Prevalence and Genetic Characterization of Powassan Virus Strains Infecting *Ixodes scapularis* in Connecticut

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Abstract. A total of 30 Powassan virus (POWV) isolates from *Ixodes scapularis* collected from Bridgeport and North Branford, CT in 2008, 2010, 2011, and 2012 and one earlier isolate from *Ixodes cookei* collected in Old Lyme, CT in 1978 were characterized by phylogenetic analysis of their envelope gene sequences. Powassan virus sequences segregated into two major groups termed the deer tick virus (DTV) and Powassan (POW) lineages. The lineage from *I. cookei* was POW. The remaining viruses from *I. scapularis* grouped with the DTV lineage. Powassan viruses from Bridgeport were nearly identical and clustered with a virus strain from a human in New York. Viruses from North Branford were homogeneous and grouped with viruses from Massachusetts, northwestern Connecticut, and Ontario. These findings suggest that POWV was independently introduced into these geographical locations in Connecticut and maintained focally in their respective environments. An improved method of isolation of POWV *in vitro* is described.

INTRODUCTION

Powassan encephalitis is a relatively rare but serious infection that has been documented in Canada, the United States, and eastern Russia and is caused by Powassan virus (POWV), *Flaviviridae: Flavivirus*, the only known member of the tick-borne encephalitis serologic complex in North America.¹ The index case was a child who died in the town of Powassan, Ontario, Canada in 1958.² Since that time, there have been at least 70 human cases, primarily in the states of New York, Minnesota, Wisconsin, Maine, Vermont, and Pennsylvania and in the Canadian Provinces of Ontario, Quebec, and New Brunswick.^{3–7} Powassan virus comprises two distinct genotypes, each with a distinct natural history and are known as POW (lineage 1) and deer tick virus (DTV) (lineage 2).^{8–10} The POW lineage is maintained in an enzootic cycle involving mainly *Ixodes cookei* and *Ixodes marxi* and medium-sized mammals, such as red squirrels, *Tamiasciurus hudsonicus*, groundhogs, *Marmota monax*, and skunks, *Mephitis* sp.,^{1,11} whereas the DTV lineage has been isolated primarily from *Ixodes scapularis*.¹ Both lineages have been isolated from ticks,^{11,12} and both have been isolated or detected in tissues from fatal human cases of encephalitis.^{2,13}

Despite the absence of reported human POWV cases in Connecticut, human disease has been recognized in the adjoining state of New York, and the virus has been isolated from ticks in Connecticut.^{11,12} In this study, we report 30 additional isolations of POWV from *I. scapularis* from two distinct geographical areas in Connecticut, an improved *in vitro* method for the isolation of this virus from ticks, the stability of two distinct genetic strains of POWV from two geographically separated populations over multiple years, and the focal nature of POWV.

MATERIALS AND METHODS

Tick collections. *Ixodes scapularis* adults were collected by dragging a flannel cloth over low-lying vegetation and remov-

ing the ticks on the cloth at two geographical separate locations in Connecticut: Lake Success Business Park in Bridgeport in southwestern, Connecticut (Fairfield County) and at the South Central Connecticut Regional Water Authority property surrounding Lake Gaillard in North Branford in south-central Connecticut (New Haven County). About 40 km separate the two sites. White-tailed deer, *Odocoileus virginianus*, are relatively abundant at both locations: i.e., 25 deer/km² in Bridgeport in the fall of 2002,¹⁴ and 40 deer/km² were reported in North Branford in 2003.¹⁵ The Bridgeport site is a privately owned 176 ha tract with mixed deciduous woodland, a lake, wetlands, and open fields.¹⁴ The North Branford site is mixed deciduous woodland with stone walls and open fields surrounding the lake.¹⁶ Adult ticks were collected in October through December of 2008, 2010, and 2011, and during April and May in 2011, and March and April in 2012. Host-seeking nymphs were collected by the same procedure described for adult ticks during May and June 2008 from two locations in Fairfield, two locations in Middlesex, and three locations in New London Counties, Connecticut. Additionally, adult ticks were removed from deer killed during the hunting season in November and December 2008 in New London, Hartford, Tolland, and Windham Counties, Connecticut.

Virus detection and isolation. Individual unfed nymphs or adults, which were alive or killed in the laboratory and frozen at –70°C, were placed in 0.5 mL microcentrifuge tubes and crushed in 50 µL phosphate-buffered saline (0.3% gelatin, 30% rabbit serum, and 1% 100 × antibiotic-antimycotic [10,000 units/mL of sodium penicillin G, 10,000 g/mL of streptomycin sulfate, and 25 µL/mL of amphotericin B; Invitrogen, Carlsbad, CA] in 0.85% saline) (PBS-G). A previously unused jumbo paper clip (5 cm size, Charles Leonard Inc., Hauppauge, NY) was used to crush each tick. Partially fed or fully fed females were ground in 250 µL PBS-G using a mortar and pestle. Ten µL of homogenate from each male, unfed female, or nymph were combined with 10 µL of homogenate from 10 other ticks to form a single pool (numbers of ticks per pool occasionally were fewer or greater than 10).¹⁷ Fed or partially fed females removed from deer were tested singly. The RNA was extracted from the homogenates according to the manufacturer's instructions using the QIAamp viral RNA mini kit protocol (Qiagen, Valencia, CA). A negative control of nucleic acid free, sterile water (Thermo Fisher Scientific Inc.,

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Waltham, MA) was used. The positive control was a 1:100 dilution of POWV (Byers Strain).

RNA from each homogenate was added to the primers and to the reagents in the Titan One Tube RT-PCR System (Roche Diagnostics, Indianapolis, IN). The primers were POW-6 (5'TT GTGTTTCCAGGGCAGCGCCA3') and ENV-A (5'GTCGA CGACGAGGTGCACGCATCTTGA3').¹² The polymerase chain reaction (PCR) mixture was pre-incubated in an Eppendorf Mastercycler (Applied Biosystems, Carlsbad, CA) at 50°C for 30 min, denatured at 94°C for 2 min, and cycled 40 times at 94°C for 15 sec, 56°C for 30 sec, and 68°C for 2 min, and then extended at 68°C for 5 min. Amplicons and an accompanying DNA ladder were visualized on a 1.5–2% agarose gel by ethidium bromide staining.

Ticks in each pool identified as containing virus were then tested individually. An additional 60 µL of PBS-G were added to each original tick homogenate. The tick was ground a second time. The RNA was extracted from each tick and tested as described previously for virus. Isolation of virus was then attempted from the homogenate of each reverse transcription (RT)-PCR-positive tick by adding 70 µL of PBS-G to the pulverized tick and again grinding the homogenate. One hundred µL of each homogenate was added to a newly confluent layer of baby hamster kidney (BHK-21 [C-13]) cells (ATTC, Manassas, VA) that had been growing in 4 mL of Minimum Essential Medium containing fetal bovine serum, glutamine, and antibiotic-antimycotic (Invitrogen) in a 25-cm² flask kept in an incubator set at 37°C with an atmosphere of 5% CO₂. Adsorption of virus onto the cells was aided by removing the growth medium, and then adding the tick-homogenate, and rocking the cell-virus mixture for 5 min. Four milliliters (mL) of growth medium was added to each flask, which was returned to the incubator. Cells were examined daily for cytopathogenic effects (CPE) 3–7 days after inoculation.

We suspected that we may not have been identifying all infected ticks by the procedure described previously. We therefore tested 1,184 ticks collected in the spring and fall of 2011 by

each of the following three methods: 1) ticks were tested by the procedure described previously; 2) the same ticks were rescreened for virus by pooling 10 µL homogenates from each of 10 ticks and directly inoculating 100 µL onto a newly confluent layer of BHK-21 cells and growing and examining those cells for CPE as described previously; and 3) the same ticks were again rescreened by pooling 10 µL homogenates from each of 10 ticks and inoculating the pooled homogenate onto a newly confluent layer of BHK-21 cells. The cells were grown for 4–5 days, and without examination for CPE, RNA was extracted from the cell medium and tested for POWV by RT-PCR. Each tick from virus-positive pools was then tested individually as follows. Seventy microliters (µL) of PBS-G were added to each of the pulverized ticks comprising each RT-PCR-positive pool. The homogenate of each tick was added to a newly confluent layer of BHK-21 cells, and the cells grown for 4–5 days. RNA was extracted from the cell medium of each inoculated flask and tested for POWV by RT-PCR.

Genetic analysis. After purification with the QIAquick PCR Purification Kit (Qiagen), the amplified 689 base-pair fragment of the envelope gene of each virus isolate was sequenced at the DNA Analysis Facility on Science Hill at Yale University. In addition, we sequenced this same gene region from a POWV isolate (AR-218-78) that was previously recovered from *Ixodes cookei* ticks collected in Old Lyme, CT during 1978.¹¹ Edited sequences were deposited in GenBank (accession nos. JX170765-JX170795). Phylogenetic relationships of one isolate of POWV from *I. cookei* and 30 isolates from *I. scapularis* collected in Connecticut were compared with each other and to 24 other POWV published GenBank-sequences from humans, ticks, and from wild mammals originating in the United States, Canada, and Russia (Table 1), and to one isolate of tick-borne encephalitis virus obtained from the Siberian region of Russia (GenBank no. AB049353). Nucleotide sequences were translated into protein and aligned by the ClustalW algorithm to preserve the integrity of codon positions. Aligned nucleotide sequences were cropped to a common length of

TABLE 1
Previously published POWV sequences used in this study

Strain	Host	Collection location	Year	GenBank no.
791A-52	<i>Dermacentor andersonii</i>	Colorado, USA	1952	AF310922
LB	Human	Powassan, Ontario, Canada	1958	NC003687
M1409	Tick	Ontario, Canada	1960s	AF310912
1427-62	Red squirrel	Ontario, Canada	1962	AF310914
64-7483	Unknown	New York, USA	1964	AF310916
64-7062	Tick	New York, USA	1964	HM440563
1982-64	Woodchuck	Ontario, Canada	1964	AF310913
M8998	Unknown	Ontario, Canada	1964	AF310911
M11665	Tick	Ontario, Canada	1965	AF310910
Spassk-9	<i>Dermacentor silvarum</i>	Spassk, Russia	1975	EU770575
12542	Fox	West Virginia, USA	1977	AF310920
T18-23-81	<i>Ixodes cookei</i>	Ontario, Canada	1981	AF310909
Nadezdinsk	Human	Nadezdinsk, Russia	1991	EU670438
IPS001	<i>Ixodes scapularis</i>	Ipswich, Massachusetts, USA	1994	AF310918
CTB30	<i>Ixodes scapularis</i>	Connecticut, USA	1994	AF310919
NFS001	<i>Ixodes scapularis</i>	Nantucket, Massachusetts, USA	1996	HM440559
R59266	Human	Ontario, Canada	1997	AF310917
SPO/B10	<i>Ixodes scapularis</i>	Spooner, Wisconsin, USA	1997	AF310921
WICF9901	<i>Ixodes scapularis</i>	Chippewa Falls Wisconsin, USA	1999	HM440558
Partizansk	Human	Partizansk, Russia	2006	EU543649
DT-NY-07	Human	New York, USA	2007	EU338402
DTVWIB08	<i>Ixodes scapularis</i>	Spooner, Wisconsin, USA	2008	HM440561
DTVWIA08	<i>Ixodes scapularis</i>	Spooner, Wisconsin, USA	2008	HM440560
DTVWIC08	<i>Ixodes scapularis</i>	Spooner, Wisconsin, USA	2008	HM440562

609 bps and analyzed by the neighbor-joining method using Molecular Evolutionary Genetics Analysis (Mega 5.0). This analysis used the maximum composite likelihood model, and support for each node was evaluated by performing 1,000 bootstrap replicates.

RESULTS

A total of 30 (18 females and 12 males) isolates of POWV was made from 1,911 adult host-seeking *I. scapularis* collected in 2008, 2010, 2011, and 2012 in Bridgeport and North Branford, CT (Table 2). Aliquots of infectious growth medium of each of the 30 isolates were frozen at -70°C . Percent infection ranged from 0 in the fall of 2008 in North Branford, CT to 4.2% in the spring of 2011 in North Branford. Virus was isolated from adult ticks collected in the fall of 2008, 2010, and 2011 and in the spring of 2011 and 2012. Virus was not isolated from 99 *I. scapularis* nymphs collected from seven different locations in Fairfield, Middlesex, and New London Counties and from 81 adult *I. scapularis* collected off white-tailed deer from 28 different locations in New London, Hartford, Tolland, and Windham Counties in Connecticut in 2008.

In 2011, we initially isolated POWV from 1 of 1,184 ticks tested by the procedure of pooling and screening 10 ticks at a time by RT-PCR, visualizing the amplified 689 base-pair fragment of the envelope gene, testing each of the 10 ticks from the positive pool by RT-PCR, and then isolating the virus by placing the homogenate of the positive tick onto a newly confluent layer of BHK-21 cells. We also placed pooled homogenates from these 1,184 ticks directly onto BHK-21 cells and examined them daily 3–7 days after inoculation for CPE. No isolation was made. Additionally, we inoculated the pooled homogenates of the 1,184 ticks onto BHK cells again, withdrew 70 μL of growth medium 3–5 days after inoculation, extracted the RNA, amplified the 689 base-pair fragment of the envelope gene, and visualized the amplicon on a 1.5–2% agarose gel by ethidium bromide staining. Homogenates of each of the 10 ticks in a positive-pool were inoculated onto a newly confluent layer of BHK-21 cells, and after 3–5 days, 70 μL of growth medium was withdrawn and tested by RT-PCR as described previously for the pooled specimens. Nineteen isolations were made from the 1,184 specimens tested by this method.

The 30 POWV isolates from Bridgeport, CT and North Branford, CT and one earlier isolate from Old Lyme, CT were subsequently characterized by phylogenetic analysis of their envelope gene sequences (Figure 1). The POWV sequences segregated into two major, monophyletic groups

termed the DTV and POW lineages. These data are consistent with previous analyses.^{8,10,18} The POW lineage included the virus isolated from *I. cookei* collected in Old Lyme, CT during 1978. The remaining Connecticut viruses were isolated from *I. scapularis*, and all grouped within the DTV lineage. Powassan virus sequences obtained from Bridgeport, CT during 2008, 2010, 2011, and 2012 were genetically identical or nearly identical to each other and clustered together with a virus strain detected from a fatal human case in New York with 92% bootstrap support. Viruses originating from North Branford, CT during 2010 and 2011 formed a genetically homogeneous subclade and together, they grouped with viruses obtained from Massachusetts, northwestern Connecticut, and Ontario, Canada with 85% bootstrap support. North Branford, CT viruses differed from Bridgeport, CT viruses by nine nucleotide substitutions and one encoded amino acid change within the sequenced portion of the envelope gene.

DISCUSSION

Our isolation of POWV from 30 individual adult *I. scapularis* is the largest reported number of isolates of this virus-complex to date. All isolates belonged to the DTV lineage, but the isolates from the Bridgeport, CT site from 2008 to 2012 were genetically nearly identical to each other and clustered with POWV detected from a human in New York state. By contrast, the isolates from 2010 and 2011 from North Branford, CT were genetically similar to one another but distinctly different from the isolates from Bridgeport, CT and clustered with a previously published isolate from northwestern Connecticut,¹² Massachusetts, and Ontario, Canada. The isolation of two distinct subclades of POWV from collection sites 40 km apart was surprising. These findings suggest to us that POWV was separately and independently introduced into both of these geographical locations, and that both subclades were focal in their respective environments. Both of these subclades of the DTV lineage segregated from the POWV isolate from *I. cookei* collected in Old Lyme, CT in New London County.¹¹ This isolate was similar to other POW lineage viruses from New York and Ontario, Canada. These findings support previous studies reporting on the focal nature and distinct transmission cycles of the POW and DTV genotypes.^{1,19,20}

Rates of seasonal infection of adult *I. scapularis* ranged from 0.8% to 1.6% from 2008 to 2012 in Bridgeport, CT and from 0.4% to 3.9% in North Branford, CT when more than 100 ticks were tested. The relative stability of the POWV sequences from the two different sites support previous observations of the stability of this monophyletic group of POWV.^{18,19} Although reasons for this stability are unknown, the relatively long life cycle of *I. scapularis* of 2 years,²¹ the vertical and transstadial transmission of the virus,²² and the possible direct transfer of the virus from infected to non-infected co-feeding ticks on small mammals may contribute to this stability.²³

It is noteworthy that efforts to isolate viruses from *I. scapularis* were initially attempted when Lyme disease was first recognized in Lyme, Old Lyme, and East Haddam, CT.²⁴ More than 1,440 *I. scapularis* were collected in 1977 from these three towns on the east side of the Connecticut River (New London County) and from nine towns on the west side of the Connecticut River (Middlesex County), and many of

TABLE 2

Prevalence of POWV-infected *Ixodes scapularis* collected in two locations in Connecticut, 2008, 2010, 2011, and 2012

Year	Location	No. positive/no. tested (%)	
		Spring (March–April)	Fall (October, November)
2008	Bridgeport	ND*	2/251 (0.8)
2008	North Branford	ND	0/42 (0)
2010	Bridgeport	ND	2/180 (1.1)
2010	North Branford	ND	5/128 (3.9)
2011	Bridgeport	2/55 (3.6)	12/794 (1.5)
2011	North Branford	4/96 (4.2)	1/239 (0.4)
2012	Bridgeport	2/126 (1.6)	ND

*ND = not done (ticks not collected).

FIGURE 1. Phylogenetic relationships of POWV sequences based on analysis of a 609 bp fragment of the envelope gene. Numbers at branch nodes represent bootstrap support for 1,000 replicates. Taxon names specify the strain number, host, location, and year (last two digits) of collection.

these specimens were tested for arboviruses. Procedures used to attempt isolation of viruses from ticks and from tissues of small mammals included intracerebral inoculation of homogenates into suckling mice, intraperitoneal inoculation of homogenates into guinea pigs and hamsters, and injection of homogenates into cultures of Vero, BHK-21, and CER (chicken embryo related cell line) cell cultures. No isolations were made by any of the methods used. These findings suggest that the DTV lineage was not prevalent in these areas of Connecticut in 1977 when Lyme disease was initially recognized. Not all of the methods used would have detected POWV, however, if present, the DTV lineage likely would have been isolated in suckling mice, a method successfully used to isolate the POW lineage from *I. cookei* collected in Old Lyme, CT in 1978.¹¹

Powassan virus was initially isolated from human brain tissue in suckling mice, which showed abnormal neurologic function.² Additional isolations of this virus from humans, wild animals, and from ticks were made similarly in the decades that followed.²⁵ The DTV lineage was first detected by dissecting salivary glands from field-collected ticks removed from carcasses of dead deer or that were fed for 4 to 5 days on a naive New Zealand rabbit in the laboratory, staining one salivary gland of each tick with Feulgen reaction, and examining the salivary glands for evidence of virus growth.^{12,26} The remaining salivary gland of each Feulgen-positive tick was pooled with those of four other ticks, placed in Hanks Balanced Salt Solution with 15% fetal bovine serum, homogenized, and intracerebrally inoculated into outbred CD-1 suckling mice. Injected mice subsequently were examined for illness. The virus was described by RT-PCR and sequencing of the amplification product. Later, the previous procedure for detecting POWV was modified by homogenizing field-collected male *I. scapularis* in PBS, pooling 5 μ L from each of six ticks, and testing the pool using appropriate primers by RT-PCR.¹⁷ Putative virus pools were injected into suckling mice for isolation of the virus. The number of individual ticks infected was determined by testing individual homogenates by RT-PCR. Isolation of POWV from RT-PCR positive ticks was later reported in BHK cells.¹⁹ Powassan virus was confirmed in BHK cells showing CPE by RT-PCR. We followed this latter procedure, but we had difficulty dependably identifying amplicons by RT-PCR and recognizing CPE in BHK-21 cells infected with putative homogenates of POWV in 25-cm² flasks. We had greater success when we initially placed tick homogenates in BHK-21 cells, withdrew 70 μ L of cell medium from 4- to 5-day-old cultures irrespective of CPE, and tested the RNA sample by RT-PCR. Homogenates of individual ticks from positive tick pools were then placed on BHK-21 cells. Cell medium from individual flasks was tested for virus 4–5 days after inoculation by RT-PCR. Isolation of virus was enhanced by this *in vitro* method by first growing virus from infected ticks in BHK-21 cells, thereby increasing viral titer, and then testing the cell medium by RT-PCR for POWV. This ability to isolate POWV from relatively large numbers of individual ticks enabled us to determine their genetic relationships with each other and with isolates reported by others.

Prevalence of Powassan encephalitis may be increasing,⁶ however reasons are unknown for the absence of reported human cases of POWV in Connecticut. Our two collecting sites were not near human habitation, and thus ticks had limited contact with humans, however, *I. scapularis* is rela-

tively common in Connecticut, and there are likely other locations where ticks are infected with POWV. The absence of active surveillance for Powassan encephalitis in humans, and a possible lower infectiousness of the DTV lineage of POWV, compared with the virus causing tick-borne encephalitis may contribute to the lack of reporting of this encephalitic disease.¹²

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