Establishment of a one-step reverse-transcription qPCR to detect Getah virus in mosquitoes and feral swine in Alabama, USA

by

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Abstract

Getah virus (GETV) is an emerging mosquito-borne alphavirus in the family *Togaviridae*, known to cause disease in a range of animal hosts including pigs, horses, and occasionally humans. The virus is endemic to several countries in Asia and the Western Pacific, where it has caused outbreaks characterized by febrile illness, reproductive failure in swine, and fever and rash in horses. Transmission occurs primarily via mosquitoes, particularly *Culex*, *Aedes*, and *Anopheles* species, some of which are also found in the United States. Although GETV has never been reported in North America, the presence of competent mosquito vectors and susceptible animal hosts raises concern over the potential for introduction and establishment. To date, no large-scale surveillance efforts have been conducted in the U.S. to assess the presence or absence of GETV in local vector or animal populations.

To address this gap, we developed a highly sensitive and specific one-step reverse transcription PCR (RT-PCR) assay targeting a conserved region of the GETV genome. The assay was first optimized and validated using synthetic GETV RNA controls to ensure diagnostic accuracy. We then applied this molecular tool in a surveillance study involving both mosquito vectors and potential mammalian amplifying hosts. A total of 1,626 mosquitoes, collected across Alabama between 2019 and 2024, were pooled and tested. In parallel, 315 tissue and blood samples (kidney, spleen, and whole blood) were collected from feral swine, a species known to develop high viremia following GETV infection and serve as a major amplifying host in endemic regions. All samples were processed for total RNA extraction, followed by RT-PCR screening for GETV.

All 1,626 mosquito pools and 315 swine samples tested negative for GETV RNA using the established RT-PCR assay. These findings suggest that detectable circulation of GETV were not

identified in our mosquito and feral pig samples collected in Alabama. This is the first study to employ molecular techniques for large-scale GETV surveillance in the U.S.

Our results provide the first molecular evidence supporting the absence of GETV in both mosquito and feral swine samples in the southeastern U.S., thereby offering valuable baseline data for future risk assessments. The significance of this work lies not only in the development of a reliable diagnostic tool but also in its application to proactive surveillance in an ecologically suitable region. Given the increasing global movement of animals and the documented expansion of arboviruses into new geographic regions, this work is timely and critical. While GETV is not currently present in the U.S., the detection of competent vectors and susceptible hosts reinforces the need for continued monitoring to enable early detection and rapid response should the virus be introduced.

This study had several limitations. First, our surveillance relied solely on molecular detection of viral RNA, which identifies only active infections and may miss previous exposures. Serological assays, which could detect past infections through antibody presence, might offer a more comprehensive understanding of viral exposure. Unfortunately, no commercial ELISA kits are currently available for GETV antibody detection. Second, our host surveillance was limited to mosquitoes and feral pigs, despite the fact that horses and humans are also susceptible to GETV infection. Third, this study focused on a single state—Alabama—and cannot be generalized to the entire U.S. To build a more comprehensive understanding of the potential GETV threat, future studies should expand surveillance to include more geographic regions, additional host species, and incorporate both molecular and serological approaches.

This study provides the first molecular evidence of the absence of Getah virus in mosquito and feral swine populations in Alabama. While the virus was not detected, the ecological presence

of competent vectors and susceptible hosts in the U.S. warrants continued and expanded surveillance efforts to monitor for potential future introductions of this emerging pathogen.

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List of Abbreviations

Ae.	*Aedes*
An.	*Anopheles*
BLAST	Basic Local Alignment Search Tool
CHIKV	Chikungunya virus
Cs.	*Culiseta*
Cx.	*Culex*
DENV	Dengue virus
ELISA	Enzyme Linked Immunosorbent Assay
GETV	Getah virus
IFA	Immunofluorescence Assay
JEV	Japanese encephalitis virus
kb	Kilobase
Ma.	*Mansonia*
MIF	Microimmunofluorescence
MLST	Multi Locus Sequence Typing
PGFE	Pulsed Field Gel Electrophoresis

Chapter 1: Literature Review

1.1 Getah virus

Getah virus, of the *Togaviridae* family and the *Alphavirus* genus, falls within the Semliki Forest virus complex. It was first isolated in 1955 from *Culex gelidus* from a rubber plantation in Malaysia.[1] Later on, it was isolated from mosquito species in Japan, Southeast Asia, China, Australia, Mongolia, and Russia. Then, the viral identification ranged from 3°N to 60 °N across Eurasia. Research has shown that GETV is amplified in the mosquito, transmitted by bites to an animal reservoir, and further spread to other uninfected mosquitos. In these animal reservoirs, GETV infection has been seen to cause fever, rashes, edema of the hindlegs, and even lymph node enlargement in horses and abortion in pigs. Disease outbreaks first occurred in horses in Japan in the 1970s and 1980s and have since totaled to at least a dozen outbreaks among horses, pigs, and cattle across Japan, India, and China.[2]

Getah virus, as it falls under the Semliki Forest virus complex, is an enveloped single-stranded, positive sense RNA virus. The viral genome is in a range of 11,000 to 12,000 nucleotides. The strand has a methylated (7-methylguanosine) cap structure at the 5' end and a variable number of poly(A) tails at the 3'end. The genome has two open reading frames (ORFs) to encode both structural and nonstructural genes. The first 2/3 of the 5' genome contains ORF1 responsible for encoding 4 viral nonstructural proteins (nsP1-nsP4). These proteins are the cause of viral RNA transcription, replication, polyprotein cleavage, and RNA capping. This is followed by the 26S RNA junction, which promotes the transcription of the intracellular subgenomic 26s RNA which contains the second-mentioned ORF. The second ORF, which lies in the final 1/3 of the genome at 3' end side, is responsible for encoding several viral structural proteins including C, E1, E2, E3,

and 6K). The protein to note is E2, as it is the primary functional protein that arbitrates viral entry into host cells, which leads to the triggering of the host's immune responses and disease.[3]

GETV is an emerging virus that has shown an increase in outbreaks, hosts, and vectors across the past couple of decades. Targeting horses and livestock, GETV is responsible for animal disease and death, as well as economic damage across livestock farms, and its variability is increasing. Despite that, as of 2025, there still is not a lot of information available concerning GETV.

1.2 GETV Expansion

GETV was first isolated as strain MM2021 from mosquito species *Cx gelidus* in October 1955 at a rubber plantation in Malaysia, and then isolated as (M6/Mag-132) in Japan the next year in July 1956 from *Cx. tritaeniorhynchus*.[4,5] The latter virus was collected in a pigpen north of Tokyo and named Sagiyama virus, but through virus cross-neutralizing antibody titer detection and viral gene phylogenetic analysis was realized to be an isolate of GETV.[4] GETV strain N544 was isolated in Australia in 1961 and strand M1 was isolated in Hainan Island, China in 1964. Up until this point, GETV had only been isolated in regions around the Pacific Ocean. In 1966, GETV was isolated in a southern region of Eurasia for the first time from *Cx tritaeniorhynchus* in Cambodia.[6] In 2000, there were 24 different strains of GETV from isolates, and now (across GenBank and other references) there are about 170 GETV strains in 2022.[2]

Geographically, GETV has spread gradually spread from tropical regions around the Pacific to temperate regions, and even instances of the Arctic tundra. GETV can now be found in a region from 1° North in Malaysia to 60° North in Russia, as well as spanning from 38° East to 140° East. This area contains the 13 countries from which GETV has been isolated (March 2022)

which include Malaysia, Japan, Australia, China, the Philippines, Vietnam, Cambodia, South Korea, India, Sri Lanka, Mongolia, and Russia.

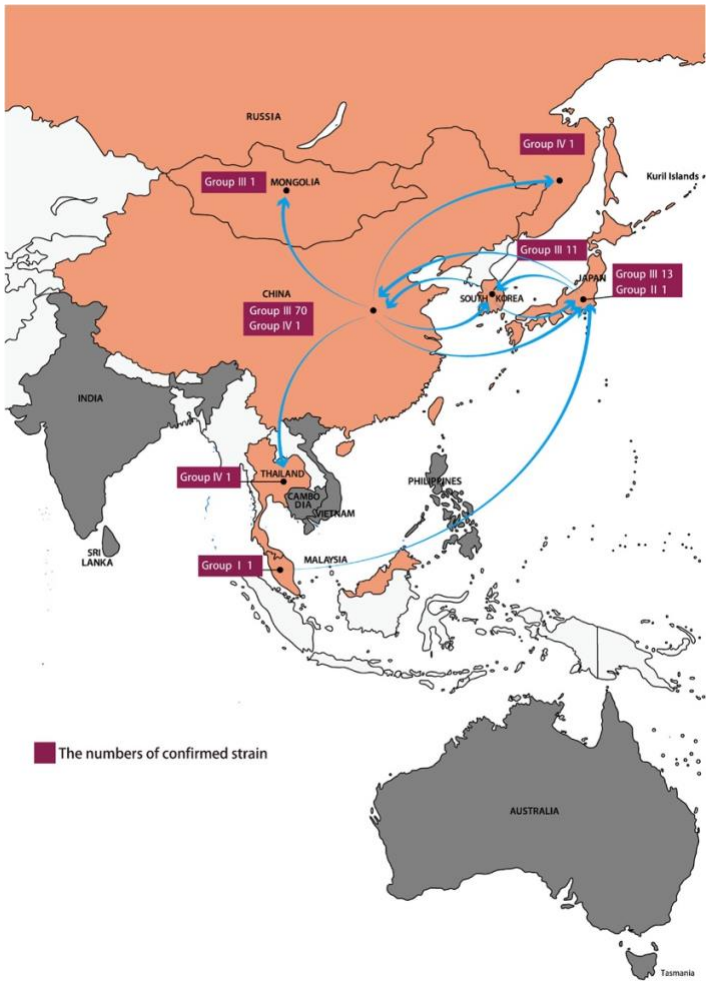

Figure 1. Global distribution and transboundary transmission of GETV. Transmission was determined using Bayesian phylogeographic inference of E2 gene sequences. Grey countries indicate that there are reports of GETV outbreaks, but no sequences in the NCBI. Orange countries indicate that there are not only reports of GETV outbreaks, but also sequences in the NCBI. The number represents all the positive cases of GETV confirmed in NCBI and Chinese magazines. Blue lines indicate transboundary transmission events with a Bayes factor > 3. Note from Shi, N., Zhu, X., Qiu, X., Cao, X., Jiang, Z., Lu, H., & Jin, N. (2021). Origin, genetic diversity, adaptive evolution and transmission dynamics of getah virus. Transboundary and Emerging Diseases, 69(4).

In China, where GETV was first isolated in 1964, the virus had spread to 22 of the 34 Chinese provinces by 2022. Since its first isolation, GETV has spread across a vast region in the Eastern hemisphere.[1,2] In recent years, China has witnessed a notable rise in GETV activity, particularly in regions such as Guangdong, Hainan, and Heilongjiang. The virus has been isolated from various mosquito species, including *Culex tritaeniorhynchus*, a common vector in agricultural areas. GETV primarily infects pigs, horses, and cattle, causing fever, rash, and reproductive disorders, which can result in significant economic losses. Equine outbreaks in China have drawn attention to the virus's impact on animal health and the potential for cross-species transmission. Recent studies have also detected GETV antibodies in wild boars and zoo animals, suggesting widespread circulation in wildlife. Surveillance efforts in China have revealed genetic diversity among circulating GETV strains, with phylogenetic analysis indicating the emergence of new lineages. Although no human cases have been definitively confirmed in China, serological evidence of past exposure has been found in some populations, raising concerns about zoonotic risk. As GETV continues to spread, enhanced vector control and surveillance are essential for mitigating its impact on public and veterinary health.

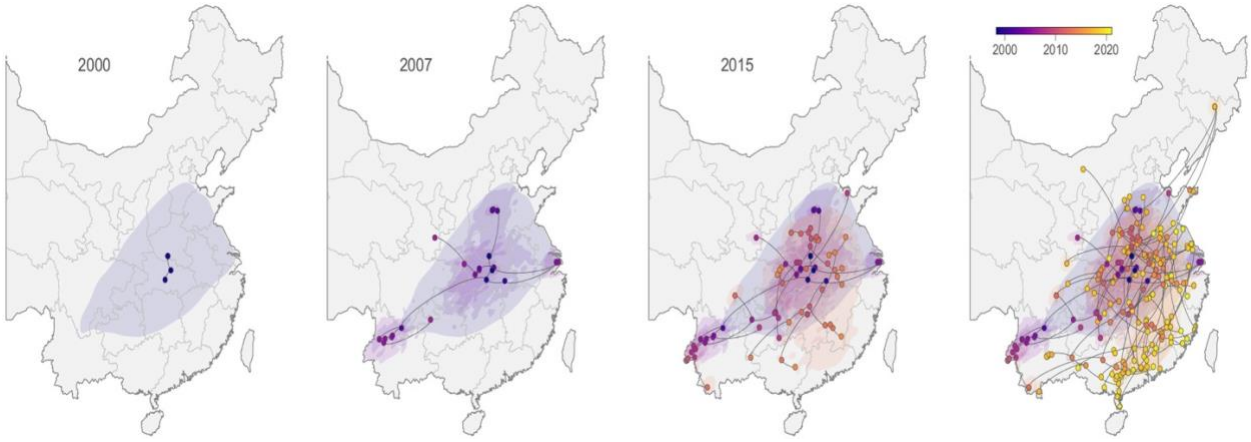

Figure 2. Dispersal history of GETV lineages in China as inferred by a continuous phylogeographic analysis. Maximum clade credibility (MCC) tree and 80% highest posterior

density (HPD) regions reflecting the uncertainty related to the phylogeographic inference and based on 1,000 trees subsampled from the posterior distribution. MCC tree nodes are colored according to their time of occurrence, and 80% HPD regions were computed for successive time layers and then superimposed using the same color scale reflecting time. In addition to the overall continuous phylogeographic reconstruction, we also mapped the dispersal history of GETV inferred until three years in the past, i.e., 2000, 2007, and 2015, which allows visualization of the progression of the virus spread." Note. From "Zhao J, Dellicour S, Yan Z, Veit M, Gill MS,He W,,Zhai X,Ji X, Suchard MA,,Lemey P, Su S,2023.Early Genomic Surveillance and Phylogeographic Analysis of Getah Virus, a Reemerging Arbovirus, in Livestock in China. J Virol97:e01091-22.

Approximately, 169 GETV strains had been identified by 2022, with 128 viral sequences being identified. 56 strains of GETV taken from animal species across 5 different countries (China, India, Thailand, Japan, and Korea). These animals included pigs (37 strains), horses (12 strains), red pandas (2 strains), wild boars (2 strains), cattle (1 strain), fox (1 strain), and blue fox (1 strain). Most of these animal isolates (47) have been found in 12 provinces in China, however, highlighting the massive spread GETV has had in its country. Notably, many of the publications referenced were published by Chinese authors.[1,2]

1.3 Evolution of GETV

The gene sequence for pathogenic protein E2 has been the target of phylogenetic analysis, and across the isolates collected from 1955 to 2022 from both insects and animals, four groups (G1-GIV) have been revealed. GI only contained one strain (MM2021), which happened to be the original strain isolated from Malaysia in 1955. Group II was similar and only included three virus strands (SAGV) isolated from Japan in 1956. Group IV includes four strains of GETV isolated from Malaysia (B254), China (YN12031), Thailand (SW), and Russia (LEIV/16275/Mag). Group

III, however, includes 80 different GETV isolates, coming from both animal specimens and bloodsucking insects. Group III has been identified to be the main pathogenic strain of GETV responsible for disease in animals.[1,7,8]

GIII GETV isolates occur in the large majority of GETV isolates (80 out of 88). While the GII isolate came from 1955, GI isolates came from 1956, and GIV isolates came from the 21st century, GIII comes from a much larger time span. GETV GIII was first isolated in 1964 and has appeared frequently in the past decades in both animals and mosquitos. Not only has its time of occurrence had a much larger range, but so has its geographical presence. While the other 3 groups have occurred in localized regions, GIII has been isolated from as south as Hainan Island in China at 19°N to as north as Mongolia at 51°N.[7,8] It is suggested that the large range of GIII isolates has occurred due to having a wider range of possible bloodsucking insects to carry and spread the virus. GIII has also been isolated across the widest range of vectors in 17 different mosquito species across all 5 known genera of vectors. It has even been isolated from non-mosquito vectors. GIII has also been the only cause of disease in animals for the past 20 years, ranging from Japanese horse isolates in 1978 to pig isolates in both Japan and China in 2015 and 2017, respectively, to blue fox isolates in China in 2017.[10] Thus, it is known to be the main disease-causing group in animals, as it has also been known to be responsible for disease in cattle and red pandas.[9,11] Figure 1 shows that GETV GIII is further divided into 3 different evolutionary branches, with the third branch being the primary perpetrator of GIIIs range across isolation time, geographical range, and possible vector.

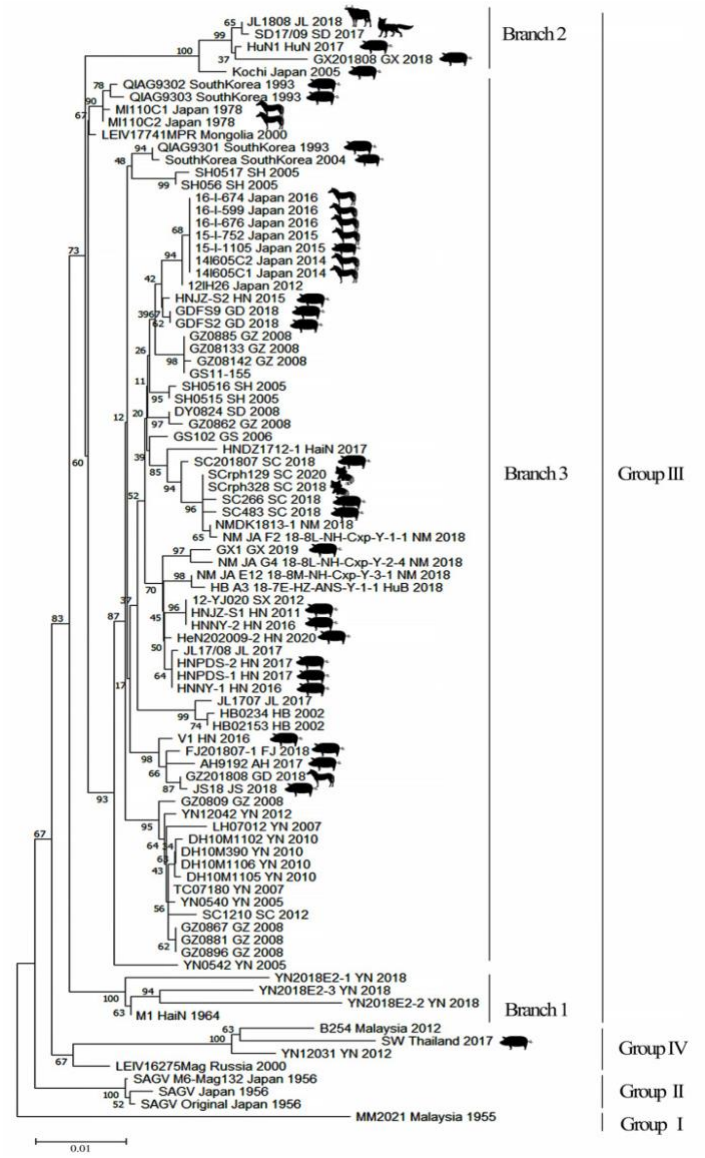

Figure 3. Phylogenetic analysis of GETV genes (E2). Using MEGA-X software, the neighbor-joining (NJ) method was used to draw the phylogenetic tree with 1000 bootstrap replicates. GETV can be divided into four groups (I–IV), of which group III can also be divided into three evolutionary branches (1–3). The animal host isolates are marked with different animal patterns on the figure and isolates without patterns were all isolated from mosquitoes." Note. From "Li B, Wang H, Liang G. Getah Virus (Alphavirus): An Emerging, Spreading Zoonotic Virus. Pathogens. 2022 Aug 20;11(8):945. doi: 10.3390/pathogens11080945.

Compared to GI, GII, and GIV, it can be concluded that GIII GETV is better suited to proliferation or further expansion in Eurasia than its counterparts due to its wider range of possible bloodsucking insects as transmission vectors and resulting wider variety of possible animals to act as virus reservoirs. Due to these conditions and recent outbreaks, GETV GIII has been declared in several journals to be "an emerging mosquito-borne virus".

Through the analysis of the molecular evolution and virus migration route of GETV, a most recent common ancestor was found, and it was then determined that the virus originated around 145 years ago (95% HPD; 75–244), and then evolved into the four aforementioned groups. GI was the first GETV (MM2021) isolated in Malaysia in 1955. GII then emerged about 60 years ago (95% HPD; 59–73), which was isolated in Japan in 1956 as SAGV. GIII viruses emerged around 50-60 years ago (95% HPD; 51–72). GIII followed two evolutionary branches, GETV (M1), which includes the oldest viruses of GIII like the isolate found in China in 1964), and other GIII viruses independent from M1. GIV is the newest group of GETV and emerged around 30 years ago (95% HPD; 16–55). This is further evidence of GETV belonging to the class of emerging viruses.[12]

Studies have been conducted on the migratory transmission route of GETV, and they show that Malaysia, Japan, and Yunnan Province in China are the three primary distribution sites. It is suggested that GETV was spread from Malaysia to Japan as early as 1917, later on from Japan to the Hainan Province of China sometime in the 1960s, and Japan to the Yunnan Province of China in the 1970s.[10] The Yunnan Province then seems to have been the source of GETV to Hebei, Shanghai, Cansu, and Sichuan provinces in China. Comparing this pattern along with the molecular genetic evolution analysis of GETV above, it is implied that GI, GII, and GIV have become stable virus populations that appear in smaller localized areas, while in contrast, GIII expands rapidly across geographical regions in the last 20 years to spread through mainland Asia.

The suggested GETV transmission and migration patterns in Asia are comparable to that of the Japanese Encephalitis virus that is prevalent in many natural hosts in the same area. [1,13]

1.4 Identification of Getan virus from mosquitoes

GETV was initially isolated from *Cx. gelidus* and *Cx. tritaeniorhynchus*, and before 2000 only four known species of Culex of the order Diptera were known vectors (*Cx. vishnui, Cx. fuscocephala*, and the two formerly mentioned). from GenBank and 41 sequences found in isolates published in Chinese and English articles. GETV had been isolated from bloodsucking insects across 5 mosquito genera and 17 mosquito species. These genera include *Culex, Armigeres, Anopheles, Aedes,* and *Mansonia* (listed in order of prevalence). Many strains have also been isolated from unclassified mosquitos, and two viral isolates have been taken from *Culicoides*, a type of bloodsucking insect known as midges. It is of growing concern as more mosquito species become vectors, particularly since many of these genera are prevalent in all corners of the globe.

1.5 Outbreaks of Getah virus in animals

The first outbreak of GETV in animals occurred in 1978 as the virus was identified in horses in Japan, despite being isolated two decades earlier in mosquitoes in the same country. Following this outbreak, further disease outbreaks occurred at an increased frequency, in not only horses, but other domesticated mammals such as pigs, cattle, and even blue foxes. Outbreaks are shown in the Table below.

Table 1. GETV outbreaks in animals

Country	Year	Animal	Death	Cases	Total # of animals
Japan	1978	Horse	0	70	455
Japan	1978	Horse	0	722	1903
Japan	1979	Horse	0	136	-
Japan	1985	Pig	8	12	-
Japan	2014	Horse	0	75	2000
Japan	2015	Horse	0	30	1992
India	1990	Horse	0	26	88
China	2017	Pig	200	1333	2915
China	2017	Blue Fox	6	25	-
China	2018	Pig	0	54	503
China	2018	Cattle	0	10	48
China	2018	Horse	0	1	-

Note. From "Li B, Wang H, Liang G. Getah Virus (Alphavirus): An Emerging, Spreading Zoonotic Virus. Pathogens. 2022 Aug 20;11(8):945. doi: 10.3390/pathogens11080945. PMID: 36015065; PMCID: PMC9416625."

1.5.1 Getah virus in horses

Horses have been shown to be common victims of GETV. In 1978, 70 of the 455 racehorses at the Miho training center in Japan became ill due to the GETV. The symptoms of these horses came to be a short-lasting fever, rash, swelling of the horses' submandibular lymph nodes, and edema of the horses' limbs that lasted around a week. The outbreak lasted for about two months as it transmitted from stable to stable.[14] This was one of two instances at a Japanese horse training center that year, with another in the Ibaraki Prefecture equestrian training center that involved GETV infection in 722 of 1903 horses. In 1990, an outbreak that lasted 21 days occurred in India described by symptoms of anorexia, depression in addition to the symptoms listed above were seen in 26 of 88 horses on a thoroughbred farm. In 2014, the same Miho training facility detected GETV

in 25 of 49 febrile horses using RT-PCR, and had another outbreak in 2015. In 2018, RT-PCR analysis of horse serum samples from Hunan yielded positive GETV as well.[15,16,17]

A study further investigated the effect of the path of infection on pathogenicity of GETV in horses by infecting subjects with either infectious blood of Getah virus isolated from horse plasma during the Japanese 1978 epizootic outbreaks. These horses developed clinical signs similar to those of the 1978 outbreak, and further intramuscular inoculation with varying amounts of virus titer demonstrated that virus at low infectivity resulted in clinical cases of skin rash while higher infectivity showed horses with enlarged submandibular lymph nodes. A skin rash was also developed amongst horses that were intranasally inoculated.

1.5.2 Getah virus in swine

Pigs are another victim of GETV. In the 1985 outbreak in Japan, 12 piglets born from the same sow were infected with GETV, and 8 of those piglets ended up dying 3 to 5 days after birth. Prior to death, the piglets also experienced symptoms of depression, tremors, and yellow-brown diarrhea. The surviving piglets all displayed hypoplasia.

These clinical symptoms were replicated in a 1987 study where 6 piglets were inoculated intramuscularly with virus material and 3 piglets were inoculated oronasally. All six of the intramuscularly inoculated piglets presented with anorexia, depression, tremor of whole body, red discoloration of the skin, trembling of the tongue, and incoordination of pelvic limbs 20 hours after inoculation. Two piglets died between 60 to 70 hours post-inoculation, while another three began dying two to three days after inoculation. Two piglets survived, with one inoculated intramuscularly and one oronasally, with the former recovering after 2 days post-inoculation and the latter only ever presenting a mild case of symptoms in the first place.

In the 2017 GETV outbreak on a pig farm in the Hunan Province of China, 1333 piglets from a batch of 2915 pigs displayed symptoms of fever, anorexia, ataxia, and tremors. 200 of the piglets then died.[18] Furthermore, 150 pregnant sows experienced stillbirths or fetal mummies. Other recorded GETV symptoms for swine include dysgenesis and severe diarrhea. GETV seems to have the largest impact on swine populations, impacting both livestock pigs and wild boars by inflicting more extreme symptoms of death, mental affliction, and childbearing issues.[19]

1.5.3 Getah virus infections in other animals

In China, GETV has also had an impact on blue fox, bovine, and red panda populations. In 2017, on a Shandong Province farm, 25 five-month-old blue foxes developed anorexia, depression, and fever, and 6 developed further neurological problems and died.[10] A cattle farm in 2018 in Jilin Province saw 10 out of 48 cattle develop fever, experience loss of appetite and depression, and test positive for GETV infection.[9] In 2020, a group of red pandas passed away in Sichuan China, and were then found to be infected with GETV.[11]

1.6 Serological survey of Getah virus infections

Since outbreaks of GETV have impacted livestock populations of horses, pigs, and cows, seroepidemiological surveys have attracted a lot of attention across China, Malaysia, Japan, South Korea, Indonesia, and even in the Southeast United States. There is currently no commercial antigen test method. Serological detection was conducted through several tests such as the popular enzyme-linked immunosorbent assay (ELISA) or complement fixation test (CFT) for example.

Etiological detection of GETV is done through viral gene amplification and virus isolation. This is done through Reverse Transcription-Polymerase Chain Reaction (RT-PCR), which is the

method conducted within this lab. This procedure includes the extraction of RNA from a sample like an animal's whole blood and the synthetization of the resulting cDNA of the viral genome through reverse transcription. This is followed by designing a pair of primers for a targeted region of the GETV gene. The gene is then amplified using the cDNA template through PCR and then compared to the presence of confirmed positive samples.

In Japan, serum neutralization tests were conducted on local horses before and after disease outbreaks for the GETV antibody and found a 6% (14/232) positive rate before the outbreak compared to a 61.2% (172/182) after. Multiple tests were also conducted on domestic pig and wild boar populations for GETV antibodies before and after an outbreak.[20] Local pig populations yielded positive rates ranging from 2.7% (1/37) to 19.1% (40/209) before and after an outbreak, while the wild boar population showed rates ranging from 3% (3/301) to 54.3% (39/70).[21] These results suggest that wild boars may be a reservoir for GETV in non-domesticated areas.

In 2018, 5 different species (chicken, duck, dairy cattle, pigs, and beef cattle) were tested in the Yunnan Province of China for GETV neutralizing antibodies. Low positive rates of 2.2% (1/46), 5.6%, (1/18) and 13.3% (2/15) were found across chicken, duck, and dairy cattle serums respectively, while positive rates of 45.9% (39/85) and 71.9% (23/32) were found in pigs and beef cattle serums, respectively.[22] Testing was done on goats in the Hainan Province in 1982 and a complement fixation test found a positive rate of 37.5% (6/16).[23] ELISA was conducted on sheep and goat specimens from Xinjiang, China in 2022, and a positive rate was found to be 11.7 (55/471) and 10.0% (47/308) respectively. [24]Seroepidemiological surveys show that GETV can be found in a variety of hosts, and has shown that livestock animals like swine, beef cattle, and sheep are especially susceptible to GETV contamination.[2]

1.7 Getah virus infections in humans

From 1979-1982, serum was collected from healthy persons and patients with unknown fever for sero-epidemiological survey of GETV. From the serum collected from patients with an unknown local fever, 26.4% (24/91) were determined positive for GETV through CFT. CFT also yielded a positive rate of 3.4% (2/58) from the healthy people's serums.[24] In the same span of time, arbovirus antibodies were being surveyed in New South Wales, Australia amongst healthy populations, and a GETV hemagglutination-inhibition test came with positive results. In parts of Asia that were formerly Soviet Union, serum collected between 1985 and 1988 also has 7% positive for GETV hemagglutination-inhibition antibodies. In Malaysia from 1961-1966, 135 samples were positive for neutralizing antibodies. So, while there have been healthy and unhealthy people from different countries testing positive for GETV antibodies, there still has been no news or evidence of GETV infection to cause human disease. While this is good news, it has been advised to carefully monitor personnel around GETV infection in horses, pigs, and cattle, as many other arboviruses have been shown to be detrimental to human health.[2,25]

1.8 Vaccine against Getah virus infections

GETV spreads mostly through insect vectors like mosquitos, and its infection can cause disease within a variety of different animals. It has further been shown in experiments that GETV can also transmitted through herds of horses through aerosols. This is problematic as most livestock animals live together in herds or packs. In response, there were efforts from groups in Japan to develop a formalin-inactivated GETV vaccine (using strain M1-110 isolated from horses) that have been partially successful.

Thoroughbred racehorses have been vaccinated against GETV since 1979 through a program involving a consisting of 2-year-old horse to receive two vaccine doses for 2-year-old horses before peak mosquito season during the months of May and June, with annual booster doses every subsequent year at around the same time. As of 2000, these vaccinated groups have had no outbreaks or signs of GETV infection. The equine vaccination even proved to have some success in emergency situations during ongoing outbreaks. In 2014, however, an equine racing center (the same facility where the 1978 outbreak occurred) that practiced this vaccination routine unfortunately saw 75 of 2000 racehorses showing clinical symptoms of GETV. 33 horses with fever were tested positive through RT-PCR and NT. The majority of the ill horses were 2-year-old horses that had only received a singular dose of the GETV but testing also saw thoroughly vaccinated horses included. This suggests that while the vaccine does protect the horses that received it, it could no longer protect against infection from the newer strains that were emerging.

There aren't many available vaccines for other species yet, but one instance includes a porcine oil emulsion-inactivated vaccine candidate prepared from strain GETV-JS18 from a porcine GETV isolate has shown promising results. Testing yielded a 100% protection rate amongst the experimental pigs, with the neutralizing antibody tire maintaining high levels for 7 months after vaccination. Currently, there are no vaccines for other animal species.[26,27]

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Chapter 2. Establishment of a reverse-transcription PCR to detect Getah virus

2.1 Introduction

Getah virus (GETV) is an emerging mosquito-borne alphavirus in the Togaviridae family, genus Alphavirus, that poses a growing threat to animal health, particularly in parts of Asia, including China. GETV primarily infects horses, pigs, and cattle, leading to febrile illness, reproductive losses, and economic burdens in the livestock and equine industries.[1,2] As GETV is RNA-based and often co-circulates with other arboviruses such as Japanese encephalitis virus (JEV), dengue virus (DENV), and Zika virus (ZIKV), the development of sensitive and specific molecular diagnostic tools is essential for accurate detection and surveillance.[3,4,5]

Reverse-transcription polymerase chain reaction (RT-PCR) has become a widely adopted method for detecting RNA viruses due to its high sensitivity and rapid turnaround.[1] To establish a reliable RT-PCR assay for GETV, the selection of specific primer and probe sequences is critical. Target regions within the GETV genome, such as the nonstructural protein 1 (nsP1) or envelope protein (E1 or E2) genes, are often used due to their relative sequence conservation among GETV strains and divergence from other alphaviruses and flaviviruses.[6]

A key challenge in assay development is ensuring the RT-PCR does not cross-amplify other related viruses. Although GETV belongs to the Alphavirus genus and not the Flavivirus genus, these viruses share ecological niches and can co-infect the same mosquito species, increasing the chance of misdiagnosis in areas with high arboviral diversity.[7] To minimize this risk, comprehensive in silico analysis must be conducted during primer design to ensure minimal homology with sequences from flaviviruses such as JEV, DENV, ZIKV, and West Nile virus

(WNV). This involves multiple sequence alignment of GETV genomic regions alongside sequences of related viruses, followed by specificity testing using tools like BLAST.[6,8]

Furthermore, laboratory validation must include testing the assay against RNA extracted from other arboviruses, either as live viruses or synthetic constructs, to confirm the absence of non-specific amplification. Inclusion of both positive GETV controls and negative controls representing co-circulating viruses is essential during assay optimization.[9]

In conclusion, establishing a GETV-specific RT-PCR requires a combination of targeted genomic selection, careful primer/probe design, and rigorous specificity validation. A reliable assay not only supports early diagnosis during outbreaks but also aids in long-term epidemiological surveillance and control efforts, especially in endemic regions where multiple arboviruses co-exist.

2.2 Materials and Methods

Primers and probes

The full genome sequences of representative Getah viruses and other related alphaviruses were retrieved from the GenBank. The alignment of these sequences identified a conserved region on nsp1 gene to design primers and probes to detect Getah virus.

GETV-up primer: 5'-GGACGTGTGACATCACCGTT-3';

GETV-reverse primer: 5'-AGGAATGGGCTVTCAGCCTC-3';

GETV-Probe: 5'- ROX CAACCCAAATGAAGGTAACCGTGGACGT BHQ2-3'.

The PCR Amplicon size is 114 base pairs:

GGACGTGTGACATCACCGTTCGCTCTTTCTAGGATCCTTTGCTACTCCACATAGTGAG
AGACAAACAACCCAAATGAAGGTAACCGTGGACGTTGAGGCTGATAGCCCATTCCT.

Thermal cycling

One-step RT-PCR established was performed in a LightCycler® 480 II real-time PCR platform. Each reaction was performed in a 20µl final volume containing 10µl of extracted RNA or plasmid DNA and 10µl master mix with a final concentration of 1µM forward primer and reverse primer, 0.2µM 6-FAM probe, 0.2µM LCRed 640 probe. Thermal cycling consisted of one reverse transcription step, 18 high-stringency step-down cycles and 30 relaxed-stringency fluorescence acquisition cycles. The reverse transcription step was at 55°C for 15 min, followed by denaturation at 95°C for 2 min. The 18 high-stringency step-down thermal cycles were 6×1 sec @ 95°C, 12 sec @ 70°C, 8 sec @ 72°C; 9×1 sec @ 95°C, 12 sec @ 68°C, 8 sec @ 72°C; 3×1 sec @ 95°C, 12 sec @ 66°C, 8 sec @ 72°C. The relaxed-stringency fluorescence acquisition cycling consisted of 30×1 sec @ 95°C, 8 sec @ 52°C, 30 sec @ 67°C and 30 sec @ 72°C.

Table 2. The reaction mixes for PCR used in this study.

Taqman PCR	1X	12X (GETV-1)	12X (GETV-2)
dH2O	3.62	43.44	43.44
5X FRET Buffer	4.4	52.8	52.8
5X Oligo Mix	1.1	13.2	13.2
PCR Nucleotide mix	0.44	5.28	5.28
Taq (Dream) DNA Polymerase	0.44	5.28	5.28
Total	10	120	120

High-resolution melting analysis

After the FRET-PCR was completed, the melting curve analysis for probes annealing to the PCR products was determined by monitoring the fluorescence from 38°C to 85°C as described previously. Data were analyzed as 640 nm: 530 nm (F4/F1) fluorescence ratios, and the first derivative of F4/F1 (-d(F4/F1)/dt) was evaluated.

Specificity of the one-step RT FRET-PCR

The PCR products were sent to DNA sequencing to confirm the established PCR's specificity. The PCR was tested also on gBlock gene fragments of other flaviviruses.

Sensitivity of the one-step RT FRET-PCR

The gBlock gene fragments, containing the amplicon regions, were created (GenScript, Nanjing, China) to serve as positive controls and quantitative standards in this study. The nucleotide fragments were prepared as quantitative standards (10^3, 10^2, 10^1, 10^0, 10^{-1}copies molecules/ µl). The sensitivity was determined using serially diluted plasmids (10^4, 10^3, 10^2, 10^1, 10^0 , 10^{-1} copies of DNA/ 20 µl PCR reaction) to determine the detection limit of the established one-step RT-PCR that could be expected with clinical samples.

2.3 Results

The quantitative standards (10^3, 10^2, 10^1, 10^0, 10^{-1}copies molecules/ µl) based on gBlock gene fragment were used to determine that the sensitivity of the established PCR was found to be one copy of the target gene per reaction. The DNA sequencing confirmed that the sequences of the designed ones. The established PCR was found to be specific, and did not amplify gene fragments representing other related alphaviruses.

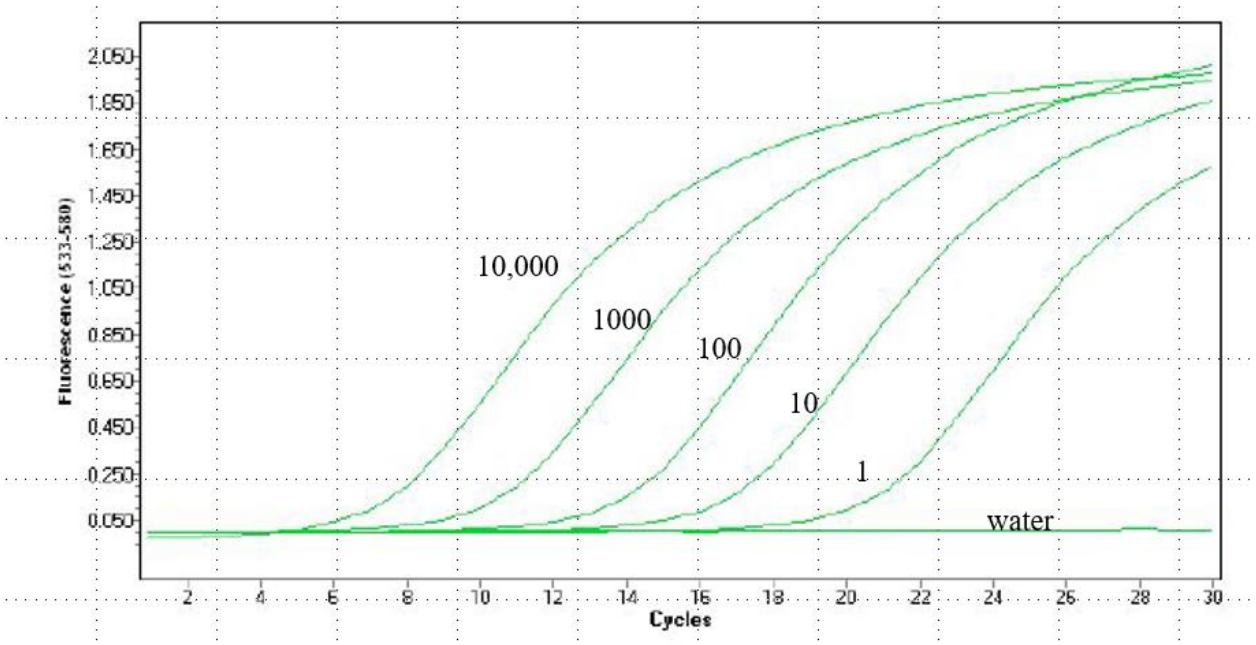

Figure 4. The sensitivity of qPCR to detect Getah virus. The plasmid quantitative standard (10^4, 10^3, 10^2, 10^1, 10^0 / reaction) containing sequence of Getah virus and the PCR negative control were detected by the one-step reverse transcription FRET-PCR established in this study.

2.4 Discussion

In this study, we successfully developed a one-step reverse transcription PCR (RT-PCR) assay for the specific detection of Getah virus (GETV). The assay demonstrates high sensitivity and specificity and offers several advantages over traditional two-step RT-PCR approaches. These features make it a promising tool for rapid diagnosis and surveillance of GETV in clinical and field settings.

One of the most notable strengths of our assay is its high sensitivity. The detection limit was determined to be as low as one copy of the gene target per reaction, underscoring its potential for early detection of GETV infections, especially in asymptomatic or low-viremia stages. High sensitivity is critical in the context of arboviral infections, where viral loads can vary widely depending on the stage of infection, host immune status, and sample type.

Another key advantage is the assay's high specificity. The primer and probe sequences were carefully designed based on conserved regions of the GETV genome, particularly regions with the lowest sequence homology to other alphaviruses. In silico analyses and laboratory testing confirmed that the assay did not amplify other closely related alphaviruses, including Chikungunya virus and Sindbis virus, which may co-circulate in some geographic regions. This high degree of specificity minimizes the risk of false positives, which is crucial for accurate diagnosis and for guiding timely control measures during outbreaks.

The use of a one-step RT-PCR format further enhances the practicality of this method. Unlike traditional two-step RT-PCR, which requires separate reverse transcription and PCR amplification steps, the one-step assay combines both processes in a single reaction. This streamlines the workflow, reduces the hands-on time, minimizes the risk of contamination, and makes the assay more suitable for high-throughput testing or resource-limited laboratories. In outbreak scenarios or field surveillance efforts, such operational efficiency is highly desirable.

Despite these advantages, the study has certain limitations that warrant attention. The primary limitation lies in the validation scope. The current validation was performed using synthetic gene targets, not RNA extracted from live or inactivated viral particles. While synthetic constructs are useful for assay optimization and preliminary sensitivity and specificity assessment, they do not fully replicate the complexity of actual clinical or field samples. The performance of the assay on total RNA extracted from infected biological specimens—such as blood, tissue, or mosquito pools—remains to be evaluated. The presence of PCR inhibitors, sample degradation, or viral RNA secondary structures could influence the assay's robustness in real-world applications.

Therefore, future work should focus on validating the assay using RNA extracted from cultured GETV and from field-collected samples. This would allow for a more comprehensive

assessment of the assay's performance, including its reproducibility, diagnostic accuracy, and utility in detecting GETV during natural infections or outbreaks. Additionally, broader testing against a larger panel of arboviruses—including flaviviruses such as Japanese encephalitis virus, dengue virus, and Zika virus—will further strengthen confidence in the assay's specificity and real-world applicability.

In conclusion, the one-step RT-PCR assay presented here is a highly sensitive, specific, and efficient method for the detection of GETV. While further validation using viral RNA from biological samples is necessary, the current findings represent a significant step forward in molecular diagnostics for GETV. Once fully validated, this assay has the potential to become a valuable tool for veterinary and public health surveillance, aiding in the early detection and control of GETV in endemic and at-risk regions.

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Chapter 3. Detection of Getah virus in mosquito and feral swine by qPCR

3.1 Introduction

Arthropod-borne viruses (arboviruses) continue to pose a global threat to human and animal health due to their ability to rapidly emerge and spread through complex vector-host dynamics. Among these, *Getah virus* (GETV) is a mosquito-borne virus that belongs to the genus *Alphavirus* within the family *Togaviridae*. First isolated in Malaysia in 1955, GETV has since been detected across several countries in Asia and parts of Australia. Although it is relatively less known compared to other alphaviruses such as Chikungunya virus or Sindbis virus, GETV has garnered increasing attention in recent years due to its expanding geographic distribution, growing host range, and its association with disease outbreaks in livestock.[1,2]

GETV is primarily transmitted by mosquitoes, including *Culex*, *Aedes*, and *Anopheles* species, many of which are widely distributed globally. The virus causes disease in a variety of animal hosts, including pigs, horses, cattle, and even some wild and zoo animals. Clinical signs in animals can include fever, skin rashes, infertility, and abortion, particularly in swine and equine populations.[3,4] Although GETV has not yet been confirmed as a significant pathogen in humans, serological evidence of GETV exposure in people has been reported in parts of Asia, raising concerns about its potential zoonotic risk.

Pigs are considered one of the primary amplifying hosts of GETV. Experimental and field studies have shown that infected pigs can develop viremia sufficient to infect mosquito vectors, thereby facilitating the maintenance and amplification of the virus in enzootic cycles. In regions such as China and Japan, where GETV activity is well-documented, outbreaks in pig farms have underscored the virus's potential to cause economic losses and impact animal health. As such, the

detection of GETV in pig populations is of epidemiological importance and may serve as an early indicator of its local transmission.[1]

Although GETV has been detected in various countries throughout Asia and Oceania, no cases or detections have been reported in the United States to date. However, several mosquito species known to be competent GETV vectors—such as *Culex tritaeniorhynchus*, *Aedes albopictus*, and *Aedes aegypti*—are present in many parts of the U.S.[1,2] Moreover, pig farming is a major agricultural industry across the country, creating the potential ecological conditions necessary for GETV transmission if the virus were introduced. Given the increasing globalization of trade and travel, as well as the documented ability of other exotic arboviruses (e.g., West Nile virus and Zika virus) to establish themselves in new regions, the risk of GETV emergence in the U.S. cannot be overlooked.[1,3]

Surveillance is a critical first step in assessing whether exotic arboviruses such as GETV are silently circulating in new environments. Molecular techniques, particularly reverse transcription polymerase chain reaction (RT-PCR), offer sensitive and specific tools for detecting viral RNA in both vectors and animal reservoirs.[5] In recent years, RT-PCR assays have been developed and refined for the detection of GETV, with some demonstrating the ability to detect extremely low levels of viral genetic material—down to a single copy of the target gene per reaction.[1,6,7] Such sensitivity is essential for early detection efforts, especially in areas where the virus may be present at low prevalence or circulating subclinically.

In this study, we employed a highly sensitive one-step reverse transcription PCR assay that we previously developed and validated for the detection of GETV. This assay was designed to amplify a conserved region of the GETV genome with a detection limit of one gene copy per reaction, and it was confirmed to be highly specific, showing no cross-reactivity with other related

alphaviruses or flaviviruses. The one-step format simplifies the workflow by combining reverse transcription and amplification in a single tube, reducing the potential for contamination and making it more suitable for routine surveillance applications.

The primary objective of this study was to apply this sensitive molecular assay to investigate the potential presence of GETV in the United States. Specifically, we sought to determine whether GETV RNA could be detected in field-collected mosquito populations and pig samples from selected U.S. locations. While there is currently no evidence to suggest that GETV is circulating in North America, this study represents a proactive approach to arboviral surveillance, driven by the need to detect potential incursions before they become established and harder to control.

Understanding whether GETV has silently entered or is being maintained in U.S. mosquito or swine populations is important not only for public and animal health preparedness but also for informing biosecurity policies and guiding future vector control strategies. Even in the absence of detection, baseline surveillance data such as those generated in this study contribute to a broader understanding of arbovirus ecology and can serve as a reference point for future investigations.

In summary, GETV remains an emerging alphavirus of veterinary and potentially zoonotic importance. Although no evidence currently links the virus to infections in the U.S., the widespread presence of competent mosquito vectors and susceptible animal hosts suggests that vigilance is warranted. By applying a highly sensitive and specific RT-PCR assay, this study aims to assess the possible presence of GETV in U.S. mosquitoes and swine, contributing valuable data to the growing global effort to monitor and manage emerging vector-borne viruses.

3.2 Materials and methods

Mosquito Collection and DNA Extraction

From 2019 to 2024, mosquito samples were collected in Auburn, Alabama, as part of a regional arboviral surveillance initiative under the Rickettsia felis Surveillance Program. Trapping sites were strategically selected to represent both human and veterinary public health risk areas and included local hospitals, farms, construction zones, public parks, residential neighborhoods, and Auburn University campus buildings.

Mosquitoes were collected during two periods: in October 2019 and again from June to September 2020. Two types of traps were used in parallel: four CO_2-baited BG-2-Sentinel traps (Biogents AG, Germany) and six UV light-baited New Standard Miniature Light Traps (John W. Hock Company, Florida, USA). Traps were deployed between 6:00 PM and 8:00 AM and positioned 3–4 feet above ground level in areas with high humidity and ample vegetation, which are conducive to mosquito activity.

A total of 2,575 adult mosquitoes were collected—560 in 2019 and 2,015 in 2020. Mosquitoes were transported live to the laboratory immediately after collection and either anesthetized at 4°C or stored at −20°C until further processing. Species identification was performed under a Nikon SMZ 800N stereo zoom microscope using morphological keys published by the Centers for Disease Control and Prevention (CDC) and the Walter Reed Biosystematics Unit. Specimens were sorted and pooled into groups of 8 to 15 individuals by species, collection site, and date, yielding a total of 275 mosquito pools. Additionally, 51 individual specimens were processed separately to confirm species identity using molecular methods. An additional 77 adult mosquitoes previously collected from Saint Kitts and Nevis were also included in this study.

Prior to homogenization, each mosquito pool was rinsed briefly in phosphate-buffered saline (PBS), followed by 70% ethanol to remove surface contaminants, and then rinsed again in PBS. Pooled mosquitoes were placed in 2 mL screw-cap tubes containing 1 mL of sterile PBS and 2.8

mm zirconia beads. Homogenization was carried out using a Precellys® 24 Tissue Homogenizer (Bertin Instruments) for two cycles of 20 seconds at 5,500 rpm. Homogenized samples were stored at −20°C until extraction of nucleic acids.

Total nucleic acids were extracted from 200 µL of homogenate using the High Pure PCR Template Preparation Kit (Roche Life Science) according to the manufacturer's protocol, with minor modifications. Samples were incubated with 200 µL of tissue lysis buffer and 40 µL of proteinase K (20 mg/mL) at 70°C for 10 minutes. After addition of ethanol, samples were passed through silica filter columns, followed by two sequential wash steps, and nucleic acids was eluted in 200 µL of elution buffer. The concentration and purity of nucleic acids were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific), and extracts were stored at −20°C.

To confirm DNA integrity and assess the quality of mosquito DNA extracts, a conventional PCR targeting the nsp1 gene was performed using both the universal Folmer primers and an in-house primer set. PCR products were purified using ExoSAP-IT™ (Applied Biosystems) and subjected to Sanger sequencing.

Feral Pig Sampling and Extraction of total nucleic acids

Feral pig (Sus scrofa) samples were collected from July 2019 to March 2020 in Bullock and Macon Counties, Alabama, as part of an interagency wildlife surveillance effort. Collection sites included private lands and the Tuskegee National Forest. Feral pigs were captured using remotely triggered corral traps constructed from steel fencing panels. Trapped pigs were euthanized following the guidelines of the American Veterinary Medical Association (AVMA) for humane animal euthanasia.

A total of 315 pigs were sampled. Three types of biological specimens were collected: whole blood (n = 276), kidney (n = 315), and spleen (n = 51). Samples were collected immediately post-mortem, placed on ice in sterile containers, and transported to the laboratory within 3 hours of collection. Solid tissues were stored in DNA/RNA Shield™ (Zymo Research) at 1:10 (w/v) ratio to preserve nucleic acids during transport and storage.

Nucleic acid extraction was performed using the High Pure PCR Template Preparation Kit (Roche) according to manufacturer instructions. For solid tissues, approximately 200–300 mg of kidney or spleen was homogenized in 1 mL of sterile PBS with zirconia beads using a Precellys® 24 homogenizer. For blood samples, 400 µL was used directly. The homogenates were digested with proteinase K and lysed with buffer at 70°C for 10 minutes. After ethanol precipitation, the lysates were processed through filter columns with wash steps, and nucleic acid was eluted in 200 µL of elution buffer. Nucleic acid samples were quantified using a NanoDrop spectrophotometer and stored at −20°C.

One-Step RT-PCR for Getah Virus Detection

A one-step reverse transcription PCR (RT-PCR) assay was developed to detect Getah virus RNA in mosquito and feral pig samples. Primers and a hydrolysis probe were designed to target a conserved 114 base pair region of the Getah virus genome, identified via alignment of publicly available sequences from GenBank using ClustalW. To ensure specificity, sequences were compared against closely related alphaviruses, including Chikungunya virus, Ross River virus, and Sindbis virus.

The primers and probe sequences were as follows:

- **Forward primer**: 5′-GGACGTGTGACATCACCGTT-3′

- **Reverse primer**: 5′-AGGAATGGGCTVTCAGCCTC-3′
- **Probe**: 5′-ROX-CAACCCAAATGAAGGTAACCGTGGACGT-BHQ2-3′

Each 20 µL RT-PCR reaction contained 10 µL of template RNA or positive control plasmid DNA, 5 µL of 4× TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems), forward and reverse primers (1 µM each), and the probe (0.2 µM). Amplification was performed on a LightCycler® 480 II Real-Time PCR System (Roche Diagnostics) under the following cycling conditions: reverse transcription at 55°C for 15 minutes; initial denaturation at 95°C for 2 minutes; followed by 18 high-stringency step-down cycles of denaturation at 95°C for 10 seconds and annealing/extension beginning at 65°C and decreasing by 1°C every two cycles; and 30 final cycles at 95°C for 10 seconds and 60°C for 30 seconds with fluorescence acquisition.

3.3 Results

All 1,626 mosquito pools and 315 swine samples tested negative for GETV RNA using the established RT-PCR assay. These findings suggest that, at the time and locations of sampling, there was no detectable circulation of GETV in the mosquito and feral pig populations in Alabama. This is the first study to employ molecular techniques for large-scale GETV surveillance in the United States (Tables 3 and 4).

Table 3. Evaluation of Getah virus in mosquitoes in this study

Collection	Year	Location	Mosquito ID	Pooled/Individual	Sample count
Phase 1	2019	Vet school campus, Dairy farm	Yes	Pooled	83
Phase 2	2020	Vet School	Yes	Pooled	218
		Vet school, Peet theatre, Chewacla and Arboratum, Town creek	Yes	Individual	208
		Chewacla Drive & Arboratum, Paces at the Estates, Town creak	No	Pooled	37
Outside of AU	2020	Ross University, St. Kitts	Not known	Not known	205
Phase 3	2024	Ridgewood, Equestrian	No	Individual	466
		Vet school, Ridgewood village, McKinley Ave, Lee Rd, Longleaf Dr.	No	Individual	409

Table 4. Evaluation of Getah virus in feral swine

Samples	Sample count	Getah virus PCR	Collection year	Location
Blood	276	00	July 2019 and March 2020	Privately owned tract of land, Bullock County; Tuskegee National Forest, Macon County
Kidney	315	00		
Spleen	51	00		
Total	642	00		

3.4 Discussion

This study represents the first large-scale molecular surveillance of Getah virus (GETV) in the United States, focusing on two key target populations: mosquito vectors and feral swine. Although all samples tested negative for GETV RNA, the significance of this work lies not in the detection of the virus, but in the foundational baseline it provides for the continued monitoring of emerging arboviruses in North America. Given the global emergence and expanding geographic range of mosquito-borne viruses in recent decades, proactive efforts like this are essential to strengthening the capacity for early detection, risk assessment, and response planning.

Getah virus, an alphavirus in the family *Togaviridae*, is endemic to parts of Asia and has been historically associated with febrile illness and reproductive losses in pigs, as well as outbreaks of fever and rash in horses and humans.[1,3,4] While GETV has not yet been reported in the Americas, the potential for introduction and establishment cannot be discounted, particularly given the international movement of animals and increasing global trade. In this context, our surveillance work provides important evidence that, at present, there is no detectable circulation of GETV among mosquitoes or feral pigs in Alabama, a state with abundant mosquito habitats, active livestock farming, and large populations of wild swine.

The negative results obtained in this study suggest that GETV has not yet been introduced into the southeastern United States. However, it is critical to note that the ecological conditions in this region could support transmission should the virus be introduced. Several mosquito species found in Alabama, including *Aedes* spp., *Culex* spp., and *Anopheles* spp., have been demonstrated to be competent vectors for GETV in laboratory experiments or implicated in natural transmission cycles in Asia. Furthermore, feral pigs, which are highly abundant and widely distributed across the southern U.S., could serve as potential amplifying hosts, just as domestic pigs do in Asia. The combination of suitable vectors and competent mammalian hosts indicates that, while GETV is currently absent, the region remains ecologically permissive for its establishment, should an introduction of the virus event occur.

This underscores the importance of continued surveillance. Monitoring programs should not be reactive but rather sustained and proactive, particularly in regions with high vector density and frequent animal-human interactions. In addition, the increasing incidence of zoonotic viruses such as West Nile virus, Eastern equine encephalitis virus, and Venezuelan equine encephalitis virus in the U.S. highlights the need for preparedness against alphaviruses that may not yet be

present but pose a credible threat due to shared vectors, climate suitability, and international connectivity.

While the findings of this study are informative, several limitations must be acknowledged. First, this surveillance effort employed only molecular detection methods, specifically RT-PCR targeting a conserved region of the GETV genome. While this method is highly specific and capable of detecting low levels of viral RNA, it is generally effective only during a narrow window of active viremia or virus presence in tissues. This is particularly relevant in field-collected samples, where the timing of infection relative to sampling is unknown. In contrast, serological assays, such as enzyme-linked immunosorbent assays (ELISAs) or virus neutralization tests, can detect past exposure through the presence of antibodies, offering a broader temporal window for surveillance. Unfortunately, no commercial ELISA kits currently exist for detecting anti-GETV antibodies, limiting the feasibility of sero-surveillance in this study. Developing accessible and validated serological tools should be a priority for future GETV surveillance efforts.

Second, while feral pigs and mosquitoes are major components of the natural transmission cycle of GETV, they are not the only relevant hosts. Horses are well-documented incidental hosts and have been affected by GETV outbreaks in multiple Asian countries.[1,8] Humans can also become infected, and while human disease is typically mild, it may go undiagnosed or misdiagnosed as other febrile illnesses. The present study did not include sampling from equine or human populations, representing a gap in our understanding of potential GETV exposure in the region. Future work should consider broader host surveillance, especially in areas with high densities of horses, wildlife, and outdoor human activity.

Third, this study was geographically limited to Alabama. Although the state provides a useful model for arboviral ecology in the southeastern U.S., the findings cannot be directly

extrapolated to other regions with different mosquito fauna, climate conditions, or host species distributions. Expanding surveillance to include additional states and ecological zones would improve our understanding of the potential for GETV emergence across the continental U.S. Multi-state collaborative efforts that combine molecular detection with serological surveillance across a range of hosts would offer the most comprehensive approach.

Finally, the samples in this study were collected over two field seasons, with mosquito collections focused in 2019 and 2020 and pig collections spanning 2019–2020. Viral circulation patterns may fluctuate seasonally or annually due to environmental factors, vector dynamics, or host immunity. Longitudinal studies across multiple years would help ensure that temporary absences are not misinterpreted as true freedom from infection.

In conclusion, while this study did not detect Getah virus in any mosquito or feral pig samples from Alabama, it contributes critical baseline data that enhances our understanding of the current status of GETV in the United States. The work highlights the importance of proactive surveillance, even in the absence of confirmed cases, and identifies key ecological and methodological considerations for future monitoring efforts. Given the presence of competent vectors and suitable hosts, the United States remains at theoretical risk for GETV emergence. Expanding surveillance to include other regions, additional host species, and serological testing would further strengthen our capacity to detect and respond to the potential introduction of this virus.

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