogen. In addition, our choice of stratified analysis as a method to control for the confounding effect of cattle numbers on BVDV seroprevalence was crude, and necessitated choice of a somewhat arbitrary cut-point for what constituted a 'large' potential exposure to cattle. More sophisticated analysis techniques such as multivariable modelling might better control for exposure of deer to cattle and would be appropriate in situations where studying BVDV was the principal aim. Finally, although a relatively high (≥ 640) IFA cutoff titer was chosen for *B. burgdorferi* to minimize false positive results, the possibility cannot be ruled out that cross-reactivity with other *Borrelia* spp. may account for some of the seropositive deer attributed to *B. burgdorferi*.

Conclusion

Our study contributes to the growing literature on WTD as potential sources and targets of disease agents shared with humans and domestic animals. With increasing documentation of the relatively common presence of EEE in WTD in some areas, it is prudent for wildlife managers, laboratory workers, and hunters to exercise care when handling nervous system tissues (e.g. when sawing off antlers) to avoid exposure to the EEE virus. In Michigan where both bovine tuberculosis and chronic wasting disease occur in free-ranging deer, hunters are already familiar with common personal protective equipment (such as wearing latex gloves) and hygienic butchering measures, and many have adopted practices necessary for their protection [48].

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Conflict of Interest

The authors declare no conflict of interest.

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