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Arboviruses in North Dakota, 2003–2006

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Abstract. To investigate arbovirus transmission in North Dakota, we collected and screened mosquitoes for viral infection by Vero cell culture assay. Seven viruses were isolated from 13 mosquito species. Spatial and temporal distributions of the important vectors of West Nile virus (WNV), Cache Valley virus, Jamestown Canyon virus (JCV), and trivittatus virus are reported. Snowshoe hare virus, Potosi virus, and western equine encephalomyelitis virus were also isolated. The risks of *Culex tarsalis* and *Aedes vexans* transmitting WNV to humans were 61.4% and 34.0% in 2003–2006, respectively, but in 2003 when the largest epidemic was reported, risks for *Ae. vexans* and *Cx. tarsalis* in Cass County were 73.6% and 23.9%, respectively. Risk of humans acquiring an infectious bite was greatest from about the second week of July through most of August. West Nile virus sequences were of the WN02 genotype. Most JCV strains belonged to a single clade of genetically related strains. Cache Valley virus and JCV were prevalent during August and early September and during July and August, respectively.

INTRODUCTION

Five arboviruses have been reported from North Dakota mosquitoes. Two of these viruses caused significant epidemics and epizootics in humans and in horses. Western equine encephalomyelitis virus (WEEV; Togaviridae: *Alphavirus*) infected 1,180 humans and caused 96 deaths in 1941.[1] Sixty-one years later in 2002, West Nile virus (WNV; Flaviviridae: *Flavivirus*) was introduced into North Dakota and caused disease in 17 humans and 577 horses followed by 617 human cases, five human deaths, and 42 equine cases during 2003.[2] Since then, North Dakota has reported human WNV cases every year and has one of the highest incidence rates of neuroinvasive disease in the country.[2,3] Other arboviruses identified in North Dakota include St. Louis encephalitis virus (SLEV; Flaviviridae: *Flavivirus*),[4] Cache Valley virus (CVV; Bunyaviridae: *Orthobunyavirus*),[4] and trivittatus virus (TVTV; Bunyaviridae: *Orthobunyavirus*).[4] Jamestown Canyon virus (JCV; Bunyaviridae: *Orthobunyavirus*), which has one of the widest geographical ranges in North America of the California serogroup of viruses, surprisingly, has previously not been documented in North Dakota.[4]

After the first report of WNV in North Dakota in 2002,[2] we established a mosquito and virus surveillance program in and around the eastern city of Fargo (Cass County) and near the town of Walcott (Richland County) in 2003–2005, and in the northwestern city of Williston in 2004–2006 (Williams County) (Figure 1). Additionally, collections were made for 3 days in July 2003 in the rural town of Lakota (Nelson County). Our objectives were to conduct an in-depth study of mosquitoes and their viruses throughout the spring and summer in selected locations in North Dakota. We report the isolation of seven viruses from 13 species of mosquitoes, the spatial and temporal distributions of the important vectors of the most abundant arboviruses, the relative risk of mosquito vectors in transmitting WNV to humans, the correlation of temporal numbers of human cases of WNV with mosquito abundance and infection rate, and a genetic analysis of WNV and JCV isolates from different mosquito species, years of isolation, and geographic locations.

MATERIALS AND METHODS

Mosquito collections and identification. Trapping of mosquitoes was done in Cass County in 2003–2005 and in Williams County in 2004–2006 (Figure 1). Fargo (46°52′38″N/96°47′21″W), a city of 110,000 people, is the county seat in Cass County and is located on the western side of the Red River, which runs along the North Dakota-Minnesota border. Williston (48°08′49″N/103°37′05″W), a city of about 18,000 inhabitants, is the county seat in Williams County and is situated along the Missouri River near the North Dakota-Montana border. Mosquitoes were also collected in 2003 in Nelson County in the vicinity of Lakota, the county seat. Lakota (48°02″34″N/98°20′10″W) is an agricultural community with < 700 inhabitants and is located in the northwestern portion of the county. Trapping in Lakota was discontinued after 3 nights because of inability to consistently obtain dry ice to supplement the traps. Mosquitoes were collected near Walcott (46°32′52″N/96°56′19″W) in Richland County, which is adjacent to the southern border of Cass County, in 2003–2005 (Figure 1). Trapping was carried out from July 4 to September 26, June 8 to October 6, May 24 to September 29, June 8 to September 7 in 2003, 2004, 2005, and 2006, respectively.

In 2003, five distinct trapping sites were established northeast (two sites in Argusville, near the Sheyenne River (47°03′08″N/96°20′10″W)) and southwest (three sites in Horace (46°45′32″N/96°54′13″W) of Fargo and one site near Walcott. Trapping was not done in Fargo because of an extensive mosquito control program initiated on the day traps were first placed in the field. Traps were often placed weekly at each location. Traps were set for 1 to 2 nights at 10 different locations near Lakota.

In 2004 and 2005, the same six trapping sites northeast and southwest of Fargo were continued. Three additional sites were established in Horace. Seven different geographically different sites were established within the City of Fargo in 2004 and 2005.

In 2004–2006, mosquitoes were trapped at six different locations immediately around Williston, ND. An additional site was trapped in 2004 and 2005. Trapping was not done

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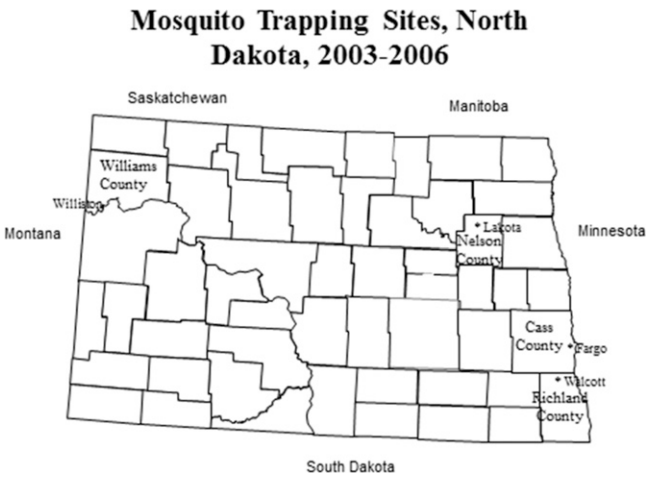

FIGURE 1. North Dakota Counties where mosquito traps were placed.

within the city because of relatively frequent spraying of insecticides for the control of mosquitoes.

Mosquitoes were collected with Centers for Disease Control and Prevention (CDC) miniature light traps baited with dry ice (model 512; John W. Hock Co., Gainesville, FL).[5] Traps were placed about 0.9 m off the ground in the afternoon or evening and collected the following morning. Mosquitoes were knocked down in the field or laboratory with dry ice or freezing temperatures, aspirated, and transferred into a 17 × 55 mm flat-bottomed shell vial that was sealed with a rubber stopper. Three layers of water and gas-proof tape were used to seal the juncture of the rubber stopper and vial. The vial was appropriately labeled and immediately stored on dry ice or in a −80°C freezer until packaged in dry ice within a Styrofoam shipping container and sent by overnight mail to The Connecticut Agriculture Experiment Station (CAES). Mosquitoes were stored at CAES in a −80°C freezer until processing. Extremely large collections were placed in 0.47 L or 0.24 L Mason jars that were sealed with three layers of water and gas-proof tape.

Mosquitoes were identified to species.[6,7] Specimens were placed on a cold platform and, with the aid of a dissecting microscope, each specimen was identified. Mosquitoes were placed into pools according to species, date, and trap location. Pools contained from 1 to 50 mosquitoes. After identification and pooling, mosquitoes were kept on regular ice until attempted isolation of virus on the same day.

Virus isolation. Mosquitoes were ground in 0.5 to 1.25 mL of phosphate-buffered saline containing 0.5% gelatin, 30% rabbit serum, and 1% 100× antibiotic-antimycotic (10,000 units μg/mL of streptomycin sulfate, and 25 μg/mL of amphotericin B, Invitrogen, Carlsbad, CA, in 0.85% saline). Mosquitoes and saline were placed in a 2.5 mL plastic centrifuge vial containing a copper BB pellet. A Vibration Mill MM 300 (Retsch Laboratory, Irvine, CA) set at 30 cycles per second and operated for 4 minutes was used inside a biosafety hood to macerate mosquitoes.

After centrifugation at 4°C for 7 minutes at 520 × *g*, a 100 μL sample of supernatant was placed onto a monolayer of Vero cells, growing in 4 mL of Minimum Essential Medium containing fetal bovine serum, glutamine, and antibiotic-antimycotic (Invitrogen), set up the day before in a 25-cm^2 flask kept in a 5% CO_2 incubator set at 37°C. Growth medium in each flask was decanted before addition of the inoculum. The flask was rocked for 5 minutes before 4 mL of new growth medium was added to the flask. After flasks were returned to the incubator, cells were examined for cytopathogenic effects (CPE) 3–7 d after inoculation.

Virus identification. Many viral isolates were identified by virus-dilution serum constant-neutralization tests. Virus dilutions from 10^{-4} to 10^{-6} were incubated with a panel of hamster antisera (1:10) directed against JCV, La Crosse virus (LACV; Bunyaviridae, *Orthobunyavirus*), snowshoe hare virus (SSHV; Bunyaviridae, *Orthobunyavirus*), Keystone virus (KEYV; Bunyaviridae, *Orthobunyavirus*), CVV, Potosi virus (POV; Bunyaviridae, *Orthobunyavirus*), Jerry Slough virus (JSV; Bunyaviridae, *Orthobunyavirus*) or hyperimmune mouse ascetic fluids (1:10) against eastern equine encephalomyelitis virus (EEEV; Togaviridae: *Alphavirus*), Highlands J virus (HJV; Togaviridae: *Alphavirus*), TVTV, WEEV, or WNV for 1 hour at 37°C. The virus–serum mixtures were then assayed for neutralizing activity by infecting Vero cell cultures and screening them for CPE.[8]

West Nile virus also was identified by a TaqMan reverse transcriptase-polymerase chain reaction (RT-PCR) assay. The QIAamp viral RNA mini kit protocol (Qiagen, Valencia, CA) was used to extract RNA from a 70 μL sample of infectious Vero cell-growth medium. The RT-PCR protocol was used to identify isolates of WNV.[9] The positive RNA control for WNV was from an isolate from *Culiseta melanura* (8094-01) that had been diluted 1:100. Its cycle threshold was 21 to 22. Double processed sterile water (Sigma, St. Louis, MO) was used as the negative control. Primers and probe for WNV used in the TaqMan RT-PCR assay were prepared by QIAGEN. Primers were WNENV-forward 1160-1180 (5′-TCAGCGATCTCTCCACCAAAG-3′) and WNENV-reverse 1229-1209 (5′-GGGTCAGCACGTTTGTCATTG-3′) with probe WNENV 1186-1207 (5′-TGCCCGACCATGG-GAGAAGCTC-3′).[9] The probe had the 5′ end labeled with FAM reporter dye and the 3′ end labeled with TAMRA quencher dye. Primers, probe, and viral RNA were added to the reagents in the TaqMan RT-PCR Ready-Mix Kit (PE Applied Biosystems, Branchburg, NJ). Each isolate was prepared for testing by adding 2.5 μL of viral RNA, 0.25 μL of 100 μM of each primer, 0.15 μL of 25 μM of probe, 12.5 μL of 2× buffer, 0.5 μL of RT-PCR enzyme, and 8.85 μL of water to a microcentrifuge tube for a total volume of 25 μL. Samples were amplified with a Smart Cycler that was run with Smart Cycler software (Cepheid, Sunnyvale, CA). Amplification included one cycle at 50°C for 20 min, one cycle at 95°C for 10 min, and 50 cycles at 95°C for 15 sec, and at 60°C for 60 sec. Isolates with a cycle threshold value of < 37 were identified as WNV.

Four *Orthobunyavirus* isolates (JCV, POTV, CVV, and TVTV) also were identified by RT-PCR. RNA was extracted from primary viral isolates using the viral RNA Kit (Qiagen), and RT-PCR was performed using the Titan One-Tube RT-PCR System (Roche Diagnostics, Indianapolis, IN). Primers BUNS+new: 5-TGACCAGTAGTGTACTCCAC-3_ and BUNS-new: 5_-CAAGCAGTAGTGTGCTCCAC-3_ that targeted the conserved terminal ends of the S-segment of the *Orthobunyavirus* genus were used as previously described.[10,11] The amplification product of each unknown virus was digested with one or more of four restriction enzymes in separate

reactions. The master mix for each restriction enzyme consisted of 13.55 μL of water, 2.0 μL 10× reaction buffer, 0.2 μL 100× BSA, and 0.25 μL of the specific restriction enzyme to which was added 4 μL of the amplified reagent and then incubated overnight at 37°C.

Each digestion product was separated on a 2% agarose gel and stained with ethidium bromide. The restriction enzymes used and their fragment sizes for specific viruses were as follows: EcoRV cut JCV at 364 bps and 627 bps, CAC81 severed POTV at 256 bps and 677 bps, SwaI cut CVV at 411 bps and 539 bps, and XhoI cut TVTV at 233 bps and 740 bps.

Alternatively for isolates that could not be specifically identified using restriction enzymes or that were not identified by virus-dilution serum constant-neutralization tests, amplification products were purified using the PCR purification kit (Qiagen) and commercially sequenced at the DNA Analysis Facility (Yale University, New Haven, CT). Edited nucleotide sequences were compared with those available on GenBank using the Blastn search algorithm http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Genetic analyses of West Nile virus. The NS3 gene of WNV was selected for analysis because this gene was previously shown to have a strong phylogenetic signal.[12] The RNA was extracted from primary viral isolates using the Viral RNA Kit (Qiagen). The RT-PCR was performed using the Titan One-Tube RT-PCR System (Roche Diagnostics, Indianapolis, IN) and primers WNV4532f (TCGCGATTAGTGCGTACACC) and WNV6516r (GAAGTGCTCAGGCATCTTTCC). Amplification was performed as follows: 1 cycle of 50°C for 30 min and 94°C for 2 min, 10 cycles of 94°C for 15 sec, 55°C for 30 sec, and 68°C for 2 min, followed by 25 cycles of 94°C for 15 sec, 55°C for 30 sec, and 68°C for 2 min + 5 sec per cycle, and 1 cycle of 68°C for 7 min. The PCR products (length = 1,984 bps) were commercially sequenced at the Yale DNA Analysis Facility and edited sequences were aligned using the ClustalW algorithm in MEGA 5.[13] A phylogenetic tree was reconstructed by maximum-likelihood (ML) analysis in MEGA using the substitution model TN93 + G. The optimal nucleotide substitution model was identified after performing ML fits of 24 different models in MEGA. Support for individual nodes was obtained by performing 1,000 bootstrap replicates.

Genetic analyses of Jamestown Canyon, snowshoe hare, and trivittatus viruses. The RNA was extracted from primary virus cultures, the S segment was amplified by RT-PCR, and amplification products were purified and sequenced according to previously described methods.[14] A total of 48 sequences were combined with another 19 sequences available on GenBank and aligned using MEGA. The final alignment consisted of 832 characters that included the nucleocapsid open reading frame and flanking portions of the 5′ and 3′ non-coding regions. Phylogenetic analysis was performed by ML analysis as described earlier for WNV sequences except using a different substitution model (T92 + G+I) that was selected after performing ML fits in MEGA.

GenBank. The WNV, JCV, SSHV, and TVTV edited nucleotide sequences were submitted to GenBank (accession nos. KM215518–KM215607).

Statistics. The field infection rates for each species of mosquito infected with a specific virus within Cass, Richland, Nelson, or Williams Counties for each week and year was determined per 1,000 specimens using the bias-corrected maximum likelihood estimation method.[15]

Relative risk of infected vectors transmitting WNV to humans in North Dakota in 2003–2006 was assessed using information on mosquito abundance, infection rate, vector competence, and biting behavior.[16] Relative abundance was determined by dividing the total numbers of a specific species captured during the 4-year study by the total number of mosquitoes collected and identified (Table 1). It is noteworthy that relative abundance was determined from numbers of mosquitoes captured in CDC miniature light traps placed relatively near the ground and may not reflect the abundance of specific species feeding on humans. Infection rate was determined using a previously published formula.[15] Vector competence (fraction of infected mosquitoes that will transmit virus in a later feeding) was obtained from previously published studies on specific species. The fraction of blood meals of specific species that contained mammalian blood published by others was used as a relative estimate of the likelihood of the species feeding on humans. Humans shelter themselves from mosquitoes and have significantly less exposure to mosquitoes compared with wild and large domestic mammals. The use of the fraction of specimens feeding on mammals is a

TABLE 1
Thirteen mosquito species infected with virus,* North Dakota, 2003–2006

Species	Number of specimens by year				Totals	Percent
	2003	2004	2005	2006		
Aedes cinereus	0	593	5,867	218	6,678	0.2
Aedes vexans	223,693	519,443	1,477,623	591,152	2,811,911	83.2
Anopheles earlei	21	6	72	79	178	< 0.1
Coquillettidia perturbans	1,021	1,574	2,199	246	5,040	0.2
Culex tarsalis	3,028	20,608	48,615	478	72,729	2.2
Culiseta inornata	983	6,336	21,566	508	29,393	0.9
Ochlerotatus dorsalis	2,511	15,279	36,953	1,375	56,118	1.7
Ochlerotatus flavescens	579	463	938	310	2,290	0.1
Ochlerotatus melanimon	403	8,381	35,559	46,306	90,649	2.7
Ochlerotatus spencerii	0	479	368	26	873	< 0.1
Ochlerotatus sticticus	176	7,555	25,985	416	34,132	1.0
Ochlerotatus triseriatus	95	56	106	0	257	< 0.1
Ochlerotatus trivittatus	16,017	94,904	135,873	12,757	259,551	7.7
Other species*	393	1,751	3,274	2,666	8,084	0.2
Totals	248,920	677,428	1,794,998	656,537	3,377,883	

*Viruses were not isolated from 21 species; these species are not listed.

TABLE 2
Viruses isolated from mosquitoes collected in Nelson, Cass, Richland, and Williams Counties, North Dakota, 2003–2006

Virus	Year and Counties										
	2003			2004			2005			2006	
	Nelson	Cass	Richland	Cass	Richland	Williams	Cass	Richland	Williams	Williams	Total
West Nile	2	37	1	19	0	13	67	0	41	6	186
Trivittatus	0	35	11	85	1	14	123	4	73	33	379
Jamestown Canyon	5	11	1	10	0	1	14	0	44	2	88
Cache Valley	1	1	0	0	0	0	53	0	96	0	151
Snowshoe Hare	0	0	0	1	0	0	3	0	0	0	4
Western Equine Encephalomyelitis	0	0	0	0	0	2	0	0	0	0	2
Potosí	0	0	0	0	0	0	6	0	0	0	6
Total	8	84	13	115	1	30	266	4	254	41	816

relative estimate of the approximation that a specific species would feed on humans.[16] All mosquito species reported in this study feed on humans.

A vector index was used to correlate weekly numbers of documented WNV-infected humans with mosquito abundance and infection rate.[17] Human cases were recorded as the number for a specific week and were plotted at the middle of that week (numbers were reported at the end of the week by the North Dakota Department of Health).[18] The average numbers of a specific vector per trap-night per week were multiplied by its infection rate (IR)/1,000 to give the vector index, the average number of infected mosquitoes per trap-night.

RESULTS

Mosquitoes. A total of 3,377,883 specimens representing 34 species were collected and identified in 2003–2006 (Table 1). Data for mosquito species (N = 21) that did not yield virus are not included. *Aedes vexans* was dominate throughout the state and represented 83.2% of the specimens identified. *Ochlerotatus trivittatus*, *Ochlerotatus melanimon*, *Culex tarsalis*, and *Ochlerotatus dorsalis* made up 7.7%, 2.7%, 2.2%, and 1.7%, respectively.

Viruses. Seven distinct viruses were identified among the 816 isolates (Table 2). Trivittatus virus (46.4%), WNV (22.8%), CVV (18.5%), and JCV (10.8%) were the most prevalent. Trivittatus virus was isolated from mosquitoes collected from June 2 through the week of October 6. The other viruses tended to be isolated from mosquitoes collected from mid to late June and early July through the second week of September. Potosi virus was an exception and was isolated during the weeks of August 18 through September 22.

Viruses were isolated from 13 species of mosquitoes (Table 3). The largest numbers of viral isolations were from *Oc. trivittatus* (N = 313), *Ae. vexans* (N = 289), and *Cx. tarsalis* (N = 131). Six different viral species were isolated from *Ae. vexans* (CVV, JCV, POTV, SSHV, TVTV, and WNV). Five different viruses were isolated from *Cx. tarsalis* (CVV, JCV, TVTV, WNV, and WEEV), *Oc. dorsalis* (CVV, JCV, POTV, TVTV, and WNV), and *Oc. trivittatus* (CVV, JCV, SSHV, TVTV, and WNV). Four and three different viruses were isolated from *Culiseta inornata* (CVV, JCV, POTV, and WNV) and *Ochlerotatus flavescens* (CVV, JCV, and WNV).

West Nile virus. This invasive Flavivirus was isolated in 2003–2006 from five genera and nine species collected in Nelson, Cass, Richland, and Williams Counties (Tables 2 and 3). The largest numbers of isolations were from *Cx. tarsalis* (N = 121) and *Ae. vexans* (N = 55). The WNV IR per thousand pooled mosquitoes for all species by year and county are shown in Supplemental Table 1. *Culex tarsalis* and *Ae. vexans* IRs by county and year ranged from 1.12 to 12.26 and < 0.01 to 0.19 per thousand specimens, respectively.

Culex tarsalis was collected from the first week of June into the first week of October (Figure 2A). Largest numbers were collected from the week June 30 through the week August 4 when numbers per trap-night averaged 134 to 239. West Nile virus was isolated from *Cx. tarsalis* from the last week of June through the second week of September. Eighty-eight percent of the isolations from *Cx. tarsalis* were made during a 7-week period from the week beginning July 14 through the week

TABLE 3
Seven viruses isolated from 13 species of mosquitoes, North Dakota, 2003–2006

Species	Viruses							
	Cache Valley	Jamestown Canyon	Potosi	Snowshoe Hare	Trivittatus	West Nile	Western Equine Encephalomyelitis	Total
Aedes cinereus	3	0	0	0	0	0	0	3
Aedes vexans	92	58	4	3	77	55	0	289
Anopheles earlei	1	0	0	0	0	0	0	1
Coquillettidia perturbans	0	0	0	0	0	1	0	1
Culex tarsalis	4	2	0	0	2	121	2	131
Culiseta inornata	27	4	1	0	0	2	0	34
Ochlerotatus dorsalis	15	8	1	0	1	1	0	26
Ochlerotatus flavescens	1	1	0	0	0	1	0	3
Ochlerotatus melanimon	6	2	0	0	1	0	0	9
Ochlerotatus spencerii	0	0	0	0	0	1	0	1
Ochlerotatus sticticus	0	1	0	0	3	0	0	4
Ochlerotatus triseriatus	0	0	0	0	0	1	0	1
Ochlerotatus trivittatus	2	12	0	1	295	3	0	313
Total	151	88	6	4	379	186	2	816

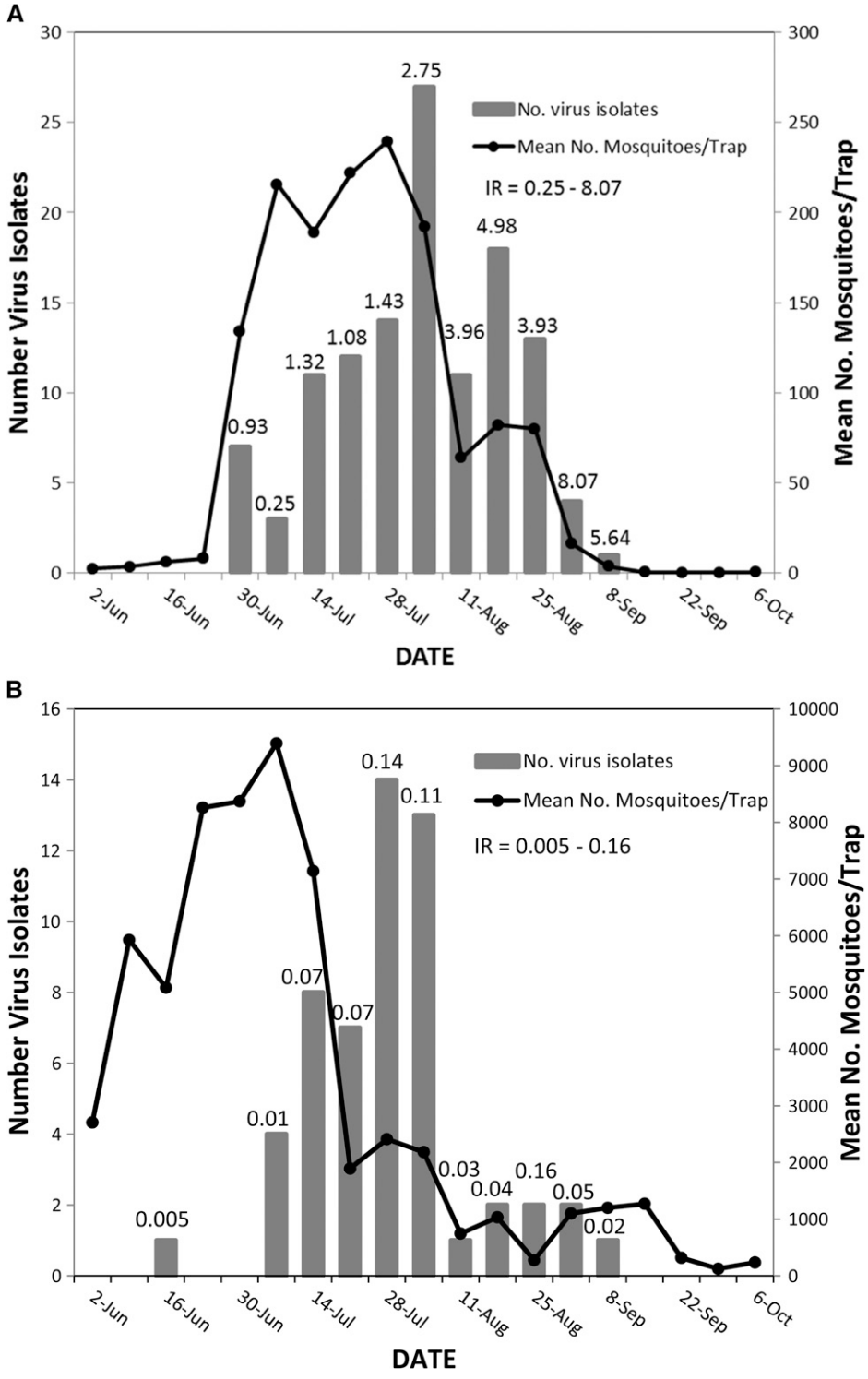

FIGURE 2. (**A**) Weekly number of West Nile virus isolations and infection rates (IR) relative to mean numbers of *Culex tarsalis* per trap-night, North Dakota, 2003–2006. (**B**) Weekly number of West Nile virus isolations and infection rates relative to mean numbers of *Aedes vexans* per trap-night, North Dakota, 2003–2006.

beginning August 25. The IRs for *Cx. tarsalis* tended to increase as the season progressed from 0.93 per thousand specimens on June 30 to 8.07 and 5.64 during the weeks of September 1 and 8.

Aedes vexans averaging 2,705 per trap-night were collected during the week of June 2 and continued to be collected through the week of October 6 (Figure 2B). Populations averaged 5,083 to 9,394 specimens per trap-night from the week of June 16 through the week of July 14. Numbers averaged 1,270 per trap-night during the week of September 15. West Nile virus was first isolated from *Ae. vexans* during the week of June 16, but consistent isolations began during the week of

July 7 and continued through the week of September 8. Eighty-four percent of the 55 isolations were obtained during a 5-week period from July 7 through the week of August 4.

Smaller numbers of isolations were made from *Coquillettidia perturbans*, *Cs. inornata*, *Oc. dorsalis*, *Oc. flavescens*, *Oc. spencerii*, *Ochlerotatus triseriatus*, and *Oc. trivittatus* (Table 3, Supplemental Table 1). West Nile virus was isolated from *Oc. dorsalis* during the week of June 16.

The risk of seven infected species for transmitting WNV to humans in North Dakota was assessed using a risk-assessment method, which used information on mosquito abundance, infection rate, vector competence, and biting behavior (Supplemental Table 2).[16] The greatest seasonal risk of 61.4% was from *Cx. tarsalis*. The second largest risk was from *Ae. vexans* with a value of 34.0%. The risks of five other species were much lower, ≤ 1.8%.

We evaluated the importance of specific species on transmission of WNV in Cass County during the years of 2003–2005. Mosquitoes were trapped at the same five trapping sites during the 3 years when numbers of human cases in Cass County were 32, 0, and 12. The risks of the five species that were infected are shown in Supplemental Table 3. The largest number of human cases was in 2003 when the risks of being bitten by infected *Ae. vexans* and *Cx. tarsalis* were 73.6% and 23.9%. In 2004 and 2005, risks of being infected by *Ae. vexans* were 24.8% and 17.3% and by *Cx. tarsalis* were 61.3% and 76.6%, respectively, when numbers of human cases were 0 and 12. The IRs for *Ae. vexans* were 9.5 and 6.3 times greater in 2003 than in 2004 and 2005, respectively. The IRs for *Cx. tarsalis* were 5.6 and 2.6 times greater in 2003 than in 2004 and 2005. These data suggest that infected *Ae. vexans* participated in vectoring WNV in North Dakota in 2003 when 617 people were documented to have been infected.

Dates of onset of humans infected with WNV and the weekly numbers of isolations of WNV from all mosquitoes for 2003–2006 are shown in Figure 3. The earliest human case was reported during the week of May 26 and the last cases were reported during the week of October 6. A steady increase in numbers of human cases began in mid-June and peaked during the last week of August with a 182 cases. West Nile virus was first isolated on June 16. Numbers of isolations increased from the last week of June through July and peaked on August 4, when 42 isolations were made, 2 to 3 weeks before the peaks of human cases. The decline of human cases beginning during the week of September 1 followed by 3 weeks the reduction in numbers of isolations that began during the week of August 11.

The weekly risk of humans being bitten by an infectious mosquito was also evaluated (Supplemental Table 4). The risks of being bitten by *Cx. tarsalis* were greater than being bitten by *Ae. vexans* and five other vector species during most weeks beginning in mid-July through August. However during this time period, the weekly risk of being bitten by infectious *Ae. vexans* plus the lesser important vectors varied from 10.3% to 57.8%.

We compared the weekly vector indexes for *Cx. tarsalis*, *Ae. vexans*, and the seven other vectors to the onset of human cases for 2003–2006 (Figure 4).[17] The weekly vector index for all vectors exceeded two only in 2003 when the largest outbreak of West Nile fever erupted with 617 cases. The weekly vector indexes for *Ae. vexans* in 2003 were greater than indexes for *Cx. tarsalis* the last 2 weeks of July through the first week of August, and were similar during the second and third weeks of August (Figure 4, Supplemental Table 5). The vector index for all species combined in 2003 exceeded one during the third week of July and exceeded two during the last

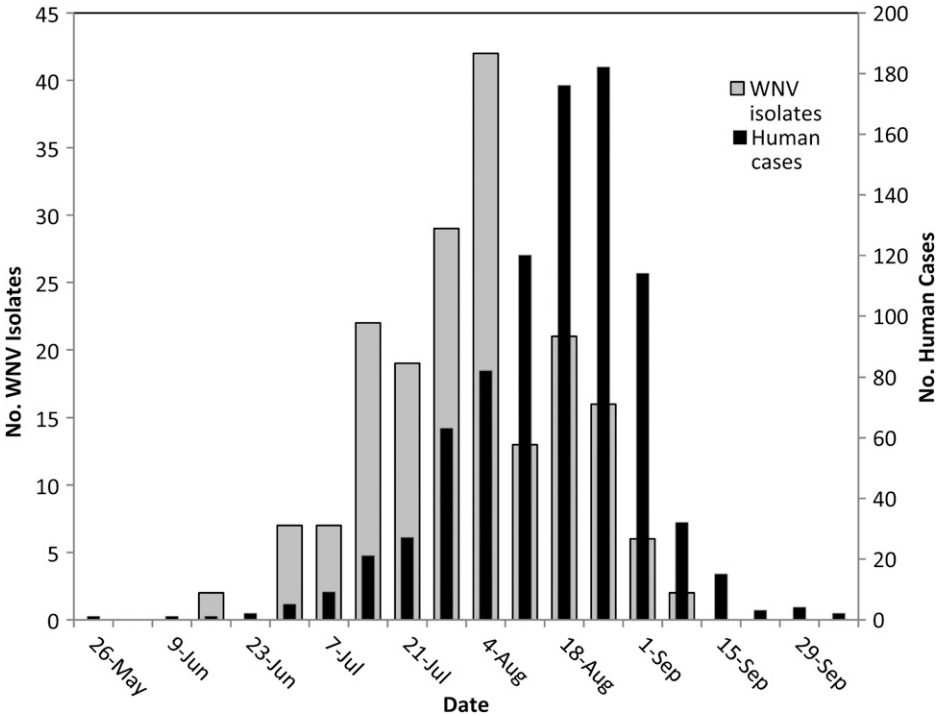

FIGURE 3. Relation between onset of human disease and total number of West Nile virus isolates from all mosquito species, North Dakota, 2003–2006.

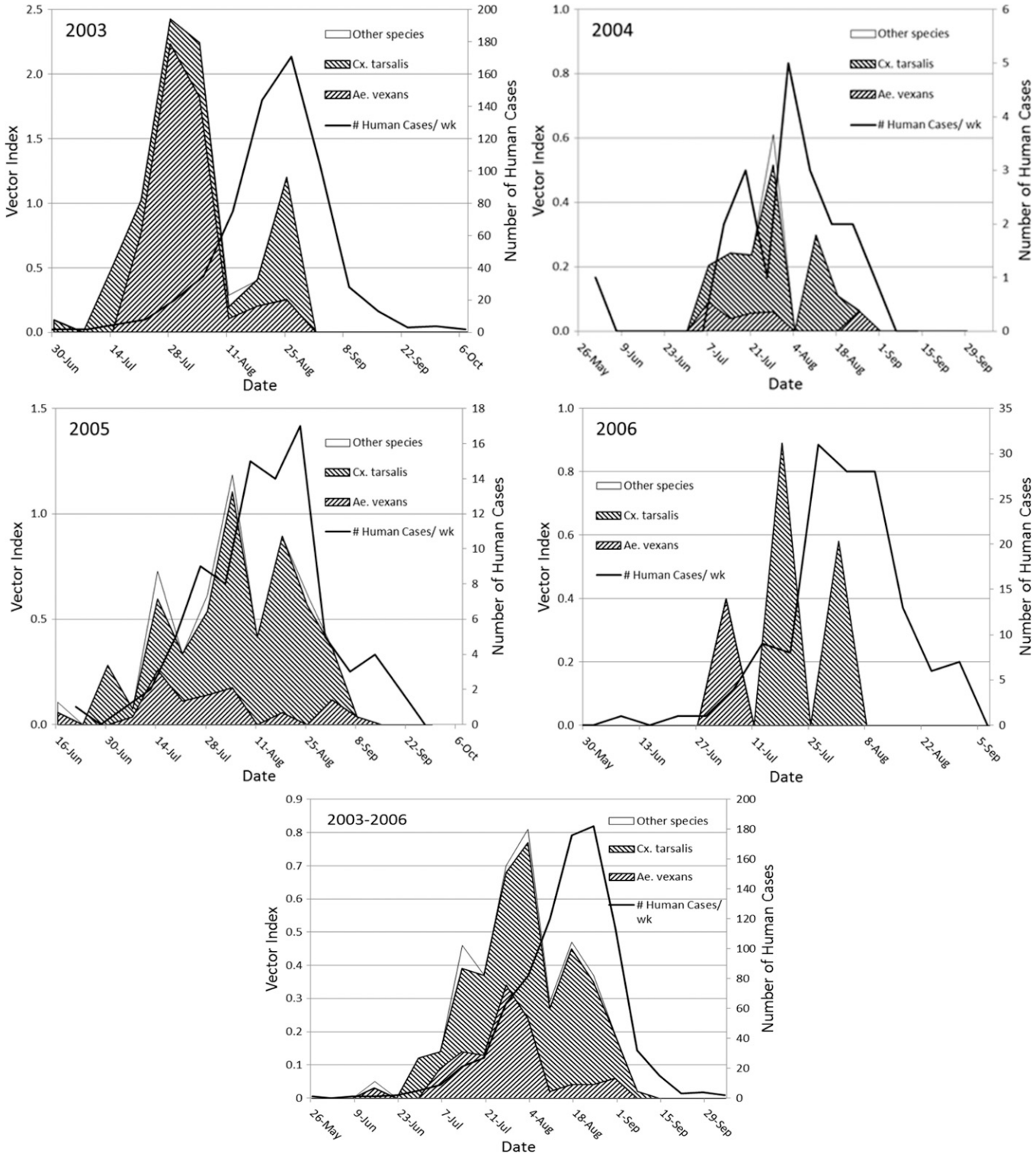

FIGURE 4. Stacked area graphs showing weekly vector index for *Culex tarsalis*, *Aedes vexans*, and seven other species relative to weekly numbers of human cases diagnosed with West Nile virus infection 2003, 2004, 2005, 2006, and 2003–2006. The other species were *Coquillettidia perturbans*, *Culiseta inornata*, *Ochlerotatus dorsalis*, *Ochlerotatus flavescens*, *Ochlerotatus spencerii*, *Ochlerotatus triseriatus*, and *Ochlerotatus trivittatus*.

week of July and first week of August. The increasing onset of human disease of 75 to 171 cases per week lagged by about 2 to 3 weeks the increasing vector index. The weekly vector indexes in the years 2004–2006, when numbers of human cases varied from 20 to 137, exceeded one only in 1 week in 2005 (Figure 4, Supplemental Tables 6–8). *Cx. tarsalis* had a higher weekly vector index compared with *Ae. vexans* during most weeks during these 3 years. The composite weekly vector indexes during 2003–2006 for *Cx. tarsalis* were similar to those for *Ae. vexans* plus the other vectors from the middle of

FIGURE 5. Phylogenetic tree depicting relationships of WNV isolates from North Dakota based on ML analysis of NS3 sequences. Branch lengths are proportional to the number of nucleotide substitutions. Numbers at node indicate bootstrap *P* values ≥ 50%.

June through the last week of July, but weekly vector indexes throughout August were higher for *Cx. tarsalis* (Figure 4, Supplemental Table 9).

Figure 5 depicts the ML tree of WNV sequences from North Dakota. The overall topology is consistent with prior analyses and shows that viruses segregate into two major groups termed the NY99 and WN02 genotypes in North America.[19,20] Earlier WNV isolates from the northeastern United States form the NY99 genotype, whereas later strains from North Dakota and the rest of the US cluster into the WN02 genotype.

Cache Valley virus. This Bunyamwera serogroup virus of the genus *Orthobunyavirus* was isolated from mosquitoes captured in 2003 and 2005 but not in 2004 and 2006 (Table 2). Single pools of *Cs. inornata* collected on July 4 in Nelson County and of *Oc. trivittatus* collected on July 22 in Cass County yielded positive Vero cell cultures in 2003. In 2005, CVV was widespread and prevalent in both eastern (Cass County) and western (Williams County) North Dakota. Pools from five genera and nine species were positive for CVV (Table 3). The largest numbers of isolations were from *Ae. vexans* ($N = 92$), *Cs. inornata* ($N = 27$), and *Oc. dorsalis* ($N = 15$). The IRs for each species by year and county are shown in Supplemental Table 10 and ranged from 0.03 in *Oc. trivittatus* to 16.55 in *Anopheles earlei* in Williams County, and from 0.04 in *Ae. vexans* to 1.94 in *Cs. inornata* in Cass County. Ninety-seven percent of the isolations were made during the weeks of August 1 through September 8 (Figure 6). The weekly IRs were relatively high during this 6-week period, ranging from 0.14 to 1.09. Most isolations of CVV were made in August and early September when mosquito populations were significantly reduced from mid-June through mid-July.

Jamestown Canyon virus. This widely distributed *Orthobunyavirus* in the United States was isolated in 2003–2006 from mosquitoes captured in four counties (Table 2). Eighty-eight isolations were made from four genera and eight species (Table 3). The largest numbers of isolations were from *Ae. vexans* ($N = 58$), *Oc. trivittatus* ($N = 12$), and *Oc. dorsalis* ($N = 8$) (Table 3). The IRs for year and county ranged from 0.01–0.08, 0.02–0.51, and 0.07–1.21 for *Ae. vexans*, *Oc. trivittatus*, and *Oc. dorsalis*, respectively (Supplemental Table 11). Isolations were made from mosquitoes collected from the week of June 30 through the week of September 8 (Figure 7). Ninety-eight percent of the isolations were from mosquitoes collected from the week of June 30 through the week beginning August 25.

Phylogenetic analysis of 44 isolates of JCV showed that the vast majority belonged to a single clade of genetically related strains (Figure 8). Forty-one isolates from seven species, four different counties, and four different years were similar to the lineage A isolates from Connecticut.[14] Three isolates sorted by phylogenetic analysis and by virus-dilution serum constant-neutralization tests were the Jerry Slough variant of JCV.

Trivittatus virus. This California encephalitis virus was the most frequently isolated virus (Table 2). The 379 isolates were from three genera and six species collected in Cass, Williams, and Richland Counties in 2003–2006 (Table 3). Two-hundred ninety-five and 77 of the isolates were from *Oc. trivittatus* and *Ae. vexans*, respectively. The IRs for *Ochlerotatus trivittatus* and *Ae. vexans* by year and county ranged from 0.70 to 5.09 and 0.01 to 0.42, respectively (Supplemental Table 12).

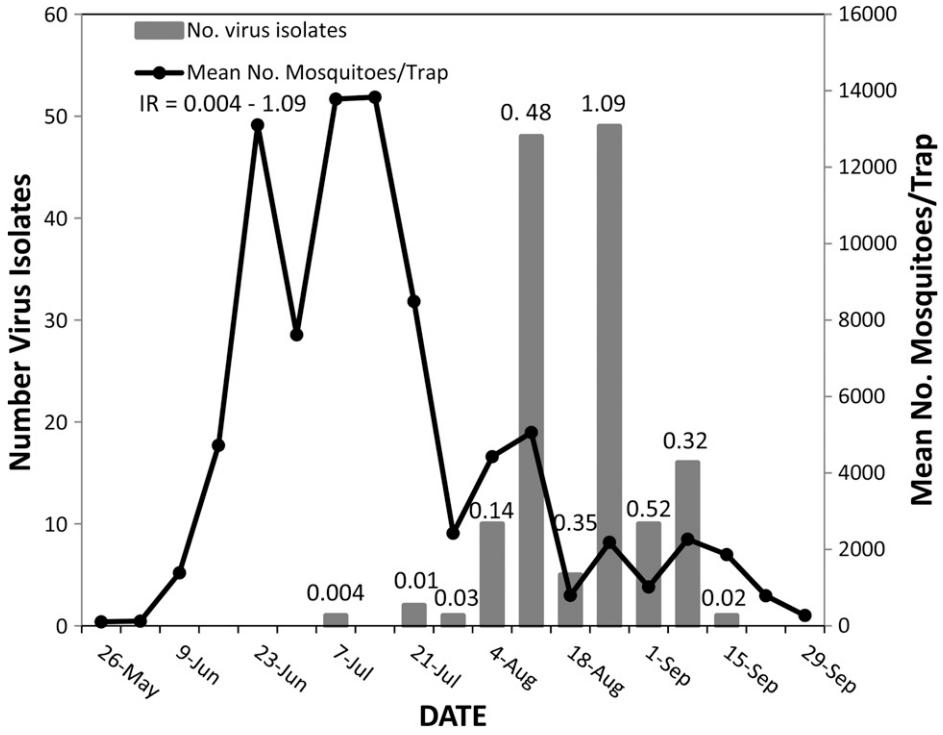

FIGURE 6. Weekly numbers of Cache Valley virus isolations and infection rates (IR) relative to the mean number per trap-night of *Aedes vexans*, *Aedes cinereus*, *Anopheles earlei*, *Culex tarsalis*, *Culiseta inornata*, *Ochlerotatus dorsalis*, *Ochlerotatus flavescens*, *Ochlerotatus melanimon*, and *Ochlerotatus trivittatus*, North Dakota, 2005.

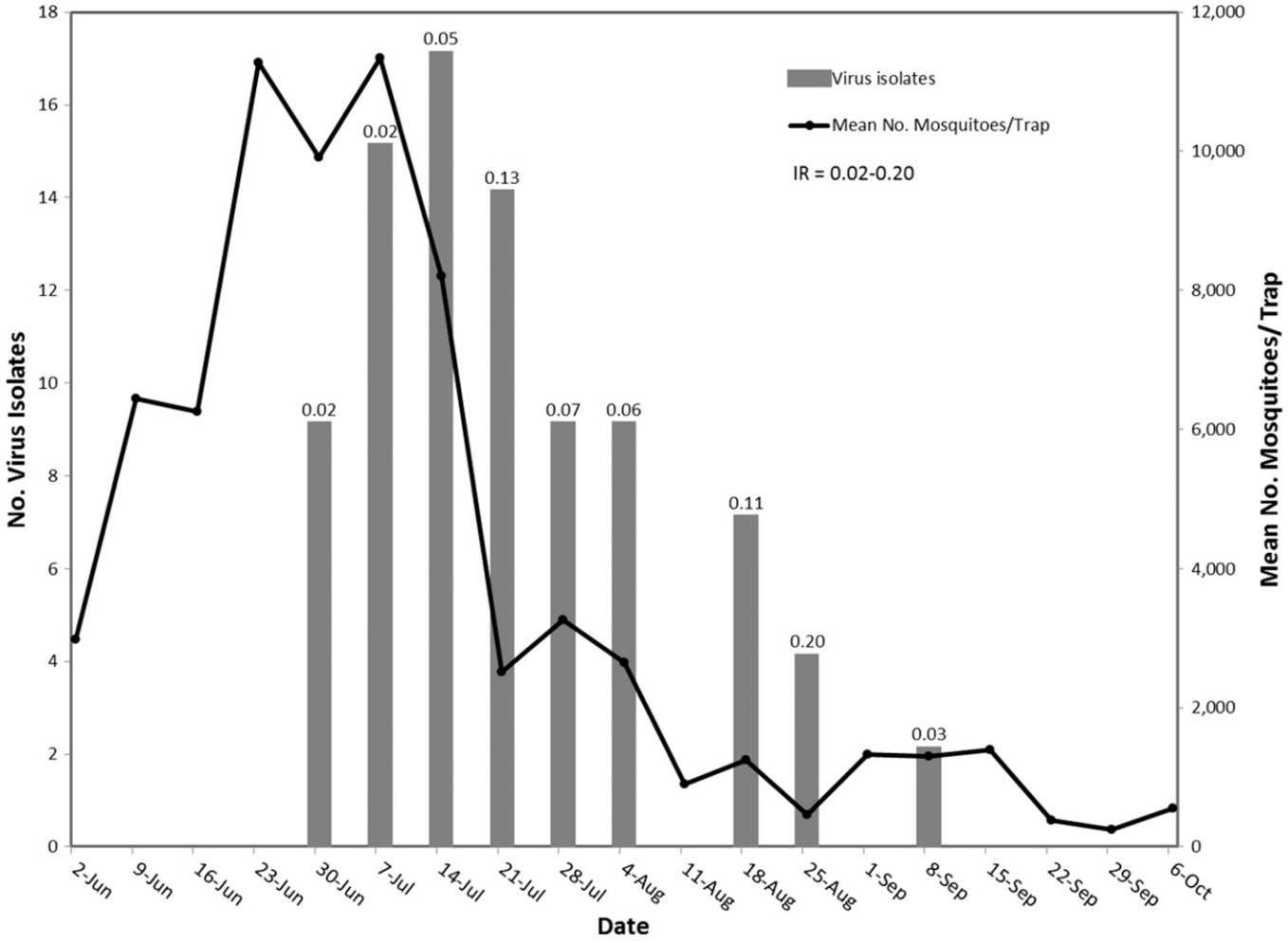

FIGURE 7. Weekly numbers of Jamestown Canyon virus isolations and infection rates (IR) relative to the mean number per trap-night of *Aedes vexans*, *Culex tarsalis*, *Culiseta inornata*, *Ochlerotatus dorsalis*, *Ochlerotatus flavescens*, *Ochlerotatus melanimon*, *Ochlerotatus sticticus*, and *Ochlerotatus trivittatus*, North Dakota, 2003–2006.

Virus was isolated from *Oc. trivittatus* from early June to early October in 2003–2006 (Figure 9). Ninety-seven percent of the isolates were from early June through the week of August 4. Weekly IRs was equal to or > 0.39. No isolates were obtained during the weeks of September 8, 15, and 29. One pool of 50 specimens was dually infected with TVTV and WNV.

Aedes vexans infected with TVTV were collected from June 8 through August 5. Two isolates from *Ae. vexans* were shown by phylogenetic analysis to be similar to the prototype strain isolated from *Oc. trivittatus* and distinctly different from JCV (Figure 8).[21]

Western equine encephalomyelitis virus. This native *Alphavirus* was isolated from two pools of *Cx. tarsalis* collected on August 3 and August 10, 2004 in Williams County (Tables 2 and 3). The IR was 0.30 (Supplemental Table 13).

Snowshoe hare virus. This California encephalitis virus was isolated in Cass County from one pool of *Oc. trivittatus* collected on July 1, 2004 and from three pools of *Ae. vexans* collected on July 13, 2005 (Tables 2 and 3, Supplemental Table 13). The IR for both species was 0.01. Two isolates from *Ae. vexans* were phylogenetically similar to the snowshoe hare virus (Figure 8).

Potosi virus. This *Orthobunyavirus* was isolated from three species collected in Cass County from August 22 through September 28, 2005 (Table 2 and 3, Supplemental Table 13). Four isolations were from *Ae. vexans* (IR = 0.01). Single isolations were from *Oc. dorsalis* (IR = 0.04) and from *Cs. inornata* (IR = 0.08).

DISCUSSION

Seven species of viruses circulated among 13 species of North Dakota mosquitoes during 2003–2006 (Tables 2 and 3). Five of the viruses cause human disease: WNV, JCV, CVV, SSHV, and WEEV. West Nile virus was isolated from four North Dakota counties in 2003–2006 (Table 2). Cache Valley virus was isolated from three counties in 2003 and in 2005. Jamestown Canyon virus was isolated from three counties in 2003–2006. Snowshoe hare virus and WEEV were isolated from one county each in 2004 and 2005 and in 2004, respectively.

Culex tarsalis and *Ae. vexans* were both important vectors of WNV in North Dakota. This virus was isolated from nine species of mosquitoes and multiple isolations were made from

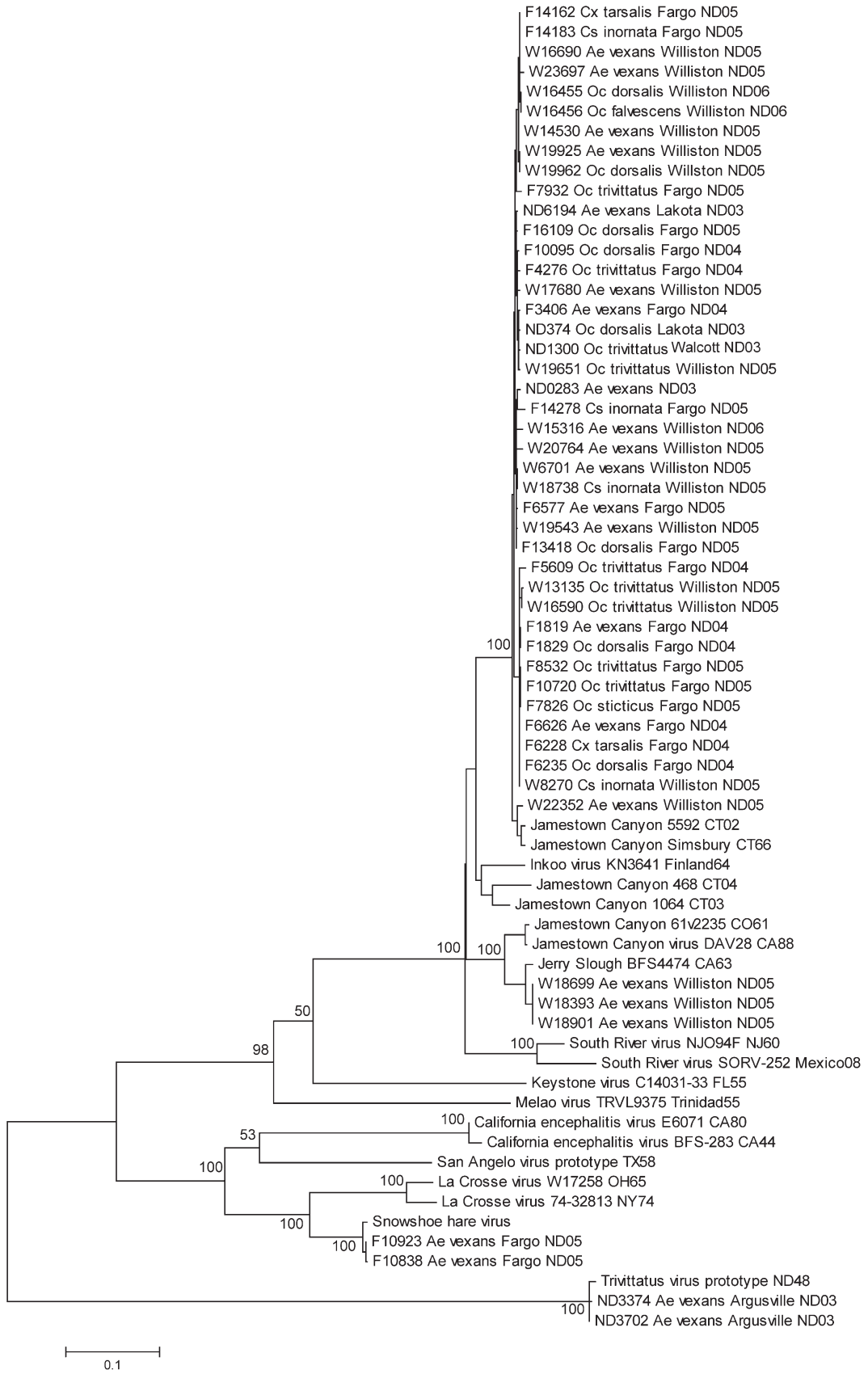

FIGURE 8. Phylogenetic tree showing relationships of Jamestown Canyon, snowshoe hare, and trivittatus viruses from North Dakota based on ML analysis of S-segment nucleotide sequences. Branch lengths are proportional to the number of nucleotide substitutions. Bootstrap *P* values ≥ 50% are given only at major nodes for clarity.

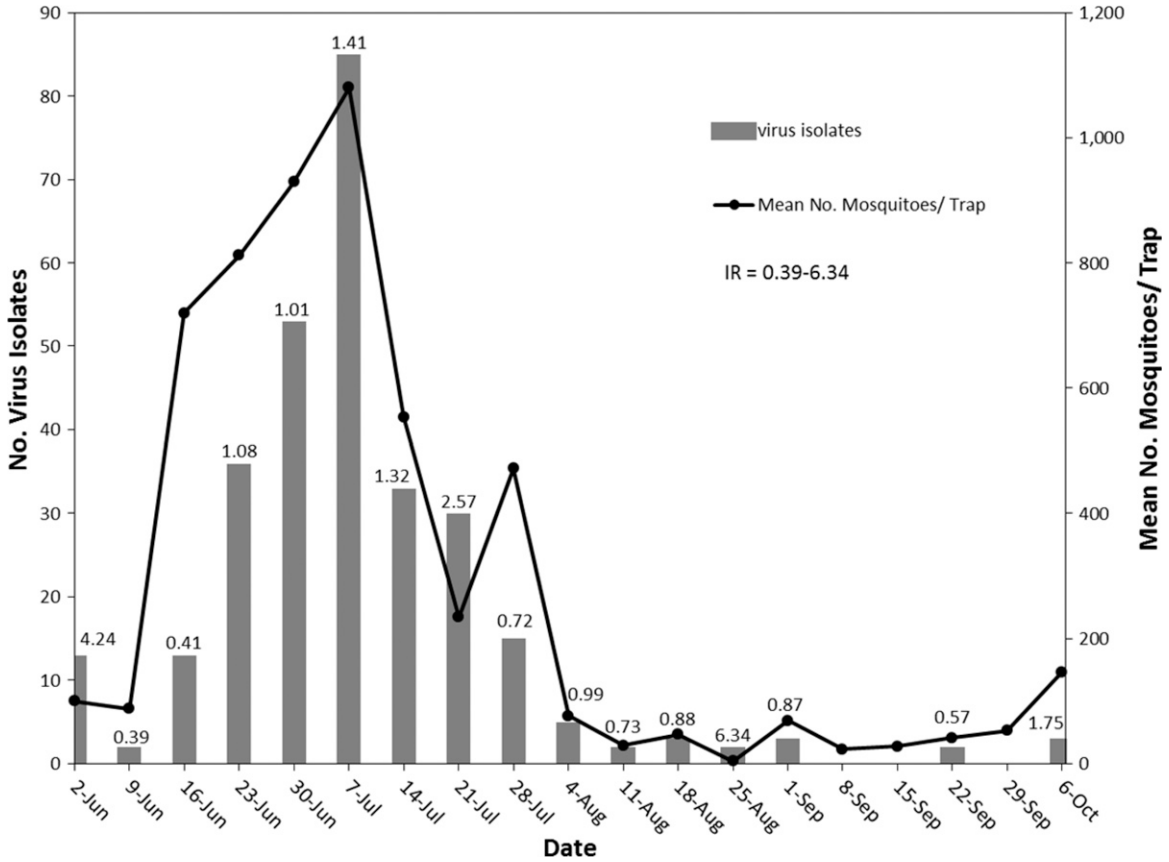

FIGURE 9. Weekly numbers of trivittatus virus isolations and infections rates (IR) relative to the mean number per trap-night of *Ochlerotatus trivittatus*, North Dakota, 2003–2006.

four species, but *Cx. tarsalis* and *Ae. vexans* accounted for 95.4% of the total risk for the years 2003–2006 (Supplemental Table 2).[16] The relatively high IR for *Cx. tarsalis* and the abundance of *Ae. vexans* accounted for their relatively high risks. *Culex tarsalis* was the more important with a risk factor of 61.4%. However, the largest epidemic of WNV infection in North Dakota was recorded in 2003, and in Cass County with 32 cases, humans had a greater risk of being bitten by infected *Ae. vexans* (73.6%) (Supplemental Table 3). The abundance and relatively high IR of 0.19 per thousand tested mosquitoes accounted for the risk of *Ae. vexans* transmitting WNV to humans in 2003. Previous laboratory and field studies identified *Cx. tarsalis* as one of the most important vectors in western United States,[22–25] including North Dakota.[26] Our current investigations confirm this conclusion, but we also documented *Ae. vexans* to be an important vector. Previously, WNV was detected only in *Cx. tarsalis* in North Dakota.[26,27] Unlike studies elsewhere in the United States where *Ae. vexans* was not as abundant,[28–30] numbers per trap-night in North Dakota averaged > 1,890 from June 2 through the week of August 4 and averaged as high as 9,393 per trap-night during the week of July 7 (Figure 2B).

Culex tarsalis feeds on both birds and mammals,[31–35] including humans.[36] It was relatively abundant in North Dakota from the end of June through the end of August, had a relatively high IR during the summer, is efficient in transmitting this virus horizontally,[22,24,25,37] and is relatively efficient in transmitting this virus vertically.[24,38,39] *Culex tarsalis* was collected during the weeks of June 2 to October 6, similar to collections made in Grand Forks, North Dakota,[26] but populations were highest from the week of June 30 through August ranging from 63.9 to 239.4 per trap-night (Figure 2A). The *Cx. tarsalis* weekly risk and vector index was dominant during August except in 2003 (Supplemental Tables 4–9, Figure 4). These data were similar to those reported in Grand Forks, ND.[26,27] The IR was near or greater than three per thousand specimens, and populations were relatively abundant (Figure 2A). The IR increased in early September, but populations were drastically reduced and risk of transmission reduced. It is noteworthy that *Cx. pipiens*, which is a relatively important vector in Illinois and Colorado,[17,40] was not prevalent in our collections in North Dakota, although we did not use gravid traps to collect mosquitoes.[41]

Aedes vexans is the most abundant mosquito in North Dakota,[42] and it is an aggressive feeder on humans.[43] Infected specimens with WNV were collected during the same weeks of summer as were *Cx. tarsalis* (Figure 2A, B), and females with a disseminated infection transmit the virus by bite.[37] It primarily feeds on mammals, particularly large mammals, and infrequently feeds on birds.[31,44–46] More than 1,850 specimens were captured per trap-night from early June through the first week of August, and more than a 1,000 specimens were collected per trap-night in mid-September. Weekly risk of being bitten by infected specimens ranged from 36.2% to 74.0% during most of July and the first week of August, but declined to 10.3–14.5% for the remainder of August (Supplemental Table 4). It increased to 35.5% and 60.0% in early September. *Aedes vexans* may become infected by feeding on small or

medium-sized mammals, which have been reported to have specific antibodies to WNV,[47] shown to produce viremias sufficient to infect mosquitoes,[48–51] and found naturally infected in the field.[52]

The risk that humans will acquire an infectious bite was greatest from the middle of July through the end of August with transmission still occurring in early September (Supplemental Table 4). The vector index was highest from the middle of July through the end of August (Supplemental Table 9, Figure 4). Increasing vector indexes preceded onset of human symptoms by 2 to 3 weeks. This time-lag results from a 7–14-day delay for symptoms to appear and a several day WNV extrinsic incubation period in the mosquito.[53,54] With the exceptions of weeks June 16, July 7, July 28, and September 8, human risk of being bitten by infected *Cx. tarsalis* during the weeks of June 30 through September 8 was greater than being bitten by other vector species, including *Ae. vexans*. Nonetheless, the weekly risk of being bitten by an infectious *Ae. vexans* plus other vector species, other than *Cx. tarsalis*, from mid-July through August ranged from 10.3% to 57.8%. Vector indexes were also relatively high for *Cx. tarsalis* during mid-July through August. The earliest human cases may have resulted from bites from *Ae. vexans* and *Oc. dorsalis*, which were relatively abundant during June. Alternatively, humans may have been bitten by *Cx. tarsalis* that overwintered as adults and were vertically infected.[24,38,39] West Nile virus has been isolated from overwintering *Cx. pipiens* and has been shown to survive winter in unfed, vertically infected *Cx. pipiens* with amplification initiated in the spring by horizontal transmission.[55,56] Human cases reported in October may have resulted from bites by infected *Cx. tarsalis* or *Ae. vexans*.

Phylogenetic analysis of WNV sequences of isolates made from 2003 to 2006 showed that strains circulating in North Dakota were members of the WN02 genotype (Figure 5). These findings are consistent with those reported elsewhere in the United States.[19,20]

Five of the viruses belong to the genus *Orthobunyavirus* in the family Bunyaviridae. Cache Valley virus, originally isolated from *Cx. inornata* collected in Cache Valley, Utah in 1956,[57] has been reported from four Canadian provinces, 22 states, including North Dakota, northern Mexico, and Jamaica.[58–60] Our isolations of CVV from *Ae. vexans*, *Cs. inornata*, *Oc. dorsalis*, and six other species (Table 3, Supplemental Table 10) are consistent with previous findings of its presence in several species of mosquitoes and from mammals, including humans.[58,61] It causes congenital defects in fetal or neonatal sheep and cattle,[62,63] including those in North Dakota,[64] has caused disease in humans,[61,65,66] and it may be responsible for birth defects in humans.[67]

Cache Valley virus was isolated in two of the four years sampled and was prevalent only in 2005 when it was isolated from 149 pools of mosquitoes collected in eastern and western North Dakota (Table 2). These data suggest that CVV becomes epizootic periodically. Although four isolations were made in July when mosquito populations of the nine infected species were highest, 96.7% of the isolations were made throughout August and the first week of September when mosquito abundance was significantly reduced compared with their numbers in June and July (Figure 6). The infection rate in August and the first week of September was relatively high ranging from 0.14 to 1.09 per 1,000 specimens tested and is the time when sheep and cattle are most likely to become infected with virus.

Jamestown Canyon virus is widely distributed in North America and causes a mild febrile illness that may cause central nervous system infection.[68,69] Human cases are relatively rare with 15 cases reported from 2004 to 2010, including one from Montana in 2009,[69] and two cases were reported during 2012.[70] Although not previously reported from North Dakota, we found this virus to be widespread. It is transmitted primarily by mammalian-feeding *Aedes* and *Ochlerotatus* mosquitoes.[71] Deer are considered the primary hosts, although other ungulates may also be involved.[71]This virus survives winter in mosquito eggs via vertical transmission.[71] Our isolation of JCV from eight species within four genera from four counties during the 4 years confirms earlier findings that several species are likely vectors (Table 2 and 3) The largest numbers of isolations were from *Ae. vexans*, but IRs were all < 0.1 per thousand specimens tested (Supplemental Table 11). Species less numerous but with larger IRs included *Cs. inornata*, *Oc. dorsalis*, *Oc. flavescens*, *Ochlerotatus melanimon*, and *Oc. trivittatus*. Species not previously reported to be infected included *Cx. tarsalis* and *Oc. flavescens*. This virus was isolated from the last week of June to the second week of September (Figure 7). Most isolates (98%) were from mosquitoes collected during the week of June 30 through the last week of August. Sequence analysis of 44 isolates from North Dakota showed that most belonged to Lineage A (Figure 8). Forty-one isolates from seven species, collected in four different counties, and four different years were similar to the lineage A isolates from Connecticut.[14] Three isolates belonged to the Jerry Slough variant.[72] Our findings suggest the JCV strains were stably maintained in North Dakota, infecting a number of different mosquito species.

Trivittatus virus, which was originally isolated in Bismarck, North Dakota in 1948,[21] was the most frequently isolated virus. Although isolated from six species, 77.8% were from *Oc. trivittatus*, confirming it as the most important vector (Table 3).[73–75] Many of the remaining isolates (20.3%) were from *Ae. vexans*. The IRs for *Oc. trivittatus* by county and year ranged from 0.70 to 5.09 and were significantly higher than the IRs for *Ae. vexans*, which ranged from 0.01 to 0.42 (Supplemental Table 12). This virus, which is transmitted horizontally and vertically by *Oc. trivittatus*,[73–76] was isolated during most weeks of the summer and fall, particularly during June and July when *Oc. trivittatus* were most abundant (Figure 9). Two isolates were genetically similar to the prototype strain (Figure 8). This virus infects humans,[77] but rarely causes clinical symptoms.[71]

Seasonal relationships between numbers of *Oc. trivittatus* infected with TVTV and mosquitoes infected with CVV were markedly different (Figures 6 and 9). Differences reflect at least in part the means by which these mosquitoes become infected. Isolations of TVTV were made weekly, with the exception of 3 weeks in September, from *Oc. trivittatus* throughout the season from early June to the first week of October (Figure 9). Vertical transmission is responsible for early season-infection, and vertical and horizontal transmission cause infection later in the season.[73–76] In contrast, horizontal transmission likely is the primary means of infection during summer for CVV. In mid-June through late July, weekly vector numbers per trap-night averaged > 4,000 to > 14,000 and few isolations were recorded (Figure 6). In late

July, after numbers of mosquitoes declined, numbers of weekly isolations in August and early September increased to as high as 50 per week. Mosquitoes collected in June and early July likely had not fed on viremic mammals, whereas relative numbers of those collected in late July and into the first week of September likely became infected by feeding on viremic mammals.

Potosi virus originally isolated from *Ae. albopictus* collected in Potosi, MO in 1989,[78] also has been isolated from several species of mammal-feeding mosquitoes in Illinois, Michigan, Ohio, the Carolinas, Connecticut, and New York.[79–84] We now report POTV in three species in North Dakota. Isolation of POTV from four pools of *Ae. vexans* and single pools of *Ae. dorsalis* and *Cx. inornata* in 2005 clearly shows its distribution extends into the northern great plains. Infected mosquitoes were collected from late August through the last week of September 2005. This virus is horizontally transmitted by several species to white-tailed deer, *Odocoileus virginianus*.[85,86] Although POTV is not known to cause human disease, related Bunyamwera viruses are known to cause febrile illness, central neurological system disease, and hemorrhagic fever.[61,87–89] It is also worth noting that POTV may co-circulate with CVV, which causes congenital defects in sheep and cattle,[62,63] during late August and September.

Snowshoe hare virus was initially isolated in 1959 in Montana from a snowshoe hare, *Lepus americanus*,[90] and subsequently from at least 23 species of mosquitoes,[91] which were collected in Alaska,[91] Montana,[91] New York,[92] Wisconsin,[92] Massachusetts,[92] and all Canadian provinces.[93,94] Our isolations of SSHV from *Ae. vexans* and *Oc. trivittatus* are the first from North Dakota. They were genetically similar to a known strain of SSHV (Figure 8). This virus is transmitted horizontally by several species of mosquitoes in the genera *Aedes*, *Ochlerotatus*, and *Culiseta*, and it is also transmitted vertically.[95,96] Snowshoe hares and squirrels are principal reservoir hosts in Canada.[93,94] Human infection ranges from absence of clinical disease to non-fatal meningitis and encephalitis.[93,94] Disease in domestic animals has occurred infrequently.[97]

Western equine encephalomyelitis virus causes disease and death in humans and horses in western and central Canada and the United States, Mexico, Guyana, Brazil, Uruguay, and Argentina.[98] One of the largest human epidemics of WEEV occurred in North Dakota in 1941.[1] Since 1955, a total of 93 cases have been documented in North Dakota, but none has been reported since 1987.[99,100] The basic transmission cycle involves *Cx. tarsalis* and passerine birds. Humans and horses are tangential hosts. Our isolation of WEEV from two pools of *Cx. tarsalis* in 2005 shows this virus still persists at low levels in North Dakota. The decline of WEEV as a human and equine health problem in North Dakota is similar to declines reported in Kern County, CA and elsewhere in North America.[101,102] Both WNV and WEEV are amplified in birds after bites by infected *Cx. tarsalis*, but WEEV has not amplified sufficiently in recent decades to infect humans and horses. Reasons for the suppression of WEEV in the years preceding and after the introduction of WNV are not known.

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