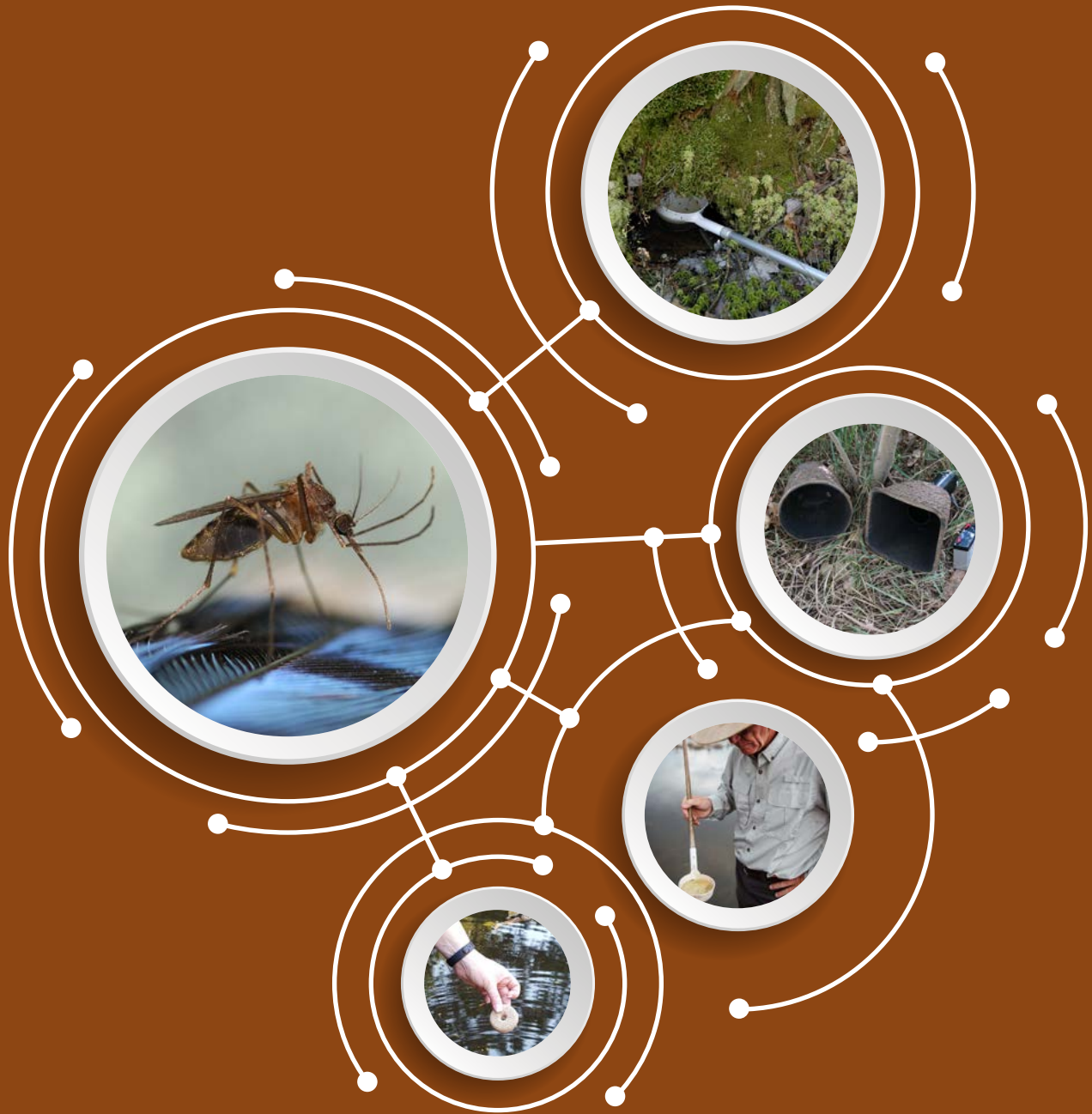


Eastern Equine Encephalitis Virus Surveillance and Control Guidelines





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About These Guidelines

Comprehensive Center for Disease Control and Prevention (CDC) guidelines for arbovirus surveillance programs in the United States were published in 1993. These guidelines detailed best practices for surveillance and control of eastern equine encephalitis (EEE), La Crosse encephalitis (LAC), St. Louis encephalitis (SLE), and western equine encephalitis (WEE). In the years since the guidelines were published, EEE has emerged as a vector-borne disease of increasing public health concern due to multiple outbreaks of neuroinvasive disease and the potential for

further expansion of EEE into new U.S. regions. Additionally, knowledge about EEE epidemiology and transmission ecology has greatly expanded. The objective of this guidance is to consolidate new knowledge and describe how this can be used to better assess EEE virus activity and mitigate its public health impact. These guidelines are meant for state and local public health officials and mosquito control personnel to aid them in the surveillance and control of EEE.



Epidemiology and Ecology

In the United States, one of the most significant mosquito-borne viruses is eastern equine encephalitis (EEE) virus. EEE virus is an alphavirus mainly cycling between birds and mosquitoes inhabiting freshwater hardwood swamps (Morris 1988).

Transmission risk is highest in lowland regions where its principal enzootic vector, the ornithophilic mosquito *Culiseta melanura*, readily occurs. Most cases occur in eastern or Gulf Coast states. As with other zoonotic arboviruses, EEE virus persists in a complex ecological web of host species, alternating between mosquito vectors and vertebrate amplification hosts, mostly birds. Habitats that pose a threat to humans and other vertebrates are those that support mosquito species that can serve as vectors and vertebrate hosts that develop viremia of sufficient magnitude to infect mosquitoes (amplification hosts). Reptiles and amphibians have also been implicated in enzootic transmission, particularly in Southern states, perhaps serving as an over-wintering refuge for EEE virus (Graham et al. 2012).

Freshwater swamp and bog habitats are a source ecosystem for EEE virus even in periods of low-level transmission (Miley et al. 2021, Skaff et al. 2017). These freshwater habitats provide pools of water for *Cs. melanura* larval growth and development and plant nectar for adult mosquito survival. In the Northeast region, EEE virus infections in mosquitoes are correlated with proximity to forested wetland habitat dominated by red maple (*Acer rubrum*) and Atlantic white cedar (*Chamaecyparis thyoides*). The dominant trees shift to bald cypress (*Taxodium distichum*) and water tupelo (*Nyssa aquatica*) in the southeast bottomlands. In the Great Lakes region, *Cs. melanura* favor sphagnum bogs for oviposition and larval development. Primary buttressed trees in these habitats include black spruce, red spruce, yellow birch, and tupelo.

In the late summer, bird populations disperse after breeding, causing declines in easily available avian blood meals. Swamp-inhabiting mosquitoes may then seek blood outside of the swamp habitats, increasing risk that the virus will spill over to infect other vertebrates and secondary vectors. Surveillance for EEE virus-infected hosts routinely detects vertebrate and vector infections within the swamp habitat, and during epizootic and

epidemic transmission outside their primary habitat. Some secondary vectors act as bridge vectors, feeding on birds and mammals and transmitting virus from enzootic to epizootic and epidemic hosts. Once spillover occurs, surveillance can detect EEE virus infections in other potential vector mosquito species.

Vectors suspected of transmitting EEE virus to horses and humans vary geographically. *Culiseta melanura* can act as both an enzootic and epidemic vector (Armstrong and Andreadis 2010). Primary bridge vectors include *Coquillettidia perturbans*, *Aedes canadensis*, *Aedes sollicitans*, and *Culex salinarius* in the Northeast and Mid-Atlantic regions (Armstrong and Andreadis 2010; Armstrong and Andreadis 2022; Crans 1977), *Cq. perturbans*, *Ae. canadensis*, *Cx. salinarius*, and *Cx. erraticus* in the Southeast (Burkett-Cadena et al. 2022), and *Cq. perturbans* in the Midwest (Nasci et al. 1993). Bridge vectors are competent to transmit virus and infected mosquitoes of these species are frequently detected during epizootic and epidemic periods. Horses and humans are considered dead-end hosts because they do not develop high enough levels of EEE virus in their blood to infect feeding mosquitoes.

The first human EEE disease cases were recognized during a 1938 outbreak in southeastern Massachusetts (Feemster 1938). Subsequent outbreaks were then reported in New Jersey during the 1950s (Goldfield and Sussman 1968). Since then, EEE cases have occurred sporadically and in small clusters, most around freshwater hardwood swamps in the Atlantic and Gulf Coast states and the Great Lakes region (Goldfield and Sussman 1968, Przelomski et al. 1988, CDC 2006). Human infections typically occur during summer months, with >80% of reported human cases having an illness onset during July through September (Lindsey et al. 2018). During 2003–2018, an average of eight EEE disease cases were reported annually in the United States (range = 4–21 cases/year) (Lindsey et al. 2018, CDC 2021a). However, in 2019, 38 cases were reported nationally (Vahey et al. 2021). The reasons for this increase are unknown but are likely related to several factors, including weather, abundance of birds, mosquitoes that can transmit the virus, human behavior, and clinical awareness and diagnostic testing practices.



Human Disease

Routes of Transmission

EEE virus is maintained in a cycle between *Cs. melanura* mosquitoes and avian hosts in freshwater hardwood swamps. Historically, *Cs. melanura* was not considered to be an important vector of EEE virus to people because it feeds almost exclusively on birds. Transmission to people requires another mosquito species to create a bridge between infected birds and uninfected mammals, such as people or horses (Morris 1988). However, recent research in northern New England detected *Cs. melanura* occasionally feeding on humans and possibly

contributing to EEE virus transmission to humans and horses (Molaei et al. 2015). Most of the bridge species are within the *Aedes*, *Coquillettidia*, and *Culex* genera. EEE virus has been documented to be transmitted through organ transplantation with one organ donor transmitting the infection to three organ transplant recipients (Pouch et al. 2019). Although not documented, EEE virus likely can be transmitted from person-to-person through blood transfusions.

Clinical Presentation and Evaluation

Most persons infected with EEE virus have no apparent illness (Davis et al. 2008, Calisher 1994). Among those who develop symptoms, the incubation period typically ranges from 4 to 10 days but can be several weeks in people who are immunocompromised (CDC 2021b, Sherwood and Oliver 2015).

Symptomatic infection is characterized by fever, chills, malaise, arthralgia, and myalgia (Calisher 1994). Most people recover completely in 1 to 2 weeks unless central nervous system involvement is present. Less than 5% of infected individuals develop meningitis or encephalitis (Morris 1988, Goldfield et al. 1968). In infants, neurologic disease often occurs soon after onset; in older children and adults, encephalitis may occur after several days of systemic illness. Signs and symptoms in patients with neuroinvasive disease include headache, confusion, focal neurologic deficits, meningismus, seizures, or coma (Feemster 1938, Przelomski et al. 1988, Deresiewicz et al. 1997, Clarke 1961, Letson et al. 1993, Ayers and Feemster 1949). Cerebrospinal fluid (CSF) findings include an initial neutrophil-predominant pleocytosis, shifting to a lymphocyte-predominance, and elevated protein levels; glucose levels are normal (Przelomski et al. 1988, Deresiewicz et al. 1997, Silverman et al. 2013). Neuroimaging shows brain lesions consistent with encephalitis, including neuronal destruction and vasculitis in the cortex, midbrain, and brain stem (Przelomski et

al. 1988, Silverman et al. 2013). Magnetic resonance imaging using T2-weighted images often show areas of increased signal in basal ganglia and thalami (Deresiewicz et al. 1997).

Persons aged >50 and <15 years seem to be at greatest risk for developing severe disease when infected with EEE virus. EEE neuroinvasive disease is estimated to have a 30% case fatality rate and results in neurologic sequelae in >50% of survivors (Feemster 1938, Goldfield and Sussman 1968, Deresiewicz et al. 1997, Letson et al. 1993, Ayers and Feemster 1949, Silverman et al. 2013, Gaensbauer et al. 2014). Death typically occurs 2 to 10 days after symptom onset but can occur much later. The neurologic sequelae can range from mild brain dysfunction to severe intellectual impairment, personality disorders, seizures, paralysis, and cranial nerve dysfunction. Many patients with severe sequelae require long-term care and die within a few years.

EEE virus disease should be considered in any person with a febrile or acute neurologic illness with a potential for recent exposure to mosquitoes, organ transplantation, or blood transfusion, particularly during the summer months in areas where virus activity has been reported. In addition to other more common causes of encephalitis and aseptic meningitis (e.g., herpes simplex virus and enteroviruses), other arthropod-borne viruses (e.g., West Nile, La Crosse, St Louis encephalitis, and Powassan) should also be considered in the differential diagnosis of suspected EEE.



Surveillance

Objectives of Arboviral Surveillance

Arboviral surveillance consists of two distinct, but complementary activities: epidemiological surveillance and environmental surveillance. Epidemiological surveillance measures human disease to quantify disease burden, detects early signs of an outbreak, and identifies information needed for timely responses, including seasonal, geographic, and demographic patterns in human morbidity and mortality. In addition to monitoring disease burden and distribution, epidemiological surveillance has been instrumental in characterizing clinical disease presentation and disease outcome, as well as identifying high-risk populations and human factors associated with serious disease. Epidemiological surveillance has also detected and quantified alternative routes of transmission to humans, such as contaminated blood donations and organ transplantation.

Environmental surveillance monitors local mosquito populations, virus activity in vectors and non-human vertebrate hosts, and other relevant environmental parameters to predict human risk and prevent outbreaks of arboviral disease in humans.

Epidemiological and environmental surveillance for arboviruses is facilitated by ArboNET, the national arbovirus surveillance system. ArboNET was developed in 2000 as a comprehensive surveillance data capture platform to monitor West Nile virus (WNV) infections in humans, mosquitoes, birds, and other animals. This comprehensive approach was essential to tracking the progression of WNV as it spread and became established across the United States, and it remains a significant source of data on the epidemiology and ecology of WNV. Since 2003, ArboNET has also collected data on other domestic and exotic arboviruses of public health significance.

In the absence of effective human vaccines for most domestic arboviruses, preventing arboviral disease in humans primarily depends on measures to keep infected vectors from biting people. A principal objective of environmental surveillance is to quantify the intensity of virus transmission in a region and provide a predictive index of human infection risk. This risk prediction, along with information about the local conditions and habitats, and virus-vector interactions that impact vector abundance and infection, can be used to inform an Integrated Vector Management (IVM) program and decisions about implementing interventions to control mosquitoes and to subsequently prevent disease.

Though epidemiological surveillance is essential for understanding arboviral disease burden, utilizing human case surveillance by itself is insufficient for predicting outbreaks. Outbreaks can develop quickly, with most human cases occurring over a few weeks during the peak of transmission. The time from human infection to onset of symptoms to diagnosis and reporting can be several weeks or longer. As a result, human case reports typically lag well behind the transmission from vectors that initiated the infection. Cases in non-human vertebrate hosts are often the first indicator of local arboviral activity. Monitoring infection prevalence in vectors and incidence in non-human vertebrate hosts and comparing these to historical environmental and epidemiological data can help identify conditions associated with human risk 2 to 4 weeks before human disease onset. This provides additional lead time for critical vector control interventions and public education programs to be put in place. The following sections describe the elements of epidemiological and environmental arboviral surveillance and how they may be used to monitor and predict risk and to trigger interventions.

Human Surveillance

Passive Surveillance and Case Investigation

Arboviral diseases are nationally notifiable conditions, and many are explicitly reportable in U.S. states and territories. Most disease cases are reported to public health authorities from public health or commercial laboratories; healthcare providers can also directly submit reports of suspected cases. State and local health departments are responsible for ensuring that reported human disease cases meet the national case definitions. The most recent

case definitions for arboviral diseases can be located on the CDC Nationally Notifiable Disease Surveillance System website (<https://ndc.services.cdc.gov/conditions/arboviral-diseases-neuroinvasive-and-non-neuroinvasive/>). For some arboviruses (e.g., WNV), presumptive viremic donors are identified through universal screening of the blood supply; case definitions and reporting practices for viremic donors vary by jurisdiction and blood services agency.



All identified human disease cases and presumptive viremic blood donors should be investigated promptly. Jurisdictions may choose to interview the patient's healthcare provider, the patient, or both depending on information needs and resources. Whenever possible, the following information should be gathered:

- Basic demographic information (e.g., age, sex, race/ethnicity, state, county of residence);
- Clinical syndrome (e.g., asymptomatic blood donor, uncomplicated fever, meningitis, encephalitis, acute flaccid paralysis);
- Illness onset date and/or date of blood donation
- If the patient was hospitalized and if they survived or died;
- Travel history in the 4 weeks prior to onset;
- If the patient was an organ donor or a transplant recipient in the 4 weeks prior to onset;
- If the patient was a blood donor or blood transfusion recipient in the 4 weeks prior to onset;
- If the patient was pregnant at illness onset;
- If the patient is an infant, were they breastfed before illness onset.

If the patient donated blood, tissues, or organs in the 4 weeks prior to illness onset, immediately inform the blood or tissue bank and appropriate public health authorities. Similarly, any infections temporally associated with blood transfusion or organ transplantation should be reported. Prompt reporting of these cases will facilitate the identification and quarantine of any remaining infected products and the identification of any other exposed recipients so they may be managed appropriately.

Passive surveillance systems are dependent on clinicians considering the diagnosis of an arboviral disease and obtaining the appropriate diagnostic test and reporting of laboratory-confirmed cases to public health authorities. Because of incomplete diagnosis and reporting, the incidence of arboviral diseases is underestimated. Where applicable, reported neuroinvasive disease cases are considered the most accurate indicator of activity in humans because of the substantial associated morbidity. In contrast, reported cases of non-neuroinvasive disease are more likely to be affected by disease awareness and healthcare-seeking behavior in different communities and by the availability and specificity of laboratory tests performed.

Enhanced Surveillance Activities

Enhanced surveillance for human arboviral disease cases should be considered when environmental or human surveillance suggests that an outbreak is suspected or anticipated. Educating healthcare providers and infection control practitioners about the need for arbovirus testing and reporting of all suspected cases could increase the sensitivity of the surveillance system. This might be accomplished by distributing print materials, participating in local hospital meetings and grand rounds, and providing lectures/seminars. Public health agencies should also work to establish guidelines and protocols with local blood collection agencies for reporting viremic blood donors. At the end of the year, an active review of medical records and laboratory results from local hospitals and associated commercial laboratories should be conducted to identify any previously unreported cases. In addition, an active review of appropriate records from blood collection agencies could be conducted to identify any positive donors that were not reported.

Environmental Surveillance

Vector-based Surveillance

Vector surveillance is an integral component of an IVM program and is the primary tool for quantifying virus transmission and human risk. The principal functions of a mosquito-based surveillance program are to:

- Collect data on mosquito population abundance and virus infection rates in those populations;
- Provide indicators of the threat of human infection and identify geographic areas of high risk;
- Support decisions regarding the need for and timing of intervention activities (e.g., enhanced vector surveillance and control, use of new technologies, and public education programs);

- Monitor the effectiveness of vector control methods, including susceptibility of target mosquitoes, to control methods used.

Mosquito-based arboviral monitoring complements disease surveillance programs by contributing fast results and data for action. Programs maintaining in-house laboratories can process mosquito samples daily, giving results within a few days. Data on vector species community composition, relative abundance, and infection rates allow programs to rapidly compute infection indices, assess risk, and respond. Maintaining mosquito surveillance over the long-term provides a baseline



of historical data to evaluate risk and guide mosquito control operations. However, the utility of mosquito-based surveillance depends both on the type and quality of data collected (e.g., number and type of traps, timing and frequency of sampling, testing procedures) and consistent effort across transmission seasons to link surveillance indices with human risk.

There are three main categories of vector surveillance: larval, adult, and transmission activity. Together, this information is used to determine where and when control efforts should be implemented. Larval surveillance involves sampling a wide range of aquatic habitats to identify the sources of vector mosquitoes and evaluating larval control measures applied. For adult mosquitoes, regular (e.g., monthly, weekly) sampling is done at fixed sites throughout the community that are representative of the habitat types present in the area. Adult mosquitoes are collected using a variety of trapping techniques, including traps for host-seeking, resting, or gravid (carrying eggs) mosquitoes seeking a place to lay eggs (oviposition site). Adult surveillance can also be used to evaluate control activities pre- and post-treatment. Transmission activity surveillance provides information on the level of infected mosquitoes in an area.

Specimen Collection and Traps

Mosquito species involved in enzootic or epidemic transmission are readily captured in CDC light traps (with or without carbon dioxide [CO₂]) and New Jersey light traps (McCardle et al. 2004). For best results the traps need to be placed in well-protected sites with very limited wind movement. Resting boxes may be used to increase the chances of capturing infected mosquitoes, and the CDC battery powered resting box traps greatly increase the number of mosquitoes captured and the consistency and ease of sampling (Panella et al. 2011). The resting populations can also be collected using backpack aspirators (e.g., modified CDC backpack aspirator <https://www.johnwhock.com/products/aspirators/modified-cdc-backpack-aspirator/>, or the lightweight battery-powered aspirator [Nasci 1981]) to remove mosquitoes from natural harborages or artificial resting structures (e.g., wooden resting boxes, red boxes, fiber pots, and other similar containers: Holderman et al. 2018).

Specimen Handling and Processing

Because mosquito-based surveillance relies on identifying virus in the collected mosquitoes through detection of viral proteins, viral ribonucleic acid (RNA), or live virus

(see **Laboratory Diagnosis and Testing**), specimens should be handled in a way that minimizes exposure to conditions (e.g., heat, successive freeze-thaw cycles) that would degrade the virus. Optimally, a cold chain should be maintained from the time mosquitoes are removed from the traps to the time they are delivered to the processing laboratory. Mosquitoes can be transported from the field in a cooler with cold packs or on dry ice, and then placed on a chill-table, if available, during sorting, identification, and pooling. Usually only female mosquitoes are tested in routine arboviral surveillance programs. If virus screening is not done immediately after mosquito identification and pooling, the pooled samples should be stored frozen (e.g., -70°C) or at temperatures below freezing for short-term storage. Although the lack of a cold chain might impact the ability to culture the virus, it does not appear to reduce the ability to detect viral RNA by reverse transcription polymerase chain reaction (RT-PCR) (Turell et al. 2002).

Vector-based Surveillance Indicators

Data derived from mosquito surveillance include estimates of mosquito species abundance and infection rate in those mosquito populations. The indices derived from those data vary in information content, ability to be compared over time and space, and association with transmission levels and levels of human risk. Five indicators that have commonly been used include: vector abundance, number of positive pools, percent of pools positive, infection rate, and vector index (Table 3.1).

Vector abundance provides a measure of the relative number of mosquitoes in an area during a particular sampling period. It is the total number of mosquitoes of a particular species collected, divided by the number of trapping nights during a specified sampling period, and is expressed as the number/trap night. Risk assessments often consider mosquito abundance because high mosquito densities can be associated with arboviral disease outbreaks (Olson et al. 1979, Eldridge 2004). For example, during a WNV outbreak in Maricopa County, Arizona in 2010, *Culex quinquefasciatus* densities were higher in outbreak compared to non-outbreak areas (Godsey et al. 2012, Colborn et al. 2013). High *Cs. melanura* and *Cq. perturbans* abundance has also been associated with elevated EEE virus activity. However, high mosquito abundance can occur in the absence of virus, and outbreaks can occur when abundance is low, but the vector infection rate is high. Vector abundance measures are used for planning IVM and monitoring the outcomes of mosquito control. Number of traps, their distribution,



and the timing of sample collection should be sufficient to obtain spatially and temporally representative data.

Number of positive pools is the total of the number of arbovirus positive mosquito pools detected in a given surveillance location and period. These may be a tally of the total positive pools separated by species or for all species tested. This indicator provides evidence of arboviral activity, particularly during field investigations and outbreak response, but is not recommended as a stand-alone indicator. Instead, data can be used to produce more informative indices (i.e., Infection Rate and Vector Index).

Percent of pools positive is calculated by dividing the number of positive pools by the total number of pools tested, expressed as a percentage. It provides a rough estimate of the rate of infection and can be used to compare activity over time and place. However, the comparative value is limited unless the number of pools tested is large and the number of mosquitoes per pool remains constant. As with the number of positive pools index, these data can be used for calculation of the, often more informative, Infection Rate and Vector Index.

The **Infection Rate** in a vector population estimates the prevalence of infected mosquitoes in the population and is a good indicator of human risk. It provides a useful, quantitative basis for comparison, allowing evaluation of changes in infection rate over time and space. Infection rate indices have been used successfully to link infection rates with human risk (Bell et al. 2005). Variable pool numbers and pool sizes can be used, while retaining comparability, but larger sample sizes improve accuracy. Two methods are commonly used to calculate infection rate:

- **Minimum Infection Rate (MIR)** for a given mosquito species is the number of positive pools divided by the total number of mosquitoes tested. MIR assumes that infection rates are low and that only one mosquito is positive in a positive pool. MIR is usually expressed as the number infected/1,000 tested. It can also be expressed as a proportion or percent positive.
- **Maximum Likelihood Estimate (MLE)** is the preferred method, particularly during outbreaks. MLE does not assume only one positive mosquito per positive pool and provides a more accurate estimate when

infection rates are high (Gu 2008). The MLE and MIR are similar when infection rates are low. The MLE requires more complex calculations than the MIR, however, a Microsoft Excel Add-In to compute infection rates from pooled data is available (<https://www.cdc.gov/mosquitoes/php/toolkit/mosquito-surveillance-software.html>).

The **Vector Index (VI)** estimates the abundance of infected mosquitoes in an area and incorporates into a single index information on presence, relative abundance, and infection rates of individual species (Gujral et al. 2007, Bolling et al. 2009, Jones et al. 2011). The VI is calculated by multiplying the average number of mosquitoes collected per trap night by the proportion infected. VI is expressed as the average number of infected mosquitoes collected per trap night in the area during the sampling period. In areas with multiple vector species, a VI is calculated for each species. Individual VIs are summed to give a combined estimate of infected vector abundance.

Increases in VI reflect increased risk of human disease and serves as a more reliable prediction measure than vector abundance or infection rate alone (Bolling et al. 2009, Jones et al. 2011, Kwan et al. 2012, Colborn et al. 2013). As with other surveillance indicators, the accuracy of the VI depends on the number of trap nights used to estimate abundance and the number of specimens tested to estimate infection rate.

Use of Vector-based Surveillance Indicators

Mosquito-based surveillance indicators have two important roles in arboviral surveillance and response programs. First, they can provide quantifiable thresholds for proactive vector control efforts and public health messaging. By identifying thresholds for vector abundance and infection rates that are below levels associated with disease outbreaks, IVM programs can institute proactive measures to maintain mosquito populations at levels below which virus amplification can occur. Second, if thresholds related to outbreak levels of transmission can be identified, surveillance can help determine when proactive measures were insufficient to dampen virus amplification and more aggressive measures, such as wide-scale aerial application of mosquito adulticides and expanded public messaging, are needed to stop an outbreak.



Table 3.1: Summary of Mosquito-based Surveillance Indicators

Index	Description	Equation
Vector Abundance	Number of mosquitoes of a particular vector species captured per trap per night	Number of a particular mosquito species captured in a night/number of traps set up that night
Number of Positive Mosquito Pools	Number of positive mosquito pools detected in a given period of time	Simple count of positive mosquito pools
Percentage of Positive Mosquito Pools	Proportion of positive mosquito pools	Number of positive mosquito pools/total number of pools tested X 100
Infection Rate	An estimate of the number of mosquitoes infected per 1,000 tested	Minimum Infection Rate (MIR) = Number of positive pools/total number of mosquitoes tested Maximum Likelihood Estimate (MLE), use links in the footnote.
Vector Index	An estimate of the abundance of infected mosquitoes in an area	\bar{N} = Number of mosquitoes per trap night for a given species \hat{P} = Estimated Infection Rate $\text{Vector Index} = \sum_{i=\text{species}} \bar{N}_i \hat{P}_i$

For MLE computations, use the mosquito surveillance software at <https://www.cdc.gov/mosquitoes/php/toolkit/mosquito-surveillance-software.html>



Animal-based Surveillance

Bird-based Surveillance

Wild birds are the primary vertebrate hosts of EEE virus and serve as the principal amplification hosts for mosquito infection. EEE epizootics precede human epidemics and in the well-established enzootic EEE virus foci, EEE antibody prevalence among wild birds ranged from 5 to 85% (Elias et al. 2017, Dalrymple et al. 1972, Stamm 1968). However, during epizootics outside the well-established enzootic EEE virus foci, similar antibody prevalence rates in local wild bird populations were observed (Hayes et al. 1962, Emord and Morris 1984, Stamm 1958, McLean et al. 1985). Some “primary” bird species, typically passerine species, show higher EEE virus reactive antibodies than other bird species and are good sentinels for routine EEE surveillance. Antibody prevalence for primary species during EEE epizootics can range from 40 to 70% (Crans et al. 1994), suggesting intense EEE virus transmission. EEE antibody prevalence in wild bird populations can decline to less than 10% after 3 consecutive non-epizootic years (Hayes et al. 1962, Emord and Morris 1984). Virus activity and antibody seroprevalence for EEE virus in local bird populations usually correlate well with the risk of human infection. Accurate monitoring of virus and antibody prevalence in wild birds should provide early warning of increased transmission that may constitute a risk to equine and human populations. Wild birds are monitored by repeated sampling of local populations to test for antibody or virus. Free-ranging adult and immature birds are captured in ground-level mist nets set at locations appropriate for the desired species. The Australian crow trap also provides an effective method for collecting birds (Tsachalidis et al. 2006). Captured birds are bled, banded, and released for possible later recapture to check for seroconversion. Recapture data also gives useful insights on movement, survival, and other population characteristics of the birds. Successful use of this technique requires a labor-intensive sampling effort because of low recapture rates. Because antibodies may persist for 2 or more years, the results from carefully identified juvenile birds may provide the most useful index of current virus activity (Smith et al. 1983). This technique requires substantial resources. In addition, it requires highly-trained personnel as well as state and federal collecting permits.

Mortality from EEE virus infection occurs in ring-necked pheasants, emus, and other exotic game bird species (Morris 1988, Saxton-Shaw et al. 2015). Some surveillance programs monitor the morbidity and mortality

in captive ring-necked pheasants as sentinels and as an indicator of EEE virus activity.

Live Bird Serology

Chicken flocks are used for West Nile (WN), western and eastern equine encephalitis, and St. Louis encephalitis (SLE) virus surveillance. Surveillance for EEE, SLE, and WN viruses can take place simultaneously to reduce costs. Like most birds, chickens are susceptible to and can tolerate EEE, SLE, and WNV infections. Chickens, especially older chickens, develop low titer viremia and, therefore, are not likely to contribute to local virus amplification. Chicken flocks can be inexpensively maintained on farms or in urban-suburban locations by residents or health officials. However, it is important to base the choice of locations for the sentinel chickens on historical records of virus activity. Spreading small groups of sentinel chickens throughout the area at risk yields more representative estimates of virus activity. Each spring, 6- to 8-week-old chickens are placed at the selected sentinel sites. Each sentinel site is stocked with 6 to 30 pretested, non-immune, individually banded chickens kept in standard sentinel sheds. Sentinel chickens are bled from the wing vein, the jugular vein, or from the heart weekly, biweekly, or monthly throughout the transmission season. Similar to wild bird surveillance, sentinel chickens were thought to be inappropriate as an early warning system for epidemic activity because the turnaround time from the field to the laboratory results was too long (Morris 1988). Currently, molecular biology-based methods such as RT-PCR and advanced serological methods such as EEE immunoglobulin M (IgM) antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) greatly shorten the turnaround time and in some locations sentinel chicken flocks may be used as early warning systems (Goodman et al. 2015). However, some studies reported failure in some locations (Crans 1986), therefore, use of sentinel chicken flocks need to be evaluated for each area.

Horses and Other Vertebrates

In areas with susceptible horse populations, surveillance for equine cases can provide a sensitive early warning system for EEE outbreaks. Horses are subject to high vector attack rates due to their field exposure. Reports by local veterinarians of equine encephalomyelitis give warning of increased arbovirus activity in an area. This can alert public health officials to investigate the



situation. Equine surveillance can be active or passive. Active surveillance requires regularly contacting large-animal veterinarians, encouraging them to report clinically suspect equine cases and to submit blood and autopsy samples for laboratory confirmation. Record sheets, containing a case history and vaccination history, must accompany samples for laboratory testing if the results are to be useful. Some limitations in using equines include EEE virus immunity from prior vaccination, movement into and out of the surveillance area, and lack of prompt reporting of morbidity or mortality by attending veterinarians.

Several studies report EEE virus antibody-positive sera among populations of free-ranging white-tailed deer, *Odocoileus virginianus*, suggesting white-tailed deer are frequently exposed to EEE virus infections (Hoff et al. 1973, Bigler et al. 1975, Tate 2005, Schmitt et al. 2007). Deer serosurveys have been utilized to study distribution ranges of EEE virus activity especially in northeastern United States (Berl et al. 2013, Mutebi et al. 2011, Mutebi et al. 2015). *Odocoileus virginianus* inhabit a geographically localized home range, often

not exceeding a 1.6 km (1 mile) radius, where they both become infected and are harvested (DeNicola et al. 2000, Marchinton and Hirth 1984). Collecting *O. virginianus* blood samples is less labor intensive because of the seasonal deer harvests, samples are collected from the carcasses when hunters bring the harvested deer to the registration station. EEE virus antibody surveillance in harvested *O. virginianus* is a potential tool for EEE surveillance and distribution mapping. Deer serosurveys may be useful for monitoring EEE virus activity but have no predictive value for human infection because deer harvesting occurs in the early fall after the EEE virus transmission season.

Similar studies have been conducted using moose and game birds in the northeastern United States (Mutebi et al. 2012, Lubelczyk et al. 2014, Elias et al. 2017). However, these studies only provide information on distribution ranges EEE virus activity and cannot be used as early warning systems.



ArboNET

ArboNET, the national arboviral surveillance system, was developed by CDC and state health departments in 2000 in response to the emergence of WNV in 1999. Since its development, ArboNET has expanded to include many other arboviruses of public health importance. ArboNET is an electronic surveillance system administered by CDC's Division of Vector-Borne Diseases (DVBD). Human arboviral disease data are reported from all states, territories, and freely associated states. In addition to human disease cases, ArboNET maintains data on arboviral infections among human viremic blood donors, non-human mammals, sentinel animals, dead birds, and mosquitoes.

Data Collected

Variables collected for human disease cases include patient age, sex, race, and county and state of residence; date of illness onset; case status (i.e., confirmed, probable, suspected, or not a case); clinical syndrome (e.g., encephalitis, meningitis, or uncomplicated fever); whether illness resulted in hospitalization; and whether the illness was fatal. Cases reported as encephalitis (including meningoencephalitis), meningitis, or acute flaccid paralysis are collectively referred to as neuroinvasive disease; others are considered non-neuroinvasive disease. Acute flaccid paralysis can occur with or without encephalitis or meningitis. Information regarding potential non-mosquito-borne transmission (e.g., blood transfusion or organ transplant recipient, breast-fed infant, or laboratory worker) and recent donation of blood or solid organs should be reported if applicable. Clinical symptoms and diagnostic testing data can also be reported.

Blood donors identified as presumptively viremic by nucleic acid amplification test (NAAT) screening of the donation by a blood collection agency are also reported to ArboNET. Case definitions have been developed for the purposes of national surveillance. The date of blood donation is reported in addition to the variables routinely reported for disease cases.

Arboviral disease in non-human mammals (primarily horses) and evidence of exposure in trapped mosquitoes, dead birds, and sentinel animals (primarily chickens) are also reported to ArboNET. Variables collected for non-human infections include species, state and county, and date of specimen collection or symptom onset.

Detailed descriptions of all variables collected by ArboNET and instructions for reporting are included in the ArboNET User Guide, which can be requested from DVBD by phone (970-261-6400) or email (dvbid2@cdc.gov).

Data Transmission

Jurisdictions can transmit data to ArboNET using one or more of four methods supported by DVBD:

1. Jurisdictions that have a commercially- or state-developed electronic surveillance system can upload records from their system using an Extensible Markup Language (XML) message;
2. Jurisdictions can upload records from a Microsoft Access database provided by CDC DVBD using an XML message;
3. Jurisdictions may enter records manually using a CDC website; or
4. Jurisdictions can report cases using an HL-7 message via the CDC National Electronic Disease Surveillance System (NEDSS), and DVBD will download records directly from NEDSS to ArboNET. ArboNET data are maintained in a Microsoft® Structured Query Language (SQL) Server® database inside CDC's firewall. Users can access data via a password-protected website but are limited to viewing data only from their own jurisdiction.

The ArboNET website and database are maintained by CDC information technology staff and are backed up nightly.

Dissemination of ArboNET Data

CDC epidemiologists periodically review and analyze ArboNET surveillance data and disseminate results to stakeholders via direct communication, briefs in Morbidity and Mortality Weekly Reports and Epi-X, comprehensive annual summary reports, and DVBD's website. CDC also produces maps of domestic and exotic arboviral activity, which are then posted on CDC's disease-specific websites. Surveillance reports are typically updated weekly during the transmission season and monthly during the off-season. A final report is usually released in the spring of the following year. CDC provides limited-use ArboNET data sets to the general public by formal request. Data release guidelines have been updated to be consistent with those developed by CDC and the Council of State and Territorial Epidemiologists (CSTE).



Limitations of ArboNET Data

Human surveillance for arboviral disease is largely passive and relies on the receipt of information from physicians, laboratories, and other reporting sources by state health departments. For viruses that can cause neuroinvasive disease, neuroinvasive disease cases are likely to be consistently reported because of the substantial morbidity associated with this clinical syndrome. In comparison, non-neuroinvasive disease cases are inconsistently reported because of a less severe spectrum of illness, geographic differences in disease awareness and healthcare-seeking behavior, and variable capacity for laboratory testing. Surveillance data for fever cases associated with neuroinvasive arboviruses should be interpreted with caution and generally should not be used to make comparisons between geographic areas or over time. Accordingly, ratios of reported neuroinvasive disease cases to non-neuroinvasive disease cases should not be interpreted as a measure of virulence in an area.

ArboNET does not routinely collect information regarding clinical signs and symptoms or diagnostic laboratory test results. Therefore, misclassification of the various syndromes caused by arboviruses cannot be detected. In addition, ArboNET does not routinely collect information regarding the specific laboratory methods used to confirm each case. Although serologic assays are relatively specific, false-positive results and cross-reactions occur between related viruses (e.g., flavivirus, such as West Nile, SLE, and dengue viruses, or California serogroup viruses,

such as La Crosse and Jamestown Canyon viruses). Positive IgM antibody results should be confirmed by additional tests, especially plaque-reduction neutralization test (PRNT). However, such confirmatory testing often is not performed. While the electronic mechanisms for data transmission allow for rapid case reporting, the inclusion of both clinical and laboratory criteria in the surveillance case definition creates delays between the occurrence of cases and their reporting. Provisional data are disseminated to allow for monitoring of regional and national epidemiology during the arboviral transmission season. However, these reports generally lag several weeks behind the occurrence of the cases comprising them, and the data may change substantially before they are finalized. For this reason, provisional data from the current transmission season should not be combined with or compared to provisional or final data from previous years.

The collection and reporting of non-human surveillance data are highly variable among states (and even between regions within states) and changes from year to year. Because of this variability, non-human surveillance data should not be used to compare arboviral activity between geographic areas or over time.

For more information about ArboNET, please contact the Division of Vector-Borne Diseases by phone: 970-261-6400 or email: dvbid2@cdc.gov.



Laboratory Diagnosis and Testing

Laboratory Diagnosis of Human Arboviral Diseases

Laboratory testing for evidence of arboviral diseases typically involves serologic and molecular testing. For several viruses where humans are an amplification host, molecular testing is more specific and can be used to confirm the diagnosis in the first week of illness. For viruses that typically are neuroinvasive, serology is more likely to be used to determine if someone was recently infected.

In most patients, infection with an arbovirus that can cause encephalitis is clinically inapparent or causes a nonspecific viral syndrome. Numerous pathogens cause encephalitis, aseptic meningitis, and febrile disease with similar clinical symptoms and presentations and should be considered in the differential diagnosis. Definitive diagnosis can only be made by laboratory testing using specific reagents. Selection of diagnostic test procedures should take into consideration patient factors (e.g., age, immune status, vaccination history), timing of infection, the range of pathogens in the differential diagnosis, the criteria for classifying a case as confirmed or probable, as well as the capability of the primary and confirming diagnostic laboratories.

Appropriate selection of diagnostic procedures and accurate interpretation of findings requires information

describing the patient and the diagnostic specimen. For human specimens, the following data must accompany sera, CSF or tissue specimens for results to be properly interpreted and reported:

1. Symptom onset date (when known);
2. Date of sample collection;
3. Unusual immunological status of patient (e.g., immunosuppression);
4. State and county of residence;
5. Travel history (especially in arbovirus-endemic areas);
6. History of prior vaccination (e.g., yellow fever, Japanese encephalitis, or tick-borne encephalitis viruses); and
7. Brief clinical summary including clinical diagnosis (e.g., encephalitis, aseptic meningitis).

Minimally, onset and sample collection dates are required to perform and interpret initial screening tests. The remaining information is required to evaluate any test results from initial screening. If possible, a convalescent serum sample taken at least 14 days following the acute sample should be obtained to enable confirmation by serological testing.

Human Diagnostic Testing

EEE virus is a Health and Human Services (HHS) Select Agent (<https://www.selectagents.gov/SelectAgentsandToxinsList.html>), and therefore, subject to strict regulations regarding its possession and use. Those intending to conduct EEE virus testing must be familiar with the complete information and specific guidance found at the Federal Select Agent Program (<https://www.selectagents.gov/index.html>) website **before** conducting EEE virus testing.

Briefly, samples determined to be positive for EEE virus must be documented and reported to the Federal Select Agent Program via Form 4 (<https://www.selectagents.gov/form4.html>) within 7 calendar days of identification, and, if not diagnosed at a registered entity, they must then be transferred to a registered Select Agent facility or destroyed. Documentation of these activities must be maintained.

Serology

The front-line diagnostic assay for laboratory diagnosis of human EEE virus infection is the IgM antibody assay. Commercially available immunofluorescence assay (IFA) kits to detect IgM or immunoglobulin G (IgG) antibodies

are often used in public health and other laboratories the United States. In addition, IgM and IgG assays developed at CDC are available in both ELISA and microsphere (IgM) immunoassay (MIA) (Basile et al. 2013) formats; protocols and limited supplies of reagents are available from CDC's DVBD Diagnostic Laboratory. CDC will provide positive controls and limited reagents considering commercial sources are available to state public health labs.

Because the IgM and IgG assays can be positive due to non-specific reactivity or rarely cross-reactivity (e.g., EEE virus is the only virus in the EEE antigenic complex in the United States, but low-level cross-reactivity might occur with other alphaviruses), they should be viewed as a presumptive positive. For a case to be considered confirmed, samples that are antibody-positive on initial testing should be evaluated by a more specific assay. Currently, the PRNT is recommended for confirming IgM serological results. Although EEE virus is a rare cause of arboviral encephalitis in the United States, several other arboviral encephalitides are present in the United States and in other regions of the world. Specimens submitted for



EEE virus testing should also be tested by ELISA and PRNT against other arboviruses known to be active or present in the area or in the region to where the patient traveled.

Virus Detection Assays

Numerous procedures have been developed for detecting viable EEE virus, EEE virus antigen, or EEE virus RNA in human diagnostic samples, many of which have been adapted to detecting EEE virus in other vertebrates and in mosquito samples. These procedures vary in their sensitivity, specificity, and time required to conduct the test. Among the most sensitive procedures for detecting EEE virus in samples are those using RT-PCR to detect EEE virus RNA in human CSF, serum, and other tissues. Real-time RT-PCR, standard RT-PCR, and nucleic acid sequence-based amplification (NASBA) amplification methods have been developed and validated for specific human diagnostic applications (Lambert et al. 2003); however, no commercially-produced or Food and Drug Administration (FDA)-approved molecular EEE virus diagnostic tests are available.

EEE virus presence can be demonstrated by isolation of viable virus from samples taken from clinically ill

patients. Appropriate samples include CSF, serum samples obtained very early in infection, and brain tissue taken at biopsy or postmortem. Virus isolation should be performed in known susceptible mammalian (e.g., Vero) or mosquito cell lines (e.g., C6/36). Mosquito origin cells may not show obvious cytopathic effect and must be screened by immunofluorescence or RT-PCR. Confirmation of virus isolate identity can be accomplished by indirect IFA using virus-specific monoclonal antibodies (MAbs) or nucleic acid detection (e.g., RT-PCR, real-time RT-PCR, or sequencing). The IFA using well-defined murine MAbs is an efficient, economical, and rapid method to identify alphaviruses isolated in cell culture. Incorporating MAbs specific for other arboviruses known to circulate in various regions will increase the rapid diagnostic capacities of state and local laboratories.

Immunohistochemistry (IHC) using virus-specific MAbs on tissue has been useful in identifying both human and veterinary cases of EEE virus infection. In suspected fatal cases, IHC should be performed on formalin fixed autopsy, biopsy, and necropsy material, ideally collected from multiple anatomic regions of the brain, including the brainstem, midbrain, and cortex.

Resources for Human Diagnostic Laboratories

Clinical Laboratory Improvements Amendments

(CLIA) certification: To maintain certification, CLIA recommendations for performing and interpreting human diagnostic tests should be followed. Laboratories performing arboviral serology or RNA-detection testing are invited to participate in the annual proficiency testing that is available from CDC's DVBD in Fort Collins, CO. To obtain additional information about the proficiency testing program and about training in arbovirus diagnostic procedures, contact the DVBD by phone: 970-261-6400 or email: dvbid2@cdc.gov.

Biocontainment: Containment specifications are available in the CDC/National Institutes of Health publication

Biosafety in Microbiological and Biomedical Laboratories (BMBL 6). This document can be found online at: <https://www.cdc.gov/labs/bmbl/>.

Shipping of diagnostic samples and agents. Shipping and transport of clinical specimens should follow current International Air Transport Association (IATA) and Department of Commerce recommendations. For more information, visit the IATA dangerous goods Web site at: <http://www.iata.org/publications/dgr/Pages/index.aspx>, and the USDA Animal and Plant Health. Inspection Service (APHIS), National Center for Imports and Exports website: <https://www.aphis.usda.gov/aphis/ourfocus/importexport>.

Non-human Laboratory Diagnosis

Identification and Pooling

Mosquitoes should be identified to species or lowest taxonomic unit. Specimens are placed into pools of 50 specimens or less based on species, sex, location, trap-type, and date of collection. If resources are limited, testing of mosquitoes for surveillance purposes can be limited to the primary vector species.

Homogenizing and Centrifugation

After adding the appropriate media, mosquito pools can be macerated or ground by a variety of techniques including mortar and pestle, vortexing sealed tubes containing one or more copper clad BBs, or by use of tissue homogenizing apparatuses that are commercially available. After grinding, samples are centrifuged, and an aliquot is removed for testing. Because mosquito pools may contain arboviruses and other pathogenic viruses which may be



aerosolized during processing, laboratory staff should take appropriate safety precautions including use of a Class II Type A biological safety cabinet, appropriate personal protective equipment (PPE) and biosafety practices.

Virus Detection

EEE virus is an HHS Select Agent, and therefore, subject to strict regulations regarding its possession and use. Those intending to conduct EEE virus testing must be familiar with the complete information and specific guidance found at the Federal Select Agent Program (<https://www.selectagents.gov/index.html>) website **before** conducting EEE virus testing.

Briefly, samples determined to be positive for EEE virus must be documented and reported to the Federal Select Agent Program via Form 4 (<https://www.selectagents.gov/form4.html>) within 7 calendar days of identification, and, if not diagnosed at a registered entity, they must then be transferred to a registered Select Agent facility or destroyed.

Multiple diagnostic methods can be used to identify EEE virus in mosquitoes including virus isolation, molecular assays, or immunoassays. Virus isolation in Vero cell culture (Armstrong et al. 2011) remains the standard for confirmation of positive pools but is time consuming and requires specialized laboratory facilities. For virus isolation, mosquito pool homogenates are added to Vero cell cultures, monitored for cytopathic effect, and identified using appropriate diagnostic assays. Aliquots are stored at -70°C to retain virus viability for future testing. Vero cell culture has an additional benefit of detecting other viruses in the mosquitoes, a feature lost using test procedures that target virus-specific nucleotide sequence or proteins (Andreadis et al. 2004, Andreadis et al. 1998). Molecular assays detect viral RNA or nucleic acids in mosquito pools quickly, with high sensitivity and specificity, and do not require cold chain or high levels of biocontainment (Lanciotti et al. 2000, Callahan et al. 2001, Lambert et al. 2003, Armstrong et al. 2012). Methods include rapid molecular assays, RT-PCR, and other nucleic acid amplification tests (e.g., multi-target RT-PCR). Assays may use different primer sets for detection and confirmation of virus in mosquito pools (Lambert et al. 2003). Antigen detection assays are less sensitive than molecular assays, but for some arboviruses (e.g., WN) can provide valuable infection rate data when employed consistently in a mosquito surveillance program. For additional details see: **Human Diagnostic Testing** and **Appendix 1**.

Laboratory Testing of Non-human Vertebrates

The choice of laboratory diagnostic tests depends on the needs, approach, and surveillance capability of a given health agency. Tests include antibody-capture ELISA, complement fixation (CF), hemagglutination inhibition (HI), and plaque reduction neutralization test (PRNT). However, few reagents are commercially available for domestic or wildlife IgM antibodies for antibody capture ELISAs. Many public health laboratories will therefore use PRNT because these are not dependent on species specific antibodies.

Serology

The same serologic techniques applied to clinically ill animals may also be used for healthy subjects for vertebrate serosurveys or for healthy sentinel animals serially-sampled as sentinels. As with human diagnostic samples, serologic results from non-human vertebrates must be interpreted with caution due to potential cross-reactivity. Cross reactions may occur between EEE and WEE antibodies in the CF and HI tests. Vaccination for EEE can also cause positive PRNT, HI, CF, and possibly IgM test results.

Specimens from horses and other domestic animals can be tested through diagnostic laboratories including U.S. Department of Agriculture's (USDA) Animal and Plant Health Inspection Service's (APHIS) National Veterinary Services Laboratory (NVSL) in Ames, Iowa. Testing can take up to several weeks to complete depending upon the type of sample submitted and the testing protocol required to obtain a definitive result. Details on the diagnostic criteria for EEE for can be found at: <https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/equine/eee-wee-vee/equine-encephalitis>. For additional details see: **Human Diagnostic Testing**.

Virus Detection

Methods for virus detection, isolation, and identification are the same as described for human and mosquito diagnostics. The most commonly used methods to detect EEE virus or viral RNA in animal populations are immunoassays, virus isolation, and molecular tests. Specimens typically are tissues or fluids from acutely ill or dead animals. Virus detection in apparently healthy animals is very low-yield and inefficient, and therefore not cost-effective, and should not be considered for routine surveillance programs. Some animals have few tissues with detectable virus particles or viral RNA at necropsy, such as horses. Others, such as certain bird species, may have fulminant infections with high viral loads in almost every tissue.



Prevention and Control

Integrated Vector Management

Prevention and control of arboviral diseases is accomplished most effectively through a comprehensive, IVM program applying the principles of Integrated Pest Management. IVM is based on an understanding of the underlying biology of the arbovirus transmission system and utilizes regular monitoring of vector mosquito populations and arboviral activity levels to determine if, when, and where interventions are needed to keep mosquito numbers below levels which produce risk of human disease, and to respond appropriately to reduce risk when it exceeds acceptable levels.

Operationally, IVM is anchored by a monitoring program providing data that describe:

- Conditions and habitats that produce vector mosquitoes;
- Abundance of vector mosquitoes over the course of a season;
- Arboviral transmission activity levels expressed as infection rate in mosquito vectors;
- Parameters that influence local mosquito populations and virus transmission.

These data inform decisions about implementing mosquito control activities appropriate to the situation, such as:

- Source reduction through habitat modification;
- Larval mosquito control using the appropriate methods for the habitat;

- Adult mosquito control using insecticides applied from trucks or aircraft when established thresholds have been exceeded;
- Community education efforts related to risk levels and intervention activities.

Monitoring also provides quality control for the program, allowing evaluation of the effectiveness of larval and adult control efforts, and causes of control failures (e.g., undetected larval sources, insecticide resistance, equipment failure).

Mosquito Control Activities

Mosquito control tools target mosquitoes at the adult or immature stage depending on program objectives (Table 5.1). Multiple species are involved in EEE virus transmission, and different populations of a single species may vary their activity based on environmental conditions. The decision to conduct mosquito control activities is based on mosquito and meteorological surveillance, established local thresholds and triggers (mosquito, human, and non-human animal), and insecticide resistance status of the target species. Mosquito control professionals should have detailed knowledge of the local mosquitoes involved in EEE virus transmission to prevent and control disease. Programs should use insecticides and other control tools registered by the U.S. Environmental Protection Agency (EPA) (<https://www.epa.gov/mosquitocontrol>) in compliance with label instructions and any local, state, and federal laws regulating their use.

Table 5.1. Summary of Mosquito Control by Life Stage, Method, and Objective

Life stage	Method	Objective	Example	Notes
Larvae	Environmental management (modification/manipulation)	Eliminate or disrupt larval aquatic habitats to reduce adult mosquitoes	<ul style="list-style-type: none"> ■ Wetland management ■ Biological control 	May be prohibited or logistically unfeasible
	Larvicide application to aquatic habitats	Kill immature mosquitoes to reduce adult populations	<ul style="list-style-type: none"> ■ Insect growth regulators (e.g., pyriproxyfen, methoprene) ■ Microbial products (e.g., <i>Bacillus thuringiensis</i> subspecies <i>israelensis</i> (Bti)/<i>Lysinibacillus sphaericus</i>/Spinosad) ■ Oils and films 	Limited data on efficacy. <i>Culiseta melanura</i> habitats may be inaccessible by ground/aerial application

Continued



Life stage	Method	Objective	Example	Notes
Adult	Ultra-low volume (ULV) insecticides	Reduce the adult mosquito population active at the time of treatment	■ Space Spray	Targets mosquitoes active at the time of application
	Residual adulticides	Residual control of mosquitoes	■ Residual treatments to surfaces ■ Barrier treatments to vegetation	Targets resting mosquitoes

Larval Mosquito Control

The objective of larval mosquito control is to reduce immature mosquito populations before they emerge as adults. This can be an efficient method of managing mosquitoes where larval sites are accessible, but habitats of EEE virus vectors are often hard to find and labor-intensive to treat. Few studies have shown efficacy of larval control methods against EEE virus vectors.

Culiseta melanura larvae develop in crypts filled with water in swamp and bog habitats. A single study showed aerial application of methoprene penetrated larval crypts and had 81% efficacy (emergence inhibition) over 5-weeks post-treatment (Woodrow et al. 1995). Temephos was also evaluated and not detected in the larval habitats (crypts) of *Cs. melanura*. Although not evaluated yet for EEE, aerial or ULV *Bacillus thuringiensis israelensis* (Bti) water-dispersible granules can penetrate foliage and water in covered areas to control other mosquitoes that occur in cryptic larval habitats (e.g., *Aedes aegypti*, *Cx. quinquefasciatus*) (Pruszynski et al. 2017). These delivery techniques may be also useful against *Cs. melanura* and the larval habitats of epizootic bridge vectors. Although further studies are needed on the efficacy and implementation of larval control of EEE virus, applying a larvicide at the same time as an adulticide application to reduce adult mosquito populations may help prevent population rebound due to newly emerged adults and mosquitoes not active at the time of application.

Larvicides (and pupacides) are applied directly to water sources or placed in areas where flooding is expected to target the aquatic habitats of vector species. Larvicide can be applied by ground or aerial dispersal methods. For small aquatic larval sites or areas that cannot be reached by vehicles, backpack sprayers and dusters are used to

apply liquid, granules, or pellets. Formulations can be short-acting (up to 2 weeks) or extended-release products (lasting more than 1 month). Larvicides may kill on contact through ingestion, or act as stomach poisons or growth regulators. Information on insecticides for larval mosquito control is available from the U.S. EPA (<https://www.epa.gov/mosquitocontrol/controlling-mosquitoes-larval-stage>).

Adult Mosquito Control

Adult mosquito control aims to reduce the abundance of biting, infected adult mosquitoes to prevent them from transmitting arboviruses to humans and to break the mosquito-host transmission cycle. Where populations are increasing above acceptable levels, adulticides are used to reduce vectors. Vector mitigation strategies should be applied quickly once arboviral activity is detected and be targeted to the local EEE virus epizootic and enzootic vectors. Programs should use insecticides registered by EPA for this purpose (<https://www.epa.gov/mosquitocontrol/controlling-adult-mosquitoes>).

Adulticides can reduce the numbers of adult mosquito vectors for EEE virus, but not enough cases occur annually to demonstrate clear impact on EEE virus transmission to humans. Indicators of high transmission risk are used to decide when to apply adulticides and often by the time aerial applications occur, transmission to humans has already occurred. Also, due to the epidemic nature of this disease, untreated areas relevant for comparison might not be available, which limits the ability to make conclusions about the efficacy of using adulticides to reduce disease (Grady et al. 1978).

Adulticiding can be conducted from the ground with backpack spray equipment, truck-mounted equipment, or



by air with fixed-wing or rotary-wing applications. Types of treatment include space-spray (e.g., ULV) adulticides and residual treatments.

- Space-spray and ULV treatments rely on mosquitoes and insecticide droplets coming into direct contact in the air column. These are temporary measures to reduce the mosquito population active at the time of treatment (FCCMC 2018). ULV formulations applied in small volumes prevent deposition and enhance degradation of the active ingredients in the environment (Bonds 2012). Mosquitoes not active at the time of application are not exposed. Because there is little to no deposition of insecticide, no residual control of mosquitoes occurs. As a result, multiple applications may be needed for sustained control (Andis et al. 1987).
- Long-lasting adulticides, also called residual or barrier treatments, can be applied to surfaces and to vegetation. To be effective, the mosquito must land on the treated surface and directly contact the insecticide. This type of application targets the resting mosquito population and is typically used in urban pest management and residential properties (FCCMC 2018).

Vector Management in Public Health Emergencies

Intensive early season adult mosquito control efforts have been shown to decrease WN viral transmission activity and result in reduced human risk (Lothrop et al. 2008). However, depending on local conditions, proactive vector management may not maintain mosquito populations at levels sufficiently low to avoid development of outbreaks. As evidence of sustained or intensified virus transmission in a region increases, emergency vector control efforts to reduce the abundance of infected, biting adult mosquitoes must be implemented. This is particularly important in areas where vector surveillance indicates that infection rates in mosquitoes are continually increasing or being sustained at high levels and evidence of infection found in other species (e.g., human or non-human mammal cases). Delaying adulticide applications until numerous human cases occur negates the value and purpose of the surveillance system.

Safety and Quality of Vector Control Insecticides and Practices

Insecticides to control larval and adult mosquitoes are registered specifically for that use by the EPA. Instructions provided on the product labels prescribe the required application and use parameters and must be carefully followed. Properly applied, these products do not negatively affect human health or the environment. In persons living in treated areas, ULV application of mosquito control adulticides does not produce any detectable biological changes indicating exposure or increase asthma or other adverse health events (Currier et al. 2005, Duprey et al. 2008, Karpati et al. 2004). The morbidity and mortality from arboviruses demonstrably exceed the risks from mosquito control practices (Davis and Peterson 2008, Macedo et al. 2010, Peterson et al. 2006).

Legal Action to Achieve Access or Control

Individually owned private properties may be major sources of mosquito production. Examples include accumulations of discarded tires or other trash, neglected swimming pools, and similar water features that become stagnant and produce mosquitoes. Local public health statutes or public nuisance regulations may be employed to gain access for surveillance and control or to require the property owner to mitigate the problem. Executing such legal actions may be a prolonged process during which adult mosquitoes are continuously produced. Proactive communication with residents and public education programs may alleviate the need to use legal actions. However, legal efforts may be required to eliminate persistent mosquito production sites.

Quality of Control

Insecticide products and application procedures (for both larval and adult control) must periodically be evaluated to ensure an effective rate of application is being used and that the desired degree of control is obtained. Application procedures should be evaluated regularly (minimally once each season) to assure equipment is functioning properly to deliver the correct dosages and droplet parameters and to determine appropriate label rates to use locally. Finally, mosquito populations should routinely be evaluated to ensure insecticide resistance is not emerging.



Records

Surveillance data describing vector sources, abundance and infection rates, records of control efforts (e.g., source reduction, larvicide applications, adulticide applications), and quality control data must be maintained and used to evaluate IVM needs and performance. Long-term data are essential to track trends and to evaluate levels of risk.

Insecticide Resistance Management

For vector control to be effective, mosquitoes must be susceptible to the insecticide selected for use. In order to delay or prevent the development of insecticide resistance in vector populations, IVM programs should include a resistance management component (FCCMC 2018). This should include routine monitoring of the status of resistance in the target populations to:

- Provide baseline data for program planning and insecticide selection before the start of control operations;
- Detect resistance at an early stage so that timely management can be implemented;
- Continuously monitor the effect of control strategies on insecticide resistance, and determine potential causes for control failures, should they occur.

Insecticide resistance may be monitored using bioassays in larvae or adult mosquitoes (Brogden and McAllister 1998). The CDC bottle bioassay is a simple, rapid, and economical tool to detect insecticide resistance by determining the time taken for an insecticide active ingredient to kill mosquito vectors. The results can help guide the choice of insecticide used for spraying. The CDC bottle bioassay can be used as part of a broader insecticide resistance monitoring program, which may include field cage tests and biochemical and molecular methods. A practical laboratory manual for the CDC bottle bioassay is available online <https://www.cdc.gov/mosquitoes/php/toolkit/cdc-bottle-bioassay.html>. For additional information, contact CDC at USBottleAssayKit@cdc.gov.

The IVM program should include options for managing resistance that are appropriate for local conditions. The techniques regularly used include the following:

- **Management by moderation.** Prevent onset of insecticide resistance by reducing overall chemical use or persistence by:
 - Using doses no lower than the lowest label rate to avoid genetic selection;

- Using chemicals of short environmental persistence and avoiding slow-release formulations that increase selection for resistance;
- Avoiding use of the same class of insecticide to control adult and immature stages;
- Applying locally; many districts treat only hot spots and use area-wide treatments only during public health alerts or outbreaks;
- Using less frequent applications; leaving generations, population segments, or areas untreated (when appropriate);
- Establishing higher thresholds for mosquito mitigation with insecticides, except during public health alerts or outbreaks.

- **Management by continued suppression.** This strategy is used in regions of high value or persistent high risk (e.g., heavily populated regions or locations with recurring outbreaks) where mosquitoes must be kept at very low densities. It involves the application of dosages within label rates but sufficiently high to be lethal to heterozygous mosquitoes that are partially resistant. If the heterozygous mosquitoes are killed, resistance will be slow to emerge. This method should not be used if any significant portion of the population in question is fully resistant. Another approach more commonly used is the addition of synergists that inhibit existing detoxification enzymes and thus eliminate the competitive advantage of these individuals. Commonly, the synergist of choice in mosquito control is piperonyl butoxide (PBO).
- **Management by multiple methodologies.** This strategy involves the use of insecticides with different modes of action in mixtures or in rotations. There are economic limitations associated with this approach (e.g., costs and logistics of switching or storing chemicals), and critical variables in addition to the insecticide mode of action that must be taken into consideration (i.e., mode of resistance inheritance, frequency of mutations, population dynamics of the target species, availability of refuges, and migration). Programs should evaluate resistance patterns routinely and the need for rotating insecticides at annual intervals.

Continuing Education

Continuing education for operational vector control workers is required to instill or refresh knowledge related to practical mosquito control. Training focusses on safety,



applied technology, and requirements for the regulated certification program mandated by most states. Training should also include information on the identification of mosquito species, their behavior, ecology, and appropriate methods of control.

Guidelines for a Phased Response

The objective of a phased response to EEE surveillance data is to implement public health interventions appropriate to the level of risk in a community (Table 5.2).

A surveillance program adequate to monitor EEE virus activity levels associated with human risk must be in place to detect epizootic transmission in advance of human disease outbreaks. Human case reports lag behind human infection events and are poor indicators of current risk levels. Effective public health action depends on interpreting the best available surveillance data and initiating prompt and aggressive intervention when necessary.

Table 5.2. Recommendations for a Phased Response to EEE Surveillance Data

Category	Probability of outbreak	Definition	Recommended response
0	Negligible or none	Off-season; adult vectors inactive; climate unsuitable	None required; may pursue source reduction and public education activities
1	Remote	Spring, summer, or fall; adult vectors active but not abundant; ambient temperature not satisfactory for viral development in vectors	Source reduction; use larvicides at specific sources identified by entomologic survey; maintain vector and virus surveillance
2	Possible	Focal abundance of adult vectors; temperature adequate for extrinsic incubation; seroconversion in sentinel hosts	Response from category 1, plus: increase larvicide use in/near urban areas; initiate selective adulticide use; increase vector and virus surveillance
3	Probable	Abundant adult vectors in most areas; multiple virus isolations from enzootic hosts or a confirmed human or equine case; optimal conditions for extrinsic incubation and vector survival; these phenomena occur early in the normal season for viral activity	Implement emergency control contingency plan: Response in category 2 plus, adulticiding in high-risk areas; expand public information program (use of repellents, personal protection, avoidance of high vector contact areas); initiate active hospital surveillance for human cases



Community Engagement

Individual-Level Actions to Reduce Risk

Without an effective vaccine for people, the best way to prevent mosquito-borne disease is by preventing mosquito bites. This can be accomplished through community-based IVM programs and by personal protection behaviors, such as:

- **Mosquito-avoidance.** Health officials may recommend residents avoid outdoor activities when high virus activity levels have been detected or when mosquitoes are most active.
- **Use of personal insect repellents.** CDC recommends using EPA-registered insect repellents or covering up with long-sleeved shirts and long pants when outside.
- **Removal of residential mosquito sources.** Once a week, residents should empty, cover, or throw out items that hold water, such as tires, buckets, planters, toys, pools, birdbaths, flowerpots, or trash containers.

Jurisdictions can promote individual and community-based prevention measures through public education and risk communication activities. Messages should acknowledge the seriousness of the disease without promoting undue fear or panic in the target population. Fear-driven messages may heighten the powerlessness people express in dealing with vector-borne diseases. Messages should be clear and consistent with the recommendations of coordinating agencies and include a call to action. Use plain language and adapt materials for lower literacy and non-English speaking audiences.

People can further reduce their risk of mosquito bites by not going outdoors when mosquitoes are biting and recommendations to avoid outdoor activity when and where high virus activity levels have been detected are a component of prevention programs. Recommendations to avoid being outdoors during peak mosquito biting times may conflict with neighborhood social patterns, community events, people's jobs or the practices of persons without air-conditioning. It is important to communicate when the important mosquito vectors are most active. Emphasize that insect repellent use is protective and should be used when outdoors, particularly during the prime mosquito-biting hours.

Additional information about personal insect repellents, including permethrin, can be found at <https://www.cdc.gov/mosquitoes/prevention/index.html>.

Information for individuals on control mosquitoes around their home can be found at <https://www.cdc.gov/mosquitoes/mosquito-control/mosquito-control-at-home.html>.

Prevention Strategies for High-Risk Groups

Audience members have different disease-related concerns and motivations for action. Proper message targeting (including use of plain language) permits better use of limited communication and prevention resources. The following are some population segments that require specific targeting. See **Human Disease** for additional information about risk groups that should be targeted.

Persons with Outdoor Exposure. People who engage in extensive outdoor work or recreational activities are at greater risk of being bitten by mosquitoes. Messages for these individuals should encourage use of insect repellent and long-sleeved shirts and pants. Local spokespersons (e.g., union officials, job-site supervisors, golf pros, sports organizations, lawn care professionals, public works officials, gardening experts) may be useful collaborators. Place messages in locations where people engage in outdoor activities (e.g., parks, golf courses, hiking trails).

People Experiencing Homelessness. Extensive outdoor exposure and limited financial resources in this group present special challenges. Application of insect repellents to exposed skin and clothing may be most appropriate prevention measures for this population. Work with social service groups in your area to educate and provide insect repellents to this population segment.

Residences Lacking Window and Door Screens. The absence of intact window/door screens might increase exposure to mosquito bites. Encourage residents to consistently use screened windows and doors to keep mosquitoes outside. Focus attention on the need to repair screens and provide access to resources to do so. Partner with community organizations that can assist older persons or others with financial or physical barriers to screen installation or repair.

Older Adults. For many mosquito-borne diseases, older adults are at greater risk for serious disease. Messages on mosquito avoidance, insect repellent use, and weekly removal of standing water where mosquitoes lay eggs around the home should be shared with this audience.



Communication and Community Engagement

At the community level, advocating for organized mosquito abatement and participating in community mobilization projects to address sources of mosquitoes such as trash, standing water, or untreated swimming pools are activities that can help protect individuals and at-risk groups.

Providing clear messages and understandable concepts promotes community understanding and acceptance. The following provides a description of selected best practices for reaching high-risk groups, offers suggestions for cultivating partnerships with media and communities, and provides select outreach measures for mobilizing communities.

Communicating About Vector Control. Public understanding and acceptance of emergency adult mosquito control operations using insecticides is critical to its success, especially where these measures are unfamiliar. Questions about the products being used, their safety, and their effects on the environment are common. Improved communication about surveillance and how decisions to use mosquito adulticides are made may help residents weigh the risks and benefits of control. When possible, provide detailed information regarding the schedule for adulticiding through newspapers, radio, government-access television, the internet, recorded phone messages, social media, or other means your agency uses to successfully communicate with its constituencies.

Community Mobilization and Outreach. Community mobilization can improve education and help achieve behavior change goals. Promote the concept that health departments and mosquito control programs require community assistance to reduce mosquito-borne disease risk. Leverage online platforms to further disseminate messages.

A community task force that addresses civic, business, public health, and environmental concerns can be valuable in achieving buy-in from various segments of the community, and in developing common messages. Community mobilization activities can include clean-up days to get rid of mosquito habitats (e.g., tires, trash). Effective community outreach also involves presenting messages in person, involving citizens in prevention and control activities, and using traditional and social media outreach. Hearing the message of personal prevention from community leaders can validate the importance of the disease and serve as a community call to action. Health promotion events and activities

reinforce the importance of prevention and control in a community setting.

Partnership with Media and the Community. It can be beneficial to cultivate relationships with the media (e.g., radio, TV, newspaper, web-based news outlets) prior to an outbreak. Obtain media training for at least one staff member and designate that individual as the organization's spokesperson. Develop key messages and a communication plan, including press releases, prior to developing products. Many communities have heard mosquito prevention and control messages repeated for several years. Getting the public's attention when risk levels increase can be a challenge. Therefore, evaluate and update mosquito bite prevention messages annually and test new messages with different population segments to evaluate effectiveness. Develop partnerships with agencies and organizations that have relationships with populations at higher risk (such as persons over 50 years of age) or are recognized as community leaders (e.g., churches, service groups). Working through sources trusted by the priority audience can heighten the credibility of and attention to messages. Partnerships with businesses that sell materials to fix or install window screens or that sell insect repellent may be useful in some settings (e.g., local hardware stores, grocery stores).

Social Media. A majority of Americans use social media which can be an inexpensive and rapid method for disseminating information to the community. Outreach can be conducted using X, Facebook, YouTube, blogs, and other websites that may reach constituents less connected to more traditional media sources. Using images or videos in your posts make them more attention grabbing. It is also best practice to include a call-to-action people can take. Provide links that direct users to webpages or other resources with more complete information.

Online Resources. The Internet has become a primary source of health information for most Americans. Encourage constituents to seek advice from credible sources. Make sure local public health agency websites are clear, accurate, and up to date. Useful information is available from a number of resources:

- The CDC web pages are updated frequently to reflect new data, findings, and recommendations. Materials on the CDC web site are in the public domain and serve as a resource for state and local health departments and other organizations.



- CDC staff can provide technical assistance in the development of audience research and strategies for public education and community outreach. Contact CDC, Division of Vector-Borne Diseases' health communications staff in Fort Collins, CO at 970-221-6400.
- The EPA is the government's regulatory agency for insecticide and insect repellent use, safety, and effectiveness. Information about mosquito control insecticides and repellents is available at <https://www.epa.gov/mosquitocontrol>. These include guidance for using insect repellents safely and a search tool to assist in finding a repellent (<https://www.epa.gov/insect-repellents/find-repellent-right-you#searchform>), which

allows the user to examine the protection time afforded by registered insect repellents containing various concentrations of the active ingredients.

- There are several non-governmental organizations that have developed useful tools and information that can be adapted for local needs. Examples include: the American Mosquito Control Association (www.mosquito.org/) and the National Pesticide Information Center (NPIC) (www.npic.orst.edu).



Appendix 1. EEE Virus Testing for Mosquito Pools (Real-Time RT-PCR)

Before conducting any EEE virus testing, note: EEE virus is an HHS Select Agent (<https://www.selectagents.gov/SelectAgentsandToxinsList.html>), and therefore, subject to strict regulations regarding its possession and use. Those intending to conduct EEE virus testing must be familiar with the complete information and specific guidance found at the Federal Select Agent Program (<https://www.selectagents.gov/index.html>) website **before** conducting EEE virus testing.

Briefly, samples determined to be positive for EEE virus must be documented and reported to the Federal Select Agent Program via Form 4 (<https://www.selectagents.gov/form4.html>) within 7 calendar days of identification, and they must then be transferred to a registered Select Agent facility or destroyed.

Testing Algorithm. All samples are screened for virus using either or both sets of the primers/probes listed below. A positive result in any of the negative controls invalidates the entire run. Failure of the positive control to generate a positive result also invalidates the entire run. A sample that is positive with one primer set and negative with the second set is classified as equivocal.

Note: At the CDC, Division of Vector-borne Diseases, Arboviral Disease Branch, the kits and protocols used by the Entomology and Ecology Team are described below; however, there are several other options for RNA extraction and real-time RT-PCR on the market.

Results Interpretation

The following algorithm is used to evaluate the results.

Positive: Ct value \leq 37
Negative: Ct value $>$ 37

PCR Plate Set-up:

1. Prepare primers and probes according to the following concentrations:
 - Primers: 100 μ M in nuclease-free water
 - Probes: 25 μ M in TE buffer
2. Real-time RT-PCR master mix should be prepared in a “clean room” physically separated from all other laboratory activities with dedicated reagents and equipment (i.e., pipettes). Combine the reagents listed below in an RNase free centrifuge tube on ice. Using

Qiagen’s Quantitect Probe RT-PCR kit (#204443), prepare master mix as follows:

Per reaction:

- 25.0 μ l master-mix
- 18.2 μ l water* (nuclease-free)
- 0.5 μ l 100 μ M forward primer
- 0.5 μ l 100 μ M reverse primer
- 0.3 μ l 25 μ M probe
- 0.5 μ l RT enzyme

Add about 5-10 reactions to your total number of samples (and account for “No template controls” (NTCs), positive controls, and negative extraction controls) and multiply number by volumes above.

Example: You have 20 samples (12 unknown samples, 2 positive controls, 2 negative controls, and 4 NTCs). Make a master mix for 25 to 30 samples.

- NTC = mix ONLY with no sample, to test mix components (PCR control)
- Negative control = extracted water (extraction control)

3. Pipette **45 μ l of master mix*** into either 0.2 ml optical (specifically for real-time assays; emission fluorescence is read through the cap) PCR tubes or a 96-well optical PCR plate. Use a reservoir and a multichannel pipette for many wells.
4. Pipette **5 μ l of RNA*** into each well. Refer to a template to ensure that the proper sample is added to the corresponding well. Do not add anything to NTC samples (master mix only).

- See RNA extraction tips on following page.

*The volume of RNA added per reaction is typically 5 μ l but can be increased (up to 25 μ l) with the appropriate adjustment of the water in the master mix. For example, if you want to test 10 μ l RNA, reduce the water per reaction to 13.2 μ l, and add 40 μ l master mix and 10 μ l RNA to each well.



**Cycling conditions
(QIAGEN conditions for Real Time RT-PCR):**

1 cycle each:

50°C for 30 min

95°C for 15 min

45 cycles:

95°C for 15 sec

60°C for 1 min (data collection step)

EEEV primers and probes. There are one published and one unpublished primer/probe sets available for the detection of EEEV RNA.

Published: Lambert et al. 2003.

EEEV 9391 F	ACACCGCACCTGATTTTACA
EEEV 9459 R	CTTCCAAGTGACCTGGTCGTC
EEEV 9414-probe	TGCACCCGGACCATCCGACCT
(unpublished)	
EEEV 1898 F	ACCTTGCTGACGACCAGGTC
EEEV 1968 R	GTTGTTGGTCGCTCAATCCA
EEEV 1919-probe	CTTGGAAGTGATGCAAATCCACTCGACA

RNA Extraction Tips

NOTES: Avoid contamination while working with RNA

- Maintain physically separated work areas; one dedicated to **pre-amplification RNA work** (RNA extraction) and the other for **master mix** production.
- Utilize dedicated/separate equipment within pre and post amplification areas; especially pipettes and centrifuges.

- Always wear gloves; even when handling unopened tubes.
- Open and close tubes quickly and avoid touching any inside portion.
- Use RNase-free plastic disposable tubes and pipet tips.
- Use aerosol block pipet tips.
- Use RNase-free water.
- Prepare all reagents on ice.

1. Solid phase samples (mosquitoes or tissues) are first homogenized in an isotonic buffer to produce a liquid homogenate. Mosquito specimens are homogenized using a copper clad steel bead (BB) grinding technique using a vortexer or mixer mill (e.g., Qiagen Tissuelyser). Homogenates are clarified by centrifugation in a microcentrifuge (e.g., Eppendorf) at maximum speed for 5 minutes to pellet any particulate material.
2. Extract RNA from the clarified supernatant using the QiaAmp viral RNA kit (QIAGEN part #52904) or another comparable kit specifically designed to purify RNA. Follow the manufacturer's protocol exactly with the following modification for mosquito specimens: include 1 additional wash/centrifugation step with AVL1 buffer, if using the Qiagen kit. Extract at least two negative controls and two positive controls along with the test specimens. The positive controls should differ in the amount of target RNA present (i.e., a pre-determined high positive and a low positive). Note: The volume of sample extracted can be greater or less than the standard volume stated in the QIAGEN protocol (140 µl) with the appropriate adjustments to all other volumes in the protocol. CDC typically extracts 100 µl.



References

- Andis MD, Sackett SR, Carroll MK, Bordes ES. Strategies for the emergency control of arboviral epidemics in New Orleans. *J Am Mosq Control Assoc.* 1987;3:125–130.
- Andreadis TG, Anderson JF, Vossbrinck CR, Main AJ. Epidemiology of West Nile virus in Connecticut, USA: A five-year analysis of mosquito data, 1999–2003. *Vector Borne Zoonotic Dis.* 2004;4:360–378.
- Andreadis TG, Anderson JF, Tirrell-Peck SJ. Multiple isolations of eastern equine encephalitis and Highlands J viruses from mosquitoes (Diptera: *Culicidae*) during a 1996 epizootic in southeastern Connecticut. *J Med Entomol.* 1998;35:296–302.
- Armstrong PM and Andreadis TG. Eastern equine encephalitis virus in mosquitoes and their role as bridge vectors. *Emerg Infect Dis.* 2010;16:1869–1874.
- Armstrong PM and Andreadis TG. Ecology and epidemiology of eastern equine encephalitis virus in the Northeastern United States: An historical perspective. *J Med Entomol.* 2022;59:1–13.
- Armstrong PM, Andreadis TG, Finan SL, Shepard JJ, Thomas MC. Detection of infectious virus from field-collected mosquitoes by Vero cell culture assay. *J Vis Exp.* 2011;2889.
- Armstrong PM, Prince N, Andreadis TG. Development of a multi-target TaqMan assay to detect eastern equine encephalitis virus variants in mosquitoes. *Vector Borne Zoonotic Dis.* 2012;12:872–876.
- Ayres JC and Feemster RF. The sequelae of eastern equine encephalomyelitis. *N Engl J Med.* 1949;240: 960–962.
- Basile AJ, Horiuchi K, Panella AJ, Laven J, Kosoy O, Lanciotti RS, et al. Multiplex microsphere immunoassays for the detection of IgM and IgG to arboviral diseases. *PLoS One.* 2013;8:e75670.
- Bell JA, Mickelson NJ, Vaughan JA. West Nile virus in host-seeking mosquitoes within a residential neighborhood in Grand Forks, North Dakota. *Vector-Borne Zoonotic Dis.* 2005;5:373–382.
- Berl E, Eisen RJ, MacMillan K, Swope BN, Saxton-Shaw K D, Graham AC, et al. Serological evidence for eastern equine encephalitis virus activity in white-tailed deer, *Odocoileus virginianus*, in Vermont, 2010. *Am J Trop Med Hyg.* 2013;88:103–107.
- Bigler WJ, Lassing E, Buff E, Lewis AL, Hoff GL. Arbovirus surveillance in Florida: Wild vertebrate studies 1965–1974. *J Wildl Dis.* 1975;11:348–356.
- Bolling BG, Barker CM, Moore CG, Pape WJ, Eisen L. Seasonal patterns for entomological measures of risk for exposure to *Culex* vectors and West Nile virus in relation to human disease cases in Northeastern Colorado. *J Med Entomol.* 2009;46:1519–1531.
- Bonds JA. Ultra-low-volume space sprays in mosquito control: a critical review. *Med Vet Entomol.* 2012;26:121–130.
- Brogdon WG, McAllister JC. Insecticide resistance and vector control. *Emerg Infect Dis.* 1998;4:605–613.
- Burkett-Cadena ND, Day JF, Unnasch TR. Ecology of eastern equine encephalitis virus in the Southeastern United States: Incriminating vector and host species responsible for virus amplification, persistence, and dispersal. *J Med Entomol.* 2022;59:41–48.
- Calisher CH. Medically important arboviruses of the United States and Canada. *Clin Microbiol Rev.* 1994;7:89–116.
- Callahan JD, Wu SJ, Dion-Schultz A, Mangold BE, Peruski LF, Watts DM, et al. Development and evaluation of serotype- and group-specific fluorogenic reverse transcriptase PCR (TaqMan) assays for dengue virus. *J Clin Microbiol.* 2001;39:4119–4124.
- CDC. Eastern equine encephalitis virus: Data and Maps for Eastern Equine Encephalitis. 2024a. <https://www.cdc.gov/eastern-equine-encephalitis/data-maps/index.html>.



CDC. Eastern equine encephalitis virus: Clinical Signs and Symptoms of Eastern Equine Encephalitis. 2024b. <https://www.cdc.gov/eastern-equine-encephalitis/hcp/clinical-signs/index.html>.

CDC. Eastern equine encephalitis—New Hampshire and Massachusetts, August–September 2005. *MMWR Morb Mortal Wkly Rep.* 2006;55:697–700.

Clarke DH. Two nonfatal human infections with the virus of eastern encephalitis. *Am J Trop Med Hyg.* 1961;10:67–70.

Colborn JM, Smith KA, Townsend J, Damian D, Nasci RS, Mutebi JP. West Nile virus outbreak in Phoenix, Arizona—2010: Entomological observations and epidemiological correlations. *J Amer Mosq Control Assoc.* 2013;29:123–132.

Crans WJ, Caccamise DF, McNelly JR. Eastern equine encephalomyelitis virus in relation to the avian community of a coastal cedar swamp. *J Med Entomol.* 1994;31:711–728.

Crans WJ. Failure of chickens to act as sentinels during an epizootic of eastern equine encephalitis in southern New Jersey, USA. *J Med Entomol.* 1986;23:626–629.

Crans WJ. The status of *Aedes sollicitans* as an epidemic vector of eastern equine encephalitis in New Jersey. *Mosq News.* 1977;37:85–89.

Currier M, McNeill M, Campbell D, Newton N, Marr JS, Perry E, et al. Human exposure to mosquito-control pesticides—Mississippi, North Carolina, and Virginia, 2002 and 2003. *MMWR Morb Mortal Wkly Rep.* 2005;54:529–532.

Dalrymple JM, Young OP, Eldridge BF, Russell PK. Ecology of arboviruses in a Maryland freshwater swamp. III. Vertebrate hosts. *Am J Epidemiol.* 1972;96:129–140.

Davis LE, Beckham JD, Tyler KL. North American encephalitic arboviruses. *Neurol Clin.* 2008;26:727–ix.

Davis RS, Peterson RK. Effects of single and multiple applications of mosquito insecticides on nontarget arthropods. *J Am Mosq Control Assoc.* 2008;24:270–280.

DeNicola AJ, VerCauteren KC, Curtis PD, Hygnstrom SE. Managing white-tailed deer in suburban environments: A technical guide. A publication of Cornell Cooperative Extension, the Wildlife Society—Wildlife Management Working Group, and Northeastern Wildlife Damage Research and Outreach Cooperative. New York (NY). 2000.

Deresiewicz RL, Thaler SJ, Hsu L, Zamani AA. Clinical and neuroradiographic manifestations of eastern equine encephalitis. *N Engl J Med.* 1997;336:1867–1874.

Duprey Z, Rivers S, Luber G, Becker A, Blackmore C, Barr D, et al. Community aerial mosquito control and naled exposure. *J Am Mosq Control Assoc.* 2008;24:42–46.

Eldridge BF. Surveillance for arthropodborne diseases. In: Eldridge BF, Edman JD, eds. *Medical Entomology*. Kluwer Academic Press; 2004:645.

Elias SP, Keenan P, Kenney JL, Morris SR, Covino KM, Robinson S, et al. Seasonal patterns in eastern equine encephalitis virus antibody in songbirds in southern Maine. *Vector Borne Zoonotic Dis.* 2017;17:325–330.

Emord DE, Morris CD. Epizootiology of eastern equine encephalomyelitis virus in upstate New York, USA. VI. Antibody prevalence in wild birds during an interepizootic period. *J Med Entomol.* 1984;21:395–404.

Feemster RF. Outbreak of encephalitis in man due to the eastern virus of equine encephalomyelitis. *Am J Public Health Nations Health.* 1938;28:1403–1410.

Florida Coordinating Council on Mosquito Control (FCCMC). In: Lloyd AM, Connelly CR, Carlson DB, eds. *Florida Mosquito Control: The state of the mission as defined by mosquito controllers, regulators, and environmental managers*. University of Florida, Institute of Food and Agricultural Sciences, Florida Medical Entomology Laboratory. 2018.

Gaensbauer JT, Lindsey NP, Messacar K, Staples JE, Fischer M. Neuroinvasive arboviral disease in the United States: 2003 to 2012. *Pediatrics.* 2014;134:e642–e650.



Godsey MS Jr, Burkhalter K, Young G, Delorey M, Smith K, Townsend J, et al. Entomologic investigations during an outbreak of West Nile virus disease in Maricopa County, Arizona, 2010. *Am J Trop Med Hyg.* 2012;87:1125–1131.

Goldfield M and Sussman O. The 1959 outbreak of eastern encephalitis in New Jersey. I. Introduction and description of outbreak. *Am J Epidemiol.* 1968;87:1–10.

Goldfield M, Welsh JN, Taylor BF. The 1959 outbreak of eastern encephalitis in New Jersey. The inapparent infection: Disease ratio. *Am J Epidemiol.* 1968;87:32–38.

Goodman CH, Russell BJ, Velez JO, Laven JJ, Bagarozzi DA Jr, Moon JL, et al. Production of a Sindbis/eastern equine encephalitis chimeric virus inactivated cell culture antigen. *J Virol Methods.* 2015;223:19–24.

Grady GF, Maxfield HK, Hildreth SW, Timperi RJ Jr, Gilfillan RF, Rosenau BJ, et al. Eastern equine encephalitis in Massachusetts, 1957-1976: A prospective study centered upon analyses of mosquitoes. *Am J Epidemiol.* 1978;107:170–178.

Graham SP, Hassan HK, Chapman T, White G, Guyer C, Unnasch TR. Serosurveillance of eastern equine encephalitis virus in amphibians and reptiles from Alabama, USA. *Am J Trop Med Hyg.* 2012;86:540–544.

Gu W, Unnasch TR, Katholi CR, Lampman R, Novak RJ. Fundamental issues in mosquito surveillance for arboviral transmission. *Trans R Soc Trop Med Hyg.* 2008;102:817–822.

Gujral IB, Zielinski-Gutierrez EC, LeBailly A, Nasci R. Behavioral risks for West Nile virus disease, northern Colorado, 2003. *Emerg Infect Dis.* 2007;13:419–425.

Hayes RO, Beadle LD, Hess AD, Sussman O, Bonese MJ. Entomological aspects of the 1959 outbreak of eastern encephalitis in New Jersey. *Am J Trop Med Hyg.* 1962;11:115–121.

Hoff GL, Issel CJ, Trainer DO, Richards SH. Arbovirus serology in North Dakota mule and white-tailed deer. *J Wildl Dis.* 1973;9:291–295.

Holderman CJ, Gezan SA, Stone AES, Connelly CR, Kaufman PE. Mosquitoes (Diptera: *Culicidae*) collected from residential yards and dog kennels in Florida using two aspirators, a sweep net, or a CDC trap. *J Med Entomol.* 2018;55(1):230–236.

Jones RC, Weaver KN, Smith S, Blanco C, Flores C, Gibbs K, et al. Use of the vector index and geographic information system to prospectively inform West Nile virus interventions. *J Am Mosq Control Assoc.* 2011;27:315–319.

Karpati AM, Perrin MC, Matte T, Leighton J, Schwartz J, Barr RG. Pesticide spraying for West Nile virus control and emergency department asthma visits in New York City, 2000. *Environ Health Perspect.* 2004;112:1183–1187.

Kwan JL, Park BK, Carpenter TE, Ngo V, Civen R, Reisen WK. Comparison of enzootic risk measures for predicting West Nile disease, Los Angeles, California, USA, 2004-2010. *Emerg Infect Dis.* 2012;18:1298–1306.

Lambert AJ, Martin DA, Lanciotti RS. Detection of North American eastern and western equine encephalitis viruses by nucleic acid amplification assays. *J Clin Microbiol.* 2003;41:379–385.

Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. *J Clin Microbiol.* 2000;38:4066–4071.

Letson GW, Bailey RE, Pearson J, Tsai TF. Eastern equine encephalitis (EEE): a description of the 1989 outbreak, recent epidemiologic trends, and the association of rainfall with EEE occurrence. *Am J Trop Med Hyg.* 1993;49:677–685.

Lindsey NP, Staples JE, Fischer M. Eastern equine encephalitis in the United States, 2003–2016. *Am J Trop Med Hyg.* 2018;98:1472–1477.

Lothrop HD, Lothrop BB, Goms DE, Reisen WK. Intensive early season adulticide applications decrease arbovirus transmission throughout the Coachella Valley, Riverside County, California. *Vector Borne Zoonotic Dis.* 2008;8:475–490.



- Lubelczyk C, Elias SP, Kantar L, Albert J, Hansen S, Saxton-Shaw K, et al. Detection of eastern equine encephalitis virus antibodies in moose (*Alces americana*), Maine, 2010. *Vector Borne Zoonotic Dis.* 2014; 14:77–81.
- Macedo PA, Schleier JJ 3rd, Reed M, Kelley K, Goodman GW, Brown DA, et al. Evaluation of efficacy and human health risk of aerial ultra-low volume applications of pyrethrins and piperonyl butoxide for adult mosquito management in response to West Nile virus activity in Sacramento County, California. *J Am Mosq Control Assoc.* 2010;26:57–66.
- Marchinton RL, Hirth DH. Behavior in White-tailed deer: Ecology and management. In: Halls LK, ed. *Wildlife Management Institute.* Stackpole Books; 1984:129–168.
- McCardle PW, Webb RE, Norden BB, Aldrich JR. Evaluation of five trapping systems for the surveillance of gravid mosquitoes in Prince Georges County, Maryland. *J Am Mosq Control Assoc.* 2004;20:254–260.
- McLean RG, Frier G, Parham GL, Francy DB, Monath TP, Campos EG, et al. Investigations of the vertebrate hosts of eastern equine encephalitis during an epizootic in Michigan, 1980. *Am J Trop Med Hyg.* 1985;34:1190–1202.
- Miley KM, Downs J, Burkett-Cadena ND, West RG, Hunt B, Deskins G, et al. Field analysis of biological factors associated with sites at high and low to moderate risk for eastern equine encephalitis virus winter activity in Florida. *J Med Entomol.* 2021;8:2385–2397.
- Molaei G, Armstrong PM, Graham AC, Kramer LD, Andreadis TG. Insights into the recent emergence and expansion of eastern equine encephalitis virus in a new focus in the Northern New England USA. *Parasit Vectors.* 2015;8:516.
- Morris CD. Eastern equine encephalitis. In: Monath TP, ed. *The arboviruses: Epidemiology and ecology*, Vol. 3. CRC Press; 1988:1–20.
- Mutebi JP, Godsey M, Smith RP Jr, Renell MR, Smith L, Robinson S, et al. Prevalence of eastern equine encephalitis virus antibodies among white-tailed deer populations in Maine. *Vector Borne Zoonotic Dis.* 2015;15:210–214.
- Mutebi JP, Lubelczyk C, Eisen R, Panella N, Macmillan K, Godsey M, et al. Using wild white-tailed deer to detect eastern equine encephalitis virus activity in Maine. *Vector Borne Zoonotic Dis.* 2011;11:1403–1409.
- Mutebi JP, Swope BN, Saxton-Shaw KD, Graham AC, Turmel JP, Berl E. Eastern equine encephalitis in moose (*Alces americanus*) in northeastern Vermont. *J Wildl Dis.* 2012;48:1109–1112.
- Nasci RS, Berry RL, Restifo RA, Parsons MA, Smith GC, Martin DA. Eastern equine encephalitis virus in Ohio during 1991. *J Med Entomol.* 1993;30:217–222.
- Nasci RS. A lightweight battery-powered aspirator for collecting resting mosquitoes in the field. *Mosq News.* 1981;41:808–811.
- Olson JG, Reeves WC, Emmons RW, Milby MM. Correlation of *Culex tarsalis* population indices with the incidence of St. Louis encephalitis and western equine encephalomyelitis in California. *Am J Trop Med Hyg.* 1979;28:335–343.
- Panella NA, Crockett RJ, Biggerstaff BJ, Komar N. The Centers for Disease Control and Prevention resting trap: a novel device for collecting resting mosquitoes. *J Am Mosq Control Assoc.* 2011;27:323–325.
- Peterson RK, Macedo PA, Davis RS. A human-health risk assessment for West Nile virus and insecticides used in mosquito management. *Environ Health Perspect.* 2006;114:366–372.
- Pouch SM, Katugaha SB, Shieh WJ, Annambhotla P, Walker WL, Basavaraju SV, et al. Transmission of eastern equine encephalitis virus from an organ donor to 3 transplant recipients. *Clin Infect Dis.* 2019;69:450–458.
- Pruszyński CA, Hribar LJ, Mickle R, Leal AL. A large scale biorational approach using *Bacillus thuringiensis israelensis* (Strain AM65-52) for managing *Aedes aegypti* populations to prevent dengue, chikungunya and Zika transmission. *PLoS One.* 2017;12:e0170079.
- Przelomski MM, O'Rourke E, Grady GF, Berardi VP, Markley HG. Eastern equine encephalitis in Massachusetts: A report of 16 cases, 1970–1984. *Neurology.* 1988;38:736–739.



Saxton-Shaw KD, Ledermann JP, Kenney JL, Berl E, Graham AC, Russo JM, et al. The first outbreak of eastern equine encephalitis in Vermont: Outbreak description and phylogenetic relationships of the virus isolate. *PLoS One*. 2015;10:e0128712.

Schmitt SM, Cooley TM, Fitzgerald SD, Bolin SR, Lim A, Schaefer SM, et al. An outbreak of eastern equine encephalitis virus in free-ranging white-tailed deer in Michigan. *J Wildl Dis*. 2007;43:635–644.

Sherwood JA, Oliver J. Eastern equine encephalitis incubation time periods of 5 and 8 days. *Pediatr Infect Dis J*. 2015;34:459–460.

Silverman MA, Misasi J, Smole S, Feldman HA, Cohen AB, Santagata S, et al. Eastern equine encephalitis in children, Massachusetts and New Hampshire, USA, 1970–2010. *Emerg Infect Dis*. 2013;19:194–201.

Skaff NK, Armstrong PM, Andreadis TG, Cheruvellil KS. Wetland characteristics linked to broad-scale patterns in *Culiseta melanura* abundance and eastern equine encephalitis virus infection. *Parasit Vectors*. 2017;10:501.

Smith GC, Francy DB, Campos EG, Katona P, Calisher CH. Correlation between human cases and antibody prevalence in house sparrows during a focal outbreak of St. Louis encephalitis in Mississippi, 1979. *Mosq News*. 1983;43:322–325.

Stamm DD. Arbovirus studies in birds in south Alabama, 1959-1960. *Am J Epidemiol*. 1968;87:127-137.

Stamm DD. Studies on the ecology of equine encephalomyelitis. *Am J Public Health Nations Health*. 1958;48:328–335.

Tate CM, Howerth EW, Stallknecht DE, Allison AB, Fischer JR, Mead DG. Eastern equine encephalitis in a free-ranging white-tailed deer (*Odocoileus virginianus*). *J Wildl Dis*. 2005;41:241–245.

Tsachalidis E, Sokos CK, Birtsas PK, Pasikou NK. The Australian crow trap and the Larsen trap: Their capture success in Greece. *Proceedings of the 2006 Naxos International Conference on Sustainable Management and Development of Mountainous and Island Areas. Vol. II*. 2006;325–329.

Turell MJ, Spring AR, Miller MK, Cannon CE. Effect of holding conditions on the detection of West Nile viral RNA by reverse transcriptase-polymerase chain reaction from mosquito (Diptera: *Culicidae*) pools. *J Med Entomol*. 2002;39:1–3.

Vahey GM, Mathis S, Martin SW, Gould CV, Staples JE, Lindsey NP. West Nile virus and other domestic nationally notifiable arboviral diseases—United States, 2019. *MMWR Morb Mortal Wkly Rep*. 2021;70:1069–1074.

Woodrow RJ, Howard JJ, White DJ. Field trials with methoprene, temephos, and *Bacillus thuringiensis* serovar *israelensis* for the control of larval *Culiseta melanura*. *J Am Mosq Control Assoc*. 1995;11:424–427.