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Isolation and Identification of Eastern Equine Encephalitis Virus in Mosquito Pools

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Abstract

The Eastern Equine Encephalitis Virus (EEEV) is transmitted to humans by mosquitoes of many genera. This virus has a high mortality rate and frequent life-altering complications for survivors of infection. The current practice for isolating and identifying the EEEV has been restricted to testing pools of 50 or less mosquitoes. A procedure for simulating one infected mosquito in a pool of 100 was established and tested. Data collected shows the threshold pool size can be increased to 100 without loss of specificity or accuracy. This increased will be met with many benefits including economic savings and increased time efficiency.

Introduction

The Eastern Equine Encephalitis virus (EEEV) is an arbovirus most commonly found in freshwater swamps of the Atlantic and Gulf Coast states along with the Great Lakes region of the northeast United States.[4,10] Specifically, most positive identifications of the EEEV have been in Florida, Georgia, Massachusetts, and New Jersey. 260 cases of EEE have been reported in the last 45 years, 66 of which have occurred in Florida[4]. On average, 6 cases of EEE appear each year with notable exceptions.[5] In 2005, 21 cases of EEE occurred in the United States, while only 3 occurred in both 2007 and 2009.

EEEV is a member of the genus *Alphavirus* and the family *Togaviridae*.[1,2] The Alphaviruses are all positive-sense single-stranded RNA viruses. Other notable Alphaviruses include Western equine encephalitis virus, Venezuelan equine encephalitis virus, and the Ross River virus. EEEV is also an arthropod-borne virus. Other notable arboviruses include West Nile virus, Yellow Fever virus, and the Dengue virus.[1]

EEE is considered to be the most deadly arthropod-borne encephalitis in North America.[1] EEE has a mortality rate of roughly 35%,[2] with higher rates found in adults older than 50 and children under the age of 15. However, permanent brain damage occurs in half the patients that survive the disease.[3] EEEV infection can occur in two fashions: systemic or encephalitis. System infections have an abrupt onset of flu-like symptoms; including fever, malaise, and chills.[3] This route usually resolves itself in 1-2 weeks. If the virus is able to cross the blood-brain barrier encephalitis may occur. This form of the disease usually manifests days after systemic symptoms. Symptoms of encephalitis include headache, nausea, convulsions, and coma. Death can occur in 2 to 10 days following these symptoms.[3] Survival is commonly met with

complications such as paralysis, cognitive impairment, severe sequelae, and seizures. Patients with severe sequelae usually die within a few years of infection. EEE infant mortality has been found to be as high as 74%, with a total mortality rate of 90% over nine years post-infection.[3] There is currently no treatment or commercially-available vaccine for EEE. Fortunately, the virus is most commonly found in areas not normally frequented by man, such as swamps and marshes.[2,4,5]

Culiseta melanura is a mosquito that transmits EEEV to birds native to freshwater swamps.[4] The bird is known as am amplifying host, that is to say it has a high enough titer of virus in its blood to pass the virus on to other mosquitoes that feed on it in the future. *Cs. melanura* poses only a small threat for direct virus transmission to humans as this is not one of their normal hosts. However, members of the genera *Aedes*[10] and *Culex* have been known to feed on freshwater swamp birds, humans, and horses.[1] These species of mosquito are therefore known as a bridge species in the transmission cycle. Humans and horses are dead-end hosts as they do not normally have a high enough titer of virus in their blood for other mosquitoes to pick up. Transmission of the virus from human to human, horse to human, or horse to horse has not been found in nature.

Detection of the EEEV in mosquito populations is a crucial task for mosquito control employees in Florida. The current practice for detection involves placing healthy “sentinel” chickens in likely areas for virus transmission and checking their blood frequently for presence of IgM antibodies to the virus.[10] Mosquito traps are placed around the chicken coops and in nearby areas and are routinely emptied into deep freezers for storage. If a chicken was to test positive then all the mosquitoes caught nearby would be tested as well to attempt to identify the particular species responsible for viral transmission. Mosquito control would quickly move in

and treat the area using methods appropriate to control the putative vector species in the hopes of stopping the virus before it is able to spread to nearby humans or animals.

Real-Time Polymerase Chain Reaction (RT-PCR) techniques are used to quantify the amount of virus present in a sample.[6,7] RT-PCR requires the use of a fluorescent probe containing a specific binding region matching a segment of the target DNA. The probe is essentially a short, specific strip of DNA with a fluorescent tag at one end and a molecule known as a "quencher" on the other. The quencher prevents the tag from fluorescing and is only removed by the 5'-3' exonuclease activity of the Taq polymerase.[6,7] For this to occur, the probe must be bound to the target DNA sequence. Throughout the RT-PCR process a laser is used to cause all bound probes to fluoresce. The degree of fluorescence is therefore indicative of the target DNA concentration. The RT-PCR process occurs in 42 cycles, although it has been shown that positives after cycle 37 are usually false.[6]

The current process for detecting EEEV in mosquito pools places a cap on 50 mosquitoes per pool. This number has been found to be most effective for detecting the West Nile virus as it has been shown that if one mosquito of the 50 is infected with the virus, there is above a 95% chance for detection.[6] Adding more mosquitoes to the pool size was shown to be detrimental to the process, most likely due to increased concentration of waste debris.

It has been hypothesized that increasing the pool size for EEEV detection can occur up to a point, after which additional increases will only be met with poorer results. Increasing the pool size could be incredibly beneficial, both financially and in a time-management sense.

Methods and Materials

Laboratory reared *Anophleles quadramaculitis* mosquitoes were divided into four pools of 50. Each pool of 50 was then homogenized in a 2mL centrifuge tube containing 1mL of BFD. These tubes were brought into a Biosafety Level-3 facility and placed on ice. 50µL of virus (at concentration $3x10^7$ PFU/mL) was added to a 2mL centrifuge tube containing 450µL of PBS, labeled "tube -1". A serial dilution was performed by micropipetting 50µL from "tube -1" to another centrifuge tube containing 450µL of PBS, labeled "tube -2". This process was repeated four times, resulting in tubes -3, -4, -5, and -6. Each tube's label corresponds to the number of dilutions it had undergone.

Tubes -1, -2, and -3 were discarded. 100µL of tube -4 was added to one of the centrifuge tubes containing 50 crushed mosquitoes. This tube was then briefly vortexed and placed in a centrifuge for 4 minutes at 4000rpm. Upon completion, 140µL of supernatant was micropipetted into a screw-top centrifuge tube containing 560µL of lysis buffer. The same steps were repeated for tubes -5 and -6. The final mosquito pool homogenate was combined with 100µL of PBS. This was then centrifuged and micropipetted into lysis buffer as before. All samples were left to sit for 15 minutes, the time needed for the virus to be fully deactivated.

All previous steps were repeated for mosquito pools of 100 and 200. Upon completion, 12 samples were created, 4 of each pool size. These samples were then placed into a QIAcube®, a machine specifically designed for RNA purification. The machine produced 60µL of purified RNA product from the original 700µL solution.

A master mix of reagents was made to prepare the samples for RT-PCR. This master mix was made up of the following reagent amounts per sample:

- 6.75µL RNAse-Free H_2O
- 12.5µL Master Buffer Mix
- 0.075µL Forward Primer
- 0.075µL Reverse Primer
- 0.10µL Probe
- 0.50µL RT-PCR Enzyme

20µL of master mix was added to 26 wells of a PCR 96-well plate. 5µL of each purified RNA product was added to two of these wells (24 total) to perform the entire RT-PCR process in duplicate. 5µL of a known positive sample was added to one well to act as a positive control. 5µL of RNAse-Free H_2O was added to the final well to act as a negative control. All 26 wells contained 25µL at this point. The 96-well plate was placed into a RT-PCR machine and set to run for 42 cycles. Upon completion, all data was collected and tabulated. ‘3^{-2}

Results

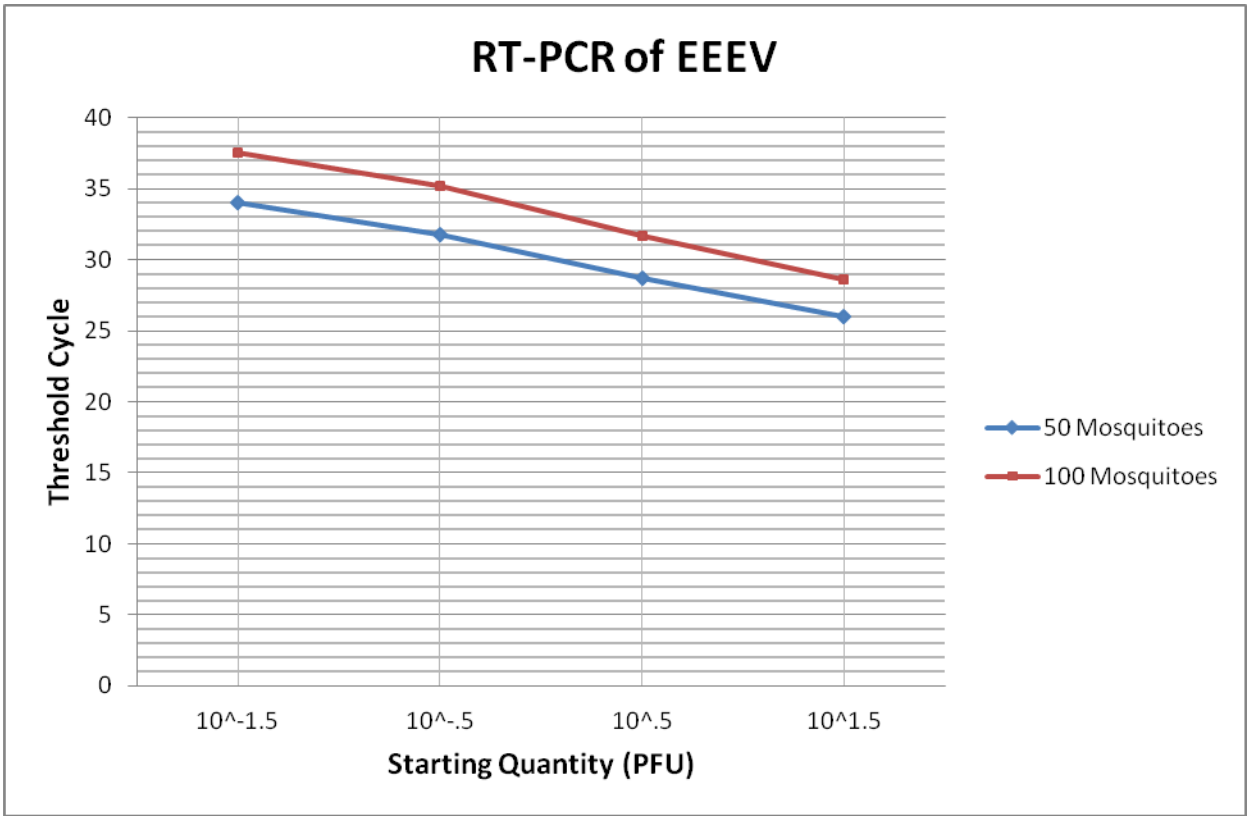

Fig. 1. Logarithmic Plot of the crossing threshold quantity of virus in the sample on the x-axis and cycle threshold on the y-axis. The x-axis is in units of Plaque Forming Units. $10^{-1.5}$ is equally to roughly 0.03 PFUs. Every consecutive quantity increases by one order of magnitude. The concentration of the virus stock solution was $3x10^{7}$. However, after purification and four steps of a dilution series it became $3x10^{1}$ ($\sim10^{1.5}$). All samples were run in duplicate and the averages were used to determine point placement.

The experiments performed are described in detail in Methods and Materials. The results of these experiments are depicted in Figure 1. After the RNA purification steps, all samples were subjected to RT-PCR. To be considered positive, a sample would need to fluoresce more than a threshold limit as detected by the RT-PCR machine. The cycle at which the samples overcame this limit is known as the threshold cycle. In theory, the lower the cycle number the more virus contained in the original sample. Along these lines, samples originally containing small amount

of virus will have a higher threshold cycle as it would take these longer to reach the same concentration as those starter with greater virus amounts. This was shown to be true as noted by the near-perfect linearity of the logarithmic values found in Figure 1.

The same serial dilution and stock concentration were used on samples in a pool of 200 mosquitoes but no isolation occurred. A second pair of primers and probes was used on all samples to determine accuracy. The results for pools of 50 and 100 were near identical to Figure 1 and there was again a negative result for virus isolation in pools of 200.

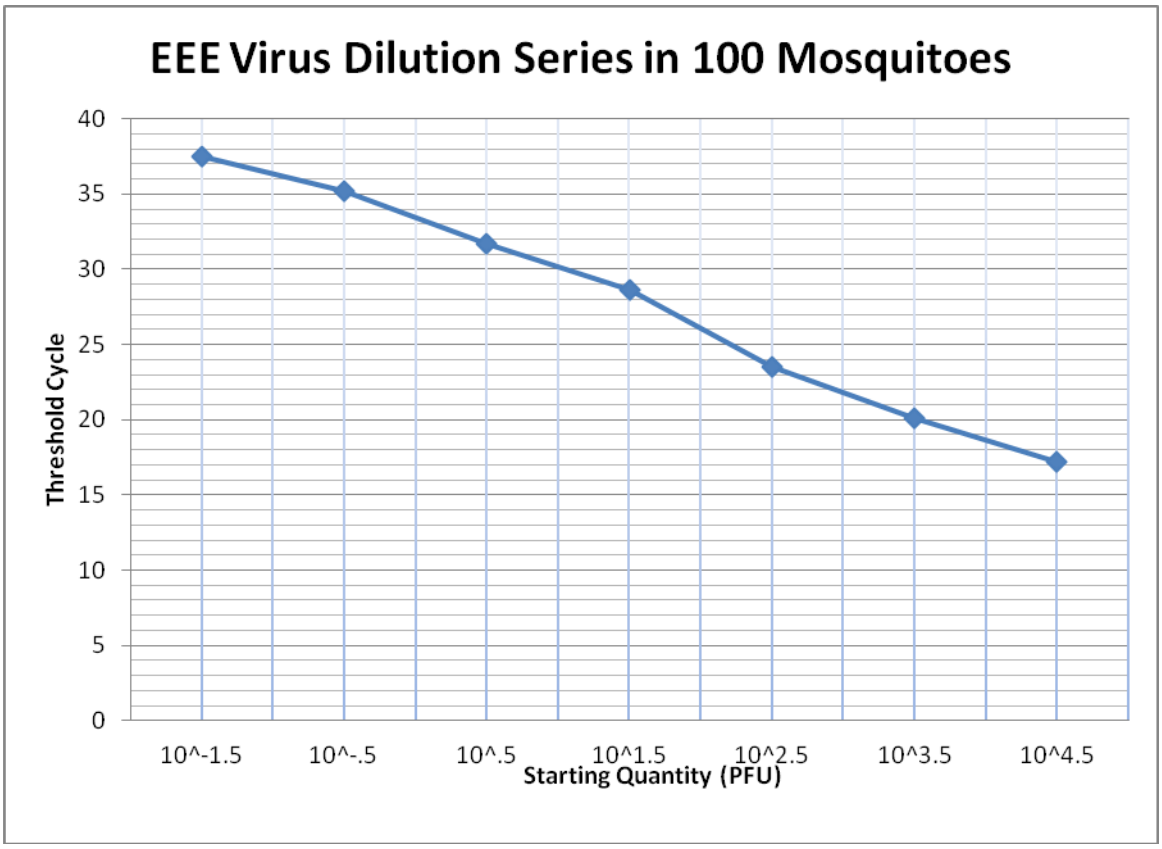

Fig. 2. Logarithmic plot of the crossing threshold quantity of virus in the sample on the x-axis and cycle threshold on the y-axis. The x-axis is in units of Plaque Forming Units. $10^{-1.5}$ is equally to roughly 0.03 PFUs. Every consecutive quantity increases by one order of magnitude. The largest concentration ($10^{4.5}$) is equally to roughly 31,000 PFUs. The lowest ($10^{-1.5}$) is equal to roughly 0.03 PFUs.

The data in Figure 2 displays a complete serial dilution series performed on pools of 100 mosquitoes. The samples containing the least amount of virus (found towards the left of the graph) have the highest threshold cycles. Similarly, the samples containing the greatest starting concentration of virus (found towards the right of the graph) have the lowest threshold cycles. All samples reaching the threshold after 37 cycles are considered to be potential false positives and must be subjected to further testing. This testing includes using a second pair of primers with matching probes.

The results of Figure 2 show a near-perfect linearity across the logarithmic differences in starting concentration. As a rule of thumb, every increase by order of magnitude should be met with a subsequent drop of threshold cycle by 2-3. That is to say, if a sample containing 10 PFUs at start reaches the threshold at 30 cycles, a sample containing 100 PFUs at start should reach the threshold between 27-28 cycles. This theory correlates well with the data displayed in Figure 2 as each increase by order of magnitude is met with (on average) a drop of 3 in the threshold cycle.

Discussion

The virus isolation and identification process (laid out in the Methods and Materials) proved to be successful in pools of 50 and 100 mosquitoes. On average, isolating any starting quantity of virus in pools of 100 took five extra cycles when compared to pools of 50. Still, it was possible to test for the virus up to 0.31 PFUs in pools of 100. Past research has shown that infected *C. melanura* can have titers in the range of $1x10^4$ PFU to $1x10^6$ PFU. Therefore, the current procedure for detection is more than adequate to identify the virus from one infeced mosquito in a pool of 100 mosquitoes.

The accuracy of these results hinges on the known concentration of the virus stock solution. This solution was labeled to be $3x10^7$ PFU/mL. However, the process allowed for the detection of the virus even if the starting quantity was in the range of 0.3 PFUs. This is similar to the detection limit of WNV in pools of 50 mosquitoes, which has been reported at 0.1 PFU.[8] It has been hypothesized that the reason that RT-PCR assays are able to detect less than 1 PFU is because RNA viruses produce many defective viral particles for every infective viral particle produced. Although the EEEV is a radically different virus, it still stands to reason that the threshold mark for detection for the two viruses would be similar.

Numerous attempts at enhancing the process to accommodate pools of 200 were met with failure. It appears that the extreme amount of waste debris produced by the process inhibits any chance of isolation. This problem is already being addressed with the use of a micro-magnet bead assay virus isolation process that has already been proven to work in pools of 200. However, this assay requires specialized reagents and overnight incubation periods. Therefore, in

some situations it is more prudent, in a fiscal and time-management sense, to utilize the normal viral RNA isolation and identification process.

A laboratory specifically funded to research EEEV may run thousands of samples a year. By proving the pool size can be safely doubled to 100, the effective cost of running samples of a population of mosquitoes is halved. It has been estimated that running a single sample from the "pool of 50 mosquitoes" phase to the RT-PCR phase costs $6.00USD. If a laboratory was to run 1,000 samples a year by the current practice, simply from a reagents standpoint it would cost in the range of $6,000. Increasing the pool size to 100 would save that lab $3,000 a year. This is, of course, not taking into account the amount of time saved by this lab. Essentially this project has succeeded in doubling the amount of samples that can be run per year while maintaining the same cost. Incredibly, all of this was done without sacrificing accuracy or specificity.

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