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Project Title: Powassan Encephalitis Virus in Deer Ticks (*Ixodes scapularis*) in Maine

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Summary

Ixodes scapularis ticks were collected from 30 towns in 11/16 Maine counties from June 2015-December 2016. Both *I. scapularis* nymphs (n= 230) and adults (n= 1499) were tested by polymerase chain reaction (PCR) for the presence of Powassan virus, subtype II (deer tick virus). In total, of 21 pools of *I. scapularis* nymphs tested, 2 were positive for the virus. Of the 203 pools of adults tested, 15 were positive for the virus. Positive pools were collected in southern regions of the state, in Wells (York County), Cape Elizabeth (Cumberland County), Standish (Cumberland County, Rockland (Knox County), and Augusta (Kennebec County). One positive pool was collected on Swan's Island (Hancock County), although no positive samples were found east of Mount Desert Island or in northern Maine, despite high numbers of ticks collected in Washington and Hancock Counties. In addition to documenting the presence of the virus in *I. scapularis* in Maine, this study also demonstrates the growing population of ticks present in eastern and western regions of Maine.

Objectives

We feel, based on the results determined so far, to have achieved these objectives proposed for this project:

- Collect up to 400 field derived ticks and test for the presence/prevalence of POW virus by RT-PCR.
- Assess the importance of the adult stage of the deer tick as vector of emerging tick-borne diseases such as POW.
- *Document study results as a technical report or peer-reviewed publication*.*

*Awaiting further analysis before drafting publication

Background

In the northeastern United States, the deer (or black-legged) tick *Ixodes scapularis* is known to transmit up to five bacterial or viral agents to people, companion animals, and livestock^{1,2}. While some, such as Lyme disease, have been a recognized public health concern for over two decades, other such as human babesiosis and anaplasmosis are now emerging in northern New England states with greater rapidity³. The most clinically serious of these 'new' agents, Powassan encephalitis virus (POWV), has begun to be more recognized in the last five years⁴, with recent reports, including one fatality, occurring in Maine⁵.

Powassan encephalitis virus was initially known from northern North America, particularly in areas of central and eastern Canada, the upper Midwest of the United States, and, prominently, the northeastern US^{6,7}.

The arthropod vectors of POWV are hard ticks, primarily of the genus *Ixodes* with reservoirs including rodents and mustelids⁸ (Ebel 2010). POWV has, to date, been found as two ecologically separate lineages: the first lineage, commonly known as traditional

Powassan, occurs in a cycle involving *Ixodes cookei* Packard and *Ixodes marxi* Latreille and their hosts, mustelids and sciurids, respectively⁸. The second lineage, referred to alternatively as deer tick virus (DTV) is transmitted by *Ixodes scapularis* through its most commonly reported reservoir host, *Peromyscus leucopus*⁹.

The range of the black-legged (or deer) tick *Ixodes scapularis* has expanded in eastern North America since the 1970's¹⁰, with reported cases of Lyme disease, anaplasmosis, and babesiosis correlated with the tick's establishment¹¹⁻¹³ (Rand et al. 2007, Walk et al. 2009, Prusinski et al. 2014). Much of southern New York state and southern New England are considered endemic for both Lyme disease and its vector (Hanincova et al. 2006) while northern regions extending in Canada are still recording the fluid distribution of the tick as it increases its range (Rand et al. 2007, Leighton et al. 2012). Both *I. cookei* and *I. marxi* populations have appeared stable while the expansion of *I. scapularis* has occurred (Smith et al. 1993, Rand et al. 2007).

Historically, cases of POWV are found in Canada (Maclean and Donahue 1959) and the northern United States, with foci in the upper Midwest and the northeast (Hinten et al. 2008). Clinically, presentations include encephalitis, meningoencephalitis, and meningitis, with an average incubation period of 8-30 days. Lesser symptoms may include headache, disorientation, and limb weakness. On average, the virus has a 10% case fatality rate (Smith et al. 1974). By many recent reports, POWV is considered an emerging infectious disease (Hinten et al. 2008), with many of the recent cases of human infection thought to be associated with the DTV lineage (Ebel 2010, Armstrong and Anderson 2012). From 1999-2004, five cases of non-fatal POWV were reported from northern New England (Hinten et al. 2008). Although anecdotal evidence, such as positive wildlife serology, hinted at POW

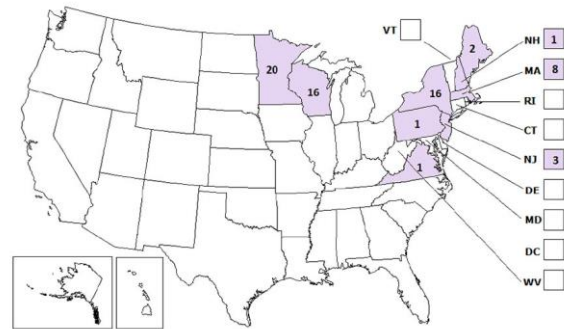


Figure 1. Powassan case reports from the United States, 2006-2015. Courtesy of US CDC.

lineage 1, no confirmation by strain typing documented which lineage of Powassan was cycling. More recently, a midcoast Maine resident died in 2013, following a bite of a fall-derived female *I. scapularis*.

Strain typing from autopsied samples linked the death to the DTV lineage of POWV (Cavanaugh et al. in press). Since that time, two additional cases of Powassan encephalitis virus have been reported from Cumberland County in 2015 and 2016, respectively. While neither case is fatal, both appear to have been acquired during the late fall season, when adult *I. scapularis* are most active. Consequently, in addition to sampling near sites of the 2013 cases, we were able to survey near the 2015 cases well for this project.

After the initial report of POWV found in the sera of deer found in New England (Nofchissey et al. 2013), and of its widespread presence in the environment of other northeast states (Dupuis et al. 2013), we followed this survey with a subsequent examination to document the distribution of POWV in questing *I. scapularis* across Maine from 2015-2016.

Materials and Methods

Field Methods. Sampling sites were chosen based on two existing criteria – 1) Known presence of *I. scapularis* ticks, which accounted for many of the sites in southern and central Maine, and 2) Sites chosen based on habitat deemed suitable for survival of *I. scapularis* populations, which was used when sampling northern, northwestern, and eastern Maine. Two regions, one in Maine's midcoast and the other in interior Cumberland County, were chosen because of proximity to reported cases of POWV. Table 1 lists sampling sites and latitude/longitude readings where ticks were collected.

Field workers flagged ticks using a 1m² corduroy flag dragged over leaf litter and brush during the nymphal season (June-July) at 15°-20°C, and in the fall adult season (October-November) at ≥10°C. Ticks were transported back the laboratory in live vials or vials filled with ETOH, and stored at 4°C.

Ticks were sorted by sex, life stage, collection site, and date collected. Each tick was placed in its own 2ml plastic centrifuge tube and immediately processed for RNA isolation.

RNA Isolation and Polymerase Chain Reaction. Individual ticks were crushed using a sterile paperclip then homogenized in 300µl TRIzol reagent (Invitrogen) with 3-5 stainless steel BB's. After homogenization, 100µl of each sample was pooled into groups of up to 10 adult ticks, 20 nymphs, and 50 larvae. Pools were adjusted to a total volume of 1,000µl TRIzol® reagent, and RNA was extracted following standard protocol (Chomczynski and Sacchi, 1987). RNA pellets were stored in 75% ethanol at -80°C and dissolved in 25ml nuclease free water (GrowCells)

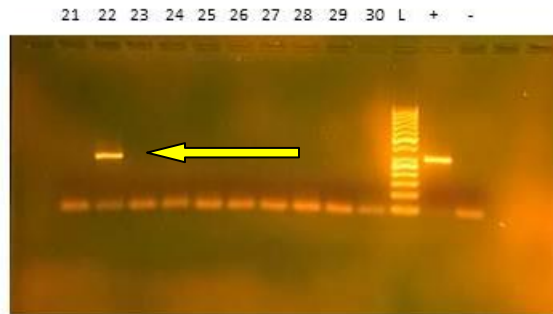


Figure 2. PCR gel of tick samples tested for Powassan virus. Positive and negative controls are on the right side of the 50bp ladder. Positive samples are highlighted by arrow.

for use in cDNA synthesis. Individual homogenates were stored in the remaining volume of TRIzol reagent at -80°C.

PCR was performed using primers previously designed to amplify both lineages of Powassan virus (Telford et al., 1997). Briefly, approximately 100ng of total RNA was used in a SuperScript™ III one-step RT-PCR reaction with Platinum™ Taq DNA polymerase following standard protocol (Invitrogen). The reaction mix was cycled for 30min at 50°C followed by 15min at 95°C and 40 cycles of 30sec at 94°C, 30sec at 55°C, and 30sec at 72°C with a final extension of 5min at 72°C. cDNA was run on a 2% agarose gel and visualized using ethidium bromide staining (Fig. 2).

Results

During the summer season of 2015 and 2016, nymphal ticks (n=250) were sampled and gathered into 22 pools, based on site and date of collection, from communities in southern Maine (Table 2). Five positive pools of nymphal *I. scapularis* were found in Standish, Cape Elizabeth, and Rockland. In both years, summer conditions were particularly dry, limiting collection of ticks during the summer season. Highest collection rates for nymphs occurred along the coast, with the sites in Rockland and Wells higher (12-23 ticks/hr) than interior areas such as Augusta and Sidney (5-8 ticks/hr).

Adult ticks, more resistant to dessication, were sampled during their seasonal peak (early October to mid-November) in 2015 and 2016. Of the 165 pools of 1161 adult ticks collected statewide (Table 3), 13 were positive for POWV. Positive samples were found in Wells, Cape Elizabeth, Standish, Rockland, Thomaston, Edgecomb, Augusta, Vassalboro, Randolph, and Swan's Island, primarily found in 2016.

Despite dry conditions in the previous summers, adult collections were robust in both years. While previous studies have documented the tick's presence in coastal areas north to southern Penobscot County, this study marks the first substantial collections from the Moosehead Lake region in Piscataquis County, the Machias area in Downeast, ME and northwestern Maine in Somerset County (Table 1).

Of particular interest was the presence of different positive bands on a small number of gels. Each row of bands should represent a particular strain of POWV, either the classic Type I or the Type II (deer tick virus strain) (Fig. 2). Finding two different bands may indicate that we have both strains currently active in Maine, something not reported for other New England states⁴.

In total, 1,729 *I. scapularis* were tested for this project (over 4x the amount proposed). As a consequence, the infection rates of POWV in individual ticks is taking far

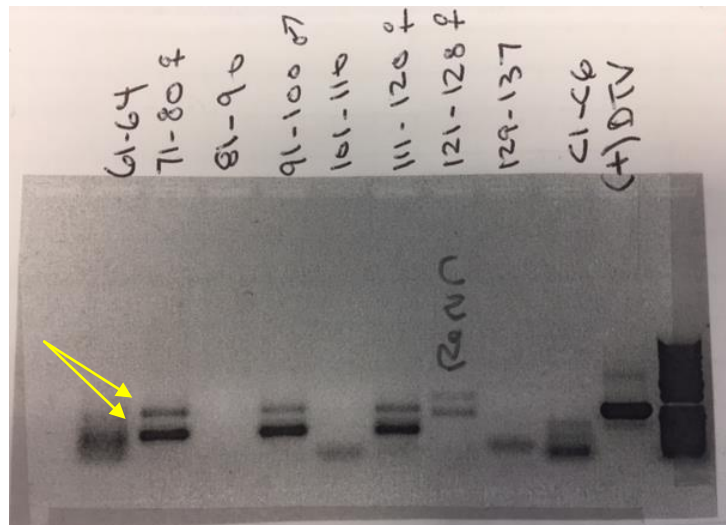


Figure 2. PCR gel delineating two positive bands, indicating two possible lineages (strains) of POWV in sample.

longer than expected, and will be sent as an addendum later in the spring of 2017.

Discussion

Based on data derived from this study, in addition to previous serological work on cervids, we have found that Powassan virus is readily present in areas of Maine, including in areas outside where human cases are reported from. Its detection in vector ticks, following three human case reports, indicates that this virus is a public health issue in Maine, and should be included in public information and outreach on tick-borne diseases, and may also be considered when the benefit of tick management programs are weighed against the costs of such programs.

When comparing rates of positivity between nymphal and adult cohort ticks, we have found infection both cohorts. Traditionally, nymphs, active June-August, have been the primary life stage of concern for tick-borne diseases such as Lyme disease and babesiosis¹. Their small size does not usually allow for detection before bacterial pathogens are transmitted.

However, recent cases of POWV from the northeast indicate that a large percentage POWV cases are diagnosed in the fall or early spring, during the adult tick season¹⁴. We found that 7% of the pools of adults tested positive for the virus. This is slightly lower than the rate of infection in pools of nymphs (9%), but the sample of positive adults was found over a larger area and encompassed more ticks tested, including sites with positive ticks near Augusta and Acadia National Park. Positive pools of nymphs were found in coastal areas, where *I. scapularis* has been long established (Wells, Cape Elizabeth), despite sampling in other towns such as the Islesboro, Augusta, and Rockland. It is expected that adults would be infected at the same rate or higher than nymphs because of their longer life and greater chance of biting and infected host (S Telford, pers. comm.). Larger sampling sizes of nymphs and the eventual infection rate examination may resolve this discrepancy. Not surprising, positive adults were also found in proximity to where human POWV cases were reported in 2013 (midcoast) and 2015 (interior Cumberland County).

Regionally, POWV cases have been reported from other northeastern states, notable VT, NH, upstate New York, and Massachusetts (Fig. 3). The results of this study indicate that these states, as well as neighboring Maritime Provinces of Canada may wish to consider the possibility that POWV is in their *I. scapularis* populations^{15,16}. While many early cases of POWV infections have been attributed to the Type I lineage of Powassan, our recovery of two positive bands on the PCR gel, in addition to findings of the Maine 2013 fatality, means that both lineages (strains) cycle here in Maine⁴⁻⁶.

Interestingly, while not found on the mainland areas of Mount Desert Island, one pool of adult ticks tested positive from Swan's Island, a site where Lyme disease has emerged in the last decade. The appearance of the virus on an offshore island highlights the importance of birds as a transport mechanism for ticks across great distances during spring migration^{18,19}.

One aim of this study was to determine if adult ticks, normally not highlighted for public health messaging about tick-borne diseases, are an important concern for POWV. Because of the epidemiological evidence that three Maine cases of POWV were contracted during the adult ticks seasons from 2013-2016, and 7% of the adult *I. scapularis* collections were positive for the virus, there should be increased public awareness of the adult tick population, particularly in fall (October to December) and early spring (March-April). In addition to homeowners working in the peridomestic environment during this time (raking

leaves) high exposure also occurs to hunters and hikers (and their companion animals) during this time of year. Rand et al. found that adult ticks also tend to attach to longer periods to two particular age cohorts – youth and older adults, indicating a greater risk of bacterial pathogens to these age groups from the bites of adults¹¹. However, as POWV has been shown, in the lab, to be transmitted in under an hour, this should be of concern to anyone spending a large amount of time outdoors, where ticks are encountered. As a consequence, personal protection measure, such as application of repellents and deterring attachment of ticks by closing gaps in clothing (cuffs of pants, etc) should be strongly advised for recreation and enforced for outdoor workers to reduce tick exposure.

Conclusions

- This pilot study demonstrates that Powassan virus is present in *Ixodes scapularis* ticks in Maine, adding this virus to a list of pathogens carried by these ticks.
- Powassan virus was found across southern Maine at several sites, but was not detected yet in northern or eastern Maine.
- Unlike bacterial pathogens, where nymphal deer ticks are the primary public health concern, adult ticks may be as important as nymphs for the transmission of Powassan virus.

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Tables

Table 1. Sites sampled for *Ixodes scapularis* in Maine, 2015-2016.

Site Name	Town	County	Latitude	Longitude
Aroostook State Park	Presque Isle	Aroostook	46.620925°	-68.013134°
Crescent Beach State Park	Cape Elizabeth	Cumberland	43.563616°	-70.236418°
Steep Falls Wildlife Management Area	Standish	Cumberland	43.828832°	-70.607335°
Pond Road	Standish	Cumberland	43.789224°	-70.499918°
Ferry Terminal	Swan's Island	Hancock	44.182936°	-68.421867°
Sand Beach (ANP)	Bar Harbor	Hancock	44.329644°	-68.180340°
Blagden Preserve	Southwest Harbor	Hancock	44.38725°	-68.369909°
Hull's Cove (ANP)	Bar Harbor	Hancock	44.408376°	-68.246220°
Savage Park	Augusta	Kennebec	44.335586°	-69.761764°
Reynolds Forest	Sidney	Kennebec	44.420607°	-69.713352°
Davidson Preserve	Vassalboro	Kennebec	44.452028°	-69.643813°
Gauge Trail	Randolf	Kennebec	44.232560°	-69.757246°
Oyster River Bog	Rockland	Knox	44.122686°	-69.153276°
Highland Path	Thomaston	Knox	44.080791°	-69.195071°
Waterman's Beach	South Thomaston	Knox	44.026052°	-69.125408°
Marsh River Preserve	New Castle	Lincoln	44.028597°	-69.555412°
River Link Trail	Edgecomb	Lincoln	44.012173°	-69.594916°
Zak Preserve	Edgecomb	Lincoln	43.944502°	-69.614642°
Dodge Point	Edgecomb	Lincoln	43.992435°	-69.566354°
Brownfield Bog Wildlife Management Area	Brownfield	Oxford	43.978986°	-70.932516°
Richardson Tract	Orrington	Penobscot	44.685818°	-68.742872°
Delano Wildlife Management Area	Monson	Piscataquis	45.336698°	-69.556762°
Little Moose Preserve (MPRL)	Moosehead Junction TWP	Somerset	45.478377°	-69.680279°
Pomerleau Park	Jackman	Somerset	45.628668°	-70.262053°
Robbins Hill Rest Area	Solon	Somerset	44.893396°	-69.811877°
Turtle Head Preserve	Islesboro	Waldo	44.389870°	-68.880302°
Machias River Preserve	Machias	Washington	44.714030°	-67.484145°
Outback Trail	Machias	Washington	44.707107°	-67.454848°
Wells Reserve	Wells	York	43.336630°	-70.545899°
Rachel Carson National Wildlife Refuge	Kittery	York	43.093330°	-70.664720°

Table 2. Results of PCR testing for POWV on *Ixodes scapularis* nymphs in Maine, 2015-2016.

Town	Lifestage	# Ticks	Pools Tested	Positive Pools	% Positive Pools
Islesboro	nymph	5	1	0	0
Wells	Nymph	68	6	1	16
Cape Elizabeth	Nymph	84	6	1	16
Augusta	Nymph	24	3	0	0
Standish	Nymph	9	2	0	0
Rockland	Nymph	40	3	0	0
		230	21	2	

Table 3. Results of PCR testing for POWV on *Ixodes scapularis* adults in Maine, 2015-2016.

Town	Lifestage	# Ticks	Pools Tested	Positive Pools	% Positive Pools
Orrington	adult	62	7	0	0
Brownfield	adult	20	2	0	0
Kittery	adult	59	7	0	0
Moosehead TWP	adult	14	2	0	0
Jackman	adult	10	1	0	0
Long Island	adult	20	2	0	0
Machias	adult	34	6	0	0
Mt. Desert Island	adult	50	5	0	0
Swans Island	adult	93	10	1	10
West Forks	adult	6	2	0	0
Wells	Adult	376	50	6	12
Cape Elizabeth	adult	242	33	4	12
Augusta	Adult	106	16	0	0
Standish	Adult	115	15	2	13
Rockland	Adult	292	45	2	4
Total		1499	203	15	

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