Transstadial Transfer of West Nile Virus by Three Species of Ixodid Ticks (Acari: Ixodidae)

JOHN F. ANDERSON,[1] ANDREW J. MAIN,[2, 3] THEODORE G. ANDREADIS,[2] STEPHEN K. WIKEL,[4] AND CHARLES R. VOSSBRINCK[2]

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ABSTRACT Larvae and/or nymphs of four species of ixodid ticks, *Ixodes scapularis* Say, *Amblyomma americanum* (L.), *Dermacentor andersoni* Stiles, and *Dermacentor variabilis* Say, were fed to completion on laboratory hamsters or mice which had been inoculated with a West Nile (WN) virus isolate from *Culex pipiens* L. captured in Connecticut USA. Maximum titers in mice and hamsters were ≈5 and two logs, respectively, lower than recorded (10 logs) in a naturally infected American crow, *Corvus brachyrhynchos* Brehm. WN virus was isolated in Vero cell culture from ticks and detected by TaqMan RT-polymerase chain reaction (PCR) in ticks that had completed their feeding as larvae or nymphs, and in *I. scapularis*, *D. andersoni*, and *D. variabilis* that had molted into the next stage of development. Naïve hosts, fed upon by nymphs that as larvae had fed on viremic hosts, did not become infected. WN virus was isolated in Vero cell culture from one female *I. scapularis* and was detected by TaqMan RT-PCR in 24 adult *I. scapularis*, one *D. andersoni*, and two *D. variabilis* adults that had fed to completion as larvae on viremic hosts and as nymphs on naïve mice or hamsters. Three species of ixodid ticks acquired WN virus from viremic hosts and transstadially passed the virus, but vector competency was not demonstrated.

KEY WORDS West Nile virus, *Dermacentor variabilis*, *Dermacentor andersoni*, *Amblyomma americanum*, *Ixodes scapularis*

THE MOSQUITO-TRANSMITTED West Nile (WN) virus occurs in Africa, Europe, Asia, and Australia (Kunjin virus) (Hayes 1989, Hubalek and Halouzka 1999) and was recently introduced into northeastern United States (Anderson et al. 1999, Lanciotti et al. 1999). WN virus, unlike most other viruses in the Flaviviridae, infects both mosquitoes and ticks and may be transmitted by soft and hard-bodied ticks (Hoogstraal 1985).

The importance of mosquitoes as vectors of WN virus is well documented. This virus has been isolated from over 40 species of mosquitoes in the Old World (Hayes 1989, Hubalek and Halouzka 1999) and isolated or detected in >20 species of mosquitoes in eastern United States (Anderson et al. 1999, 2001; Lanciotti et al. 1999, Nasci et al. 2001, White et al. 2001, Andreadis et al. 2001, Bernard et al. 2001, Kulasekera et al. 2001, Marfin et al. 2001). Vector competency was documented first with African and Eurasian mosquitoes (Philip and Smadel 1943, Kitaoka 1950, Taylor and Hurlbut 1953) and more recently with North American mosquitoes (Turell et al. 2001).

Additionally, this virus also has been recovered in Africa and Eurasia from the soft-bodied ticks *Ornithodoros capensis* Neumann (Mirzoeva et al. 1974, L'vov et al. 1975) and *Argas hermanni* (Audouin) (Schmidt and Said 1964). Laboratory studies have documented transmission of WN virus by *O. savignyi* (Audouin) (Hurlbut 1956), *O. moubata* (Murray) (Whitman and Aitken 1960), *O. erraticus* (Lucas) and *O. capensis* (Vermeil et al. 1960), and *A. arboreus* Hoogstraal (Kaiser and Kohls) (Abbassy et al. 1993). While WN virus has been isolated from field-collected hard-bodied ticks *Hyalomma asiaticum* Schulze and Schlottke, *H. detritum* Schulze, *H. marginatum* Koch, *Rhipicephalus turanicus* Pomerantsev and Matikashvili, *R. bursa* Canestrini and Fanzago, *Amblyomma variegatum* (Fabricius), *Ixodes ricinus* (L.), and *Dermacentor marginatus* Sulzer (Shalunova et al. 1968, Chumakov et al. 1968, 1974, Berezin 1971, Sokolova et al. 1973, Mirzoeva et al. 1974, Lvov et al. 1975, Darwish and Hoogstaal 1981, Hayes 1989, and Hubalek and Halouzka 1999), vector competency has not been documented (Whitman and Aitken 1960).

The importance of ticks in the natural history of West Nile virus is unknown (Hoogstraal 1985). Unlike mosquitoes, ticks have not has been reported to be associated with human disease. However, ticks, particularly soft-bodied species, are relatively long lived

[1] Department of Entomology, The Connecticut Agricultural Experiment Station, 123 Huntington Street, New Haven, CT 06511 (e-mail: John.F.Anderson@po.state.ct.us).

[2] Department of Soil and Water, The Connecticut Agricultural Experiment Station, 123 Huntington Street, New Haven, CT 06511.

[3] American University in Cairo, 113 Kasr El-Aini, Cairo, Egypt.

[4] Center for Microbial Pathogenesis, School of Medicine, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030.

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and could harbor the virus for years. The isolation of this virus from *A. hermanni* infesting pigeon houses in Egypt during winter suggests that this species could serve as a host for the virus during winter (Schmidt and Said 1964).

Ixodid ticks are relatively common in temperate areas of the United States, and several feed on humans. We therefore examined whether four species of hard-bodied ticks known to feed on humans (*Ixodes scapularis* Say, *Dermacentor andersoni* Stiles, *Dermacentor variabilis* Say, and *Amblyomma americanum* [L.]) could acquire, transstadially pass, and transmit this virus to naïve laboratory animals. *I. scapularis* and *A. americanum* frequently feed on birds in their juvenile stages and could acquire the virus while feeding on viremic birds. The two *Dermacentor* species are almost exclusively mammalian parasites and rarely have been recorded feeding on birds. However, WN virus has also been documented to infect domestic and wild mammals such as horse (*Equus caballus* L.), striped skunk (*Mephitis mephitis* [Schreber]), eastern chipmunk (*Tamias striatus* [L.]), eastern gray squirrel (*Sciurus carolinenesis* Gmelin), and domestic rabbit (*Oryctologus cuniculus* [L.]) (Marfin et al. 2001, Kulasekera et al. 2001, Anderson et al. 2001). *Dermacentor* ticks could therefore, become infected while feeding on infected mammals.

Materials and Methods

Unfed *I. scapularis* larvae, *D. andersoni* larvae and nymphs, *D. variabilis* larvae and nymphs, and *A. americanum* larvae and nymphs were fed to completion on 21–27 d-old C3H/HeN mice or 3–6 wk-old Syrian hamsters inoculated with passage two of WN virus strain 2741 isolated from *Culex pipiens* L. in Connecticut in 1999 (Anderson et al. 1999). Larval *I. scapularis* were hatched from eggs laid in the laboratory from fully fed female ticks off white-tailed deer, *Odocoileus virginianus* Boddaert, killed during the fall hunting season or from field collected questing adult females fed on rabbits in the laboratory. Larval and nymphal *D. variabilis*, *D. andersoni*, and *A. americanum* were obtained from laboratory colonies that had been maintained on mice and rabbits. Unfed larvae and nymphs were 4–12 wk old when placed on host animals.

Colony ticks and laboratory emerged *I. scapularis* used in these studies were free of known infectious pathogens. *D. andersoni* and *D. variabilis* colonies had never been exposed to *Rickettsia rickettsii* during their more than 5 yr of maintenance in the laboratory. Colorado tick fever virus is not transovarilly transferred by *D. andersoni. Borrelia burgdorferi*, *Babesia microti*, and *Anaplasma phagocytophila* are not transovarially transferred *by I. scapularis. B. burgdorferi* has been reported to be present in progeny of infected females (Piesman et al. 1986, Magnarelli et al. 1987), but larvae have never been documented to infect hosts during feeding, and *B. burgdorferi* has never been cultured from unfed larvae.

Virus titers equal to 5×10^6 and 2.5×10^6 plaque-forming units (pfu) per milliliter were inoculated intraperitoneally into Syrian hamsters and C3H/HeN mice, respectively. Ticks were allowed to attach to mice or hamsters 0, 1, or 2 d before animals had been inoculated with virus. All experiments were carried out within a biological safety hood.

After ticks had attached to a host, each animal was placed into a standard aluminum cylindrical cage suspended over a whitish colored pan containing water. Ample food and water were provided. Engorged ticks, which had fallen off the animal and into the water, were placed into rearing vials with adequate moisture and kept in a bell jar sealed with petroleum jelly at room temperature under a photoperiod of 16 h of light and 8 h of darkness. *I. scapularis* were maintained at a RH of 98%; the other three species were kept at a RH of 94%.

Mice and hamsters were euthanized after completion of feeding. Virus isolation attempts were made from blood and from tissues of brain, spleen, and kidney of some but not all inoculated mice and hamsters to verify infection.

Attempts to isolate virus from ticks were made from day zero after completion of feeding as a larva or nymph to after the tick had molted into the next developmental stage and in a few experiments molted into the adult stage. Some larvae that fed on viremic hosts where tested for virus in the adult stage. These larvae molted into nymphs, fed on naive hosts, and then molted into adults.

Plaque-forming units of virus in the blood and antibody titers in inoculated animals were determined by inoculating each of fifteen 22 d old C3H/HeN mice with 2.5×10^6 pfu and twenty-one 22-d old hamsters with 5×10^6 pfu. Heparinized blood was drawn from each of three mice 36, 60, 84, and 104 h postinoculation and from one mouse at 128 h after inoculation (two mice died). Blood was drawn from each of three hamsters on days one through six and from two hamsters on day 7 (one hamster died). The uncoagulated blood sample taken from each mouse and the serum from each hamster were tested for plaquing viruses in a Vero cell-cultured 6-well assay. Blood or serum that had been frozen at $-80°$ was serially diluted from 10^{-1} to 10^{-6} in phosphate-buffer saline containing gelatin (PBS-G) (0.5% gelatin, 30% rabbit serum, antibiotic and antimycotic). Diluted blood or serum (100 μl) was placed in duplicate wells in each of the two 6-well plates containing a confluent layer of cells established 3 d earlier and from which minimal essential medium (MEM) (Life Technologies, Rockville, MD) had been decanted. Inoculated cells were incubated for 1 h at 37°C in 5% CO_2 with periodic rocking. Cells were then overlaid with MEM containing 1% agar. Cells were incubated at 37°C in 5% CO_2 and overlaid 4 d after application of blood or serum to Vero cells with a 0.004% solution of neutral red mixed with MEM containing 1% agar. Plaques were counted 24 h later.

To compare viremias in laboratory rodents with that of a naturally infected American crow, *Corvus brachyrhynchos* Brehm, a serum sample from a dying WN virus infected American crow captured in Meriden,

CT 12 September 2000 was tested for plaquing viruses as described previously.

Antibody levels in inoculated mice and hamsters were determined by plaque reduction neutralization tests (Beaty et al. 1995). A constant inoculum of 100 pfu of WN virus strain 2741 was tested against twofold dilutions from 1:10–1:320 of mouse and hamster inactivated sera. Blood and sera were diluted in PBS-G. Mixtures of virus and blood or serum were incubated for 1 h at 37°C before inoculation onto three-day old Vero cells. Each blood or serum dilution was replicated. Inoculated cells were incubated for 4 d and over-laid with neutral-red agar. Plaques were counted 1 d later. Titers were recorded at 80% reduction or greater of pfu.

Attempts to isolate virus from ticks were as follows. Each tick or group of up to five ticks was surface cleansed with 70% isopropal alcohol and triturated either in a mortar containing alundum using a pestle or in 1.5 ml centrifuge vial containing a copper BB pellet. Ticks were triturated in 1.0–1.5 ml of PBS-G within a biological safety hood. Ticks in the centrifuge vials were milled at 25 cycles per second for 4 min in a Retsch Laboratory Vibration Mill MM 300 (Irvine, CA). All samples were centrifuged at 4°C for 10 min at 520 $\times g$ and the supernatant passed through a 0.22u filter. There were 100 ul from each tick sample inoculated onto a monolayer of Vero cells set up the day before in a 25-cm^2 flask at 37°C in 5% CO_2. Cells were examined for cytopathogenic effect up to 7 d after inoculation. Although WN virus was the only virus used in these studies and inoculated in host animals, we extracted RNA from Vero-cell positive ticks and documented the virus as WN by TaqMan reverse transcriptase (RT)-PCR (Lanciotti et al. 2000).

Ticks that were negative and positive in Vero cell culture were tested for the presence of WN virus by TaqMan RT-PCR as follows. RNA was extracted from a 140 μl supernatant sample of each centrifuged triturated tick using the QIAamp viral RNA kit (QIAGEN, Valencia, CA) and stored at −80°C. Negative and positive control samples were included in each test. The negative control was double-processed tissue culture water (Sigma, St. Louis, MO). The positive control was *Cx. pipiens* isolate 8770. Primers were WNENV-forward 1160 (TCAGCGATCTCTCCACCAAAG) and WNENV-reverse 1229 (GGGTCAGCACGTTTGTCATTG). WNENV-probe 1186 (TGCCCGACCATGGGAGAAGCTC) was used with 5′ end labeled with the FAM reporter dye and the 3′ end labeled with the TAMRA quencher dye. A 25 μl reaction volume using the TaqMan RT-PCR Ready-Mix Kit (PE Applied Biosystems) was prepared with 2.5 μl of RNA, 0.25 μl of each primer, 0.15 μl of probe, 12.5 μl of two X buffer, 0.5 μl RT-PCR enzyme, and 8.85 μl of water. Samples were amplified in a Smart Cycler operated by Smart Cycler software (Cepheid, Sunnyvale, CA). Samples were subjected to one cycle of 50°C for 20 min, 95°C for 10 min, and then 50 cycles of 95°C for 15 s and 60°C for 60 s. Specimens with a cycle threshold value of <37 were considered to be infected with virus.

Results

Viremia and Antibody Titers in Mice, Hamsters, and an American Crow. Virus pfu were highest 1.5–2 d after inoculation in mice and hamsters. Neutralization antibodies were detected in mice and hamsters 3–3.5 d after infection. Mean numbers of pfu per mouse blood 1.5, 2.5, 3.5, 4.5, and 5.5 d after infection were 7.5×10^5, 2.1×10^4, 6.6×10^2, 0, and 0, respectively. In hamsters on days 1 through 7 post infection, mean pfu were 2.1. $\times 10^7$, 2.5×10^8, 1.2×10^7, 1.7×10^3, 8.8×10^2, 1.5×10^2, and 0, respectively. Mouse plaque reduction neutralization titers 1.5 d through 4.5 d post inoculation were, respectively, 0, 0, 1:3.3, and 1:20. Mean plaque reduction neutralization titers in hamsters on days 1 through seven post infection, were 0, 0, 1:13, 1:133, 1:267, 1:213, and 1:240, respectively. Mice and hamsters began dying on day 5 and 7 after inoculation of WN virus, respectively. Before death, mice and hamsters exhibited loss of appetite, disorientation, somnolence, and loss of mobility.

Viral titer from the serum drawn from a dying American crow was 3.1×10^{10} pfu per milliliter.

Transstadial Passage of WN Virus. The four species of ticks used in our experiments acquired virus during feeding (Table 1). WN virus was recovered in Vero cell culture from triturated specimens shortly after they had completed feeding as larvae or nymphs. WN virus also was transstadially passed in three species. Virus was cultured from nymphs of *I. scapularis*, *D. andersoni*, and *D. variabilis*, which had fed on viremic hosts in their larval stages. One *D. andersoni* nymph and one *I. scapularis* nymph tested positive by Vero cell culture 103 d and 128 d, respectively, after completion of feeding as larvae on infected hamsters. Additionally, virus was cultured in Vero cells from adult males of *D. andersoni* that fed on a virus-infected hamster and mouse as nymphs. Usually, <10% of the ticks that initially fed on viremic hosts and subsequently molted produced positive cultures of virus in Vero cells.

A greater proportion of ticks tested positive for WN virus when analyzed by TaqMan RT-PCR than by Vero cell culture (Table 1). For example, 35 of 39 culture-negative 122 d-old nymphs of *I. scapularis* that fed as larvae on infected hamsters tested positive in experiment 1. In experiment 3, 22 of 35 groups of unfed *I. scapularis* nymphs contained WN virus RNA, including the five groups of culture-positive ticks. Yet in other experiments, such as experiment 9, results by TaqMan RT-PCR were similar to Vero cell analyses.

Transmission Experiments. Five to 15 *I. scapularis* nymphs that had fed as larvae on WN virus infected hamsters or mice were placed on each of nine naïve hamsters and 11 naïve mice. One to eight nymphs fed to completion on each host. Nymphs were from cohorts where larval specimens tested positive for WN virus by Vero cell culture. All naïve animals were healthy, and none exhibited neurological disease such as lethargy, loss of appetite, disorientation, loss of mobility, or tremors for the 10–18 d animals were kept. None of the animals died. WN virus was not isolated

Table 1. Number of *I. scapularis*, *A. americanum*, *D. andersoni*, and *D. variabilis*, infected with West Nile virus, as determined in Vero cell culture and TaqMan RT-PCR, after feeding to completion as larvae or nymphs on infected hamsters or mice

Species and stage	Experiment #	# days ticks placed on host before inoculation	Host[a]	Ratio of infected ticks to number tested at intervals (days) after completion of feeding[b]				Molted to next stage[d]
				0–5	6–12	11–15	16–25	
I. scapularis larva	1	0	H	10/12	7/12	ND[c]	5/11	5/62 (35/39)
I. scapularis larva	2	0	M	4/10 (4/4)	5/6 (2/11)	0/6 (0/5)	0/6 (0/3)	0/7
I. scapularis larva	3	1	H	30/30 (36/36)	17/23 (22/24)	0/8 (3/9)	0/8	5/165 (22/35)
A. americanum larva	4	1	H	1/2	1/2	ND	ND	0/1
A. americanum nymph	5	0	M	ND	ND	ND	ND	0/17
D. andersoni nymph	6	0	M	ND	ND	ND	ND	2/3
D. andersoni larva	7	1	H	5/6	0/4	ND	ND	1/39 (0/15)
D. andersoni nymph	8	1	H	2/2	0/1	ND	ND	0/7
D. andersoni nymph	9	2	H	ND	ND	ND	ND	1/11 (1/10)
D. variabilis larva	10	0	M	0/1	0/8	ND	ND	0/118
D. variabilis larva	11	1	H	3/3	1/4	ND	ND	1/12
D. variabilis nymph	12	1	H	0/1	1/2	ND	ND	0/13 (2/13)

[a] H, hamster; M, mouse.
[b] Data without parentheses represents Vero cell analysis. Data in parentheses represents TaqMan RT-PCR analysis.
[c] Not Done.
[d] The interval in days from the time ticks completed their feeding on infected mice or hamsters to when they were killed by trituration in each experiment was as follows: Experiment 1, 22–128; Experiment 2, 86–91; Experiment 3, 75; Experiment 4, 104; Experiment 5, 53–115; Experiment 6, 53; Experiment 7, 17–103; Experiment 8, 103; Experiment 9, 88–90; Experiment 10, 18–43; Experiment 11, 16–108; Experiment 12, 58–99.

from blood or from tissues of brain, heart, spleen, and kidney of euthanized animals. Neutralization antibodies were not detected in sera from 14 of the tested animals.

Ten to 15 *D. variabilis*, *D. andersoni*, and *A. americanum* nymphs that had fed as larvae on infected hamsters were each placed on naïve mice. Larval specimens tested in Vero cell culture were documented to be infected with WN virus. The mouse infested with *D. andersoni* nymphs died before all nymphs completed feeding. WN virus was not isolated from its tissues. Mice parasitized by nymphal *D. variabilis* and *A. americanum* were healthy for 28 d before they were euthanized. WN virus was not isolated from tissues of brain, heart, kidney, or spleen or from blood. Neutralizing antibodies were not detected in the sera from the mice parasitized by *D. variabilis* and *A. americanum.*

Forty-five nymphs of *I. scapularis*, five nymphal *D. andersoni*, and eight nymphs of *D. variabilis*, which had previously engorged on infected hamsters as larvae, fed on naïve mice or hamsters and were tested for virus after they had molted to adults. Virus was isolated in Vero cell culture from one of 31 females and none of the 14 *male I. scapularis* tested. Virus was not recovered from adult *D. andersoni* and *D. variabilis.* However, 13 of 16 female and 11 of 14 male *I scapularis*, one of three male and 0 of four female *D. andersoni*, and two of eight female *D. variabilis* were positive by TaqMan RT-PCR.

Discussion

All four species, *I. scapularis*, *A. americanum*, *D. variabilis*, and *D. andersoni*, acquired the virus while feeding on infected animals, but virus titers decreased rapidly in the days following completion of feeding. While transstadial passage of the virus from larvae to nymphs was documented in *I. scapularis*, *D. variabilis*, and *D. andersoni*, and from nymphs to adult in *D. andersoni*, naive host animals fed upon by nymphs did not become infected. In one instance, WN virus was cultured from an adult *I. scapularis* that had fed to completion on an infected hamster as a larva and a naïve mouse as a nymph. The mouse remained healthy, and virus was not isolated from its tissues. These findings were similar to those reported by Whitman and Aitken (1960) with *D. variabilis.*

WN virus strain 2741 causes systemic infection and invades the central nervous system in C3H/HeN mice (Wang et al. 2001), and like WN virus strain 385–99 from a snowy owl that died in New York City (Xiao et al. 2001), causes systemic disease in hamsters. Viremia and neutralizing antibody titers following animal inoculation were similar to that described for hamster inoculation with WN virus (Xiao et al. 2001). However, viremia in mice and hamsters were ≈5 and two logs, respectively, lower than that recorded in a naturally infected American crow. Lower viremias than what we detected in the American crow may have contributed to our inability to document competency. Alternatively, WN virus may not infect salivary tissues of ixodid ticks or be passed through salivary ducts during feeding.

Detection levels of WN virus RNA by TaqMan RT-PCR were as few as 0.1 pfu (Lanciotti et al. 2000). Thus, this assay is more sensitive than Vero cell culture for identifying the presence of this virus. The greater number of ticks testing positive by this analysis suggests that WN virus may be more prevalent in ticks that had fed on infected mice and hamsters than indicated by Vero cell culture.

Immature ticks take 2.5 to >4 d to complete feeding (Balashov 1972). Considerable quantities of blood are ingested, particularly during the final ≈24 h of feeding (Kemp et al. 1982). Inasmuch as considerable quan-

tities of antibodies to WN virus could also be imbibed along with virus, many of the ticks used in our experiments were placed on hosts before infection and thus completed feeding before appearance of detectable antibody. Even so, transmission of virus during feeding was not documented. Larger numbers of infected ticks, which fed in a previous stage on hosts with virus titers approaching 10^{10} pfu, ought to be tested for competency.

Larval *I. scapularis* feed on birds in August and September in northeastern United States (Anderson and Magnarelli 1984), the time of year when WN virus is most prevalent in mosquitoes and birds. Therefore, relatively large numbers of larval *I. scapularis* will likely feed on viremic birds and ingest the virus. Fully fed larvae survive the winter in the duff layer of soil (Spielman et al. 1985). Our data show that WN virus will survive throughout the larval stage as well as the larval/nymphal molt, in at least some ticks. WN virus may therefore be able to survive the winter in fully fed larvae. While there is no evidence that *I. scapularis* is a competent vector, birds preen themselves and could ingest infected attached nymphs, which like larvae also readily feed on birds. Since transmission of WN virus by ingestion has been reported in laboratory mice (Odelola and Odcye 1977) and suggested by Garmendia et al. (2000) in a naturally infected hawk, the possibility remains that birds could become infected by feeding on ticks infected with WN virus.

At least 16 species of argasid ticks and 37 species of ixodid ticks have been reported feeding on birds in North America and Mexico (Doss et al. 1974, Durden and Keirans, 1996). It is possible that one or more of the argasid species and perhaps even some of the ixodid species will be shown to be vector competent. Ticks are secondary to mosquitoes as vectors, but the role of ticks in the ecology of WN virus is worth pursuing.

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