An Assessment for the Presence of Powassan virus in *Ixodes scapularis* Nymphs from Locations in Virginia, Maryland, New Jersey, Pennsylvania, New York, and Connecticut

by

Scott Mueller

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Name of Candidate: CPT Scott Mueller
Master of Science in Public Health Degree
March 21, 2013

DISSERTATION AND ABSTRACT APPROVED:

DATE: MAR 21, 2013

PASS ✓

Dr. David Florin
DEPARTMENT OF PREVENTIVE MEDICINE AND BIOMETRICS
Committee Chairperson

✓

Dr. Richard Johnson
DEPARTMENT OF PREVENTIVE MEDICINE AND BIOMETRICS
Thesis Advisor

✓

Melissa Miller
U.S. Army Public Health Command Region-North, Fort Meade, MD
Committee Member

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ABSTRACT

An Assessment of Powassan virus Presence in *Ixodes scapularis* Nymphs from Locations in Virginia, Maryland, New Jersey, Pennsylvania, New York, and Connecticut

Scott Thomas Mueller, Master of Science in Public Health, 2013

Thesis directed by: LCDR David Florin, Assistant Professor, Preventive Medicine and Biometrics

Powassan virus (POWV) is a naturally occurring tick-borne flavivirus in North America that can cause severe encephalitic illness and death in humans. Cases of the disease have increased significantly over the last 10 years. Many of these cases have been attributed to deer tick virus (DTV), a distinct genotype of POWV. Deer tick virus is transmitted by *Ixodes scapularis* (Say 1821), an aggressive tick that readily bites humans. This study collected *I. scapularis* from sites along the U.S. East Coast, including locations south of where DTV has been previously reported in ticks. The collected ticks were individually homogenized with a portion of each homogenate pooled into groups of 10 by collection site. A reverse transcriptase polymerase chain reaction (RT-PCR) was conducted to amplify portions of the DTV ns-5 gene for the purpose of detecting the presence of the DTV virus. Nine of the study's 11 sites, including the two most southern sites in Virginia, contained ticks infected with DTV. New state records for the presence of DTV in *I. scapularis* are reported for New Jersey, Pennsylvania, Maryland, and Virginia. The results of this study indicate that DTV is circulating in ticks far south of what has previously been documented.

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CHAPTER 1: Background of Powassan virus

General Description of Powassan virus and its Genotypes

Powassan virus (POWV) is a member of the Tick-Borne Encephalitis (TBE) serogroup in the family Flaviviridae (24). The virus was first described and named by McLean and Cobb in 1959 after it was discovered in the brain of a five year old boy who had died of encephalitis (56). Powassan virus is the only known tick-borne flavivirus in North America capable of producing human infection (43). The maintenance of this pathogen in nature involves an enzootic transmission cycle with *Ixodes spp.* ticks and small mammals. Powassan virus has a single stranded, positive-sense RNA genome that codes for three structural and seven non-structural proteins (54). The structure and replication of POWV are believed to be like other TBE members (28).

Two genotypes or lineages of POWV have been identified and characterized. The "prototype" lineage is called POW1, or Lineage I. The second lineage was discovered in the late 1990s (76) and is referred to as deer tick virus (DTV), POW2, or Lineage II. The POW1 and DTV genotypes circulate in separate enzootic transmission cycles, each exploiting its own distinct ecological niche (30). Lineage I and II of Powassan virus will also be referred hereafter as the POW1 and DTV genotypes, respectively. The two genotypes comprise the viral species Powassan virus, additionally referred in this text as POWV.

Distribution of POWV and its Genotypes

The geographic distribution of POWV is widespread, occurring in far eastern Russia, Canada, and the United States. Knowledge on the distribution of the virus stems primarily from the occurrence of clinical cases (28). Although full ranges for the POW1 and DTV genotypes are not well characterized, the ranges are generally associated with the tick vectors and the vertebrate reservoir. In general, POW1 is found in eastern Russia, Canada, and the northern and western U.S.; DTV is known to circulate in the north-central and northeast U.S.

Multiple isolations and seroepidemiological studies have confirmed POW1 circulates in the Primorsky krai region of eastern Russia (48). In Canada, POW1 occurs in Nova Scotia, Quebec, Ontario, and British Columbia (2; 57-59). Deer tick virus was found in Ontario when it was determined the cause (49) of a fatal case of POWV that had previously been attributed to POW1 (39). In the United States both of the POWV genotypes occur. It is believed POW1 circulates in the northeast, north-central, and western US (2; 44). Deer tick virus has been found in either infected ticks or patients with encephalitis in New York, Connecticut, Massachusetts, West Virginia, Wisconsin, and Minnesota (9; 11; 30; 32; 76; 78). Oftentimes when POWV is reported where the ranges of POW1 and DTV overlap, the genotype is not determined or stated. As an example, a recent description of four human cases of POWV from Minnesota and North Dakota did not indicate what genotype was the cause (68). Similarly, in 2011 the Pennsylvania Department of Health reported the first human case of Powassan virus in the state but did not specify whether the illness was attributed to POW1 or DTV (5).

PHYLOGENY

The relationships between Powassan virus and other tick-borne flaviviruses, as well as the relatedness within the virus' genotypes have been evaluated in numerous studies (8; 32; 49; 65). Antigenic and genetic work indicate POWV is similar to other closely related tick-borne flaviviruses (28). Phylogenetic studies also suggest that

POWV derived from a Eurasian ancestor common to TBE viruses that migrated to North America on birds or animals crossing the Bering land bridge. The virus later diverged into the current POW1 and DTV genotypes (32; 49; 52; 65; 88; 89). Evidence of greater genetic diversity in DTV had indicated the genotype is the older of the two (32). Recent phylogenetic analysis using more robust techniques provided a different estimate of DTV and POW1 divergence, showing both lineages simultaneously radiating approximately 200 years ago (65). Currently, neither DTV nor POW1 can be definitively identified as the ancestral clade. Nucleotide sequence analysis of Russian POW1 strains show they have a low substitution rates compared to North American POW1 strains and are most similar to those from eastern Canadian (52). This evidence suggests that POWV was introduced to Russia from Canada sometime in the last century, quite possibly through the fur trade (52).

Soon after the discovery of DTV, phylogenetic studies suggested the virus was not a distinct species but rather a separate genotype of POW1 (8; 32; 49). The degree of DTV's relatedness to POW1 was assessed based on serology and nucleotide sequence similarity. Hemagglutination inhibition (HI) tests and cross-neutralization experiments demonstrated the two genotypes were indistinguishable based on serology (8). Molecular analysis has showed a close similarity between DTV and POW1. The full genome of DTV is only one base shorter than POW1, there is only a 6% amino acid difference, and the compared nucleotide sequence identity is 84% (49). However, genetic variation is at least three times greater between DTV and POW1 than within the lineages for certain genes evaluated (32). The gene coding for the envelope protein contained the most variation between POW1 and DTV, being 13 times greater than variation within the genotypes. That stated, the genetic diversity that exists between DTV and POW1 is still within what has been reported for other flavivirus species, further suggesting they are not distinct species (8; 32; 49).

Evidence of the different host/vector preferences of DTV and POW1 indicate the two genotypes do exploit their own distinct ecological niches. The two lineages operate in similar cycles (tick/small mammal) but utilize different species. The ticks *Ixodes cookei* (Packard 1869) and *Ixodes marxi* (Banks 1908) have been found to be vectors of POW1 (19; 51), while *Ixodes scapularis* (Say 1821) has been identified as a vector of DTV (76). Various species in the Tribe Marmotini, including woodchucks (*Marmota monax* Linneaus 1758), squirrels, and skunks (family Mephitidae) have been implicated as hosts important in the maintenance of POW1 (53; 59), whereas white-footed mice (*Peromyscus leucopus* Rafinesque 1818) are thought to be the critical host for the perpetuation of DTV in nature (29). The ecology, evolution, and geographic distributions of DTV and POW1 are closely tied to those of their respective hosts and vectors. Although ecological differences between DTV and POW1 exist, the consensus opinion has emerged that the two are distinct lineages, or genotypes, of the species Powassan virus (8; 32; 49).

The POW1 Genotype: Description, Vector, and Host

The POW1 genotype was first isolated in 1952 from *Dermacentor andersoni* (Stiles 1908) ticks collected in Colorado (77). The virus was not described and named, however, until it was reported by McLean and Cobb in 1959 (56). They identified Powassan virus in the brain of a 5-year old boy who died of encephalitis in the town of

Powassan, Ontario. The POW1 genotype has been found to circulate in transmission cycles involving small to medium sized mammals and one or more tick species (2; 53).

Vector

Powassan virus has been reported in a number of tick species in North America and Russia. The POW1 lineage has been reported as the genotype transmitted by all POWV carrying ticks except *I. scapularis* (28), which carries DTV. Most reports on tick species infected with POWV were published before the DTV lineage was discovered with modern genetic techniques; therefor the virus genotype was never determined. Although *I. scapularis* has been a competent vector for POW1 in laboratory studies (21), no fragment of the POW1 genotype has been detected in field caught *I. scapularis*. Therefore it remains to be seen whether or not the species is a vector of the POW1 lineage.

In North America, Powassan virus considered to be of the POW1 genotype has been isolated from *Ixodes spinipalpis* (Hadwen & Nuttall 1916), *Ixodes angustus* (Neumann 1899), *Dermacentor variabilis* (Say 1821), *I. cookei*, *I. marxi* and *D. andersoni* (2; 41; 57; 60). However, not all of these have been determined as competent vectors important in the maintenance of POW1 in nature. The two tick species incriminated with perpetuating the enzootic cycle of POW1 in North America are *I. cookei* and *I. marxi*, with the former being most important (28). Known as the groundhog or woodchuck tick, *I. cookei* may be found east of the Rocky Mountains and commonly occurs in the Northeastern and Midwestern U.S., and southern Canada (64). The groundhog tick has been reported to be moderately to highly host specific to groundhogs (26; 34; 47). *I. marxi*, commonly called the squirrel tick, has been found east and west of the Mississippi River in 18 states and in Canada (19; 51). It prefers to feed on red squirrels (*Tamiasciurus hudsonicus* Erxleben 1777), but can also be found on other squirrels, chipmunks, foxes, raccoons and snowshoe hares (10; 18; 19; 51).

In far eastern Russia, the POW1 genotype has been isolated from *Ixodes persulcatus* (Schulze 1930), *Haemaphysalis consinna* (Koch 1844), *Haemaphysalis neumanni* (Dönitz 1905), and *Dermacentor silvarum* (Olenev 1927)(48; 50). The POW1 genotype has also been isolated from the mosquitoes *Anopheles hyrcanus* (Pallass 1771) and *Aedes togoi* (Theobald 1907)(46); however, these species are not considered important in transmission or maintenance of the genotype. More knowledge is needed to understand of the importance of host and vector species for POW1 in eastern Russia.

Host

Woodchucks, red squirrels, and skunks have been incriminated as important hosts in the perpetuation of the POW1 genotype in nature (2; 13; 28; 44; 53; 57; 59-61). Evidence of POWV infection, such as isolations of the virus or positive serology, has been found in a variety of animals including rodents, carnivorous mammals, birds, frogs, turtles, mosquitoes, and snakes (2; 4; 7; 13; 42; 48; 57; 83). The low seroprevalences of antibodies to POW1 in many species indicate they may be unimportant and/or dead-end hosts. Limited information on infection rates in many species also precludes determining their role in the amplification and persistence of POW1 in nature. High antibody titers to POW1 have been found in groundhogs, squirrels, and skunks in eastern and western Canada and the U.S. northeast, suggesting they are critical hosts in the maintenance of the virus (53; 59). Currently, woodchucks, skunks, and squirrels remain the hosts recognized as important in the persistence of POW1 transmission (28).

The DTV Genotype: Description, Vector, and Host

The DTV genotype was discovered in 1996 when collected *I. scapularis* were found infected with a virus similar to POWV (76). The ticks had been collected in Massachusetts and Connecticut from deer shot by hunters and from vegetation. Direct sequencing of amplification products revealed that the observed virus was similar to Powassan virus (POW1), yet distinct enough to be differentiated. The new virus was provincially named "deer tick virus", or DTV (76), and was later determined to be a genotype of Powassan virus.

Vector

The only known vector of DTV is *I. scapularis*, commonly known as the deer or black-legged tick. Originally found in ticks collected from Massachusetts and Connecticut (76), DTV has since been isolated from field caught *I. scapularis* in Rhode Island, Wisconsin, New York, and Minnesota (9; 29; 78). Where DTV has been found, collected *I. scapularis* ticks have been observed with 1-5% infection rates (1; 29; 76; 78). Currently no other tick species have been reported infected with DTV. *Ixodes scapularis* ticks transmit and maintain the pathogens that cause Lyme disease, anaplasmosis, and human babesiosis (6; 28; 66; 73). They are aggressive and opportunistic hematophages and readily feed on humans. The *I. scapularis* tick is a well-recognized public health threat that has demonstrated its potential in facilitating emerging diseases (28).

The two-year life cycle of deer ticks depicted in Figure 1 consists of the egg, larva, nymph, and adult stages. Eggs hatch in mid-summer into 6-legged larvae, and subsequently seek and take their first blood meal from a small vertebrate hosts (e.g. white-footed mice or chipmunks). After feeding, the larvae molt into nymphs and overwinter to search for their next blood meal in the spring. Nymphs have 8 legs and seek a larger host such as squirrels to feed on. Deer tick nymphs are opportunistic and will take a blood meal from a variety of hosts including humans. After a feeding period of 3 to 4 days, they will detach and subsequently molt into their adult forms. The adult ticks become active in mid-fall seeking even larger hosts, preferably white-tailed deer. The female adults feed for up to a week until they become massively engorged, whereas the males do not take such a large meal. Mating can occur on the host or after detachment on the leaf litter. The female will lay approximately 3000 eggs in the early spring, which will hatch later in the summer and commence the next generation cycle (35).

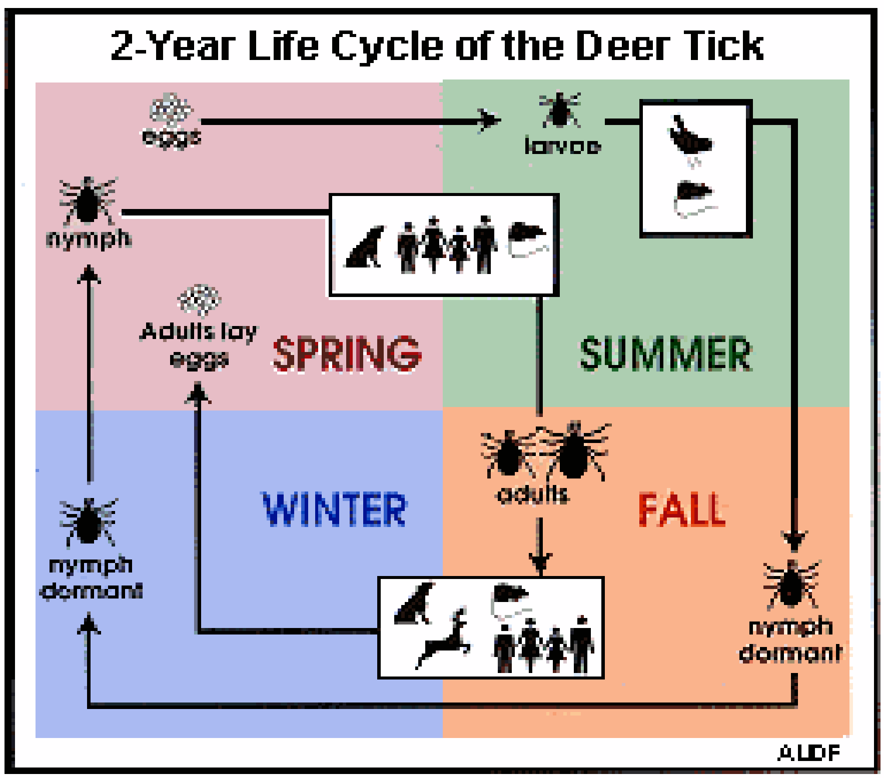


Figure1. The two-year life cycle of *Ixodes scapularis* ticks
(figure source: http://www.aldf.com/deerTickEcology.shtml (35))

Expansion in both the geographic distribution and population abundances of *I. scapularis* has been attributed to the ecological changes in the northeastern and midwestern U.S. during the last century (6; 55; 73). White-tailed deer are considered the keystone host for *I. scapularis* (86; 87). The deforestation that occurred in the 19th century eliminated almost all of the deer in the Northeast, and it is believed the deer tick populations were reduced as well (6). In the 20th century, abandoned fields transformed into eastern deciduous forests through natural succession, and deer populations rebounded throughout the Northeast. The ideal habitat and keystone host for *I. scapularis* were restored, and the range and populations of deer ticks grew (6). Today, deer ticks are both a nuisance and a public health threat in many areas of the eastern and midwestern United States (Figure 2). The CDC's Lyme Disease Incidence Map (Figure 3) shows the areas where abundant *I. scapularis* populations and *Borrelia burgdorferi* result in high cases of disease (17).

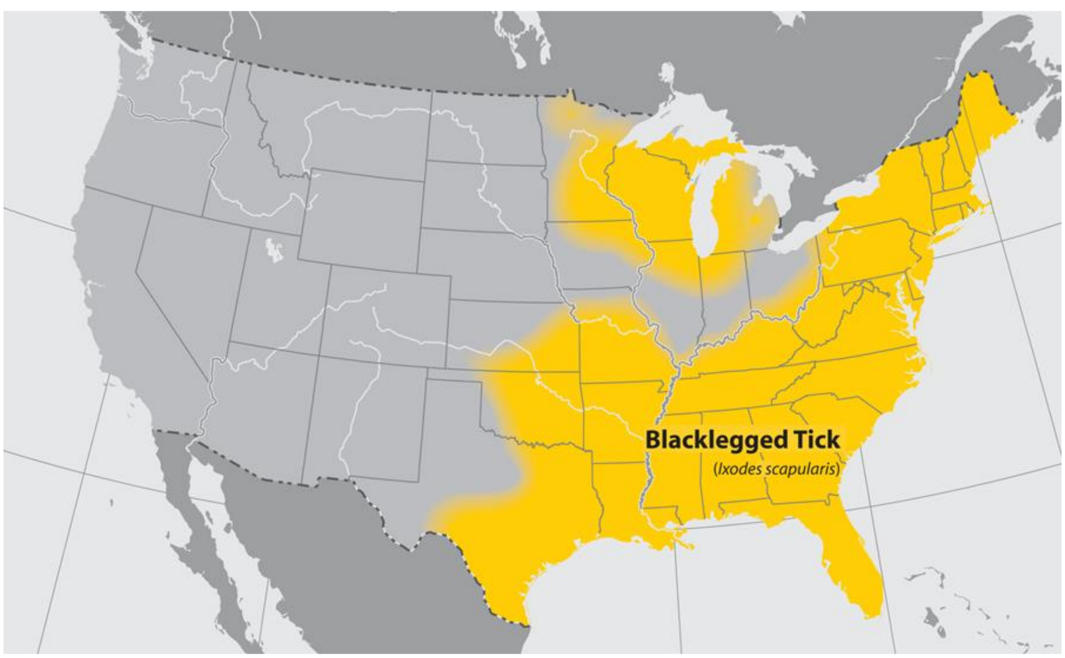


Figure 2: Approximate Distribution of *I. scapularis* in the United States (source: http://www.cdc.gov/ticks/maps/blacklegged_tick.html (15)).

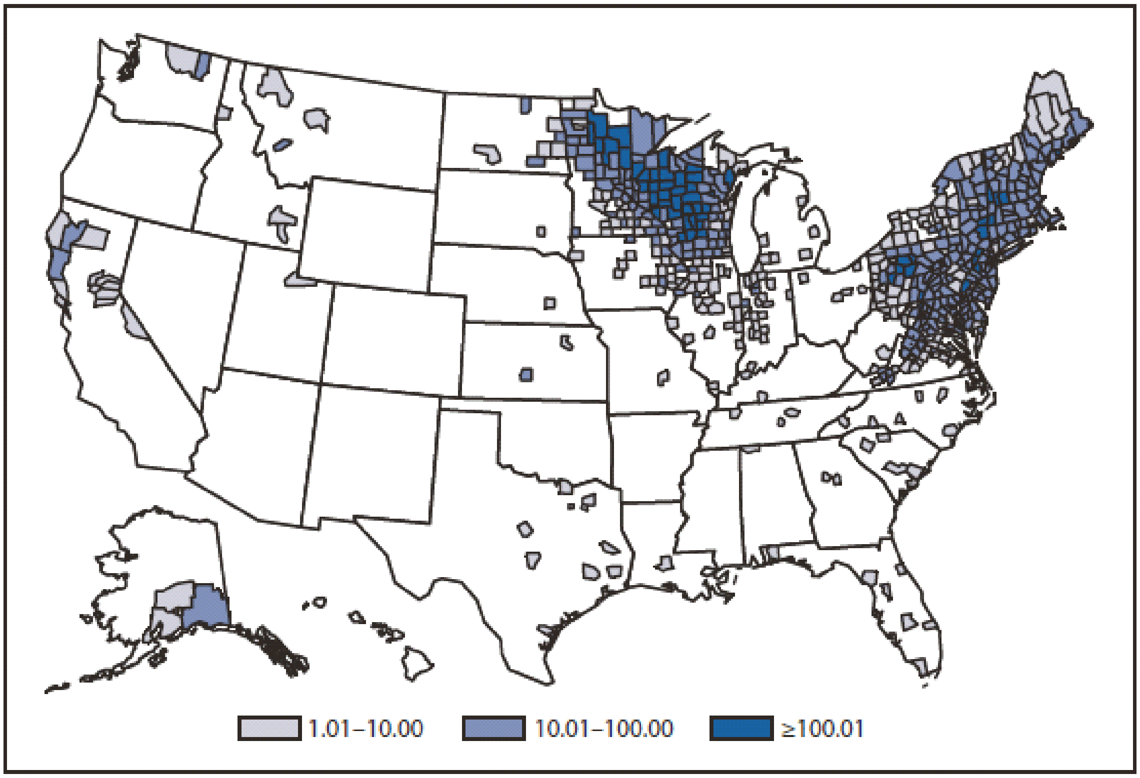


Figure 3: CDC's 2010 Lyme Disease Incidence Map of the United States: Values indicate incidence of Lyme disease per 100,000 population in each county for 2010 (17).

Host

Limited work has been conducted to determine what potential hosts are capable and/or important in DTVs maintenance in nature. One study looked at the enzootic transmission cycle of DTV, evaluating white-footed mice and meadow voles (*Microtus pennsylvanicus* (Ord 1815) (29). Powassan virus that was believed to be the DTV genotype was found in white-footed mice and deer ticks, but not in voles. Positive ticks were collected at sites with seroreactive white-footed mice, but not from sites where no seroreactive mice were found. This suggested that DTV may be maintained by the white-footed mouse/*I.scapularis* transmission cycle (29). If *P. leucopus* are indeed a preferred blood meal source for nymphal *I. scapularis* as well as the critical host for DTV maintenance in nature, it is worth mentioning *I. cookei* ticks do not readily feed on white-footed mice (26).

TRANSMISSION DYNAMICS

The transmission dynamics of POW1 and DTV in nature are not well studied or thoroughly understood. Transovarial and transtadial transmission of POW1 has been observed in *I. scapularis* under laboratory conditions (21), yet wild caught *I. scapularis* have never been found infected with this lineage. *Ixodes scapularis* nymphs that fed as larvae on DTV infected mice were observed to have a 22% transtadial transmission rate (31) suggesting that transovarial and transtadial transmission may be important in maintaining DTV in nature. Ebel et al. (31) also discovered that DTV can be transmitted in as little as 15 minutes after attachment. The short attachment time required for transmission could have important public health implications. Ebel's 2010 review of Powassan virus highlights the need for studies evaluating the types of transmission (vertical and horizontal) in DTV and POW1 that contribute to their perpetuation in nature (28).

HUMAN DISEASE AND PUBLIC HEALTH CONCERNS

Powassan encephalitis is the severe and debilitating disease caused by POWV (3). While it occurs infrequently, the high case fatality rate of Powassan encephalitis (10-15%) and long term neurological sequelae of its survivors underscore the severity of this disease (2; 67). Incubation periods have been reported ranging from 8 to 34 days (39). Clinical presentations of both genotypes of POWV are encephalitis, meningoencephalitis, and aseptic meningitis (28). The first five reported cases of Powassan encephalitis were reviewed to determine a detailed clinical understanding of the disease (72). Prodromal symptoms were found to include sore throat, headache, sleepiness and disorientation. Encephalitis developed later and was associated with respiratory distress, vomiting, prolonged and sustained fever, possible convulsions, and paralysis (52; 72). Approximately 50% of Powassan encephalitis survivors experienced long term neurological sequelae (39; 43) including hemiplegia, severe headaches, memory problems, and wasting (2; 28).

The DTV genotype was originally thought to be mild or asymptomatic (76); however, three Powassan encephalitis cases have since been attributed to DTV infection (33; 49; 75). Of these, two cases resulted in death directly attributable to DTV and the remaining patient died five weeks after the onset of illness from a pulmonary embolism. In animal models, the virulence and symptoms observed for both POW1 and DTV inoculated mice were similar, although symptoms appeared slightly later in DTV inoculated mice (8). The low numbers of POWV cases observed coupled with the limited number of determined POWV precludes identifying which lineage produces more symptomatic and/or severe infections. However, the human cases attributed to DTV and animal model studies give evidence that DTV produces similar disease to POW1 with severe implications (28).

Laboratory diagnosis of Powassan encephalitis can be made through the presence of POW1 or DTV IgM antibodies in serum or cerebrospinal fluid (CSF), or detecting either genotype's RNA in CSF (33). The POW1/DTV antibodies are considered specific if their titers are ≥4-fold higher than WNV or St. Louis encephalitis antibody titers (43). While most laboratories do not specifically test for Powassan virus (5), testing for the pathogen is available in some state's public health laboratories and the CDC (33). The genotype responsible for infection is determined by genomic sequencing (33). The majority of Powassan encephalitis cases that have been reported do not include genetic sequence information to determine the virus genotype (33).

There is no specific therapy for cases of Powassan encephalitis. Treatment consists of supportive care for the disease's symptoms (24). Patients should be hospitalized, as they need careful monitoring of their fluids through the course of the disease. Interferon and ribavirin are drugs that have shown to be effective in cell cultures and infected animal experiments for closely related flaviviruses (12; 45). These drugs were administered to a recent case of Powassan encephalitis, however the patient later died from the illness (33).

Prevention

The methods for preventing Powassan virus infection are similar to those for other tick-borne diseases and include tick habitat avoidance, the use of repellents, and, to a lesser extent, body checks to remove ticks. Preventing POWV infection is best achieved by avoiding the ticks that transmit the pathogen (82). Human contact with the vectors of POW1, *I. cookei* and *I. marxi*, is infrequent due to their host specificity (26; 47; 53). Exposure to DTV transmitted by *I. scapularis*, however, is more likely due to the species' opportunistic feeding habits on a wide-range of hosts including humans (28). Precautions should be taken when outdoors in areas with endemic *I. scapularis* populations, especially during the spring, early summer, and fall months when these ticks are most active. Light-colored clothing that covers exposed skin, with pants tucked into enclosed shoes reduces contact with ticks (36). Avoiding *I. scapularis* habitat including wooded areas with heavy leaf litter, bushy areas, and high grass helps to limit exposure (16).

Additionally, the use of repellents containing DEET (20% or more), and permethrin on clothing is recommended to deter host seeking ticks (16).

While removing attached ticks is a recommended method for preventing Lyme disease, it may not be as effective in preventing POWV transmission. The time from tick attachment until efficient transmission of the DTV genotype in mice has been observed to be less than 15 minutes (31). This sharply differs from the agent of Lyme disease, *Borrelia burgdorferi,* which has a required time before transmission of approximately 24 hours (71). The significantly shorter duration of attachment required for DTV transmission does not allow a window of reduced risk for infection even if the tick is removed within a short period of time post attachment.

Powassan encephalitis: An Emerging Disease

A recent increase in human disease from POWV has drawn interest in determining the potential public health impact of the virus (28). Only 27 Powassan encephalitis cases were reported in the U.S. and Canada from 1959-1998, an average of 0.7 cases/year (43). Over the last decade there has been a sharp increase in human disease from POWV infection. Nine serologically confirmed Powassan encephalitis cases occurred from 1999-2005 (1.3 cases/year) in the U.S. alone, resulting in questions on the virus' state as an emerging infectious disease (43). The incidence of disease from POWV has since increased again, with at least 49 cases in the U.S. from 2005-2012 (68; 74). Table 1 illustrates the rapid increase in cases from 2002-2011. Although still comparatively rare, evidence indicates POWV is an emerging public health concern in areas of the U.S. (9; 68).

Table 1: Human cases of Powassan encephalitis from 2002-2012 as reported by the USGS (74).

Year	Reported POWV cases
2002	1
2003	-
2004	1
2005	1
2006	1
2007	7
2008	2
2009	6
2010	8
2011	16
2012	8

The geographic distribution of Powassan encephalitis cases appears to be expanding in the U.S. Historically occurring in the Northeast, many of the recent cases have occurred westward in Wisconsin, Minnesota, and North Dakota (9). Four cases were reported from a single teaching hospital in North Dakota in 2011 (68). Additionally, a southern expansion of POWV disease may well be underway. The first recognized case of human illness from POWV in Pennsylvania was reported in 2011 (5). In Virginia, the first reported case of POWV occurred in 2009 (37; 74).

Different causes have been hypothesized for the recent increase in POWV disease cases as well as the observed geographic expansion of where they are occurring. Elevated awareness of arboviruses may be responsible for the higher numbers of reported illnesses from POWV. The introduction of West Nile Virus (WNV) led to an increase in testing for POWV and other encephalitis-causing arboviruses (43; 67). POWV cases that have been identified in Maine and Vermont were direct results of requests for WNV testing (67). The initial wave of WNV occurred between 1999 and 2006, invoking unique demands on the nation's public health resources and a heightened emphasis on arbovirus surveillance and reporting. The drastic increase in Powassan encephalitis cases, however, did not begin until 2007. Prior to this rise in Powassan encephalitis cases, the number of WNV cases appeared to be stabilizing or decreasing throughout much of the U.S. (74). While WNV emphasis may have contributed to the identification of some Powassan cases, other factors appear to be responsible for the recent surges in Powassan encephalitis cases.

The DTV genotype of POWV could be responsible for a large proportion of the increase in human disease (28). The vectors of POW1, *I. cookei* and *I. marxi*, are host specific and rarely attack humans, and thus present a low public health risk (Smith, 1992). DTV, on the other hand, is transmitted by the aggressively biting and opportunistic *I. scapularis*. As *I. scapularis* will readily feed on humans, people are more likely to be bitten by them compared to *I. cookei* (Ebel 2010). These feeding behaviors, along with the expansion of *I. scapularis* populations, may be the driving the rise in Powassan encephalitis cases. The introduction and establishment of enzootic transmission of DTV may occur during the colonization of *I. scapularis* in a new environment, or after the tick population has become well-established. The low infection rate of DTV in ticks (1-5%) may contribute to its delay in occupying these newly available environments recently colonized by deer ticks. This could explain why Powassan encephalitis cases have just recently escalated despite abundant vector populations throughout the Northeast for the last 25 years. The locations of Powassan encephalitis cases provide additional evidence incriminating DTV as the culprit for the recent human disease from POWV. Most of the recent cases of Powassan encephalitis from 2005-2012 have occurred in areas endemic for *I. scapularis* (33). Many case reports have either identified or implicated DTV as the agent responsible for the observed illness (9; 33; 37; 75). The feeding habits of I. scapularis, the increased distribution of the tick, and its association with recent encephalitis cases suggests DTV is responsible for the increased incidence of the Powassan encephalitis cases.

CALLS FOR ECOLOGICAL STUDIES:

Many of the mechanisms and dynamics involved in the maintenance and transmission of Powassan virus are unknown. A relatively small number of field studies have been conducted on either of the genotypes, and a thorough understanding of their distinct ecologies is lacking. Recent reviews and case reports on Powassan virus have emphasized the need for future research on POWV, particularly the DTV genotype (28; 68; 75). Ecological investigations such as entomological surveys have been proposed to assist in identifying where high densities of ticks and POWV exist (75). A recent review on Powassan virus, inclusive of both the POW1 and DTV genotypes, emphasized the need for additional field studies to understand the basic interactions involved with the vectors, their hosts, and the environment (28). Some of the specific questions regarding DTV that need to be addressed include:

- What hosts are involved in the maintenance and amplification of DTV?
- Where does the virus circulate within its host/vector's ranges?
- What types and rates of transmission occur with DTV?
    - Vertical (i.e. transovarial, transtadial)
    - Horizontal
        - Vector to vector (i.e. Co-feeding, Sexual)
        - Vector to vertebrate (i.e. salivarian)

Conducting research that answers these and other questions on the basic interactions that occur between POWV's genotypes and their hosts will provide a more thorough understanding of their importance and potential burden to public health (28).

Understanding the extent of POWV's distribution, particularly the DTV genotype, is important to assessing the public health implications of this pathogen. While still rare, the recent escalation in human cases coupled with the severity of this disease is a cause for concern and warrants attention. The few studies in the Northeast that isolated DTV in field caught *I. scapularis* were conducted at locations in eastern New York and north into Massachusetts. Abundant populations of *I. scapularis* occur far south of where these DTV studies collected ticks. The DTV lineage of POWV might be circulating in these more southern *I. scapularis* populations and may have a much broader geographic area than has been observed. The 2009 northern Virginia Powassan encephalitis case that was believed to have been transmitted by an *I. scapularis* tick (37) indicates at least one area of DTV enzootic transmission may exist south of where the pathogen was previously known to occur. A more detailed understanding of the geographic distribution and prevalence of DTV is needed to help determine where it may be capable of producing human disease.

STUDY GOALS AND AIMS

The goal of this multi-state study on the U.S. East Coast was to determine the presence or absence of DTV in areas known to have high *I. scapularis* densities. The presence of DTV was determined by detecting the virus in collected *I. scapularis* nymphs. The study's sampling locations included areas far to the south of where published DTV isolations have been reported.

Study Aims:

- Determine if DTV is present in collected *I. scapularis* ticks at study sites in New York, Connecticut, New Jersey, Pennsylvania, Maryland, and Virginia.
- If present, determine the infection prevalence of DTV in the *I. scapularis* ticks collected.

CHAPTER 2: Methods

TICK SAMPLING METHODS

The main objective of the tick sampling efforts during the spring and early summer of 2012 was to collect the required number of *I. scapularis* ticks needed for the study. From January – March, 2012, a sampling agenda was developed that focused on addressing the study's aim of whether DTV was present in the tick populations at the sites sampled. This required determining various aspects of the sampling strategy including the sample size needed at each site, what defined a sampling event, what data were to be recorded in the field, how the ticks were to be sampled, and how they would be preserved. Due to the associated risks in collecting ticks known to transmit disease, preventive measures were also planned prior to sampling efforts beginning.

Determining the required sample size (number of ticks) at each site was necessary before the DTV phase began. DTV infection rates in *I. scapularis* have consistently been reported between 1-5%. This study calculated 230 ticks were required to detect a 1% DTV infection rate with a power of 90%. The sample size was increased from 230 to 250 to provide extra ticks at each site if needed. The sample size formula and calculation for this study is given (25):

Sample size formula: $\mathrm{n} = \frac{\log \beta}{\log \mathrm{p}}$, where n = sample size needed,
β = probability of Type II error
p = prevalence of non-infected

For a Power = 90, β = 0.1, p = 0.99

$$\mathrm{n} = \frac{\log 0.1}{\log 0.99} = 229.3$$

Describing what defined a single collecting event had important implications for how data and ticks would be gathered and organized. A sampling event was considered a tick collection that occurred in one day at a specific site for a period of time. If the collector returned the next day to collect ticks, it was considered a separate sampling event. Ticks were sampled during the daylight hours anytime between roughly 0900 and 1800 Eastern. For each sampling event, all captured *I. scapularis* were placed in a vial containing 95% ethanol with a label indicating the date and location. All other tick species, considered “bi-catch”, were combined in a separate vial with alcohol and labeled in a similar manner. Preserving the ticks in 95% ethanol was based off a recent study that evaluated storage methods for mites later used in molecular studies (20). Corriveau and others (2009) demonstrated that 95% preservation at room temperature generated good quality DNA after eight months of storage.

Collection Data Sheets

Information on site locations, habitats, weather, sampling methods used, and total time for sampling were recorded on Tick Collection Data Sheets (Appendix 1). Upon arrival at a sampling site and prior to collecting ticks, the following information was recorded:

- The site name and GPS coordinates (Fourtrex®, Garmin Ltd., Kansas City, MO)
- Weather information including an estimated temperature, wind speed, and degree of cloudiness (i.e. sunny, partly cloudy, partly sunny, or overcast)
- The start time and number of people collecting

Once the tick collection was complete, the end time was recorded and the total time of the sampling event was determined. The collection methods utilized (e.g. drag, flag) were documented, including an estimate of the proportion of the time each was used. Significant weather changes from the start of sampling were also recorded. Information from the data sheets were later transferred to an Excel spreadsheet.

Personal Protection Measures

All of the sampling locations were in high risk areas for Lyme disease, so precautions were taken to prevent exposure to tick bites. The uniform worn during all sampling events consisted of permethrin treated short-sleeved coveralls tucked into permethrin treated boots (Figure 4a). Duct tape was applied around boot cuffs to inhibit ticks from crawling inside (Figure 4b). Upon the completion of sampling, the duct tape, boots, and coveralls were removed and examined for ticks. Away from the tick collection site, a body check was conducted to find and remove any ticks. During four months of extensive sampling in areas infested with *I. scapularis* and *Amblyomma americanum* (Linnaeus 1758) ticks, only one tick bite was known to have occurred on the subject researcher. An *I. scapularis* nymph was found attached to the collector's forearm while in the field, where it was promptly removed with forceps and then preserved in alcohol. The short attachment time reduced any potential for Lyme disease, and no other disease symptoms were observed.

4(a)

4(b)

Figure 4. Uniform worn during sampling events consisted of permethrin treated coveralls (4a) tucked into permethrin treated boots with duct tape around boot cuffs (4b)

Sampling Devices and Collecting Techniques

Three different sampling devices were evaluated during the study; a CDC drag, a constructed tick drag, and a constructed tick sweep.

The CDC drag (Catalogue #2840T, Bioquip®, Rancho Dominguez, CA) is a widely used device for tick collecting (Figure 5a). It consists of a 23x45" sheet made of muslin sewn into a Dacron sail tape hem. The sheet is attached to a 1" dowel. The drag is pulled with a cord that is attached to the ends of the dowel. As the drag is pulled along the ground, questing ticks attach to the fabric. The CDC drag was used by dragging it along the ground for a length between 15 to 40 yards. The drag would then be picked up and the sheet would be viewed to detect if ticks were present. Ticks were removed from the sheet using masking tape and stored in a plastic bag. *I. scapularis* ticks and the bi-catch ticks were placed on separate pieces of masking tape. Once all ticks were removed from the sheet, the process would start again with another 15 to 40 yard drag. After sampling was complete at the site, all the ticks collected and stored on pieces of masking tape were transferred to vials of ethanol using forceps.

The constructed tick drag was similar in concept to the CDC drag; however it was larger and made of different materials. The drag consisted of a 34”x40” white sheet of dense cotton fabric that was sewn onto a ½”x2” oak board. Nine 2” metal washers were sewn into the trailing end of the drag to ensure the fabric lay smoothly on the ground when pulled. This constructed drag was pulled for 15-40 yard lengths, and ticks were removed in the field the same as described above for the CDC drag.

The constructed tick sweep (Figure 5b) was based on the design described by Carroll and Schmidtmann’s (14). A 20” long x 1½” wide pole was cut down its center lengthwise for the insertion of the rectangular shaped sweep cloth. An 18”X36”, 100% cotton rectangle bilayer cloth with a vinyl layer in between (Baby-R-US® “Water proof flannel mattress pad”, RN# 67391, Item # 6739017K7) served as the sweep and was placed between the two cut sections that were secured together. This sweep cloth was soft and provided good texture to collect ticks, yet was also durable to withstand repeated use. The 20” pole and sheet were attached on an angle to a 5’ pole, resulting in what looked similar to an ice hockey stick. The orientation and motion of the sweep was similar to how an ice hockey stick is handled. The collector held the longer pole and maneuvered the sweep along the ground. The sheet was swept slowly back and forth across the leaf litter approximately 4 to 8 times and then checked for ticks. As with the other sampling devices, the ticks were removed with masking tape and stored in plastic bags until sampling was complete.

5(a)

5(b)

Figure 5. Sampling devices evaluated included: (a) a CDC tick drag (picture source: www.bioquip.com); (b) a constructed tick sweep.

Sampling Phases

Ticks were collected at locations in Virginia, Maryland, Pennsylvania, New Jersey, New York, and Connecticut from late March through mid-June, 2012. Two phases of tick sampling were conducted; Test sampling and DTV tick sampling.

Test Sampling Phase

Test sampling was conducted from late March through April to monitor tick activity, identify good *I. scapularis* nymph habitat, and evaluate collection methods. A number of sites in Maryland and Virginia were used for the Test sampling (Table 2), some of which were not retained for the DTV sampling phase. Being in the south of the study's geographic area, the test sampling locations served as a monitor of local tick activity and an indicator to when the study should transition to the DTV phase. The evaluation of various environments for their potential to produce *I. scapularis* nymphs was done by comparing collection data from different habitats. Deciduous and coniferous forest stands, forest edge ecotones, and tall grass fields are examples of habitats sampled during the Test phase. The sampling devices (drags and sweep) were evaluated during this phase by comparing their ease of use, practicality, and tick collection rates (# of ticks/hour).

Table 2. Sites used during the Test Sampling Phase.

Test Tick Collection Sites

State	Site Name	County
Maryland		
	Fort George G. Meade	Anne Arundel
	Patuxent Wildlife Refuge - North Tract	Anne Arundel
	Patuxent Wildlife Refuge - South Tract	Prince George's
Virginia		
	Bull Run Regional Park	Fairfax
	Algonquin Regional Park	Loudoun
	Banshee Reeks Nature Preserve	Loudoun
	Hemlock Overlook Regional Park	Fairfax

DTV Sampling Phase

The ticks collected in the DTV sampling phase in May and June were from 11 field locations from Connecticut to northern Virginia (Table 3). All ticks that were tested for DTV were collected in this phase. Ticks were collected within 500 meters from the GPS coordinates recorded at each site. In an effort to distribute the collection locations across the study's geographic area, sites were no closer than 20 miles from each other. Locations were picked to include states that had previously reported positive DTV infected *I. scapularis* (CT (1; 76), NY (78)), and states south of where the virus has been reported to circulate (PA, MD, VA, NJ). Within each state, efforts were taken to select sites in areas with high populations of *I. scapularis*. This was achieved by reviewing literature available on tick collections and distributions, conducting an on-line search, and contacting personnel familiar with tick research from numerous private and government entities. Personnel from county health departments, environmental departments on Department of Defense (DOD) installations, and private wildlife/nature preserve organizations all contributed information that helped determine the location of collection sites.

A detailed habitat study of the collecting sites was not possible given time and financial constraints. Generally, the habitats of all the tick collection sites are similar. The sites are hardwood woodlands with open understories and decomposing leaf litter on the ground (Figure 6). The trees common at these woodlands included beech, maple, oak, ash, and hickory. All sites have a gently rolling topography.

Table 3. Sites used during the DTV Sampling Phase.

DTV Tick Collection Sites

State	Site Name	GPS coordinates
Connecticut		
	Naval Base New London	N 41° 24.504, W° 072 05.434
New York		
	United States Military Academy (USAMA)	N 41° 21.357, W° 074 02.511
	Louis Calder Center	N 41° 07.650, W° 073 43.803
Pennsylvania		
	Pennypack Ecological Restoration Trust	N 40° 07.415, W° 075 04.222
	Fort Indiantowntown Gap	N 40° 25.670, W° 076 34.010
New Jersey		
	Naval Weapons Station Earle	N 40° 16.811, W° 074 09.081
	Joint Base McGuire-Dix-Lakehurst	N 40° 00.433, W° 074 37.781
Maryland		
	Aberdeen Proving Ground	N 39° 24.321, W° 076 17.245
	Fort George G. Meade	N 39° 05.888, W° 076 45.235
Virginia		
	Banshee Reeks Nature Preserve	N 39° 01.961, W° 077 35.921
	Hemlock Overlook Regional Park	N 38° 45.999, W° 077 24.432

Figure 6. Example of the open understory hardwood woodland habitat sampled for ticks during the DTV sampling phase. The sites are hardwood woodlands with open understories and decomposing leaf litter on the ground (Figure 6).

Tick Collection Sites

In Connecticut, the single site where ticks were collected was located at Naval Base New London (NBNL) near the town of Groton. This submarine and training base consists of 687 acres along the Thames River, and employs over 9,500 military and civilian personnel (63). Less than 15 miles away and within the same county is the town of Lyme, made well-known by the tick-borne disease that took its name. Ticks were collected on the northwest side of the installation, in a heavily wooded area east of Shark Blvd. and north of NBNL's golf course. Being the site furthest north in the study, NBNL was the last location to be sampled.

In New York, ticks were collected at Fordham University's Louis Calder Center and the United States Military Academy (USMA) at West Point. The Louis Calder Center is a 113 acre biological research station located north of New York City in Westchester County (80). In 2007 and 2008, four human cases of POWV were reported in the county (74). The highest published infection rate of the DTV lineage in ticks was from a site in Westchester County (78). Many studies on *I. scapularis* have been conducted at the Louis Calder Center (22; 23). Dr. Thomas Daniels from the research station's Vector Ecology Laboratory was quite supportive, providing advice and extensive access to sample on the property. Ticks were collected in a wooded area to the west of Calder Lake. The other tick collection site in New York was located in Orange County at the USMA. The mission of the USMA is to educate and train cadets to graduate as commissioned officers in the US Army (81). This training includes field exercises conducted in areas with high *I. scapularis* populations. Ticks were collected in a woodlot approximately 700 ft. east of the intersection of Highway 293 and Mine Rd.

Cadet training was heard and observed in the woods surrounding the tick collection site during the two days of sampling.

The two collection sites in Pennsylvania were located in the Pennypack Ecological Restoration Trust (Pennypack) and Fort Indiantown Gap (Fort IG). The Pennypack Ecological Restoration Trust is an 809-acre private conservancy land-trust located 15 miles northeast of Philadelphia that is open to the public (79). The property contains over 10 miles of trails through meadows, fields, and woodlands on portions of Pennypack Creek. Ticks were collected in a mature woodlot approximately 500 ft. north of Old Welsh Rd. along the conservancy's Bethayres Trail. Fort Indiantown Gap is an 18,000 acre National Guard Training Center located 22 miles northeast of Harrisburg in central Pennsylvania. The installation reports training of over 100,000 military personnel annually (38). The tick collection site at Fort IG was a small woodlot in the cantonment area located on the north side of Service Rd. between Wiley and Smathers Roads.

Naval Weapons Station Earle (NWSE) and Joint Base McGuire-Dix-Lakehurst (JBMDL) were the two locations where ticks were collected in New Jersey. NWSE is a 10,000 acre military facility located in Monmouth County where previous tick research on *I. scapularis* had been conducted (70). At NWSE, sampling was conducted in the cantonment area near Colt's Neck. The specific location was a small woodlot inside Laurelwood Loop within an unoccupied housing area. JBMDL is located 18 miles southeast of Trenton and consists of 42,000 acres (85). The installation's environmental director suggested ticks were abundant throughout JBMDL. Furthermore, his accounts of JBMDL employees who had developed debilitating cases of Lyme disease indicated *I. scapularis* populations were present. In the cantonment area of Fort Dix, the tick collection site was in a woodlot to the northwest of the intersection of New Jersey Rd. and 10^{th} St.

In Maryland ticks were collected from sites at the U.S. Army's Aberdeen Proving Ground (APG) and Fort George G. Meade. Located in Hartford County, APG consists of over 80,000 acres along the Chesapeake Bay stretching from the Susquehanna River in the north to the Gunpowder River at its southern boundary (84). Ticks were collected in a wooded area northwest of an unoccupied housing community along Skippers Point Circle in the Edgewood area of APG. Fort Meade is located approximately 40 miles southwest of APG between Baltimore and Washington D.C. The post employs over 56,000 military and civilian employees that provide information, intelligence, and cyber services to DOD and other federal agencies (62). Ticks were collected from a woodlot north of the intersection of Mapes Rd. and O'Brien Rd.

The Virginia locations used for the DTV sampling phase were Banshee Reeks Nature Preserve and Hemlock Overlook Regional Park, both in the north part of the state. Banshee Reeks is a 725 acre nature preserve in Loudoun County consisting of successional fields, wetlands, hardwoods forests, and riverine habitat (69). The county is endemic for abundant populations of *I. scapularis* and its associated diseases. Over 25% of Lyme disease reported in Virginia is from Loudoun County (40). The collection site at Banshee Reeks was in the woods surrounding the old grain mill off the main road through the preserve. Hemlock Overlook Regional Park is located in Fairfax County and contains mostly mature woodlands on rolling and hilly terrain. In addition to the general outdoor activities the park provides to the public, it also has a challenge course that attracts 20,000 – 30,000 users annually. Ticks were collected at Hemlock overlook to the southwest of the public parking area off Yates Ford Rd.

LABORATORY METHODS

All laboratory work including confirmation of field tick species identifications, homogenization of ticks, RNA isolations, reverse transcriptase polymerase chain reactions (RT-PCR), and gel electrophoresis was conducted at the U.S. Army's Public Health Command Region – North (PHCR-North) at Fort Meade, MD. A concerted effort was given to best match molecular techniques reported in DTV literature with the capabilities of the laboratory at PHCR-North. If determined applicable, molecular products and protocols already used by PHCR-North laboratory personnel were given preference when developing this study's DTV assay. The final assay included methods described in previous DTV literature, techniques and products utilized at PHCR-North, and some methods unique to the study. The positive control used in this study was graciously provided by Dr. Sam R. Telford III from the Cummings School of Veterinary Medicine at Tufts University, North Grafton, MA. The control consisted of 60 µl of suckling mouse brain suspension with 10^4-10^5 adult mouse LD50 of DTV per mL. Additionally, Dr. Telford provided nine *I. scapularis* nymphs that had fed as larvae on infected mice. Two of these ticks were found positive for DTV using the study's assay, confirming its ability to detect the pathogen. Amplification products were submitted to the USUHS Biomedical Instrumentation Center for sequencing. Efforts taken to prevent contamination included wearing nitrile gloves, using separate rooms for isolations and RT-PCR, and temporally processing ticks based upon collection site.

Identification of Ticks

All of the collected ticks were identified, counted, and recorded in an Excel spreadsheet database for later analysis. Ticks in the bi-catch vials were identified to species with the aid of a dissecting scope (20-40X) and morphological tick keys (18; 27). The count data from each sampling event consisted of:

- Number of nymphs for each species
- Number of adult ticks for each species
- Number for each gender of the adult ticks
- Total # of individuals (adults + nymphs) for each species

The field identified *I. scapularis* nymphs were confirmed prior to being homogenized for molecular testing. Durden and Kieran's key (27) for the nymphs of the genus *Ixodes* was used to confirm the field identifications of all collected *I. scapularis.* The date of confirmation identification and the total number of nymphs for each field sampling event was recorded in the database.

Tick Homogenization

Nymph *I. scapularis* ticks were individually homogenized to liberate viral RNA for later isolation. To protect against cross-contamination between sites, ticks from separate sampling sites were not homogenized at the same time. The sample number assigned to each tick and the date it was homogenized was recorded in the tick database.

Ticks were homogenized individually in 2.0 mL MaxyClear™ SnapLock microcentrifuge tubes (Axygen®, Union City, CA). Between 80 and 160 ticks were individually homogenized at a time. The microtubes needed for the total number of ticks to be processed were arranged in blocks. One steel 4.5mm BB (Daisy, Rogers, AR), 430 µl of AVL buffer (Qiagen, Valencia, CA) and 20 µl of Proteinase K (Worthington

Biochemical Corp., Lakewood, NJ) were added to the tubes. The *I. scapularis* nymphs previously identified were kept in a petri dish containing 95% ethanol. Individual ticks were gently picked up with forceps and rinsed with a steady stream of de-ionized water dispensed from a wash bottle for a minimum of 5 seconds. After being rinsed, the ticks were placed in their individual tubes with the AVL buffer, BB, and Proteinase K. A sample code was assigned to each tick/tube that consisted of abbreviations for the state and site where the tick was collected, as well as a distinct number (001-250). The tubes were labeled with the sample code using a permanent marker. Table 4 shows examples of sample codes for study sites. Ticks were allowed to incubate at room temperature in the tubes for a minimum of 30 minutes to allow the Proteinase K to break down cell proteins.

Table 4. Sample codes for select study sites.

State	**Site**	**Sample Code**	**Ticks Labeled**
VA	Hemlock Overlook Regional Park	VAHO	VAHO001-VAHO250
VA	Banshee Reeks Nature Preserve	VABR	VABR001-VABR250
MD	Fort Meade	MDFM	MDFM001-MDFM250
MD	Aberdeen	MDAB	MDAB001-MDAB250
NJ	McGuire-Dix-Lakehurst	NJMD	NJMD001-NJMD250
PA	Fort Indiantown Gap	PAIG	PAIG001-PAIG250
PA	Pennypack Ecol. Rest. Trust	PAPP	PAPP001-PAPP250

The ticks were homogenized using a TissueLyser (Qiagen). The microtubes were loaded into the TissueLyser and subsequently shaken for 6 minutes at 20 oscillations/sec. The BB inside each microtube eviscerated the nymphal tick during the process, creating a 450 µL solution of AVL, Proteinase K and liberated tick contents and parts. The tubes were individually opened and the BBs were removed using a magnetic stir bar covered with a kimwipe® (Kimberly Clark Worldwide Inc., Roswell, GA). The BB was taken off the kimwipe and discarded. The stir bar was then re-positioned on a clean portion of the kimwipe prior to removing the next tubes BB to prevent cross-contamination. The tubes were then either centrifuged and arranged on blocks for subsequent RNA isolation, or placed in white storage boxes and put in a -20°C freezer for isolation at a later time.

RNA Isolation

The tick homogenate samples were pooled into groups of 10 and isolated using the QIAamp® Viral RNA Mini Kit (Qiagen). Successful isolation of deer tick virus from field caught *I. scapularis* ticks using the Viral RNA Mini Kit has recently been reported (1). The Mini Kit contains detailed instructions along with the following proprietary reagents and supplies needed to isolate the viral RNA:

- QIAamp® Spin Columns
- Collection Tubes (2 mL)
- Buffer AVL
- Buffer AW1
- Buffer AW2
- Buffer AVE
- Carrier RNA (poly A)

Prior to isolation, the tick homogenates were organized in blocks to allow for the use of a multichannel pipette for pooling. The Qiagen spin columns, collection tubes, 1.5 mL sterile tubes, and 1.5 mL unsterile tubes were arranged in blocks to prepare for pooling and subsequent isolation (Figure 7). The pooling of samples occurred as 14 µL samples from each of 10 tick homogenates (140 µL total) was combined into one 1.5 mL microcentrifuge tube containing 560 µL of AVL/carrier RNA. The 250 tick homogenates from each site thus became 25 pooled samples per site to be isolated. The samples were then isolated per the manufacturers protocol, with each pooled sample yielding 60 µL of eluate containing purified RNA.

The 2,746 ticks tested combined into 275 pooled samples and were labeled and recorded as samples P1-P275. The 1.5 mL sterile microcentrifuge tubes containing the RNA templates were labeled with their corresponding pooled sample names. The tick database was used to record the tick homogenates that comprised each pooled sample. The dates of homogenization and isolation were recorded for the samples as well. Table 5 provides an example of how tick homogenates and pooled samples were documented in the tick database.

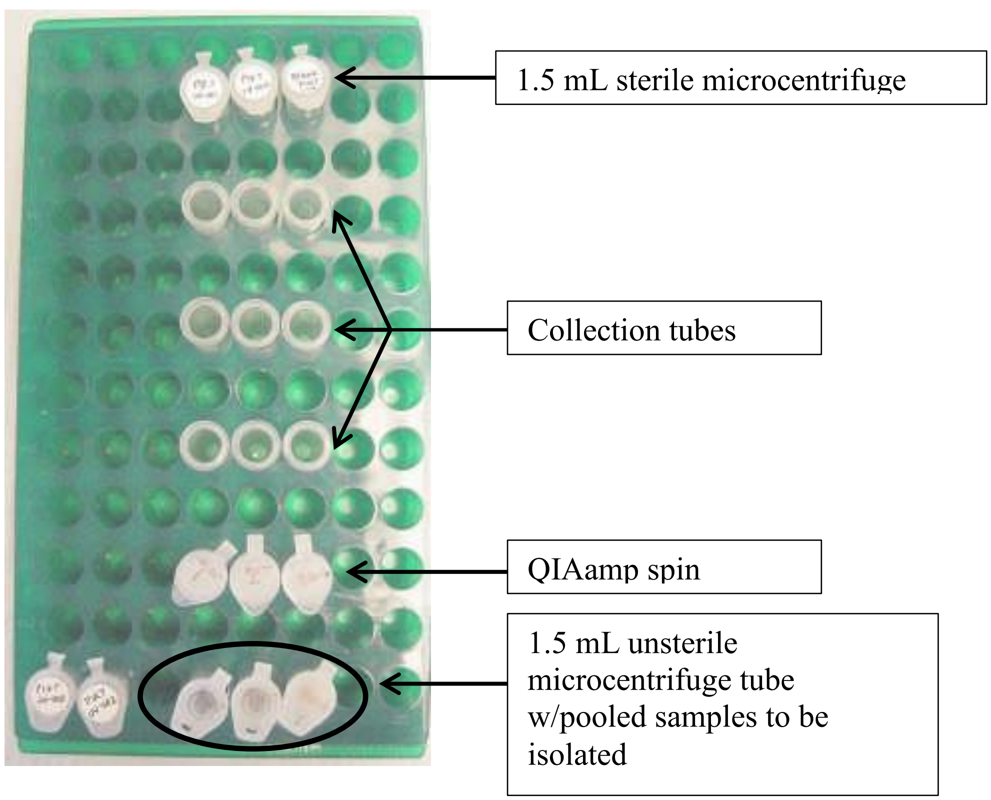


Figure 7. Arrangement of the Qiagen spin columns, Collection tubes, 1.5 mL sterile tubes, and 1.5 mL unsterile tubes for pooling and subsequent isolation. Picture from Public Health Command Region – North isolation protocol.

Table 5. Data management of tick homogenates and pooled samples.

Tick Sample	Date collected	Date Identified	Date homogenized	Date Pooled & Isolated	Pool number	Date PCR	Pooled PCR Results
VABR001	5/12/2012	1/28/2012	1/28/2012	1/28/2012	126	2/1/2013	Negative
VABR002	5/12/2012	1/28/2012	1/28/2012	1/28/2012			
VABR003	5/12/2012	1/28/2012	1/28/2012	1/28/2012			
VABR004	5/12/2012	1/28/2012	1/28/2012	1/28/2012			
VABR005	5/12/2012	1/28/2012	1/28/2012	1/28/2012			
VABR006	5/12/2012	1/28/2012	1/28/2012	1/28/2012			
VABR007	5/12/2012	1/28/2012	1/28/2012	1/28/2012			
VABR008	5/12/2012	1/28/2012	1/28/2012	1/28/2012			
VABR009	5/12/2012	1/28/2012	1/28/2012	1/28/2012			
VABR010	5/12/2012	1/28/2012	1/28/2012	1/28/2012			

Polymerase Chain Reaction

A two-step RT-PCR was conducted using an aliquot from the isolated pooled samples to amplify an approximately 300 base pair (bp) segment of the DTV ns5 gene (29). Aliquots of all pooled samples from a site had RT-PCRs conducted in the same run. Illustra™ Ready-To-Go™ RT-PCR beads (GE Healthcare, Pittsburg, PA) provided the following reaction reagents and supplies used:

- RT-PCR beads: when bought to a final volume of 50 µL, each bead dissolved resulting in a PCR reaction with 2.0 units of Taq DNA polymerase, 60 mM KCl, 10 mM Tris-HCL, 1.5 mM $MgCl_2$, 200 µM of each dNTP, Maloney Murine Leukemia Virus Reverse Transcriptase (FPLCpure™), RNAguard™ Ribonuclease Inhibitor (porcine) and stabilizers, including RNase/DNase-Free BSA
- 0.2 mL microcentrifuge tubes
- $pd(N)_6$: random primer used to generate the first-strand complimentary DNA (cDNA)

Previous research detecting DTV in ticks using RT-PCR utilized different sets of forward and reverse primers (11; 29; 76; 78). These were reviewed to determine the best set of previously validated primers optimal for use with the RT-PCR beads. The primers used by Ebel et al. (29) that amplify an approximate 300-bp length segment of the ns-5 gene were chosen as they met the primer length (15-30 bp) and GC content (~50%)

requirements for the RT-PCR beads. The primer sequences were confirmed by a Primer Blast in GeneBank and are listed below:

- forward ns5-f primer: 5'-GGCCATGACAGACACAACAGCGTTTG-3'
- reverse ns5-2 primer: 5'-GAGCGCTCTTCATCCACCAGGTTCC-3'

These primers were ordered from the USUHS Biomedical Instrumentation Center and received as lyophilized product. The forward and reverse primers were suspended with laboratory grade water into their 144 molar (M) and 213M stock concentrations, respectively. From these, dilutions of 500 µL of 5M concentrations were made for both the forward and reverse primers.

RT-PCR Procedure

The first step of the RT-PCR procedure consisted of combining the reaction reagents and generating cDNA. The 0.2 mL tubes containing the RT-PCR beads were arranged in a cooler block and marked with labels corresponding to the pooled template RNA samples. A master mix of the total water and pd(N)$_6$ required for all the reactions was made in a 1.5 mL microcentrifuge tube. A 43 µL aliquot of this master mix was dispensed into each 0.2 mL tube, subsequently dissolving the RT-PCR bead inside. A 2 µL aliquot from each pooled RNA sample was then added to its corresponding 0.2 mL tube, bringing the total reaction volume to 45 µL. The 0.2 mL tubes were closed, placed in a PTC-200 Thermal Cycler (MJ Research™, Boston, MA) and incubated at 42˚C for 20 minutes to generate cDNA. This was followed by incubating at 95˚C for 5 minutes to inactivate the reverse transcriptase and denature the template.

The second step of this RT-PCR consisted of adding the forward and reverse primers and amplifying the targeted gene. For each reaction tube already containing 45 μL of reagents, 2.5 μL of both the forward and reverse primer dilutions (5 μL total) was required. This resulted in a 0.25M concentration of forward and reverse primer in the 50 μL reaction. A primer master mix was made in a 1.5 mL microcentrifuge tube by adding the total volumes of both the forward and reverse primers required for all the reactions. The tubes were removed from the Thermal Cycler, briefly centrifuged, and placed in a block. The caps were opened and a 5 μL aliquot of primer master mix was dispensed into each reaction tube. For each reaction, caps were carefully opened and new pipette tips were used to prevent contamination. The caps were then closed and the tubes were loaded back into the Thermal Cycler for amplification of the target gene. The reactions underwent 40 cycles of denaturing, annealing, and elongation, followed by a final extension step. The times and temperatures for these steps are listed below:

Amplification - 40 cycles of:

- Denaturing – 45 seconds at 94˚C
- Annealing – 40 seconds at 40˚C
- Elongation – 1 minute at 72˚C

Final extension – 6 minutes at 72˚C

Once complete, the Thermal Cycler held the reaction tubes at 4˚C. The reactions were removed, placed in a cooler block, and transported to another room with a dedicated space for gel electrophoresis.

Gel Electrophoresis

Amplified products from RT-PCR were visualized on 2.2% agarose FlashGel® cassettes (Lonza, Rockland, ME) utilizing the Flashgel dock system (Lonza). Each cassette consisted of 32 wells, allowing all 25 amplified samples from a site, along with positive and negative controls to be visualized on one gel. The wells were flooded as per the manufacturer's protocol. For each reaction tube, 4 µL of amplified product was added and mixed with 5 µL of de-ionized water and 2 µL of loading die. A 3 µL aliquot of this mixture was loaded into a well on the gel cassette. A 50 bp ladder was included in wells on the sides of the cassette. The cassette was loaded into the Flashgel dock, and a low current (130-160V) was applied. The UV light and camera in the Flashgel dock allowed the agarose cassette to be observed and photographed with a computer as the DNA migrated across the gel. The lanes below each well were observed for the presence of a band around 300 bp in length. If present, bands were characterized as either being strong, moderate, weak, or extremely weak (Figure 8). Strong and moderate banding for at least one pool was considered evidence of possible DTV presence. Additionally, the presence of 5 weak bands on a gel was viewed as evidence of possible DTV. Once complete, the gel cassette was discarded in accordance with PHCR-North's Chemical Hygiene Plan. Amplification samples observed having strong to moderate bands on their gel lanes of approximately 300 bp in length were identified as pools potentially positive for deer tick virus. Isolation and RT-PCR of the individual ticks from these potentially positive pools was planned. Any individual positive ticks were then sequenced by the USUHS Biomedical Instrumentation Center to confirm the DTV sequence and thus the presence of the pathogen.

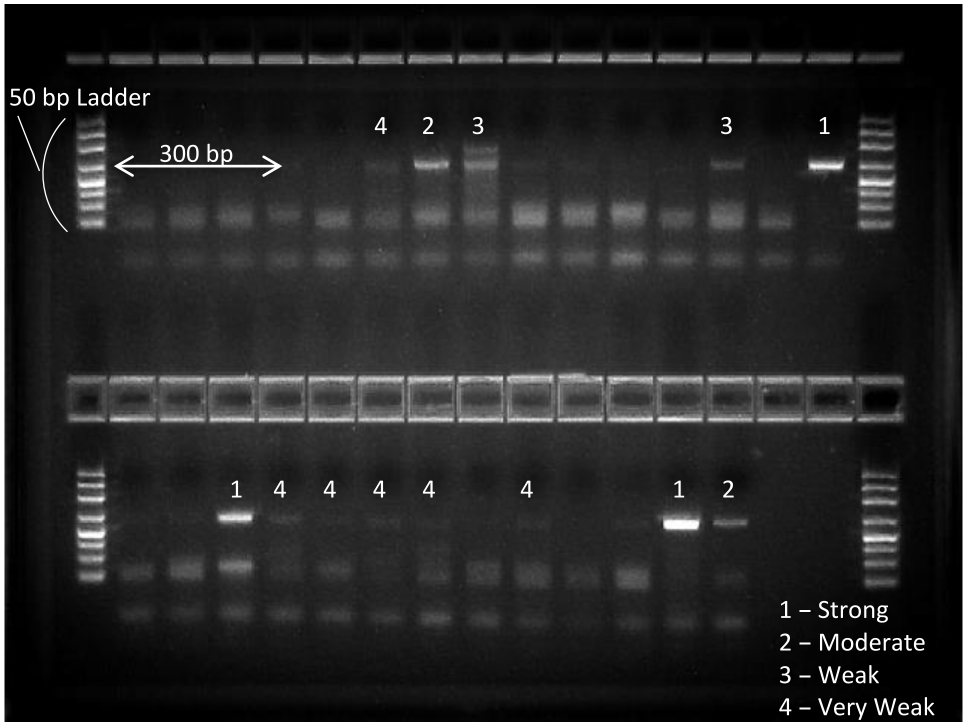


Figure 8: Electrophoresis gel with bands labeled as strong (1), moderate (2), weak (3), or very weak (4). 50 bp ladders are shown on side well lanes, with the 300 bp line indicated.

CHAPTER 3: Tick Sampling Results and Discussion

TICK SAMPLING RESULTS

During both the Test and DTV phases, a total of 7,374 ticks were collected in over 93 hours of sampling. The total number of *A. americanum*, *I. scapularis*, and *D. variabilis* ticks collected from all sites during the study is listed in Table 6. *Amblyomma americanum* was the most abundant tick (4,394) due to its prevalence in the southern sites of the study. However, *A. americanum* was not collected at any of the sampling locations in Pennsylvania, New York, or Connecticut. The second most collected tick, *I. scapularis* (2,941), was found in all states and in considerable numbers at each DTV sampling site. *Ixodes scapularis* was the dominate species collected in states with previously reported DTV infected ticks (Pennsylvania, New York, and Connecticut). A total of 39 adult *D. variabilis* ticks were collected at sites from New York to Virginia. Four *Haemophysalis leporispalustris* (Packard 1869) were also collected, all from sites in New Jersey and Maryland. The count data for all of the study's sites are shown in Appendix 2.

Table 6: Total counts from all sites for the three most collected tick species.

I. scapularis			*A. americanum*			*D. variabilis*		
Nymphs	Adults		Nymphs	Adults		Nymphs	Adults	
	Males	Females		Males	Females		Males	Females
2907	17	17	3975	246	173	0	19	20
Total *I. scapularis* = 2941			Total *A. americanum* = 4394			Total *D. variabilis* = 39		

Test Sampling Phase

Test sampling was conducted from late March through April, with 372 ticks collected in just under 22 hours of sampling. The total number of *A. americanum*, *I. scapularis*, and *D. variabilis* ticks collected during the Test Phase is listed in Table 7.

Amblyomma americanum were the predominate tick collected during this phase, probably due to their abundance at these southern study sites in Maryland and Virginia. Additionally, the Test sampling was conducted in early spring, prior the rise in nymph *I. scapularis* activity.

Table 7: Total counts for the three most collected tick species during the Test Phase of sampling.

I. scapularis			*A. americanum*			*D. variabilis*		
Nymphs	Adults		Nymphs	Adults		Nymphs	Adults	
	Males	Females		Males	Females		Males	Females
31	8	10	153	95	67	0	4	4
Total *I. scapularis* = 49			Total *A. americanum* = 315			Total *D. variabilis* = 8		

The Test phase also served to evaluate tick sampling devices, identify *I. scapularis* nymph habitat, and monitoring tick activity. The constructed tick sweep was quickly identified as the sampling device of choice as it collected more ticks of every species during a given sampling event. Of the various habitats sampled during the Test phase, hardwood woodland sites produced almost all of the *I. scapularis* nymphs collected. The activity of *I. scapularis* nymphs increased at the end of April, prompting the transition to the DTV sampling phase at the beginning of May. Evaluation data on sampling devices, *I. scapularis* habitat, and tick activity were not recorded in detail, thus restricting thorough quantitative analysis. The observations made during the Test phase greatly facilitated the completion of critical planning decisions for the DTV sampling phase.

DTV Sampling Phase

DTV sampling was conducted from early May through mid-June, with 7,002 ticks collected in just over 71 hours of sampling time. The total number of *A. americanum*, *I. scapularis*, and *D. variabilis* ticks collected during the DTV Phase is listed in Table 8. Both *A. americanum* and *I. scapularis* were prevalent at all sites. Collection rates (#ticks caught/hour) for *I. scapularis* and *A. americanum* ticks for all sampling events are listed in Appendix 3, sorted by the highest to lowest values for *I. scapularis*. The average collection rates for *I. scapularis* and *A. americanum* ticks at each site are listed in table 9. The site sampled at Pennypack (PA) produced the highest collection rates for *I. scapularis*, while Hemlock Overlook Regional Park (VA) had the lowest. Areas with high rates for one species did not preclude high rates for the other. Compared to all other sites, NWS Earle (NJ) had the highest and second highest collection rates of *A. americanum* and *I. scapularis*, respectively.

Table 8: Total counts for the three most collected tick species during the DTV Phase.

I. scapularis			*A. americanum*			*D. variabilis*		
Nymphs	Adults		Nymphs	Adults		Nymphs	Adults	
	Males	Females		Males	Females		Males	Females
2876	9	7	3822	151	106	0	15	16
Total *I. scapularis* = 2892			Total *A. americanum* = 4079			Total *D. variabilis* = 31		

Table 9: Average collection rates for *I. scapularis* and *A. americanum* ticks at each site.

State	Site	Sampling Time (min.)	***I. scapularis***		***A. americanum***	
			Ticks collected	Ticks/hour	Ticks collected	Ticks/hour
PA	Pennypack	120	268	134.0	0	0.0
NJ	NWS Earle	215	279	77.9	604	168.6
CT	Groton	230	258	67.3	0	0.0
PA	Fort IG	250	250	60.0	0	0.0
NY	West Point	270	269	59.8	0	0.0
MD	Fort Meade	290	257	53.2	328	67.9
VA	Banshee Reeks	410	252	36.9	487	71.3
NY	Louis Calder	485	283	35.0	0	0.0
NJ	JB MDL	460	255	33.3	920	120.0
MD	Aberdeen	545	261	28.7	615	67.7
VA	Hemlock Ov.	1125	260	13.9	1125	60.0
	Totals:	73.3 hours	2892	39.5 ticks/hr	4079	55.6 ticks/hr

DISCUSSION OF TICK SAMPLING RESULTS

Sampling efforts met the objective of collecting the required number of *I. scapularis* ticks (n=230) at each site. Test sampling was critical in refining the tick sampling methods and monitoring tick activity for the start of the next phase. The collector had established a defined sampling protocol and had become familiar with *I. scapularis* nymph habitat by the start of the DTV sampling phase. This resulted in efficient sampling during the DTV phase with the required number of ticks caught at every site.

During the Test Phase the tick sweep was quickly identified as the best sampling device for use in the study. The tick sweep's productive results and ease of use were a critical discovery that assisted in effective sampling during the DTV phase. The sweep was easier to maneuver around vegetated areas, fallen logs, and other structure in the woods compared to the tick drags. It was also the most productive device evaluated as it appeared to catch the highest number of *I. scapularis* or *A. americanum* ticks during any sampling event. Furthermore, the sweep was less laborious than the drags, enabling the collector to sample for a much longer time. These characteristics are very useful for tick studies requiring expedient collection of *I. scapularis* or *A. americanum* ticks.

The tick collection rates varied for *I. scapularis* and *A. americanum* across the sites. *A. americanum* were not collected at any of the five northern sites from Pennsylvania, New York, or Connecticut of the study. While the southern sites did have lower collection rates for *I. scapularis*, they were not inversely correlated with *A. americanum* collection rates. The high *I. scapularis* collection rates at the Pennypack (PA) and NWS Earle (NJ) sites indicate they may be focal locations of deer tick activity. The low collection rate for Hemlock Overlook provided the study with a site that had a lower *I. scapularis* abundance. If DTV were to be found at Hemlock Overlook, it would suggest the pathogen could circulate in areas without high abundances of *I. scapularis* far south of where it has previously been reported.

CHAPTER 4: Laboratory Results and Discussion

LABORATORY RESULTS

In total, 2,746 *I. scapularis* nymphs from the study's 11 sites were individually homogenized, pooled, and tested for the presence of DTV. One electrophoresis gel was made for each site that included all of its 25 pooled samples. These gels are shown in Appendix 4 with the pooled sample numbers indicated in their respective lanes. A summary of the number bands and their intensity, or strength, is given in Table 10. Five sites had a strong band present, four of which were from states (Virginia, Pennsylvania, and New Jersey) where no DTV has been reported in ticks. Some degree of banding at the 300 bp length was observed in at least one pool at every site. Two of the sites (Groton and NWS Earle) did not meet the study's criteria for having evidence of DTV in ticks. However, these sites did have some weak and/or very weak bands. The PCR amplification products for the positive control and Pool 24 (Calder Center, NY) were sequenced and matched the target DTV gene. This indicates the strong band on the Calder Center, NY gel is from at least one positive tick in Pool 24. Many of the gels showed evidence of possible contamination. The presence of 300 bp bands on a gel often occurred in groups of sequential order. Sometimes one of the bands was the strongest, flanked by a number of weak positives (Figure 9).

Table 10: Number and degree of bands observed for the study sites.

State:	Site:	Strong	Moderate	Weak	Very Weak	Total Bands Observed
		Degree of Banding				
VA	Banshee Reeks	1	1	2	8	12
	Hemlock Overlook		7	2	3	12
MD	Fort Meade		4	2	10	16
	Aberdeen		1	1	6	8
NJ	MDL	1	7	6	3	17
	NWS Earle				2	2
PA	Fort IG	2	4	4	4	14
	Pennypack	1	2	1	7	11
NY	Calder Center	1				1
	West Point			7	7	14
CT	Groton			2	3	5

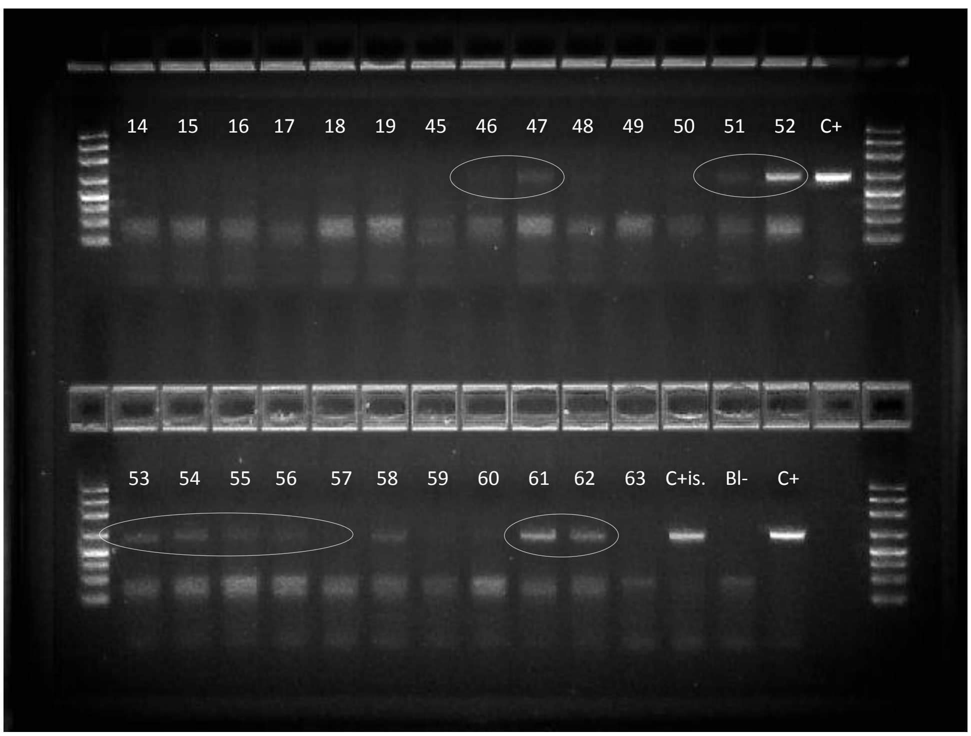


Figure 9. Possible contamination in the Pennypack, PA site. Sequential pool samples have positive 300 base pair bands (circled in white). Lack of contamination in wells adjacent to positive controls and no target band in the isolation blank indicate contamination occurred during storage or homogenization.

Discussion of Laboratory Results

The laboratory results show evidence that DTV may be present at most of the sites in the study. Sequence results from the positive pool from the Calder Center site in New York indicate DTV is circulating at that location. Many of the sites with strong gel banding at the target gene's location are located far south of previously reported DTV infected ticks. Although current results look promising, several aspects of the laboratory results prevent the final determination on the status of DTV presence.

The strength of banding is weak for many of the pooled samples, raising concern on whether the band was indeed the target amplification product. Similar weak banding, however, has been observed in gels from a RT-PCR using the same primers this study used (Figure 10) (29). The weak band (lane six) in the Ebel et al. (29) gel was from a pool of five ticks collected on Nantucket Island, MA. Sequencing confirmed the pool contained a DTV infected tick. The weak bands observed in this study have not been analyzed yet. The pending sequence results may confirm that these weak bands, and the stronger bands observed, are indeed proof of DTV presence at these sites.

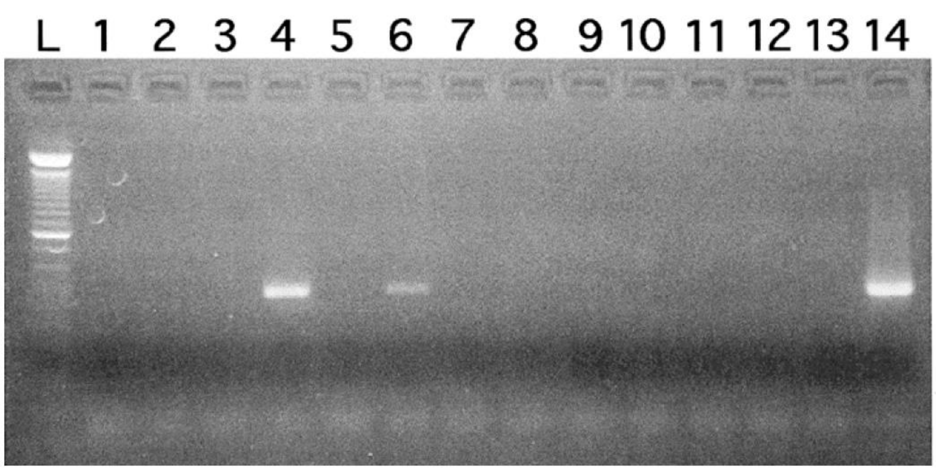


Figure 10. Image from Ebel et al. (29). Electrophoresis gel from a RT-PCR using the same primers used in the current study. Lanes 4 and 6 are positive pools (5 ticks/pool). Lane 14 is the positive control (DTV infected mouse brain).

The possible contamination that may have occurred raises concerns on what pools were true positive samples. A review of tick storage methods, laboratory procedures, and laboratory results was conducted to assess what might have caused contamination. The various possible contamination sources have different implications on how the study's results can be interpreted. Positive control contamination of the tick pools would render any results invalid, precluding any determination of either the presence or prevalence of DTV infection in ticks. While tick to tick contamination would prohibit an estimate of DTV infection prevalence, the presence or absence of the pathogen at a site could still be determined. The numerous positive pools would be the result of at least one DTV infected tick from the site, inferring the presence of the pathogen. The steps in processing the ticks (Figure 11), from storage through electrophoresis preparation, were reviewed to determine if positive control or tick-to-tick contamination could have been present.

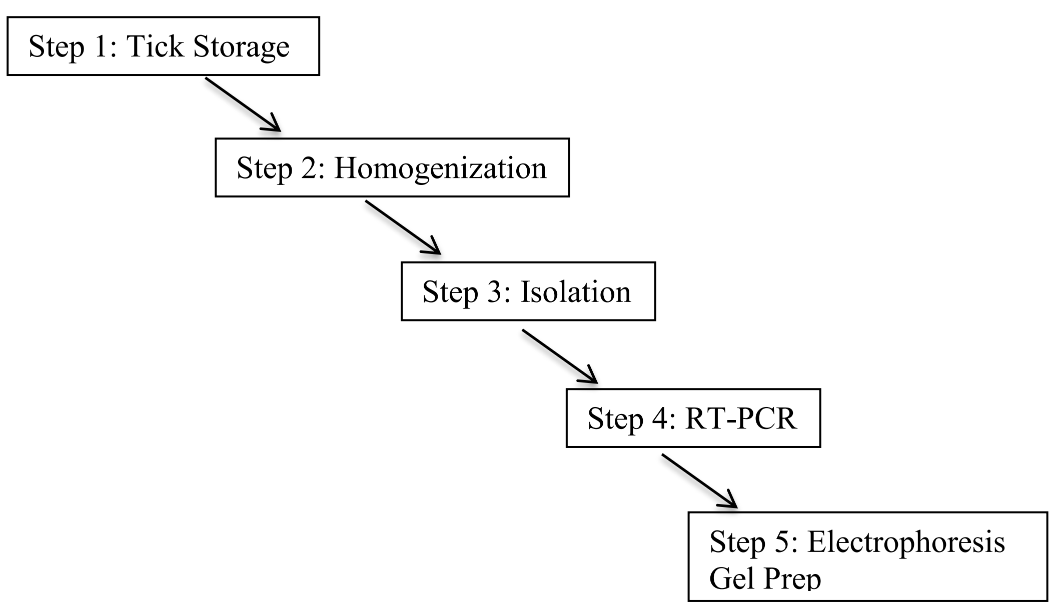


Figure 11: Tick processing steps reviewed for possible tick-to-tick or positive control contamination.

During Step 1, all field caught *I. scapularis* from a sampling event were removed from the masking tape that they were initially collected on and placed in one vial with 95% ethanol for storage until laboratory processing. Some ticks had legs broken off or were slightly damaged when being transferred from the tape to the storage vial. This was especially true in instances where ticks were unable to be transferred for some time (i.e. 12-24 hours). Deer tick virus infected hemolymph from a damaged tick could have possibly contaminated other ticks in the vial. This may have subsequently resulted in numerous false positive bands of varying intensities for the ticks in that vial. Tick-to-tick contamination during storage was therefore deemed possible.

During homogenization (Step 2), ticks were placed in microcentrifuge tubes with BBs, eviscerated, and then centrifuged. During the process of opening and closing tubes to remove BBs, tick homogenate may have contaminated the gloves of the handler and subsequently transferred to the following tubes. Concerted efforts were made to eliminate this type of tick-to-tick contamination, but the possibility of it occurring did still remain.

During the pooling and isolation, RT-PCR, and electrophoresis gel steps, the primary concern was on whether the positive controls contaminated the tick pools. During pooling and isolations, the positive control (C+ is.) was only introduced after the pooling of ticks was complete and their vials were closed. It seems unlikely that the possible contamination observed was due to the isolation positive control. In many of the isolations no positive control was included. If contamination was to have occurred from the isolation C+, it would most likely show as positive bands in the samples surrounding it. These samples would be the last pooled sample for the site (pool closest to the C+ is.

on the gel) or the isolation blank for the site. The Pennypack, PA gel was an example of a site where the lack of positive bands surrounding the C+ lanes show the possible contamination was not due to the isolation positive control (Figure 12). Similarly, other site's electrophoresis gels showed no evidence of contamination from their isolation positive controls.

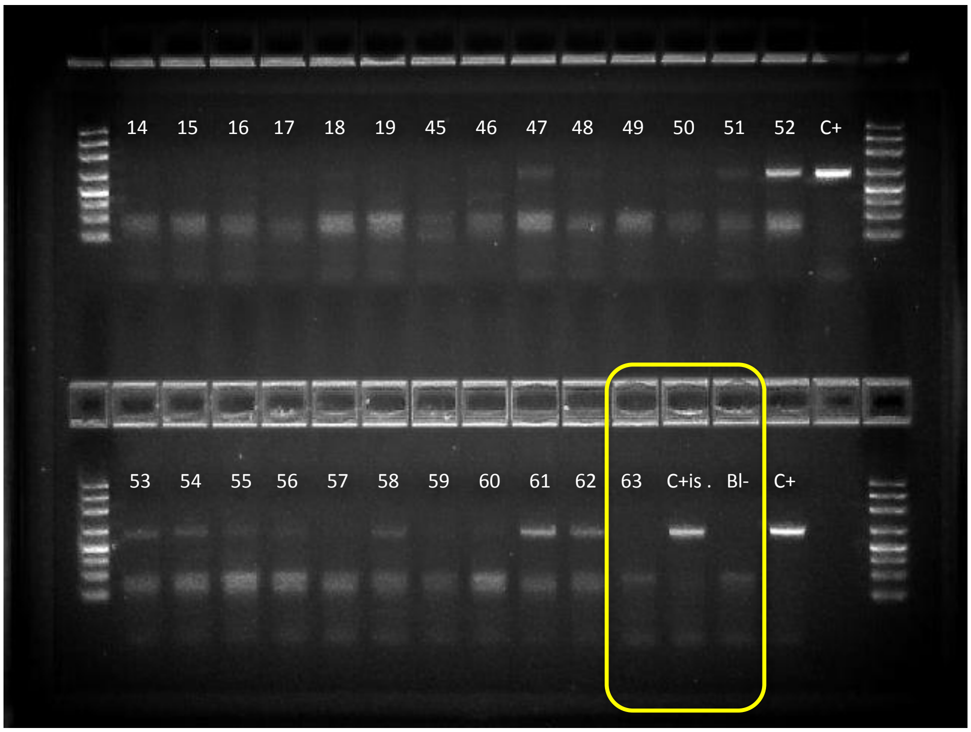
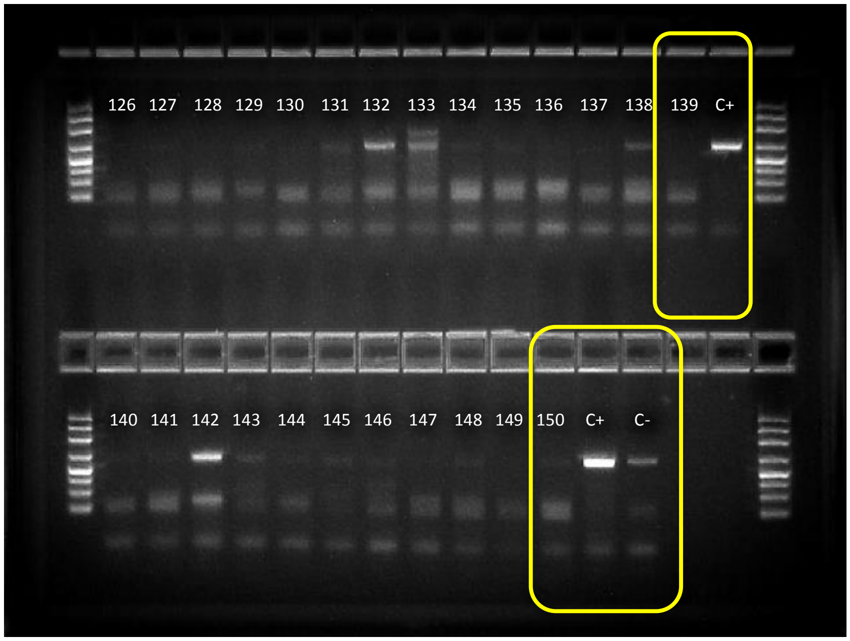


Figure 12: Electrophoresis gel from the Pennypack, PA site showing the lack of contamination from the isolation positive control (C+is.). The closest samples during isolation, pool 63 (63) and the isolation blank (Bl-) have no bands.

Contamination from the RT-PCR control (C+ PCR) also appears to have been unlikely. The C+ PCR was introduced during RT-PCR after all of the pooled samples were prepared and their 0.2 mL tube caps were closed. When adding the forward and reverse primer during the second PCR step, the positive control and PCR negative control were not opened until after the primers were added to the pooled samples and their caps were closed. Electrophoresis gels were also prepared in this manner, with all pooled samples prepared and loaded into the gel wells prior to the positive and negative PCR controls being opened. In one instance, evidence of contamination of the negative control from the positive control was observed. This is seen in the Banshee Reeks (VA) and Hemlock Overlook (VA) sites where the same amplified positive and negative controls were used for both (Figure 13). The lack of a band in the lanes to the other side of the PCR C+ indicates the contamination was confined to the negative control. All other gels showed no evidence of PCR C+ contamination.



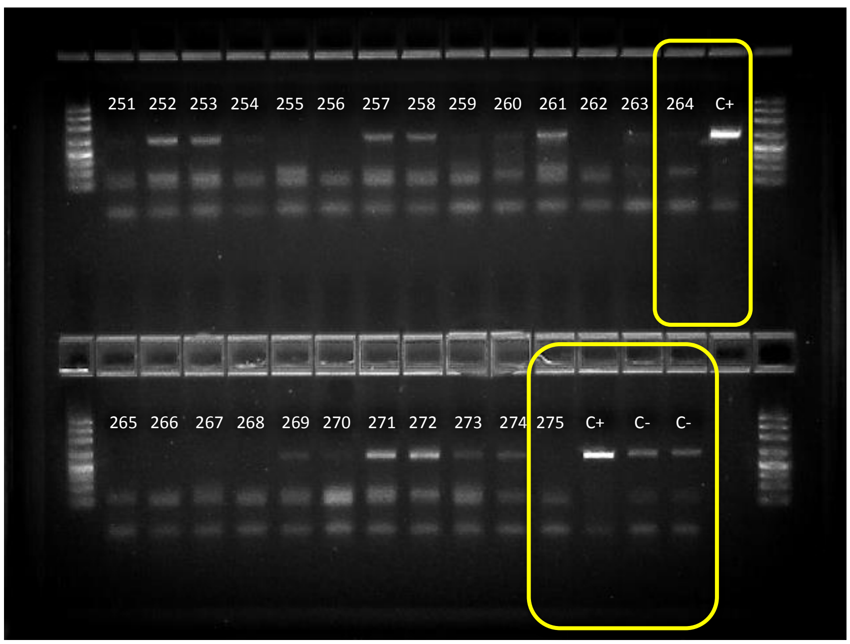


Figure 13: While Evidence of negative control (C-) contamination from the positive control (C+), no contamination is seen immediately left of C+.

The possible contamination observed appears to be most likely due to tick-to-tick contamination. The lack of contamination adjacent to the isolation and PCR positive controls indicates they were probably not the source of contamination. As previously mentioned, while tick-to-tick contamination does inhibit prevalence rates to be determined, DTV infection prevalence may still be ascertained. The 25 possibly tick-to-tick contaminated pools at each site essentially represent one large pool. Any positive pool therefore would indicate the presence of DTV in ticks at the site. A more thorough investigation on whether contamination did occur and to what extent is needed. Isolating ticks from positive pools individually would be the first step in assessing this. Until this is done, confident interpretations of the results of this study are difficult to make.

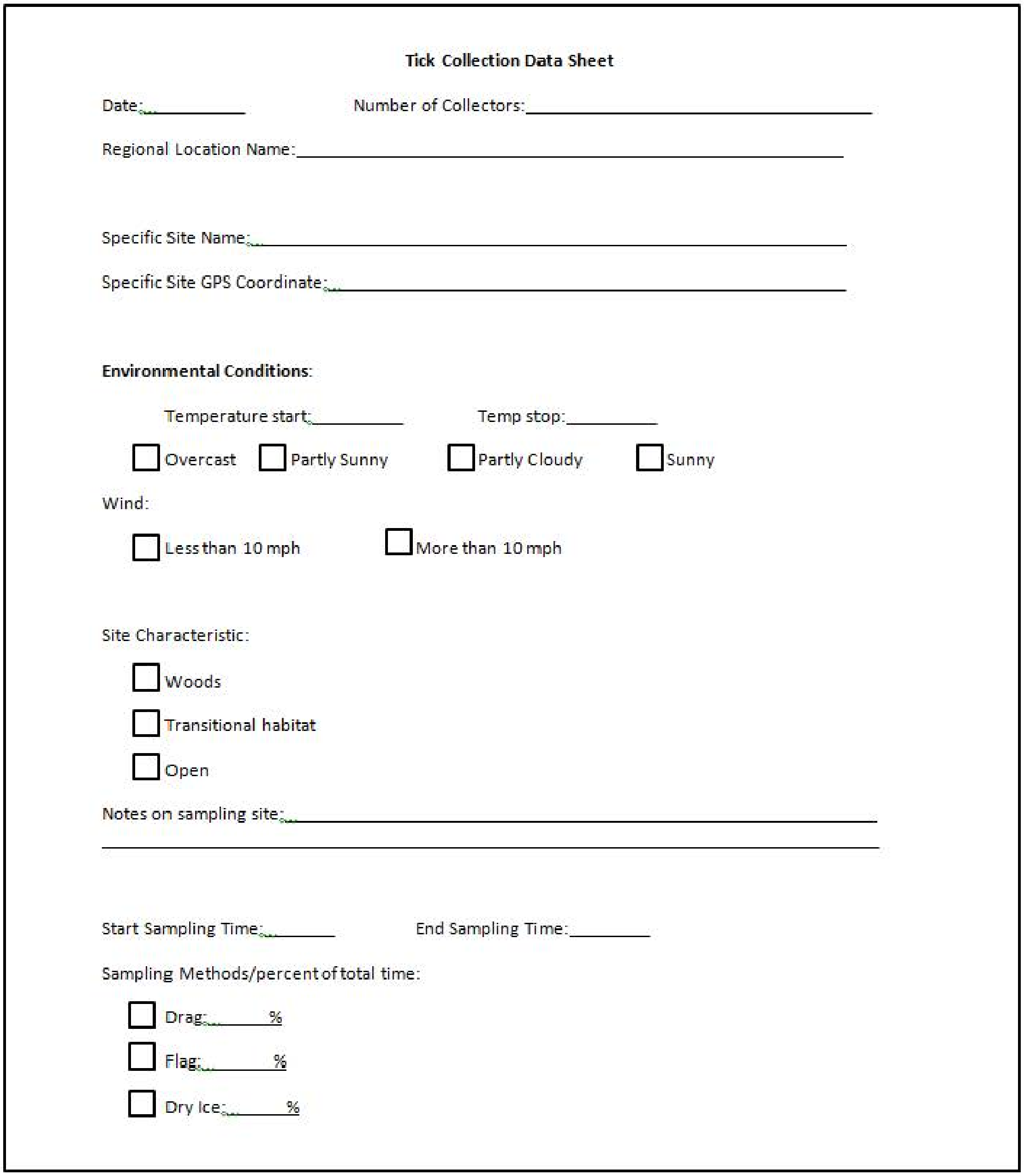
CHAPTER 6: Conclusion

The goal of this multi-state study on the U.S. east coast was to determine the presence or absence of DTV in areas known to have high *I. scapularis* densities. Additionally, the prevalence of DTV infection in the *I.scapularis* populations sampled was to be determined. While possible contamination issues prevented the study from estimating prevalence rates, results indicate DTV infected ticks were present at the study sites. One positive pool has been sequenced and confirmed as positive for DTV. Ticks from DTV positive pools are currently being tested individually and will be sequenced to confirm DTV presence. These sequence results are expected to be similar to the positive results from the Calder Center site and the positive control. If these sequence results confirm the presence of DTV at these sites, this study will have found DTV infected ticks in areas far south of what has been previously reported.

If more southern populations of DTV infected ticks do exist, the question exists as to of why human disease from this virus is almost absent. Interestingly, the states where DTV was originally found, Massachusetts and Connecticut (76), do not have a single historical case of Powassan encephalitis on record (74). Recent expansion in the geographic distribution of human cases of Powassan encephalitis indicates the virus may be establishing itself in new locations. While unconfirmed, it appears the Virginia Powassan encephalitis case in 2009 (37) may have been due to DTV. The transmission dynamics of the DTV have not been well studied, so predicting whether the pathogen will produce disease at new locations is difficult at best. That said, the geographic expansion and increased number of cases from Powassan encephalitis since 2007 should be a cause for concern, especially in areas where DTV is known to circulate.

The escalation in human cases of Powassan encephalitis is especially alarming considering the severity of the disease. If the upward trend in the number of Powassan encephalitis cases continues along with the expansion of where these cases are occurring, this emerging disease will warrant a significant amount of attention. In providing evidence of DTV infection in ticks at multiple locations far south of what has previously reported, this study suggests there is a potential for future Powassan encephalitis cases in areas where it has not occurred historically.

Appendix 1. Data entered on the Tick Collection Data Sheet at each sampling event included administrative and environmental information.



Tick Collection Data Sheet

Date:__________ Number of Collectors:______________________________

Regional Location Name:____

Specific Site Name:____

Specific Site GPS Coordinate:____

Environmental Conditions:

Temperature start:_________ Temp stop:_________

☐ Overcast ☐ Partly Sunny ☐ Partly Cloudy ☐ Sunny

Wind:

☐ Less than 10 mph ☐ More than 10 mph

Site Characteristic:

☐ Woods

☐ Transitional habitat

☐ Open

Notes on sampling site:____

____

Start Sampling Time:________ End Sampling Time:________

Sampling Methods/percent of total time:

☐ Drag:______%

☐ Flag:______%

☐ Dry Ice:______%

Appendix 2. Count Data for all sampling conducted during the Testing and DTV Phases.

Count Data for all Tick Sampling

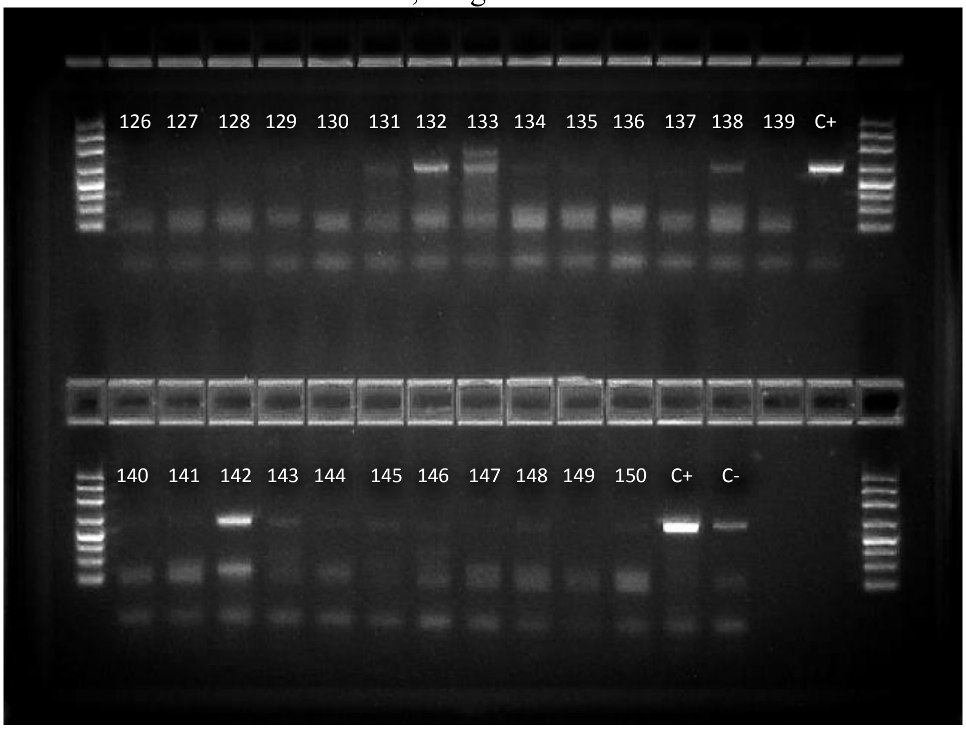
State	Site	Date	*I. scapularis*			*A. americanum*			*D. variabilis*		
			Nymphs	Adults		Nymphs	Adults		Nymphs	Adults	
				Males	Females		Males	Females		Males	Females
MD	Fort Meade	3/28/2012	0	1	2	5	24	12	0	0	0
MD	Fort Meade	3/29/2012	0	0	0	9	8	12	0	0	1
MD	Pat. C. Tract	3/30/2012	0	0	0	0	3	2	0	0	0
MD	Pat. C. Tract	3/30/2012	0	0	0	0	3	2	0	0	0
MD	Pat. C. Tract	3/30/2012	0	0	0	0	1	0	0	0	0
MD	Pat. C. Tract	3/30/2012	0	0	0	0	0	0	0	0	0
MD	Pat. C. Tract	3/30/2012	0	0	0	0	6	1	0	0	0
VA	Hemlock Overlook	4/3/2012	0	0	2	20	3	5	0	0	0
VA	Bull Run Park	4/3/2012	0	0	0	0	0	0	0	0	0
MD	Fort Meade	4/3/2012	0	0	0	13	8	3	0	0	0
MD	Pat. C. Tract	4/13/2012	0	0	0	0	1	3	0	0	0
MD	Pat. C. Tract	4/13/2012	0	0	0	0	3	1	0	0	0
MD	Pat. N. Tract	4/13/2012	0	2	0	1	2	1	0	0	0
MD	Pat. N. Tract	4/13/2012	0	0	1	0	4	1	0	0	0
VA	Algonquin	4/14/2012	0	0	0	1	0	0	0	0	0
VA	Algonquin	4/14/2012	0	0	0	0	0	0	0	0	0
MD	Fort Meade	4/21/2012	2	0	0	62	11	4	0	0	0
MD	Pat. N. Tract	4/24/2012	1	3	4	1	3	4	0	2	0
VA	Banshee Reeks	4/27/2012	27	2	1	39	13	16	0	2	3
MD	Fort Meade	4/28/2012	1	0	0	2	2	0	0	0	0
PA	Fort IG	5/11/2012	47	0	1	0	0	0	0	0	0
VA	Banshee Reeks	5/12/2012	164	0	1	274	38	21	0	5	5
NJ	JB MDL	5/17/2012	134	0	1	507	17	10	0	0	0
NJ	JB MDL	5/18/2012	120	0	0	359	17	10	0	0	1
MD	Fort Meade	5/19/2012	122	0	0	79	7	3	0	0	0
MD	Fort Meade	5/20/2012	62	0	0	37	1	1	0	0	0
PA	Fort IG	5/21/2012	199	1	2	0	0	0	0	0	0
PA	Pennypack	5/23/2012	177	0	0	0	0	0	0	0	0
PA	Pennypack	5/24/2012	91	0	0	0	0	0	0	0	1
NY	West Point	5/28/2012	193	3	2	0	0	0	0	1	0
NY	West Point	5/29/2012	69	2	0	0	0	0	0	0	0
NY	Louis Calder Center	5/31/2012	142	3	0	0	0	0	0	0	0
VA	Banshee Reeks	6/4/2012	87	0	0	110	25	19	0	8	6
VA	Hemlock Overlook	6/5/2012	81	0	0	317	6	4	0	0	0
MD	Fort Meade	6/6/2012	73	0	0	196	4	0	0	0	2
MD	Aberdeen	6/7/2012	107	0	0	263	2	7	0	1	1
MD	Aberdeen	6/8/2012	154	0	0	333	4	6	0	0	0
VA	Hemlock Overlook	6/9/2012	59	0	0	249	4	3	0	0	0
NJ	NWS Earle	6/11/2012	175	0	0	367	10	12	0	0	0
NJ	NWS Earle	6/12/2012	104	0	0	212	1	2	0	0	0
VA	Hemlock Overlook	6/14/2012	71	0	0	257	7	6	0	0	0
VA	Hemlock Overlook	6/15/2012	49	0	0	262	8	2	0	0	0
NY	Louis Calder Center	6/18/2012	90	0	0	0	0	0	0	0	0
NY	Louis Calder Center	6/19/2012	48	0	0	0	0	0	0	0	0
CT	Groton	6/20/2012	145	0	0	0	0	0	0	0	0
CT	Groton	6/21/2012	113	0	0	0	0	0	0	0	0

Appendix 3: Sampling times and collection rates (Ticks/Hour) for *I. scapularis* and *A. americanum*

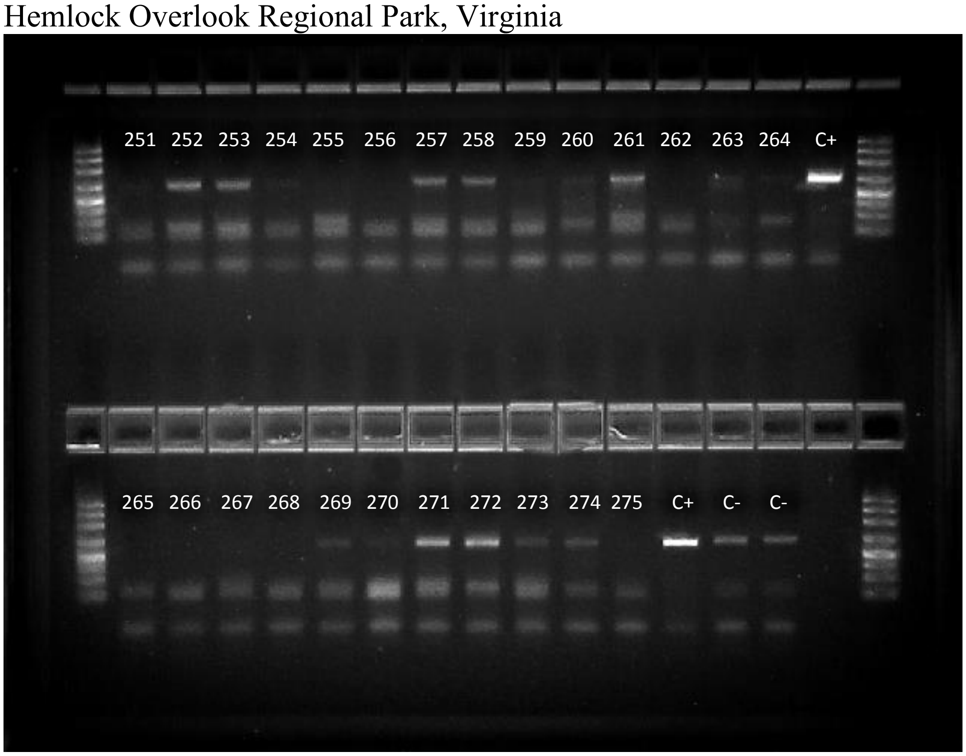
State	Site	Date	Sampling Time (min.)	***I. scapularis***		***A. americanum***	
				Ticks collected	Ticks / hour	Ticks collected	Ticks / hour
PA	Pennypack	5/23/2012	60	177	177.0	0	0.0
PA	Pennypack	5/24/2012	60	91	91.0	0	0.0
NJ	NWS Earle	6/11/2012	120	175	87.5	389	194.5
CT	Groton	6/20/2012	105	145	82.9	0	0.0
MD	Fort Meade	5/19/2012	105	122	69.7	89	50.9
PA	Fort IG	5/21/2012	180	202	67.3	0	0.0
NJ	NWS Earle	6/12/2012	95	104	65.7	215	135.8
NY	West Point	5/29/2012	65	71	65.5	0	0.0
NY	West Point	5/28/2012	205	198	58.0	0	0.0
CT	Groton	6/21/2012	125	113	54.2	0	0.0
VA	Banshee Reeks	6/4/2012	105	87	49.7	154	88.0
MD	Fort Meade	5/20/2012	75	62	49.6	39	31.2
PA	Fort IG	5/11/2012	70	48	41.1	0	0.0
NY	Louis Calder Center	6/19/2012	70	48	41.1	0	0.0
MD	Fort Meade	6/6/2012	110	73	39.8	200	109.1
NY	Louis Calder Center	6/18/2012	145	90	37.2	0	0.0
NJ	JB MDL	5/17/2012	225	135	36.0	534	142.4
MD	Aberdeen	6/8/2012	260	154	35.5	343	79.2
VA	Banshee Reeks	5/12/2012	305	165	32.5	333	65.5
NY	Louis Calder Center	5/31/2012	270	145	32.2	0	0.0
NJ	JB MDL	5/18/2012	235	120	30.6	386	98.6
MD	Aberdeen	6/7/2012	285	107	22.5	272	57.3
VA	Hemlock Overlook	6/9/2012	225	59	15.7	256	68.3
VA	Hemlock Overlook	6/5/2012	310	81	15.7	327	63.3
VA	Hemlock Overlook	6/15/2012	230	49	12.8	272	71.0
VA	Hemlock Overlook	6/14/2012	360	71	11.8	270	45.0

Appendix 4: Electrophoresis gels for all study sites. Numbered lanes refer to the tick pool tested. Positive Controls for isolation and PCR are labeled C+is. and C+. Negative controls for PCR are labeled Bl- and C-.

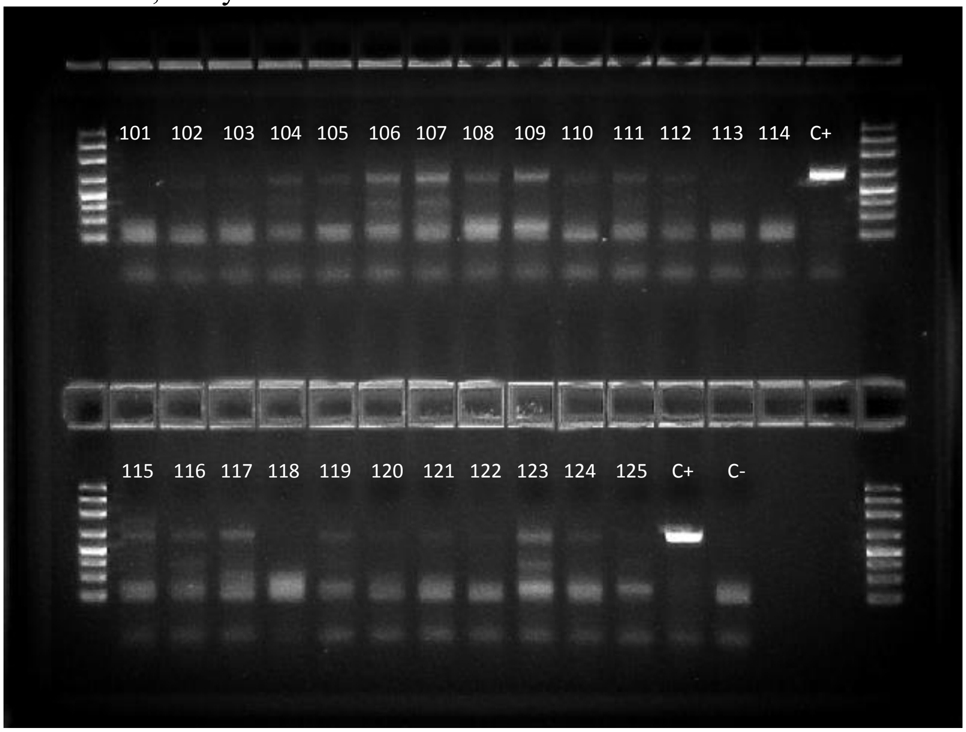
Banshee Reeks Nature Preserve, Virginia



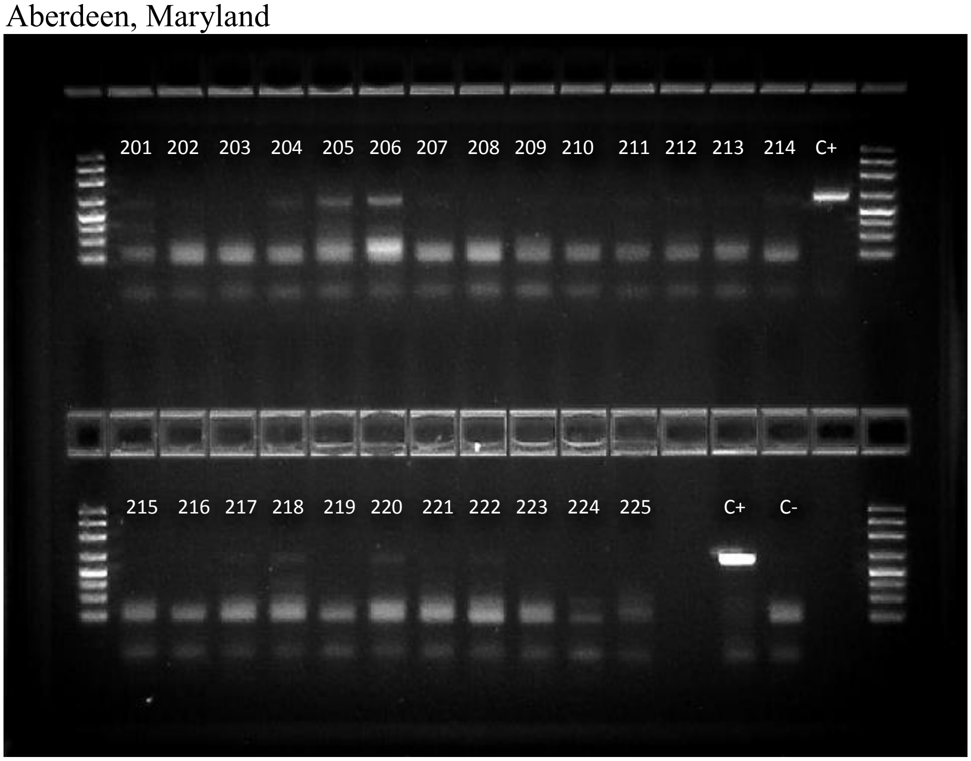
Hemlock Overlook Regional Park, Virginia



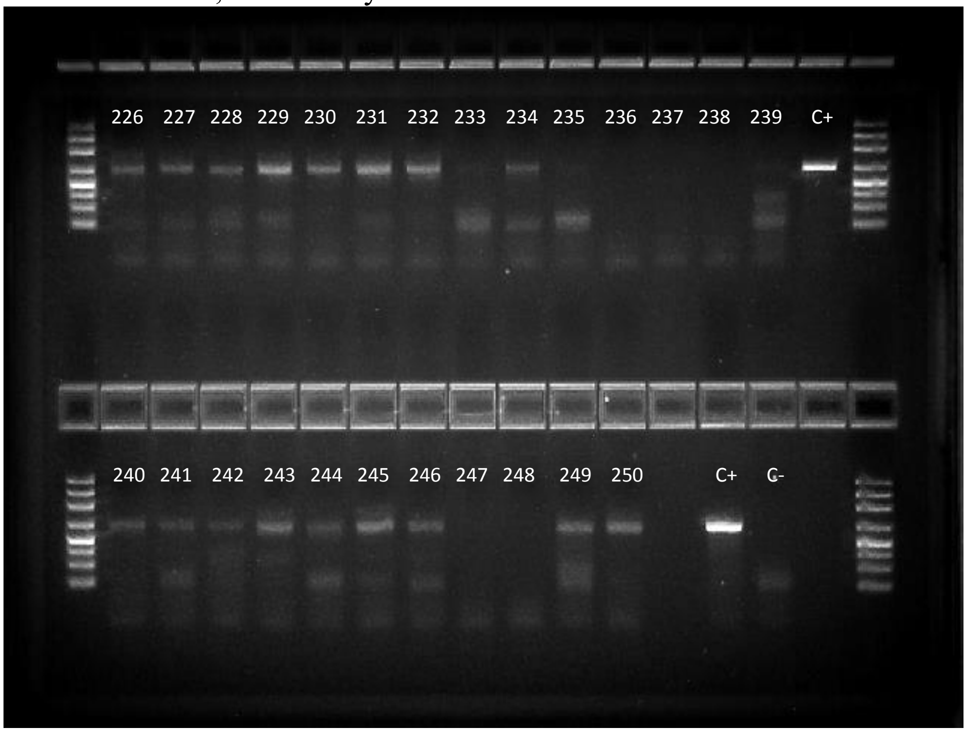
Fort Meade, Maryland



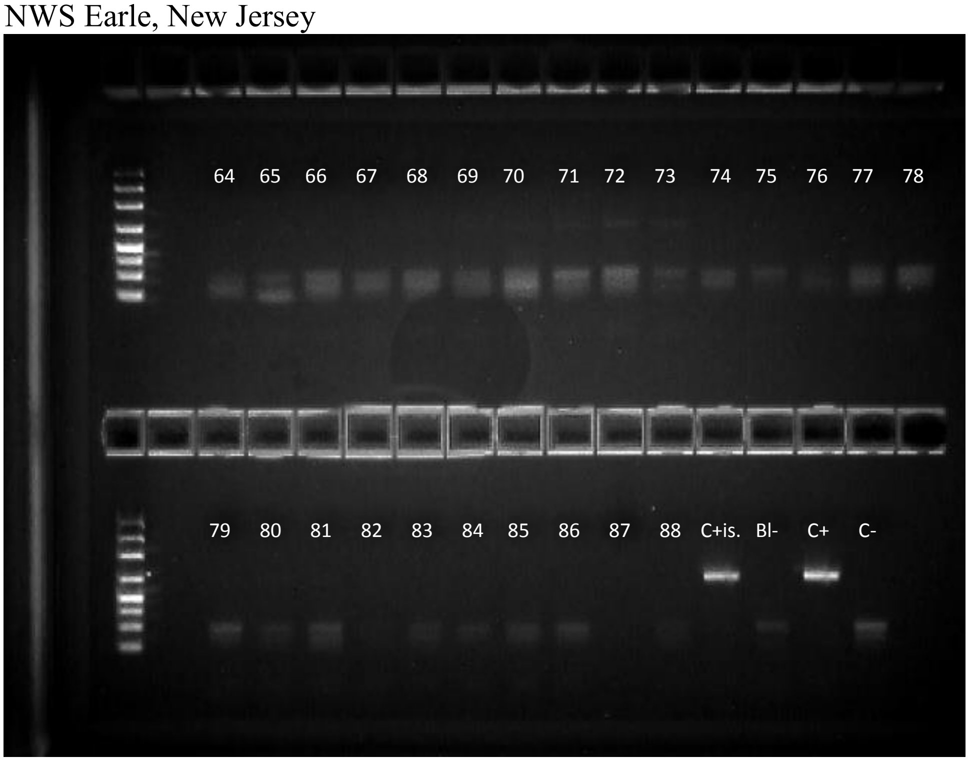
Aberdeen, Maryland



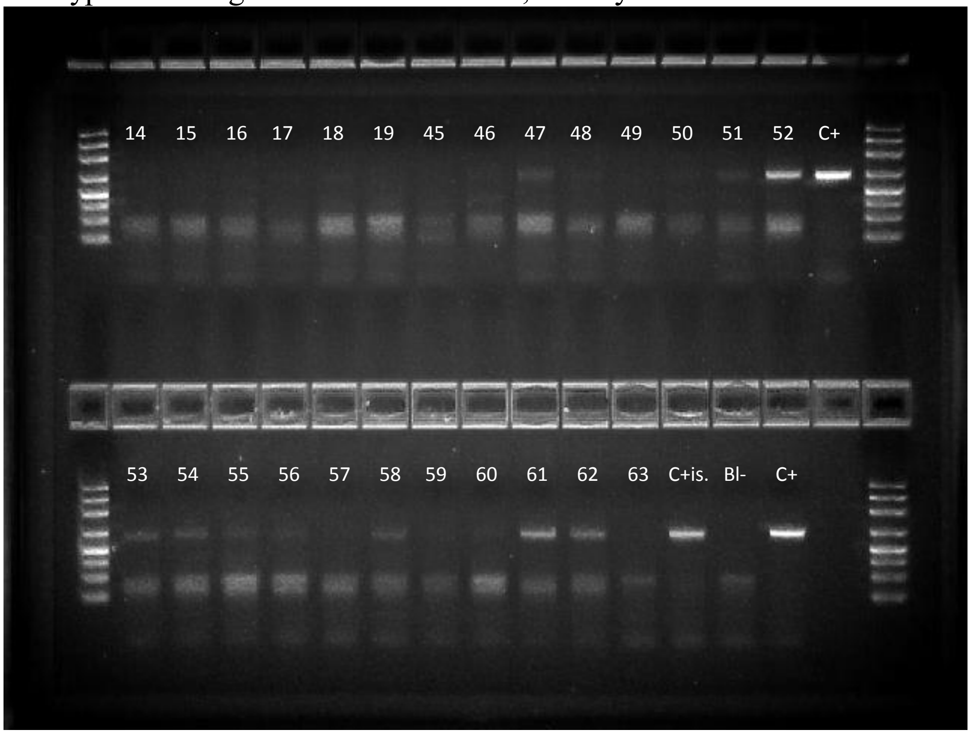
Joint Base MDL, New Jersey



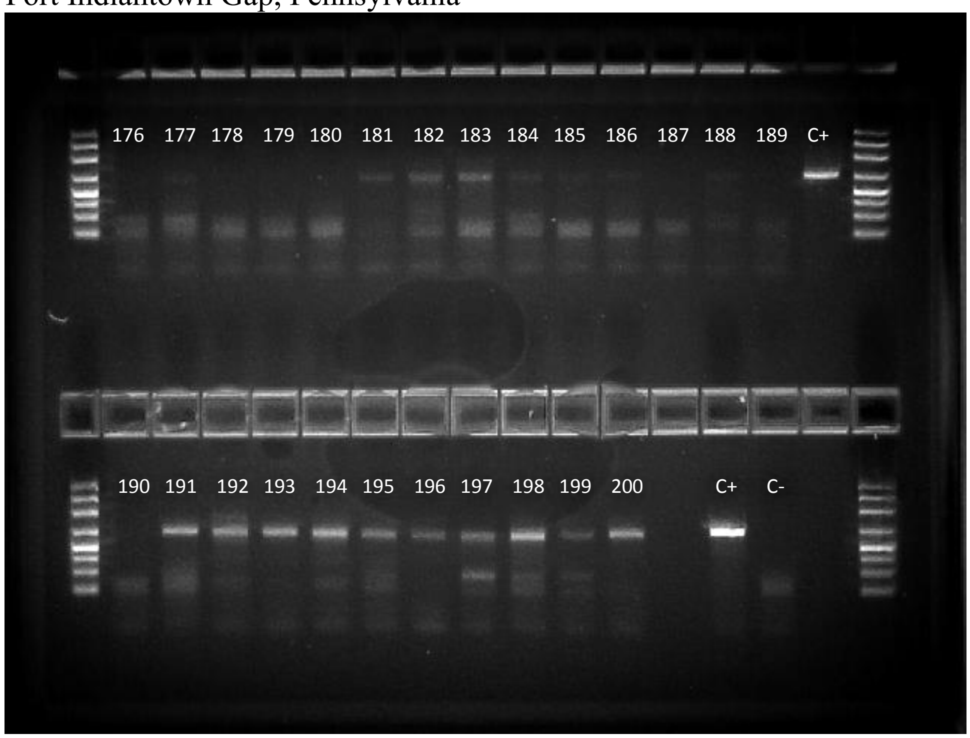
NWS Earle, New Jersey



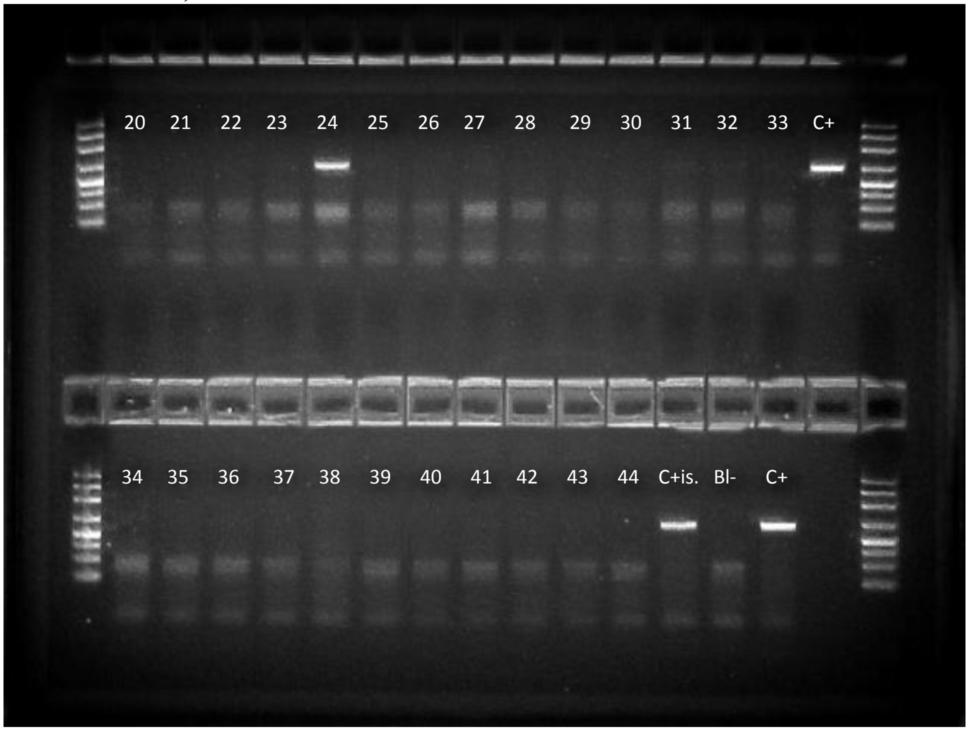
Pennypack Ecological Restoration Trust, Pennsylvania



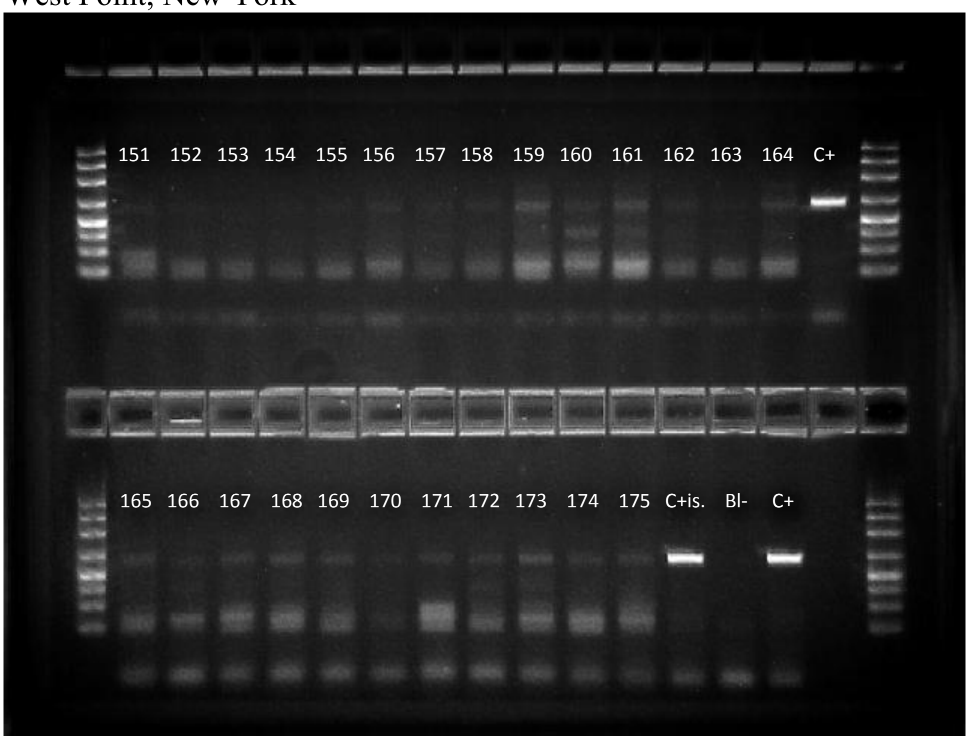
Fort Indiantown Gap, Pennsylvania



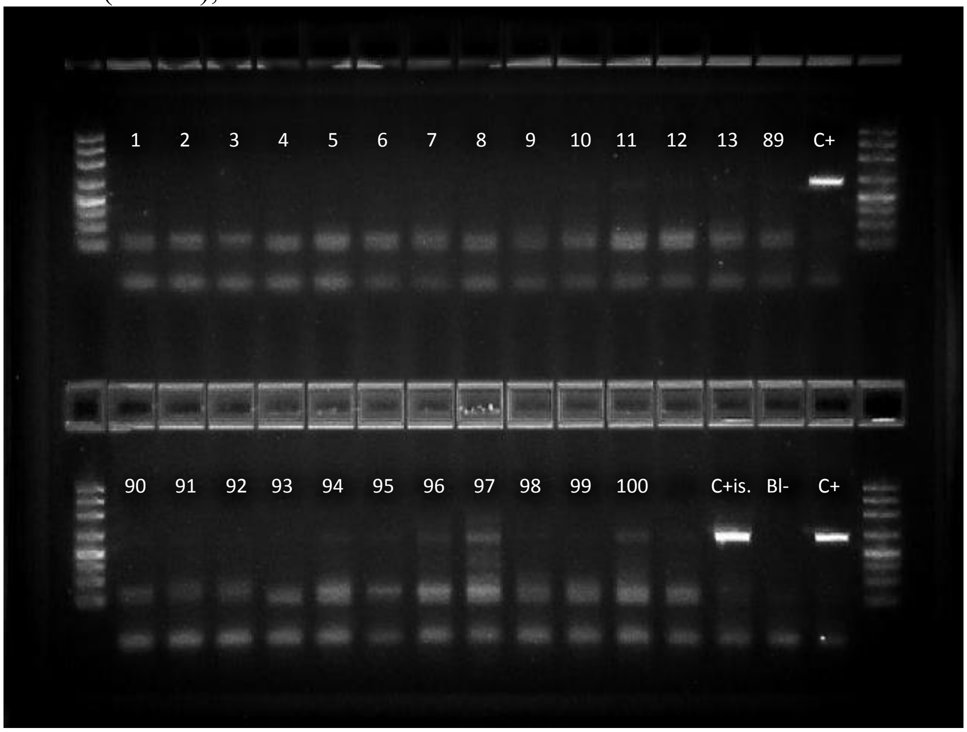
Calder Center, New York



West Point, New York



Groton (NBNL), Connecticut



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