


Spatial and temporal expansions of Eastern equine encephalitis virus and phylogenetic groups isolated from mosquitoes and mammalian cases in New York State from 2013 to 2019

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ABSTRACT

Surveillance for the emerging infectious disease Eastern equine encephalitis, and its causative virus in mosquitoes, continued within New York State from 2013 to 2019. There were increases in geographic area and number of consecutive years, with cases in four mammalian species, and virus in 11 mosquito species. The first cases in a goat and in an emu were reported. The first detection of virus in *Aedes cinereus* was reported. Virus in phylogenetic group NY4 was isolated from a horse and from mosquitoes 6 kilometers and 13 days apart in 2013. Phylogenetic groups NY4 and NY5 were found 15 days apart in two towns 280 kilometers distant in 2013. Within four adjacent counties there was a pattern of overlap, where four had NY5, two adjacent counties had NY6, two adjacent counties had NY7, and one county had NY5, NY6, and NY7, reducible to a Euler diagram. Virus in phylogenetic group NY5, found within an 11-kilometer wide area in New York State, was related to FL4 found in Florida 1,398 kilometers distant. This was consistent with a phylogenetic group originating in Florida, then being moved to a specific location in New York State, by migratory birds in consecutive years 2013 and 2014.

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Introduction

EEE cases humans and other vertebrates

Eastern equine encephalitis (EEE) is a potentially deadly disease for humans and other vertebrates, without curative treatment [1]. From 2009 to 2018, 72 human cases have been reported in 21 states [1]. This protean disease in vertebrates has emerged in new geographic areas within the United States [2]. In Maine, from 2001 to 2009 geographic area increased to five counties, in which cases in vertebrates were reported in horses, wild birds, pheasants, and one llama [3]. Maine, in 2014, had its first human case [4]. In New Hampshire, in 2005, the first human case in this State was reported [5]. The first discovery of antibodies against EEE virus in moose in Vermont was in 2010 [6]. The first discovery of antibodies against EEE virus in white-tailed deer in

Vermont was also in 2010 [7]. In Vermont, in 2011, the first cases in animals were reported [7]. Within New York State, from 2002 to 2012, there has been an increase in geographic area, from five counties to twelve counties, in which cases have been reported in humans, horses and wild birds [8]. In Arkansas, in 2013, the first human case in this State was reported [9]. In Canada, in 2008, EEE was diagnosed in animals [10], in the Province of Quebec, which borders New York State. In 2016, Canada had its first reported human case, which was in the Province of Ontario [11], which also borders New York State.

EEE virus in mosquito vectors

The first discovery of EEE virus in mosquitoes in Maine occurred in 2005 and was in *Culiseta melanura*

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(Coquillett) [12]. The first detection of EEE virus in mosquitoes in Vermont was in 2012 and was in *Culiseta melanura* [13]. The first detection of EEE virus in mosquitoes in New Hampshire was in 2005 and was in *Culiseta melanura*, *Culiseta morsitans* (Theobald), *Coquillettidia perturbans* (Walker), *Culex pipiens* L., and *Aedes cinereus* Meigen [5]. Within New York State EEE virus was not detected in mosquitoes in any of the years from 1998 to 2002. Then, EEE virus was detected in mosquitoes every year from 2003 to 2012 [14], which included the period of time, 2005–2012, when EEE virus emerged in the nearby states of Maine, Vermont, and New Hampshire.

EEE virus is thought to be transmitted to horses and humans by mosquitoes, such as the genera *Aedes*, *Coquillettidia*, and *Culex* [15,16]. The genus *Culiseta* is an enzootic vector among birds [17]. *Culiseta melanura* is the first species of mosquito in which EEE virus is detected each season in our geographic region of central New York State [18]. EEE virus has appeared in new species of mosquitoes [14].

In New York State, EEE disease emerged first in pheasants in 1952 [19]; next in horses in 1970 [20] with a total of 151 horse cases through 2012 [8,15,18,21]; then in humans in 1971 [22] with a total of six human cases through 2012 [23]. Previous surveillance for EEE virus in mosquitoes from 1971 to 2012 found virus in 11 mosquito species [14,24]. This present work describes aspects of this disease and virus in our subsequent surveillance.

Materials and methods

From 2013 to 2019, ongoing surveillance for vertebrate cases, mosquito vectors, and EEE virus, was conducted by county health departments and the New York State Department of Health, exclusive of the five counties of New York City. This surveillance programme has been described [14,21,24]. When an EEE case was reported in a new town, a mosquito surveillance site was initiated nearby. Once a trap site was proven productive for the capture of mosquito vectors it was maintained in subsequent years [24].

Data were gathered on vertebrate cases, mosquito vectors, and EEE virus, as follows.

Human cases of suspected encephalitis, including EEE, were reported and investigated under the State of New York – Title 10 Public Health Law, Part 2, Section 1. Cases were confirmed according to CDC definitions [25]. For human cases, medical history included any travel outside the county of residence. Other causes of viral encephalitis were excluded by laboratory testing [26,27]. Animal cases of suspected encephalitis, including EEE, were reported and investigated by county health departments and contracted veterinarians under State of New York, Agriculture and Markets Law, Article 5, Section 73. New York State

Department of Agriculture and Markets completed case investigations. Veterinary history including travel was recorded. Other causes of viral encephalitis, including rabies, were excluded by laboratory testing [28] by Wadsworth Center of the New York State Department of Health. Veterinary test results were often available within two days.

Mosquito surveillance was conducted annually during the active transmission season, May to October. Common adult mosquito collection methods were used: carbon dioxide-baited Centers for Disease Control light traps, gravid traps, and diurnal resting shelters. Beginning in 2016, for the purpose of screening, particularly *Aedes* species, for Zika virus, some county health departments opted to add BG-Sentinel traps (Biogents, Regensburg, Germany) and Fay-Prince traps. Collections were made once or twice per week, customarily. Sites selected for surveillance contained suitable habitat for mosquito species considered as vectors of EEE virus and West Nile virus and other arboviruses. Mosquito specimens were first visually sorted. Males were discarded. Females were identified to species and confirmed with a dissecting microscope. Female mosquitoes were grouped by species, physiologic status (non-blooded, blooded, gravid), collection method, date, and site. Groups of 10–60 specimens were placed in vials, referred to as pools [24]. Pools were held on dry ice and submitted for testing.

Arbovirus testing of mosquitoes was conducted in laboratories at the Wadsworth Center of the Department of Health of the State of New York. Mosquito pools of all genera, except *Culex*, were placed on Vero cells for culture of virus. The supernatants of Vero cultures which had cytopathology were extracted and tested for arboviruses by multiplex real-time quantitative reverse transcription polymerase chain reaction, qRT-PCR [29]. The multiplex contained two sets of primers for Eastern equine encephalitis virus and primers for eight other viruses [29]. *Culex pipiens*, *Culex restuans* Theobald, and *Cx. pipiens-restuans* group were tested only by multiplex real-time quantitative reverse transcription polymerase chain reaction, due to the high specificity for EEE virus and high frequency in these species of Flanders virus, making tissue culture cost- and time- prohibitive [29]. If a *Culex* pool tested positive by multiplex real-time quantitative reverse transcription polymerase chain reaction, it was placed on Vero cells for culture.

As sub-set analyses, phylogenetic information was used as published in 2018 by Tan and others [30] and from GenBank data sets [31]. Genetic analysis, including extraction of ribonucleic acid, RNA, synthesis of complementary deoxy-ribonucleic acid, DNA, sequencing, assembly of genome, and inference of phylogenies from a maximum clade credibility analysis for some viruses isolated in New York State in 2013 and 2014 were performed as described [30].

For virus from mosquitoes from New York State, dates of specimen collection, geographic locations, and mosquito species information were obtained from County collection records. For virus from horses from New York State, dates of specimen collection and geographic locations were obtained from County and State collection records. For virus from Florida, dates of specimen collection, geographic locations, and host species information were obtained from GenBank [31]. Published results of phylogenetic groups from Florida were as described [30].

Results

EEE cases in vertebrates

In the fifty-seven counties in New York State (excludes New York City), monitored for EEE disease in vertebrates, thirty-eight cases were reported (Table 1, Figure 1(A)). Of these, were five cases in humans, 31 cases in horses, one case in a goat and one case in an emu. The five cases in humans resided in two counties, Onondaga and Oswego. Reportedly, no

human case traveled outside their county of residence. The 31 cases in horses occurred in ten counties. Reportedly none of these horses had recently traveled outside their town of residence. Vaccination information is presented in Table 1. Forty-two per cent [16 of 38] of the vertebrate cases occurred in towns which previously had no cases. For the first time, the counties of Cattaraugus, Chautauqua, Ontario, Wayne, and Franklin reported cases of EEE (Table 1, Figure 1(A)).

EEE virus in mosquitoes

Trapping for mosquitoes was conducted in nineteen of the 57 counties outside of New York City (Table 2). These counties submitted 31 mosquito species, in eight genera, for testing: *Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, *Culiseta*, *Orthopodomyia*, *Psorophora*, and *Uranotaenia*. EEE virus was detected in eleven mosquito species, in five genera: *Cs. melanura* (295 pools), *Coquillettidia perturbans* (17 pools), *Culiseta morsitans* (4 pools), *Aedes canadensis* [*Ochlerotatus*

Table 1. Chronology of cases of Eastern equine encephalitis in regions, counties, and towns of New York State from 2013 to 2019.

Year	Date ^{a, b}	Vertebrate	Vaccination ^c	Region	County	Town	Tally ^d
2013	7 Sep ^b	Horse	Unknown	Western	Chautauqua	Kiantone	1
2014	29 Jul ^a	Human	No	Central	Onondaga	Cicero	1
	1 Aug ^a	Horse	By Owner	Central	Oswego	Schroepfel	5
	27 Aug ^b	Horse	Unknown	Central	Oswego	Palermo	9
	2 Sep ^a	Horse	No	Western	Wayne	Rose ^e	1
	4 Sep ^a	Horse	No	Western	Wayne	Rose ^e	2
	6 Sep ^a	Horse	No	Central	Madison	Lenox	1
	6 Sep ^a	Horse	No	Western	Wayne	Rose ^e	3
	8 Sep ^b	Horse	No	Central	Oneida	Trenton	1
	10 Sep ^b	Horse	No	Central	Oswego	Constantia	6
	13 Sep ^a	Human	No	Central	Onondaga	Van Buren	1
	13 Sep ^b	Horse	No	Central	Oneida	Vienna	7
	17 Sep ^b	Horse	Unknown	Western	Wayne	Galen	1
	30 Sep ^a	Horse	By Owner	Northern	St Lawrence	Lisbon	1
	10 Oct	Horse	No	Central	Oneida	Verona	3
2015	24 Aug ^a	Human	No	Central	Onondaga	Salina	1
	1 Sep ^a	Horse	Unknown	Northern	Franklin	Waverly	1
	4 Sep ^a	Human	No	Central	Onondaga	Clay	1
	12 Sep ^a	Human	No	Central	Oswego	Albion	1
	12 Sep ^b	Horse	Unknown	Western	Wayne	Galen	2
	21 Sep ^b	Horse ^e	No	Western	Wayne	Macedon	1
	22 Sep ^a	Horse	No	Northern	St Lawrence	Hopkinton	1
2016	22 Aug ^a	Horse	No	Southern	Ulster	Kingston	1
	21 Oct ^b	Horse	No	Western	Wayne	Galen	3
2017	27 Sep ^a	Horse	No	Central	Oswego	West Monroe	9
2018	8 Aug ^a	Horse	No	Central	Oswego	West Monroe	10
	3 Oct ^b	Horse	No	Central	Oswego	Williamstown	5
2019	13 Oct ^a	Horse	No	Western	Chautauqua	Sherman	1
	10 Aug ^a	Horse	By Owner	Central	Oswego	West Monroe	11
	12 Aug ^a	Horse	No	Central	Oswego	Amboy	7
	22 Aug ^a	Goat	No	Central	Oswego	Palermo	
	25 Aug ^a	Horse	No	Central	Oswego	Amboy	8
	27 Aug ^a	Horse	No	Central	Oswego	Albion	14
	27 Aug ^a	Horse ^f	No	Western	Ontario	Farmington	1
	13 Sep ^a	Emu ^f	Unknown	Southern	Sullivan	Forestburg	
	23 Sep ^a	Horse	No	Central	Oneida	Lee	2
	1 Oct ^a	Horse ^f	No	Central	Oswego	Williamstown	6
12 Oct ^a	Horse	No	Western	Cattaraugus	Randolph	1	

^aDate of onset.

^bDate of death.

^cThere were no horses that were vaccinated with documentation by a veterinarian.

^dTally is the consecutive and cumulative number of human or horse cases, respectively, in each town since 1970.

^eThese three cases in the Town of Rose resided on the same farm.

^fImmunologic testing was on blood, not on nervous tissue.

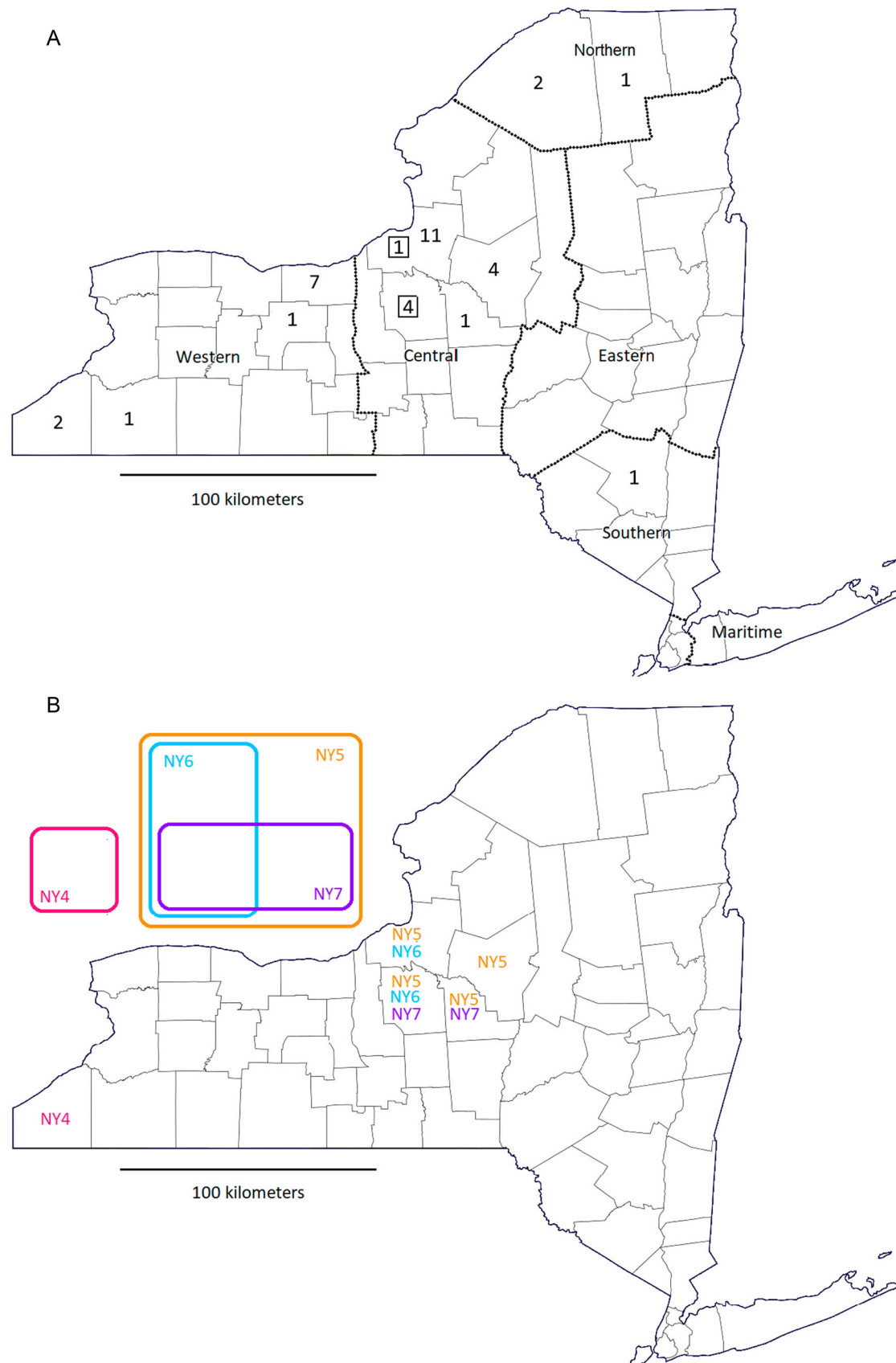


Figure 1. Part A. Map of New York State showing numbers of EEE cases in humans (in boxes) or horses (not in boxes) within counties during years 2013–2019, using data from Table 1. Black dotted lines show boundaries of Western, Central, Eastern, Northern, Southern, and Maritime Regions. Part B. Map of New York State showing phylogenetic groups of EEE virus within counties, isolated from mosquitoes or horses, during years 2013–2014, using data from Table 6. Colours: magenta NY4, orange NY5, turquoise NY6, purple NY7 are also used in Table 6. The inset shows Euler diagram of a pattern of phylogenetic groups in four counties in the Central Region.

canadensis] (Theobald) (3 pools), *Anopheles punctipennis* (Say) (2 pools), *Anopheles quadrimaculatus* Say (2 pools), *Cx. salinarius* Coquillett (3 pools), *Cx. pipiens-restuans* group (2 pools), *Aedes cinereus* (1 pool), and *Aedes vexans* (Meigen) (1 pool).

Mosquitoes with EEE virus were detected in each of the seven years of surveillance: 29 July to 26 September 2013; 1 July to 24 September 2014; 25 June to 23 September 2015; 17 August to 31 August 2016; 26 July to 26 September 2017; 30 July to 20 September 2018; 8 July to 24 September 2019. The frequency distribution over time of the number of poolings with EEE virus during the season of transmission, summed for the seven years was: June (1 pool), July (49 pools), August (201 pools), September (74 pools), and October (5 pools).

Among all 31 species of mosquitoes, the number of pools tested each year ranged from 5933 to 6643 (average 6278) (Table 2). The average annual number of mosquito specimens in each pool ranged from 34 to 37 (average 35). For *Cs. melanura* alone, the number of pools submitted annually ranged from 265 to 653 (average 538), and the average annual number of mosquito specimens in each pool ranged from 22 to 34 (average 31).

Mosquitoes with EEE virus were found in eight counties: Chautauqua, Madison, Oneida, Onondaga, Oswego, Orange, Rockland, and Suffolk (Table 2). Within these counties, EEE virus was detected in 16 towns (Table 3). Of these 16 towns, five had no prior detections of EEE virus in mosquitoes: Kiantone, New Haven, Orangetown, Salina, and Warwick. In these five towns, same-site trapping and testing had been performed for: 7, 1, 4, 11 and 18 years, respectively, before virus was detected.

Clustering

Clustering of cases could be seen in some instances. A human case of EEE coincided with the timing of detections of virus in mosquitoes, in 2014 (Table 1). In 2015, two human cases in Onondaga County (Table 1) were within 12 km of a wooded wetland where virus was detected in *Cs. melanura* (collected 18 August to 15 September), *Cq. perturbans* (collected 20 August), and *Oc. canadensis* (collected 1 September).

In the Western Region in Kiantone Town there was proximity of one horse case to infected mosquitoes. This horse case (Table 1) was approximately 6 kilometres from an ongoing surveillance site where virus was detected in mosquitoes, both in 2013 (Table 2). There were three horse cases from the same farm, in Rose Town, Wayne County and one horse case in the adjacent town, Galen, all cases occurring between 2 and 17 September 2014 (Table 1). No EEE virus was detected from mosquitoes collected on or near the premises of these cases (Table 2). The case of horse EEE in

Lenox Town (Table 1) was adjacent to Sullivan Town where virus was detected in mosquitoes 21 July to 12 August, including *Aedes cinereus* (collected 6 August). Cases in horses in West Monroe Town in 2017, 2018, and 2019 (Table 1) coincided with detections of virus in mosquitoes from surveillance sites within this town. The case of EEE in a goat (onset 22 August) coincided with the detection of EEE virus in *Cs. melanura* mosquitoes (collected 21 August) within the same town.

EEE virus phylogenetic groups

Of the 155 isolates of EEE virus from mosquitoes and cases in 2013 and 2014, phylogenetic group assignment was made and was available for 29 of these isolates. Seven phylogenetic groups, NY1 to NY7, of EEE virus were determined for New York State from 2003 through 2014, as published in 2018 by Tan and others [30]. The present surveillance period was 2013–2019, and phylogenetic group data were available for 2013 and 2014. Year of first and last appearance, from 2003 through 2014, and numbers of isolates for each phylogenetic group were tabulated (Table 4). Seven phylogenetic groups, FL1 to FL7, of EEE virus were determined for Florida from 1991 through 2014, as published in 2018 by Tan and others [30]. Year of first and last appearance, from 1991 through 2014, and numbers of isolates for each phylogenetic group were tabulated (Table 5). Date, region, county, town, and host and vector species for 29 phylogenetically assigned individual isolates of EEE virus in New York State from 2013 through 2014 are shown in Table 6 and Figure 1(B). Date, county, and host species of ten phylogenetically assigned individual isolates of EEE virus in Florida from 2013 through 2014 are shown in Table 7.

Discussion

Humans

Locations of human cases

Statewide, since 1983, there has been no increase in the number of counties (two) where human cases have been found [8,21–23]. Within these two counties, since 1983, the number of towns having human cases has increased from two to nine. During the seven years of surveillance, 2013–2019, the five towns with their first cases in humans constituted geographic spread within the Central Region. Geographic areas adjacent to New York State, specifically Connecticut, Massachusetts and New Jersey [32], and the province of Quebec, Canada [11], had human cases from 2013 to 2016. Notably, there were no human EEE cases in New York State in 2019, a year during which in ten other states of the United States there were 38 confirmed cases [1,33].

Table 2. Regions and counties of New York State where mosquitoes tested positive for Eastern equine encephalitis virus from 2013 to 2019.

Region ^a	County	2013		2014		2015		2016		2017		2018		2019	
		Positive ^b	Tested ^c	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested
Western	Cattaraugus	0	49	0	308	0	310	0	347	0	85	0	89	0	105
	Chautauqua ^d	32	365	2	269	0	229	0	167	0	84	0	101	0	57
	Erie	0	710	0	461	0	707	0	348	0	382	0	438	0	363
	Niagara ^e	0	43	0	4	0	33	0	0	0	2				
	Wayne ^e			0	58	0	177	0	85	0	97	0	59	0	56
Central	Madison	1	253	5	259	14	315	0	129	1	239	1	237	2	247
	Oneida	0	215	0	211	1	205	0	120	0	120	0	88	0	306
	Onondaga	9	674	30	786	22	747	0	478	1	747	6	646	14	648
	Oswego	11	721	50	742	21	657	5	478	30	635	17	512	40	548
Northern	Clinton ^e							0	51	0	93	0	68	0	73
	Franklin ^e														
	StLawrence ^e														
Southern	Orange	0	194	0	229	0	300	0	292	0	326	1	367	0	305
	Putnam ^e							0	28						
	Rockland	0	648	0	681	0	587	0	631	0	613	0	711	1	743
	Ulster ^e							0	16						
Maritime	Westchester	0	397	0	232	0	238	0	336	0	386	0	391	0	243
	Nassau	0	666	0	856	0	614	0	536	0	584	0	784	0	808
	Suffolk	0	1472	0	1453	0	1524	0	1891	4	1789	0	1728	9	1515
Totals		53	6407	87	6549	58	6643	5	5933	36	6182	25	6219	66	6017

^aCentral, Eastern, Western, Northern, Southern, and Maritime Regions were comprised of 13, 15, 3, 17, 7, and 2 counties, respectively.

^bNumber of pools testing positive for EEE virus.

^cNumber of pools tested for EEE virus.

^dFrom 2007 to 2012, surveillance in a 600-acre wetland preserve resulted in no detections of EEE virus in 536 pools totaling 16,505 individual mosquitoes, including 125 pools totaling 2856 individual mosquitoes in the genus *Culiseta*. From 2013 to 2019, surveillance at this site continued.

^eA blank space means mosquito testing was not conducted because mosquitoes were not collected by this county in this year.

Table 3. Towns in New York State where mosquitoes tested positive for Eastern equine encephalitis virus from 2013 to 2019.

Region ^a	County	Town	Mosquitoes testing positive (number of pools)	
Central	Madison	Sullivan	24	
		Oneida	Verona	1
	Onondaga	Cicero	76	
		Clay	2	
		Lysander	3	
	Oswego	Salina ^b	1	
		Albion	6	
		Constantia	9	
		Hastings	51	
		New Haven ^b	7	
		Palermo	6	
West Monroe		95		
Eastern	None	None		
Western	Chautauqua ^c	Kiantone ^b	34	
Southern	Orange ^d	Warwick ^b	1	
Maritime	Suffolk	Rockland ^e	Orangetown ^b	1
		Riverhead	13	

^aCentral, Eastern, Western, Northern, Southern, and Maritime Regions were comprised of 13, 15, 3, 17, 7, and 2 counties, respectively.

^bTown had no prior detections of EEE virus in mosquitoes.

^cCounty had no detections of EEE virus in mosquitoes prior to 2013.

^dCounty had no detections of EEE virus in mosquitoes prior to 2018.

^eCounty had no detections of EEE virus in mosquitoes prior to 2019.

Timing of human cases

Over the past 49 years, the frequency of cases has increased approximately, from one case every 10 years (1971, 1983, 2009) to one case every year (2009, 2010, 2011, 2014, 2015) [7,21–23]. Within a given year having cases, the number of cases has trended from one per year to three per year. These case counts may be seen in the context of a range of three to 15 cases per year in the United States from 2009 to 2018 [1] then, 38 cases in 2019 [33].

Horses

Locations of horse cases

Statewide, during these seven years of surveillance, 31 cases in horses occurred in 23 towns. Of these 23 towns, 10 had previously reported a case [8] and 13 towns had not.

Regionally, previously unaffected regions had cases of disease in horses. In the Western Region, previously

from 1970 to 2012, EEE had not been reported in horses or any vertebrates [8,24]. During 2013–2019, disease occurred, in horses, for the first time, in this region (Table 1). The number of horse cases (11) in this region was 35 per cent of the total number of horse cases (31) in New York State from 2013 to 2019. In the Western Region, the index horse case was 613 kilometres from the index horse case in New York State, which was in the Maritime Region [20].

Within previously affected regions, there was geographic expansion to previously unaffected towns. In the Central Region and Northern Region were towns with their first cases in horses (Table 1) [8]. One case, in Franklin County, was approximately 455 kilometres from the index horse case, in Suffolk County [20].

Timing of horse cases

There has been a 50-year surveillance period (1970–2019) in New York State for cases in horses. It was noteworthy that there were no horse cases during the 11 years from 1992 to 2002 [8]. In comparison, there were horse cases every year during the 17 years from 2003 to 2019 [8] (Table 1), with the exception of 2007.

Goat

The case of EEE in a goat in 2019 (see Table 1) was the first documented in New York State. The goat resided in a town where infected mosquitoes had been found, between 2013 and 2019 (See Table 3), in a county where infected mosquitoes had been found between 2003 and 2012 [14]. This town and county had cases in horses between 1992 and 2012 [8]. A case of EEE in goat occurred in Massachusetts in 2019 [34] and in a goat in Florida in 2017 [35].

Emu

The case of EEE in an emu, the first documented in New York State, in 2019 (see Table 1) resided in

Table 4. Phylogenetic groups of Eastern equine encephalitis virus collected in New York State from 2003 to 2014.

Phylogenetic groups in New York State ^a	Year of first isolate in this group ^b	Year of last isolate in this group ^b	Calculated time of first appearance of the most recent common ancestor of this group ^c	Calculated time of last appearance of the most recent common ancestor of this group ^c	Number of isolates in this group ^d
NY1	2003	2007	2001	2003	46
NY2	2004	2004	2002	2003	4
NY3	2008	2010 ^e	2005	2006	64
NY4	2013	2013	2012	2013	7
NY5	2013	2014	2012	2013	13
NY6	2014	2014	2012	2013	6
NY7	2014	2014	2013	2014	3

^aPhylogenetic or “monophyletic groups defined for ... New York” NY1 to NY7 from a “time-scaled phylogeny of a maximum clade credibility analysis based on complete genome sequences” [30].

^bInset diagram ... shows the time spans of these monophyletic groups, including the range of the sampling time” [30].

^c“mean of the tMRCA” mean of the time of the most recent common ancestor [30].

^dTime-scaled phylogeny of EEEV. A maximum clade credibility (MCC) tree of complete genome sequences of EEEV is shown [30].

^eOur records stated that GenBank KX000181 isolate of EEE virus was collected not on 6 August 2014 but was collected on 6 August 2010. The laboratory identification included number 10370362, where the first two digits represent the year 2010, or 10, the second two digits represent a county code 37 and the last four digits are the mosquito pooling number. The GenBank [31] information on specimen number KX000181 was available at: www.ncbi.nlm.nih.gov/nuccore/KX000181 and accessed 10 January 2020.

Table 5. Phylogenetic groups of Eastern equine encephalitis virus collected in Florida from 1991 to 2014.

Phylogenetic groups in Florida ^a	Year of first isolate in this group ^b	Year of last isolate in this group ^b	Calculated time of first appearance of the most recent common ancestor of this group ^c	Calculated time of last appearance of the most recent common ancestor of this group ^c	Number of isolates in this phylogenetic group ^d
FL1	1991	2008	1989	1991	4
FL2	2001	2009	1991	1995	6
FL3	2001	2002	1996	1999	3
FL4	2001	2014	1997	2000	5
FL5	2002	2001	1998	2000	3
FL6	2002	2002	1987	1992	3
FL7	2003	2010	1988	1993	6

^aPhylogenetic or "monophyletic groups defined" as FL1 to FL7 from a "time-scaled phylogeny of a maximum clade credibility analysis based on complete genome sequences" [30].

^bInset diagram ... shows the time spans of these monophyletic groups, including the range of the sampling time" [30].

^c"Mean of the tMRCA" mean of the time of the most recent common ancestor [30].

^d"Time-scaled phylogeny of EEEV. A maximum clade credibility (MCC) tree of complete genome sequences of EEEV is shown" [30].

Forestburg Town, adjacent to Thompson Town, where infected *Cs. melanura* mosquitoes had most recently been found in 2004 [14]. In Forestburg Town, a case of EEE occurred in an eagle in 2004 [8]. In Thompson Town, a case of EEE occurred in a horse, also in 2004 [8]. Emus can be reservoirs of EEE virus [36].

Mosquitoes

Species of mosquitoes infected

In New York State, this was the first time that EEE virus was detected in the mosquito species *Aedes cinereus*. This brings the total number of

mosquito species to 12: *Cs. melanura*, *Cs. morsitans*, *Cq. perturbans*, *Ae. canadensis*, *Cx. pipiens*, *Cx. restuans*, *Cx. salinarius*, *Ae. vexans*, *Psorophora ferox* (von Humboldt), *An. punctipennis*, *An. quadrimaculatus*, and *Ae. cinereus* [14]. *Aedes cinereus* is common in New York State [37]. Previously, in New York State, *Ae. cinereus* has been collected in light traps and tested for arboviruses without EEE virus being detected [14,24]. The potential of this species to transmit EEE virus to hosts during blood-feeding is unknown and requires further study [38]. Study of naturally blood-fed *Ae. cinereus* identified mammals as host sources [39,40].

Table 6. Equine encephalitis virus isolate and phylogenetic group locations in New York State from 2013 and 2014.

Nr	Date of Collection (yyyy-mm-dd)	Region of State	County	Town	Species from which virus was isolated	GenBank Identification Number	Phylogenetic Group ^{a,b}
1	2013-07-29	Western	Chautauqua	Kiantone	<i>Culiseta melanura</i>	KX000148	NY4 ^c
2	2013-08-05	Western	Chautauqua	Kiantone	<i>Culiseta melanura</i>	KX000084	NY4
3	2013-08-05	Western	Chautauqua	Kiantone	<i>Culiseta melanura</i>	KX000113	NY4
4	2013-08-07	Central	Oswego	Hastings	<i>Culiseta melanura</i>	KX000197	n a
5	2013-08-13	Central	Onondaga	Cicero	<i>Culiseta melanura</i>	KX000083	NY5
6	2013-08-14	Western	Chautauqua	Kiantone	<i>Culiseta melanura</i>	KX000103	NY4
7	2013-08-22	Central	Madison	Sullivan	<i>Culiseta morsitans</i>	KX000167	NY5
8	2013-08-23	Western	Chautauqua	Kiantone	<i>Culiseta morsitans</i>	KX000073	NY4
9	2013-08-25	Western	Chautauqua	Kiantone	<i>Anopheles punctipennis</i>	KX000139	NY4
10	2013-09-07	Western	Chautauqua	Kiantone	<i>Equus caballus</i>	KX000108	NY4
11	2013-09-10	Central	Oswego	West Monroe	<i>Culiseta melanura</i>	KX000178	NY5
12	2013-09-10	Central	Onondaga	Cicero	<i>Culiseta melanura</i>	KX000194	NY5
13	2013-09-12	Central	Oswego	Hastings	<i>Culiseta melanura</i>	KX000071	n a
14	2013-10-01	Central	Onondaga	Cicero	<i>Culiseta melanura</i>	KX000142	NY5
15	2013-10-03	Central	Onondaga	Cicero	<i>Culiseta melanura</i>	KX000118	NY5
16	2014-07-16	Central	Oswego	West Monroe	<i>Culiseta melanura</i>	KX000135	NY6
17	2014-07-29	Central	Onondaga	Cicero	<i>Aedes canadensis</i>	KX000093	NY5
18	2014-07-29	Central	Onondaga	Cicero	<i>Culiseta melanura</i>	KX000212	NY7
19	2014-07-30	Central	Madison	Sullivan	<i>Culiseta melanura</i>	KX000109	NY5
20	2014-07-31	Central	Oswego	West Monroe	<i>Culiseta melanura</i>	KX000092	NY6
21	2014-07-31	Central	Onondaga	Cicero	<i>Coquillettidia perturbans</i>	KX000223	NY5
22	2014-08-05	Central	Onondaga	Cicero	<i>Coquillettidia perturbans</i>	KX000133	NY5
23	2014-08-07	Central	Onondaga	Cicero	<i>Coquillettidia perturbans</i>	KX000149	NY7
24	2014-08-21	Central	Onondaga	Cicero	<i>Culiseta melanura</i>	KX000072	NY6
25	2014-08-27	Central	Oswego	Palermo	<i>Equus caballus</i>	KX000137	NY6
26	2014-09-01	Central	Oswego	New Haven	<i>Culiseta melanura</i>	KX000160	NY6
27	2014-09-02	Central	Oswego	West Monroe	<i>Coquillettidia perturbans</i>	KX000111	NY6
28	2014-09-03	Central	Onondaga	Cicero	<i>Culiseta melanura</i>	KX000060	NY5
29	2014-09-03	Central	Oswego	Hastings	<i>Culiseta melanura</i>	KX000076	n a
30	2014-09-06	Central	Madison	Lenox	<i>Equus caballus</i>	KX000126	NY7
31	2014-09-08	Western	Wayne	Rose	<i>Equus caballus</i>	KX000121	n a
32	2014-09-08	Central	Oneida	Trenton	<i>Equus caballus</i>	KX000143	NY5
33	2014-09-24	Western	Chautauqua	Kiantone	<i>Culiseta melanura</i>	KX000184	n a
34	2014-10-03	Central	Onondaga	Cicero	<i>Culiseta melanura</i>	KX000116	NY5

^aPhylogenetic or "monophyletic group" assignment as obtained from analysis [30].

^bInitials "n a" means that these isolates were not assigned to a phylogenetic group.

^cColours: magenta NY4, orange NY5, turquoise NY6, purple NY7 were also used in Figure 1(B).

Table 7. Equine encephalitis virus isolate and phylogenetic group locations in Florida State from 2013 and 2014.

Nr	Date of collection (yyyy-mm-dd)	County	Species from which virus was isolated	Isolate identification number	GenBank identification number	Phylogenetic group ^a
1	2013-01-08	Levy	<i>Equus caballus</i>	USA/I13-003-E/2013	KU840292	FL4
2	2013-04-08	Wakulla	<i>Morus genus</i>	USA/I13-020-D-A/2013	KU840298	FL4
3	2013-04-08	Wakulla	<i>Morus genus</i>	USA/I13-021-F/2013	KU840339	FL4
4	2013-05-26	Alachua	<i>Corvus species</i>	USA/I13-026-A-A/2013	KU840352	FL4
5	2013-09-27	Jackson	Cervidae family	USA/I13-042-B-A/2013	KU840374	FL4
6	2013-12-12	Osceola	<i>Balearica regulorum</i>	USA/I13-052-D-A/2013	KU840341	FL4
7	2014-06-18	Alachua	<i>Equus caballus</i>	USA/I14-023-D-A/2014	KU840301	FL4
8	2014-06-21	Alachua	<i>Equus caballus</i>	USA/I14-021-B-A/2014	KU840338	FL4
9	2014-06-23	Levy	<i>Equus caballus</i>	USA/I14-022-D-A/2014	KU840323	FL4
10	2014-08-18	Levy	<i>Equus caballus</i>	USA/I14-041-B-A/2014	KU840355	FL4

^aPhylogenetic, or monophyletic, group assignment as obtained from analysis [30].

Locations of infected mosquitoes

Within the Central Region, in New Haven Town, EEE virus was, for the first time, detected in mosquitoes (see Table 3). This was the same town in which, for the first time, a human case occurred in 2011 [8,23]. This constituted geographic spread within this Central Region [18].

Within the Western Region, previously, from 1971 to 2012, EEE virus was not detected in mosquitoes [14,24]. For the first time, EEE virus was detected in mosquitoes in 2013 and 2014 in the County of Chautauqua (see Table 2).

Within the Southern Region, in Rockland and Orange Counties, for the first time, EEE virus was detected in mosquitoes (see Table 2). The species was *Cx. pipiens-restuans* in the County of Rockland. The species was *Cs. melanura* in the County of Orange. The site with these mosquitoes was 32 kilometres from the location of horses with EEE in 1990 [24] and 2003 [8]. In this same county, EEE in pheasants occurred in 1952 [19].

Within the Maritime Region, in Suffolk County, EEE virus was detected in mosquitoes in 2017 and 2019 (see Table 2). EEE virus was not detected in mosquitoes for a four-year interval from 2009 to 2012 [14]. In association, in this region, EEE cases in vertebrates were not reported for a 15-year interval 2004–2012 [8] and from 2013 to 2019 (see Table 1). The first horse cases reported in New York State occurred in Suffolk County, in 1970 [20].

Statewide, from 1998 to 2002, no virus in mosquitoes was detected [14]. Then, beginning in 2003 [14], virus in mosquitoes was detected in every year through 2019 (see Table 2).

Timings of infected mosquitoes

During 1998–2002, EEE virus was not detected in mosquitoes [14]. From 2003 to 2012, EEE virus was detected in mosquitoes each year [14]. With the detection of EEE virus in mosquitoes in 2013 through 2019, there have been 17 consecutive years of virus in mosquitoes. This was the longest consecutive annual presence of EEE virus in mosquitoes in New York State. Statewide, there has been geographic expansion of

horse cases, from 15 counties as of 2012 [8] to 20 counties as of 2019.

Virus

Phylogenetics and epidemiology of virus

Molecular epidemiology of EEE virus in New York State, using various vocabulary, has been described: An analysis of un-passaged isolates of virus revealed a “consensus genotype” and “minority genotypes” [41]. Among viruses isolated during the 1970s, the rate of nucleotide substitution increased an order of magnitude, from 10⁻⁵ to 10⁻⁴ [42]. A publication in 1994 described a 1974 “division” into two “monophyletic groups” [42]. What had been described as a “division” into two “monophyletic groups” in 1994 [42] was, in a publication in 2018, described as a 1979 “branching” at a “major node” [30]. During two consecutive years, 1990 and 1991, the same “monophyletic group” was detected in the Central Region of New York State [42]. In this Central Region, 16 virus isolates, from 2003 to 2005, given specimen numbers from “NY03c” through “NY05f” [43], had “genetic similarity” among themselves and were assigned to “Onondaga03 clade” [43]. This “genetic similarity” or “clade” named in 2008 [43] was, in a publication in 2018, subsequently named “phylogenetic group” [30]. The specimens numbered “NY03c” to “NY05f” [43] were subsequently assigned to “phylogenetic group” “NY1” or as “NY1 strains” [30]. “Phylogenetic group” “NY1” was determined to have been present from 2003 to 2007 [30].

Publications proposed “overwintering” of virus [42,44] as an explanation for a phylogenetic group detected during a subsequent year [43]. However, the absence of detectable virus in those mosquitoes that had been collected and tested prior to 25 June [14] is an observation that is not consistent with the idea that virus can “overwinter” in mosquitoes in New York State. Hosts proposed for a virus life cycle include wild birds [45] and snakes [46].

Movement of EEE virus from Florida to New York State, has been hypothesized [43]. A subsequent publication showed additional phylogenetic evidence for the idea of movement of virus into New York State [30].

NY4, NY5, NY6, and NY7 were the phylogenetic groups isolated in New York State in 2013 and 2014 (see Table 6) [30]. FL4 was the only phylogenetic group isolated in Florida in 2013 and 2014 (see Table 7) [30]. NY4, NY5, and FL4 were descended from a common ancestor virus [30]. In 2013, NY4 was also isolated in Kiantone Town in Chautauqua County in New York State (see Table 6). In 2013, FL4 was isolated in five counties in Florida (see Table 7). The distance between, for example, Alachua County (Table 7) in Central Florida and Kiantone Town in Chautauqua County in Western New York State was 1398 kilometres. NY4 was detected in Western New York State from July to September 2013. Replication, within New York State, of virus in a phylogenetic group can be proposed as an explanation for there being more than one isolate of such a group during the months of July through October.

NY5 was detected in the Central Region from August to October 2013, and the following year NY5 was detected from July to October 2014. This would imply movement of the phylogenetic group NY5 into New York State during two sequential years. In New York State virus has never been detected in mosquitoes collected before 25 June [14]. Movement from Florida to New York State, in sequential years, of virus in the same phylogenetic group can be proposed as an explanation for the detection of virus in the same group during those years. Also, movement of one phylogenetic group, from Florida to New York State, then from New York State to Florida, and then again from Florida to New York State can be proposed. In 2013, NY4 was found in Kiantone Town, and NY5 was found in Cicero (see Table 6). The distance between Kiantone Town and Cicero Town was 280 kilometres. The time interval between the first detection of NY4, on 29 July 2013, and the first detection of NY5, on 13 August 2013, was 15 days (see Table 6). On 25 August 2013, in Kiantone Town, virus in phylogenetic group NY4 was isolated from mosquitoes and on 7 September 2013 from a horse (see Table 1). These NY4 phylogenetic isolates were in a temporal proximity of 13 days. The horse and the mosquitoes were in spatial proximity of 6 kilometres. These results increased the resolution of geo-spatial and movement and location for a phylogenetic group.

Phylogenetic group NY5 was related to phylogenetic group FL4 [30]. In one town, Cicero, in New York State, NY5 was isolated in two consecutive years, 2013 and 2014 (see Table 6). This town has an east-west longitudinal distance of 11 kilometres, compared to a latitudinal distance of 1398 kilometres to Alachua County in Florida (see Table 7) where FL4 has been isolated. This observation is consistent with virus in Florida being moved to New York State by migratory birds. Migratory birds in this area of New York State have been found to have EEE virus [15,47]. Individual

migratory birds have been banded and shown to return to the same woodlot in sequential years [47]. This was consistent with virus in a phylogenetic group in Florida being moved to a specific 11-kilometre location, in two sequential years, in New York State. Within four counties (see Figure 1(B)), in 2013 and 2014, for isolates for which phylogenetic groups were assigned, there was a pattern. All four counties had NY5; one county had only NY5, two adjacent counties had NY6, two adjacent counties had NY7, one county had NY5, NY6, and NY7. This pattern can be expressed in a Euler diagram (see Figure 1(B) inset). The durations of the appearances of phylogenetic groups NY1, NY2, and NY3 were 5, 1, and 3 years, respectively (see Table 4). Both in the same phylogenetic group [30], FL4 persisted for 13 years in Florida (see Table 5), but group NY4 persisted for 1 year in New York State (see Table 4). It is not known how long NY5, NY6, and NY7 may continue to appear in New York State (see Table 4).

Limitations

Case count totals may have varied among publications, due to differences in clinical recognition of suspected cases. Case count totals may vary depending on differences in interpretations of state health codes or federal definitions [25]. Some cases were identified before the establishment of case definitions [48]. Confirmation of cases may have varied among different testing methods: *in vivo*; *in vitro* cell culture; antibody titers; tissue pathology; or nucleic acid DNA or RNA polymerase chain reaction; any of which may vary in sensitivity or specificity and also have time periods during the course of disease when such a test may be expected to be appropriate or not appropriate, for example presence or absence of antibody or nucleic acid in body fluids [23]. Owners of horses have not been obligated to report illness or to make tissue available for testing. Because rabies and EEE are both viral encephalitides, concern about rabies prompts many owners to report ill animals and consent to specimen acquisition by veterinarians. The numbers of mosquitoes submitted were lower in some locations at times because of variations in staffing. The testing of fewer mosquitoes can result in the inability to obtain low values of minimum infection rate (MIR), thus reduced sensitivity. This would explain the apparently incongruous observation of EEE in horses but no EEE virus in mosquitoes. Viral detection by polymerase chain reaction, PCR, has the limitation that host blood in mosquitoes has been thought to contain a factor that can inhibit polymerase chain reaction [49,50]. Testing by cell culture can be limited if the virus is no longer viable. It was not possible to test all mosquitoes collected. The testing laboratory set a weekly limit to the numbers of

mosquito pools that could be tested. Each county chose the mosquito species and specimens to submit. Despite these potential issues, we think there have not been substantial changes in the methods of surveillance or of laboratory testing from 1971 to 2019. Phylogenetic results on specimens after 2014, that is from 2015 to 2019, were not available.

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