Epidemiology, Ecology, and Evolution of Emerging Arboviruses in Panama,

A One Health Approach

By

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Dedication

To all those who have suffered and will suffer from arboviral infections and their consequences and to my loving family.

Acknowledgments

This research has flourished through the guidance and support of many; from whom I have gained life lessons, resilience, friendship, collaboration, tenacity, integrity, theory, and technique. Foremost, among them, to my mentors Professors Christl A. Donnelly, and Nuno R. Faria: I am thankful for every idea, collaboration, life lesson and guidance you have provided during my DPhil journey. While I would like to highlight the unique contributions and support that each of you has brought to my academic journey and personal odyssey, space constraints allow me to only to list your names but each of you knows how important were your contributions in my personal and academic growth: Nikos Vasilakis, Amy Y. Vittor, Kathryn Hanley, Robert B. Tesh, Scott C. Weaver, Zulma Cucunubá, William Marciel, Kelly Charniga, Bernardo Gutierrez, Oliver Pybus, Janie Messina, Moritz Kraemer, Josefrancisco Galué, Carlos Lezcano, Luis Felipe Rivera, Yelissa Juarez, Xacdiel Rodriguez, Jesse Waggoner, Hilda Guzman, Joe Croock, Andrea Kastner, Heather Green, Patty Aguilar, Rolando Torres, Juan Miguel Pascale, Nestor Sosa, Adam Hendy, Mariana Garcia, Yaneth Pittí, Ingra Morales Claro, Filipe Romero Rebello Moreira, Ester Sabino, Ben Lambert, Rene Pereyra Elias, Caterina Felici, Carmella Gaston, Darci R. Smith.

My profound appreciation extends to Gorgas Memorial Institution, my host institution in Panama, where I have undertaken all laboratory experiment and managed all field collections. I also want to acknowledge, The Panamanian National Secretary of Science, SENACYT-Panamá (Grants: FID-16-201, FID-080, FID-2021-160) and CREATE-NEO-CREID-NIAID From the National Institutes of Health from The United States of America (grant:1U01AI151807) and The Clarendon Scholarship from Oxford University, for their generous funding that propelled my research and DPhil forward. Last but not least, Lincoln College, you have not only facilitated my DPhil journey, with scholarship and support, but have also provided me with a family that enriched my days with tea, cake, majestic gowns, exquisite dinners, harmonious concerts, Emily Carr parties, barbeques, and camaraderie. The memories crafted amid the historic walls of Oxford will remain etched in my soul. To this institution and the city, I bid a fond adieu, carrying a treasure trove of memories… Oxford I will miss you!!!

Abstract

Epidemiology, Ecology, and Evolution of Emerging Arboviruses in Panama, A One Health Approach

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This thesis investigates the emergence and increased incidence of Madariaga virus (MADV) and Venezuelan equine encephalitis virus (VEEV) in Panama, two arthropod-borne zoonotic pathogens that have historically been associated with equine and human disease respectively. In particular, the 2010 outbreak in the Darien, eastern Panama, marked a significant rise in human and equine neurological infections, highlighting the concurrent circulation of MADV and VEEV. The central hypothesis posits these viruses as emerging threats, with their increased incidence linked to ecological shifts in potential vertebrate hosts and mosquitoe vectors. The objectives of this thesis are five-fold and encompass evaluating the emergence of MADV and VEEV in Panama, assessing post-2010 incidence, developing real-time diagnostic tools for enhanced molecular surveillance, elucidating the roles of different host and vector species in their transmission, and implementing a One Health approach for active arboviral surveillance in the Darien Gap located in the isthmus of Panama, connecting North and South American continents. Across multiple chapters, this thesis explores transmission dynamics, phylogenetics, immune responses, molecular diagnostics, hosts and mosquitoe vectors, and a proposes a proactive pathogen surveillance strategy to enhance our understanding of emerging arboviruses and improve preparedness against future outbreaks.

Statement of contribution and associated publications

My thesis comprises articles that have been published in peer-reviewed academic journals or are currently in submission. These articles cover a wide range of topics and stem from collaborative work involving members and collaborators of The Gorgas Memorial Institute of Health Studies in Panama, Imperial College London, the University of Texas Medical Branch, Emory University, the University of Florida, and the University of Oxford. Although detailed author contributions are outlined after each article, the following is a summary of my contributions to each chapter.

Chapter 1 – Endemic and Epidemic Human Alphavirus Infections in Eastern Panama: An Analysis of Population-Based Cross-Sectional Surveys

This chapter consists of a publication accepted and published in final form in the *American Journal of Tropical Medicine and Hygiene*. I was joint first author (with ZMC) and responsible of study design, funding acquisition, project administration, recruitment strategy, laboratory procedures, and data collection prior to my DPhil studies. In Oxford I have conducted data analysis, modelling (with ZMC, BL), interpretation of results (with ZMC, BL, CAD), and writing of the manuscript (with ZMC, CAD). The article can be accessed at the following link: https://www.ajtmh.org/view/journals/tpmd/103/6/article-p2429.xml

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Chapter 2 – Clinical and Serological Findings of Madariaga and Venezuelan Equine Encephalitis Viral Infections: A follow up Study 5 Years After an Outbreak in Panama

This chapter consists of a publication accepted and published in final form in the *Open Forum Infectious Diseases Journal*. I was joint first author (with YP). I was responsible of study design, funding acquisition, project administration, recruitment strategy, laboratory procedures, data collection prior my DPhil studies. In Oxford I have conducted statistical analysis, interpretation of results and writing of the manuscript. YP undertook all laboratory experiments. The article can be accessed in the following link. https://academic.oup.com/ofid/article/7/9/ofaa359/5894900

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Chapter 3 – Real-time RT-PCR for Venezuelan equine encephalitis complex, Madariaga, and Eastern equine encephalitis viruses: application in human and mosquito public health surveillance in Panama

This chapter consists of one publication that is accepted and published in final form in the *Journal of Clinical Microbiology*. I was responsible for the study design, funding acquisition, project administration, recruitment strategy, mosquito collections, laboratory procedures, and data collection before my DPhil studies. I also established prospective febrile surveillance to detect active Venezuelan equine encephalitis and Madariaga infections in the Darien region during my DPhil. In Oxford I have conducted phylogenetic analysis, interpretation of results and wrote the original draft of the manuscript (with edits from JJW and NRF). Article can be accessed in the following link: https://journals.asm.org/doi/10.1128/jcm.00152-23

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Chapter 4 – Madariaga and Venezuelan equine encephalitis virus seroprevalence in rodent enzootic hosts in Eastern and Western Panama.

This paper has been accepted with minor revisions in the *American Journal of Tropical Medicine and Hygiene*. I was joint first author with JFG who undertook laboratory experiments and data curation. Study design, funding acquisition, project administration, recruitment strategy, rodent collections, and data collection were undertaken by LCC. In Oxford, I conducted statistical analysis, interpreted of results, and wrote the original manuscript.

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Chapter 5 – A One Health surveillance to detect active arboviral circulation and potential host and vectors.

This chapter involves a multicentric, multidisciplinary collaborative effort. I was responsible for study design, funding acquisition, project administration, recruitment strategy, Trinidad trap design, mosquito collections, laboratory procedures, genome sequencing, and data collection before my DPhil studies. During my DPhil studies I have undertaken additional mosquito and hamster testing with a newly developed Real-Time- RT-PCR, I also have conducted phylogenetic analysis, mosquito and host analysis, and interpretation of results and writing of the manuscript.

In addition to the articles above there are six other published articles on epidemiology and evolution of emerging infectious diseases that I have undertaken as collaborations during my

DPhil. All of the references to these articles in their final form have been added as appendices to this document.

As Jean-Paul's supervisors, we hereby certify that the statement of contribution listed above is a fair representation of his work.

Professor Nuno R. Faria

Professor Christl A. Donnelly

January 12, 2024

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Introduction

1. Arboviruses

Arthropod-borne viruses (arboviruses) are a diverse group of viruses with world-wide distribution that are maintained in nature through biological transmission mediated by infection and replication within hematophagous arthropods, such as mosquitoes, ticks, sandflies, midges, and susceptible vertebrate hosts[1,2]. This designation comprises a heterogenous group that include taxa from a wide variety of RNA viruses: Togaviruses, Flaviviruses, Bunyaviruses, Nairoviviruses, Thogotoviruses, Orbiviruses, Reoviridae and the Asfaviruses, the only known DNA arbovirus species[1]. Today, more than 500 arboviruses are recognised, with approximately 100 being recognised as human pathogens[3]. The term arbovirus was first coined early in the 1940s by laboratory researchers when referring to animal viruses transmitted to a vertebrate host by a hematophagous arthropod vector[2,4], but the history of arboviral disease research dates back much further[5,6]. According to the World Health Organization (WHO), arboviruses are defined as viruses that replicate and are transmitted by a hematophagous arthropod to a vertebrate host, also including direct transmission of these agents[2,4].

Early discovery of arboviruses relied heavily on observation and experimentation[7,8]. Yellow fever virus (YFV) was the first documented arbovirus in the 17th century[5–8]. In the late 19th and early 20th centuries, scientists including Carlos Finlay and Walter Reed confirmed that a mosquito transmitted yellow fever, revolutionizing the understanding of arboviral diseases[5,6]. When an arboviral outbreak occurred, scientists would typically isolate the suspected virus from the blood or tissues of infected patients. Cell culture and animal inoculation were instrumental in this process[6]. The advent of electron microscopy in the 1930s allowed for the direct visualisation of viruses[3,4,6–8]. In the later part of the 20th century, molecular methods such as Polymerase Chain Reaction (PCR) and DNA sequencing revolutionized arbovirus discovery and detection by enabling the identification and characterisation of viral genetic material[6,7].

The Rockefeller Foundation played a seminal role in the early discovery and study of arboviruses[5–8]. In the early 20th century, it funded important research programs to control yellow fever[5,6]. Max Theiler, supported by the Foundation, developed the first effective vaccine for yellow fever in 1937, for which he was awarded the Nobel Prize in Physiology or Medicine

in 1951[5–8]. Through the International Health Division, the Rockefeller Foundation also established laboratories in various countries to conduct field research on arboviruses and supported scientists who made remarkable contributions to the understanding and control of these diseases[5–7].

Arbovirus classification and taxonomy

Initially, arboviruses were classified based on the type of arthropod vector, geographical distribution, and clinical manifestations[7]. Arbovirus classification was developed by Jordi Casals and others at the Rockefeller Foundation using the complement fixation test originally developed for *Mycobacterium tuberculosis* investigations, and later, confirmation by neutralization tests[7]. Arboviruses were classified by a distinction in antibody cross-reactivity of fourfold or more in dual directions, meaning the difference between the heterologous and homologous antibody titres from two distinct viruses[7,9]. A discrepancy of fourfold or more in a single direction indicated a subtype. Meanwhile, antigenic variants were only discernible using specific serological evaluations, such as kinetic hemagglutination inhibition techniques[9,10]. Subsequent development of the DNA amplification by PCR, sequencing techniques and phylogenetic studies of arboviruses greatly contributed to arbovirus classification[6,7,11].

Arbovirus ecology, epidemiology, and transmission cycles

Arboviruses reside within complex ecological systems involving interactions between the virus, the arthropod vector, and the vertebrate host[10]. The prevalence of arboviruses is influenced by various ecological factors such as climate, habitat, vector density, and host availability. For instance, climate change has been associated with shifts in the geographic distribution of arboviral diseases due to alterations in vector habitats[12–14].

Mosquitoes and ticks are the primary vectors for arboviruses. These vectors have distinct breeding and feeding habits which influence the ecology of arboviruses. Mosquitoes, for example, breed in stagnant water and are, therefore, more prevalent in humid and wet environments[15]. Ticks, on the other hand, tend to thrive in wooded and grassy areas[16]. Uncovering the habitats and behaviour of these vectors is crucial for better understanding the epidemiology of arboviral diseases[15,16].

The transmission of arboviruses generally involves cycling between arthropod vectors and vertebrate hosts. There are several different transmission cycles including urban, sylvatic or enzootic, and epizootic or rural cycles. Dengue (DENV), yellow fever (YFV), Zika virus (ZIKV), and chikungunya viruses (CHIKV), among others, are arboviruses that are typically associated with urban transmission cycles[17,18]. In the urban cycle, humans are the primary vertebrate hosts, and arboviruses are transmitted between humans and urban-adapted vectors, such as *Aedes aegypti* and *Aedes albopictus* mosquitoes[17,18] as shown in **Figure 1.**

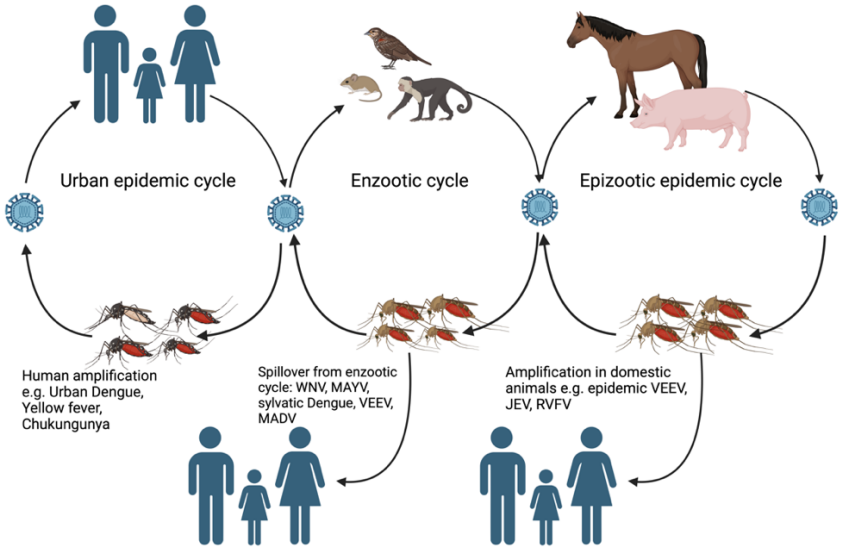

Figure 1. Arbovirus transmission Cycles. *Arboviruses typically exhibit three primary transmission cycles: urban, enzootic (or sylvatic), and epizootic (or epidemic). Urban cycle: in this cycle, humans are the primary vertebrate host and direct human-vector transmission occurs (e.g. urban DENV, YFV, ZIKV and CHIKV). Enzootic cycle: spillover to humans occurs when enzootic vectors or bridge vectors bite wild animals and humans, e.g., West Nile virus (WNV), Mayaro virus (MAYV), Madariaga virus (MADV) and enzootic Venezuelan equine encephalitis virus (VEEV). Epizootic cycle: secondary amplification in domestic animals leads to increased exposure and large epidemic in human and animal populations, e.g., epizootic VEEV*[19,20]*, Japanese encephalitis virus (JEV)*[21] *and Rift Valley fever virus (RVFV)*[22]*. The figure was constructed using BioRender (https://www.biorender.com).*

In the sylvatic or enzootic cycle, arboviruses are transmitted between non-human vertebrates, such as rodents, birds or monkeys, and arthropod vectors, typically in a forest or jungle environment[10,17]. This cycle is primarily responsible for maintaining active circulation of arboviruses in nature. Enzootic or epizootic bridge vectors mediate spillover infections between wild vertebrate hosts and humans[17,19,20], e.g., West Nile virus (WNV)[23], Mayaro virus (MAYV)[24], enzootic VEEV[19,20] and MADV[25,26].

Epizootic cycles refer to a phase where pathogens, primarily transmitted among wild animals, experience secondary amplification within domestic animal populations facilitated by what are known as bridge vectors[19,20]. Bridge vectors are vectors that can transfer pathogens from one species to another, bridging the gap between wild and domestic transmission cycles[19,20]. In this context, human infections are often a consequence of spillover events where humans are exposed to the pathogen due to their proximity or interaction with affected domestic animals[19,21,22].

VEEV is predominantly an arbovirus transmitted among equines, but during epizootic outbreaks, the virus can be transmitted to humans by mosquitoes that have bitten and fed upon infected equines[19,20,27]. These mosquitoes act as bridge vectors, transmitting the virus from the primary equine hosts to humans[19,20,27]. Similarly, Japanese encephalitis virus (JEV) is primarily maintained in a cycle involving wading birds and *Culex* spp. mosquitoes in rural rice-growing and pig-farming communities[21]. However, during its epizootic phase, the virus can be introduced into human populations, often due to bridge vectors that have fed upon infected birds or pigs[21]. Rift Valley fever virus (RVFV) offers another illustrative example. Under normal circumstances, RVFV is transmitted among wild animals by *Aedes* spp. mosquitoes[22,28]. However, during periods of heavy rainfall, the number of these mosquitoes can surge, leading to an increased transmission among domestic animals like sheep, cattle, and goats. Humans can then contract the virus through direct contact with infected animals or via bridge vectors that have bitten these animals[22,28]. The increased exposure of humans to these viruses during their epizootic or epidemic cycles has historically been associated with larger, more widespread outbreaks[19,21,28,29]. Recognizing and understanding these cycles is crucial for predicting and mitigating potential epidemics, particularly in regions where these diseases are endemic or have the potential to emerge and cause epidemics in human populations[29].

Arbovirus epidemiology

Arboviruses exhibit a broad global distribution, yet their prevalence demonstrates a notable heterogeneity influenced by geographical parameters, climatic conditions, and vector proliferation[10,12,13]. Dengue fever, attributable to DENV, manifests as an endemic disease in over 100 countries, mostly in the tropics and subtropics[30]. WNV, originally discovered in Africa, has expanded its range to encompass regions including Europe, the Middle East, and the Americas[31]. This widespread dispersal accentuates the inherent diversity and adaptability intrinsic to arboviruses and their associated vectors[10].

The evolution of arboviruses is closely linked to their interactions with both vertebrate hosts and arthropod vectors[10]. Genetic modifications within the arboviral genomes, which may confer the ability to infect and replicate within novel host species or vectors, can potentiate the geographical dispersion and emergence of arboviral diseases[10]. A case in point is the CHIKV's genetic adaptation to the *Aedes albopictus* mosquito, a factor implicated in its global transmission[32]. Furthermore, the evolutionary dynamics of arboviruses is modulated by specific ecological determinants[10]. Variation in climate and land utilization patterns influence the geographical distribution and population density and distribution of vectors[12,13]. Fluctuations in factors like temperature, precipitation, and relative humidity govern mosquito breeding and longevity, with downstream implications on the transmission dynamics of arboviruses[12,13]. From an epidemiological perspective, population immunity emerges as a key driver of arbovirus transmission[33]. Regions characterized by naïve populations or low immunity levels against a designated arbovirus exhibit heightened vulnerability to outbreaks and subsequent epidemic spread[33].

Human mobility also plays a crucial role in the transmission and spread of arbovirus infections. Arboviruses, which are primarily transmitted to humans through the bites of infected arthropods, such as mosquitoes, are significantly influenced by patterns of human movement[34,35]. As viremic individuals travel, whether for tourism, business, forced migration or displacement, they can inadvertently introduce arboviruses to new regions by acquiring infections in areas where these viruses are endemic[34]. Moreover, long-distance anthropogenic transport of adult mosquitoes, larvae and pupae can further contribute to the expansion of vectors into novel locations[36–38]. Human mobility and transport activities can thus facilitate the introduction and establishment of mosquitoes vectors and novel arboviruses in non-endemic areas. Furthermore, human mobility within endemic regions can exacerbate the transmission of arboviruses[34,35,39]. Infected individuals may carry the virus from one location to another, leading to the dispersal of arboviral strains. In regions where arboviruses are endemic, local movement within and between communities can contribute to the rapid transmission of diseases like dengue, chikungunya, and Zika[40].

Surveillance and public health measures are put in place to mitigate the impact of arbovirus transmission, which occurs due to a variety of factors mentioned above. These strategies can include vector control, education on preventive measures, and early detection of cases to prevent the introduction and spread of arboviruses in new areas[39]. Understanding the dynamic

relationship between human mobility and arbovirus transmission is vital for the development of robust public health policies and interventions to manage and control these infectious diseases[34,39–41].

Arbovirus diagnostic and laboratory surveillance

For decades, serological tests, particularly the Enzyme-Linked Immunosorbent Assay (ELISA) and the Plaque Reduction Neutralization Test (PRNT), have been at the core of arbovirus diagnosis[7,42]. These tests detect virus-specific antibodies like IgM and IgG, and measure neutralizing antibodies, respectively[42]. However, cross-reactivity among related viruses sometimes hampers the accuracy of serological tests[43].

On the other hand, virus isolation in cell culture has long been hailed as the gold standard in arbovirus diagnostics[42]. This technique identifies isolated viruses through various methods, such as observing cytopathic effects, employing immunofluorescence, or leveraging molecular methods. But its demanding nature and time-consuming processes mean this technique is not always the go-to method for swift diagnosis[44].

Serological monitoring includes the detection of immunological markers to evaluate history of past exposure to arboviruses. Serological surveys can help assess the risk of future outbreaks and reconstruct past transmission dynamics[45,46]. It involves testing blood samples for antibodies against specific viruses indicating past or recent infection[46]. Examples of typical serological tests for arboviruses conducted in public health laboratories include PRNT, enzyme ELISA and hemagglutination inhibition test HI.

The advent of the Reverse Transcription Polymerase Chain Reaction (RT-PCR) brought a more sensitive and specific means to detect arbovirus RNA[42]. By converting viral RNA to complementary DNA (cDNA) and amplifying targeted genetic markers, this method has become indispensable in diagnostic laboratories worldwide. The more advanced real-time RT-PCR, which amplifies and detects genetic material concurrently, is one of the most used diagnostic methods to detect arboviral RNA in public health laboratories worldwide[42]. In public health laboratory settings, molecular nucleic acid amplification tests, such as RT-PCR, are routinely used for the direct detection of viral RNA in samples collected during the acute phase of infection[47]. This is particularly useful for the identification of known emerging or re-emerging arboviruses[48].

Emerging tools like Loop-Mediated Isothermal Amplification (LAMP) offer hope for resource-challenged settings. As a nucleic acid amplification technique, LAMP is a rapid, isothermal nucleic acid amplification technique and operates at a constant temperature, eliminating the need for thermal cyclers[49]. With ongoing development, LAMP assays could soon provide a cost-efficient alternative for diagnosing arboviruses[49]. Lastly, the accessibility of Rapid Diagnostic Tests (RDTs) has been a game-changer, especially in areas with limited resources[50]. These lateral flow assay-based tests yield results in about 30 minutes[50]. Although they might not be as sensitive or specific as laboratory-based counterparts, they are particularly useful in diagnosing arboviruses like DENV[43,51].

More recently, the rise of next-generation sequencing (NGS) technologies has uncovered a new era in viral diagnostics[6]. NGS technologies has brought about a transformative era in viral diagnostics and research. NGS provides a rapid and cost-effective sequencing of viral genomes, facilitating the study of viral genomics, evolution, and pathogenicity[52]. It also allows for metagenomic analysis of clinical samples, aiding in the identification of multiple viruses without prior knowledge[53]. NGS plays a crucial role in developing diagnostic assays, epidemiological surveillance, and response to viral outbreaks[52–54]. As NGS technologies advance, their impact on viral diagnostics and research is expected to grow even further[52,54].

Targeted genome sequencing focuses on partial or whole genome sequencing of known pathogens, enabling researchers to undertake genomic epidemiology studies, functional or evolutionary studies of known pathogens[53]. In contrast, untargeted pathogen genome sequencing, typically refers to agnostic sequencing of genetic material present in a sample without a priori knowledge of the pathogen causing infection. This method is also referred to as metagenomic and is a valuable tool for virus discovery and identification of emerging pathogens[53]. While the choice between these approaches depends on the specific research goals, available resources, and the level of detail required for the analysis, rapid untargeted sequencing of viral genomes is especially advantageous for detecting novel or divergent virus strains[6,55]. However, untargeted sequencing approaches are not yet routinely used in public health laboratories[53].

Arbovirus mechanics of evolution and emergence

Central to arbovirus genetic diversity is their rapid evolution predominantly driven by the rapid pace of viral mutations accumulated across viral genomes. RNA-based arboviruses like DENV,

YFV, ZIKV, and CHIKV lineages accumulate on average around 2×10^{-4} subs/nt/year to 8×10^{-4} subs/nt/year across their genomes per year[56,57]. This is attributed to the inherent inability of their RNA-dependent RNA polymerases (RdRp) to proofread, culminating in frequent errors during genome replication[58]. As a result, these viruses typically manifest as quasispecies, a complex mixture of genetically related variants[59]. Such vast genomic diversity can influence vector preference, disease progression and viral pathogenesis, as virus variants with advantageous mutations may gain prominence in particular environmental pressures[59]. Additional drivers for arbovirus evolution include recombination and reassortment[58]. While recombination involves genetic material exchange between two viral genomes, reassortment deals with segment swapping and occurs in segmented viruses[58]. Both mechanisms create new genetic concoctions that can lead to phenotypically distinct viral strains[58].

Extrinsic selective pressures, emanating from sources such as host immunity, vector competence, and environmental dynamics, also play important roles in arbovirus evolution[10]. Positive selection can select mutations that increase viral fitness parameters, like replication efficiency or immune evasion[10,60]. A pertinent example are mutations in the envelope glycoprotein 2 (E2) gene of VEEV ID strains, which were linked to its increased virulence and viremia in South America[20,27,61]. In contrast, purifying selection tends to weed out detrimental mutations. Another facet of arbovirus evolution is genetic drift, particularly important during vector-host transitions, known as population bottlenecks[62]. Such events can cause arbitrary shifts in viral genetic landscapes, potentially resulting in fixation of mutations at the population-level[62].

Overall, the evolutionary dynamics of arboviruses involve a complex interplay of genetic, host, ecological, and environmental factors[10,60]. A detailed understanding of these dynamics is important to anticipate and counteract the emergence of new arboviral threats[60]. This knowledge can support the strengthening of surveillance infrastructures and promote the creation of vaccines and adaptive treatments that take into account the evolutionary processes of arboviruses[10,60].

Arbovirus surveillance strategies

Traditional surveillance methods for arboviruses encompass several techniques, each with its distinct advantages and limitations. Passive surveillance relies on the routine reporting of arbovirus cases by healthcare providers to public health authorities[63]. Although cost-effective,

its efficacy can be compromised due to underreporting and communication delays. In contrast, active surveillance represents a more proactive approach[63]. Public health authorities actively gather data, screening specific populations and consistently collecting and testing samples from both humans and vectors[63]. Despite being resource-intensive, active surveillance strategies typically provide more timely and reliable data[63]. Moreover, sentinel surveillance further refines data collection by focusing on a network of selected reporting sites, such as hospitals or clinics[64]. While it produces data of superior quality compared to passive surveillance, its scope may not fully represent the broader population[64].

Arbovirus vector surveillance strategies

In the field of arbovirus control, vector surveillance stands paramount[65,66]. It involves detailed tracking of the abundance and distribution of vectors, primarily mosquitoes and ticks, coupled with testing for viral presence[65]. Such data is critical to identify key vector species involved in arbovirus transmission, transmission risks and strategize vector control measures[65,66].

Technological advancements in arbovirus surveillance

Remote sensing and Geographic Information Systems (GIS) have also become important tools for arbovirus surveillance and research in endemic areas[67,68]. These technologies allow for mapping of cases, mosquito vectors, reservoirs distributions together with environmental data, such as temperature and rainfall, which affect vector competence, distribution, viral replication and transmission[12,67]. Furthermore, the use of digital data offers new possibilities for surveillance and response to arbovirus outbreaks. This includes real-time monitoring of Internet user queries, search for news and social media usage patterns[69]. For example, dengue and chikungunya disease monitoring using near real-time Google Trends search query data has revealed promising results in monitoring transmission of these diseases[70,71].

One Health approach in arbovirology

Arboviruses and therefore arbovirus transmission cycles comprise a variety of key factors, such as vector population, host population, climate, etc., that influence these dynamics[12]. Arbovirus surveillance and outbreak response require a unifying approach capable of integrating information from different sources. This is essential for preventing and responding effectively to arboviral threats. In this regard, the One Health approach is an important integrated, targeted, and unifying strategy that aims to achieve sustainable balance and optimize the health of

humans, domestic animals, wild animals and ecosystems[72]. Such broadened surveillance aids in discerning arbovirus circulation in its early stages, precluding potential human outbreaks[73].

Whitin the One Health paradigm, it becomes imperative to monitor shifts in climate, vegetation, and land use, as these could precipitate arbovirus outbreaks[74]. Combination of environmental and ecological data may help to refine predictive or distribution models for both arboviruses and zoonotic viruses[75]. For a holistic surveillance system, compilation of data across human, animal, and environmental health sectors is pivotal. This integrative approach requires collaborations across diverse disciplines, including epidemiology, veterinary sciences, geography, climate, virology, zoology, ecology, among others[76].

Although the One Health approach is a promising strategy in arbovirology and, more generally, pathogen surveillance, its implementation is challenging[76]. Challenges encompass logistical obstacles, budget constraints, and potential communication gaps between sectors[72,76]. Greater emphasis on capacity building and collaborative surveillance is necessary in endemic areas or hotspots of arbovirus outbreaks[77], which will be key to implement a successful One Health approach and potentially improve the detection and control of arboviral threats[78].

Technological innovations, such as portable untargeted genomic sequencing and cross-source data analysis, promise to revolutionize surveillance to improve public health decision-making[79]. However, standardised frameworks for One Health data, metadata and analysis remain poorly defined[72,76].It is also vital that training and capacity-building initiatives focus on interdisciplinary One Health competencies, complemented by public policies that reinforce integrated surveillance initiatives[78].

Arbovirus vaccine development

The development of effective vaccines has become a critical long-term strategy to protect populations at risk of contracting arboviruses. However, the pace of vaccine development varies for different arboviruses. For example, the yellow fever vaccine, established in the 1930s, stands out as one of the most effective countermeasures against arboviruses, providing long-lasting immunity after a single dose[80].

In contrast, DENV vaccine development encounters additional challenges, notably the imperative to confer protection against its four distinct serotypes[81]. Although the CYD-TDV

vaccine (Dengvaxia) has received licensure in multiple nations, its successful implementation is challenged by several factors, including the elevated risk of severe dengue in seronegative recipients[81,82]. This risk might arise from the vaccine's ability to simulate a silent primary DENV infection, potentially escalating the severity upon a subsequent natural viral exposure[81–83]. Moreover, the intricate nature of DENV immunity poses additional challenges in inducing balanced immune defences against multiple serotypes[81]. The phenomenon of antibody-dependent enhancement (ADE) further complicates the DENV vaccine development pathway[84].

Although there are not licensed ZIKV vaccines, several candidates have been developed, including those bases in mRNA platform and live-attenuated vaccines[85]. Some of these have progressed to phase 2 clinical evaluations[85,86]. Similarly, CHIKV vaccine efforts span from virus-like particles and live-attenuated forms to subunit vaccines[86], with several vaccine candidates demonstrating potential in preliminary clinical studies[87].

Chikungunya licensed vaccine

The CHIKV (VAL1553) Valneva vaccine received FAD approval in December 2023. This represents a promising advancement in the field of virology. The VAL1553 vaccine provides effective protection against CHIKV, a mosquito-borne alphavirus responsible for debilitating febrile illness in humans[87]. Developed through an innovative recombinant DNA technology, this vaccine employs a virus-like particle (VLP) with a recombinant CHIKV strain with a nsP3 deletion. Proteins are synthesized and self-assembled to mimic the native virus's morphology without the genetic material necessary for replication[88]. This vaccine expresses CHIKV envelope proteins, specifically the E1 and E2 glycoproteins, to induce a robust immune response. Through rigorous preclinical and clinical trials, the vaccine has demonstrated efficacy in eliciting both humoral and cellular immune responses[87].

Alphavirus encephalitis vaccines and control strategies

In terms of control and prevention, prompt vaccination of equines in regions with a history of VEE is vital, with the presently available live-attenuated TC-83 vaccine being the most effective strategy[19]. Nonetheless, swift responses to outbreaks are often hampered by inadequate surveillance systems[19,20]. For human populations, personal protection measures including the use of repellents containing N, N-Diethyl-meta-toluamide (DEET) and clothing treatments with Permethrin are recommended[89], while certain common control methods are

deemed unsuitable due to the behaviour of enzootic VEEV vectors[90]. A promising trivalent vaccine targeting WEEV, VEEV, and EEEV has recently undergone phase-one testing, demonstrating encouraging results in both toxicity and immunogenicity for the future prevention and control of equine encephalitis[91].

The significant economic costs associated with vaccine research and development often clash with the fact that arboviral epidemics occur mostly in low-income nations, underscoring the need for sustainable, equitable and regional financial strategies for vaccine development[92,93].

Vector surveillance and control strategies

Vector control is a fundamental component of arbovirus control. Vector control strategies include the use of insecticides, removal of breeding sites, and biological control using, for example, Wolbachia-infected mosquitoes to reduce competent vector populations[94].

Mosquito traps and surveillance strategies play a crucial role in monitoring and controlling arbovirus vectors, which are responsible for transmitting diseases such as dengue, Zika, and chikungunya. One commonly used mosquito trap is the CDC light trap, which utilizes a light source to attract mosquitoes into a collection bag[95]. Another effective method is the gravid trap, designed to target female mosquitoes seeking oviposition sites by using water containers as attractants[96]. Additionally, BG Sentinel traps incorporate a combination of attractants, including carbon dioxide and specific mosquito attractants, enhancing their efficacy. Furthermore, ovitraps strategically placed in potential breeding sites allow for the collection of mosquito eggs, providing valuable information on vector distribution[97].

Surveillance systems for detecting arbovirus cases and monitoring vector populations are crucial. These systems enable the implementation of timely control measures during outbreaks[94]. Educating communities about arbovirus transmission and promoting preventive behaviours, such as using bed nets are essential to reduce human vector contact, eliminate breeding sites, and reduce mosquito reproduction[98,99].

Cross-border collaboration for arbovirus surveillance and control is vital, as vectors, reservoirs and viruses do not respect political boundaries[100]. International cooperation can facilitate the sharing of surveillance data, technical expertise, and resources[101].

Vector control strategies

Controlling mosquito populations during arbovirus outbreaks is imperative for preventing the transmission of diseases[102,103]. One commonly employed strategy is the application of insecticides, with pyrethroids being among the most widely used due to their efficacy and relatively low toxicity to humans[102,103]. Ultra-low volume (ULV) space spraying is a common method, involving the release of small droplets of insecticide into the air to reach flying mosquitoes. Additionally, larvicidal treatments targeting mosquito breeding sites, such as standing water containers, employ insect growth regulators or microbial agents to disrupt the development of mosquito larvae[102,103]. Integrated vector management (IVM) strategies combine various control measures, including community engagement, source reduction, and the use of insecticide-treated bed nets and screens[103]. The deployment of genetically modified mosquitoes, such as those with reduced vector competence or sterile males, and mosquitoes infected with the symbionts Wolbachia is also under exploration[104,105], with encouraging results[106]. The successful control of arbovirus vectors during outbreaks requires a comprehensive, multidisciplinary approach that addresses both adult mosquitoes and their breeding habitats, minimizing the risk of disease transmission[103]. Regular monitoring and adaptation of control strategies based on vector surveillance data are essential components of effective vector control programs.

2. Alphaviruses

The Alphaviruses (*Alphavirus*, *Togaviridae*) encompass a group of enveloped and single-stranded RNA viruses with 11 to 12 kilobase genomes that encode four non-structural proteins (nsP1-4) and five structural proteins (**Fig. 2**) (C, E3, E2, 6K/TF, and E1)[107,108]. Alphaviruses are largely transmitted by arthropods, especially mosquitoes, and have a broad geographical distribution, excluding Antarctica[109].

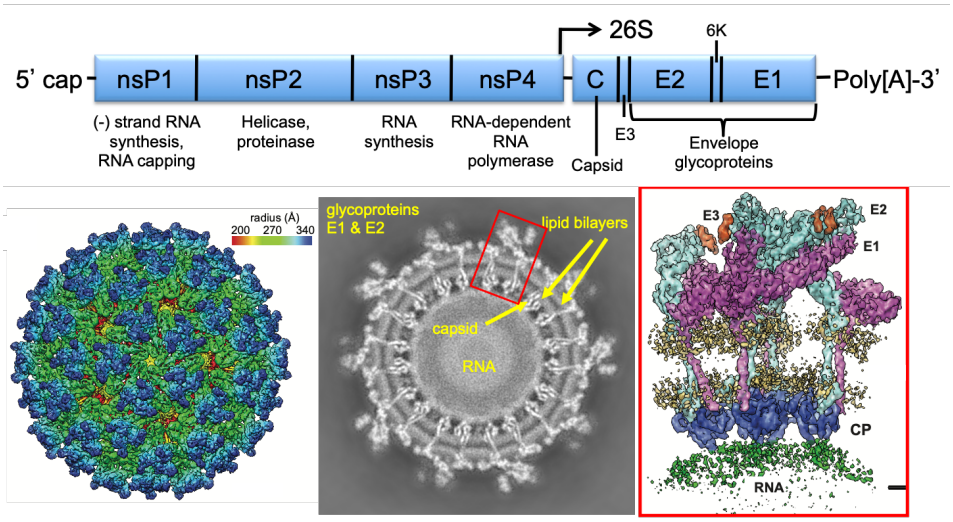

Figure 2. Alphavirus genome and viral particle with structural proteins*. Single stranded RNA genome of alphaviruses showing two main open reading frames that codify structural and non-structural viral proteins*[110]*. The E1, E2 and E3 contain important antigenic sites subjected to immunological selective pressure. Adapted from: Zang et al., 2011, EMBO Press, https://doi.org/10.1038/emboj.2011.261*[110]*.*

There are 30 identified viral species within the Alphavirus genus which can be classified into 10 groups based on their antigenic or genetic relationships. Complexes such as Barmah Forest, Ndumu, Middelburg, and Semliki Forest are predominantly found in the Old World. New World alphaviruses also include members of the Semliki Forest Antigenic Complex, namely alphaviruses such as MAYV, Una virus (UNAV), the recently introduced CHIKV[109,111,112], while the western equine encephalitis virus antigenic complex (WEEV) includes the Aura (AURAV), Trocara virus (TROV), eastern equine encephalitis virus (EEEV), and VEEV[111,112]. Some alphaviruses such as the EEEV have extensive distribution in the Americas[109,111,112], while others such as MAYV and AURAV, are confined to neotropical areas[24,111,112].

Alphaviruses display a wide range of host interactions and disease presentations[109,111]. For instance, the Semliki Forest virus complex is linked to primates and symptoms like fever, rash, and joint pain[109,111]. On the other hand, the WEEV and EEEV complexes predominantly affect birds, while the VEEV complex use predominately rodents as reservoirs, with all causing forms of encephalitis in humans and equines[109,111]. The transmission of alphaviruses is primarily zoonotic, with *Culex* and *Aedes* mosquitoes being the primary vectors[111–113]. Depending on the virus, transmission may involve non-human primates, birds, or rodents. Human transmission,

particularly for CHIKV, can occur both in rural to urban settings, with humans and non-human primates acting as hosts[114].

Despite many alphaviruses causing mild illnesses in humans, some lead to severe, potentially fatal conditions[46]. Their epidemiology, however, remains poorly understood in large part due to clinical misdiagnoses, especially in regions where viruses like ZIKV and DENV are also prevalent[115]. Comprehensive understanding of these viruses, their transmission, evolution, and diagnostic tools, is crucial for disease management and prevention [115].

2.1. Venezuelan equine encephalitis virus complex

Initially identified in 1936 in Venezuela[116], alphaviruses belonging to the VEE complex mainly circulate among rodents and mosquitoes in certain areas of Central and South America, Mexico, and Florida, posing a re-emerging health threat as evidenced in several recent outbreaks[19,20].

The VEE complex is closely related to the eastern equine encephalitis (EEE) complex and can be classified in around fourteen known antigenic subtypes with distinct epidemiological characteristics[11]. The most epidemiologically relevant VEE complex subtypes are the IAB and IC epizootic subtypes, which have been linked to high case-fatality rates in horses and major epidemics involving thousands of human cases[19,20]. The infection generally presents with influenza-like symptoms can be particularly severe, causing neurological symptoms in a 10-14% fraction of those affected[20,117].

From an evolutionary perspective, phylogenetic studies have shown a close relationship between several VEE complex strains (**Fig. 3**), supporting the hypothesis that epizootic strains result from the acquisition of adaptive mutations in enzootic strains when these jump to new host species[118]. This evolutionary pathway seems to be influenced by changes in the E2 glycoprotein surface charge altering its interaction with cellular receptors[61].

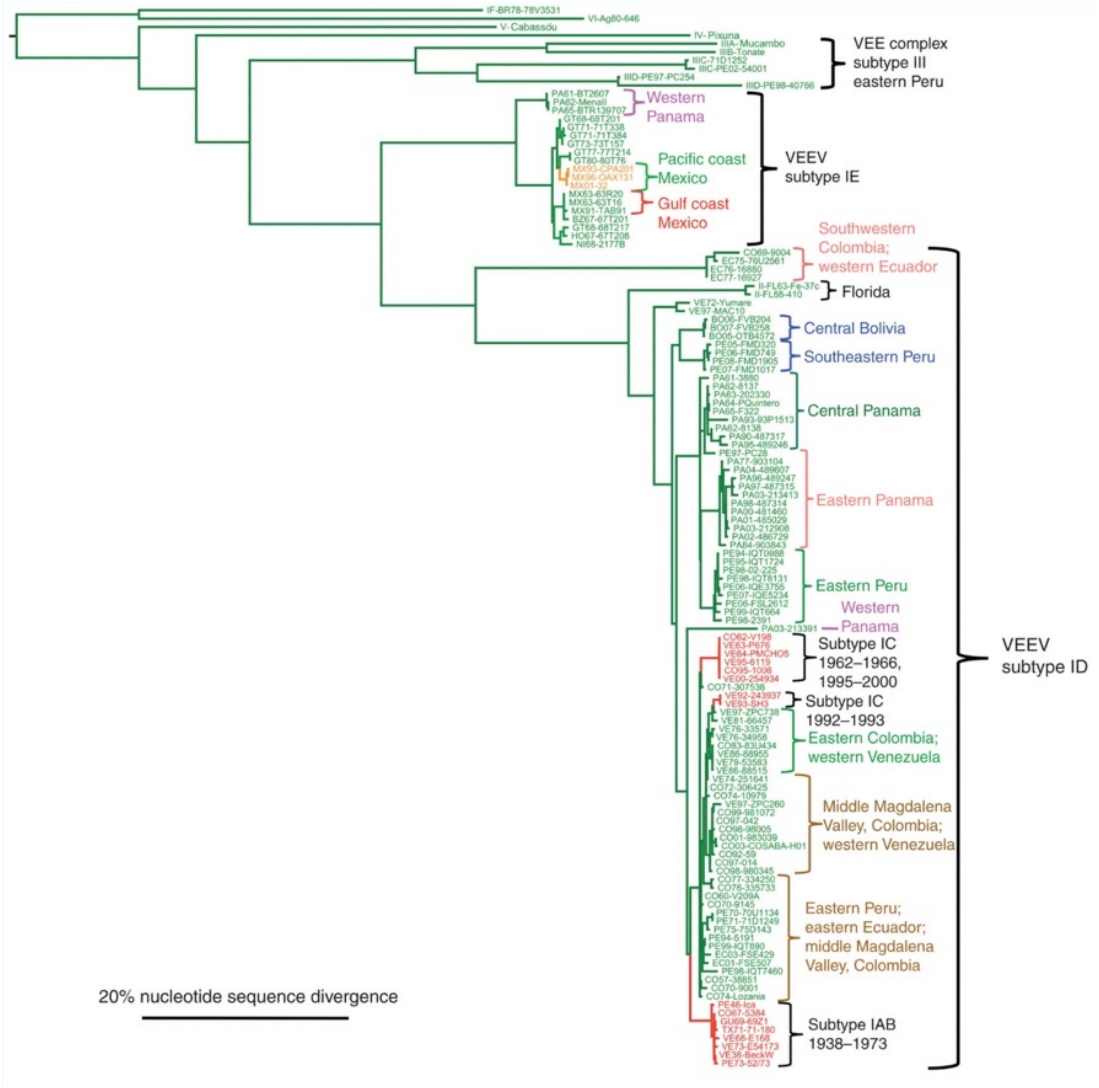

Figure 3. Phylogenetic tree of VEEV complex subtypes. *Phylogenetic tree showing evolutionary relationship of VEEV complex subtypes. Internal branches of enzootic subtypes are presented in green while epizootic subtypes are highlighted in red. Figure adapted from Aguilar et al, 2011, Future Virol. doi: 10.2217/FVL.11.5*[20].

The transmission cycle of the VEE complex remains poorly understood, with horses playing a crucial role in amplifying the virus during outbreaks and a diverse range of other animals acting as hosts[19,20]. The transmission is known to not occur directly from human to human but considering the behavioural traits of certain mosquito species and the expansion of human settlements, the potential for future urban transmission of VEE complex viruses cannot be overlooked[10,60].

The vectors facilitating the spread of VEE complex epizootic strains are mainly mosquitoes, with different species playing pivotal roles in various outbreaks[119]. The mosquito species *A*. (*Ochlerotatus*) *taeniorhynchus* stands out as a significant epizootic vector, particularly in coastal regions that witnessed large outbreaks in Central and South America[19,119]. Enzootic transmission, on the other hand, occurs mainly among *Zygodontomys*, *Sigmodon* and *Proechimys* rodent genera that act as primary reservoirs[19,120]. A group of mosquitoes species within the *Culex* genus, specifically from the subgenus *Melanoconion*, are identified as the main vectors in VEE enzootic transmission cycle[19,121].

2.2. Eastern equine encephalitis virus complex

The EEE virus complex (EEEV) comprises virus strains transmitted by mosquitoes and can be found throughout the Americas[11]. In North America, this virus is found along the eastern seaboard of the U.S. and Canada, with certain strains also present in Mexico Northern regions and the Caribbean[11]. In North America, it typically affects around 5-6 individuals annually, with swamp environments being the most common areas of transmission[11]. The virus primarily circulates between the bird species of the Passeriformes order and specific mosquito species like *Culiseta melanura*[122,123]. Case fatality rate in humans is around 30% and up to 90% in horses[124–126]. In 2019, the United States experienced its largest EEEV outbreak, registering 36 cases with a 35% case-fatality rate[126]. This is in stark contrast to the average of 5-6 annual cases reported between 2003 to 2018[126]. The reasons for the recent increase of EEEV cases remain unclear[126].

In South America, EEEV was first identified in Argentina in 1933[11,127]. Recent findings have shown that North American EEEV and South American EEEV lineages exhibit differences in their ecological, epidemiological, and genetic characteristics, leading to the reclassification of the South American EEEV lineages as MADV[11]. This virus is named after its original discovery site in Argentina and is currently characterised by three primary genetic lineages, South American EEEV lineages, II-IV that have spread across several countries (**Fig. 4**)[11]. Earlier data suggested that MADV was not harmful to humans but led to high mortality rate in horses[128–130]. Despite significant human exposure, especially during outbreaks, MADV human cases were not observed in regions like Panama and Argentina until recently[129,130]. Notably, the first human MADV outbreak was recognized in Panama in 2010 and resulted in several human and equine severe cases and fatalities[131].

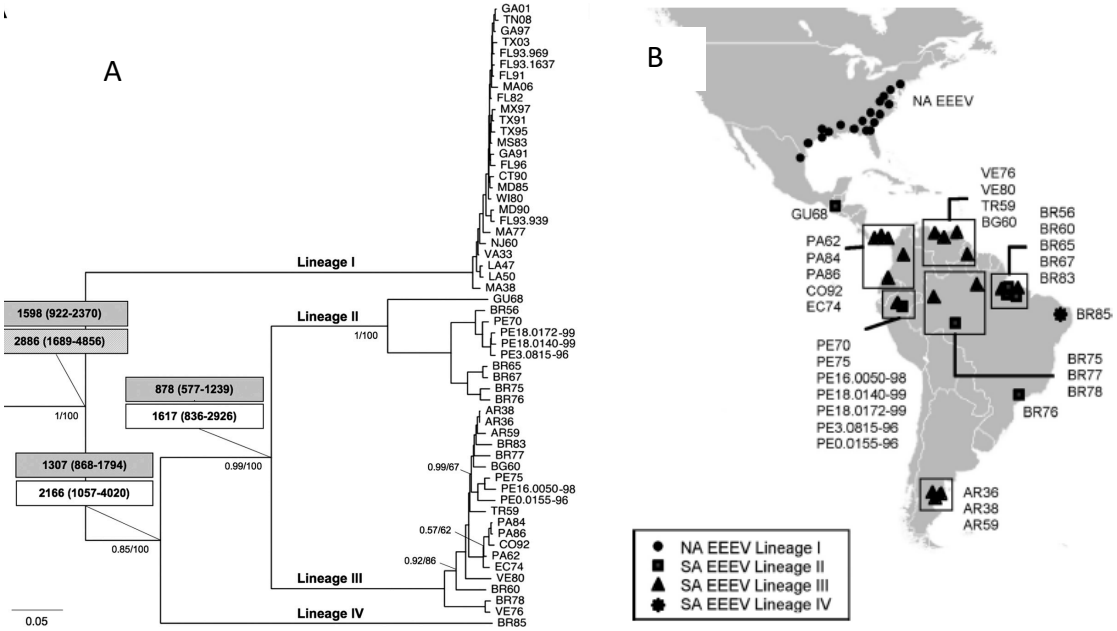

Figure 4. Phylogenetic tree and distribution of EEEV antigenic complex*. A) Phylogenetic tree of Eastern equine encephalitis showing EEEV (NA EEEV Lineage I) and MADV (SA EEEV lineages II-IV).* ***B)*** *Distribution of strains of the EEEV antigenic complex. SA= South America, NA = North America. Figure adapted from Arrigo et al, 2010, J Virol, doi: 10.1128/JVI.01586-09*[11].

Mosquitoes species like *Culex* (*Mel*.) *taeniopus* and *C.* (*Mel.) pedroi* are believed to be the main mosquito vectors in transmitting VEEV and MADV across Central and South America[132–134]. The principal host for VEEV and MADV include rodents[25,26,135], while recent evidence Panama supports this hypothesis that rodents are potential host for MADV, there is some evidence that also suggests that lizards could act as MADV hosts[136]. However, the MADV enzootic cycle remains poorly understood and evidence for potential MADV hosts remains incomplete (**Fig. 5**).

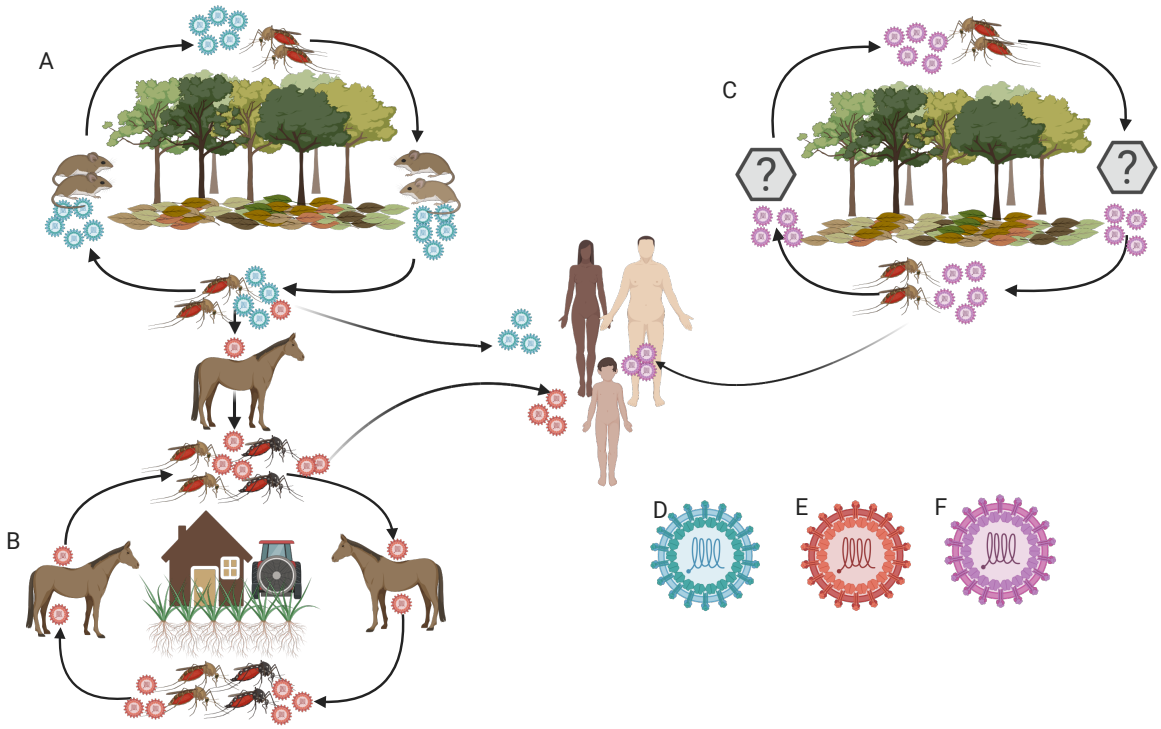

Figure 5. Overview of VEEV and MADV transmission cycles. *Overview of VEEV and MADV transmission cycles. A. VEEV enzootic cycle involves sylvatic rodents and mosquitoes. B. VEEV epizootic cycle involves horses as amplifying hosts and epizootic mosquitoes. C. Madariaga enzootic cycle involves Culex Mel. spp. mosquitoes. Question marks highlight the unknow amplifying host species associated with the sylvatic MADV transmission cycle. D. VEEV enzootic subtypes. E. VEEV epizootic subtypes. F. MADV subtypes.*

2.3 VEE complex and MADV epidemiology in Panama, Central America

VEEV epizootics in South America have been characterized by explosive epidemics and expanding geographical spread from South to North America involving thousands of human and equine cases[20,27]. The emergence of VEEV epizootic subtypes is believed to occur through mosquito or equine adaptive mutations from an enzootic ancestor[61,137]. In Panama, enzootic VEEV has been exclusively associated with human disease since 1960[138]. Subsequently, human cases have been identified through the dengue surveillance system[117], highlighting the interconnectedness of arboviral surveillance systems in capturing the emergence of zoonotic pathogens.

Figure 6. Timeline of VEEV and MADV historical events in Panama. *Human cases, outbreaks, surveillance, mosquito detection and animal detection for MADV and VEEV in Panama are presented.*

In Panama, human MADV cases are typically identified following equine case detections, usually at the onset of the rainy season. Most human MADV cases have been detected using viral isolation, RT-PCR generic alphavirus detection, ELISA, sanger sequencing and hemagglutination inhibition test, in cases detected in the Darien Province, near the Colombian border[131]. In Panama, MADV seems to be particularly severe in children, with a median age of

infection being around five years and a mortality rate of approximately 10%[131]. Patients with MADV generally experience early symptoms like fever and headaches[139,140]. As the disease progresses, more severe neurological symptoms manifest, including disorientation, sleepiness, seizures, and even coma[131]. Outside of Panama, there are very few MADV-related human cases, possibly due to immunity factors, inherent virus characteristics and incomplete surveillance[131].

Diagnosing MADV using serological assays is particularly challenging in endemic regions due to the simultaneous circulation of several alphaviruses, leading to cross-reactions[131]. Additionally, the presence of viruses with similar symptoms further complicates clinical and laboratory interpretations. A combination of techniques, including viral isolation, antibody tests, and RNA detection, is vital for accurate diagnosis (**Fig. 7**)[131].

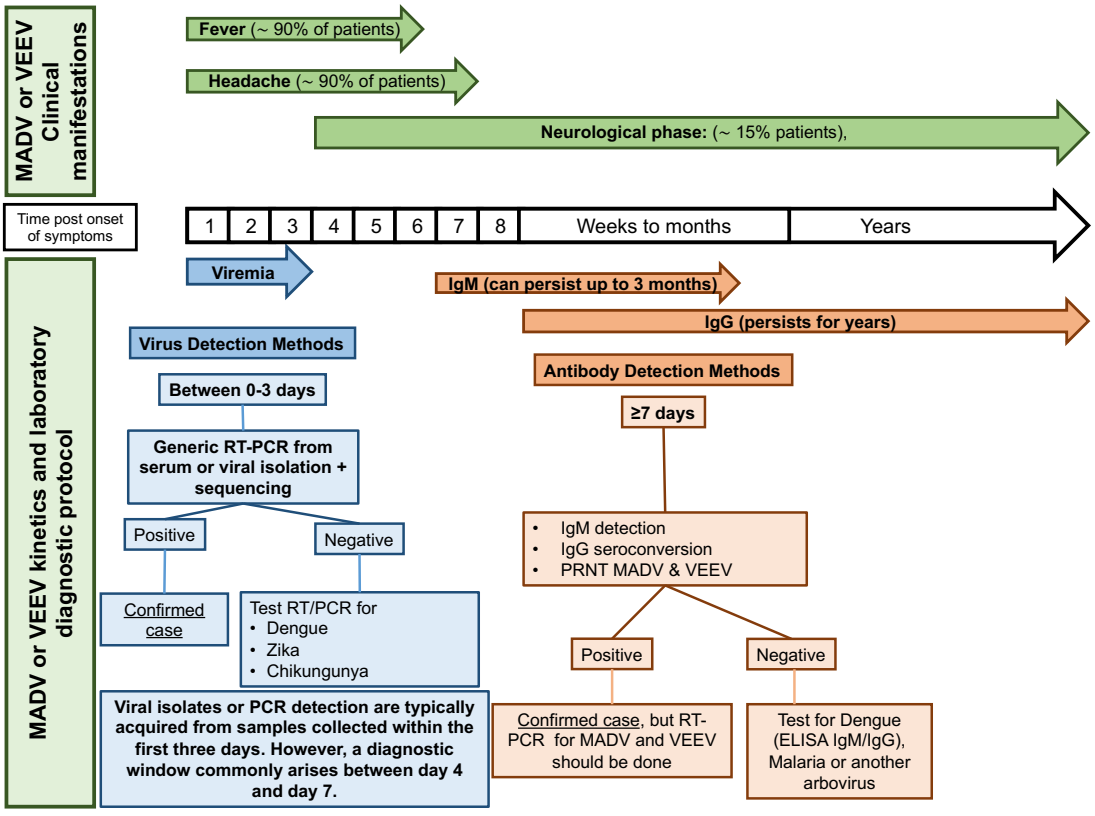

Figure 7. Diagnostic algorithm of MADV and VEEV in endemic regions. *Virological, molecular and serological test used for alphavirus diagnosis in endemic areas with co-circulation of VEEV and MADV based in daily clinical evolution of patients.*

To date, molecular specific molecular test to detect MADV remain unavailable. Molecular detection involves the use of pan-alphavirus primers, which are designed to amplify a 400-500

nucleotide region of alphavirus genomes. Following this amplification, identification of the specific viral complex or species complex is typically achieved through sequencing or the use of nested polymerase chain reaction (PCR)[141]. Such methods are laborious and increase opportunities for laboratory contamination.

3. Significance and aims of this thesis

MADV is an emergent arthropod-borne zoonotic pathogen that was primary linked with equine disease although extensive epidemiological investigation in the Americas[128–130]. However, in 2010 an increase of human and equine neurological infections was associated with MADV and VEEV simultaneous circulation in the remote eastern province of Darien, Panama[131]. ***My central hypothesis posits that MADV and VEEV are emerging alphaviruses with sustained and increased incidence over recent decades in eastern Panama, and these increased risks are associated with changes in the ecology of potential vertebrate host and mosquitoes vectors***.

The aims of this thesis were five-fold:

1. To determine the extent to which human MADV infections, along with the concurrent circulation of VEEV, represent emerging arbovirus events, in Panama.
2. To determine MADV and VEEV incidence after 2010, including temporal antibody dynamics and long-term neurological consequences of infection caused by these viruses in Panama.
3. To develop novel real-time RT-PCR tools to detect both VEEV and MADV virus and implement a febrile surveillance in Panama.
4. To clarify the role of different rodent and enzootic vectors species in their enzootic and epizootic transmission in Panama.
5. To pilot a prospective One Health approach to detect active arboviral circulation in human, vertebrate hosts and enzootic mosquitoes vectors in the Darien Gap, Panama.

The studies presented in this thesis were designed to examine arboviral transmission from the perspective of the human host, the enzootic mosquitoes vectors, the vertebrate hosts and the virus. In Chapter 1, my research sheds light on the emergence and intensification of VEE complex and MADV as potential public health threats in Central America. Particularly, my research focuses on investigating historical transmission patterns and dynamics of MADV and VEEV prior the 2010 outbreak. In addition, I explore the risk of MADV and VEEV infection and investigate mosquitoes vectors during an outbreak response. In addition, I also explore

seroprevalence and historical dynamics of the emerging alphavirus Mayaro and Una virus. In Chapter 2, I investigate the dynamics of the immune response in affected individuals, and the lasting neurological impacts of the infections. In Chapter 3, I develop and implemented molecular diagnostic and febrile surveillance to detect MADV and VEEV infections in Panama. In Chapter 4, I investigate the role of rodents in arbovirus transmission cycles and identify the primary vectors responsible for transmitting novel arboviruses. Finally, in Chapter 5, my research showcases a forward-looking One Health strategy to actively identify and monitor the spread of emerging arboviruses in human populations, mosquitoes vectors and animal reservoir, ensuring better preparedness and potential mitigation against future arboviral outbreaks.

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Chapter 1 – Endemic and Epidemic Human Alphavirus Infections in Eastern Panama: An Analysis of Population-Based Cross-Sectional Surveys

This original research chapter comprises a single publication focused on reconstructing the historical dynamics of alphavirus infection in Panama. It involves the exploration of alphavirus risk factors, the investigation of potential neurological consequences, and an examination of vector distribution near households of confirmed alphavirus cases. This chapter has been published in its final form in *The American Journal of Tropical Medicine and Hygiene*.

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Endemic and Epidemic Human Alphavirus Infections in Eastern Panama: An Analysis of Population-Based Cross-Sectional Surveys

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Abstract. Madariaga virus (MADV) has recently been associated with severe human disease in Panama, where the closely related Venezuelan equine encephalitis virus (VEEV) also circulates. In June 2017, a fatal MADV infection was confirmed in a community of Darien Province. We conducted a cross-sectional outbreak investigation with human and mosquito collections in July 2017, where sera were tested for alphavirus antibodies and viral RNA. In addition, by applying a catalytic, force-of-infection (FOI) statistical model to two serosurveys from Darien Province in 2012 and 2017, we investigated whether endemic or epidemic alphavirus transmission occurred historically. In 2017, MADV and VEEV IgM seroprevalences were 1.6% and 4.4%, respectively; IgG antibody prevalences were MADV: 13.2%, VEEV: 16.8%, Una virus (UNAV): 16.0%, and Mayaro virus: 1.1%. Active viral circulation was not detected. Evidence of MADV and UNAV infection was found near households, raising questions about its vectors and enzootic transmission cycles. Insomnia was associated with MADV and VEEV infections, depression symptoms were associated with MADV, and dizziness with VEEV and UNAV. Force-of-infection analyses suggest endemic alphavirus transmission historically, with recent increased human exposure to MADV and VEEV in Aruza and Mercadeo, respectively. The lack of additional neurological cases suggests that severe MADV and VEEV infections occur only rarely. Our results indicate that over the past five decades, alphavirus infections have occurred at low levels in eastern Panama, but that MADV and VEEV infections have recently increased—potentially during the past decade. Endemic infections and outbreaks of MADV and VEEV appear to differ spatially in some locations of eastern Panama.

INTRODUCTION

Alphaviruses (*Togaviridae: Alphavirus*) are important zoonotic, single-stranded RNA arthropod-borne viruses. Clinically, alphaviruses are associated with febrile, severe and sometimes fatal disease in the Americas.[1] Among the most important alphaviruses are eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV), and members of the Semliki Forest antigenic complex. These viruses have caused explosive epidemics of human encephalitis and arthritogenic disease in Latin American tropical regions.[2,3]

Eastern equine encephalitis virus has recently been reclassified as two different species: EEEV in North America and Madariaga virus (MADV) in other parts of Latin America[4]—each with different predispositions to cause human disease.[5] In 2010, we reported severe neurologic diseases in humans associated with MADV infection in Panama.[6] The mechanism underlying this outbreak remains unknown, but age-specific seroprevalence data obtained during the 2010 and 2012 studies suggest recent MADV emergence in Panama.[7,8] Venezuelan equine encephalitis virus epizootic subtypes IAB and IC are associated with explosive human and equine epidemics/epizootics, which occur chiefly in South and Central America.[2] Those epizootic subtypes emerge from enzootic ID subtype ancestors because of viral adaptations for infection of equids and mosquitoes that allow it to spread rapidly among human and animal populations.[9] In Panama, enzootic subtypes ID and IE circulate in eastern-central and western Panama, respectively, where the natural cycle occurs in mosquitoes (subgenus *Melanoconion*) and sylvatic rodents.[10]

The Semliki Forest alphavirus complex includes Mayaro virus (MAYV) and Una virus (UNAV) that are mostly found in the Amazon region of Peru, Brazil, and Venezuela. Mayaro virus is characterized by fever and arthralgia, which can persist for years.[11] However, UNAV has not been associated with human disease. In the Americas, sizeable human MAYV outbreaks have most often been reported in the Amazon Basin, although recently, this virus was isolated from a febrile child in Haiti, suggesting it may be moving beyond its established territory.[12] Una virus has been detected at low levels during epidemiological studies and surveillance,[13,14] but because this virus has rarely been associated with human disease, the risk

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to people living in endemic Latin America remains unclear.[15] Both MAYV and UNAV are vectored by forest mosquitoes: *Haemagogus janthinomys* mosquitoes are the primary vectors of MAYV,[15] whereas *Psorophora ferox* and *Psorophora albipes* mosquitoes are thought to be the main vectors of UNAV.[16,17] The MAYV enzootic cycle is also known to involve nonhuman primates as amplification hosts.[15,18]

In June 2017, a fatal MADV infection was confirmed in the Mogue community in Darien, the most eastern province of Panama, prompting field investigations. Here, we use seroprevalence data collected during this survey to determine population exposure and to characterize factors associated with seroprevalence for MADV and other alphaviruses. By combining seroprevalence survey data from 2012 with that from the recent survey, we also attempted to determine whether alphaviruses emerged recently or were present historically.

MATERIALS AND METHODS

We reconstructed the epidemiological dynamics of MADV and VEEV using data from cross-sectional surveys undertaken in 2012 and 2017 in Darien Province villages (Figure 1). We also identified factors associated with alphavirus exposure, measured as IgG seroprevalence. Maps were constructed using the GPS coordinates collected during the investigation using ArcGIS package online version (Argis Solutions, Inc., Denver, CO). Land use shapes were validated by the Ministry of Environment (https://www.miambiente.gob.pa).

2012 serosurvey. The original 2012 study was conducted by the Gorgas Memorial Institute of Health Studies (GMI) to estimate prevalence and to identify risk factors for zoonotic diseases in Panama.[8] The study included five villages (Figure 1). A total of 897 participants were surveyed, but only 774 sera were available for laboratory testing. In Tamarindo, 176 participants were surveyed, 167 in Aruza, 250 in El Real, 130 in Mercadeo, and 174 in Pijibasal/Pirre 1-2. All available samples were tested to detect neutralizing antibodies against MADV and VEEV using a plaque reduction neutralization test (PRNT). Non-antibody detection against UNAV was addressed during this study. Details of this survey have been described previously.[8] Specific characteristics of the study sites are given in the Supplemental Materials.

2017 serosurvey. On June 30, 2017, a fatal human MADV case was confirmed with viral isolation in Mogue village (Figure 1). This was followed by a collaborative initiative between the Panamanian Ministry of Health and the GMI for outbreak investigation and response. From July 18 to 22, 2017, 83.3% of inhabitants (250 of 300) were surveyed, including members from all households. Each participant was interviewed using a standardized epidemiological form to record occupation, activities, livestock, and crop holdings. Other details are given in the Supplemental Materials and Figure S1. Human sera collected in 2017 were tested using alphavirus genus-specific real-time reverse transcription polymerase chain reaction (RT-PCR)[19] and by ELISAs to detect IgM and IgG antibodies against MADV and VEEV. Positive sera were then confirmed using the PRNT with the same method as in the 2012 serosurvey.[8] ELISA antigens were prepared from EEEV (prepared by Robert Shope at the Yale Arbovirus Research Unit in August 1989)- and VEE complex virus (strain 78V-3531)-infected mouse brain. For the PRNT, we used chimeric Sindbis virus (SINV)/MADV—shown to be an accurate surrogate for MADV in these assays[20]—and VEEV vaccine

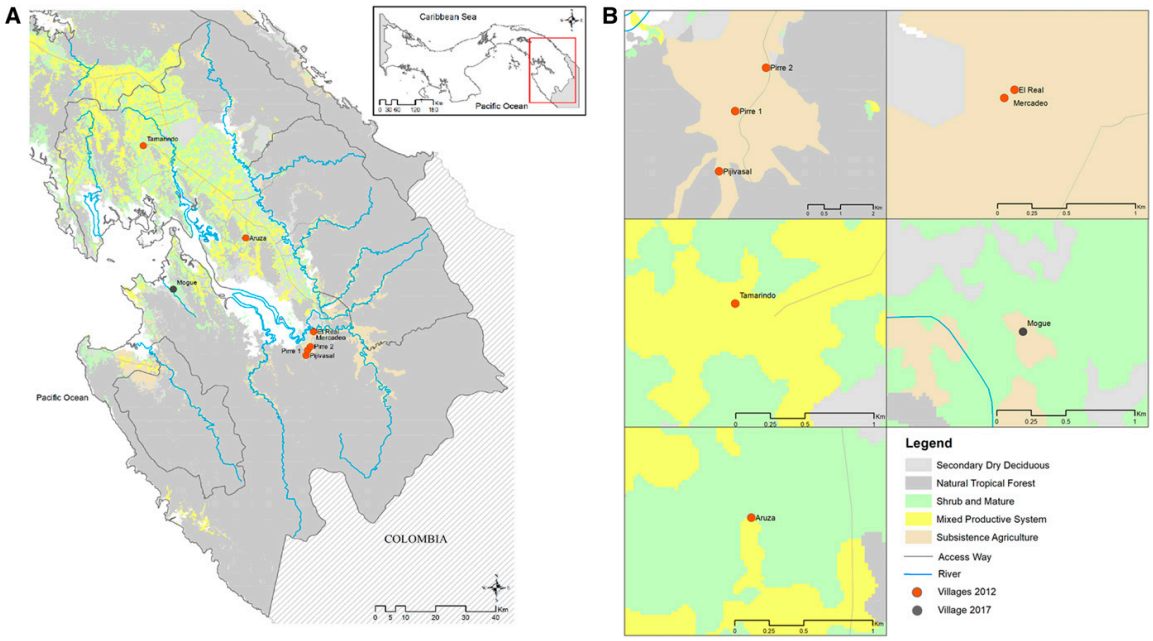

FIGURE 1. Map of the study sites in eastern Panama: (**A**) Sampling sites in the Darien Province in eastern Panama. (**B**) Zoom-in projection of sampling sites on a land-use layer. This figure appears in color at www.ajtmh.org.

strain TC83. In addition, sera were tested for MAYV, UNAV, and Chikungunya virus (CHIKV) by using the PRNT using wild-type strains (MAYV-ARV-0565, UNAV-BT-1495-3, and CHIKV-256899). "Plaque reduction neutralization test$_{80}$" was positive to more than one virus at a titer of ≥ 1:20, and there was less than a 4-fold difference in titers.

Mosquito collection and testing in 2017. Mosquitoes were collected during 2 consecutive days in Mogue from July 19 to 21 using 10 traps: five CDC light traps were baited with octanol, and five Trinidad traps were baited with laboratory mice. Traps were placed outdoors in peridomestic areas at the edge of the vegetation, from 18:00 to 06:00. Trapped mosquitoes were collected early in the morning and placed in cryovials for storage in liquid nitrogen and transportation to the GMI. Mosquitoes were maintained cold, sorted to species level using taxonomic keys,[21] and grouped into pools of 20 individuals.

Mosquito pools were homogenized in 2 mL of minimum essential medium supplemented with penicillin and streptomycin and 20% fetal bovine serum using a TissueLyser (Qiagen, Hidden, Germany). After centrifugation at 12,000 rpm for 10 minutes, 200 μL of the supernatant was inoculated in each of two 12.5-cm^2 flasks of vero cells. Samples were passaged twice for cytopathic effect confirmation. The original mosquito suspensions were used for RNA extraction and tested using alphavirus genus–specific RT-PCR.[19]

Statistical methods. *Associated symptoms and risk factors analysis.* We conducted separate analyses for MADV, VEEV, and UNAV; in each case, the outcome variable was the presence/absence of antibodies against the virus, as determined by a PRNT$_{80}$ titer ≥ 1:20. The associations between each outcome and self-reported symptoms in the last 2 weeks were tested using chi-squared and Fisher exact tests; $P < 0.05$ was considered significant. The associations between each outcome and independent variables were estimated using generalized estimating equations for logistic regression models[22] and were expressed as odds ratios (ORs). The most parsimonious model was obtained with the log likelihood ratio test variable selection.[23] Univariable and multivariable ORs were calculated with 95% CIs.

Force-of-infection (FOI) analysis. To investigate the endemicity and/or recent emergence of three alphaviruses (VEEV, MADV, and UNAV), we combined age-structured seroprevalence data from both surveys (i.e., from 2012[8] to 2017), which encompassed seven sites (Pirre 1-2, Pijibasal, Mercadeo, Tamarindo, El Real, Aruza, and Mogue) where either human or equine cases of VEEV or MADV have occasionally been reported. See Figure 1 and Supplemental Materials for a detailed description of these sites.

The historical FOI was estimated using a catalytic model,[24] where the number of seropositive individuals in each sample was modeled using a binomial distribution,

$$n(a,t) \sim B(N, P[a,t]).$$

Here, $n(a,t)$ is the number of seropositive individuals and $p(a,t)$ is the underlying seroprevalence; in both cases, a denotes age and t denotes time; N is sample size. By making assumptions about $p(a,t)$ (described in the following), we tested whether MADV, VEEV, and UNAV transmission rates have historically been constant over time ("constant FOI" model) or have varied—for example, because of recent introduction of these viruses ("time-varying FOI" model).

For a constant FOI (λ), we modeled seroprevalence for age a in year t (i.e., the time when the serosurvey occurred) as,

$$p(a,t) = 1 - \exp(-\lambda a).$$

For a time-varying FOI (λ_t), we modeled seroprevalence for age a as,

$$p(a,t) = 1 - \exp\left(-\sum_{i=t-a+1}^{t} \lambda_i\right).$$

In this framework, we assume no seroreversion (loss of antibodies over time), no age dependence in susceptibility or exposure,[25] and the mortality rate of infected individuals is the same as for susceptible individuals. The models were estimated in a Bayesian framework using Stan's no-U-turn sampler.[26,27] Details of priors and model simulations and

TABLE 1
Characteristics of the 243 study participants with complete data from the 2017 survey

Characteristic	*N* (%)
Gender	
Male	120 (49.4)
Female	123 (50.6)
Ages (years)	
2–11	80 (32.9)
12–30	82 (33.7)
≥ 31	81 (33.3)
House members*	4 (2–6)
Activities	
Main occupation	
Student	122 (50.2)
Farmer/rancher	48 (19.8)
Homemaker/occupation at home	73 (30.0)
Breeding poultry	45 (18.5)
Fishing for consumption	7 (2.9)
Contact with pastures	78 (32.1)
Contact with crops	123 (50.6)
Clearing vegetation	80 (32.9)
Working in agriculture	86 (35.4)
Working in pastures	24 (9.9)
Working in grain deposits	21 (8.6)
Working in sawmills/forest	33 (13.6)
Working in chicken coops	58 (23.9)
Working in pigsties	44 (18.1)
Washing clothes in ravines or rivers	111 (45.7)
Taking bath in natural water source	211 (86.8)
House-level features	
Total houses	59
House floor material	
Wood	55 (93.2)
Other	4 (6.8)
House with walls	29 (49.2)
House window material	
Concrete (ornamental blocks)	42 (71.2)
Wood	17 (28.8)
Roof material of house	
Tin roof	28 (47.5)
Straw thatched	31 (52.5)
Vegetation around the house	25 (42.4)
Rice cultivation around the house	4 (6.8)
Corn cultivation around the house	3 (5.1)
Waste disposal methods	
Burying	5 (8.5)
Burning	43 (72.9)
Other	11 (18.6)
Rain water	57 (96.6)

* Range.

packages used are provided in Supplemental Materials and Figures S1–S7. Median of the posterior distribution of the parameters and their corresponding 95% credible intervals (95% CrIs) are presented.

Ethics. The outbreak investigation was undertaken during a public health outbreak response, and ethical approval for use of surveillance data and cross-sectional surveys was given by the GMI Ethics Committee (IRB #0277/CBI/ICGES/15 and IRB #047/CNBI/ICGES/11). The written informed consent of participants was obtained. All identifying information of participants was removed, and confidentiality was strictly respected. The animal component of this study was approved by the GMI Committee of Care and Use of Animals (001/05 CIUCAL/ICGES, July 4, 2005) and conducted in accordance with law number 23 of January 15, 1997 (Animal Welfare Guarantee) of the Republic of Panama.

RESULTS

Characteristics of the study population. In 2017, 250 participants belonging to 59 houses were surveyed, with complete risk factor data available for only 243 individuals (97.2%). Ages ranged from 1 to 97 years, and females comprised 51% of surveyed individuals. Further characteristics of the surveyed population are given in Table 1.

In 2012, a total of 826 participants were surveyed, but only 774 sera were available for laboratory testing. The risk factors determined from this serosurvey have previously been published.[8]

Alphavirus detection and seroprevalence in 2012 and 2017. In 2012, the overall neutralizing antibody seroprevalence was 4.8% (95% CI: 3.4–6.5) for MADV and 31.6% (95% CI: 28.3–35.0) for VEEV.

In 2017, the overall neutralizing antibody seroprevalence was MADV: 13.2% (95% CI: 9.2–18.0), VEEV: 16.8% (95% CI: 12.4–22.0), UNAV: 16.0% (95% CI: 11.7–21.1), and MAYV: 1.2% (95% CI: 0.3–3.5). No evidence of CHIKV infection was found. Neutralizing antibody seroprevalence to more than one virus was observed in 3.6% (95% CI: 1.6–6.7) of participants. The proportion of subjects with both MADV and VEEV antibodies was 3.7% (df = 1; Pearson chi-square = 3.43; test for independence $P = 0.064$), both UNAV and VEEV antibodies 3.7% (df = 1; Pearson chi-square = 0.91; test for independence $P = 0.340$), and both MADV and UNAV antibodies 2.9% (df = 1; Pearson chi-square = 0.97; test for independence $P = 0.325$). Only one subject presented antibodies against these three viruses. IgM prevalence was: MADV 1.6% (95% CI: 0.4–4.2) and VEEV 4.4% (95% CI: 2.2–7.8). Concurrent MADV and VEEV IgM were observed in 0.8% of individuals (95% CI: 0.1–2.9). Viral RNA was not detected in sera.

Associated symptoms and risk factors. Exposure to MADV was significantly associated with self-reported dizziness, fatigue, depression, and difficulty cooking. Having VEEV neutralizing antibodies was associated with dizziness and insomnia (Table 2). Participants older than 11 years were more likely to test positive for UNAV antibodies, with those older than 30 years being the most likely (Tables 3 and 4). Having a house with walls reduced the risk of testing positive for UNAV antibodies (Tables 3 and 4). The most parsimonious multivariable model revealed that being older and having vegetation around the house were positively associated with MADV antibody prevalence (Table 4). Washing clothes in ravines or rivers was also positively associated with VEEV antibodies in the multivariable model (Table 4).

Enzootic vectors. In 2017, a total of 113 mosquitoes across 10 species were collected: *Culex* (*Culex*) *coronator* (36.3%), *Cx.* (*Melanoconion*) *pedroi* (14.2%), *Cx.* (*Mel.*) *spissipes* (10.6%), *Cx.* (*Cx.*) *nigripalpus* (10.6%), *Cx.* (*Mel.*) *vomerifer* (8.8%), *Cx.* (*Cx.*) *declarator* (5.3%), *Cx.* (*Mel.*) *adamesi* (2.7%), and *Cx.* (*Mel.*) *dunni* (2.7%). The overall mean number

TABLE 2
Symptoms and signs associated with UNAV, MADV, and VEEV exposure (neutralizing antibodies)

Symptom		UNAV*		MADV*		VEEV*	
	N (%)§	*n* (%)†	*P*-value‡	*n* (%)†	*P*-value‡	*n* (%)	*P*-value‡
Fatigue	85 (35.0)	14 (35.0)	0.998	15 (48.4)	0.094	19 (45.4)	0.125
Difficulty with concentration	60 (24.7)	13 (32.5)	0.210	10 (32.3)	0.296	11 (26.2)	0.804
Memory loss	58 (23.9)	12 (30.0)	0.320	11 (35.5)	0.104	13 (31.0)	0.236
Confusion	41 (16.9)	10 (25.0)	0.133	6 (19.4)	0.693	11 (26.2)	0.076
Dizziness	72 (29.6)	**18 (45.0)**	**0.020**	12 (38.7)	0.236	**18 (42.9)**	**0.039**
Seizures	5 (2.1)	2 (5.0)	0.191	2 (6.5)	0.123	2 (4.8)	0.207
General weakness	65 (26.7)	15 (37.5)	0.093	**13 (41.9)**	**0.041**	13 (31.0)	0.499
Paralysis	11 (4.5)	3 (7.5)	0.396	1 (3.2)	1.000	4 (36.4)	0.102
Difficulty ambulating	29 (11.9)	5 (12.5)	0.540	5 (16.1)	0.302	8 (19.1)	0.118
Headache	110 (45.3)	22 (55.0)	0.176	15 (48.4)	0.709	21 (50.0)	0.498
Insomnia	33 (13.6)	3 (7.5)	0.313	**9 (29.0)**	**0.012**	**12 (28.6)**	**0.002**
Depression	22 (9.1)	5 (12.5)	0.285	**6 (19.4)**	**0.044**	2 (4.8)	0.228
Irritability	16 (6.6)	3 (7.5)	0.732	2 (6.5)	1.000	4 (9.5)	0.490
Difficulty cooking	23 (9.5)	5 (12.5)	0.473	**6 (19.4)**	**0.044**	6 (14.3)	0.241
Difficulty cleaning	28 (11.5)	5 (12.5)	0.832	6 (19.4)	0.144	5 (11.9)	0.932
Difficulty working	25 (10.3)	3 (7.5)	0.776	6 (19.4)	0.075	6 (14.3)	0.348
Fever	6 (2.5)	1 (2.5)	1.000	0 (0.0)	1.000	1 (2.4)	0.173
Chills	2 (0.8)	1 (2.5)	0.303	0 (0.0)	1.000	0 (0.0)	1.000
Emesis	1 (0.4)	0 (0.0)	1.000	0 (0.0)	1.000	1 (2.4)	0.173
Diarrhea	1 (0.4)	0 (0.0)	1.000	0 (0.0)	1.000	1 (2.4)	0.173

MADV = Madariaga virus; UNAV = Una virus; VEEV = Venezuelan equine encephalitis virus. $n = 40$ with UNAV antibodies; $n = 31$ with MADV antibodies; $n = 42$ with VEEV antibodies; $n = 243$ participants in total.
* Based on plaque reduction neutralization test results.
† Proportion of those with antibodies that reported symptoms.
‡ Results with $P < 0.05$ are shown in boldface type.
§ Overall proportion of participants with symptoms.

TABLE 3

Independent factors associated with the seroprevalence of UNAV, MADV, and VEEV neutralizing antibodies in univariate generalized estimating equations for logistic regression models (n = 243)

	UNAV*			MADV*			VEEV*		
	Univariate analysis			Univariate analysis			Univariate analysis		
Factor	OR	95% CI	P-value†	OR	95% CI	P-value†	OR	95% CI	P-value†
Gender									
Male	Ref.	–	–	Ref.	–	–	Ref.	–	–
Female	0.76	0.39–1.51	0.436	0.92	0.44–1.92	0.817	1.77	0.92–3.42	0.087
Age-group (years)									
2–11	Ref.	–	–	Ref.	–	–	Ref.	–	–
12–30	2.35	0.69–8.00	0.170	**6.50**	**2.49–31.89**	***0.021***	2.68	0.93–7.73	0.067
31–97	**9.59**	**3.15–29.17**	< ***0.001***	**12.00**	**2.49–57.75**	***0.002***	**5.91**	**2.16–16.20**	***0.001***
Activities									
Main occupation									
Student	Ref.	–	–	Ref.	–	–	Ref.	–	–
Farmer/rancher	**8.24**	**3.38–20.11**	< ***0.001***	2.43	0.94–6.31	0.068	**3.40**	**1.36–8.47**	***0.009***
Housewife/at home	2.48	0.92–5.04	0.053	1.98	0.82–4.82	0.198	**4.21**	**1.86–9.52**	***0.001***
Breeding poultry	0.92	0.38–2.24	0.858	**2.35**	**1.01–5.50**	***0.048***	1.45	0.65–3.25	0.366
Walking/playing through pastures	0.77	0.36–1.64	0.499	1.36	0.61–3.01	0.451	1.34	0.66–2.73	0.418
Walking/playing through crops	1.23	0.62–2.44	0.546	**2.64**	**1.15–6.03**	***0.021***	1.75	0.87–3.41	0.117
Clearing vegetation	1.85	0.93–3.71	0.080	**2.25**	**1.05–4.84**	***0.037***	1.76	0.90–3.46	0.100
Working in agriculture	**3.02**	**1.51–6.04**	***0.002***	1.83	0.86–3.90	0.114	**2.56**	**1.31–4.98**	***0.006***
Working in sawmills/forest	2.19	0.93–5.17	0.073	1.25	0.44–3.56	0.664	2.07	0.89–4.52	0.092
Working in chicken coops	1.27	0.58–2.73	0.545	2.20	0.98–4.93	0.054	1.13	0.53–2.44	0.750
Working in pigsties	1.40	0.61–3.19	0.422	0.63	0.20–1.94	0.420	2.08	0.94–4.58	*0.069*
Washing clothes in ravines or rivers	1.40	0.75–2.32	0.337	1.74	0.81–3.75	0.152	**3.11**	**1.53–6.33**	***0.002***
Taking bath in natural water source	1.08	0.39–3.04	0.871	2.34	0.53–10.25	0.259	1.95	0.60–6.42	0.269
House level									
House with walls	**0.47**	**0.39–3.04**	***0.042***	1.83	0.83–4.02	0.133	0.78	0.37–1.64	0.515
House window material									
Concrete‡	Ref.	–	–	Ref.	–	–	Ref.	–	–
Wood	0.68	0.28–1.66	0.397	0.59	0.20–1.74	0.341	0.89	0.37–2.15	0.799
Roof material house									
Tin roof	Ref.	–	–	Ref.	–	–	Ref.	–	–
Straw thatched	0.93	0.47–1.86	0.853	1.61	0.72–3.63	0.249	1.42	0.68–2.59	0.349
Vegetation around the house	0.64	0.31–1.35	0.245	**2.94**	**1.24–5.26**	***0.006***	1.18	0.56–2.49	0.653
Waste disposal methods									
Burying	Ref.	–	–	Ref.	Ref.	–	Ref.	–	–
Burning	1.21	0.42–3.54	0.721	0.23	0.03–2.02	0.189	1.20	0.37–3.87	0.755
Other	1.28	0.48–3.44	0.616	0.89	0.28–2.84	0.846	0.89	0.29–269	0.846

MADV = Madariaga virus; OR = odds ratio; UNAV = Una virus; VEEV = Venezuelan equine encephalitis virus. Results with $P < 0.05$ are shown boldface and bold-italic type.
* Based on plaque reduction neutralization test results.
† Results with $P < 0.05$ are shown in boldface type.
‡ Ornamental blocks.

of females per trap night was 6.7 in the Trinidad traps compared with 4.6 in the CDC traps. No viruses were detected in samples from mosquitoes.

Alphavirus FOI. For each virus, we fit both constant and time-varying FOI models to the seroprevalence data (see Methods) to describe the per capita rate at which susceptible individuals become infected per year. Because the constant FOI model is effectively nested within the time-varying FOI model, we report on whether the latter model improved the fit relative to the former.

TABLE 4

Independent factors associated with the seroprevalence of UNAV, MADV, and VEEV neutralizing antibodies in multivariable generalized estimating equations for logistic regression models (n = 243)

	UNAV*			MADV*			VEEV*		
	Multiple regression			Multiple regression			Multiple regression		
Factor	OR	95% CI	P-value†	OR	95% CI	P-value†	OR	95% CI	P-value†
Age-group (years)									
2–11	Ref.	–	–	Ref.	–	–	Ref.	–	–
12–30	2.39	0.70–8.15	0.164	**6.28**	**1.27–31.00**	**0.024**	1.83	0.61–5.53	0.279
≥ 31	**9.98**	**3.27–30.48**	**< 0.001**	**12.64**	**2.61–60.19**	**0.002**	**4.53**	**1.61–12.74**	**0.004**
House with walls	**0.43**	**0.20–0.94**	**0.035**	–	–	–	–	–	–
Washing clothes in ravines or rivers	–	–	–	–	–	–	**2.65**	**1.24–5.63**	**0.011**
Vegetation around the house	–	–	–	**2.96**	**1.25–6.98**	**0.013**	–	–	–

MADV = Madariaga virus; OR = odds ratio; UNAV = Una virus; VEEV = Venezuelan equine encephalitis virus.
* Based on plaque reduction neutralization test results.
† Results with $P < 0.05$ are shown in boldface type.

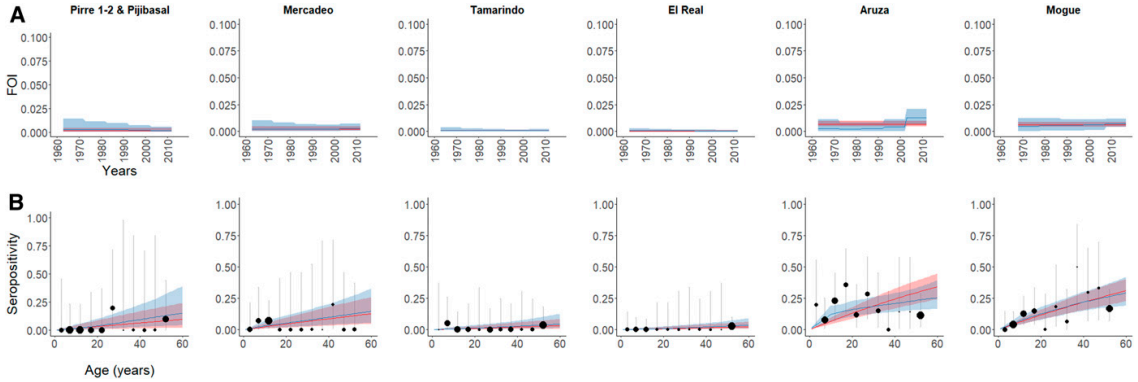

FIGURE 2. Force-of-infection (FOI) models fitted to Madariaga virus (MADV) seroprevalence data. (**A**) (Top panels) estimated constant (red) vs. time-varying FOI (blue) for MADV in eastern Panama over 50 years and (**B**) (bottom panels) fitted and observed seroprevalence. Red lines represent the estimated constant FOI and blue lines the estimated time-varying FOI. In each case, the shading represents 95% credible intervals from the model. The circles' radii in the lower panels indicate sample size in each 5-year age-group, and the vertical lines represent 95% CIs for observed seroprevalence. This figure appears in color at www.ajtmh.org.

Our results indicate temporal and geographic heterogeneity in the human population's exposure to MADV (Figure 2), VEEV (Figure 3), and UNAV (Figure 4). The highest estimated seroprevalence of each of the three viruses in younger than 10-year-olds (an indirect metric of recent transmission) was estimated for VEEV in Pirre 1-2 and Pijibasal at a posterior median of 44.8% (95% CrI: 34.9–55.0), followed by UNAV in Mogue at 5.6% (95% CrI: 4.1–7.5) and by MADV in Aruza at 4.7% (95% CrI: 3.2–6.7).

For MADV, in six of the seven locations, there was no evidence of time-varying transmission (Table 5); but in one location, Aruza, FOI was estimated as 0.012 (95% CrI: 0.006–0.021) (Figure 2A) in the latest decade analyzed (2002–2012)—a multiple of 4.6 and 5.3 times (ratio of posterior medians) the values estimated for 1992–2012 and 1982–1992, respectively (Figure 2B).

For VEEV, in six of the seven locations, there was no statistical support for time-varying transmission (Table 5). For the constant model, we estimated an annual FOI of 0.08 (95% CrI: 0.06–0.11) for VEEV in Pirre 1-2 and Pijibasal, corresponding to seroprevalence reaching 75% in 15-year-olds and almost 100% by 60-year-olds (Figure 3A). However, from the relatively small sample (only 75 subjects), it is unclear whether these results are due to consistently high endemic transmission or recent introductions and/or recent outbreaks. For one location, Mercadeo, a time-varying FOI model fit the data best. In this case, FOI in the most recently analyzed decade (2002–2012) was estimated at 0.04 (95% CrI: 0.03–0.06)—an increase of 1.5 times (ratio of posterior medians) over the previous decade (1992–2012) and 3.1 times compared with that of 1972–1992 (Figure 3B).

For UNAV, only tested in Mogue, a constant model fit the data best with an FOI estimated at 0.008 (95% CrI: 0.006–0.011) (Figure 4). No changes or increases in the incidence of UNAV associated with epidemics were observed, and infections occurred constantly during the analyzed period of time.

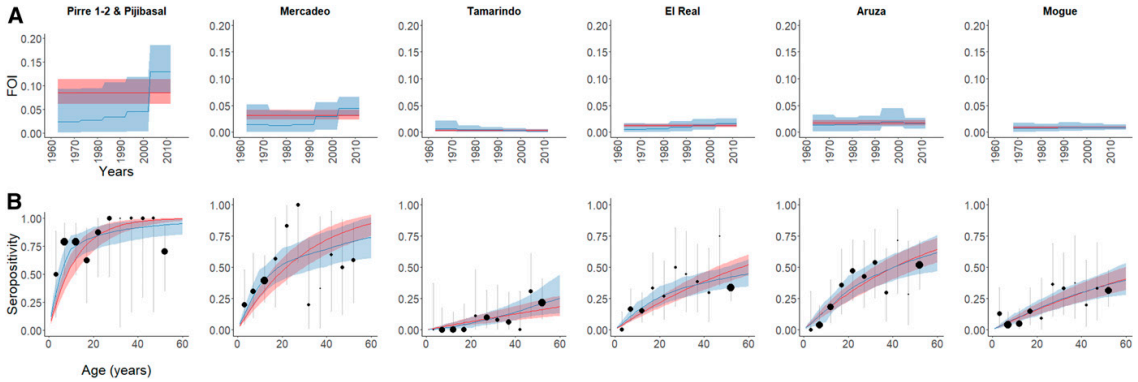

FIGURE 3. Force-of-infection (FOI) models fitted to Venezuelan equine encephalitis virus (VEEV) seroprevalence data. (**A**) (Top panels) estimated constant (red) vs. time-varying FOI (blue) for VEEV in eastern Panama over 50 years and (**B**) (bottom panels) fitted and observed seroprevalence. Red lines represent the estimated constant FOI and blue lines the estimated time-varying FOI. In each case, the shading represents 95% credible intervals from the model. The circles' radii in the lower panels indicate sample size in each 5-year age-group, and the vertical lines represent 95% CIs for observed seroprevalence. This figure appears in color at www.ajtmh.org.

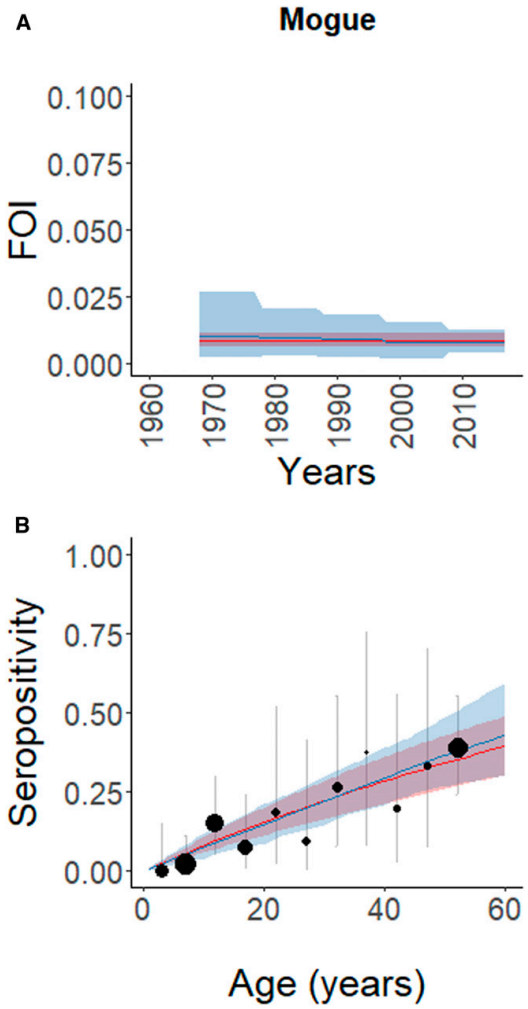

FIGURE 4. Force-of-infection (FOI) models fitted to Una virus (UNAV) seroprevalence data. (**A**) (Top panels) estimated constant (red) vs. time-varying FOI (blue) for UNAV in eastern Panama over 50 years and (**B**) (bottom panels) fitted and observed seroprevalence. Red lines represent the estimated constant FOI and blue lines the estimated time-varying FOI. In each case, the shading represents 95% credible intervals from the model. The circles' radii in the lower panels indicate sample size in each 5-year age-group, and the vertical lines represent 95% CIs for observed seroprevalence. This figure appears in color at www.ajtmh.org.

DISCUSSION

By analyzing data from recent cross-sectional seroprevalence studies, we reconstructed alphavirus transmission in eastern Panama. Historical transmission rates indicated endemic transmission of VEEV, MADV, and UNAV in humans with increased human exposure during the past decade. Here, we show evidence of acute IgM antibody responses against MADV and VEEV in people without signs of neurologic disease, suggesting asymptomatic infections or mild disease. To our knowledge, this is the first evidence of human infection with UNAV in Panama, although its circulation was reported during the 1960s in mosquitoes (*Ps. ferox* and *Ps. albipes*) collected in western Panama.[16] To our knowledge, our results demonstrate the highest seroprevalence of UNAV reported in the literature as for July 2020.[14,28]

Using catalytic FOI model fit to age-stratified seroprevalence data, we reconstructed 50 years of historical transmission rates for VEEV and MADV for seven locations in Darien Province. In most locations, the data indicated consistent endemic transmission of these viruses. In two locations—Mercadeo (for VEEV) and Aruza (for MADV), there was evidence of a recent increase in human exposure. These results suggest that MADV and VEEV incidences differ geographically. The observed FOI profile suggests that VEEV infections increased in Pirre 1-2 and Pijibasal and Mercadeo, locations surrounded by tropical forest, whereas MADV infections increased mostly in Aruza, a formerly forested area converted to agricultural land over 30 years ago.[29] Although ecological changes could be associated with the increased exposure to MADV in Aruza, it is unclear which drivers could also explain the simultaneous increase in VEEV we estimated.

Only 3.6% of participants had antibodies to more than one alphavirus. Mixed alphavirus antibody responses in Peru[5] and Panama[8] suggest cross-protective immunity. However, the mechanism of cross-protection and whether some alphaviruses induce a stronger heterologous response than others remain unclear.

The MADV seroprevalence in 2017 was greater for those living with vegetation around the house, contrasting with previous evidence in 2012, suggesting possible change in exposure risk.[8] However, characteristics of houses in Mogue in 2017 may differ from areas that were surveyed in 2012.[8] Potential MADV vectors within the *Culex* (*Melanoconion*) subgenus[30] were found during our peri-domestic investigation in Mogue. This finding of vectors near houses with surrounding vegetation as a risk factor supports the hypothesis that MADV infections can occur near houses. This contrasts with VEEV risk factors, which include washing clothes in ravines or rivers, suggesting that VEEV seropositivity is associated with human incursion into the gallery forest, a potential natural habitat for development of larvae of the main vectors *Culex* (*Melanoconion*) spp.[30]

Having a house with walls was associated with lower UNAV seroprevalence in Mogue. This suggests that UNAV infections can also occur outside the forest, where the main vector *Ps. ferox* and nonhuman primates are believed to maintain the enzootic cycle.[15,16,18] *Psorophora* spp. have been also found in disturbed areas of Panama,[31] indicating potential changes in the vector habitat usage.

Alphaviral exposure was associated with several self-reported neurological and constitutional sequelae. Specifically, weakness, insomnia, depression, and dizziness were commonly associated with prior MADV, VEEV, and UNAV exposure. Depression and other neurological symptoms have also been observed after neurotropic flavivirus infections in North America.[32] However, the role of several alphaviruses in long-term neurological impairment is still unknown. This highlights the need to further investigate the long-term ramifications of alphaviral infection with objective testing (e.g., neuropsychological testing and imaging).

TABLE 5
Comparison of constant vs. time-varying FOI for UNAV, MADV, and VEEV in 2012 and 2017

Place	Virus*	Sample size	Age classes	Constant FOI model		Time-varying FOI model		Comparison		
				elpd	se	elpd	se	$elpd_{diff}$	se	*P*-value†
Pirre and Pijibasal	MADV	74	11	–4.98	1.92	–5.43	1.62	–0.45	0.46	0.835
Mercadeo	MADV	103	11	–9.19	2.40	–9.36	2.09	–0.17	0.50	0.634
Tamarindo	MADV	176	11	–6.33	2.85	–6.78	2.57	–0.45	0.33	0.916
El Real	MADV	251	11	–3.48	1.90	–3.55	1.59	–0.06	0.33	0.577
Aruza	MADV	167	11	–30.12	5.23	**–24.27**	**3.32**	5.86	2.10	0.003
Mogue	MADV	243	11	–20.92	3.16	–21.26	3.14	–0.35	0.29	0.880
Pirre and Pijibasal	VEEV	73	11	–25.15	11.38	–18.58	7.08	6.56	4.56	0.075
Mercadeo	VEEV	103	11	–26.07	2.32	**–22.26**	**2.31**	3.81	0.87	**< 0.001**
Tamarindo	VEEV	176	11	–14.01	2.54	–13.18	2.37	0.83	0.71	0.120
El Real	VEEV	251	11	–27.68	5.89	–25.34	4.23	2.35	1.94	0.112
Aruza	VEEV	167	11	–20.98	1.87	–20.98	1.79	0.00	0.40	0.503
Mogue	VEEV	243	11	–21.53	2.68	–22.24	2.80	–0.70	0.36	0.976
Mogue	UNAV	243	11	–17.84	1.78	–18.63	2.22	–0.78	0.67	0.880

elpd = expected log predictive density for an out-of-sample data point; $elpd_{diff}$ = difference in elpd between the two models; FOI = force-of-infection; MADV = Madariaga virus; UNAV = Una virus; VEEV = Venezuelan equine encephalitis virus; se = standard error.
* Based on plaque reduction neutralization test results.
† Based on comparing *z* statistics with standard normal quantiles; results with $P < 0.05$ (shown in boldface) indicate the time-varying FOI model significantly outperformed the constant FOI model.

Alphaviral RNA was not detected in samples from either humans or mosquitoes, although field surveys and collection were performed soon after the confirmation of a fatal MADV infection in the community. Although sample size is always a limiting factor in attempts to identify ongoing infections, these results suggest that these alphaviruses may be short-lived peripherally, or produce low viremia.[7] Low MAYV seroprevalence was also detected in our earlier research,[7] indicating little human exposure to this virus in Panama.

Our study has several limitations. Clinical outcomes statistically associated with exposure to these alphaviruses represent exploratory and causal inference studies that should be followed up with more comprehensive assessments. Our study only obtained preliminary data during an outbreak response to generate hypotheses. However, mosquito collections were only performed over 2 days, and the number of collected mosquitos does not allow us to draw conclusions about active viral circulation. The collection of few mosquito vectors near houses suggests close contact between vectors and humans. The use of both CDC traps baited with octanol and Trinidad traps enhanced our ability to capture alphavirus enzootic vectors.[33] The sample size used in these serosurveys only allowed us to describe general trends in the FOI over time. Also, we cannot exclude cross-reactivity or age-dependency in exposure or susceptibility. More precise estimates would require an increased sample size and, ideally, longitudinal data collection.

In summary, we investigated alphavirus transmission in Panama using age-specific seroprevalence data to look back over five decades. Our results suggest that human alphavirus infections may have gone undetected by the Panamanian surveillance system, and hint that the MADV and VEEV outbreaks in 2010 may have been due to a common increase in enzootic circulation. The antibody seroprevalence we determined for UNAV is the highest reported in Latin America. Taken together, these results coupled with potential symptoms of MADV and VEEV infection underscore the importance of developing comprehensive arboviral surveillance in Latin American enzootic regions.

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Chapter 2 – Clinical and Serological Findings of Madariaga and Venezuelan Equine Encephalitis Viral Infections: A Follow-up Study 5 Years After an Outbreak in Panama

This original research chapter comprises a publication that focused on a five-year follow-up study involving 65 patients from the original 2010 alphavirus outbreak in eastern Panama. The study aims to characterize neurological consequences, analyze antibody dynamics, and assess seroconversion rates. The chapter has been published in its definitive form in *The Open Forum Infectious Diseases Journal*.

Open Forum Infectious Diseases

MAJOR ARTICLE

Clinical and Serological Findings of Madariaga and Venezuelan Equine Encephalitis Viral Infections: A Follow-up Study 5 Years After an Outbreak in Panama

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Background. Human cases of Madariaga virus (MADV) infection were first detected during an outbreak in 2010 in eastern Panama, where Venezuelan equine encephalitis virus (VEEV) also circulates. Little is known about the long-term consequences of either alphavirus infection.

Methods. A follow-up study of the 2010 outbreak was undertaken in 2015. An additional survey was carried out 2 weeks after a separate 2017 alphavirus outbreak in a neighboring population in eastern Panama. Serological studies and statistical analyses were undertaken in both populations.

Results. Among the originally alphavirus-seronegative participants (n = 35 of 65), seroconversion was observed at a rate of 14.3% (95% CI, 4.8%–30.3%) for MADV and 8.6% (95% CI, 1.8%–23.1%) for VEEV over 5 years. Among the originally MADV-seropositive participants (n = 14 of 65), VEEV seroconversion occurred in 35.7% (95% CI, 12.8%–64.9%). In the VEEV-seropositive participants (n = 16 of 65), MADV seroconversion occurred in 6.3% (95% CI, 0.2%–30.2%). MADV seroreversion was observed in 14.3% (95% CI, 1.8%–42.8%) of those who were originally seropositive in 2010. VEEV seroconversion in the baseline MADV-seropositive participants was significantly higher than in alphavirus-negative participants. In the population sampled in 2017, MADV and VEEV seroprevalence was 13.2% and 16.8%, respectively. Memory loss, insomnia, irritability, and seizures were reported significantly more frequently in alphavirus-seropositive participants than in seronegative participants.

Conclusions. High rates of seroconversion to MADV and VEEV over 5 years suggest frequent circulation of both viruses in Panama. Enhanced susceptibility to VEEV infection may be conferred by MADV infection. We provide evidence of persistent neurologic symptoms up to 5 years following MADV and VEEV exposure.

Keywords. Madariaga virus; neurological sequelae; Panama; Venezuelan equine encephalitis virus.

Madariaga (MADV; formerly known as South American eastern equine encephalitis) and Venezuelan equine encephalitis viruses (VEEV) are single-stranded RNA arthropod-borne zoonotic viruses (*Togaviridae: Alphavirus*), with circulation throughout much of the Americas [1]. Enzootic subtypes of the VEE antigenic complex are associated with human endemic, and sometimes fatal, infections in the Americas [2]. Human infections with these subtypes occur via spillover from enzootic cycles that involve sylvatic rodents and mosquitoes of the subgenus Culex (Melanoconion). VEEV epizootic/epidemic subtypes (IAB, IC) are associated with large and explosive equine-amplified epidemics in South America, and the available evidence suggests that epizootic strains evolve from enzootic ancestors via mosquito- or equine-adaptive mutations [3]. In Panama, enzootic/endemic VEEV subtype ID infection is highly prevalent in the easternmost province of Darien, resulting in up to 75% seroprevalence in some villages [4]. In this setting, MADV first emerged in the human population in 2010 in the eastern province of Darien, Panama [5]. Enzootic VEEV (subtype ID) was simultaneously circulating in the same area, causing significant neurologic morbidity and mortality [5].

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Clinically, most human VEEV infections are symptomatic with dengue or influenza-like illness [6]. VEEV is underdiagnosed in Latin America, where it has been estimated that 0.1%–7% of dengue cases are in fact VEEV infections [2]. In the Americas, 30–50 million dengue infections were estimated to have occurred in 2010 [7]. By extension, then, VEEV could account for 300 000 to 3.5 million cases per year. Around 15% of VEEV cases develop neurologic disease, of which 1% are fatal [2]. VEEV infection manifests most frequently as a self-limited febrile illness, with a smaller portion of cases involving encephalitis, seizures, spontaneous abortions, and gastrointestinal hemorrhage [2, 8–10].

In contrast to VEEV, only 3 human cases of MADV were identified in the Americas before the 2010 outbreak in Panama, despite extensive research and epidemiologic surveillance in enzootic areas [11–13]. Mosquito vectors of MADV are also members of the subgenus *Culex* (*Melanoconion*). However, the main reservoir in Latin America is still unknown [14]. We previously reported on the acute clinical presentation, which included a nonspecific febrile illness, meningoencephalitis, and encephalitis usually accompanied by seizures, hemiparesis, and psychomotor retardation [5]. The severe symptoms were primarily seen in children.

As MADV is an emerging virus in Latin America, its long-term sequelae remain unknown. Our literature search revealed little information about long-term neurologic sequelae of enzootic VEEV infections. Following an outbreak in Texas in 1971, Bowen et al. (1976) described signs and symptoms in 86 patients hospitalized with VEEV [6]. None of the affected children reported any sequelae, but 7 of 9 adults examined 9 months later complained of fatigue.

The objective of this study was to estimate seroconversion rates and clinical consequences of MADV and VEEV infections by following up on probable and confirmed cases and their household contacts identified during the 2010 outbreak. We also examine the neurological symptoms of a neighboring population according to alphavirus exposure status. This is the first report, to our knowledge, of the long-term neurological symptoms following MADV, and only the second such report for VEEV.

METHODS

This study was undertaken in the easternmost province of Darien, Panama, which borders Colombia. Alphaviral encephalitis outbreaks were reported in Darien in 2010 [5] and 2017 [15]. A follow-up study of patients (suspected, probable, or confirmed) from the 2010 outbreak and their household contacts was undertaken in 2015. Detailed information on the 2010–2015 cohorts is provided in Tables 1 and 2. An additional population survey was undertaken in 2017 in Mogue, a community in Darien that was not included in the passive surveillance efforts of 2010. Samples were processed at Panama's reference laboratory, the Gorgas Memorial Institute, located in Panama City.

Table 1. Characteristics of the Study Populations in the 2010 Outbreak Cohort Studied in 2015 (n = 65) and the Mogue Study in 2017 (n = 243)

Characteristic		2015, No. (%)	2017, No. (%)
Sex			
	Male	37 (56.9)	120 (49.4)
	Female	28 (43.1)	123 (50.6)
Age (tercile), y			
	1–17	24 (36.9)	129 (53.1)
	19–43	19 (29.2)	64 (26.3)
	45–90	22 (33.9)	50 (20.6)
Occupation			
	Professional	3 (4.6)	8 (3.3)
	Technician	1 (1.5)	1 (0.4)
	Construction worker	2 (3.1)	0 (0.0)
	Housewife	15 (23.1)	56 (23.1)
	Student	15 (23.1)	93 (38.4)
	Rancher	13 (20.0)	48 (19.8)
	Farmer	11 (16.9)	48 (19.8)
	Preschool-aged child	1 (1.5)	24 (9.9)
	Other	12 (18.5)	8 (3.3)

Human Survey and Clinical Evaluation in 2015

Participants were located using records from 2010. Upon obtaining written consent, each participant was interviewed and examined by a physician using a demographic and focused medical history questionnaire. The presence or absence of neurological symptoms within the preceding 2 weeks was noted. Blood samples were obtained by peripheral venipuncture. Serum samples were placed in cryogenic tubes for storage in liquid nitrogen and then transported to the Gorgas Memorial Institute for analysis.

Human Survey and Clinical Evaluation in 2017

The survey was carried out from July 18 to 22, 2017, 3 weeks after confirmation of a fatal MADV case on June 30, 2017. A cross-sectional outbreak investigation was undertaken, and blood sampling and epidemiological surveys were carried out including demographic characteristics, potential risk factors,

Table 2. MADV and VEEV Seroprevalence by Survey Year as Determined by Plaque Reduction Neutralization Tests

Year	Alphavirus Seronegative, No. (%)	MADV Seropositive, No. (%)	VEEV Seropositive, No. (%)	Dual Infection, No. (%)
2010 (n = 65)	35 (53.8)	14 (21.5)	16 (24.6)	0
2015 (n = 65)	29[a] (44.6)	18[a] (27.7)	24 (36.9)	6 (9.2)

Abbreviations: MADV, Madariaga virus; VEEV, Venezuelan equine encephalitis virus.

[a]There were 28 alphavirus-seronegative participants, but 1 MADV seroreversion rendered that individual also seronegative. The other individual with MADV seroreversion became VEEV positive in 2015.

and clinical information. The presence or absence of neurological symptoms within the preceding 2 weeks was evaluated and recorded by a physician. Detailed information on surveys, laboratory testing, risk factors, and serologic results have been previously described [15]. The purpose of this 2017 survey was to describe alphaviral epidemiology and neurological sequelae in a community in which a fatal MADV case was confirmed. The epidemiological results are reported separately [15]. We include the 2017 neurological symptom survey here to validate the findings from our 2010 cohort.

Patient Consent Statement

An outbreak investigation in 2017 was undertaken during a public health outbreak response, and ethical approval for alphavirus and encephalitis surveillance and investigation was obtained from the Gorgas Memorial Institute Committee (IRB, CBI/ICGES/2015 and CIB/ICGES/2018). Study participation was voluntary, and written informed consent was obtained from adults (18 years of age and older) and from parents or guardians of children aged 2 to 17 years. In addition, verbal assent was obtained from children aged 7 to 12 years, and written informed consent was obtained from children aged 13 to 17 years.

Laboratory

Samples were also tested in duplicate for IgM and IgG antibodies against MADV and VEEV using enzyme-linked immunosorbent assays (ELISAs) for recent and past antibody response detection [16]. All samples were also run using virus-specific plaque-reduction neutralization tests (PRNTs). For the PRNT, a positive sample was reported as the reciprocal of the highest dilution that reduced plaque counts by >80% ($PRNT_{80}$). ELISA antigens were prepared from eastern equine encephalitis virus (EEEV) using the sucrose-acetone technique (prepared by Dr. Robert Shope at the Yale Arbovirus Research Unit in August 1989) and VEE complex strain 78V-3531 from infected mouse brain. Strains used for the PRNT were the chimeric SINV/MADV [17], MADV wild-type strain PA2010-247168 (UTMB/WRCEVA), and TC83, an attenuated vaccine strain of VEEV closely related to the subtype ID strains that circulate in Panama [18].

Case Definition

We deemed IgM-positive samples to represent infection within 12 months. While there are no studies to our knowledge on the persistence of alphavirus IgM, dengue IgM wanes for most people by 1 year [19]. Samples positive for IgM and negative for IgG were classified as recent infection (within 2 weeks). Samples positive for IgG alone indicated past exposure. Due to substantial cross-reactivity between MADV and VEEV by ELISA, virus-specific infection was determined by $PRNT_{80}$ (a titer of ≥1:20 was determined to be positive). In the event that PRNTs for both viruses were positive, we deemed a 4-fold difference in titers to discriminate between the causative virus vs cross-reactivity. If less than a 4-fold difference was present, we classified this as exposure to both viruses.

Statistical Analysis

Seroconversion rates for both viruses were calculated for each alphaviral exposure group from the 2010 cohort and reported with exact binomial 95% confidence intervals. The probability of seroconversion by alphaviral exposure group was determined by calculating risk ratios with Fisher exact *P* values given the small sample sizes. To determine whether neurological sequelae were reported at higher frequencies in the alphavirus-exposed group, we conducted a univariate logistic regression analysis, followed by a multivariable logistic regression controlling for sex and age. The outcome variable, alphaviral exposure, was defined as having been exposed to MADV, VEEV, or both viruses using the laboratory criteria described above. *P* values with alpha <.05 were considered significant. All analyses were undertaken using the statistical package Stata, version 14 (StataCorp, College Station, TX, USA).

RESULTS

Characteristics of the Population

During the original 2010 outbreak, 190 cases with febrile or neuroinvasive disease and 72 household contacts were included. In the 2015 follow-up study, 165 of these participants were excluded due to incomplete baseline information, and an additional 32 cases could not be located. A total of 65 cases were ultimately included in the 2015 follow-up serosurvey (Supplementary Figure 1). Characteristics of the 2015 and Mogue 2017 study populations are described in Table 1. There was a slight preponderance of men compared with women in the 2015 follow-up study, with an even age distribution. The most frequent occupational categories included housewife, student, and farmer. In contrast, the Mogue 2017 population was more heavily weighted toward younger participants (53.1% younger than 17 years), with a correspondingly higher rate of students.

Alphavirus Epidemiological and Serological Profiles

At baseline in 2010, 14 (21.5%) of the 65 participants were seropositive for MADV only, 16 (24.6%) were seropositive for VEEV only, and 0 were seropositive for both as determined by PRNT (Table 3).

Between 2010 and 2015, MADV seroconversion occurred in 6 participants. One of these participants was MADV IgM positive (and IgG positive). VEEV seroconversion occurred in 8 participants. One of these participants was VEEV IgM positive (and IgG positive). Among the 35 participants who were alphavirus-seronegative in 2010, seroconversion was observed for MADV in 5 of 35 (14.3%; 95% CI, 4.8%–30.3%) and for VEEV in 3 of 35 (8.6%; 95% CI, 1.8%–23.1%) by 2015 (Table 2).

Table 3. Neutralizing Antibody Titers Measured in 2010 and 2015 Among the 6 Individuals Found to Have Antibodies to Both MADV and VEEV in 2010

	Neutralizing Antibodies			
	2010		2015	
ID	MADV	VEEV	MADV	VEEV
37	1:20	1:640	<1:20	1:160
40	1:20	1:640	<1:20	1:320
44	<1:20	1:640	<1:20	1:80
44[a]	1:20	1:640		
47	1:40	1:160	<1:20	1:80
49	1:80	1:640	<1:20	>1:640
54	1:20	1:640	<1:20	1:320
54[a]	1:40	1:640		

Abbreviations: MADV, Madariaga virus; VEEV, Venezuelan equine encephalitis virus.
[a]Second convalescent serum sample obtained ~15 days after onset of illness.

Among the 14 participants who were MADV-seropositive in 2010, seroconversion to VEEV was observed in 2015 in 5 of 14 (35.7%; 95% CI, 12.8%–64.9%). Only 1 of the 16 participants who were VEEV-seropositive in 2010 seroconverted to MADV by 2015 (6.3%; 95% CI, 0.2%–30.2%). Seroreversion (disappearance of detectable antibodies) was observed for MADV in 2 of 14 participants (14.3%; 95% CI, 1.8%–42.8%). No VEEV seroreversions were observed. The risk ratio for seroconversion to MADV in VEEV-seropositive (in 2010) participants vs previously MADV- and VEEV-seronegative subjects was 0.4 (95% CI, 0.1–3.4; Fisher exact $P = .38$). The risk ratio for seroconversion to VEEV in MADV-seropositive (in 2010) subjects vs MADV- and VEEV-seronegative subjects was 4.2 (95% CI, 1.1–15.1; Fisher exact $P = .03$).

A total of 6 participants were found to have neutralizing antibodies to both MADV and VEEV in 2010, though in each instance titers were at least 4-fold greater against VEEV (Table 3). Convalescent serum sample results (>15 days after symptom onset) were obtained for 2 of these participants (ID 44 and 54) in 2010. These demonstrate a rise in MADV titer in relation to the acute samples. Each of these participants with low MADV titers became MADV $PRNT_{80}$–negative (≥1:20) by 2015 (Table 3).

The mean age of MADV seroconverters was 42.0 years, spanning a large range (10 to 75 years). Most were men (n = 4, 66.7%), and half were cattle ranchers (n = 3, 50.0%, compared with 20.0% of the total sample). Cases were clustered around the township Meteti, located on the Panamerican Highway in the middle of Darien Province. The mean age of VEEV seroconverters (range) was lower, at 14.1 (7–25) years. The cases were evenly distributed between men and women. This group consisted of mostly school-age children (n = 6, 75.0%) and did not include any cattle ranchers or farmers. Three of the participants were also from the township of Meteti; others were scattered throughout the province.

Seroprevalence in the Mogue Cross-Sectional Survey

A total of 243 individuals were surveyed in Mogue, Darien Province, during 2017, with seroprevalence of 31/243 (12.8%) for MADV and 42/243 (17.3%) for VEEV. The MADV-positive participants had a mean age (range) of 35.3 (5–78) years and an equal distribution of men (n = 16) and women (n = 15). Most positive participants were housewives (n = 11, 35.5%), farmers (n = 9, 29%, and students (n = 9, 25.8%). The VEEV-positive participants had a mean age (range) of 37.9 (2–97) years and were mostly women (n = 26, 61.9%). The majority were housewives (n = 19, 45.2%), followed by farmers (n = 12, 28.6%) and students (n = 8, 19.1%).

Neurological Symptoms

Memory loss, dizziness, fatigue, difficulty concentrating, confusion, depression, irritability, myalgia, insomnia, seizures, and impairment in activities of daily living were more frequent in participants exposed to VEEV and/or MADV (Table 4; Supplementary Figure 2). We repeated this survey in a different population in Darien, Panama, in 2017, with roughly similar results (Table 4, right-hand columns). After adjusting for sex and age, the association with prior alphaviral (MADV and/or VEEV) exposure and seizures (OR, 14.5; 95% CI, 1.6–130.1), irritability (OR, 2.7; 95% CI, 1.1–6.3), insomnia (OR, 2.5; 95% CI, 1.3–5.0), and memory loss (OR, 1.9; 95% CI, 1.1–3.3) remained statistically significant (Table 5).

DISCUSSION

We provide new clinical and epidemiological findings on human infection with MADV and VEEV. Our seroconversion results suggest that MADV has become endemic, with co-circulation of VEEV in eastern Panama. Participants positive for MADV in 2010 had higher rates of VEEV seroconversion than those who were originally seronegative for alphaviruses. On the other hand, participants with prior exposure to VEEV tended to seroconvert to MADV at lower rates than those who were originally alphavirus seronegative. These data suggest that MADV exposure leads to enhanced susceptibility against VEEV, but not vice versa. The mechanism underlying this enhanced susceptibility to VEEV following MADV exposure is unclear. Increased risk of acquiring alphavirus sympatric infections may reflect their similar enzootic habitat and overlapping epidemiological risk of acquisition [5]. However, we have observed differences in MADV and VEEV vector and host usage, as well as different geographic distributions of disease [4, 15]. The seemingly increased susceptibility to VEEV conferred by prior exposure to MADV may also be explained by immune interference. Alphavirus vaccine studies have demonstrated that sequential vaccination with eastern equine encephalitis (EEE) and western equine encephalitis (WEE) vaccines before live-attenuated VEE vaccination resulted in a diminished VEEV-neutralizing antibody response

Table 4. Frequency of Neurological Signs and Symptoms (%) Reported in 2 Study Populations in Darien, Panama

Symptoms	Original Outbreak Cohort (n = 65)				2017 Mogue Study (n = 243)			
	MADV (n = 13)	VEEV (n = 17)	MADV & VEEV (n = 7)	No Alphaviral Exposure (n = 28)	MADV (n = 22)	VEEV (n = 33)	MADV & VEEV (n = 9)	No Alphaviral Exposure (n = 179)
Memory loss	6 (46)	8 (47)	4 (57)	8 (29)	8 (36)	10 (30)	3 (33)	37 (21)
Headache	5 (42)[a]	7 (41)	4 (57)	11 (39)	9 (41)	15 (45)	6 (67)	80 (45)
Dizziness	5 (38)	6 (35)	4 (57)	5 (18)	7 (32)	13 (39)	5 (56)	47 (26)
Fatigue	4 (31)	5 (29)	3 (43)	4 (14)	10 (45)	14 (42)	5 (56)	56 (31)
Confusion	5 (38)	5 (29)	1 (14)	2 (7)	3 (14)	8 (24)	3 (33)	27 (15)
Depression	2 (17)	5 (29)	3 (43)	3 (11)	5 (23)	1 (3)	1 (11)	15 (8)
Irritability	4 (31)	5 (29)	1 (14)	1 (4)	1 (5)	3 (9)	1 (11)	11 (6)
Myalgia	2 (17)	3 (18)	3 (43)	4 (14)	8 (36)	9 (27)	4 (44)	40 (22)
Insomnia	3 (23)	4 (24)	2 (29)	3 (11)	3 (14)	6 (18)	6 (67)	18 (10)
Difficulty with activities of daily living	3 (23)	1 (6)	1 (14)	1 (4)	6 (27)	6 (18)	2 (22)	20 (11)
Weakness	1 (8)	3 (18)	1 (14)	2 (7)	9 (41)	9 (27)	4 (44)	43 (24)
Seizures	2 (15)	0 (0)	0 (0)	0 (0)	2 (9)	2 (6)	0 (0)	1 (0.6)
Paralysis	1 (8)	0 (0)	0 (0)	0 (0)	1 (5)	4 (12)	0 (0)	6 (3)
Total	13	17	7	28	22	33	9	179

Exposure status reflects seropositivity measured in 2015 for the original outbreak cohort and seropositivity measured in 2017 for the Mogue study. Note participants whose MADV titers seroreverted in 2015 are classified as MADV positive.

Abbreviations: MADV, Madariaga virus; VEEV, Venezuelan equine encephalitis virus.

[a]One nonrespondent in this category yielded a denominator of 12.

[20, 21]. To our knowledge, this is the first epidemiological study to demonstrate asymmetric human cross-immunity findings, even though these viruses were identified almost a century ago in the 1930s [2,14].

In contrast, VEEV infection appears to generate a robust and durable immune response. Not only do titers remain detectable 5 years after exposure, but the low rate of seroconversion to MADV during this period suggests the presence of cross-protective immunity. The alphaviral vaccine literature provides ample evidence of in vivo examples of cross-protection. For example, hamsters inoculated with an attenuated strain of VEEV experienced a 37% reduction in mortality when subsequently exposed to western equine encephalitis virus (WEEV) and a 59% reduction in mortality when inoculated with EEEV [22]. However, passive transfer of neutralizing antibodies was not shown to confer protection to heterologous viruses [13, 23]. Cross-protection may therefore be attributable to a cellular or humoral response mediated by non-neutralizing antibodies [24].

Seroreversion was documented in 2 of the 14 2010 MADV-positive individuals. It is possible that neutralizing antibody titers waned over time and dropped below our limits of detection. Whether these individuals are newly at risk for MADV infection remains uncertain. In the case of hepatitis B, for example, it was demonstrated that in the face of declining antibody titers following vaccination, hepatitis B infection rates rose in Senegalese children [25].

Memory loss, dizziness, fatigue, difficulty concentrating, confusion, depression, irritability, myalgia, insomnia, and impairment of daily living activities were more frequent in participants exposed to VEEV and/or MADV. After adjusting for sex and age, increased seizures, irritability, insomnia, and memory loss remained statistically significant. Seizures and paralysis were mainly observed in severe cases of MADV encephalitis during the 2010 outbreak. However, seizures were not present in any of the 2010 VEE participants, in contrast to reports of VEE sequelae in children in Colombia [26]. Indeed, on the whole, our 2010–2015 cohort of VEEV-positive participants did not present with severe cases of neurological disease. This may reflect variations in virulence among VEEV strains [27], though there are historical reports of severe and fatal cases of enzootic VEE in Panama [18].

Seven of 9 adults reported fatigue 9 months after a VEEV outbreak in Texas in 1971 [6]. Our results more closely resemble descriptions of sequelae after WEEV infection [28], as well as West Nile virus [29, 30]. Memory loss, learning impairments, and behavioral changes are noted in approximately half of patients after acute illness due to neurotropic alphaviruses [31]. While there does not appear to be any precedent for cognitive testing of participants exposed to alphaviral infections, there have been several such studies in patients who recovered from West Nile virus (WNV). For example, Murray et al. [32] noted

Table 5. Univariate and Multivariable Logistic Regression Analysis of the Presence of Self-Reported Signs and Symptoms by Alphaviral Exposure (n = 308)

Symptoms	No. (%)	Unadjusted Odd Ratio	95% CI	*P* Value	Adjusted Odds Ratio[a]	95% CI	*P* Value
Seizures	7 (2.3)	13.21	1.56–111.3	.018	14.46	1.61–130.1	.017
Paralysis	12 (3.9)	2.14	0.68–6.83	.195	1.5	0.43–5.30	.525
Difficulty walking	29 (9.4)	1.10	0.49–2.47	.808	0.87	0.36–2.10	.767
Irritability	27 (8.8)	2.44	1.10–5.41	.028	2.68	1.14–6.27	.023
Depression[b]	35 (11.4)	1.92	0.94–3.91	.074	1.73	0.81–3.65	.154
Impairment in ADL	40 (13.0)	2.08	1.06–4.09	.032	1.47	0.71–3.03	.297
Insomnia	45 (14.6)	2.81	1.48–5.35	.002	2.52	1.28–4.97	.008
Confusion	54 (17.5)	1.88	1.03–3.41	.040	1.64	0.87–3.10	.128
Weakness	72 (23.4)	1.34	0.77–2.32	.298	1.18	0.66–2.11	.564
Myalgia	73 (23.7)	1.52	0.88–2.63	.131	1.10	0.61–1.98	.757
Memory loss	84 (27.3)	2.31	1.38–3.90	.002	1.89	1.09–3.28	.022
Dizziness	92 (29.9)	1.87	1.12–3.10	.016	1.48	0.87–2.54	.148
Fatigue	1001 (32.8)	1.61	0.98–2.65	.063	1.27	0.74–2.16	.376
Headache	137 (44.6)	1.05	0.65–1.70	.840	0.86	0.51–1.45	.582

Abbreviations: ADL, activities of daily living.

[a]Adjusted by age and sex.

[b]n = 307.

that at 8 years from initial infection (neuroinvasive and febrile WNV), 40% of patients still reported sequelae. We suspect that the true burden of MADV and VEEV extends substantially beyond the acute phase of illness. Understanding the full scope of such sequelae is paramount in this region, given the high prevalence of MADV and VEEV exposure.

Interestingly, the majority of MADV- and VEEV-positive individuals in our study did not recall having encephalitis or severe neurological signs/symptoms. Other studies have also suggested that the majority of the encephalitic alphaviral infections present as a self-limited febrile illness [2,33]. Thus, the high rates of self-reported neurological sequelae in this study suggest that long-term neurological sequelae occur even after mild to moderate clinical presentations.

Our study has several limitations. Because this follow-up study was undertaken 5 years after the outbreak, we were not able to determine the timing of seroconversion during the 5-year period before data collection. It is therefore possible that the majority of the seroconversions occurred shortly after the 2010 outbreak. However, we did note that 1 of the participants presented with MADV IgM in 2015, suggesting recent infection. In addition, our sample size was small because our cohort was generated from participants originally tested during an outbreak, and there were many participants who could not be traced. Therefore, our data may not be generalizable. Longitudinal studies of a larger population are important to validate the seroconversion rates and cross-immunity we describe here. Furthermore, there may have been bias in reporting neurological signs and symptoms stemming from the fact that the participants were selected based on their inclusion in the 2010 outbreak studies. We sought to counteract this by including neurological symptom data from a separate cross-sectional study conducted in 2017.

In summary, our results demonstrate that MADV remains in circulation and is an important human pathogen in Panama. We further describe other novel findings, such as the decay in MADV antibodies in some individuals, the possibility of cross-protective immunity conferred by VEEV but not by MADV, and increased susceptibility to VEEV conferred by prior MADV exposure. These findings have implications for vaccine development and merit further study. In addition, we provide preliminary information on the persistence of neurologic symptoms following MADV and VEEV infection. Future investigation into the duration and magnitude of such sequelae, as well as underlying mechanisms and risk factors, may benefit not only those affected by these viruses but also the larger population exposed to similar neurotropic arboviruses.

Supplementary Data

Supplementary materials are available at Open Forum Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Chapter 3 – Real-time RT-PCR for Venezuelan equine encephalitis complex, Madariaga, and Eastern equine encephalitis viruses: application in human and mosquito public health surveillance in Panama

This original research chapter was published in its final in the *Journal of Clinical Microbiology*. The objective of this Chapter was to devise a novel method for enhanced alphavirus molecular diagnosis and surveillance. I validated the newly developed Real-Time RT-PCR using historical clinical and mosquitoes samples and initiated an alphavirus febrile surveillance in Eastern Panama.

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Real-time RT-PCR for Venezuelan equine encephalitis complex, Madariaga, and Eastern equine encephalitis viruses: application in human and mosquito public health surveillance in Panama

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ABSTRACT Eastern equine encephalitis virus (EEEV), Madariaga virus (MADV), and Venezuelan equine encephalitis virus complex (VEEV) are New World alphaviruses transmitted by mosquitoes. They cause febrile and sometimes severe neurological diseases in human and equine hosts. Detecting them during the acute phase is hindered by non-specific symptoms and limited diagnostic tools. We designed and clinically assessed real-time reverse transcription polymerase chain reaction assays (rRT-PCRs) for VEEV complex, MADV, and EEEV using whole-genome sequences. Validation involved 15 retrospective serum samples from 2015 to 2017 outbreaks, 150 mosquito pools from 2015, and 118 prospective samples from 2021 to 2022 surveillance in Panama. The rRT-PCRs detected VEEV complex RNA in 10 samples (66.7%) from outbreaks, with one having both VEEV complex and MADV RNAs. VEEV complex RNA was found in five suspected dengue cases from disease surveillance. The rRT-PCR assays identified VEEV complex RNA in three *Culex* (*Melanoconion*) *vomerifer* pools, leading to VEEV isolates in two. Phylogenetic analysis revealed the VEEV ID subtype in positive samples. Notably, 11.9% of dengue-like disease patients showed VEEV infections. Together, our rRT-PCR validation in human and mosquito samples suggests that this method can be incorporated into mosquito and human encephalitic alphavirus surveillance programs in endemic regions.

KEYWORDS Venezuelan equine encephalitis, Madariaga virus, Eastern equine encephalitis virus, alphavirus, rRT-PCR

New World alphaviruses (*Togaviridae*, genus *Alphavirus*) are a diverse group of mosquito-borne viruses that can cause severe disease in humans, including the Venezuelan equine encephalitis virus complex (VEEV complex), Madariaga virus (MADV), and Eastern equine encephalitis virus (1, 2). These persist in sylvatic-enzootic cycles throughout the Americas and are transmitted to humans by *Aedes* spp., *Psorophora* spp., and *Culex* spp. mosquitoes (2, 3).

Serologic and molecular evidence points to widespread VEEV complex infections in tropical Central and South America, indicating potential commonality yet significant underdiagnosis (2). At least 14 different viral subtypes within the VEEV complex have been identified to date (2), some associated with large equine and human outbreaks (VEEV subtypes IAB and IC) (1, 2). While most infections in humans are asymptomatic or subclinical, patients may develop acute febrile illness with headache, myalgias, arthralgias, nausea, and vomiting (4, 5). Cases can progress to encephalitis and result in long-term neurological effects (5, 6).

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MADV, once considered a variant of EEEV, is an emerging virus that was first associated with large outbreaks in 2010 in the Darien province of Panama (5), where VEEV subtype ID has also been detected (7). MADV was primarily linked to equine disease, with a few human cases in Trinidad and Tobago and Brazil before the Panama outbreak (8, 9). This contrasts with North American EEEV, associated with severe and fatal human cases (3). MADV detection methods are limited, and its prevalence outside Darien province is not well understood (10). MADV's geographic expansion to Northeast Brazil and Haiti highlights its potential for new areas (11, 12).

Accurate detection of VEEV complex, MADV, and EEEV during the acute phase is hindered by non-specific clinical signs and limited diagnostic tools. Antigen-based methods are unavailable, and serology requires paired samples to confirm diagnosis (1, 5). Current molecular tests lack optimal performance characteristics necessary for routine testing (13–19), and assay design is challenged by VEEV complex genetic variability (2). VEEV complex and MADV are often misdiagnosed as dengue virus due to similar symptoms during the acute phase (2). Common molecular tests involve pan-alphavirus primers amplifying a 400–500 nucleotide genome region, followed by sequencing or nested PCR for identification (5, 13, 14, 16, 18–20). These methods are labor-intensive and prone to contamination. Pan-alphavirus primers and conventional reverse transcription polymerase chain reaction (RT-PCR) chemistry may be less sensitive than real-time RT-PCR (rRT-PCR), with few reported rRT-PCR methods differentiating the VEEV complex and MADV (21).

The study aimed to design rRT-PCRs for the VEEV complex and MADV, with a secondary goal of developing a duplex MADV/EEEV rRT-PCR. These assays were evaluated using clinical samples from a Panama alphavirus outbreak and disease surveillance. Additionally, viral species, subtype, and genotype characterization were done using metagenomic sequencing on rRT-PCR-positive samples from humans and mosquitoes collected during the 2015 and 2022 outbreaks in Panama.

MATERIALS AND METHODS

VEEV complex, EEEV and MADV rRT-PCR design

Distinct alignments were established for the VEEV complex, EEEV, and MADV using comprehensive genome sequences from the NCBI GenBank (22) and aligned with MegAlign software (DNASTAR, Madison, WI, USA). The VEEV complex alignment encompassed complete genomes from Cabassou, Everglades, Mosso das Pedras, Mucambo, Pixuna, Rio Negro, Tonate, and VEEV subtypes (IAB, IC, ID, and IE). This compilation occurred in 2016 (n = 121 sequences), with a similar one for MADV in 2019 (n = 32). Employing Primer3 software (primer3.ut.ee), primers and probes were designed to contain ≤1 degenerate base and to align ≥95% with available sequences for each virus (Table 1). *In silico* validation details can be found in supplemental material.

rRT-PCR assay performance and optimization

Primer and probe sets were evaluated in singleplex reactions containing 200 nM of each oligonucleotide and genomic RNA or quantified ssDNA containing the target region. Primer/probe sets were selected to generate the most sensitive detection based on cycle threshold (Ct) values, with preserved specificity. Primer and probe concentrations in the final reaction were then adjusted between 100 and 400 nM to optimize assay sensitivity. For VEEV, a total of four primers are mixed in a single reaction (Table 1). Additional validation, conditions, and lower limit of detection (LLOD) are given in the supplemental material (Fig. 1).

Protocol validation with acute human samples

Acute human samples used in the protocol validation were collected in communities of Darien, the easternmost province in Panama, during three alphavirus outbreaks in 2015

TABLE 1 Primers and probes in the VEEV and MADV/EEEV rRT-PCRs[a]

Name	Sequence[b]	Concentration (nM)[c]	Location (5′–3′)[d]	Sequences fully matching[e]
VEEV				
VEEV forward 1	GAAAGTTCACGTTGAYATCGAGGA	200		
VEEV forward 2	GAAGGTTCACGTTGAYATCGAGGA	200	44–67	156/159 (98)
VEEV reverse 1	GCTCTGGCRTTAGCATGGTC	200		
VEEV reverse 2	GCTCTAGCRTTAGCATGGTC	200	144–163	159/159 (100)
VEEV probe	5′-FAM-TTGAGGTAGAAGCHAAGCAGGTC-BHQ-1–3′	400	112–134	158/159 (99)
MADV/EEEV				
ME forward	GAGATAGAAGCMACGCAGGTC	400	121–141; 99–119	31/32 (97); 1/449 (100)
ME reverse	TGYTTGGAATGCGTGTGC	400	255–272; 233–250	32/32 (100); 9/449 (98)
MADV probe	5′-FAM-CATCGAAAGCGAAGTGGACC-BHQ-1–3′	200	195–214	31/32 (97)
EEEV probe	5′-CFO560-TGAGGGAGAAGTGGAYACAGACC-BHQ-1–3′	400	176–198	6/449 (99)

[a]BHQ, black hole quencher; CFO560, CAL Fluor Orange 560; and FAM, Fluorescein.
[b]Probe sequences listed 5′′-fluorophore-sequence-quencher-3′′.
[c]Concentration in the final reaction mixture.
[d]Location in the following complete genome sequences: VEEV strain VEEV/Homo sapiens/GTM/69Z1/1969/IAB (Aaccession number KC344505.2); MADV strain Homo sapiens/Haiti-1901/2016 (MH359233.1); EEEV strain EEEV/Culiseta melanura/USA/SL13-0764-C/2013 (Aaccession number KX029319.1).
[e]Displayed as number of complete genome sequences without a mismatch in the primer/probe sequence over all complete genome sequences aligned (%). Genomes downloaded on 22 Sept. 2021. Data shown for the combination of forward and reverse VEEV primers.

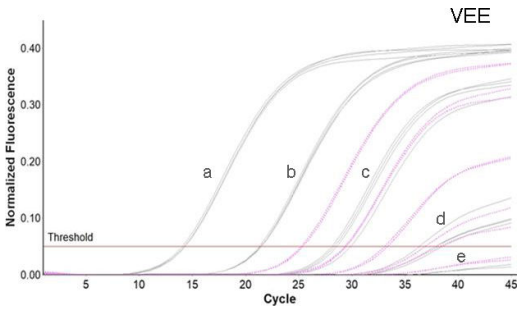


FIG 1 VEE amplification curves across a range of concentrations. Amplification curves are shown across a range of concentrations for the VEE complex rRT-PCR with ssDNA (gray curves, subtype IAB) and RNA (pink dotted curves, subtype IC). ssDNA was tested in quadruplicate at 8.0, 6.0, 4.0, 2.0, and 1.0 $\log_{10}$ copies/µL (labeled a–e, respectively). 10-fold dilutions of VEEV subtype IC RNA were tested in duplicate starting at the highest concentration available (5.0 $\log_{10}$ copies/µL).

and 2017. Cases identified in 2015 and 2017 were detected in the communities of Metetí, Cemaco, Tucutí, Yaviza, Nicanor, La Palma, and El Real de Santa María (Fig. 2A). The Darien province borders Colombia and encompasses the Darien Gap, and the Darien National Park, a UNESCO-designated World Heritage Site (23).

Patient recruitment in 2015 and 2017

Febrile patients were identified during an enhanced surveillance program by our outbreak response team using house-by-house visits during the 2015 and 2017 outbreaks. Blood samples were drawn from patients who met the case definition during the outbreak investigation.

Prospective acute disease surveillance in 2021 and 2022

In 2021, surveillance for emerging pathogens was established in Panama as part of the USA-National Institute of Allergy and Infectious Diseases, Centers for Research in Emerging Infectious Diseases Network initiative. The Coordinating Research on Emerging Arboviral Threats Encompassing the Neotropics (CREATE-NEO) in Panama undertakes

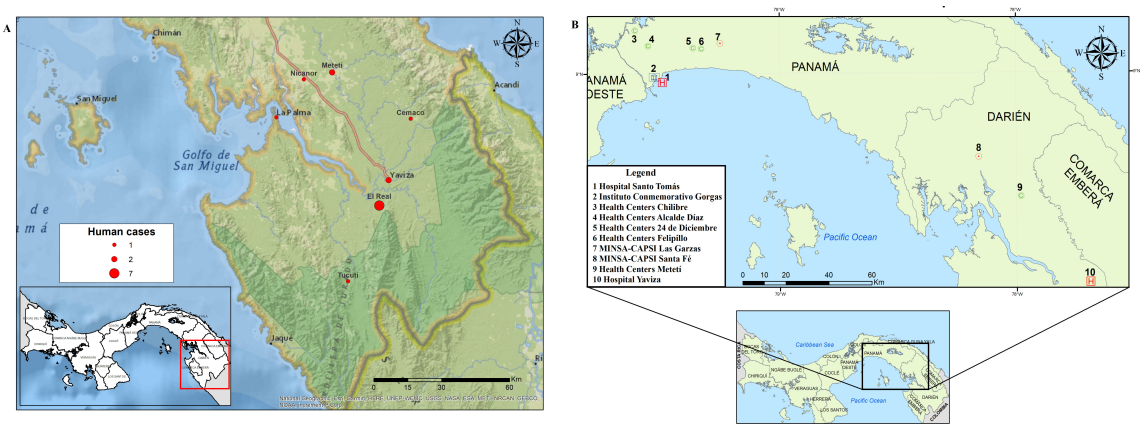


FIG 2 Map with the distribution of VEEV human cases in Darien province in 2015 and 2017, and health centers in Panama and Darien provinces. (A) Distribution of VEEV cases used for protocol validation. Red dots represent the number of cases reported by locality. (B) Distribution of health centers used for prospective febrile surveillance in Panama and Darien provinces. The map was created with ArcGIS Desktop 10.6 using shapefiles from Esri [World Countries Generalized (2021); https://www.arcgis.com/home/item.html?id=2b93b06dc0dc4e809d3c8db5cb96ba69]. Data sources for the shapefiles include Esri, Garmin International Inc., U.S. Central Intelligence Agency, and National Geographic Society (24).

acute febrile surveillance across 10 health centers in Panama and Darien Provinces (Fig. 2B) (https://www.utmb.edu/createneo/home/create-neo-home). Additional information of inclusion criteria is provided in the supplemental material.

Laboratory testing for acute disease surveillance

Acute samples (0–5 days) were first screened against DENV, CHIKV, and ZIKV virus using rRT-PCR as described previously (25), followed by testing with the newly designed MADV/VEEV rRT-PCR.

Mosquito collection

Mosquitoes were collected in a forested area (100 × 100 meters) in El Real de Santa María during the 2015 outbreak response. CDC light traps were employed over a 12-hour period (6:00 p.m. to 6:00 a.m.), positioned 1.5 meters above the ground level. These traps, equipped with octanol and CO2 as bait, were utilized for the encephalitis vector survey. Captured mosquitoes were anesthetized, identified to species using taxonomic keys (26), and preserved in liquid nitrogen. Specimens were grouped by species, with a maximum of 20 individuals per pool for subsequent analyses.

Viral isolation from mosquito pools

Mosquito pool homogenates were prepared with 20–50 mosquitoes in 2 mL of minimum essential medium supplemented with penicillin and streptomycin, and 20% fetal bovine serum (FBS), homogenized using a Tissue Lyser (Qiagen, Hidden, Germany) and centrifuged at 12,000 rpm for 10 min. A total of 200 µL of serum or mosquito homogenate was inoculated in each of two 12.5-cm^2 flasks of Vero cells (*Cercopithecus aethiops* kidney normal cells, ATCC CCL-81). Vero cells were supplemented with 10% FBS for growth and maintained with 2% FBS and 1% penicillin/streptomycin at 37°C. Samples were passed twice and monitored for cytopathic effect. All viral isolations were undertaken in the biosafety level-3 containment laboratory at the Gorgas Memorial Institute in Panama City.

Generic alphavirus RT-PCR for human and mosquito samples

Viral RNA was extracted from human serum and mosquito pool homogenates using QIAamp RNA viral extraction kit (Qiagen, Valencia, CA, USA). Viral RNA from mosquitoes was also extracted using the Macherey-Nagel extraction kit (Düren, Germany). Volume for extraction was 160 and 200 µL for human serum and mosquito pool homogenates, whereas elution volume was 60 and 50 µL, respectively. Serum and mosquito homogenates were tested in 25 µL reactions for alphaviruses using a universal alphavirus RT-PCR, as previously described (19). Antibody response was assessed in all human serum samples from 2015 as described previously (27), further details are provided in the supplemental material.

Viral metagenomic sequencing

To confirm virus species, subtype, and genotype, we sequenced seven selected VEEV complex rRT-PCR positive mosquito and human samples from 2015 and 2022 using SMART-9N metagenomic sequencing as previously described (28). Additional information is provided in the supplemental material.

VEEV phylogenetic analysis

All available VEEV genome sequences, in GenBank, representing all antigenic complexes were selected to construct the alignment; duplicated sequences, partial sequences, and overlapping sequences were removed. Finally, the novel complete or near complete VEEV genome sequences (n = 7) were aligned with 132 representative VEEV genomes retrieved from NCBI GenBank using MAFFT version 7 (29). Selection of the best-fitting nucleotide substitution model and maximum likelihood phylogenetic reconstruction were performed with IQ-Tree v2.2.0.3 (30). Statistical robustness of the tree topology was assessed with 1,000 ultrafast bootstrap replicates.

RESULTS

rRT-PCR analytical evaluation

Primers and probes for the VEE complex singleplex and MADV/EEEV duplex rRT-PCRs are shown in Table 1 along with the optimized final reaction concentrations. The dynamic range for each assay extended from 2.0 to 8.0 $\log_{10}$ copies/µL (Fig. 1; Fig. S1). For the VEEV complex assay, the linear range was evaluated with ssDNA for subtypes IAB and IV and RNA from subtype IC (2.0–5.0 $\log_{10}$ copies/µL; Figure 2). The 95% LLODs, expressed in copies/µL, were: VEEV subtype IAB, 120; VEE subtype IV, 110; MADV, 19; and EEEV, 19. Assay exclusivity was evaluated by testing genomic RNA from VEEV subtype IC, EEEV, and a set of arboviruses, including flavi-, bunya-, and alphaviruses on a single run of the VEEV complex and MADV/EEEV rRT-PCRs. VEEV complex and EEEV only yielded signals in the respective assays for these viruses. None of the other tested viruses generated a signal in either assay. In addition, none of the 56 serum samples from Georgia, USA, or Asunción, Paraguay, tested positive in either assay.

Validation with clinical samples

A total of 15 febrile patients from the 2015 and 2017 alphavirus outbreaks who met the suspected or probable case definition were used to validate the new molecular assays. Previously, a total of eleven (11/15) acute serum samples collected during the 2015 and 2017 alphavirus outbreaks had tested positive using a generic alphavirus RT-PCR and were confirmed later by sequencing as VEEV-ID infections (17). In 2021, a second round of generic alphavirus RT-PCR using the same set of primers was run on these 15 stored samples, and all of them tested negative. Notably, using the newly designed rRT-PCR, we were able to detect 10 VEEV complex RNA positive samples (Ct range: 27–38), including two samples that had tested negative at the initial screening in 2017 (Table 2). Three of the VEEV complex rRT-PCR-positive samples were also anti-VEEV IgG and IgM positive,

TABLE 2 Characteristics and laboratory results of the samples used for protocol validation patients and clinical samples and laboratory results[a]

Code	Township	Age[b]	Sex	Symptoms onset	Days of symptoms	RT-PCR-alpha (2015)	RT-PCR-alpha (2021)	rRT-PCR-VEE	Ct values	IgM-VEEV	IgM-MADV	IgG-VEEV	IgG-MADV	PRNT-veev[c]	PRNT-MADV[c]
258384	El Real	0–9	M	Aug. 2015	0	**pos**	neg	**pos**	29.3	neg	neg	neg	neg	<1:20	<1:20
267738	Cemaco	0–9	M	July 2017	3	neg	neg	**pos**	37.8	**pos**	neg	neg	neg	<1:20	<1:20
267411	Tucuti	0–9	F	July 2017	5	neg	neg	neg	–[d]	pos	neg	pos	pos	1:40	1:40
258380	El Real	0–9	F	Aug. 2015	1	**pos**	neg	neg	–	neg	neg	neg	neg	<1:20	<1:20
267410	Yaviza	0–9	F	July 2017	2	neg	neg	neg	–	pos	neg	pos	neg	<1:20	<1:20
258657	Yaviza	10–19	M	Sept. 2015	0	**pos**	neg	**pos**	28	neg	neg	neg	neg	<1:20	<1:20
258535	Nicanor	20–29	F	Sept. 2015	2	**pos**	neg	neg	–	neg	neg	neg	neg	<1:20	<1:20
258401	La Palma	20–29	M	Aug. 2015	2	**pos**	neg	**pos**	29	neg	neg	neg	neg	<1:20	<1:20
258395	Metetí	30–39	M	Aug. 2015	2	neg	neg	**pos**	37	**pos**	neg	neg	neg	<1:20	<1:20
258399	El Real	30–39	M	Aug. 2015	1	**pos**	neg	**pos**	26	neg	neg	neg	neg	<1:20	<1:20
258385	El Real	30–39	M	Aug. 2015	2	**pos**	neg	**pos**	37	neg	neg	neg	neg	<1:20	<1:20
258398	El Real	30–39	M	Aug. 2015	0	**pos**	neg	**pos**	27	neg	neg	**pos**	neg	<1:20	<1:20
258536	Metetí	30–39	F	Sept. 2015	2	**pos**	neg	neg	–	neg	neg	neg	neg	<1:20	<1:20
258386	El Real	30–39	M	Aug. 2015	5	**pos**	neg	**pos**	34	neg	neg	neg	neg	ND	ND
258379	El Real	≥40	F	Aug. 2015	2	**pos**	neg	**pos**	31	neg	neg	neg	neg	<1:20	<1:20

[a]Acute samples selected from the 2015 and 2017 alphavirus outbreaks in Darien Province. Ct, cycle threshold; neg, negative; pos, positive; and ND, not done.
[b]Age categories in years.
[c]Base on PRNT-80.
[d]–, not done and postive results and higligthed in bold.

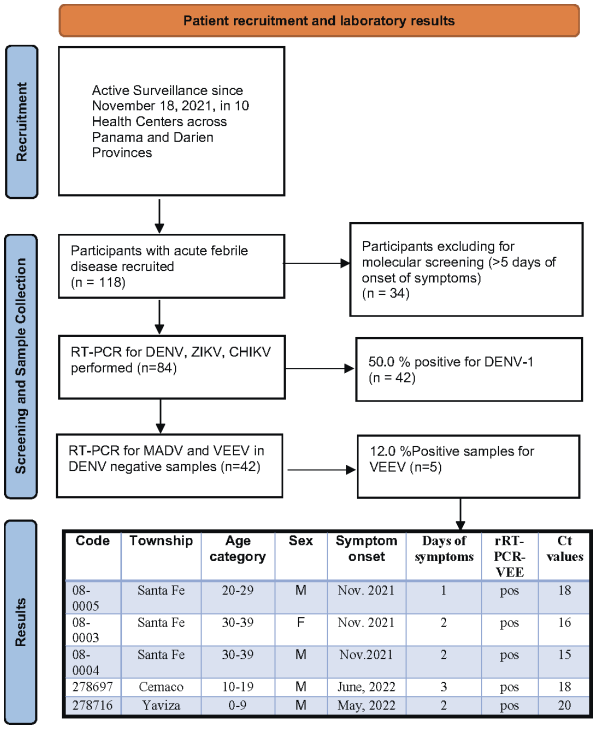
with only 0, 2, and 3 days since the onset of symptoms, respectively (Table 2). One sample was rRT-PCR positive for both VEEV and MADV viruses.

Prospective disease surveillance

A total of 118 febrile patients were recruited from 16 November 2021 to 1 December 2022. Of these, 84 (71.2%) were acute patients with the onset of symptoms ranging from 0 to 5 days. A total of 42 patients (50.0%) were DENV1 positive. We detected VEEV RNA (Ct range: 15–20) in five patients (11.9%; 95% CI: 4.0–25.6) with suspected dengue infection, one of which was from a fatal case in 2022. Details and results of disease surveillance are presented in Fig. 3.

Viral detection in mosquito pools

A total of 1,307 mosquitoes belonging to 35 species and 12 genera were collected in the community of El Real de Santa Maria, Panama, during a period of 5 days in 2015 (Table 3). The most abundant mosquito species was *Coquilletidia venezualensis* (37.5%, *n* = 490 of 1,307) and *Culex Melanoconion vomerifer* (34.4%, *n* = 450 of 1,307). Mosquito species, number of individuals, and pools are shown in Table 3. Of 150 mosquito pools, 3 *Cx.* (*Mel.) vomerifer* mosquito pools tested positive for VEEV by rRT-PCR (Ct range: 26–30). Two of these rRT-PCR-positive pools also yielded viral isolates.



Code	Township	Age category	Sex	Symptom onset	Days of symptoms	rRT-PCR-VEE	Ct values
08-0005	Santa Fe	20-29	M	Nov. 2021	1	pos	18
08-0003	Santa Fe	30-39	F	Nov. 2021	2	pos	16
08-0004	Santa Fe	30-39	M	Nov.2021	2	pos	15
278697	Cemaco	10-19	M	June, 2022	3	pos	18
278716	Yaviza	0-9	M	May, 2022	2	pos	20

FIG 3 Flowchart of patient recruitment, characteristics, and RT-PCR results of febrile patients detected throughout disease surveillance. Febrile patients were recruited from 16 November 2021 to 1 December 2022, in 10 health care centers in Panama and Darien provinces.

TABLE 3 Mosquito species collected during the 2015 outbreak in El Real de Santa Maria, Panama

Mosquito species	N	(%)	Pools[a]	VEE-rRT-PCR positive	MADV-rRT-PCR positive	Viral isolates
Coquillettidia venezuelensis	490	37.5	29	0	0	0
Culex (Melanoconion) vomerifer	450	34.4	27	3	0	2
Culex (Melanoconion) pedroi	32	2.4	4	0	0	0
Aedes serratus	31	2.4	7	0	0	0
Aedes sp.	30	2.3	5	0	0	0
Culex (Melanoconion) sp.	30	2.3	6	0	0	0
Culex (Culex) interrogator	27	2.1	5	0	0	0
Anopheles trianulatus	23	1.8	2	0	0	0
Aedes eupoclamus	14	1.1	4	0	0	0
Culex (Culex) nigripalpus	14	1.1	3	0	0	0
Culex (Culex) sp.	14	1.0	4	0	0	0
Culex (Melanoconion) atratus	14	1.0	1	0	0	0
Culex (Melanoconion) adamesi	13	1.0	3	0	0	0
Others[b]	125	9.6	50	0	0	0
Total	1307	100	150	3	0	2

[a]Numbers of mosquito pools.
[b]Species <1% abundance are listed as others.

TABLE 4 Metadata and sequencing statistics for selected VEEV complex RNA positive samples[c]

ID	Collection year	Location	Host species	Percent genome coverage 20×	Percent Nt identity with genome reference[a,b]	Percent identity with strain 3880[c]	*C* values
700677	2015	Darien	*Culex* (*Mel.*) *vomerifer*	100	89.8	92.1	27
700680	2015	Darien	*Culex* (*Mel.*) *vomerifer*	100	89.8	92.2	31
700732	2015	Darien	*Culex* (*Mel.*) *vomerifer*	100	90	92.3	26
258379	2015	Darien	Human	99.9	89.6	92	31
258398	2015	Darien	Human	70	88.7	90.7	27
258401	2015	Darien	Human	90.6	87.7	90	29
278716	2022	Darien	Human	45.98	88.1	90	20

[a]Genbank accession no. NC_001449.1.
[b]Nt = Nucleotide.
[c]Panamanian VEEV ID subtype prototype strain 3880, GenBank accession no. L00930.1.

VEEV subtype identification

Three mosquito pools and four human samples (including one from a fatal case in 2022), which tested positive for the new VEEV complex rRT-PCR, were sequenced using a virus-untargeted approach (28). The 20-fold genome coverage per base pair ranged from 45% to 100% (Table 4). The percentage of genome identity with the VEEV reference strain ranged from 87.7% to 90.0% (Table 4), while identity with the Panamanian VEEV ID subtype prototype strain 3880 ranged from 96% to 97% (Table 4). Maximum likelihood phylogenetic analysis indicated that the new viral genomes cluster together with historical Panamanian VEEV ID subtype strains within the Panama/Peru genotype (bootstrap statistical support = 100; Fig. 4).

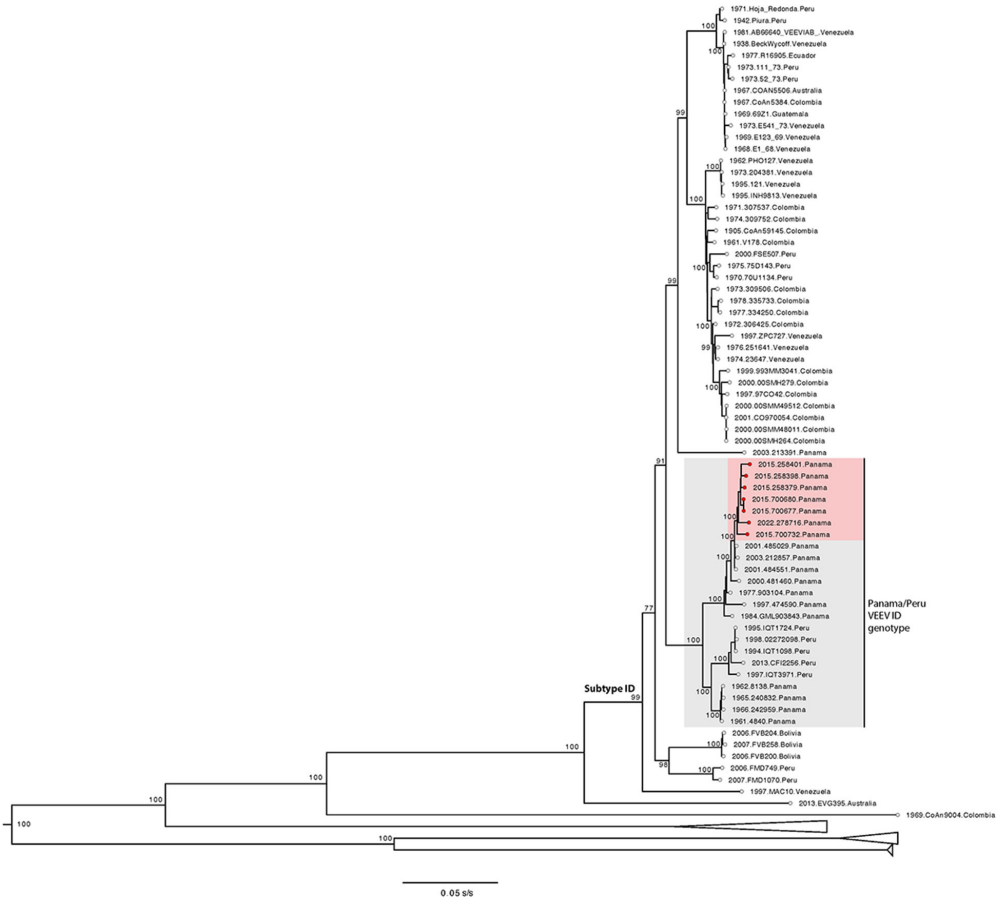


FIG 4 VEEV complex maximum likelihood phylogenetic tree. Maximum likelihood phylogeny was estimated using 139 complete or near complete VEEV genomes. Publicly available Panamanian VEEV ID subtype strains are highlighted in gray (*n* = 15), and genomes generated in this study (*n* = 7) are highlighted in red. Bootstrap statistical support is shown for selected nodes. NCBI GenBank accession numbers for the new VEEV genomes are: OR644785, OR644786, OR644788, OR644801, OR644802, OR644803.

DISCUSSION

Encephalitic alphaviruses have been detected throughout the Americas and may account for a significant proportion of non-dengue acute febrile illness (1, 2, 5, 9). Assays for their molecular detection, although existing (14–20), are often time-consuming, involving multiple PCR rounds or subsequent genome sequencing limited to well-equipped facilities (14–20). Co-circulation and the potential for co-infection with these viruses further complicate their identification, especially when clinical presentations are similar, and convenient methods for detecting VEEV complex and MADV are lacking (19). In Panama, for instance, both VEEV subtype ID and MADV have been identified, with co-circulation detected along the Colombian border (5–7). Typically, cases are identified during the neurological phase of the disease (5, 31), where the virus is cleared from serum, necessitating reliance on serological testing. Given that alphaviruses can induce IgM responses lasting 2–3 months, anti-VEEV or anti-MADV IgM detection alone could lead to misdiagnosis without seroconversion (5, 31).

We have developed singleplex and duplex rRT-PCRs for detecting VEEV complex, MADV, and EEEV viral RNA in clinical and mosquito samples. These assays identified VEEV

ID subtype and MADV in samples previously negative using a reference RT-PCR (19). We also identified a VEEV ID subtype—MADV co-infection, highlighting an advantage of our VEEV complex and MADV/EEEV rRT-PCRs over prior methods. Co-infection cases are epidemiologically significant and may have clinical relevance if associated with more severe disease (5). Our rRT-PCR assays can be rapidly integrated into testing algorithms in endemic regions. The current rRT-PCR detects VEEV ID subtype RNA within the initial 5 days of symptoms, preceding IgM and IgG antibody responses which usually manifest after 5–7 days following symptom onset (32). Intriguingly, three patients with detectable VEEV complex RNA were also VEEV IgM and IgG-reactive, suggesting possible VEEV re-infections with potential implications for vaccine development. However, early IgM responses cannot be ruled out, necessitating further research on alphavirus humoral immunity.

Through our prospective disease surveillance in Panama, we have demonstrated a notable prevalence of alphavirus detection. About 11.9% of individuals exhibiting symptoms similar to dengue have been found to have VEEV infections. These findings align with earlier assessments indicating that roughly 10% of clinical dengue cases in endemic countries can be attributed to VEEV infection (2). Moreover, this suggests a co-circulation of alphaviruses alongside other endemic arboviral infections, including dengue. Given the clinical similarities between VEEV complex infections and dengue, there exists the potential for underestimating the true burden of VEEV-related disease (2).

VEEV ID subtype RNA was found in *Cx*. (*Mel*.) *vomerifer* mosquito pools trapped during the 2015 outbreak in El Real de Santamaria. These mosquitoes were previously implicated as VEEV ID subtype vectors (2). Two pools yielded viral isolates. Notably, pan-alphavirus conventional RT-PCRs failed to detect viral RNA in these pools, suggesting the new rRT-PCR's heightened sensitivity for VEEV complex RNA detection in mosquitoes. Neither MADV nor EEEV infections were detected in mosquitoes using various methods. A similar pattern emerged from past outbreak investigations by our group in Panama (27, 33). Interestingly, MADV detection frequency in *Culex* (*Mel*.) spp. mosquitos is low in Panama (34, 35), unlike the endemic region of Iquitos, Peru, where MADV in the enzootic vector *Culex* (Mel.) *pedroi* is frequent (9, 36). Reasons for this variation in MADV and VEEV ID subtype frequency in Panama and MADV and VEEV in Panama vs Iquitos remain uncertain, possibly involving vector competence or viral competition, even enhanced VEEV ID subtype transmission via insect-specific viruses (37).

While our assays were validated with a limited number of human and mosquito samples, prospective surveillance allowed further validation. Unlike previous methods relying on plasmids, viral isolates, or a few human serum samples (13–21), we validated with human serum, mosquitoes, and post-mortem tissue samples. Our approach failed to detect two samples previously positive using standard alphavirus generic primers (19). Interestingly, a subsequent generic alphavirus RT-PCR in 2017 also failed to reamplify the former positives, possibly due to viral RNA degradation over time (38)

An rRT-PCR based on 33 VEEV sequences was reported by Vina-Rodriguez et al. but excluded other VEEV complex species and lacked clinical evaluation (21). Our assays used more complete genome sequences, with *in silico* primer and probe alignment to contemporary sequences. Untargeted metagenomic sequencing confirmed VEEV ID subtype detection using VEEV complex primers; this subtype has been detected in central and eastern Panama regions (7). These findings highlight molecular and genomic approaches' potential to enhance the detection of acute encephalitis alphavirus infections, even in archived samples.

Further prospective testing is necessary for comprehensive clinical performance characterization, including quantitative diagnostics and challenging assays with interfering substances. Limitations include the design requiring two separate assays for three viruses due to overlapping optimal design targets. However, the two rRT-PCRs can be executed simultaneously, improving lab workflow. The VEEV complex assay can also

be multiplexed with rRT-PCRs for other neurotropic arboviruses without performance loss (Jesse J. Waggone, unpublished data).

We developed sensitive and specific VEEV complex, MADV, and EEEV rRT-PCRs, surpassing available molecular methods. These assays detect VEEV-MADV co-infections, VEEV human infections, potential VEEV reinfections, and active VEEV viral circulation in mosquitoes during alphavirus outbreaks. Implementing these assays in endemic regions may enhance neurotropic alphavirus identification and characterization.

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Jean-Paul Carrera, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft | Dimelza Araúz, Formal analysis, Investigation, Writing – review and editing | Alejandra Rojas, Conceptualization, Formal analysis, Investigation, Writing – review and editing | Fátima Cardozo, Conceptualization, Formal analysis, Investigation | Victoria Stittleburg, Conceptualization, Data curation, Formal analysis, Validation | Ingra Morales Claro, Data curation, Formal analysis, Methodology, Validation | Josefrancisco Galue, Data curation, Formal analysis, Investigation, Supervision | Carlos Lezcano-Coba, Data curation, Formal analysis, Investigation, Validation, Writing – review and editing | Filipe Romero Rebello Moreira, Data curation, Formal analysis, Visualization | Luis Felipe -Rivera, Formal analysis, Investigation, Writing – review and editing | Maria Chen-Germán, Investigation, Methodology, Resources, Validation | Brechla Moreno, Investigation, Methodology, Resources, Supervision | Zeuz Capitan-Barrios, Conceptualization, Project administration, Supervision, Writing – review and editing | Sandra López-Vergès, Conceptualization, Funding acquisition, Supervision, Writing – review and editing | Juan Miguel Pascale, Conceptualization, Funding acquisition, Supervision, Writing – review and editing | Ester C. Sabino, Methodology,

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DATA AVAILABILITY

All the data used for human and mosquito validation are contained within the manuscript. Accession numbers for the newly generated genomes are OR644785, OR644786, OR644788, OR644801, OR644802, OR644803. Accession numbers and strain information of sequences used for primer design are shown in the supplemental material.

ETHICS STATEMENT

The use of human samples for protocol validation was approved by the Panamanian Ministry of Health (protocol number 2077), Gorgas's Institutional Review Board (IRB) (protocol: 335/CBI/ICGES/21), Emory University IRB (IRB00097089), and the Ethics Committee of the Instituto de Investigaciones en Ciencias de la Salud, Universidad Nacional de Asunción (P06/2017). Prospective disease surveillance was approved by Gorgas's IRB (protocol: 073/CBI/ICGES/21).

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental file 1. Additional information. (JCM00152-23-s0001.docx). Addicional information of recruitment and methods used in the study.
Table S1. Accession Numbers (JCM00152-23-s0002.xlsx). Genome accession numbers used in primer design.

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Chapter 4 - Madariaga and Venezuelan equine encephalitis virus seroprevalence in rodent enzootic hosts in Eastern and Western Panama

In this original research chapter, my goal was to elucidate the roles of rodents as potential enzootic alphavirus reservoirs in two endemic foci in Panama. For this I have conducted comprehensive investigations to clarify potential alphavirus vertebrate host by analyzing alphavirus seroprevalence in different rodent species collected across different environments, contributing valuable insights to the understanding of alphavirus ecology in the region. This manuscript has been submitted to *The American journal of Tropical Medicine and Hygiene* and has been provisionally accepted with minor suggestions.

Madariaga and Venezuelan equine encephalitis virus seroprevalence in rodent enzootic hosts in Eastern and Western Panama

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Abstract

While rodents are primary reservoirs of Venezuelan equine encephalitis virus (VEEV), their role in Madariaga virus (MADV) transmission remains uncertain, particularly given their overlapping geographic distribution. This study explores the interplay of alphavirus prevalence, rodent diversity, and land use within Darien and Western Panama provinces. A total of three locations were selected for rodent sampling in Darien Province: Los Pavitos, El Real de Santa Maria and Santa Librada. Two sites were selected in Western Panama province: El Cacao and Cirí Grande. We used plaque reduction neutralization tests to assess MADV and VEEV seroprevalences in 599 rodents of 16 species across five study sites.

MADV seroprevalence was observed at higher rates in Los Pavitos (Darien Province), 9.0%, 95% CI: 3.6-17.6, while VEEV seroprevalence was elevated in El Cacao (Western Panama province), 27.3%, 95% CI: 16.1-40.9, and El Real de Santa María (Darien Province), 20.4%, 95% CI: 12.6-29.7. Species like *Oryzomys coesi*, 23.1%, 95% CI: 5.0-53.8, and *Transandinomys bolivaris,* 20.0%, 95% CI: 0.5-71.6 displayed higher MADV seroprevalences

than other species, whereas *Transandinomys bolivaris*, 80.0%, 95% CI: 28.3-99.4, and *Proechimys semispinosus*, 27.3%, 95% CI: 17.0-39.6, exhibited higher VEEV seroprevalences. Our findings provide support to the notion that rodents are vertebrate reservoirs of MADV and reveal spatial variations in alphavirus seropositivity among rodent species, with different provinces exhibiting distinct rates for MADV and VEEV. Moreover, specific rodent species are linked to unique seroprevalence patterns for these viruses, suggesting that rodent diversity and environmental conditions might play a significant role in shaping alphavirus distribution.

Introduction

Madariaga (MADV) and Venezuelan equine encephalitic (VEEV) viruses (*Alphavirus* genus*, Togaviridae* family) are closely related arthropod-borne zoonotic RNA viruses associated with the human and equine disease throughout Latin America [1]. Most VEEV human-reported infections are symptomatic, and cases usually present with fever, headache, chills, and arthralgia [2,3]. Around 14% of febrile cases develop severe neurological complications [2]. VEEV case fatality ratio is estimated to be around 10% [2]. MADV human infection is less well documented. In Panama, MADV was first reported in the former Panama Canal Zone in a horse in 1936 [4]. Equine MADV epizootics were then reported across Panama, from the Azuero Peninsula in Central Panama to the Chepo district in North Panama, in 1947, 1958, 1962, 1973 and 1986 [5–7]. An equine epizootic in the absence of human disease was also observed in Argentina in 1981 [8]. In Iquitos, in the Peruvian Amazon, a febrile surveillance study found that 2% of participants were MADV IgM positive, indicating a low level of human exposure [9].

In 2010, 13 human MADV cases were reported during an outbreak of encephalitis in the Darien Province, at the eastern end of Panama [10]. Prior to this, a single case of human encephalitis had been reported in Brazil [11] and two MADV infections had been reported in Trinidad and Tobago [12]. MADV human infections during the 2010 Panama outbreak presented with fever and headache, and rapidly developed neurological symptoms and complications [10] with an estimated case fatality ratio of around 10% [10]. A recent report in Haiti showed that MADV human cases can present as a mild febrile disease with rash and conjunctivitis resembling symptoms observed during dengue disease [13]. Similarly, human serosurveys undertaken in Panama suggested that the majority of MADV and VEEV infections are asymptomatic or cause mild disease [3,14]. Nonetheless, follow-up studies of these individuals have demonstrated that clinical sequelae of MADV and VEEV can persist for years after infection[15]. Thus, the burden of both encephalitic alphaviruses could extend well beyond the acute febrile or neurological

disease, such as described for arthritogenic alphavirus[16]. There are no VEEV- or MADV-specific treatments or licensed vaccines for use in humans. Diagnostic tests of human infections are typically performed using pan-alphavirus and/or virus-specific reverse transcription-polymerase chain reaction (RT-PCR) approaches, plaque reduction neutralization tests and viral isolation.

Mosquitoes within the subgenus *Culex (Melanoconion)* are believed to be the principal enzootic vectors of both VEEV and MADV. Previous studies in the Peruvian Amazon and Panama have shown frequent detection and isolation of MADV in *Culex* (*Mel.*) *pedroi* [17,18] *and Culex (Mel.) taeniopus taeniopus* [7,19]. Furthermore, vector competence studies and analysis of blood feeding patterns show that *Culex (Mel.)* spp. predominantly feed on rodents in the wild [2,18,19]. Indeed, experimental and field investigations suggest that several rodent species may act as host species for VEEV, including those within the genera *Sigmodon*, *Oryzomys*, *Zygodontomys*, *Heteromys*, *Peromyscus*, and *Proechimys* [2,20,21].

However, the vertebrate hosts for MADV remain poorly understood. Studies in wild rodents and marsupials in Brazil detected viremia in *Oryzomys* sp. (rice rat) and *Didelphis marsupialis* (common opossum) *[22–24]*. MADV antibodies have also been detected in lizards and bats in Panama [14,25]. Experimental studies in *Sigmodon hispidus* (cotton rat) and evolutionary analyses further support that rodent species may be a key amplifying host for MADV [26,27].

The geographic and temporal overlap of MADV and VEEV outbreaks in Panama suggests that these viruses occupy similar enzootic transmission cycles[10]. Recent studies suggest that rodent species collected in agricultural areas of Darien Province were most likely to have MADV antibodies, while rodents with VEEV antibodies were principally found in sylvatic or forested areas [14]. To elucidate the roles of distinct rodent species as hosts for alphaviruses, we conducted an assessment of MADV and VEEV seroprevalence within rodent populations. Additionally, we investigated the potential correlation between seroprevalence rates, rodent diversity, and the patterns of land use and land coverage across five distinct enzootic foci located in the Darien and Western Panama provinces.

Materials and methods

Ethics statement

The capture, use, and euthanization of wild rodents was evaluated and approved by the Institutional Animal Care and Use Committee of the Gorgas Memorial Institute for Health Studies (010/ CIUCAL/ICGES18) and the Panamanian Ministry of Environment (SC/A-21-17, ANAM) using the criteria established in the "International Guiding Principles for Biomedical Research Involving Animals" developed by the Council for International Organizations of Medical Sciences (CIOMIS). The study was conducted in accordance with Law No. 23 of January 15, 1997 (Animal Welfare Guarantee) of the Republic of Panama.

Collection sites

Rodent trapping efforts were undertaken in 2011 and 2012 in Darien and Western Panama Province (Figure 1). A total of three locations were selected for rodent sampling in Darien Province: Los Pavitos, El Real de Santa Maria and Santa Librada (Figure 1). Two sites were selected in Western Panama Province: El Cacao and Cirí Grande (Figure 1). The main economic activities in both regions are agriculture and cattle farming. Collection sites were selected based on previous reports of confirmed human and equine encephalitic alphavirus infection in 2001, 2004 and 2010[6].

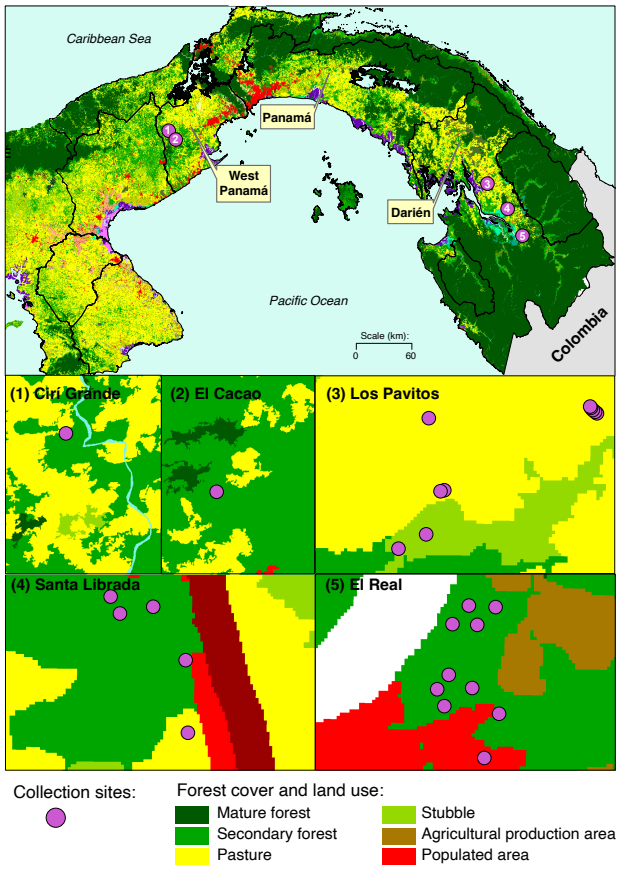

Figure 1. *Study site and small mammal species. Study site map using the land use and land coverage (LULC) shapes [46]. Classification of categories using the 2012 land use and land coverage shape. LULC categories were represented across all collection sites.*

Land use and land coverage classification

Georeferenced coordinates of collection sites were mapped onto the 2012 land use and land coverage (LULC) classification map obtained from the Panamanian Ministry of Environment [28] (Figure 1). The 2012 LULC classification was based on 5m resolution Rapid Eye Satellite Imagery [29].

Small mammals trapping

From June to November 2011 and March to April 2012, small mammals were collected using Sherman traps baited with a mixture of rice, corn, sorghum, and peanut butter. In the field, traps were placed and maintained from 6:00 PM and then checked soon after 6:00 AM. For this study, a total of 100 Sherman traps were placed in three linear transects of approximately 125m during three consecutive nights at each location. Traps were placed within houses and in the peri-domiciliary area of previously confirmed VEEV cases. Peri-domiciliary setting includes grasslands, and crop fields as well as wooded areas near homes in each of the selected locations. Trapped animals were euthanized using halothane and identified using taxonomic keys or using

the field guide to the mammals of Central America [28]. Blood samples were collected from the retro-orbital sinus. Heart, liver, spleen, lung, and kidney tissues were then harvested. All samples were immediately placed into liquid nitrogen before transportation to the Gorgas Memorial Institute (GMI) for testing. Animal carcasses were deposited in the Vertebrate Museum of the University of Panama and the Zoological Collection of the GMI (Panama City, Republic of Panama).

Laboratory methods

Alphavirus serology in small mammals

Rodent blood samples were screened in a 1:20 dilution using virus-specific plaque reduction neutralization tests (PRNTs) for VEEV and MADV viruses and then titred. A positive sample was considered as the reciprocal of the highest dilution that reduced plaque counts by >80% (plaque reduction neutralization test, $PRNT_{80}$), as previously described [14]. For PRNT, we used the wild-type MADV strain GML-267113, isolated from a fatal human case in Panama in 2017 [30], and the VEEV vaccine strain TC83. MADV and VEEV seroprevalence was estimated along with 95% confidence intervals (95% CIs) by mammalian species, year of collection, and collection site.

Viral isolation and molecular testing

Rodent tissues were used to prepare a 10% tissue suspension with 2 mL of minimum essential medium supplemented with penicillin and streptomycin, and 20% fetal bovine serum and homogenized using a Tissue Lyser (Qiagen, Hidden, Germany). After centrifugation at 17,709 x g for 10 minutes, 200 µL of the supernatant were inoculated into each of two 12.5 cm^2 flasks of Vero cells (African green monkey-ATCC CCL-81, USA). Samples were passaged twice for cytopathic effect confirmation.

Rodent tissue and cell culture supernatant were used for viral RNA extraction using the QIaAmp RNA viral extraction kit (Qiagen, Valencia, CA) and tested for alphaviruses using reverse transcription-polymerase chain reaction (RT-PCR) assays, as previously described [31].

Statistical methods

Diversity and similarity analysis

We estimated the absolute and relative abundance of small mammals in the collection sites of Darien Province and Western Panama Province during 2011 and 2012. To compare the diversity of small mammals within collection sites we used the Shannon-Wiener index (H)[32]. Lower values of H correspond to lower diversity. We also used Simpson's diversity index 1-D (SDI), which ranges from 0 (least diversity) to 1 (maximal diversity) [33]. Margalef's index was used to measure species richness, with higher values corresponding to greater species richness [34]. Diversity analysis was undertaken using the statistical package PAST version 4.03[35]. Finally, a pairwise analysis of species by location was also undertaken. P-values and 95% CIs were adjusted for multiple comparisons using Tukey's honestly significant difference (HSD) test, based on the possible pairs of means and studentized range distribution[36].

Factors associated with alphavirus seroprevalence

Rodent species were grouped at the genus level to account for the small sample size. Rodent species, VEEV (n=296) and MADV (n=292) seropositivity, and LULC classification were used for univariate logistic regression analysis. To evaluate risk factors at the community and genus level, we conducted separate univariate analyses for MADV and VEEV; in each case, the outcome variable was the presence/absence of antibodies against the virus, as determined by a $PRNT_{80}$ titer >1:20. The associations between each outcome and independent variable (community, genus and LULC) were estimated using logistic regression and were expressed as odds ratios (ORs). Univariable and multivariable ORs were calculated with 95% CIs. Statistical analyses were undertaken using the package STATA version 14.1 (College Station, TX).

Results

Rodent abundance across study sites

We collected a total of 559 rodents between 2011 and 2012, with specimens belonging to 13 genera and 16 species (Figure 2 A and B, Supplementary Table 1). Most rodents were captured during 2011 (71.8% of all collections, *n* = 430/599). In general, the majority of rodents were captured within the Darien Province (87.6% of all collections, *n* = 525/599), specifically in El Real (33.7%, *n* = 202/599), followed by Los Pavitos (27.6%, *n* = 165/599) and Santa Librada (26.4%, *n* = 158/599) (Supplementary Table 1).

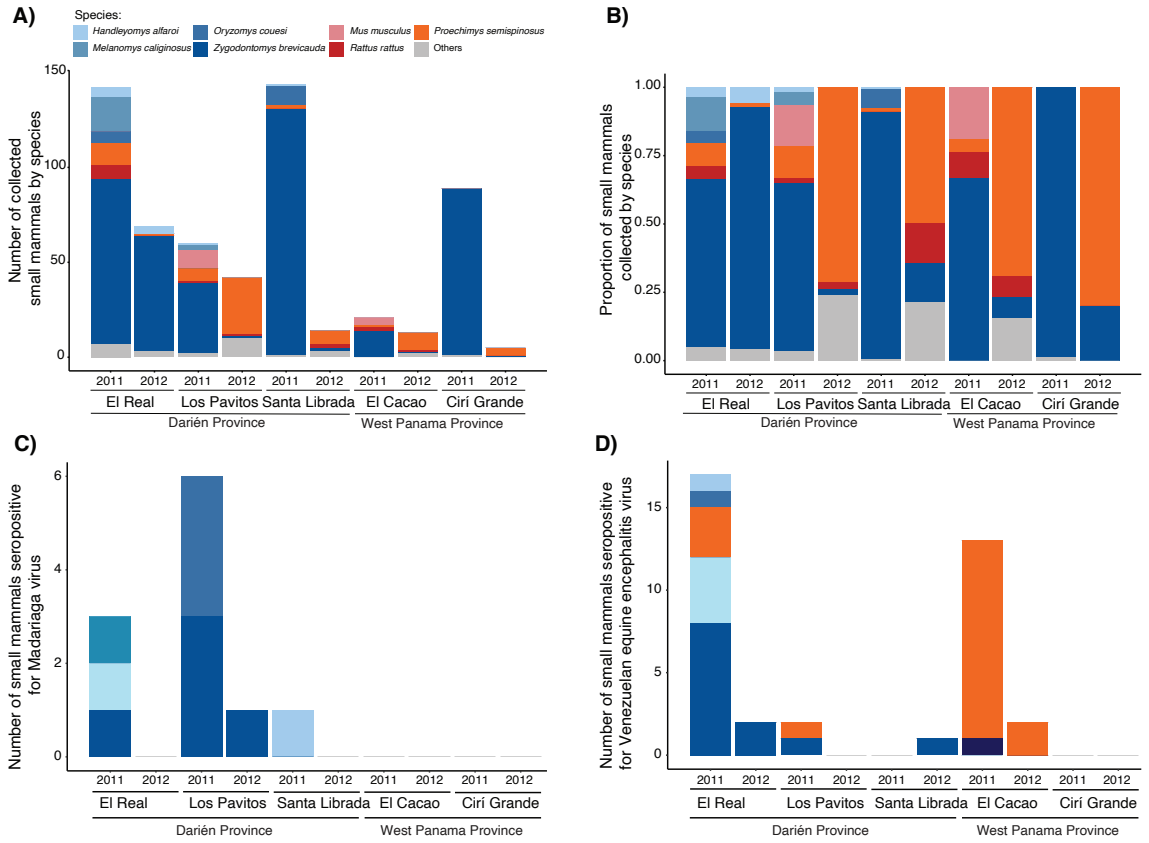

Figure 2. Alphavirus seropositivity in small mammals collected across study sites in Panama. *A) Number of sampled small mammal species by site and year. B) Proportion of sampled small mammals by site and year. C) Number of small mammals seropositive for Madariaga virus (MADV). D) Number of small mammals seropositive for Venezuelan equine encephalitis virus (VEEV).*

The short-tailed cane mouse (*Zygodontomys brevicauda*) was the most abundant species identified across study sites (70.5% of trapped animals, *n* = 402/599), followed by the Central American spiny rat (*Proechimys semispinosus*, 12.2%, *n* = 73/599), dusky rice rat (*Melanomys caliginosus,* 3.5%, *n* = 21/599), marsh rice rat (*Oryzomys couesi*, 2.7%, *n* = 16/599), the black rat (*Rattus rattus*, 2.3%), house mouse (*Mus musculus,* 2.2%, *n* = 13/599), Alfaro's rice rat (*Handleyomys alfaroi*, 1.8%, *n* = 11/599), long-whiskered rice rat (*Transandinomys bolivaris*, 1.5%, *n* = 9/599), and the cotton rat (*Sigmodon hirsutus*, 1.3%, *n* = 8/599). Species with abundance ≤ 1% are shown in Supplementary Table 1.

Highest rodent diversity and richness in the Darien Province

We estimated rodent diversity in each study site using the Simpson's diversity index (1-D) and the Shannon-Wiener (H) index. The locations of El Real de Santa Maria [1-D=0.60; H=1.42] and El Cacao Maria [1-D=0.53; H=1.13] in the Darien Province showed the highest rodent

diversity. Lower species diversity was observed in Cirí Grande [1-D=0.46; H=0.96], Los Pavitos [1-D=0.23; H=0.57] and Santa Librada [1-D=0.11; H=0.29]. El Real de Santa Maria had the highest species richness accordingly with Margalef index [M=1.88] and Santa Librada presented the lowest species richness [M=0.79] (Table 1 and Supplementary Table 2).

Table 1. **Characteristics of collection sites, small mammal diversity and alphavirus seroprevalence.** The total of small mammals included in the analysis was 599 from a total of 16 species.

Sites	**Western Panama Province**			**Darien Province**	
Location	El Cacao	Cirí Grande	El Real de Santa María	Los Pavitos	Santa Librada
Latitude and Longitude	8,76613; -80,01681	8,87128; -80,05327	8,13021; -77,72737	8,47052; -77,95490	8,15778; -77,69180
Forest cover/ land use	Secondary forest	Secondary forest	Secondary forest	Pasture	Secondary forest
***No. rodents captured (n)**	56	18	202	165	158
****No. rodent species (n)**	6	5	11	7	5
†VEEV seroprevalence	15/55 (27.3, 95% CI: 16.1-40.9)	0/17 (0.0%, 95% CI: 0.0-19.5)	19/94 (20.2%, 95% CI:12.6-29.7)	2/81 (2.4%, 95% CI:0.3-8.6)	1/49 (2.0%, 95% CI:0.0-10.8)
††MADV seroprevalence	0/55 (0.0%, 95% CI:0.0 - 6.0)	0/17 (0.0%, 95% CI: 0.0- 19.5)	3/94 (3.2%, 95% CI:0.6-9.0)	7/78 (9.0%, 95% CI:3.6-17.6)	1/48 (2.1%, 95% CI:0.0-11.8)
Simpson's diversity (Ds)	0.53	0.46	0.60	0.23	0.11
Shannon-Wiener (H)	1.13	0.96	1.42	0.57	0.29
Margalef index (M)	1.24	1.38	1.88	1.18	0.79

†VEEV: n=296
†† MADV: n=292

Species similarity at the community level

Based on pairwise analyses, species composition was similar in Santa Librada and Los Pavitos in Darien Province [Contrast =0.5; 95% CI: -0.5-1.4; p=0.639], and El Cacao and Cirí Grande in the Western province. Greater differences in species composition were observed between Darien and Western provinces (Table 2). Species compositions were generally most similar

within provinces, with the exception of El Cacao and El Real de Santa Maria. These sites had the largest smallest differences in species composition [Contrast =-1.8; 95% CI: -3.1-0.5; p=0.001], despite these sites being in different provinces (Table 2).

Table 2. Pairwise comparison of rodent species by collection site.

Sites	Contrast	95% CI	P-value
Cirí Grande vs El Real	**-2.3**	**-4.4 - - 0.2**	**0.023**
El Cacao vs El Real	**-1.8**	**-3.1- - 0.5**	**0.001**
Los Pavitos vs El Real	**2.1**	**1.2 - 3.0**	**<0.001**
Santa Librada vs El Real	**2.6**	**1.6 - 3.5**	**<0.001**
El Cacao vs Cirí Grande	0.5	-1.8 - 2.8	0.974
Los Pavitos vs Cirí Grande	**4.4**	**2.3 - 6.5**	**<0.001**
Santa Librada vs Cirí Grande	**4.9**	**2.8 - 7.0**	**<0.001**
Los Pavitos vs El Cacao	**3.9**	**2.6 - 5.2**	**<0.001**
Santa Librada vs El Cacao	**4.4**	**3.0 - 5.7**	**<0.001**
Santa Librada vs Los Pavitos	0.5	-0.5 - 1.4	0.639

Viral active circulation

No active alphavirus circulation was detected by means of RT-PCR or viral isolation. However, we note that two strains of Madrid virus (genus, *Orthobunyavirus*, family, *Peribunyaviridae*) were isolated from two specimens of *Zygodontomys brevicauda* trapped in El Real de Santa Maria. These strains are not analyzed in this study.

Widespread alphavirus seroprevalence in rodents across Panama

The overall MADV and VEEV seroprevalence in small mammals were 3.8% (95% CI: 2.0-7.0; *n* = 11/292) and 12.5% (95% CI: 8.9-16.8; *n* = 37/296), respectively (Supplementary Table 3 and 4. VEEV seroprevalence was higher in 2011 (16.2%, 95% CI: 11.4-22.1; *n* = 32/197)

compared to 2012 (5.1%, 95% CI: 1.6-11.3; *n* = 5/99) (Supplementary Table 6). MADV seroprevalence dropped from 4.6% (95% CI: 2.1-8.6; *n* = 9/194) in 2011 to 2.0% (95% CI: 0.2-7.0; *n* = 2/98) in 2012 (Supplementary Table 5). VEEV seroprevalence was widespread across the Western Province and Darien Province with the highest seroprevalence found in El Cacao (27.3%, 95% CI: 16.1-40.9; n=15/55) in the Western Province, followed by El Real de Santamaria (20.4%, 95% CI: 12.6-29.7; n=19/94) in the Darien Province (Table 1, Table 3). MADV seroprevalence was higher in rodents collected in Los Pavitos (9.0%, 95% CI: 3.6-17.6 18; n=7/78), followed by El Real (3.2%, 95% CI: 1.0-9.0; n=3/94) and Santa Librada (2.1%, 95% CI: 0.0-11.0; n=1/48) (Table 1 and Table 3). No evidence of MADV viremia or antibodies was found in rodents collected in the Western Province (0%, 95% CI: 0.0-5.0; n= 0/72).
Oryzomys couesi (23.1%, 95% CI: 5.0-53.8; n=3/13) and *Transandinomys bolivaris* (20.0%, 95% CI: 0.5-71.6 72; n=1/5) had the highest MADV seroprevalence (Supplementary Table 3), while *Transandinomys bolivaris* (80.0%, 95% CI: 28.3-99.4; n=4/5) and *Proechimys semispinosus* (27.3%, 95% CI: 17.0-39.6; n=18/66) had the highest VEEV seroprevalence (Supplementary Table 4).

Table 3. Seroprevalences by virus, collection sites and year of trapping.

Sites	MADV				VEEV			
	2011†		2012*		2011††		2012**	
	n/N (%)	95% CI	n/N (%)	95% CI	n/N (%)	95% CI	n/N (%)	95% CI
El real	3/72 (4.2)	0.01 - 0.12	0/22 (0.0)	0.00 - 0.15	17/72 (23.6)	0.14 - 0.35	2/22 (9.1)	0.01 - 0.29
Los pavitos	6/60 (10.0)	0.04 - 0.21	1/18 (5.6)	0.00 - 0.27	2/63 (3.2)	0.00 - 0.11	0/18 (0.0)	0.00 - 0.19
Santa librada	0/9 (0.0)	0.00 - 0.34	1/39 (5.6)	0.00 - 0.13	0/9 (0.0)	0.00 - 0.34	1/40 (2.5)	0.00 - 0.13
El cacao	0/41 (0.0)	0.00 - 0.86	0/14 (0.0)	0.00 - 0.23	13/41 (31.7)	0.18 - 0.48	2/14 (14.3)	0.18 - 0.43
Cirí grande	0/12 (0.0)	0.00 - 0.26	0/5 (0.0)	0.00 - 0.52	0/12 (0.0)	0.00 - 0.26	0/5 (0.0)	0.00 - 0.52

†Seroprevalence total of MADV by 2011: n=9/194; 4.6%, 95% CI (0.02 to 0.09)
**Seroprevalence total of MADV by 2012: n=2/98; 2.0 %, 95% CI (0.00 to 0.07)*
††Seroprevalence total of VEEV by 2011: n=32/197; 16.2%, 95% CI (0.11 to 0.22)
***Seroprevalence total of VEEV by 2012: n=5/99; 5.1%, 95% CI (0.02 to 0.11)*

Factors associated with alphavirus seroprevalence in rodents

MADV seroprevalence was independent of collection site, but Los Pavitos (OR=0.1; 95% CI: 0.0-0.4; p=0.002) and Santa Librada (OR=0.1; 95% CI: 0.0-0.6; p=0.017) were protective

factors for VEEV seropositivity when compared with El Real de Santa María (Table 4). Univariate analysis by rodent taxa revealed that the odds of MADV seropositivity was 9.0 times greater in *Orizomys* (OR=9.0; 95%CI: 1.9-43.2; p=0.006) compared to the reference *Zygodontomys*. The odds of VEEV seropositivity in *Proechimys* (OR=4.6; 95%CI: 2.1-10.2; p<0.001) were significantly higher than in the reference, *Zygodontomys* (Table 4). At the univariate level, pasture was significantly associated with MADV seropositivity when compared to the secondary forest (OR=5.2; 95% CI: 1.5 -18.2; p=0.01). In contrast, the risk of VEEV seropositivity was significantly decreased in pastures when compared with secondary forest (OR=0.1; 95% CI: 0.3 - 0.6; p=0.031) (Table 4).

Table 4. Univariable logistic regression. Associated factors with MADV and VEEV seroprevalence.

Variables	MADV*			VEEV*		
Collection Site	**OR**	**95% CI**	**P-value†**	**OR**	**95% CI**	**P-value†**
El Real de Santa María	**Ref.**	-	-	**Ref.**	-	-
Los Pavitos	3	0.8 – 12.0	0.122	**0.1**	**0.0 - 0.4**	**0.002**
Santa Librada	0.6	0.1 - 6.4	0.708	**0.1**	**0.0 - 0.6**	**0.017**
El Cacao	-	-	-	1.5	0.7 - 3.2	0.323
Environment						
Secondary forest	**Ref.**	-	-	**7.7**	**1.8 - 32.7**	**0.006**
Pasture	**5.2**	**1.5 - 18.2**	**0.01**	**Ref.**	-	-
Genus						
Zygodontomys	**Ref.**	-	-	**Ref.**	-	-
Melanomys	2.3	0.3 - 21.3	0.460	-	-	-
Handleyomys	5.0	0.5 - 49.7	0.170	2.0	0.2 - 18.4	0.524
Transandinomys **	7.5	0.7 - 79.9	0.095	49.0	5.1 - 473.8	0.001
Oryzomys	**9.0**	**1.9 - 43.2**	**0.006**	1.0	0.1 - 8.5	0.985
Proechimys	-	-	-	**4.6**	**2.1 - 10.2**	**<0.001**
Sigmodon	-	-	-	1.8	0.2 - 15.4	0.614

MADV = Madariaga virus; OR = odds ratio; VEEV = Venezuelan equine encephalitis virus.
**Based on plaque reduction neutralization test results.*
*** Small simple size, n=5*
†Results with P < 0.05 are shown in boldface type.

Discussion

Our findings support the hypothesis that wild rodents serve as reservoirs for both MADV and VEEV[14,26,27]. Our results show that MADV seropositivity was confined to the Darien Province, whereas VEEV seropositivity was pervasive across the examined study sites. Rodents captured within areas characterized by pasture exhibited an elevated likelihood of MADV seropositivity in contrast to those within secondary forest environments. Conversely, rodents captured within secondary forest areas displayed an increased likelihood of VEEV seropositivity.

Overall, we observed that MADV seropositivity was lower in rodents compared to VEEV (3.8% vs. 12.5%). Our seroprevalence results agree with separate surveillance efforts carried out in other regions in the Darien Province during 2012[14]. Higher frequency of VEEV detection compared to MADV has been observed in rodents, mosquitoes and humans[30,37]. This pattern may be due to intrinsic differences among VEEV and MADV, viral fitness, in vector competence, viral competition within the vector, or asymmetric cross-protective immunity[15,38].

Weaver et al. has previously suggested that the genera with the greatest evidence of participation in the enzootic transmission of VEEV were *Sigmodon*, *Oryzomys*, *Zygodontomys* and *Proechimys*[2,39]. We found that *Transandinomys bolivaris* and *Proechimys semispinosus* had the highest VEEV seroprevalence in Panama (80.0% and 27.3%, respectively). Both species have been implicated as VEEV reservoirs in prior studies[2]. Moreover, the highest MADV seroprevalence was found in *Oryzomys couesi*, *Transandinomys bolivaris* and *Handleyomys alfaroi* (23.1%, 20.0% and 14.3%, respectively). We also observed that in different communities of the Darien province, *Zygodontomys brevicauda* and *Transandinomys bolivaris* presented the highest MADV seroprevalence (8.3% and 3.1%)[14].

Proechimys semispinosus and *Transandinomys bolivaris*, the rodent species identified in this study with the highest VEEV seroprevalence in the Darien Province, are often found in secondary and primary forests[14]. *Oryzomys couesi* and *Transandinomys bolivaris*, the rodents with the highest MADV seroprevalence, are found in grasslands and agricultural areas. *Oryzomys couesi* is a semi-aquatic species that is adaptable to different environmental conditions[40,41]. Herbaceous habitats, permanent and semi-permanent wetlands appear to be an important factor for the distribution of this rodent[40,41]. It is likely that this plasticity favors MADV transmission in pasture or agriculture settings. However, it is unclear if the ecological conditions found in Darien support the development of Culex (*Mel.*) spp., or possibly other bridge vectors. The ecological profiling of the *Cx*. (*Mel*.) *spp*., done during the 1970s, suggest

these species develop their cycles in floating plant water[42]. More recent findings have discovered species near human settlements and in secondary forests[30,43], suggesting changes in their ecology.

VEEV was more prevalent in rodents captured in the communities of El Cacao in the Panama Western province and in El Real de Santa Maria (27.3% and 20.2%) located in the Darien province. Rodent diversity and richness were also higher in El Real de Santa Maria and El Cacao. Notably, El Real de Santa María is also among the regions with the highest VEEV human incidence[14,30]. Los Pavitos had the highest MADV rodent seroprevalence (9.0%), and we also observed that the risk of MADV increased in pasture compared with the secondary forest. Interestingly, Los Pavitos is a community on the Pan-American Highway where the first MADV human and equine cases were reported during the 2010 outbreak[10]. Human serosurveys have shown that the risk of human VEEV infection is associated with activities in the forest, which supports a sylvatic cycle for VEEV[14,30]. Previous studies have also shown that human MADV infection risk is associated with farming and cattle ranching activities, suggesting that MADV transmission occurs predominantly in areas with agricultural activity[14,30].

It is important to note that no MADV-seropositive rodents were observed in the El Cacao and Cirí Grande communities in the Western Panama Province. This observation is in agreement with recent serological evidence of MADV in rodents and humans being restricted to the Darien Province[10]. However, it is in contrast with pre-1990s reports of MADV showing widespread circulation across Panama[5–7]. It is unclear why contemporary MADV transmission is limited to the Darien Province, but perhaps these earlier outbreaks represented epizootic expansion from a stable enzootic focus in eastern Panama[44] Evidence of geographic expansion of MADV has also been previously observed in Panama[5,6]. High rates of MADV in rodents were recorded previously near El Real de Santa Maria in the small, heavily forested community of Pijibasal[14]. This community is in the Darien Gap National Park, suggesting that the MADV enzootic cycle also occurs in forested areas[14]. Overall, MADV and VEEV seroprevalence levels appear to differ spatially, and our results suggest that MADV seroprevalence was greater in places with low rodent diversity and pasture, while VEEV seroprevalence was greater in places with rodent high diversity and secondary forest. However, cross-protection immunity has also been proposed as a potential mechanism to explain these differences[14,15]

The limitations of this study include a lack of precise information on the environment where the rodents were collected, which means we could not describe the micro-ecological conditions linked to the distribution and prevalence of infection in rodents. Finer-scale analyses to understand the effect of land use and land cover in diversity and alphavirus seroprevalence are currently underway by our group using additional rodent data from Darien Province. Little volume of sample is also available for testing for alphaviruses in small animals, which makes laboratory testing challenging in some individuals or even other taxa. Moreover, future cross-sectional rodent surveys will allow us to identify the temporal drivers of transmission and improve our understanding of the seasonal dynamics of VEEV and MADV across Panama[45].

In summary, our study corroborates the hypothesis that wild rodents act as reservoirs for both MADV and VEEV, offering unique seropositivity patterns[14]. We observed distinct geographical distributions, with MADV seropositivity concentrated in the Darien Province and VEEV seropositivity prevalent across the surveyed sites. *Transandinomys bolivaris* and *Proechimys semispinosus* exhibited the highest VEEV seroprevalence, while *Oryzomys couesi*, *Transandinomys bolivaris*, and *Handleyomys alfaroi* showcased elevated MADV seroprevalence. Furthermore, ecological differences in habitat preference were linked to seroprevalence patterns, with secondary forests associated VEEV with seropositivity and agricultural environments associated with MADV seropositivity.

Areas with lower rodent diversity and pasture environments correlated with increased MADV seropositivity. In contrast, regions characterized by higher rodent diversity and secondary forests were associated with heightened VEEV seroprevalence. These patterns align with observed human infection risks[14,30], supporting the potential impact of rodent-driven transmission in specific ecological contexts.

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Chapter 5 – A One Health surveillance to detect active arboviral circulation and potential host and vectors.

This research chapter involves a project where I piloted a prospective One Health arboviral surveillance to detect active circulation and potential hosts and vectors. The study focused on assessing human, vertebrate hosts, and enzootic mosquitoes vectors in the Darien Gap, Panama. By adopting a comprehensive One Health perspective, I also aimed to provide valuable insights into the dynamics of arboviral circulation and potential interconnections between human, animal, and vector populations in this region.

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A One Health surveillance to detect active arboviral circulation and potential host and vectors

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Abstract

A One Health arboviral surveillance program was implemented from 2017 to 2019, in two communities of Eastern Panama. Trinidad traps baited with vertebrate hosts and CDC light traps were used to detect active arboviral circulation. Field rodent collections were undertaken during 2011-2012. Over 14,000 mosquitoes were collected using Trinidad and CDC light traps, with *Culex* (*Mel.*) *spissipes* being the most abundant species. Several mosquitoes species exhibiting anthropophilic behavior were successfully attracted and blood meal analysis showed active feeding on various vertebrates by key mosquitoes species, altogether implicating these mosquitoes as potential bridge vectors for zoonotic arboviruses. Orthobunyaviruses were detected exclusively in mosquitoes collected using Trinidad traps. Seventeen virus genomes were generated from thirteen virus isolates recovered from symptomatic sentinel animals and two from short-tailed cane mice (*Zygodontomys brevicauda*) caught in the field. Madrid virus and three new viruses - Aguas Calientes, Aruza, and Matusagaratí within the *Orthobunyavirus*

group were identified. Matusagaratí virus presented as a genetic reassortment between Madrid virus and an unknown virus. Structural modelling of the Gc glycoproteins of these strains suggest structural similarity to known orthobunyaviruses Gc structures, revealing potential antigenic regions. The simultaneous identification of key components of arboviral enzootic transmission cycles may have implications for zoonotic outbreak investigations and spillover surveillance.

INTRODUCTION

Arthropod-borne viruses (Arboviruses), constitute a diverse group of pathogens that rely on arthropod vectors, such as mosquitoes and ticks, to transmit them between vertebrate hosts[1,2]. These viruses have a significant impact on both human and animal public health, causing a substantial global burden of morbidity and mortality[2,3]. The World Health Organization (WHO) estimates that approximately 100-400 million cases of dengue occur annually worldwide[4]. Dengue, Zika, Yellow fever, and Chikungunya viruses exemplify sylvatic arboviruses that have adapted to human hosts, evolving into epidemic viruses[5–8]. These pathogens persist as significant threats to global public health.

Traditional arboviruses surveillance often relies on the clinical diagnosis of specific symptoms or laboratory testing after an individual has fallen ill[9–12]. This method can be slow and is reliant on the affected individual seeking medical care[10–13]. Furthermore, it may not capture asymptomatic or mild cases, leading to underreporting[10–13]. In animals, passive surveillance through the monitoring of clinical cases in livestock or wildlife populations is commonly used[11,14]. This approach involves the reporting of sick animals to veterinary authorities for further investigation. It is often reactive and may not capture asymptomatic infections[11,14]. The limitations of these traditional surveillance methods include significant underreporting due to reliance on the presence of clinical symptoms and signs in both humans and animals, respectively[11,14]. Asymptomatic or mild infections often go undetected, leading to an incomplete picture of arbovirus circulation[11,14].

Additionally, detecting and confirming arbovirus infections through traditional methods can be time-consuming, hindering the rapid response required to contain outbreaks and prevent further transmission[9]. Moreover, traditional surveillance systems may not readily identify spillover events, or the emergence of new zoonotic arboviruses, which can have serious public health

consequences[15]. The integration of human and animal surveillance data is often limited, impeding the understanding of the complex ecological and epidemiological dynamics of zoonotic arbovirus transmission.[11]

To overcome these constraints and enhance our capacity for early detection and response to arbovirus spillover events, we have devised pioneering approaches rooted in the One Health surveillance framework. Aiming to discern active zoonotic arbovirus circulation, elucidate the potential involvement of reservoir hosts and vectors, and ultimately bridge crucial gaps in our comprehension of arbovirus transmission dynamics, thus strengthening our ability to swiftly identify potential arboviral threats to both human and animal populations.

MATERIALS AND METHODS

Ethical considerations. Animal research in Panama was conducted in compliance with regulatory protocols. Authorization was granted by the Panamanian Ministry of Environment under protocol SC/A-21-17, dating from February 2017. Furthermore, the Institutional Animal Care and Use Committee (IACUC) at the Gorgas Memorial Institute of Health Studies oversaw the research, operating under protocols 004/CIUCAL/ICGES/11 from 2011 and 010/CIUCAL/ICGES18 from 2018, which adhered to the provisions of law No. 23 of January 15, 1997, governing animal welfare in the Republic of Panama.

Rodent trapping effort. Field rodent trapping efforts were undertaken in 2011 and 2012 in Darien Province and Western Panama Province. Additional information on trapping methods is provided in the supplementary material and described elsewhere [16]. A total of three locations were selected for rodent sampling in Darien province: Los Pavitos, El Real de Santa Maria and Santa Librada. Two sites were selected in Western Panama province: El Cacao and Cirí Grande. Detailed spatial location of rodent collections is provided in **Fig. 1A**.

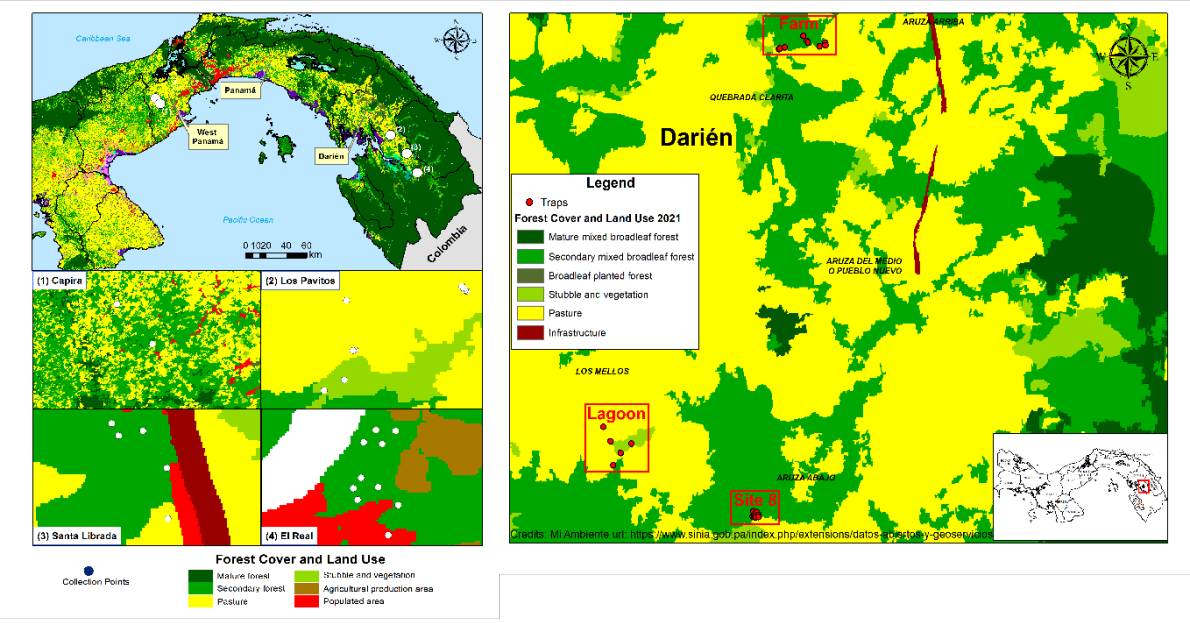

Figure 1. Map of the study sites area for rodent trapping in 2011 and 2012 in Western and Eastern Panama and mosquito collection 2017-2018 in Eastern Panama. *Collection sites and distribution of Trinidad and CDC light traps are shown. Relative mosquitoes abundance is show by different colors.*

Location of the mosquitoes collections using Trinidad and CDC light traps. Mosquitoes collections were conducted in the eastern Darién province, Panama, which adjoins Colombia and encompasses the Darién Gap, and is home to the Darién National Park, the largest tropical forest in Central America, and a UNESCO-designated World Heritage Site[17]. This location falls within the tropical forest classification. For the study, two specific communities were chosen: Aruza, comprising around 400 residents and situated amidst the Filo del Tallo hydrological reserve, and Aguas Calientes, with approximately 150 inhabitants, bordering the Matusaragatí Lagoon, a significant wetland within Darién Province (**Fig. 1B**).

Sampling strategy. Mosquitoes collection with modified Trinidad traps no. 17 and sentinel animals. We employed modified animal-baited Trinidad Traps that comprise three main components: the lid, the net, and the bait cage. The lid, with a 30 cm diameter, was constructed from plywood with a durable plastic top layer, painted white on both sides. The net was made of white, translucent polyester fabric, attached at the top and bottom by a 10-gauge wire ring, also 30 cm in diameter, with a 10 cm elastic seam at the net's bottom for bait cage and mosquito access.

The bait cage had a cylindrical shape, 10 cm in height, crafted using 4 cm polyvinyl chloride (PVC) tubing and 1 cm x 1 cm wire mesh. Two PVC rings, each 2 cm wide, were used for affixing the wire mesh to the PVC, one at the top and another 4 cm from the bottom. Both the cage floor and lid were constructed using wire mesh. For cages housing rodents or hamsters, the floor was positioned 3 cm above the PVC tubing, while in cages accommodating chickens, the floor was flush with the PVC tubing to provide more space (**Supplementary Fig. 1**).

Our Trinidad trap assessments were conducted exclusively in Aruza in 2017, whereas collections in 2018 and 2019 covered three locations across Aruza and Aguas Calientes communities in the Darien province. These sites were selected based on previous arbovirus detections in 2010, 2012, and 2015-2017. Traps were randomly positioned within 100 x 100-meter sections (Fig. 1B, Site 8). Aruza encompassed two distinct environments: (1) a farm and (2) a regrown and secondary forest patch encircled by cattle ranches, known as "Site 8." In Aguas Calientes, sampling occurred within the Matusagaratí Lagoon area. Concurrently, Alphavirus outbreaks were reported in 2017 and 2019.

Trinidad traps were positioned approximately 1.5 meters above ground level, utilizing various animal sources as sentinels. Specifically, in July 2017, three-week-old mice were used as bait. In June 2018, traps were baited with hamsters, chickens, and toads, while in 2019, hamsters were exclusively used. In July 2017, three traps were deployed for three consecutive days. In June 2018, twelve traps were set up for five consecutive days, and in 2019, ten traps were placed for six consecutive days.

Animals were daily monitored for clinical signs, and if observed, they were euthanized for tissue collection. Additionally, approximately 200 µl of blood from sentinel mice sampled in 2017 was collected and preserved on Whatman FTA® micro cards, stored at room temperature for approximately 5 years.

Mosquitoes were collected from 6:00 pm to 6:00 am at each site, utilizing mechanical aspirators. The specimens were flash-frozen with dry ice in the field, then transferred from collection tubes into cryovials, stored in liquid nitrogen, and transported to the Gorgas Memorial Institute (GMI) in Panama City, where they were further preserved at -80°C. More comprehensive sampling details are available in the supplemental material.

Mosquitoes collections with CDC light traps. In 2018 and 2019, CDC light traps were deployed at each site (Farm, Site 8, and Matusaragatí) from 6:00 pm to 6:00 am. Five CDC traps (BioQuip Products, Rancho Dominguez, CA) operated for 5 days in both 2018 and 2019, totaling 25 trap days each year. The traps were placed at a 1.5 m height above ground level and baited with octanol and CO2. Collected mosquitoes were subsequently flash-frozen, stored in liquid nitrogen, and transported to Gorgas Memorial Institute (GMI). Mosquitoes species from Trinidad and CDC traps were morphologically identified [18], and grouped in pools of 20-50 mosquitoes based in trap type and species.

Mosquito blood meal analysis. In 2019, blood-engorged mosquitoes were collected at three sites (Farm, Site 8, and Matusaragatí, see **Fig. 1B**) to ascertain vector-host associations. Collection methods included aspirators and resting shelters[19,20]. Blood from engorged abdomens was smeared onto FTA cards to preserve host DNA[21]. Small FTA card sections were precisely excised using mini snap-blade utility knives, with blade sections discarded between each FTA card to prevent contamination. Each sample was placed in a separate 1.5 mL microcentrifuge tube. DNA extraction utilized the Instagene Matrix kit (BioRad, Hercules, CA), followed by PCR amplification targeting vertebrate genes (16s ribosomal RNA, cytochrome b, and cytochrome C oxidase subunit I (COI) gene[22,23]). Overlapping Sanger sequencing was conducted, and sequences were host species-identified through GenBank Basic Local Alignment Search Tool[24].

Viral isolation. Mosquitoes pools and tissues from sentinel animal were used to prepare 10% suppressions with 2 mL of minimum essential medium supplemented with penicillin and streptomycin, and 20% fetal bovine serum using a TissueLyser (Qiagen, Hidden, Germany). After 12,000 rpm centrifugation for 10 min, 200 µL of the supernatant were each inoculated in each of two 12.5-cm^2 flasks of Vero cells. Samples were passaged twice for cytopathic effect (CPE) confirmation. Furthermore, the liver, spleen, and kidneys obtained from a total of 599 wild rodents collected in the Western and Eastern regions of Panama during 2011 and 2012 were used in an attempt to isolate the virus. Specifics regarding rodent trapping efforts, collection sites, and species can be found elsewhere [16] and supplementary material.

Arbovirus molecular screening. Rodent tissue, sentinel animal tissue, mosquitoes suspensions and supernatant of cells with evidence of CPE were screened using genus-specific primers to detect viral RNA from the three major arbovirus genera known to circulate in

Panama including alphaviruses, orthoflaviviruses and phleboviruses as described previously[25–27].

Electron Microscopy. For ultrastructural analysis, infected Vero cells were fixed for at least 1 hour in a mixture of 2.5% formaldehyde prepared from paraformaldehyde powder and 0.1% glutaraldehyde in 0.05M cacodylate buffer (pH 7.3), to which 0.03% picric acid and 0.03% CaCl2 were added. The monolayers were washed in 0.1M cacodylate buffer, and cells were scraped off and processed further as a pellet. The pellets were post-fixed in 1% OsO4 in 0.1M cacodylate buffer (pH 7.3) for 1 h, washed with distilled water, and en bloc stained with 2% aqueous uranyl acetate for 20 min at 60°C. The pellets were dehydrated in ethanol, processed through propylene oxide, and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on a Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL), stained with lead citrate, and examined in a Philips 201 transmission electron microscope at 60 kV.

Construction of RNA library and whole genome sequencing. The supernatant of Vero cells with evidence of CPE were used for RNA extraction using QIAamp viral RNA extraction kit (Qiagen, Germany) and treated with DNase I (DNA-Free; Ambion, Austin, TX). Libraries for sequencing were prepared using the Next Ultra II RNA Prep Kit (New England Biolabs, https://www.neb.com) according to the manufacturer's protocol. In brief, we fragmented ≈ 50–100 ng of RNA for 15 min, followed by cDNA synthesis, end repair, and adapter ligation. After 6 rounds of PCR with independent primer pools, libraries were analyzed using an Agilent Bioanalyzer (https://www.agilent.com) and quantified using quantitative PCR. We pooled and sequenced samples using a paired-end 75-base protocol on an Illumina platform (Illumina, Inc., https://www.illumina.com) using the NextSeq 550 High-Output Kit.

Sequence assembly and analysis. Reads were processed using Trimmomatic version 0.36[28] to remove low-quality base calls and any adapter sequences. We used the de novo assembly program ABySS[29] to assemble the reads into contigs by using several sets of reads from 25,000 to 2 million reads pairs and *K*-mer values ranging from 20 to 40. We compared contigs >400 bases long against the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) nucleotide collection using Nucleotide BLAST[24]. We also used BLAST to compare the translated contigs against viral proteins from the NCBI database. Contigs showing similarity to virus genomes or proteins were selected for further analysis. Reads were mapped to the

selected contigs using Bowtie2[30] with the local parameter and visualized with the Integrated Genomics Viewer[31] to confirm correct assemblies and to identify and repair any errors. A total of 11,241,772 reads were obtained from 13 isolates (eleven isolates from sentinel animals and two from wild rodents). Detailed sequence statistics for each sample are provided in **Supplementary Table 1**.

***Orthobunyavirus* Real Time RT-PCR design**. A multiplex rRT-PCR assay was designed based on whole genome sequences for three novel bunyaviruses found in Darién Province: Aruza, Aguas, Matusagaratí, and for Madrid, which had not yet been detected in the region. Initially, three singleplex assays were designed for detection of 1) Aruza, 2) Aguas Calientes, and 3) Madrid and Matusagaratí viruses. The latter assay detected but did not differentiate Madrid and Matusagaratí viruses. Using Primer 3 software, five to seven unique primer-probe sets were designed for the small (S) genome segment for each virus. Singleplex assays were evaluated with genomic RNA from type strains, and the most sensitive singleplex tests, characterized by lower cycle threshold (Ct) values and higher normalized fluorescence, were combined to develop a single-reaction multiplex assay. Primer-probe combinations were then combined to create a single-reaction multiplex assay. Final primer and probe sequences for each virus are shown in **Supplementary Table 2**. The multiplex assay was evaluated with varying concentrations of type strain viruses to confirm no cross-reactivity or loss in sensitivity (**Supplementary Table 3**). Specificity for Aguas Calientes assay could not be fully confirmed as there were no samples that solely contained Aguas Calientes virus. Additional information of assay performance, optimization, and analytical evaluation are shown in supplementary material.

***Orthobunyavirus* rRT-PCR**. Mosquito 10% suspension was used to extract viral RNA using QIAamp Viral RNA Mini kit (Qiagen, Hidden, Germany). For detection of viral RNA in dried-blood spots, a section of 0.5 cm^2 was cut and eluted in 100µl of phosphate-buffered saline at 400 rpm and 4°C, overnight. Extracted viral RNA from mosquitoes suspensions and dried blood spot were then screened for Aruza, Aguas Calientes and Madrid/Matusaragatí viruses using the Orthobunyavirus multiplex rRT-PCR assay described above.

Phylogenetic analysis. A total of 33 homologous genome sequences for each segment (L, M, S) were retrieved from NCBI GenBank[32] and aligned at the amino acid level with our 17 full genome sequences using the MUSCLE algorithm[33]. Sequences used for phylogenetic analysis

represent virus species from the Group C, Guamá and Capim Orthobunyavirus serogroups. The final length of the aligned datasets was 265 amino acid sites for the S segment, 1496 for the M segment, and 2296 for the L segment. For each dataset, amino acid model selection was conducted using the Model Finder tool[34] implemented in the IQTREEv2[35] software using the Bayesian Information Criteria[34]. Finally, maximum likelihood phylogenies were reconstructed for each dataset, S, M, L using the LG+I+G4+F1x4 best-fitting amino acid substitution model. Robustness was assessed with 1000 bootstrap replicates and using the SH-aLRT test available in IQTREEv2[34,35]. Clades were considered statistically robust if bootstrap and SH-aLRT values were both ≥ 80%.

Pairwise amino acid sequence comparisons. Pairwise amino acid sequence comparisons (p-distances) were performed only with sequences of the RNA-dependent RNA polymerase for taxonomic, as recommended by the International Committee on Taxonomy of Viruses (ICTV) on the *Peribunyaviridae*[36].

Multiple sequence alignment and structure modeling analyses of the Gc glycoprotein from the newly viral isolates. Multiple amino acid sequence alignments were constructed using the N-terminal half (residues Gln465-Trp874) and C-terminal half (residues Lys881-Ile1306) of the Gc glycoprotein from Schmallenberg virus (SBV; GenBank Accession Number: CCF55030) and our newly 17 *Orthobunyavirus* genomes (seven representative unique sequences were used). The sequence alignments were calculated with MultAlin[37], plotted with ESPript[38], and annotated with the previously reported structures of SBV Gc (pre-fusion N-terminal half, PDB ID: 6H3S [39]; post-fusion C-terminal half, PDB ID: 7A56[40]). The N-terminal half of the Gc from isolate strain 705358 was modeled structurally using AlphaFold2[41]. The structure model was generated as a monomer and subsequently superimposed onto the trimeric structure of La Crosse virus (LACV) Gc head domain (PDB ID: 6H3W[39]) to generate a trimeric pre-fusion model.

RESULTS

Virus discovery and characterization of new orthobunyaviruses

Viral isolates and sentinel animals. In 2017, one mouse presented with signs of illness after two days of being placed in the trap in Site 8, Aruza. In 2018, two hamsters from Site 8 showed signs of disease after two and three days of being placed in the trap, respectively. The most

observed signs were chills and lethargy. These three symptomatic sentinel animals yielded a total of 11 viral isolates (**Table 1**). Additionally, two wild-captured short-tailed cane mice (*Z. brevicauda*) trapped in 2011 in El Real de Santa Maria yielded the isolation of two viral strains (**Table 1**). Cytopathic effect was observed in Vero cells after 4-7 days post-inoculation (**Supplementary Fig. 2A-D**). Alphavirus, Phlebovirus, and Orthoflavivirus molecular tests on sentinel animal tissues, tissues collected from wild caught rodent, mosquitoes suspensions and supernatant of cells with evidence of CPE produced negative results.

Table 1. Summary of the host and tissue samples that yield viral isolates.

No.	Year	Host	Animal code	Tissue	Sample code	Tissue code	Strain designation	Passage history	Virus
1	2017	Mouse	3	liver	1	701673	705358	Passage 4	Madrid
2	2017	Mouse	3	heart	2a	701674A	705359	Passage 4	Madrid
3	2017	Mouse	3	heart	2b	701674B	705360	Passage 4	Aguas Calientes
4	2017	Mouse	3	spleen	3a	701675A	705361	Passage 4	Aguas Calientes
5	2017	Mouse	3	spleen	3b	701675B	705362	Passage 4	Madrid
6	2018	Hamster	10	kidney	4a	702150A	705363	Passage 2	Aruza
7	2018	Hamster	10	kidney	4b	702150B	705364	Passage2	Madrid
8	2018	Hamster	10	liver	5	702151	705365	Passage 2	Aruza
9	2018	Hamster	10	heart	6a	702152A	705366	Passage 2	Aruza
10	2018	Hamster	10	heart	6b	702152B	705367	Passage 2	Madrid
11	2018	Hamster	10	spleen	7	702153	705368	Passage 2	Aruza
12	2018	Hamster	8	liver	8	702154	705369	Passage 2	Matusagaratí
13	2018	Hamster	8	spleen	9	702155	705370	Passage 2	Matusagaratí
14	2018	Hamster	8	heart	10	702156	705371	Passage 2	Matusagaratí
15	2018	Hamster	8	kidney	11	702157	705372	Passage 2	Matusagaratí
16	2011	Z. brevicauda	-	spleen	1_1	ER49-11	705373	Passage 2	Madrid
17	2011	Z. brevicauda	-	spleen	2_1	ER59-11	705374	Passage 2	Madrid

Electron Microscopy. In ultrathin sections of infected Vero cells, viral particles isolated from samples from Matusagaratí and Aguas Calientes were in intercellular spaces at the cell surfaces. They were 80-100 nm in diameter and had a bunyavirus morphology (**Fig. 2 A and B**). Viral particles from samples from Aruza were 70-75 nm in diameter and were also observed in extracellular spaces (**Fig. 2 C**). In Vero cells infected with samples from Aguas Calientes virus larger virus-like particles with fuzzy surface were observed. They were 125-150 nm in diameter and localized free in the cytosol (**Fig. 2 D**) or closer to the plasma membrane (**Fig. 2 E**).

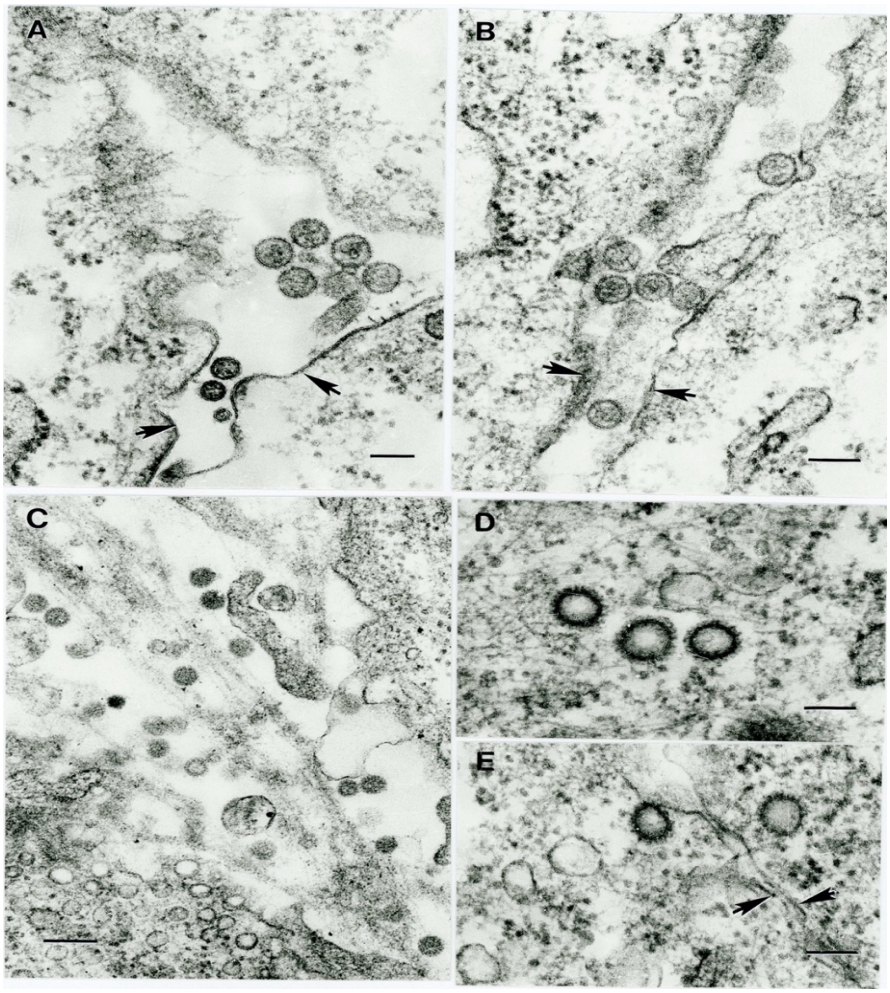

Figure 2. Ultrastructure of viruses Aguas Calientes (701674, 701675), Aruza (702150) and Matusagaratí (702152) in Vero cell monolayers. *(**A**) Viral particles of Matusagaratí virus (702152) 80-100 nm in diameter in extracellular space between two cells. Arrows indicate cell membranes. Bar = 100 nm. (**B**) Viral particles of Aguas Calientes virus (701674) 80-100 nm in diameter in extracellular space between two cells. Arrows indicate cell membranes. Bar = 100 nm.; (**C**) Viral particles with 70-75 nm in diameter of Aruza virus (702150) in extracellular spaces between the cells. Bar = 200 nm; (**D, E**) Virus-like particles 125-150 nm in diameter deeper in the cytosol (**D**) or close to the cell surface (**E**). Arrows indicate plasma membranes of two adjacent cells. Bars = 100 nm.*

Sequencing of novel viruses

A total of eleven tissues from tow hamsters and one mouse yield cytopathic effect on Vero after 4-8 days post inoculation, then supernatants was collected and used for viral RNA extraction and sequencing using Illumina platform. Details of type of tissues that produce cytopathic effect are provided in table one. A total of 2 million reads were obtained. Detailed information of number of reads per sample is provided in **Supplementary Table 4**. Finally, fifteen whole genomes were obtained from cell culture supernatant (**Table 1**).

Phylogenetic analysis of the L segment (RNA-dependent RNA polymerase). A multiple alignment of deduced aminoacidic (aa) RNA-dependent RNA polymerase sequences was built using the newly generated data and 33 homologous publicly available genome sequences from the Group C, Guama, and Capim serocomplexes within the Orthobunyavirus group, which were retrieved from GenBank[24]. Madrid virus strains obtained from sentinel #3 (705358, 705359, and 705362), from sentinel #10 (705364, 705367), and from a wild short-tailed cane mouse (*Z. brevicauda*) (705373, 705374) all clustered with SH-aLTR support within serogroup C, alongside the original prototype Madrid virus strain BT4075 (Accession Number: KF254779) isolated in Panama in 1956. Likewise, Matusagaratí virus strains (705369, 705370, 705371, and 705372) clustered together with Madrid virus in the L segment. Aruza virus strains (705363, 705365, 705366, and 705368) clustered with maximum phylogenetic support within the Guama serogroup, alongside the Mahogany Hammock virus. Aguas Calientes virus strains (705360 and 7053661) clustered with highest support within the Capim serogroup, forming a separate clade alongside with Guajara and Capim viruses (**Fig. 3A**).

Phylogenetic analysis of M segment (glycoprotein). A multiple alignment of the deduced aa glycoprotein sequences was constructed using newly generated data, in addition to 33 homologous genome sequences from the Group C, Guama, and Capim serocomplexes within the Orthobunyavirus group. Virus strains from sentinel #3 (705358, 705359, and 705362), sentinel #10 (705364 and 705367), and wild short-tailed cane mouse (*Z. brevicauda*) (705373 and 705374), all exhibited a phylogenetic clustering within serogroup C, and were most closely related to the original Madrid virus prototype strain BT4075. Matusagaratí virus strains from sentinel#8 (705369, 705370, 705371, and 705372) also clustered within serogroup C, but were most closely related to the Vinces virus prototype strain isolated in Ecuador in 1976 (**Fig. 3B**). Aruza virus strains from sentinel#10 (705363, 705365, 705366, and 705368), clustered phylogenetically within the Guama serogroup, alongside Moju virus (**Fig. 3B**). Aguas

Calientes virus strains from sentinel#3 (705360 and 7053661) clustered phylogenetically within the Capim serogroup, alongside with Guajara virus and Capim virus (**Fig. 3B**).

Phylogenetic analysis of the S segment (nucleoprotein). A multiple alignment of the deduced aa nucleoprotein sequences was constructed using newly generated data and 33 homologous genome sequences obtained from the Group C, Guama, and Capim serocomplexes within the Orthobunyavirus group, as above. Madrid virus strains from sentinel#3 (705358, 705359, and 705362), from sentinel#10 (705364 and 705367) and from the wild short-tailed cane mouse (*Z. brevicauda*) (705373 and 705374), as well as and Matusagaratí virus strains from sentinel#8 (705369, 705370, 705371, and 705372) all clustered phylogenetically within serogroup C, alongside the original prototype Madrid virus strain BT4075. Aruza virus strains from sentinel#10 (705363, 705365, 705366, and 705368) clustered phylogenetically with maximum support within the Guama serogroup, alongside Mahogany Hammock virus. Aguas Calientes virus strains from sentinel#3 (705360 and 7053661) clustered phylogenetically within the Capim serogroup, alongside with Guajara virus and Capim virus (**Fig. 3C**).

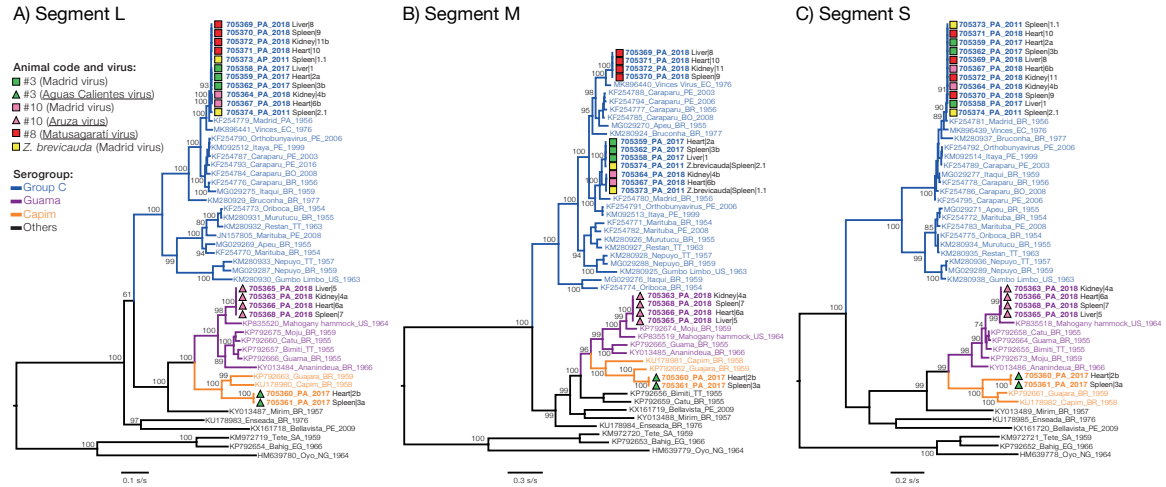

Figure 3. ***A.*** *Maximum likelihood phylogenetic tree based on segment L (RNA dependent polymerase) of the Group C, Guama and Capim serocomplexes from the Orthobunyavirus group. The tree topology was inferred using the LG+I+G4+F1x4 model. Bootstrap values (%) are shown in internal nodes. Strains identified in this study are highlighted in red.* ***B.*** *Maximum likelihood phylogenetic tree based on of the deduce aa of the segment M (glycoprotein) sequence of the Group C, Guama and Capim Serocomplexes from the Orthobunyavirus group. tree topology was inferred using the LG+I+G4+F1x4 model. Strains identified in this study are highlighted in bold. Bootstrap values are shown in internal nodes.* ***C.*** *Maximum likelihood phylogenetic tree of the segment S (nucleoprotein) deduced aa sequence of the Group C, Guama and Capim Serocomplexes from the Orthobunyavirus group. The tree topology was*

inferred using the LG+I+G4+F1x4 model. Strains identified in this study are highlighted in red. Bootstrap values are shown in internal nodes. Trees were estimated using IqTree v2.

Orthobunyavirus pairwise aminoacidic distance and taxonomy. Based on the ICTV criteria we used the deduced aa segment L (RNA-dependent RNA polymerase) sequences to classify newly orthobunyavirus species[36]. Madrid virus strains (705358, 705359, 705362, 705364, 705367, 705373, and 705374) share 100% of identity between them and share 99% of identity with the prototype strains BT4075 of Madrid virus isolated in Panama during 1956 in Bocas del Toro province. Matusagaratí virus strains (705369, 705370, 705371, and 705372) share 98% aa identity with Madrid virus based on segment L (RNA-dependent RNA polymerase) sequences. Of note, in the segment M (glycoprotein), Matusagaratí strains shared 79% and 92% aa identity with Madrid and Vinces virus, respectively. Aruza virus strains (705363, 705365, 705366, and 705368) share 92% aa identity with Mahogany Hammock virus' segment L. Aguas Calientes virus strains (705360 and 7053661) share 84% of aa identity with both Guajara virus and Capim viruses in segment L (pairwise distances for all strains can be found in Supplementary Table 5).

Overall, we identified three potentially new viruses named here as Aruza virus, Aguas Calientes virus and Matusaragati virus, with the latter being the result of reassortment between Madrid virus (segments L and S) and an unknown virus in segment M (**Fig. 3** and **Table 1**). Viral designations should be considered putative and were selected based on the name of communities where these viruses were discovered.

Comparison of orthobunyavirus isolates with structurally characterized Gc glycoproteins. Of the 17 *Orthobunyavirus* genome sequences generated in this study, 7 unique amino acid Gc glycoprotein sequences were identified. Alignment of these 7 representatives Gc amino acid sequences revealed sequence identities ranging from 28-32% with Schmallenberg virus (SBV) Gc, 32-34% with Oropouche virus (OROV) Gc, 31-33% with Bunyamwera (BUNV) Gc, and 32-33% with Lacrosse encephalitis virus (LACV) Gc [42]. High-resolution structural information describing orthobunyaviral glycoproteins [39,40] is limited to the N-terminal half of Gc of SBV (PDB ID: 6H3S), OROV (PDB ID: 6H3X), BUNV (PDB ID: 6H3V), and LACV (PDB ID: 6H3W) in the pre-fusion conformation. The pre-fusion N-terminal half of the Gc (residues 465-874; 408 residues) forms an α-helical head domain and two β-strands rich stalk subdomains [39]. The C-terminal half of the Gc from SBV (PDB ID:

7A56) and LACV (PDB ID: 7A57) in post-fusion conformation has also been elucidated and forms a class II fusion protein architecture [40] (residues 881-1,306; 420 residues).
SBV Gc structures were used for annotating and analyzing the alignment of the new Gc sequences (**Supplementary Figs. 4 and 5**). Consistent with the structural variability of the N-terminal half of orthobunyaviral Gc, amino acid comparison of isolates with SBV Gc revealed lower sequence identities (19-26%) compared the membrane-proximal C-terminal half of Gc (36-38%). Combined with the observation that all 20 cysteine residues and 10 disulfide bonds are conserved between the isolates and SBV Gc (**Supplementary Fig. 4**), it is likely that C-terminal half of Gc of the identified isolates adopt a class II fusion protein fold[40] and undergoes a shared mechanism of membrane fusion during host-cell entry[39,43].

Comparison of the N-terminal region of the Gc reveals that 15 out of 20 cysteine residues and 6 out of 10 disulfide bonds from the 7 isolates are conserved with SBV Gc (**Supplementary Fig. 5**). Of the 5 cysteine residues not conserved with SBV Gc, all are conserved with the OROV Gc sequence, suggestive that the isolates identified here exhibit the same overall tertiary structure with previously reported orthobunyavirus pre-fusion Gc structures[39].

To support the hypothesis that the Gc glycoproteins of the identified isolates are structurally similar to previously characterized orthobunyaviral glycoproteins, a model of the N-terminal half of the Gc (strain 705358, Madrid virus, isolated from sentinel#3) was generated using AlphaFold2[41]. As expected, the resulting model resembled the pre-fusion structure of SBV Gc [39] and constituted a head domain and two tandem stalk subdomains (**Fig. 4A**). Rigid fitting of the model into an electron cryo-tomography map (EMD-2352)[44] of the pre-fusion BUNV Gc spike was consistent with a tripodal architecture (**Fig. 4B**).

The SBV Gc head domain has been previously structurally characterized in complex with two neutralizing monoclonal antibodies (mAbs), termed 1C11 and 4B6 (PDB ID: 6H3T; 6H3U) [39]. Mapping of the positions of the mAb 1C11 and mAb 4B6 epitopes onto the AlphaFold-derived model revealed the solvent accessible sites at the apical and basal regions of the head domain, respectively (**Fig. 4C**). Interestingly, the positioning of the mAb 4B6 suggests a clash with an adjacent protomer within the trimer, as presented on the lattice surface. By analogy to structural studies of the neutralizing mAb, P-4G2, in complex with the Gc of Puumala hantavirus[45,46], it seems possible that mAb 4B6 disrupts higher-order lattice formation on the orthobunyavirus surface.

Overall, these combined observations suggest that the envelope surface of our orthobunyaviral isolates exhibit highly similar architectures to those observed in previously structurally characterized orthobunyaviruses.

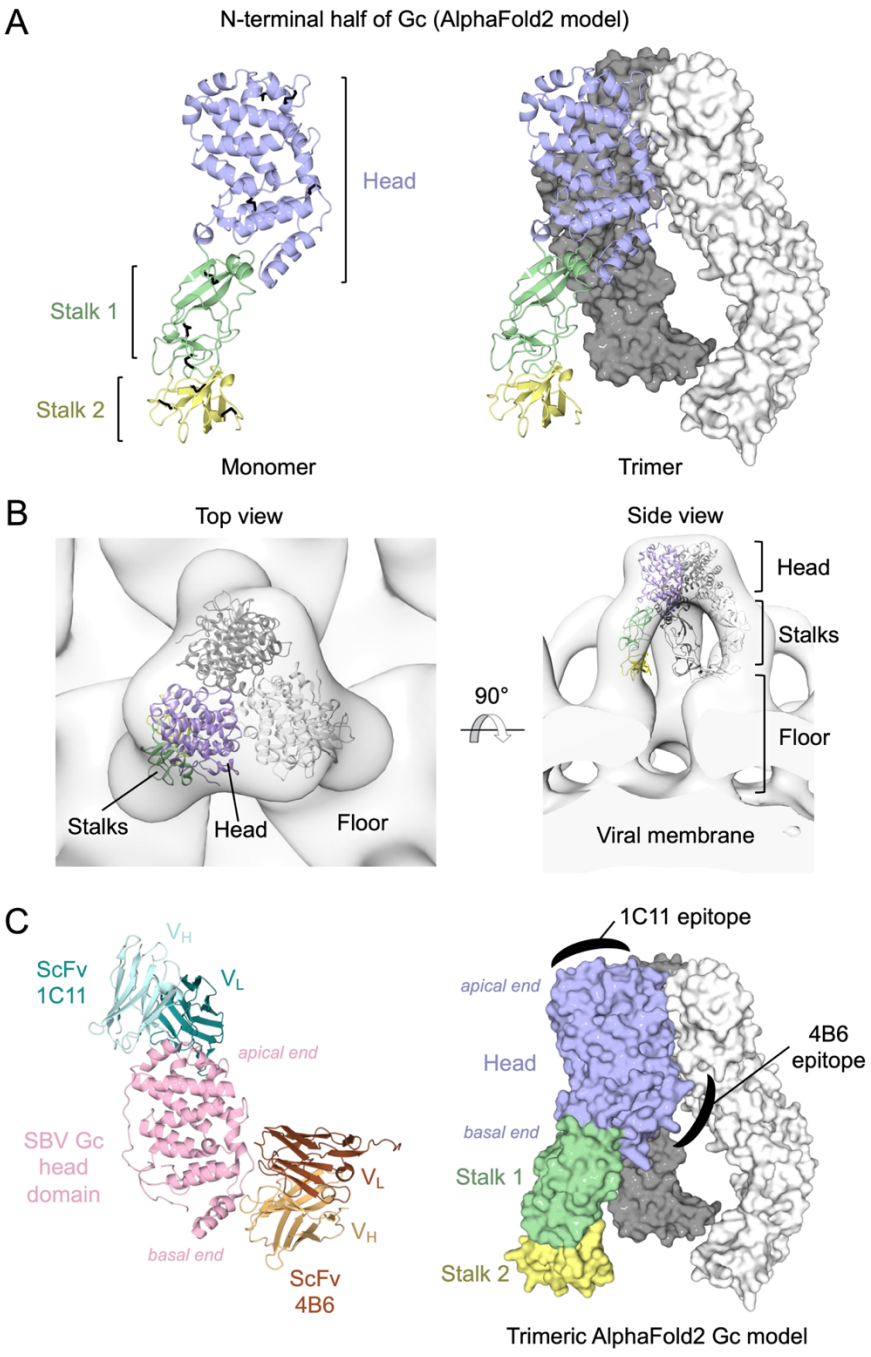

Figure 4. Structure modeling of the N-terminal half of Orthobunyaviral Gc glycoproteins. *The N-terminal half of the isolate Gc (strain 705358) was structurally modeled using AlphaFold2[41] (c (**A**) (Left) Monomeric model of the N-terminal half of Gc in cartoon representation. The head domain is colored purple, and the stalk subdomains are colored green and yellow, respectively. Conserved disulfide bonds are shown as black sticks. (Right) Trimeric model of the N-terminal half of Gc. For visual clarity, one protomeric copy is shown as cartoon while the other two are shown in surface representation (white and grey). (**B**) Rigid fitting of*

*the trimeric Gc model (cartoon) into an electron cryo-tomography map (grey; EMD-2352)[44]. The head and stalk domains form a tripodal pyramid architecture. The C-terminal 'floor' domain which connects the N-terminal half to the viral membrane was not modeled. (**C**) Antibody epitope mapping. (Left) Structure[39] of SBV Gc head domain (pink) in complex with single-chain variable fragment (ScFv) 1C11 (cyan; PDB ID: 6H3T) and 4B6 (brown; PDB ID: 6H3U). V_H and V_L denote variable heavy and variable light chains. (Right) Mapping of 1C11 and 4B6 epitopes onto the AlphaFold2-derived model of the isolate Gc N-terminal half (surface representation) in trimeric form. The antibody targeted sites are indicated with filled, black crescents.*

Mosquito and wild rodent diversity

Abundance and diversity of mosquitoes species collected with Trinidad traps. A total of 11,256 individual mosquito representing 9 genera and 30 species were collected from a total of 15 modified Trinidad No. 17 traps placed in a forested area of Aruza (**Fig. 2A**). Total number of individual mosquitoes identified and mosquitoes species per sentinel animal are summary as follows: mouse sentinel: 1,347 mosquitoes from 25 species; hamster sentinel: 9,078 mosquito from 17 species; chicken-baited traps: 815 mosquitoes from 15 species; toad-baited traps: 16 mosquito from 6 species (**Fig. 1A**).

Cx. (*Mel.*) *spissipes* was the most abundant species collected with sentinel-baited traps, with a total of 3,896 individuals representing 34.61% of the total individual mosquitoes collected. It was also the most abundantly collected species using mice- (24.57%) and hamster-baited traps (37.63%). Other abundant species included *Ma. (Man.) amazonensis* (24.49%), *Coq. (Rhy.) venezuelensis* (21.13%), and *Culex (Mel.) pedroi* (8.58%). The most abundant species collected using chickens was *Coq. (Rhy.) venezuelensis* (46.38%) while the most abundant species collected using toads was *Ma. (Man.) amazonensis* (31.25%) (**Fig. 2B**).

Abundance and diversity of mosquitoes species collected with CDC light traps. A total of 3,330 individual mosquito representing 6 genera and 36 species were collected using CDC light traps placed in a forested area during 2018 and 2019 (**Fig. 2B**). The most abundant mosquito species collected was *Cq.* (*Rhy.*) *venezuelensis* (43.4%), followed by *Cx.* (*Cux.*) *nigripalpus* (10.1%).

Wild rodent abundance and diversity. Between 2011 and 2012, a total of 559 rodents were collected, representing 13 genera and 16 species (**Supplementary Table 4**). Most collections occurred in 2011, accounting for 71.8% of all captures (n = 430/599). Notably, the Darien

Province was the primary location for rodent collection, constituting 87.6% of the total collections (n = 525/599), specifically in El Real (33.7%, n = 202/599), followed by Los Pavitos (27.6%, n = 165/599), and Santa Librada (26.4%, n = 158/599) (**Supplementary Table 4**). The short-tailed cane mouse (*Zygodontomys brevicauda*) emerged as the predominant species, accounting for 70.4% of all trapped animals (n = 422/599) (**Supplementary Table 4**).

Orthobunyavirus potential vectors and host and host-vectors interactions

Orthobunyavirus potential vertebrate host. Orthobunyaviruses were only isolated in rodent species, either sentinel or wild animals. In sentinel mice, using viral isolation a next generation sequencing orthobunyaviruses were found at a rate of 33.3%, (95% CI: 0.8-90.5; n=1/3). In sentinel hamsters, at a rate of 13.3% (95% CI:1.6-40.4; n=2/15). In wild rodents, rate of detection was 0.33% (95% CI: 0.0-1.2; n=2/599) or 0.4% (95% CI: 0.0-1.7; n=2/422) if we consider only *Z. brevicauda*. Overall, during 2017 and 2018, orthobunyaviruses were found in sentinels at a rate of 33.3% (95% CI: 0.8-90.5; n=1/3) and 40% (95% CI: 5.2-85.3, n=2/5), respectively. No orthobunyavirus detection was done during 2019.

Orthobunyavirus RNA detection in mosquitoes and dried blood spots. Using a newly developed orthobunyavirus rRT-PCR, a total of 1,091 mosquito pools were tested for orthobunyavirus RNA. Detailed analytical evaluation of the new orthobunyavirus rRT-PCR is provided in **Supplementary Material**. A total of 947 pools (86.8%) were formed by mosquitoes collected using Trinidad traps, and 144 pools were formed by mosquitoes collected from CDC light traps. Only mosquitoes pools obtained from collections using Trinidad traps produced rRT-PCR positive results. Overall, Aruza virus was detected in 2.5% (95% CI: 1.6 – 3.7; n=24/947) of the pools, Aguas Calientes virus in 2.3% (95% CI: 1.6-3.7; n=22/947) and Madrid/Matusaragatí in 0.4% (95% CI: 0.1-10.8; n=4/947) of the pools.

At the level of mosquitoes species, Aruza virus was detected in 4.7% (95% CI: 2.3-8.2; n=11/233) of the *Culex* (*Mel*.) *spissipes* pools, 3.0% (95% CI:1.0-10.0; *n*=5/163) of the *Cq.* (*Rhy.*) *venezuelensis* pools, 5.4% (95% CI:1.4-13.2; n=4/74) of the *Culex* (*Mel*.) *pedroi* pools, 8.3% (95% CI: 1.0-2.7; n=2/24) of the *Culex* (*Mel*.) *vomerifer* pools, and 5% (95% CI:0.3-24.8; n=1/20) of the *Cx.* (*Mel*.) *adamesi* pools. Aguas calientes vírus was detected in *Cx (ads.) amazonensis*. Madrid/Matusaragati was detected inCx (Mel.) pedroi, *Cq.* (*Rhy.*) *venezuelensis* and *Cx (ads.) amazonensis*. Detailed information on positive mosquitoes pools by species and rRT-PCR cycle thresholds (Ct values) is provided in **Fig. 5C** and **Supplementary Table 3**.

Matusaragatí virus was detected by rRT-PCR in a dried blood spot of a 2017 sentinel mouse after 5 years of storage at room temperature.

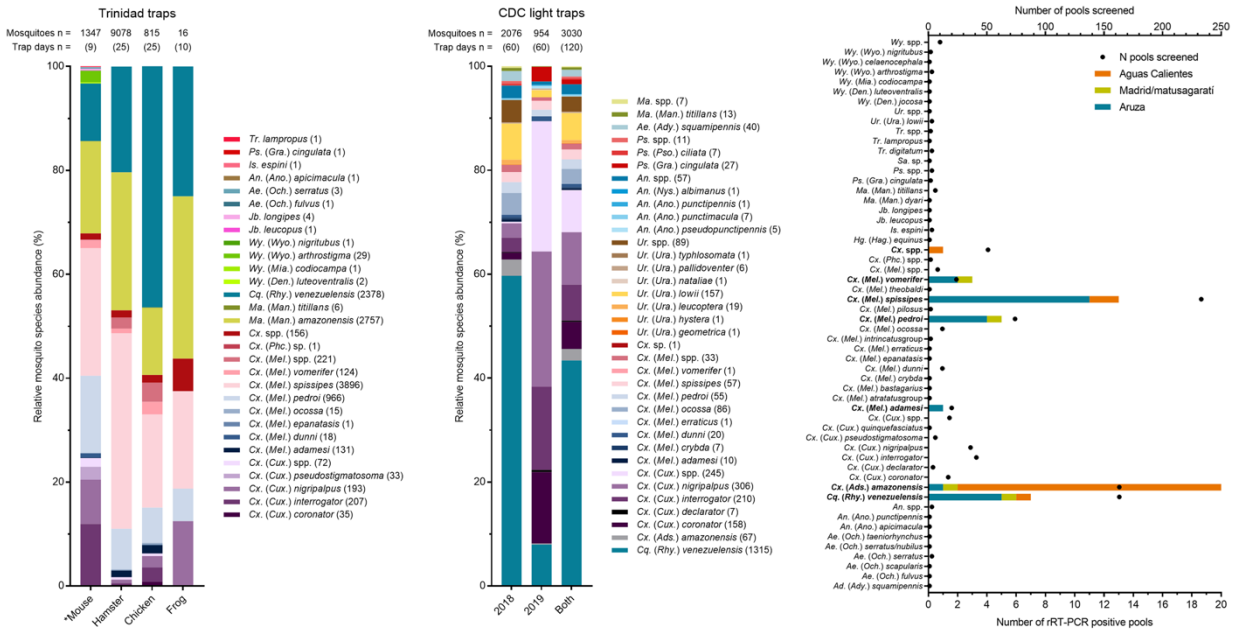

Figure 5. Mosquito relative abundance by, host type of trap Trinidad or CDC light trap and number of mosquito pool screened and Orthobunyavirus rRT-PCR positive frequency.

A. Relative Mosquito abundance per host for Trinidad trap collections. B. Relative Mosquito abundance per host for CDC light trap collections and C. Number of mosquito pools screened and Orthobunyavirus rRT-PCR positive pools. Number of mosquito (Mosquito n =) and relative sampling effort (Trap days n =) with each host shown above bar. Number of individuals per species included in parentheses next to species name; sp. = single species, spp. = potentially multiple species. Abbreviated names are given in full in the dataset at supplementary materials.

Mosquito host-feeding patterns. Fifty-six blood meals from eight mosquitoes species produced readable sequences (71.8%). These blood meals were from *Anopheles* (*Arribalzaga* group) spp. (n=1), *Psorophora cingulata* (n=1), and the majority (n=54) were from species of *Culex* (51.7%, n=29/54), of identified blood meals were from two species of the Spissipes section of *Melanoconion*, *Culex pedroi* (n=9), and *Culex spissipes* (n=20).

From the ten engorged *Cx. pedroi* identified here, nine produced an amplicon that resulted in usable sequences (>95% match in GenBank). Eight were derived from mammals, including horse (*Equus caballus,* n=6), cattle (*Bos taurus,* n=*2*), and Brazilian cottontail (*Sylvilagus brasiliensis*, n=1). From the 23 engorged *Cx. spissipes,* 20 produced a usable sequence. *Culex spissipes* blood meals were derived from a diverse range of vertebrate hosts, including mammals (n=11), birds (n=8) and reptiles (n=1). Avian hosts for *Culex spissipes* included grey-necked wood rail (*Aramides cajaneus* n=5), chicken (*Gallus gallus*, n=1), Ecuadorian Trogon (*Trogon melanurus,* n=1), and rufous-tailed jacamar (*Galbula ruficauda,* n=1). Mammalian hosts for *Culex spissipes* included cattle (n=8), Brazilian cottontail (n=2) and white-footed capuchin (*Cebus albifrons* n=1). The reptile-derived blood meal was from red-headed basilisk (*Basiliscus galeritus*). *Culex* (*Culex*) *nigripalpus* blood meals were derived from avian hosts (chicken, n=5; grey-necked wood rail, n=2; and Rufescent tiger heron, n=1) and mammals (cattle, n=3; and horse, n=1). *Culex* (*Culex*) *saltanensis* fed upon mammals, including cattle (n=2), horse (n=2) and Brazilian cottontail (n=1). *Culex* (*Aedinus*) *amazonensis* blood meals were from cattle (n=2). Unidentifiable specimens of *Melanoconion* section *Culex spp*. females were from red-headed basilisk (n=3), green iguana (n=2), and Garland anole (n=1). *Anopheles* (Arribalzaga group) spp. and *Psorophora cingulata* fed upon cattle (n=1) and horse (n=1), respectively.

DISCUSSION

We employed a One Health surveillance approach, including host-seeking and traditional mosquito traps, untargeted genomic analyses, wild rodent collections, and mosquitoes blood meal detection, to actively identify the presence of emerging arboviruses, potential hosts, and vectors in an enzootic focus. Our efforts included attempting to detect naturally infected rodents with arboviruses and led to the isolation of a total of 13 viral strains from one sentinel mouse, two sentinel hamsters, and two wild-caught specimens of *Z. brevicauda*. Deep sequencing efforts and subsequent phylogenetic analysis revealed a total of seventeen genomes within the Group C, Guama, and Capim orthobunyavirus serocomplexes. Based on the ICTV orthobunyavirus species classification criteria[36], we identified three new orthobunyaviruses, which we tentatively name here as Aruza, Aguas Calientes and Matusagaratí. We also detected the circulation of Madrid virus, an orthobunyavirus associated with human disease in Panama. To the best of our knowledge, only one single strain of Madrid Orthobunyavirus has been identified so far[47]. This strain was isolated from a 36-year-old man with severe disease manifestations that required hospitalization. The man was a technician from Gorgas Memorial

Laboratory, Panama, who was likely infected while conducting field entomological surveys in Bocas del Toro Province[47]. Notably, Matusaragatí virus was identified as a novel reassortant virus, resulting from genetic reassortment between Madrid virus and a yet unidentified virus. Reassortment events have been extensively documented in orthobunyaviruses and, in some instances, have been associated with the emergence of pathogenic viral strains[48].

Morphologically, our potential novel orthobunyaviruses were similar to the previously described Sinu virus which was classified as an Orthomyxovirus[49]. Our structural modeling analysis suggests that the Gc glycoproteins in our isolates likely conform to a structural architecture that is consistent with previously characterized orthobunyavirus Gc structures [39,40,44]. Further, we find that the membrane-distal N-terminal half of the Gc is less conserved than that of the membrane-proximal C-terminal half. This is consistent with the constrained functionality of the C-terminal region of the glycoprotein, which likely forms a class II fusion glycoprotein fold[40], and the potential for the N-terminal, membrane-distal region to be under stronger adaptive pressure by the antibody immune response[39]. Further studies that clarify the receptor(s) utilized during host-cell entry will facilitate prediction of whether the host-cell recognition and entry pathway(s) utilized by the viruses isolated herein are conserved with other orthobunyaviruses. Such information is essential for rationalizing the tropism characteristics of these pathogens and understanding the determinants of zoonotic transmission.

From an ecological perspective, despite our mosquito collection strategy across three distinct collection sites, orthobunyaviruses were exclusively identified in Site 8, which is characterized by its secondary forest habitat. Increased alphavirus infection in secondary forest habitats have previously been observed in other studies conducted within the Darien province[16,50]. In terms of host selection, despite our utilization of four types of sentinel animals, orthobunyaviruses were exclusively isolated from mice, hamsters, and from wild caught short-tailed cane mice (*Z. brevicauda*). This emphasizes the critical role of rodents as essential enzootic hosts for these viruses[51,52].

Our comparison of active arboviral detection methods and Trinidad sentinel-baited traps showed the latter as significantly superior to the collection of reservoirs in the field. Their remarkable efficiency in detecting arboviruses, achieved with a limited number of sentinel animals, starkly contrasted with the demanding task of collecting and analyzing over 600

individuals to identify two orthobunyavirus strains from naturally infected rodents. This highlights the practical advantages of using our Trinidad traps in arbovirus surveillance programs, potentially enhancing the overall effectiveness of vector-borne disease monitoring efforts within a One Health context.

Trinidad traps attracted several mosquitoes species, including *Cx.* (*Mel.*) *pedroi, Cx. (Mel.) spissipes, Cq. (Rhy.) venezuelensis, and Ps. (Gra) cingulata*, all of which have been implicated as enzootic arbovirus vectors with evidence of anthropophilic behavior[53–55]. *Cx. (Mel.) spissipes, Cq. (Rhy.) venezuelensis*, and *Cx.* (*Mel.*) *pedroi*. *Cx.* (*Mel.*) *spissipes* along with *Ma. (Man.) amazonensis*, were among the most abundant collected species in mice and hamster baited traps. The blood meal analysis also revealed that Cx. *(Mel.) spissipes, Ps. (Gra) cingulata and Cx.* (*Mel.*) *pedroi* were also actively feeding from mammals, birds, and reptiles. Altogether, these results support the hypothesis that these mosquitoes species are generalist feeders as they fed from a variety of vertebrates[55]. These mosquitoes species were also captured using CDC light traps; however, successful molecular detection of orthobunyaviruses was exclusively achieved in mosquitoes species collected with Trinidad traps. Is common to observe a low rate of naturally infected mosquitoes during investigations[56,57].

The observed variation between Trinidad and CDC light traps in detecting infected mosquito could be influenced by multiple factors as random sampling effects and relative trapping effort[58]. We also observed substantial differences in the number of mosquitoes captured using Trinidad traps compared to CDC traps, which may help to explain the relatively low rate of orthobunyavirus detection in mosquitoes collected with CDC traps. However, previous efforts of mosquitoes collections in the same region and undertaken by our group were also unsuccessful in detecting arboviral circulation when collecting larger number of mosquitoes with CDC traps [59]. Experimental infections have shown that impregnating traps with chemical compounds excreted by rats enhances the attraction of female mosquitoes compared to traps lacking these compounds [60]. This suggests that the presence of a host increases the effectiveness of attraction. When considering our results as a whole, they suggest that Trinidad traps not only enhance the likelihood of trapping mosquitoes vectors but also yield higher rates of viral detection.

This study presents certain limitations. While Trinidad traps offer insights into potential reservoirs or vectors, experimental evidence is essential to make definitive conclusions about

the host and vector capacity of the infected animal and mosquito. Nevertheless, this study provides an approach that preliminarily narrows down the number of potential vectors and reservoirs by providing patterns of species-frequency infection. The use of sentinel animals that undergo a viremic phase, attracting blood-seeking mosquitoes, could introduce confounding factors by attracting non-vectors. This might potentially yield misleading evidence regarding the vector capacity of these mosquitoes. To mitigate this, we conducted mosquitoes collections twice daily to minimize the duration of contact with sentinel animals. Moreover, it is worth noting that only a limited number of mosquitoes species tested positive, indicating a potential vector competence pattern.

In conclusion, our study provides valuable insights into the presence and diversity of orthobunyaviruses in an enzootic focus. It underscores the critical role of rodents as enzootic hosts and the effectiveness of Trinidad traps in arbovirus surveillance. These findings contribute to our understanding of arbovirus ecology and can inform vector-borne disease monitoring efforts in the region. Further experimental evidence is needed to definitively characterize host and vector capacity, and to validate the patterns of species-frequency infection observed.

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Discussion, Conclusions, and Future Directions

MADV and VEEV have emerged as notable zoonotic pathogens within the Americas, posing a significant public health burden for both human and veterinary sectors[1–3]. In the historical context of Panama, MADV has been associated with equine disease, exhibiting a high case fatality ratio ranging from 30% to 50% since 1936[4,5]. Despite extensive surveillance efforts in endemic communities, not only in Panama but also in Argentina and Peru, human infections by MADV have remained rare[5–7], with only two human cases reported in Trinidad and Tobago[8], and one in Brazil[9].

On the other hand, VEEV epizootics in South America have been characterized by explosive epidemics and expansive geographical spread from South to North America involving thousands of human and equine[1,3]. The emergence of VEEV epizootic subtypes is believed to occur through mosquito or equine adaptive mutations from an enzootic ancestor[10,11]. In Panama, enzootic VEEV has been exclusively associated with human disease since 1960[12]. Subsequently, human cases have been identified through the dengue surveillance system[13], highlighting the importance of interconnectedness of arboviral surveillance systems in capturing the emergence of zoonotic pathogens.

A notable paradigm shift occurred in 2010 within the eastern province of Darien, where a human outbreak of MADV was identified, signifying a departure from historical trends[2]. VEEV outbreaks demonstrated a spatial overlap of the MADV during 2010[2]. Subsequently, a sustained spatial overlap of the MADV and VEEV outbreaks has been consistently observed in eastern Panama[14–16]. The primary aim of my research was to determine the extent to which

human MADV infections, along with the concurrent circulation of VEEV, represent emerging arbovirus events, in Panama. ***My central hypothesis posited that MADV and VEEV are emerging alphaviruses with sustained and increased incidence over recent decades in eastern Panama, and these increased risks are associated with changes in the ecology of potential vertebrate host and mosquitoes vectors***.

My research sought to elucidate MADV and VEEV epidemiology, including historical transmission dynamics, identifying risk factors of infection, antibody dynamics, long-term neurological consequences, and ecology of potential vertebrate hosts and mosquitoes vectors, while also developing diagnostic tools and implementing prospective alphavirus febrile surveillance; and additionally, b) to piloting a One Health approach for conducting enzootic and spillover arboviral surveillance in eastern Panama. The research was designed to study each component of arbovirus transmission, encompassing the human, the virus, the vertebrate host, the mosquitoes vectors, and the environment.

Reconstructing historical alphavirus dynamics and epidemiology

The MADV and VEEV outbreaks in Panama changed the paradigm of endemic alphavirus infection in Panama and, more broadly, in Latin America[2]. If both viruses are behaving as emerging zoonotic pathogens, questions arise regarding the factors contributing to the emergence of human cases of MADV and the co-circulation of VEEV. While the confirmation of MADV and VEEV emergence in Panama is a crucial aspect of this thesis, we also investigated whether genetic adaptive mutations, ecological shifts, or concurrent rises in enzootic circulation of both viruses underlined alphavirus emergence in Panama[2]. Our genomic analysis showed no vector or equine mutations in the VEEV strains isolated in 2010[2]. In the analysis of the structural polyprotein open reading frame (ORF), MADV strains isolated during

the 2010 outbreak were distinct among each other, differing by 19 nucleotides (0.2%). Specifically, isolate 247188 contained 28 and 36 nucleotides changes (0.2% and 0.3%) when compared to the 1984 and 1986 isolates, respectively. Additionally, isolate 247168 displayed differences of 4 and 10 nucleotides changes (0.3% and 0.8%) compared to the 1984 and 1986 isolates, respectively[2]. None of these differences are known to affect virulence or modify transmissibility. Experimental infection and genetic reverse methods using murine models were employed to assess changes in MADV virulence and transmissibility. No differences in viral replication or virulence were observed between the 2010 MADV strains when compared to strains from1984 and 1986, respectively (Scott Weaver, Personal Communication).

Alphavirus Catalytic Force of Infection Models

A powerful approach to investigate historical exposure to a pathogenic agent is by testing serum samples available in biobanks using serological assays. However, the inaccessibility and incomplete epidemiology surveillance in the remote Darien Province, limited the access to historical serum samples to test the hypothesis of MADV and VEEV recent emergence.

To overcome the limited access to biobank samples and test the hypothesis of recent emergence, I employed a mathematical framework to estimate force of infection (FOI) parameters. These transmission models provide a dynamic assessment of the varying risk of infection across different age groups within a population, incorporating age-specific exposure patterns and serological data[17]. Dividing the population into distinct age groups, I inferred historical trends in infection risk by fitting the model to observed seroprevalence data[17], thereby shedding light on the dynamics of MADV and VEEV epidemics and shifts in transmission patterns.

Utilizing age-stratified data from two population-wide cross-sectional seroprevalence studies employing neutralizing antibodies across seven distinct communities in Darien and constant and time-varying catalytic FOI models, I was able to reconstruct around 50 years of MADV and VEEV transmission dynamics. Our results indicate temporal and geographic heterogeneity in the human population's exposure to MADV and VEEV over the past five decades. The detailed analysis of seroprevalence estimates among individuals younger than 10 years old, serving as an indirect indicator of recent transmission, yielded noteworthy conclusions. First and foremost, the data underscore substantial variability in the exposure rates to VEEV and MADV across different locations. Particularly striking is the observation that Pirre 1-2 and Pijibasal exhibit the highest estimated seroprevalence for VEEV with a posterior median of 44.8% (95% CrI: 34.9–55.0). This suggested a considerably elevated level of recent transmission in these areas. On the other hand, the seroprevalence of MADV, while generally lower, is notable in Aruza at 4.7% (95% CrI: 3.2–6.7). These findings not only highlight the varying dynamics of recent transmission for both VEEV and MADV but also indicated potential hotspots of viral activity.

In six out of the seven locations studied, there was no evidence of time-varying transmission for MADV. This implied a relatively low and stable transmission pattern in most of the examined locations over the analyzed periods. However, a notable exception was Aruza, where the FOI for MADV was estimated as 0.012 (95% CrI: 0.006–0.021) during the most recent decade (2002–2012). Significantly, this represented a substantial increase, being 4.6 and 5.3 times higher (ratio of posterior medians) than the values estimated for the preceding decades (1992–2002 and 1982–1992, respectively). This finding showed recent increased transmission of MADV and suggests escalating risk of MADV transmission in 2002-2012. The analysis of VEEV transmission in seven locations yielded valuable insights. In six of these locations,

statistical support for time-varying transmission is lacking, suggesting a relatively stable transmission pattern over the periods studied. Under a constant FOI model, Pirre 1-2 and Pijibasal exhibit an annual FOI estimate of 0.08 per decades (95% CrI: 0.06–0.11), indicating a high seroprevalence in older age groups, reaching 75% in 15-year-olds and nearly 100% by age 60. However, the uncertainty in these estimates due to a small sample size (only 75 subjects) raises questions about whether they indicated consistent endemic transmission or recent introductions and/or outbreaks. In contrast, for Mercadeo, a time-varying FOI model provided the best fit, indicating a dynamic transmission pattern. In this scenario, the FOI during the most recent decade (2002–2012) was estimated at 0.04 (95% CrI: 0.03–0.06), marking a 1.5-fold increase over the previous decade (1992–2002) and a notable 3.1-fold increase compared to the period from 1972 to 1992. This suggested a substantial rise in VEEV transmission in Mercadeo in recent years.

FOI models showed evidence of low MADV and VEEV endemic transmission, in eastern Panama. However, these findings implied that while overall patterns may suggest stability, specific locations, such as Aruza and Mercadeo, demonstrated potential recent emergence and spatial heterogeneity in the transmission dynamics of MADV and VEEV in the Darien region of Panama, respectively.

Una virus (UNAV): Unearthing a Historical Virus

I sought to identify risk factors for alphavirus infection in eastern Panama. Using cross-sectional survey and seroprevalence data generated from the Mogue community of Darien, I conducted an extensive exploration for additional potential emerging alphaviruses in the region, specifically targeting MAYV and UNAV. Both viruses exhibit close genetic relatedness and belong to the Semliki virus complex within the Alphavirus group. UNAV and MAYV have

been previously identified in naturally infected mosquitoes in Panama[18,19], and there have been recent reports of increased MAYV activity and outbreaks in South America[20]. Given the shared features with vectors and vertebrate hosts of epidemic arboviruses like YFV, there is a suggestive potential for MAYV to emerge as the next epidemic arbovirus[20]. Subsequently, I conducted a focused assessment of neutralizing antibodies for MAYV and UNAV. Interestingly, the seroprevalence of MAYV in the Mogue community was observed to be low, consistent with our prior efforts to assess human exposure in the Darien region [21]. In contrast, UNAV seroprevalence was identified at 16% in this region, a marked contrast with the lower evidence of exposure observed in Latin America[22]. UNAV has predominantly been detected in mosquitoes and potential non-human primate reservoirs. [23,24]. UNAV seroprevalence in Mogue for a posterior median was 5.6% (95% CrI: 4.1–7.5). For a constant model fit the data best with an FOI estimated at 0.008 per decade (95% CrI: 0.006–0.011). No changes or increases in the incidence of UNAV associated with epidemics were observed, and infections occurred constantly throughout the analyzed period.

Little information is available regarding the epidemiology of UNAV, rendering my research a singular opportunity to advance our understanding of this alphavirus. Decades after the detection of UNAV in mosquitoes in Latin America[18,19], I provide evidence of a high seroprevalence of UNAV and endemic transmission dynamics in Mogue based on the Force of infection estimates, within the population of Darien Province. It is important to note that Mogue is a remote indigenous Emberá community in the Darien Province, and the characteristics of this community should not be generalized to other regions, even within Darien. However, the fact that our study unveils the highest seroprevalence of UNAV reported in the literature as of November 2023 highlights an urgent need for exhaustive research to

uncover the full spectrum of UNAV's transmission dynamics, host range, and potential reservoirs.

MADV, VEEV and UNAV risk factors of infection

To evaluate the risk factor of alphavirus infection I used data generated from a cross-sectional serosurvey and antibody results from population in Mogue community, Darien Province during 2017. The characterisation of risk factors associated with MADV and VEEV transmission highlighted a notable difference in their circulation dynamics; the former being substantially associated with peri-domestic environments, and the latter with human activities closer to gallery forests. Conversely, we noted a lower UNAV seroprevalence associated with the physical structure of houses, indicating a potential role of household structures in reducing viral exposure. It is important to highlight that risk factors for MADV and VEEV were exclusively assessed in the Mogue community, which exhibits an endemic transmission pattern, as indicated by the FOI analysis. Interestingly, a distinct set of risk factors for MADV and VEEV was identified, such as the presence of bushes around the house being a risk factor for MADV infection in the Mogue community. This finding contrasts with a 2012 study that identified bushes as a protective factor against MADV infection. Worth noting is that the 2012 study included Aruza, a community with an epidemic transmission pattern as indicated by the FOI analysis [25].

Alphavirus seroconversion rates and antibody dynamics

I aimed to determine whether MADV and VEEV transmissions persisted after the 2010 outbreak and to quantify their incidences rates. Additionally, I sought to estimate antibody persistence. To address these questions, I followed a cohort of 65 patients recruited during the 2010 outbreak in 2015 for my DPhil. The analysis of this data revealed ongoing MADV and

VEEV transmission, as indicated by a high rate of seroconversion to both viruses. Notably, sero-reversion (loss of antibodies) was observed in MADV. It is currently unclear whether individuals previously infected with MADV for which antibody decay was observed are at risk for reinfection. In contrast, VEEV antibodies appeared to persist for an extended period (up to five years). Intriguingly, asymmetric cross-protection and enhanced susceptibility were observed. While individuals with prior VEEV infection seemed protected against MADV infections, those with prior MADV infections appeared to have an increased risk of VEEV infection. Future studies should address the impact of antibody decay and asymmetric cross-protection and susceptibility in alphavirus dynamics and potential for re-emergence in endemic regions.

Alphavirus long-term neurological consequences

I aimed to gain a deeper understanding of the long-term consequences of MADV and VEEV following acute infection. Given the documented evidence of enduring neurological changes in survivors of West Nile virus infection[26]. I conducted follow-up studies on former cases from the 2010 outbreak in Darien, specifically investigating the neurological sequelae of MADV or VEEV survivors. This involved direct clinical evaluations of patients. Additionally, I undertook a cross-sectional survey of neurological outcomes in the Mogue community in 2017 during an outbreak response. Clinical evaluations were conducted by a physician, and signs and self-reported symptoms of neurological impairments were documented in a survey designed for this study. Subsequently, I merged both datasets and performed logistic regression analysis to assess the association of MADV and VEEV seropositivity with neurological consequences. Seizures (OR, 14.5; 95% CI, 1.6–130.1), irritability (OR, 2.7; 95% CI, 1.1–6.3), insomnia (OR, 2.5; 95% CI, 1.3–5.0), and memory loss (OR, 1.9; 95% CI, 1.1–3.3) remained

for over five year and were more common in those patients MADV or VEEV with a prior acute alphaviral infection (MADV or VEEV) compared with alphavirus negatives.

The results of neurological assessment and self-reported symptoms suggest that the impact of equine encephalitis can persist in individuals well beyond the acute phase of the infection. These findings could have implications for the endemic areas of alphaviruses in Latin America. However, the use of structured questionnaires and more comprehensive clinical evaluations, including brain tomography or magnetic resonances, is required to confirm these findings. In summary, my results suggested that the impact of alphavirus encephalitis may be considerably greater than currently understood.

MADV and VEEV molecular diagnostic

The burden of encephalitic alphaviruses in Latin America largely remains unknown, possibly due to the similarity in clinical presentation with other endemic arboviruses such as dengue. Additionally, the absence of specific tools to detect MADV and the lack of incorporation of members of the VEEV complex in primers designed for VEEV diagnostics contribute to this challenge. To address this gap, I designed a new method to detect members of the EEEV and VEEV complexes. This method utilized available complete genomes and was validated with biobanked clinical and mosquitoes samples, as well as prospective samples obtained from a febrile surveillance I developed in Darien Province. The newly developed rRT-PCR assay demonstrated highly efficient results in detecting both MADV and VEEV infections in human and mosquitoes samples, including the ability to identify coinfections. I also confirmed these results by using untargeted metagenomic strategies and reconstructed VEEV phylogenetic analysis. VEEV strains identified by the rRT-PCR in human cases and mosquitoes collected during 2015, belonged to the ID subtype, Panama/Peru genotype, previously identified in

Panama[2,13]. I anticipate that the newly developed tool could be implemented in endemic countries, providing valuable evidence of acute infections, the natural history of the disease, and the burden of encephalitic alphaviruses in Latin America.

MADV and VEEV prospective febrile surveillance

MAD and VEEV infections are typically identified when neurological diseases manifest[2,3]. However, our seroprevalence studies and household contact investigations during outbreaks suggest that the majority of MADV and VEEV infections are mild or potentially asymptomatic[14,15,21,25]. Seeking to enhance surveillance, I implemented a febrile surveillance program in nine Health Centers across Panama City and Darien Province. Utilizing the newly developed rRT-PCR test for encephalitic alphaviruses, we tested serum samples from febrile patients seeking medical care. The test aimed to detect endemic arbovirus infections such as DENV, ZIKV, and CHIKV, with additional screening for MADV and VEEV infections. We identified a 11.9% incidence of VEEV in patients clinically diagnosed with DENV infection. This finding emphasizes that the burden of alphaviruses in Panama and potentially Latin America are underestimated. Notably, all cases were mild infections, resembling DENV or malaria infections. This discovery is crucial for Latin American countries considering epidemiological link to estimate dengue incidence in cluster cases. In fact, our VEEV cases were identified during a DENV outbreak.

MADV and VEEV vertebrate host

While strong evidence suggests that rodents are the primary vertebrate host for VEEV, MADV host species remained unknow. Both viruses use *Culex* (*Mel.*) spp mosquitoes as main vector, and these mosquitoes are highly catholic on feeding on rodents[3]. Theorical, epidemiological and experimental pathology also of MADV infection also suggest rodents as potential

reservoirs for this virus[25,27,28]. To elucidate the role of rodents as potential vertebrate hosts for MADV and VEEV, I conducted an analysis using available data on rodents collected in the aftermath of the 2012 outbreak, spanning the years 2011 and 2012 in Darien and the western Panama Provinces. The results of this study presented in Chapter 4 demonstrated that rodents indeed serve as vertebrate reservoirs for both MADV and VEEV in Darien, revealing spatial variations in MADV and VEEV seropositivity among rodent species. Notably, different provinces exhibited distinct rates for MADV and VEEV. Additionally, MADV and VEEV seropositivity in rodents varied in accordance with the pasture and secondary forest environment, aligning with the spatial heterogeneity observed using catalytic FOI models presented in Chapter 1. Collectively, these findings suggest that while MADV and VEEV are endemic in the Darien Province, spatial heterogeneity persists even within the same endemic region.

MADV and VEEV mosquitoes vectors

Culex Melanocion spp. are believed to be the primary vector for both MADV and VEEV[3,5,29,30]. While ecological investigations of these mosquitoes vectors in Panama dated back to the 50-70s, none of these studies were undertaken in the endemic region of Darien[18,29,31,32]. To elucidate the contemporaneous ecology of potential mosquitoes vectors for MADV and VEEV in the Endemic Darien Province, I conducted mosquito trapping efforts during the Alphavirus outbreaks in 2015 and 2017 in Darien Province. I also implemented the use of sentinel hamsters and conducted mosquito blood meal analyses in 2018 and 2019, which is further discussed in the following section. Extensive ecological studies of *Culex* (*Mel*.) spp. undertaken by Galindo in Panama shows a predominance of larvae and adults in aquatic and sylvatic environments[18,31,32]. Our results of adult mosquitoes collections undertaken during outbreak responses were able to identify *Culex* (*Mel*.) spp. in peri-domestic areas of houses of confirmed

cases, suggesting the potential coexistence of vectors and humans. Only VEEV was detected and isolated in mosquitoes collected during the 2015 outbreak response. Although our team has conducted several mosquitoes collections since 2010 in Panama, we were not able to detect active viral circulation of MADV in mosquitoes. This low proportion of vector infectivity by MADV was also observed by Galindo and others[5,29]. Additionally, efforts should be made to identify potential bridge vectors for MADV. It's important to note that, to date, infected mosquitoes vectors have not been detected during human MADV outbreaks in Panama. Future vector ecology studies should include the search and characterization of *Culex* (*Mel*.) spp breeding sites in surrounding areas of houses to better understand potential changes in vector ecology.

Arbovirus enzootic surveillance

Arboviral emergence and spillover typically occur in remote settings with inadequate surveillance[33]. Even when surveillance systems are prepared, signal of transmission is often detected when transmission is already established in human and animal populations[34]. I aimed to pilot a One Health-based surveillance approach to rapidly detect arboviral spillover in the Darien Province. I employed traditional tools such as sentinel mosquito Trinidad traps, various vertebrate hosts, and combination of classical virology, next-generation sequencing, mosquito blood-feeding analysis, and structural analysis, I successfully implemented this surveillance strategy. This approach enabled the detection of active arbovirus circulation, helped guiding the deployment of an active febrile surveillance strategy with the Ministry of Health in Panama. These results revealed the presence of at least 15 strains of Orthobunyaviruses in the Darien Province, resulting in the identification of three potential new viruses and Madrid virus. The development of targeted multiplex Real-Time RT-PCR allowed for the detection of potential vectors, and the analysis of mosquito blood meals suggested the potential bridge vector

capacity of infected mosquitoes, as they fed on a variety of animals. There are significant ethical challenges associated with using animals as sentinel attractants for viruses, and I have carefully considered the implications. Using sentinel animals, we detected fifteen strains of orthobunyaviruses. However, only two strains of Madrid virus were detected in 599 wild rodents captured in the same region. Thus, sentinel hamsters seem to be more effective in detecting active viral circulation, and the number of animals use is considerably lower when compared to the number of wild animals required to achieve similar or higher effectiveness in detecting active circulation.

Conclusions

Collectively, the findings of my research underscore the recent emergence of MADV and VEEV in eastern Panama, shedding light on potential long-term neurological consequences, ongoing transmission post 2010 outbreak, and the dynamics of antibodies, as well as revealing insights into asymmetric cross-protection and susceptibility of MADV and VEEV. Additionally, I developed tools and prospective strategies to enhance alphavirus surveillance, enabling the detection of a high incidence of VEEV infection in patients clinically diagnosed with dengue. The results of mosquitoes vectors and rodent distribution in Darien supports the hypothesis of ecological changes in the enzootic cycle of MADV and VEEV transmission and indicates potential changes in vector and host ecology. The spatial heterogeneity observed in transmission within the Darien region further contributes to the nuanced understanding of the alphavirus landscape. The emergent patterns of MADV and VEEV in Panama seem to be influenced more by ecological changes than genetic factors, deepening our comprehension of potential additional mechanisms of alphavirus emergence. Lastly, the implementation of a One Health arboviral surveillance approach allowed for the detection of active arboviral circulation and insights into potential hosts and vectors.

Despite the wealth of information generated and analysed during this thesis is not without limitations. The predominantly cross-sectional nature of my approach may introduce bias in understanding the underlying factors influencing alphavirus transmission and susceptibility in Panama. Given the prevalence of alphavirus infections in small communities in Darien, the study's power to draw robust conclusions may be limited. It is crucial to acknowledge that future neurological evaluations should include extensive and objective assessments. Despite these limitations, my dissertation contributes significantly to the body of knowledge and provides valuable insights into alphavirus emergence in remote and rural areas of Latin America.

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A. Appendix to Chapter 1

Additional details on the 2017 sero-survey

Mogue

The community of Mogue, on the banks of the Mogue River (**Fig. 1**) in the Province of Darien, which is the home of the Parque Nacional del Darien, the largest national park in Central America and a UNESCO Biosphere Reserve. Yearly rainfall ranges between 1,800 and 4,500mm. The mean maximum temperature is 28°C during the rainy season and 31°C in the dry season 20. The main subsistence activities are fishing, corn cultivation, and tourism. The total population of Mogue is estimated to be around 300 inhabitants.

Details about recruitment

In most instances, nonparticipation was due to the inability of researchers to locate the household residents. Residents 1 year of age or older were eligible for inclusion. Each participant was interviewed using a standardized epidemiological form to record occupation, activities, livestock and crop holdings. Older adults and children were interviewed with the help of a close family member when needed. Household-level information (e.g. house structure) was observed and recorded directly by the interviewer where possible. Trained phlebotomists collected 10 ml of blood from persons 9 years of age and older (3 ml for children 1–8 years-old) by peripheral venipuncture using standard aseptic technique. The samples were processed on-site within 6 hours by centrifugation to separate serum, then stored in liquid nitrogen and transported to the GMI for laboratory testing.

Details about risk factors questionnaire

Independent variables included age, sex, main occupation, activities including breeding poultry, fishing, cutting bushes, walking/playing in paddocks or crops, working in agriculture, paddocks, grain deposits, sawmills/forest, chicken coops, and pigsties, washing in rivers and bathing. Self-reported symptoms were recorded and included weakness, fatigue, difficulty focusing, memory loss, confusion, dizziness, seizures, fatigue, paralysis, difficult walking, headache, insomnia, depression, irritability, difficulty cooking, difficulty cleaning, difficulty working, fever, chills, vomit, and diarrhea. Other variables related to the house features were floor, wall, roof and window materials, types of crops grown, shrubs surrounding the home, waste management, and water supply.

Additional details on the 2012 sero-survey

For the 2012 survey sites were selected accordingly with previous report of human or equine encephalitis cases due to VEEV of MADV. This survey included the following areas seven areas:

Pijibasal
The town of Pijibasal is located within the district of Pinogana, 12 km South East of El Real de Santa María. It is surrounded by fields and the Pirre River on the western margin. This town has a grid arrangement, delimited by a concrete path that connects the houses and the school in the periphery. The houses are elevated two meters above the ground, using the ground below them as a deposit for storage chicken coops. A total of 63 inhabitants of the Embera ethnic group make up this town, which is linked by means of a dirt road, the landscape is made up of paddocks and stubble on both banks, it is divided by 4 streams (only one bridge of cement is available in the first) without prominent elevations in the field. The ecosystem between El Real and Pijivasal is similar along the road.

Pirre 2
Located on the road that connects El Real and Pijivasal. The village is composed of 21 inhabitants of countryside origin, dedicated to agriculture and livestock. Their homes are built with wood, palms and zinc. Some people work cutting wood in the forest and in paddocks with native and improved pastures, plus rice, corn, yucca and plantain crops for family subsistence.

Pirre 1
On the side of the road is this town with 56 inhabitants of countryside origin. Like Pirre 2, paddocks and stubble are observed on both banks. In some houses, they raise pigs and chickens in small pens, as well as large paddocks in the periphery, although with a small number of livestock.

Mercadeo
Mecadeo is located on the banks of the Tuira River, its population is 132 inhabitants of the Embera ethnic group, its houses are grouped along a path and built with wood and stalks, raised on the ground about two meters. In the surroundings of the town, we find stubble and subsistence crops of rice, corn, plantain and yucca.

El Real

The inhabitants are of Negroid, Embera and Latin origin. We can find houses built with different styles and materials (wood, blocks, zinc, etc.). Its population according to the 2010 census is made up of 555 inhabitants. They work at fishing, trade and other informal activities. Access is by boat through the Chucunaque River, the Tuira River and the Pirre River. The surroundings of the town are made up of large areas of paddocks, composed of native grasses and improved for the breeding of cattle. In these grazing areas there are scattered timber and fruit trees. On the edges of the rivers and streams the gallery forests have native trees that do not exceed 30 meters in height.

Tamarindo

This community is located about 12 km from the village of Santa Fe, District of Chepigana, province of Darién. Most of its inhabitants come from the central provinces specifically Herrera and the South of Veraguas. There are 203 inhabitants according to the 2012 census. Many are engaged in agriculture as the main source of income; the livestock area is minimal.

There are paddocks with native and improved grass, maize and subsistence rice crops, as well as small plots of yucca and fruit trees. There is also some scattered stubble with trees that do not exceed 15 meters in height. There are also small patches of forest on the slopes of the hills that surround the town and gallery forest in the rivers and streams.

Aruza

Aruza is a community located in the township of Rio Iglesias, district Chepigana close to Metetí and located within three protected areas by law 1) Filo del Tallo Hydrological Reserve, 2) Canglón Forest Reserve and 3) Matusagaratí Lagoon. The population is about 154 inhabitants. The Aruza area is surrounded by paddocks with native grass in the flat areas, corn crops and subsistence rice in the garden of houses, in addition, plots less than one hectare of yucca and banana. There is a small amount of stubble with trees that do not exceed 15 meters in height. Part of the Laguna de Matusagaratí wetland has been invaded and converted into paddocks and rice and oil palm cultivation areas. The secondary forests surrounding the town are located in the highlands and the lowlands are flooded in the rainy season.

Additional details on the Force of Infection (FOI) Analysis

Models were fitted on a Bayesian framework using Stan's No-U-Turn Sampler [1] with four Markov chains and 20,000 iterations on each and with 50% of these iterations discarded as "warm-up".

Prior distributions
Prior distribution for constant FOI model

The prior distribution for the FOI estimate in the constant model is based on a uniform distribution

$$FOI \sim uniform(0,2)$$

Prior distributions for time-varying FOI model

The prior distribution for FOI estimate in the time-varying FOI model follows a $student\ t$ distribution informed by the FOI estimate from the previous decade:

$$FOI \sim student\ t\ (\nu, FOI_{previous\ decade}, \sigma)$$
$$\sigma \sim Cauchy\ (0,1)$$
$$\nu \sim Cauchy\ (0,1)$$
$$FOI_{first\ decade} \sim normal\ (0,1)$$

Convergence and Posterior Predictive Checks

Convergence was assessed by the use of R^ statistic, which measures the "within chain" variability (W) and compares to the "between chains" variability (B):

$$\hat{R} = \sqrt{\frac{W + \frac{1}{n}(B - W)W}{W}}$$

This method assumes that the chains have been simulated in parallel, each with different starting points, which are overdispersed with respect to the target distribution.

If this metric is large, this suggests that either estimate of the variance can be further decreased by more simulations[2].

It is expected that in convergence, B → W and thus R^ → 1.

A value of $\hat{R} < 1.1$ was considered enough to achieve convergence. We show the convergence plots for the two instances where a time-varying FOI model fit the data the best (See Figures S2 and S3).

We also performed posterior predictive checks and examined the residuals (See Figures S4 – S6).

Model comparison

To assess predictive performance of the different models we used a method which uses Pareto-smoothed Importance sampling to approximate the leave-one-out cross-validation estimate of the expected log predictive density for an out-of-sample data point (elpd), as used from the *loo* stan package[2]. We then compared the elpd from both models (constant vs time-varying) to obtain the $\mathrm{elpd}_{\mathrm{diff}}$. To determine whether this difference is significant we calculated the *z* score and compared it with the corresponding value from a standard normal distribution as recommended by Lambert [3].

$$Pr\ = \left(z \geq \frac{\mathrm{elpd}_{\mathrm{diff}}}{\mathrm{se}}\right)$$

Supplementary Figures, Mogue Coomunity; Darien Province.

Figure S1. Photographs of the study sites.

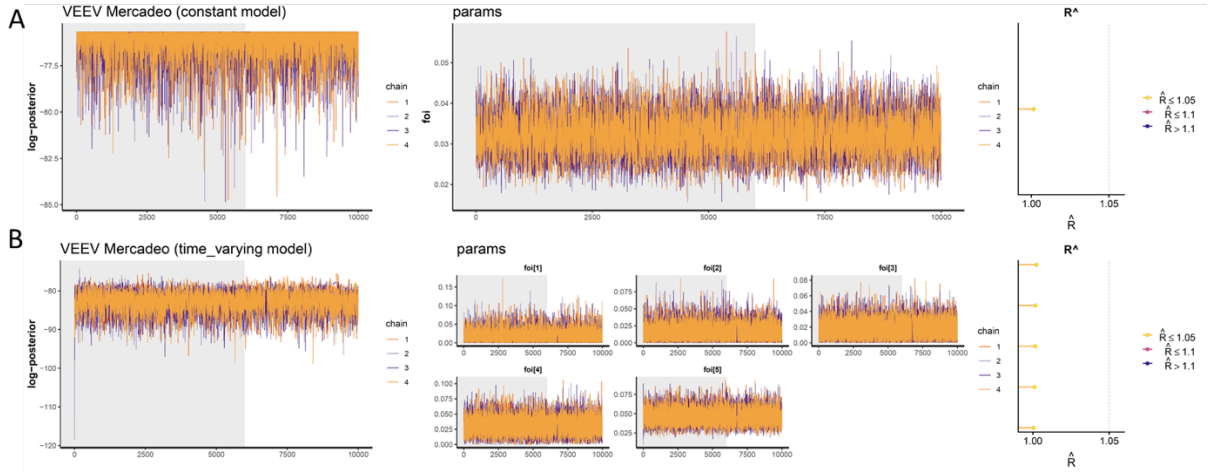

Figure S2. Convergence plots for VEEV FOI models in Mercadeo. *Convergence plots for the FOI models for VEEV in Mercadeo, presenting the log posterior, posterior distribution of the parameters and Rˆ values, for A) the constant FOI model and B) the time-varying FOI model.*

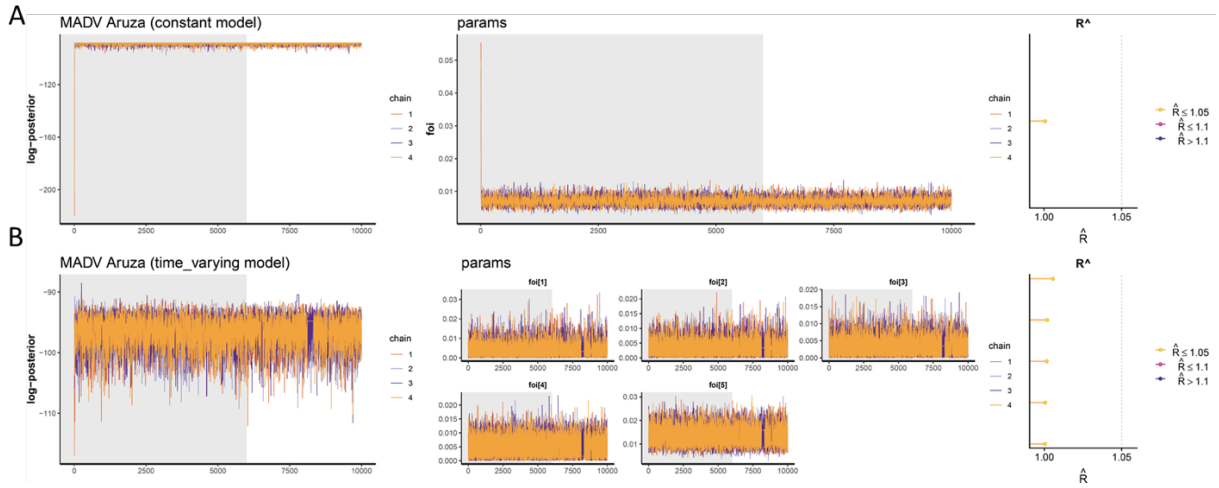

Figure S3. Convergence plots for MADV FOI models in Aruza. *Convergence plots for the FOI models for MAVD in Aruza, presenting the log posterior, posterior distribution of the parameters and Rˆ values, for A) the constant FOI model and B) the time-varying FOI model.*

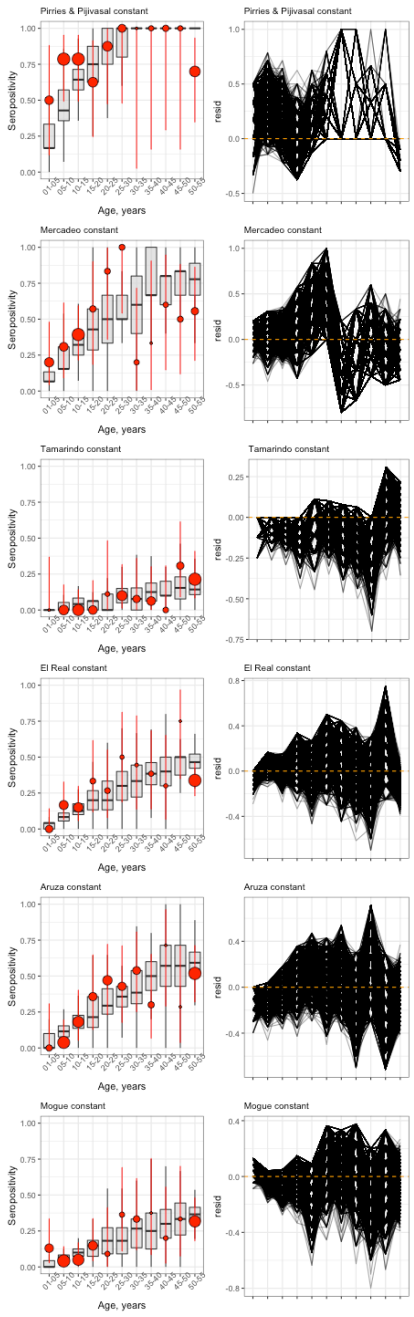

Figure S4. Posterior predictive checks and model residuals for VEEV for constant FOI models. *The left panels show the observed sero-prevalence data (red points represent mean and lines its 95% confidence intervals) and the model fitting (grey boxplots) The right panels show residuals of the model. Each row represents a location.*

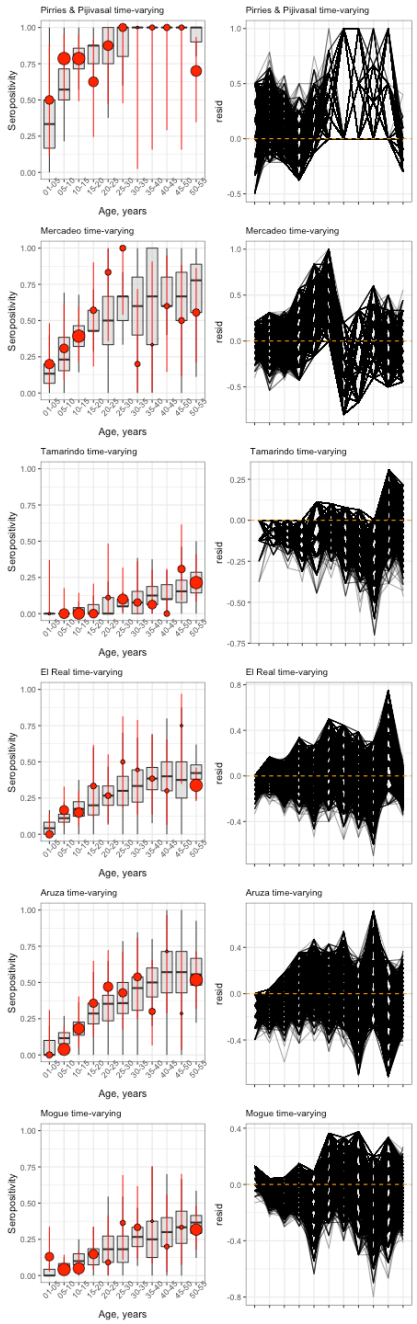

Figure S5. Posterior predictive checks and model residuals for VEEV for time-varying FOI models. *The left panels show the observed sero-prevalence data (red points represent mean and lines its 95% confidence intervals) and the model fitting (grey boxplots) The right panels show residuals of the model. Each row represents a location.*

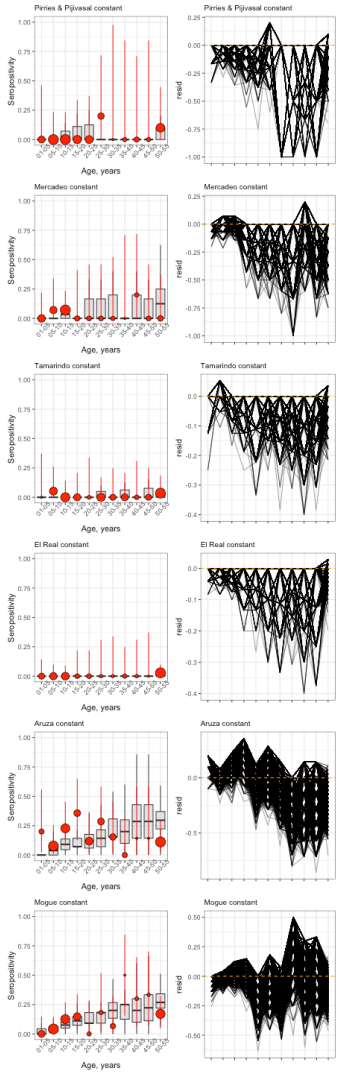

Figure S6. Posterior predictive checks and model residuals for MADV constant FOI models. *The left panels show the observed sero-prevalence data (red points represent mean and lines its 95% confidence intervals) and the model fitting (grey boxplots) The right panels show residuals of the model. Each row represents a location.*

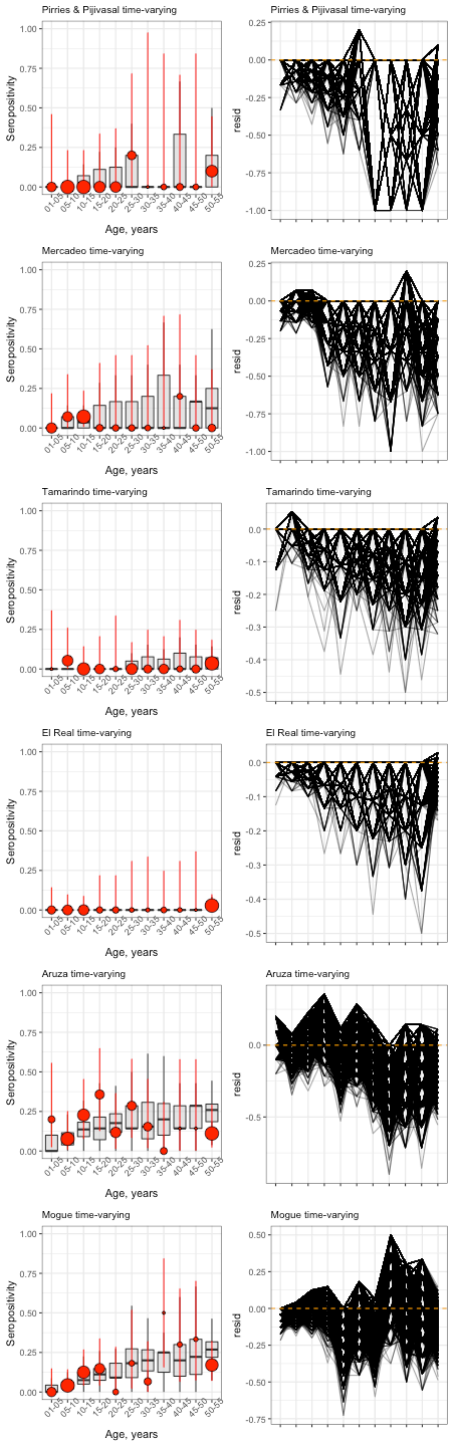

Figure S7. Posterior predictive checks and model residuals for MADV time-varying FOI models. *The left panels show the observed sero-prevalence data (red points represent mean and lines its 95% confidence intervals) and the model fitting (grey boxplots) The right panels show residuals of the model. Each row represents a location.*

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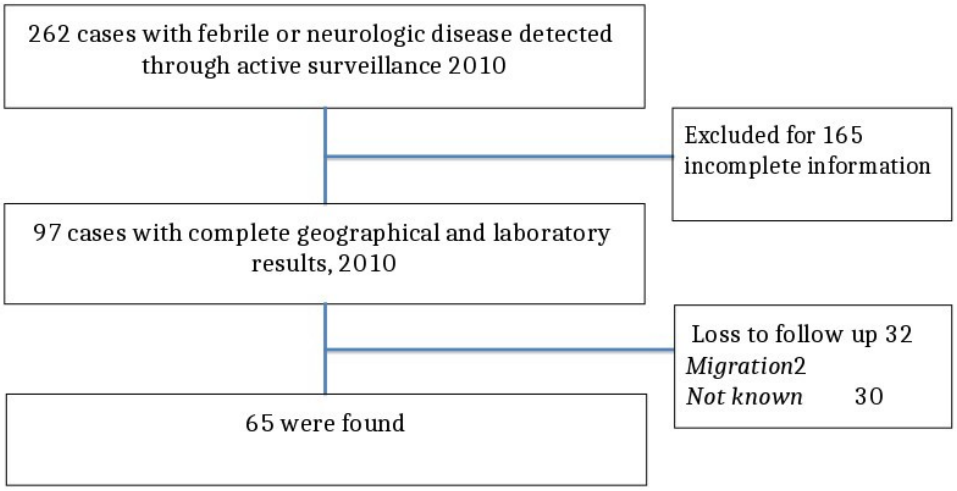
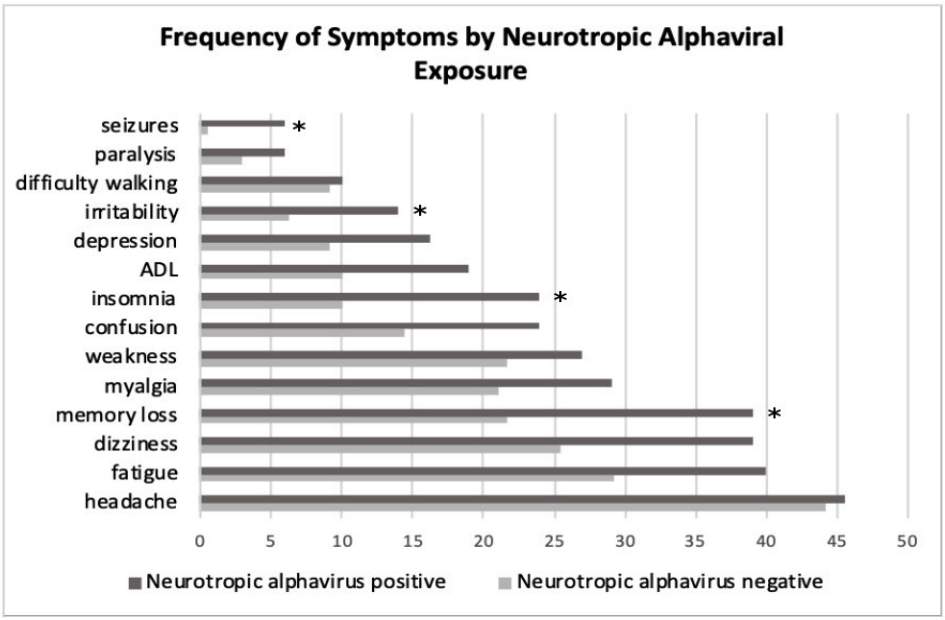
B.Appendix to Chapter 2



Supplementary Figure 1. Epidemiological flowchart and serocoverstion in the 2010 oiginal outbreak cohort.



Supplementary Figure 2. Frequency of self-reported signs and symptoms amongst the 2010 outbreak cohort studied in 2015 and the 2017 Mogue study population, combined (n=308). *Significant differences in rates ($p<0.05$), controlled for age and sex, are denoted with (*).*

C. Appendix to Chapter 3

Real-time RT-PCR for Venezuelan equine encephalitis complex, Madariaga and Eastern equine encephalitis viruses: application in human and mosquito public health surveillance

rRT-PCR in silico validation

In silico primer/probe specificity was checked by aligning sequences in BLAST (blast.ncbi.nlm.nih.gov) against (i) all available sequences and (ii) only alphavirus sequences while excluding the VEEV complex or MADV, respectively. Due to the similarity between MADV primers and EEEV sequences, all available EEEV complete genome sequences (n=441) were aligned and separate MADV and EEEV probes were designed for an rRT-PCR duplex assay. Alignments for each virus were repeated with all sequences available in September 2021 to confirm primer and probe sequences in contemporary strains.

rRT-PCR optimization

Primers were obtained from Integrated DNA Technologies (IDT, Coralville, Iowa); probes were obtained from Biosearch Technologies (Hoddesdon, United Kingdom). VEEV subtype IC and EEEV genomic RNAs were purchased from Vircell Microbiologists (Granada, Spain). Quantified Ultramer ssDNA containing the assay target region was obtained for all viruses (IDT, Coralville, Iowa) to evaluate assay performance. For ssDNA synthesis, target region sequences were selected from specific strains of VEE subtype IAB (Accession number KC344505.2) and subtype IV (Pixuna virus, Accession number NC_038673.1), MADV (Accession numbers MH359233.1 and KJ469626.1), and EEEV (Accession number KX029319.1).

rRT-PCR reaction and cycling conditions

rRT-PCRs were performed in 25 µL reactions using the SuperScript III Platinum One-Step Quantitative RT-PCR Kit (Thermo Fisher, Waltham, MA) with 5 µL of the nucleic acid template. The analytical evaluation was performed on a Rotor-Gene Q instrument (Qiagen, Germantown, MD), and the validation with serum and mosquitoes pool samples was performed on an ABI7500 (Thermo Fisher). Cycling conditions were consistent with

previous laboratory protocols: 52 °C × 15 min, 94 °C × 2 min, and 45 cycles of 94 °C × 15 s, 55 °C × 40 s (acquired in all channels), and 68 °C × 20 s (1-4).

rRT-PCR thresholds were set based on testing with the final reaction mixtures, as described previously(23, 24). The dynamic range of each assay was determined by testing synthesized targets from each reference strain in quadruplicate at 8.0, 6.0, 4.0, 2.0, and 1.0 $\log_{10}$ copies/µL. For the VEEV complex, the lower limit 95% detection (95% LLOD) was estimated by testing 10 replicates of 2-fold serial dilutions from 200 to 25 copies/µl. For MADV and EEEV, 95% LLOD was estimated from results of replicate testing in the dynamic range study. Probit analyses were performed using MedCalc, v20.013 (MedCalc Software, Belgium) to estimate LLOD.

rRT-PCR Assay Specificity

Specificity was evaluated by testing 56 serum samples from locations without known transmission of VEEV or MADV. These included 8 samples collected from patients in Georgia, USA, without known travel history, and 48 samples from individuals with an acute febrile illness in Asunción, Paraguay. The latter samples have been described in detail elsewhere (25). Total nucleic acids were extracted from 200µL of serum on an EMAG instrument (BioMérieux, Durham, NC), eluted in 50 µL and tested with the VEEV complex and MADV/EEEV rRT-PCRs.

rRT-PCR Assay exclusivity

Assay exclusivity was evaluated by testing genomic RNA from the following viruses (strain in parentheses, if designated): Rift Valley fever (h85/09); Zika (ZIJV; MR766); dengue virus serotype 1 (DENV1, Hawaii 1944), DENV2 (NGC), DENV3 (Sleman/78), and DENV4 (H241); chikungunya virus (CHIKVR80422); Mayaro virus (MAYV; ARV 0565, INHRR 11a-10); yellow fever virus (YFV; 17D and Asibi strains); West Nile virus (WNV; NAL); St. Louis encephalitis virus (SLEV; GML 902612, CorAn 9275); tick-borne encephalitis virus (TBEV; Japanese encephalitis virus (JEV); Semliki Forest virus (SFV); Ross River virus (RRV); Getah virus (GETV); Barmah Forest virus (BFV); and Una virus (UNAV) (5).

Outbreak case definition

The definition of a suspected case included fever and headache, while a probable case was defined as a suspected case plus somnolence, lethargy, or convulsions. Blood samples were centrifuged in the field, and serum was stored in liquid nitrogen for transportation to the Gorgas Memorial Institute of Health Studies in Panama City.

Inclusion criteria for Prospective surveillance

Cases, without malaria, human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis virus (HCV), and >5 and <75 years old, presenting with no more than 7 days with rash, and at least one of the following symptoms: fever, myalgia, arthralgia, periarticular edema, and conjunctivitis were recruited, evaluated and interviewed, to obtain clinical, and demographics characteristics and ethic consent at each health center.

Alphavirus serology of 2015 clinical samples

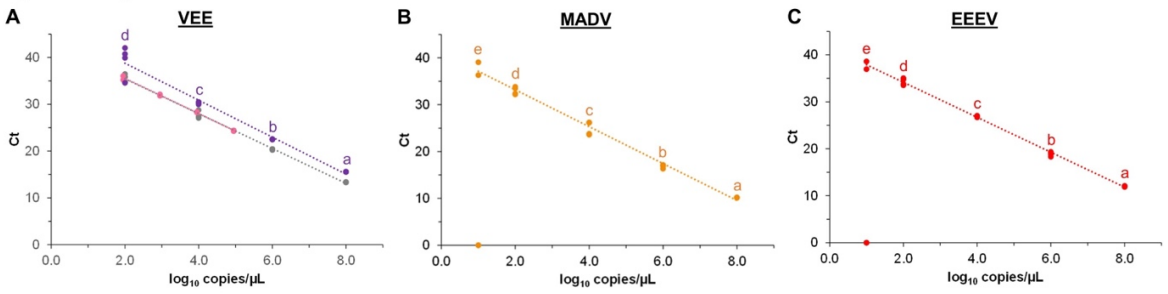
All human serum samples were tested in duplicate for IgM antibodies to MADV and VEEV antigen using an enzyme-linked immunosorbent assay (ELISA) and confirmed by a plaque-reduction neutralization test (PRNT). For the ELISA, sucrose-acetone antigens were prepared from MADV- (prepared by Dr. Robert Shope at the Yale Arbovirus Research Unit in August 1989) and VEEV- (strain TC-83) infected mouse brain. For the PRNT, we used chimeric Sindbis virus SINV/MADV (derived from Brazilian MADV strain BeAn436087 and shown to be an accurate surrogate for MADV in these assays (29) and TC83, an attenuated vaccine strain of VEEV closely related to subtype ID strains that circulate in Panama (7). The neutralizing antibody titer was determined as the reciprocal of the highest dilution that reduced plaque count by 80% ($PRNT_{80}$).

Metagenomic sequencing

Viral RNA was treated to remove residual DNA with TURBO DNase (Thermo Fisher Scientific, USA) and concentrated with Zymo RNA clean & concentrator-5 (Zymo Research, USA) following the protocol instructions. cDNA synthesis and PCR was performed as described previously(1). PCR products were then purified using AMPure XP beads (Beckman Coulter, UK) and quantified according to manufacturer's instructions with Qubit

dsDNA High Sensitivity assay (Life Technologies, USA) and Qubit 3.0 instrument (Life Technologies, USA).

cDNAs were pooled using the EXP-NBD104 (1–12) and EXP-NBD114 (13–24) Native Barcoding Kits (ONT, UK). Sequencing libraries were generated using the SQK-LSK109 Kit (ONT, UK). 50 ng of the final libraries were loaded onto FLO-MIN106 flow cells on the MinION device (ONT, UK) and sequenced using MinKNOW with the standard 48-hour run script. FASTQ files were demultiplexed and trimmed using Gruppy V5.0.16. (Oxford Nanopore, Oxford, United Kingdom), and then aligned and mapped to the reference genome (GenBank accession no. NC_001449.1) using minimap2 version 2.28.0 (2)and converted to a sorted BAM file using SaMtools 3 (http://www.htslib.org). NanoStat version1.1.24 (https://pypi.org/project/NanoStat/) was used to compute the number of raw reads and minimum contig length to cover 50 percent of the genome (N50) of the aligned reads. Genome visualization was undertaken with Tablet 1.19.05.28 (3), and to compute the number of mapped reads, percentage of genome coverage, and coverage depth. Variants were detected with medaka_variants and the consensus sequence were built using margin_medaka_consensus (Oxford Nanopore, Oxford), United Kingdom. Genome regions with <20x coverage were masked.



Supplementary Figure 1. Dynamic range of assays for VEE complex (A), MADV (B) and EEEV (C). *The dynamic range for each assay was established by testing ssDNA in quadruplicate at 8.0, 6.0, 4.0, 2.0 and 1.0 log_{10} copies/µL (labelled a-e, respectively).*

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D. Appendix to Chapter 4

Ecological features of encephalitic alphavirus in Panama

Supplementary Table 1. Absolute and relative rodent abundance by year and collection site.

Species	Years‡		Collection sites†				
	2011	**2012**	**El Real**	**Los Pavitos**	**Santa Librada**	**El Cacao**	**Cirí Grande**
	n/N (%)	**n/N (%)**	**n/N (%)**	**n/N (%)**	**n/N (%)**	**n/N (%)**	**n/N (%)**
Didelphis marsupialis	1/430 (0.2)	0/169 (0.0)	0/202 (0.0)	0/165 (0.0)	0/158 (0.0)	0/56 (0.0)	1/18 (5.6)
Liomys adspersus	4/430 (0.9)	0/169 (0.0)	0/202 (0.0)	0/165 (0.0)	0/158 (0.0)	4/56 (7.1)	0/18 (0.0)
Marmosa robinsoni	1/430 (0.23)	0/169 (0.0)	1/202 (0.5)	0/165 (0.0)	0/158 (0.0)	0/56 (0.0)	0/18 (0.0)
Marmosa sp.	1/430 (0.2)	0/169 (0.0)	0/202 (0.0)	0/165 (0.0)	0/158 (0.0)	0/56 (0.0)	1/18 (5.6)
Melanomys caliginosus	18/430 (4.2)	3/169 (1.8)	21/202 (10.4)	0/165 (0.0)	0/158 (0.0)	0/56 (0.0)	0/18 (0.0)
Mus musculus	0/430 (0.0)	13/169 (7.7)	9/202 (4.5)	4/165 (2.4)	0/158 (0.0)	0/56 (0.0)	0/18 (0.0)
Handleyomys alfaroi	6/430 (1.4)	5/169 (3.0)	6/202 (3.0)	1/165 (0.6)	4/158 (2.5)	0/56 (0.0)	0/18 (0.0)
Transandinomys bolivaris	6/430 (1.4)	3/169 (1.8)	5/202 (2.5)	1/165 (0.6)	3/158 (1.9)	0/56 (0.0)	0/18 (0.0)
Oryzomys sp.	16/430 (3.7)	0/169 (0.0)	6/202 (3.0)	10/165 (6.1)	0/158 (0.0)	0/56 (0.0)	0/18 (0.0)
Ototylomys phyllotis	0/430 (0.0)	2/169 (1.2)	2/202 (1.0)	0/165 (0.0)	0/158 (0.0)	0/56 (0.0)	0/18 (0.0)
Proechimys semispinosus	53/430 (12.3)	20/169 (11.8)	19/202 (9.4)	3/165 (1.8)	1/158 (0.6)	37/56 (66.1)	13/18 (72.2)
Rattus rattus	9/430 (2.1)	5/169 (3.0)	8/202 (4.0)	2/165 (1.2)	0/158 (0.0)	3/56 (5.4)	1/18 (5.6)
Rattus sp.	2/430 (0.5)	0/169 (0.0)	1/202 (0.5)	0/165 (0.0)	1/158 (0.6)	0/56 (0.0)	0/18 (0.0)
Sigmodon hirsutus	5/430 (1.2)	3/169 (1.8)	0/202 (0.0)	0/165 (0.0)	0/158 (0.0)	8/56 (14.3)	0/18 (0.0)
Sigmodon sp.	1/430 (0.2)	0/169 (0.0)	0/202 (0.0)	0/165 (0.0)	0/158 (0.0)	1/56 (1.8)	0/18 (0.0)
Zygodontomys brevicauda	307/430 (71.4)	115/169 (68.1)	124/202 (61.4)	144/165 (87.3)	149/158 (94.3)	3/56 (5.4)	2/18 (11.1)

‡Rodent abundance total by year: 2011 n=430 and 2012 n=169; abundance total n=599

†Rodent abundance total by collection site: El Real n=202, Los Pavitos n=165, Santa Librada n=158, El Cacao n=56 and Cirí Grande n=18

Supplementary Table 2. Diversity indexes.

Index	Collection sites				
	El Real	**Los Pavitos 2**	**Santa Librada 3**	**El Cacao**	**Cirí Grande 1**
Simpson_1-D	0.60	0.23	0.11	0.53	0.46
Shannon_H	1.42	0.57	0.29	1.13	0.96
Margalef_M	1.88	1.18	0.79	1.24	1.38

Supplementary Table 3. Similarity index.

Jaccard index	Collection sites				
	El Real	**Los Pavitos**	**Santa Librada**	**El Cacao**	**Cirí Grande**
El Real	100%				
Los Pavitos	78%	100%			
Santa Librada	56%	50%	100%		
El Cacao	27%	33%	25%	100%	
Cirí Grande	33%	43%	33%	60%	100%

Supplementary Table 4. Similarity index.

Sorensen-Dice index	Collection sites				
	El Real	**Los Pavitos**	**Santa Librada**	**El Cacao**	**Cirí Grande**
El Real	100%				
Los Pavitos	88%	100%			
Santa Librada	71%	67%	100%		
El Cacao	43%	50%	40%	100%	
Cirí Grande	50%	60%	50%	75%	100%

Supplementary Table 5. Seroprevalences by sites and year.

Sites	MADV				VEEV			
	2011†		2012*		2011††		2012**	
	n/N (%)	95% IC	n/N (%)	95% IC	n/N (%)	95% IC	n/N (%)	95% IC
El Real	3/72 (4.2)	0.01 - 0.12	0/22 (0.0)	0.00 - 0.15	17/72 (23.6)	0.14 - 0.35	2/22 (9.1)	0.01 - 0.29
Los Pavitos 8.9	6/60 (10.0)	0.04 - 0.21	1/18 (5.6)	0.00 - 0.27	2/63 (3.2)	0.00 - 0.11	0/18 (0.0)	0.00 - 0.19
Santa Librada	0/9 (0.0)	0.00 - 0.34	1/39 (5.6)	0.00 - 0.13	0/9 (0.0)	0.00 - 0.34	1/40 (2.5)	0.00 - 0.13
El Cacao	0/41 (0.0)	0.00 - 0.86	0/14 (0.0)	0.00 - 0.23	13/41 (31.7)	0.18 - 0.48	2/14 (14.3)	0.18 - 0.43
Cirí Grande	0/12 (0.0)	0.00 - 0.26	0/5 (0.0)	0.00 - 0.52	0/12 (0.0)	0.00 - 0.26	0/5 (0.0)	0.00 - 0.52

†Seroprevalence total of MADV by 2011: *n=9/194; 4.6%, 95% IC (0.02 to 0.09)*
****Seroprevalence total of MADV by 2012:*** *n=2/98; 2.0 %, 95% IC (0.00 to 0.07)*
††Seroprevalence total of VEEV by 2011: *n=32/197; 16.2%, 95% IC (0.11 to 0.22)*
*****Seroprevalence total of VEEV by 2012:*** *n=5/99; 5.1%, 95% IC (0.02 to 0.11)*

Supplementary Table 6. Seroprevalence by sites.

Sites	MADV		VEEV	
	n/N (%)	95% IC	n/N (%)	95% IC
El Real	**3/94 (3.2)**	**0.00 - 0.09**	**19/94 (20.2)**	**0.13 - 0.30**
Los Pavitos	**7/78 (9.0)**	**0.04 - 0.18**	**2/81 (2.4)**	**0.00 - 0.09**
Santa Librada	**1/48 (2.1)**	**0.00 - 0.11**	**1/49 (2.0)**	**0.00 - 0.11**
El Cacao	**0/55 (0.0)**	**0.00 - 0.06**	**15/55 (27.3)**	**0.16 - 0.41**
Cirí Grande	**0/17 (0.0)**	**0.00 - 0.20**	**0/17 (0)**	**0.00 - 0.20**

Supplementary Table 7. Seroprevalences of MADV by species and year.

Species	MADV					
	Total†		2011*		2012**	
	n/N (%)	95% IC	n/N (%)	95% IC	n/N (%)	95% IC
Didelphis marsupialis	0/1 (0.0)	0.00 - 0.98	0/1 (0.0)	0.00 - 0.98	0/0 (0.0)	0.00 - 1.00
Liomys adspersus	0/4 (0.0)	0.00 - 0.60	0/4 (0.0)	0.00 - 0.60	0/0 (0.0)	0.00 - 1.00
Marmosa sp.	0/1 (0.0)	0.00 - 0.98	0/1 (0.0)	0.00 - 0.98	0/0 (0.0)	0.00 - 1.00
Melanomys caliginosus	1/14 (7.1)	0.00 - 0.34	1/14 (7.1)	0.00 - 0.34	0/0 (0.0)	0.00 - 1.00
Mus musculus	0/7 (0.0)	0.00 - 0.41	0/0 (0.0)	0.00 - 1.00	0/7 (0.0)	0.00 - 0.41
Handleyomys alfaroi	1/7 (14.3)	0.00 - 0.58	0/3 (0.0)	0.00 - 0.71	1/4 (25.0)	0.00 - 0.81
Transandinomys bolivaris	1/5 (2.0)	0.00 - 0.72	1/4 (25.0)	0.00 - 0.81	0/1 (0.0)	0.00 - 0.98
Oryzomys sp.	3/13 (23.1)	0.05 - 0.54	3/13 (23.1)	0.05 - 0.54	0/0 (0.0)	0.00 - 1.00
Proechimys semispinosus	0/66 (0.0)	0.00 - 0.05	0/50 (0.0)	0.00 - 0.06	0/16 (0.0)	0.00 - 0.21
Rattus rattus	0/9 (0.0)	0.00 - 0.34	0/5 (0.0)	0.00 - 0.52	0/4 (0.0)	0.00 - 0.60
Rattus sp.	0/2 (0.0)	0.00 - 0.84	0/2 (0.0)	0.00 - 0.84	0/0 (0.0)	0.00 - 1.00
Sigmodon hirsutus	0/7 (0.0)	0.00 - 0.41	0/4 (0.0)	0.00 - 0.60	0/3 (0.0)	0.00 - 0.71
Sigmodon sp.	0/1 (0.0)	0.00 - 0.98	0/1 (0.0)	0.00 - 0.98	0/0 (0.0)	0.00 - 0.98
Zygodontomys brevicauda	5/155 (3.2)	0.01 - 0.07	4/92 (4.4)	0.01 - 0.11	1/63 (1.6)	0.00 - 0.09

†Seroprevalence of MADV 11/292 (3.8%)
**Seroprevalence of 2011 n= 9/194 (4.6%)*
***Seroprevalence of 2012 n=2/98 (2.0%)*

Supplementary Table 8. Seroprevalences of VEEV by species and year.

Species	VEEV					
	Total†		2011*		2012**	
	n/N (%)	95% IC	n/N (%)	95% IC	n/N (%)	95% IC
Didelphis marsupialis	0/1 (0.0)	0.00 – 0.98	0/1 (0.0)	0.00 – 0.98	0/0 (0.0)	0.00 – 1.00
Liomys adspersus	0/4 (0.0)	0.00 – 0.60	0/4 (0.0)	0.00 – 0.60	0/0 (0.0)	0.00 – 1.00
Marmosa sp.	0/1 (0.0)	0.00 – 0.98	0/1 (0.0)	0.00 – 0.98	0/0 (0.0)	0.00 – 1.00
Melanomys caliginosus	0/14 (0.0)	0.00 – 0.23	0/14 (0.0)	0.00 – 0.23	0/0 (0.0)	0.00 – 1.00
Mus musculus	0/7 (0.0)	0.00 – 0.41	0/0 (0.0)	0.00 – 1.00	0/7 (0.0)	0.00 – 0.41
Handleyomys alfaroi	1/7 (14.3)	0.00 – 0.58	1/3 (33.3)	0.00 – 0.91	0/4 (0.0)	0.00 – 0.60
Transandinomys bolivaris	4/5 (80.0)	0.28 – 0.99	4/4 (100.0)	0.40 – 1.00	0/1 (0.0)	0.00 – 0.98
Oryzomys sp.	1/13 (7.7)	0.00 – 0.36	1/13 (7.7)	0.00 – 0.36	0/0 (0.0)	0.00 – 1.00
Proechimys semispinosus	18/66 (27.3)	0.17 – 0.40	16/50 (32.0)	0.20 – 0.47	2/16 (12.5)	0.02 – 0.39
Rattus rattus	0/9 (0.0)	0.00 – 0.34	0/5 (0.0)	0.00 – 0.52	0/4 (0.0)	0.00 – 0.60
Rattus sp.	0/2 (0.0)	0.00 – 0.84	0/2 (0.0)	0.00 – 0.84	0/0 (0.0)	0.00 – 1.00
Sigmodon hirsutus	0/7 (0.0)	0.00 – 0.41	0/4 (0.0)	0.00 – 0.60	0/3 (0.0)	0.00 – 0.71
Sigmodon sp.	1/1 (100.0)	0.03 – 1.00	1/1 (100.0)	0.03 – 1.00	0/0 (0.0)	0.00 – 1.00
Zygodontomys brevicauda	12/159 (7.6)	0.04 – 0.13	9/95 (9.5)	0.04 – 0.17	3/64 (4.7)	0.00 - 0.13

†Seroprevalence 37/296 (12.5%)
**Seroprevalence of 2011 n=9/194 (4.6%)*
***Seroprevalence of 2012 n=2/98 (2.0%)*

F. Appendix to Chapter 5

Mosquito relative trapping effort in Trinidad traps. Mice: 3 traps x 3 days = 9 trap days in June 2017. In June 2018, hamsters: 5 traps x 5 days= 25 traps/day, Chickens- 5 traps x 5 days = 25 traps/days were baited, and 2 traps x 5 days were baited toads = 10 trap/days. In October 2019, hamsters 10 traps x 6 days = 60 trap/days

Rodent trapping strategy in 2011-2012 in Eastern and Western Panama. Rodent trapping effort in 2011 and 2012 was described before in Chapter 4. In summary, an extensive initiative to undertint alphavirus epidemiology and ecology was launched to capture rodents in the regions of Darien and Western Panama province. Within Darien province, three specific locations, namely Los Pavitos, El Real de Santa Maria, and Santa Librada, were strategically chosen for rodent sampling. Simultaneously, in Western Panama province, two distinct sites, El Cacao and Cirí Grande, were identified for similar endeavors.

Rodent trapping took place during the periods from June to November 2011 and March to April 2012, utilizing Sherman traps baited with a mixture consisting of rice, corn, sorghum, and peanut butter. In the field, traps were strategically deployed at 6:00 PM and subsequently assessed shortly after 6:00 AM. For the purposes of this study, a total of 100 Sherman traps were meticulously positioned along three linear transects of approximately 125 meters each, continuously over three consecutive nights at each designated location. These traps were strategically placed within households and the peri-domiciliary surroundings of previously confirmed cases of Venezuelan equine encephalitis virus (VEEV), with the peri-domiciliary environment encompassing grasslands, crop fields, as well as wooded areas proximate to residences within each of the selected study locations.

Subsequent to capture, the animals were euthanized using halothane, followed by meticulous identification through the application of taxonomic keys. Blood samples were drawn from the retro-orbital sinus, and critical organs such as the heart, liver, spleen, lung, and kidney were carefully excised for further analysis. Immediate preservation in liquid nitrogen ensured the preservation of sample integrity during transportation to the Gorgas Memorial Institute

(GMI) for comprehensive testing. Deceased animal specimens were respectfully housed within the Vertebrate Museum of the University of Panama and the Zoological Collection of the GMI, both located in Panama City, Republic of Panama.

rRT-PCR assay performance and optimization. All rRT-PCR reactions were performed on a Rotor-Gene Q instrument (Qiagen, Germantown, MD) using 20µL reactions of the Luna Probe One-Step RT-qPCR Kit (No ROX; New England Biolabs, Ipswich, MA) and 5µL of genomic RNA from type strains. Cycling conditions for the singleplex and monoplex reactions were the following: 52°C x 15min, 94°C x 2min, and 45 cycles of 94°C x 15sec, 55°C x 40sec (acquisition), and 68°C x 20sec.

Analytical evaluation. The linear range of the multiplex assay was evaluated with synthesized, quantified ssDNA (Integrate DNA Technologies, Coralville, IA) and reference RNA. RNA in the eluates was quantified with ssDNA and then a dilution series was tested to establish linearity with RNA and the 95% lower limit of detection (LLOD). Four concentrations (8.0, 6.0, 4.0, and 2.0 $\log_{10}$ copies/µL) were run in quadruplicate on a single run of the assay (**Supplementary Table 4**). Linear regression of Ct values versus concentration was performed in Excel, and the assay's linear range was defined as the range of concentration over which $R^2 \geq 0.99$.

Assay exclusivity was evaluated with clinical samples collected from a dengue outbreak in Paraguay in 2019-2020. All samples had previously tested negative for Zika, chikungunya, and dengue viruses. Assay exclusivity was evaluated using genomic RNA from a set of flaviviruses, bunyaviruses, and alphaviruses (**Supplementary Table 4**).

Orthobunyavirus rRT-PCR analytical evaluation. Using the new derived Orthobunyavirus whole genomes primers and probes were designed and rRT-PCRs were optimized. The linear range of each assay included in the multiplex rRT-PCR extended from 2.0 to 8.0 $\log_{10}$ copies/µL of eluate (Fig 3. A-F). Lower limits of 95% detection, expressed as copies/µL of eluate were the following: Aguas Calientes, 16.1; Aruza, 2.5; and Matusagaratí/Madrid, 10.0. During evaluation of assay exclusivity, no signal was observed from any tested orthoflavivirus, alphavirus, or bunyavirus (**Supplemental Table 1**). No clinical samples tested for evaluation of specificity (n=39) was positive.

SUPPLEMENTARY RESULTS

Supplementary Table 1. Coverage and total proportion of reads mapped to each virus segment.

Segment	Length	Reads mapped	Percent of total reads
Sample_2a_L	7254	758983	2.21
Sample_2a_M	4639	736130	2.15
Sample_2a_S	1152	308620	0.9
Sample_2b_L	7009	452629	1.32
Sample_2b_M	4805	428604	1.25
Sample_2b_S	1075	241806	0.71
Sample_3a_L	7016	1314305	5.51
Sample_3a_M	4806	1124204	4.72
Sample_3a_S	1091	403867	1.69
Sample_3b_L	7269	345533	1.45
Sample_3b_M	4652	342042	1.44
Sample_3b_S	1152	152671	0.64
Sample_4a_L	7040	1247985	5.39
Sample_4a_M	4579	870429	3.76
Sample_4a_S	1149	506601	2.19
Sample_4b_L	7026	21529	0.09
Sample_4b_M	4675	17822	0.08
Sample_4b_S	1173	9689	0.04
Sample_6a_L	7026	549107	2.39
Sample_6a_M	4590	362597	1.58
Sample_6a_S	1143	255835	1.11
Sample_6b_L	7034	348053	1.51
Sample_6b_M	4679	302782	1.32
Sample_6b_S	1164	139949	0.61

Supplementary Table 2. Optimized primer-probe sequences for orthobunyavirus multiplex rRT-PCR.

Primer-Probe Name		***Sequence (5′ →3′)[a]***	***Concentration (nM)[b]***
Primers			
Aruza (Moju)	Forward	CCTCGAACTGGCACCAAATG	400
	Reverse	GCGATTGGGTTCACGATGTT	400
Matus/Madrid[c]	Forward	TCGAATTTTCTTCCTCCGCG	400
	Reverse	GTTTGATGCATTTCCGGGGT	400
Aguas	Forward	CGTCAGGTGGCAGAAGGTAA	400
	Reverse	GTGACTTCTCTAGCAGGGGT	400
Probes	Aruza (Moju)	5'-FAM-AGACTGGCTGCAGCAGAAAT-BHQ-1-3'	200
	Matus Madrid	5'-CFO560-TTGGCAGTTGGAAGGTCGAG-BHQ-1-3'	200
	Aguas	5'-CFR610-CCGGACTTGGCCTACAATGT-BHQ-1-3'	200

Abbreviations: BHQ, black hole quencher; FAM, fluorescein; CFO560, Cal Fluor orange 560; CFR610, Cal Fluor red 610.

[a] Probe sequences are listed in the following order: 5'-fluorophore-sequence-quencher-3'.

[b] Concentration in the final reaction mixture

[c] Combined assay for Matus and Madrid viruses

Supplementary Table 3. Viral genomic RNA tested to establish assay exclusivity for novel orthobunyavirus multiplex rRT-PCR.

Genus	***Species***
Flavivirus	Dengue virus
	Zika virus
	St. Louis encephalitis virus
	Japanese encephalitis virus
	Yellow fever virus
Alphavirus	Una virus
	Mayaro virus
	Chikungunya virus
	Ross River virus
Bunyavirus	Rift Valley fever virus
	Oropouche virus

Supplementary Table 4. Comparison of cycle threshold (Ct) values for singleplex and multiplex rRT-PCR assays.

Sample (Code)	***Singleplex Ct***	***Multiplex Ct***
Aruza (702150)	21.50	21.72
Aruza (702150), 1:100 dilution	36.19	36.75
Aruza (702151)	25.90	26.04
Aruza (702151), 1:100 dilution	32.51	32.52
**Aguas (701674)*	28.73	31.69
**Aguas (701674), 1:100 dilution*	33.64	37.52
**Aguas (701675)*	27.59	30.21
**Aguas (701675), 1:100 dilution*	33.74	37.45
Matus/Madrid (702154)	22.99	24.98
Matus/Madrid (702154), 1:100 dilution	29.49	31.72
Matus/Madrid (702156)	30.88	31.43
Matus/Madrid (702156), 1:100 dilution	28.21	30.44

*Sample also contained Madrid virus.

Supplementary Table 5. Absolute frequency of mosquito' species collected by sentinel animal.

Genus_WRBU	Subgenus_WRBU	Species_WRBU	Abbreviated_name_Wilkerson	Mouse	Hamster	Chicken	Frog
Aedes	*Ochlerotatus*	*fulvus*	*Ae.* (*Och.*) *fulvus*	1	0	0	0
Aedes	*Ochlerotatus*	*serratus*	*Ae.* (*Och.*) *serratus*	3	0	0	0
Anopheles	*Anopheles*	*apicimacula*	*An.* (*Ano.*) *apicimacula*	0	1	0	0
Coquillettidia	*Rhynchotaenia*	*venezuelensis*	*Cq.* (*Rhy.*) *venezuelensis*	149	1847	378	4
Culex	*Culex*	*coronator*	*Cx.* (*Cux.*) *coronator*	0	28	7	0
Culex	*Culex*	*interrogator*	*Cx.* (*Cux.*) *interrogator*	161	24	22	0
Culex	*Culex*	*nigripalpus*	*Cx.* (*Cux.*) *nigripalpus*	115	58	18	2
Culex	*Culex*	*pseudostigmatosoma*	*Cx.* (*Cux.*) *pseudostigmatosoma*	33	0	0	0
Culex	*Culex*	*spp.*	*Cx.* (*Cux.*) spp.	23	45	4	0
Culex	*Melanoconion*	*adamesi*	*Cx.* (*Mel.*) *adamesi*	1	117	13	0
Culex	*Melanoconion*	*dunni*	*Cx.* (*Mel.*) *dunni*	11	6	1	0
Culex	*Melanoconion*	*epanatasis*	*Cx.* (*Mel.*) *epanatasis*	1	0	0	0
Culex	*Melanoconion*	*ocossa*	*Cx.* (*Mel.*) *ocossa*	0	12	3	0
Culex	*Melanoconion*	*pedroi*	*Cx.* (*Mel.*) *pedroi*	200	710	55	1
Culex	*Melanoconion*	*spissipes*	*Cx.* (*Mel.*) *spissipes*	331	3416	146	3
Culex	*Melanoconion*	*vomerifer*	*Cx.* (*Mel.*) *vomerifer*	21	83	20	0
Culex	*Melanoconion*	*spp.*	*Cx.* (*Mel.*) spp.	0	191	30	0
Culex	*Phenacomyia*	*spp.*	*Cx.* (*Phc.*) spp.	1	0	0	0
Culex	.	*spp.*	*Cx.* spp.	16	127	12	1
Isostomyia	.	*espini*	*Is. espini*	1	0	0	0
Johnbelkinia	.	*leucopus*	*Jb. leucopus*	1	0	0	0
Johnbelkinia	.	*longipes*	*Jb. longipes*	4	0	0	0
Mansonia	*Mansonia*	*amazonensis*	*Ma.* (*Man.*) *amazonensis*	239	2408	105	5
Mansonia	*Mansonia*	*titillans*	*Ma.* (*Man.*) *titillans*	1	4	1	0
Psorophora	*Grabhamia*	*cingulata*	*Ps.* (*Gra.*) *cingulata*	0	1	0	0
Trichoposopon	.	*lampropus*	*Tr. lampropus*	1	0	0	0
Wyeomyia	*Dendromyia*	*luteoventralis*	*Wy.* (*Den.*) *luteoventralis*	2	0	0	0
Wyeomyia	*Miamyia*	*codiocampa*	*Wy.* (*Mia.*) *codiocampa*	1	0	0	0
Wyeomyia	*Wyeomyia*	*arthrostigma*	*Wy.* (*Wyo.*) *arthrostigma*	29	0	0	0
Wyeomyia	*Wyeomyia*	*nigritubus*	*Wy.* (*Wyo.*) *nigritubus*	1	0	0	0
			Total	1347	9078	815	16

Supplementary Table 6 Absolute frequency of mosquito' species collected in CDC traps by year of collection.

Genus_WRBU	Subgenus_WRBU	Species_WRBU	Abbreviated_name_Wilkerson	2018	2019	Total
Aedeomyia	*Aedeomyia*	*squamipennis*	*Ae.* (*Ady.*) *squamipennis*	40		40
Anopheles	*Anopheles*	*pseudopunctipennis*	*An.* (*Ano.*) *pseudopunctipennis*		5	5
Anopheles	*Anopheles*	*punctimacula*	*An.* (*Ano.*) *punctimacula*	7		7
Anopheles	*Anopheles*	*punctipennis*	*An.* (*Ano.*) *punctipennis*		1	1
Anopheles	*Nyssorhynchus*	*albimanus*	*An.* (*Nys.*) *albimanus*		1	1
Anopheles		*spp.*	*An.* spp.	51	6	57
Coquillettidia	*Rhynchotaenia*	*venezuelensis*	*Cq.* (*Rhy.*) *venezuelensis*	1239	76	1315
Culex	*Aedinus*	*amazonensis*	*Cx.* (*Ads.*) *amazonensis*	65	2	67
Culex	*Culex*	*coronator*	*Cx.* (*Cux.*) *coronator*	27	131	158
Culex	*Culex*	*declarator*	*Cx.* (*Cux.*) *declarator*	3	4	7
Culex	*Culex*	*interrogator*	*Cx.* (*Cux.*) *interrogator*	57	153	210
Culex	*Culex*	*nigripalpus*	*Cx.* (*Cux.*) *nigripalpus*	58	248	306
Culex	*Culex*	*spp.*	*Cx.* (*Cux.*) spp.	6	239	245
Culex	*Melanoconion*	*adamesi*	*Cx.* (*Mel.*) *adamesi*	10		10
Culex	*Melanoconion*	*crybda*	*Cx.* (*Mel.*) *crybda*	7		7
Culex	*Melanoconion*	*dunni*	*Cx.* (*Mel.*) *dunni*	11	9	20
Culex	*Melanoconion*	*erraticus*	*Cx.* (*Mel.*) *erraticus*	1		1
Culex	*Melanoconion*	*ocossa*	*Cx.* (*Mel.*) *ocossa*	86		86
Culex	*Melanoconion*	*pedroi*	*Cx.* (*Mel.*) *pedroi*	43	12	55
Culex	*Melanoconion*	*spissipes*	*Cx.* (*Mel.*) *spissipes*	41	16	57
Culex	*Melanoconion*	*spp.*	*Cx.* (*Mel.*) spp.	27	6	33
Culex	*Melanoconion*	*vomerifer*	*Cx.* (*Mel.*) *vomerifer*		1	1
Culex		*spp.*	*Cx.* sp.	1		1
Mansonia	*Mansonia*	*titillans*	*Ma.* (*Man.*) *titillans*	12	1	13
Mansonia		*spp.*	*Ma.* spp.	7		7
Psorophora	*Grabhamia*	*cingulata*	*Ps.* (*Gra.*) *cingulata*		27	27
Psorophora	*Psorophora*	*ciliata*	*Ps.* (*Pso.*) *ciliata*	7		7
Psorophora		*spp.*	*Ps.* spp.	11		11
Uranotaenia	*Uranotaenia*	*geometrica*	*Ur.* (*Ura.*) *geometrica*	1		1
Uranotaenia	*Uranotaenia*	*hystera*	*Ur.* (*Ura.*) *hystera*	1		1
Uranotaenia	*Uranotaenia*	*leucoptera*	*Ur.* (*Ura.*) *leucoptera*	19		19
Uranotaenia	*Uranotaenia*	*lowii*	*Ur.* (*Ura.*) *lowii*	144	13	157
Uranotaenia	*Uranotaenia*	*nataliae*	*Ur.* (*Ura.*) *nataliae*		1	1
Uranotaenia	*Uranotaenia*	*pallidoventer*	*Ur.* (*Ura.*) *pallidoventer*	4	2	6
Uranotaenia	*Uranotaenia*	*typhlosomata*	*Ur.* (*Ura.*) *typhlosomata*	1		1
Uranotaenia		*spp.*	*Ur.* spp.	89		89
			total			3030

Supplementary Table 7. Absolute frequency of mosquito species pools positive for Orthobunyaviruses.

Orthobunyavirus positives results								
Code	Host	Specie	Aruza	Madrid/Matus	Aguas	ct Aruza	ct Madrid/Matus	ct Aguas
701484	Mosquito	*Culex amazonensis*	0	0	**1**			23
704766	Mosquito	*Culex amazonensis*	0	0	**1**			20
704767	Mosquito	*Culex amazonensis*	0	0	**1**			15
704828	Mosquito	*Culex amazonensis*	0	0	**1**			20
704927	Mosquito	*Coquillettidia venezuelensis*	0	**1**	0		19	
704982	Mosquito	*Culex vomerifer*	0	**1**	0		21	
704989	Mosquito	*Culex pedroi*	**1**	0	0	18		
705012	Mosquito	*Culex spissipes*	**1**	0	0	22		
705032	Mosquito	*Culex amazonensis*	0	**1**	0		18	
705037	Mosquito	*Culex spissipes*	**1**	0	0	22		
705042	Mosquito	*Culex pedroi*	**1**	0	0	25		
705043	Mosquito	*Coquillettidia venezuelensis*	**1**	0	0	24		
705044	Mosquito	*Culex spissipes*	**1**	0	0	21		
705045	Mosquito	*Culex spissipes*	**1**	0	0	26		
705046	Mosquito	*Culex amazonensis*	**1**	0	0	22		
705048	Mosquito	*Culex adamesi*	**1**	0	0	18		
705049	Mosquito	*Culex spissipes*	**1**	0	0	18		
705050	Mosquito	*Coquillettidia venezuelensis*	**1**	0	0	20		
705051	Mosquito	*Culex vomerifer*	**1**	0	0	18		
705054	Mosquito	*Culex pedroi*	**1**	0	0	16		
705055	Mosquito	*Coquillettidia venezuelensis*	**1**	0	0	26		
705057	Mosquito	*Culex spissipes*	**1**	0	0	26		
705060	Mosquito	*Coquillettidia venezuelensis*	**1**	0	0	17		
705061	Mosquito	*Culex spissipes*	**1**	0	0	18		
705062	Mosquito	*Culex spissipes*	**1**	0	0	17		
705073	Mosquito	*Culex spissipes*	**1**	0	0	24		
705074	Mosquito	*Culex spissipes*	**1**	0	0	25		
705113	Mosquito	*Culex pedroi*	0	**1**	0		19	
705137	Mosquito	*Culex amazonensis*	0	0	**1**			17
705153	Mosquito	*Culex amazonensis*	0	0	**1**			18
705181	Mosquito	*Culex amazonensis*	0	0	**1**			16
705184	Mosquito	*Culex amazonensis*	0	0	**1**			26
705195	Mosquito	*Culex amazonensis*	0	0	**1**			24
705198	Mosquito	*Culex amazonensis*	0	0	**1**			14
705199	Mosquito	*Culex amazonensis*	0	0	**1**			24
705200	Mosquito	*Culex amazonensis*	0	0	**1**			21
705213	Mosquito	*Culex amazonensis*	0	0	**1**			25
705214	Mosquito	*Culex amazonensis*	0	0	**1**			26
705229	Mosquito	*Culex amazonensis*	0	0	**1**			17
705231	Mosquito	*Coquillettidia venezuelensis*	0	0	**1**			19
705232	Mosquito	*Culex amazonensis*	0	0	**1**			27
705233	Mosquito	*Culex spissipes*	0	0	**1**			25
705234	Mosquito	*Culex amazonensis*	0	0	**1**			26
705235	Mosquito	*Culex spissipes*	0	0	**1**			28
705243	Mosquito	*Culex sp.*	0	0	**1**			29
705266	Mosquito	*Culex amazonensis*	0	0	**1**			15
705319	Mosquito	*Culex vomerifer*	**1**	0	0	22		
705321	Mosquito	*Culex spissipes*	**1**	0	0	18		
705322	Mosquito	*Culex pedroi*	**1**	0	0	20		
705323	Mosquito	*Coquillettidia venezuelensis*	**1**	0	0	19		

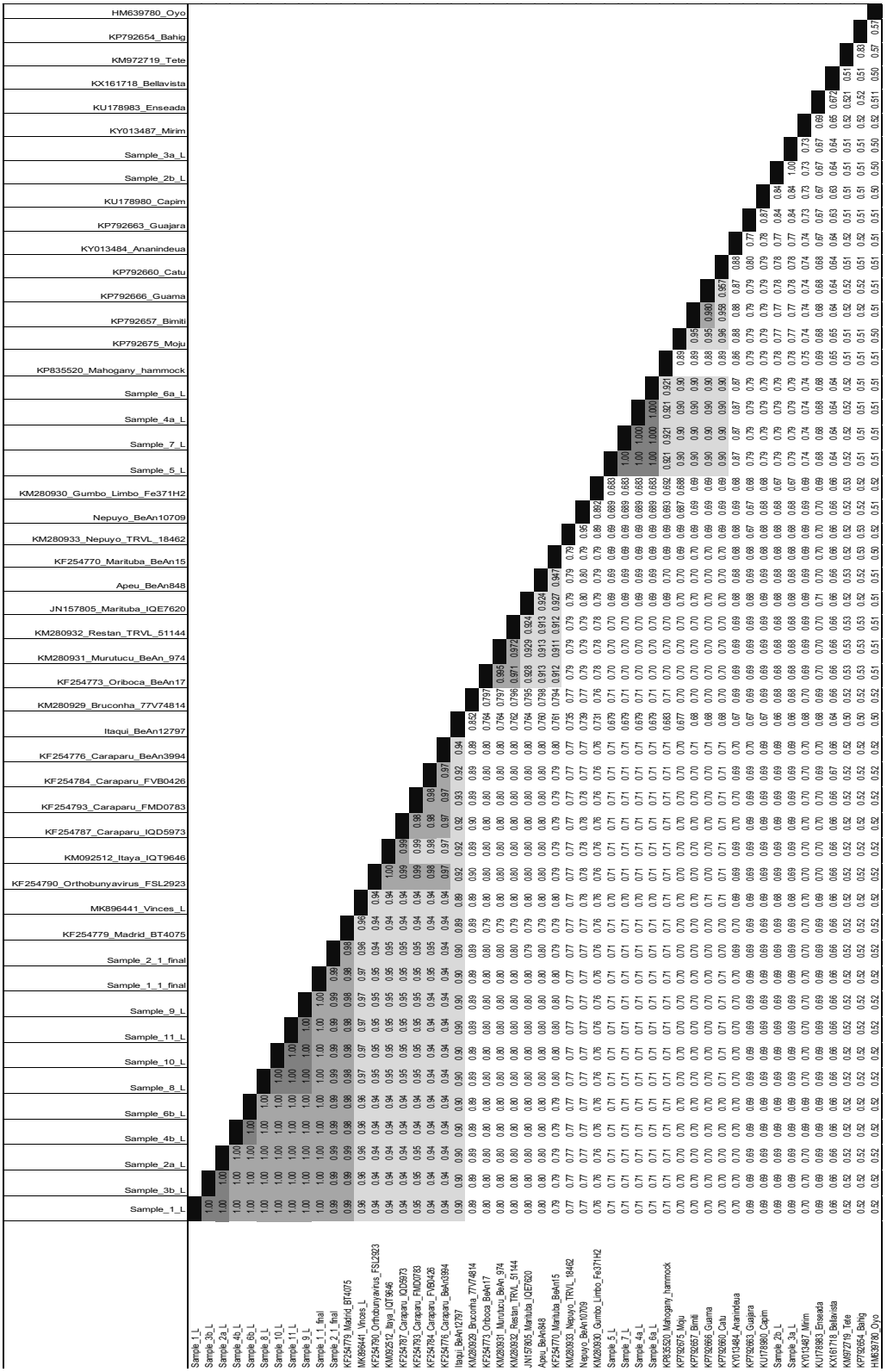
Supplementary Table 8. Absolute and relative rodent abundance by year and collection site.

Species	Years‡		Collection sites†				
	2011	**2012**	**El Real**	**Los Pavitos**	**Santa Librada**	**El Cacao**	**Cirí Grande**
	n/N (%)	**n/N (%)**	**n/N (%)**	**n/N (%)**	**n/N (%)**	**n/N (%)**	**n/N (%)**
Didelphis marsupialis	1/430 (0.2)	0/169 (0.0)	0/202 (0.0)	0/165 (0.0)	0/158 (0.0)	0/56 (0.0)	1/18 (5.6)
Liomys adspersus	4/430 (0.9)	0/169 (0.0)	0/202 (0.0)	0/165 (0.0)	0/158 (0.0)	4/56 (7.1)	0/18 (0.0)
Marmosa robinsoni	1/430 (0.23)	0/169 (0.0)	1/202 (0.5)	0/165 (0.0)	0/158 (0.0)	0/56 (0.0)	0/18 (0.0)
Marmosa sp.	1/430 (0.2)	0/169 (0.0)	0/202 (0.0)	0/165 (0.0)	0/158 (0.0)	0/56 (0.0)	1/18 (5.6)
Melanomys caliginosus	18/430 (4.2)	3/169 (1.8)	21/202 (10.4)	0/165 (0.0)	0/158 (0.0)	0/56 (0.0)	0/18 (0.0)
Mus musculus	0/430 (0.0)	13/169 (7.7)	9/202 (4.5)	4/165 (2.4)	0/158 (0.0)	0/56 (0.0)	0/18 (0.0)
Handleyomys alfaroi	6/430 (1.4)	5/169 (3.0)	6/202 (3.0)	1/165 (0.6)	4/158 (2.5)	0/56 (0.0)	0/18 (0.0)
Transandinomys bolivaris	6/430 (1.4)	3/169 (1.8)	5/202 (2.5)	1/165 (0.6)	3/158 (1.9)	0/56 (0.0)	0/18 (0.0)
Oryzomys sp.	16/430 (3.7)	0/169 (0.0)	6/202 (3.0)	10/165 (6.1)	0/158 (0.0)	0/56 (0.0)	0/18 (0.0)
Ototylomys phyllotis	0/430 (0.0)	2/169 (1.2)	2/202 (1.0)	0/165 (0.0)	0/158 (0.0)	0/56 (0.0)	0/18 (0.0)
Proechimys semispinosus	53/430 (12.3)	20/169 (11.8)	19/202 (9.4)	3/165 (1.8)	1/158 (0.6)	37/56 (66.1)	13/18 (72.2)
Rattus rattus	9/430 (2.1)	5/169 (3.0)	8/202 (4.0)	2/165 (1.2)	0/158 (0.0)	3/56 (5.4)	1/18 (5.6)
Rattus sp.	2/430 (0.5)	0/169 (0.0)	1/202 (0.5)	0/165 (0.0)	1/158 (0.6)	0/56 (0.0)	0/18 (0.0)
Sigmodon hirsutus	5/430 (1.2)	3/169 (1.8)	0/202 (0.0)	0/165 (0.0)	0/158 (0.0)	8/56 (14.3)	0/18 (0.0)
Sigmodon sp.	1/430 (0.2)	0/169 (0.0)	0/202 (0.0)	0/165 (0.0)	0/158 (0.0)	1/56 (1.8)	0/18 (0.0)
Zygodontomys brevicauda	307/430 (71.4)	115/169 (68.1)	124/202 (61.4)	144/165 (87.3)	149/158 (94.3)	3/56 (5.4)	2/18 (11.1)
Total	430/599 (71.8)	169/599 (28.2)	202/599 (33.7)	165/599 (27.5)	158/599 (26.3)	56/599 (9.3)	18/599 (3.0)

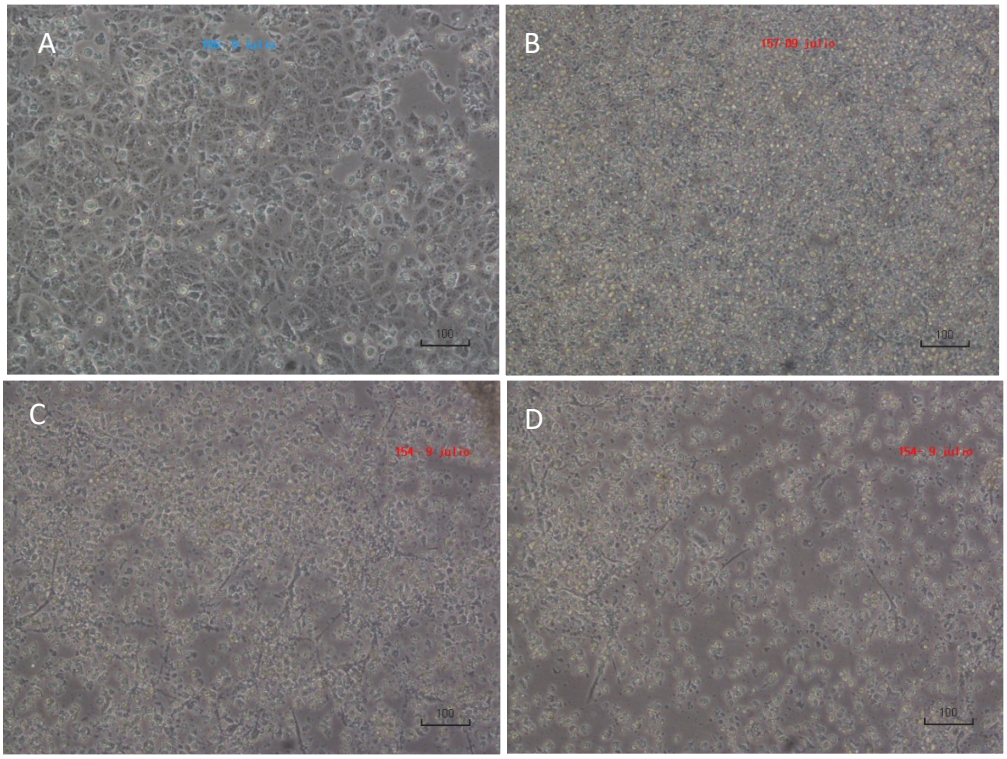
‡Rodent abundance total by year: 2011 n=430 and 2012 n=169; abundance total n=599

†Rodent abundance total by collection site: El Real n=202, Los Pavitos n=165, Santa Librada n=158, El Cacao n=56 and Cirí Grande n=18

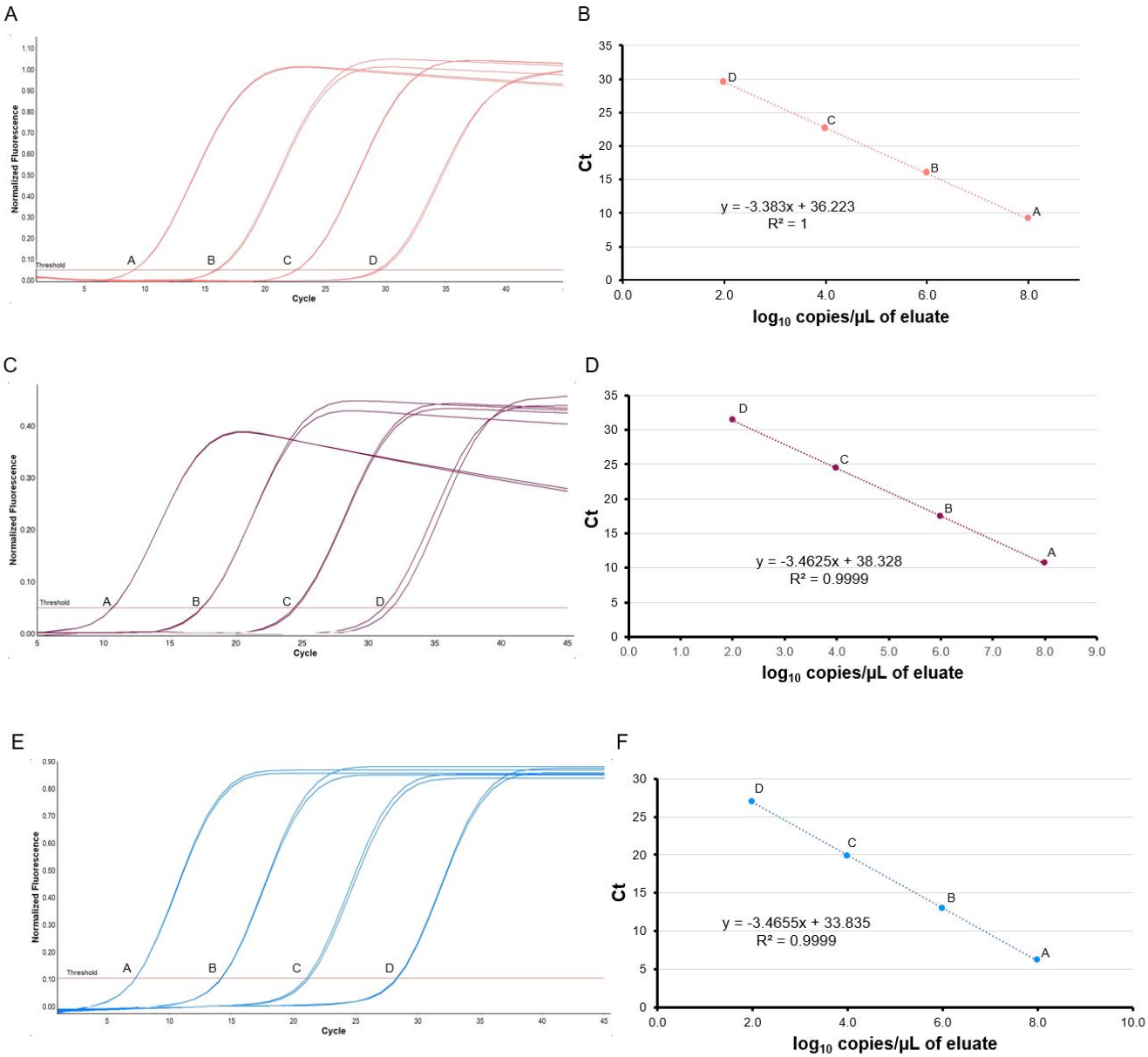
Supplementary Table 9. Animo acids pairwise distance of the RNA-dependent RNA polymerase



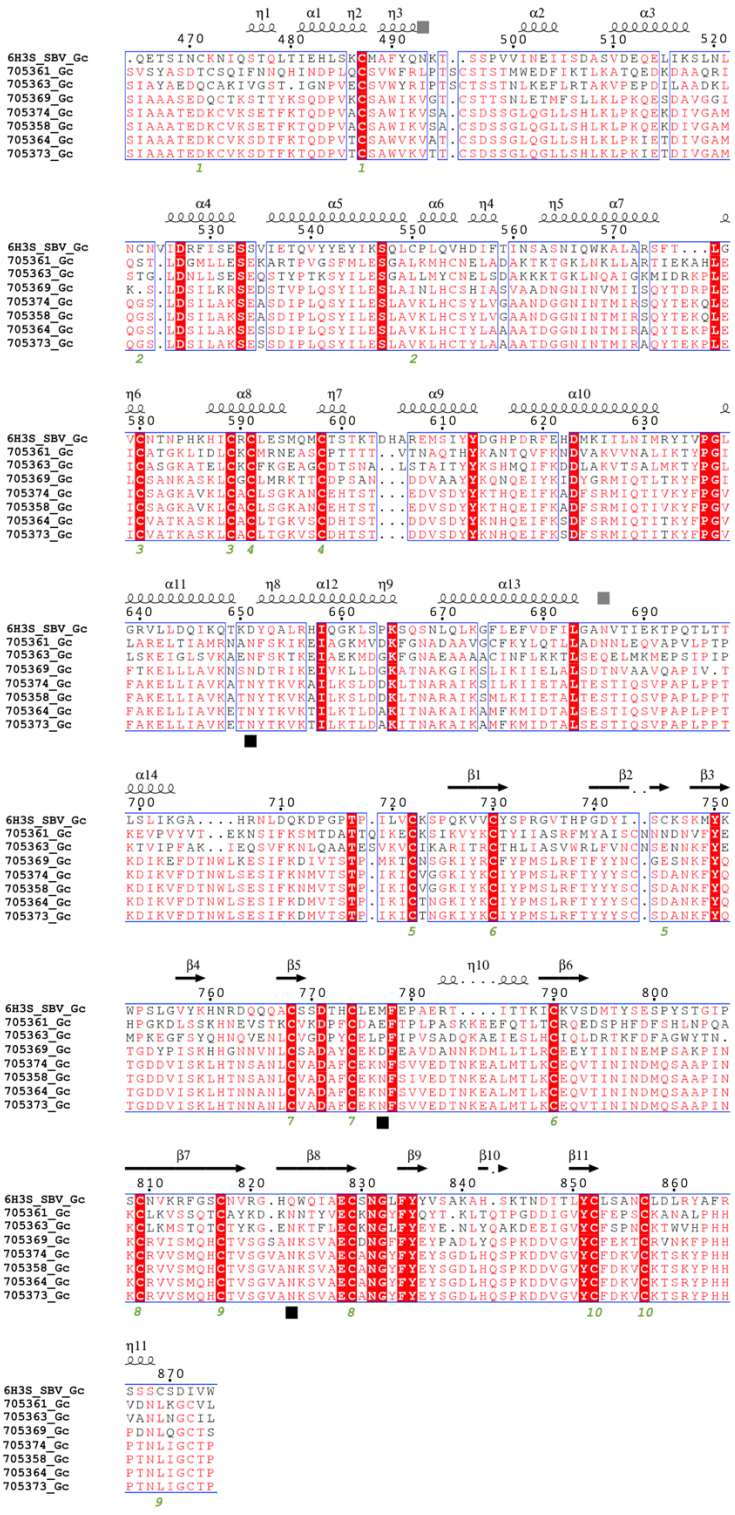
Supplementary Figure 1. Trinidad trap baited with a hamster in site 8.



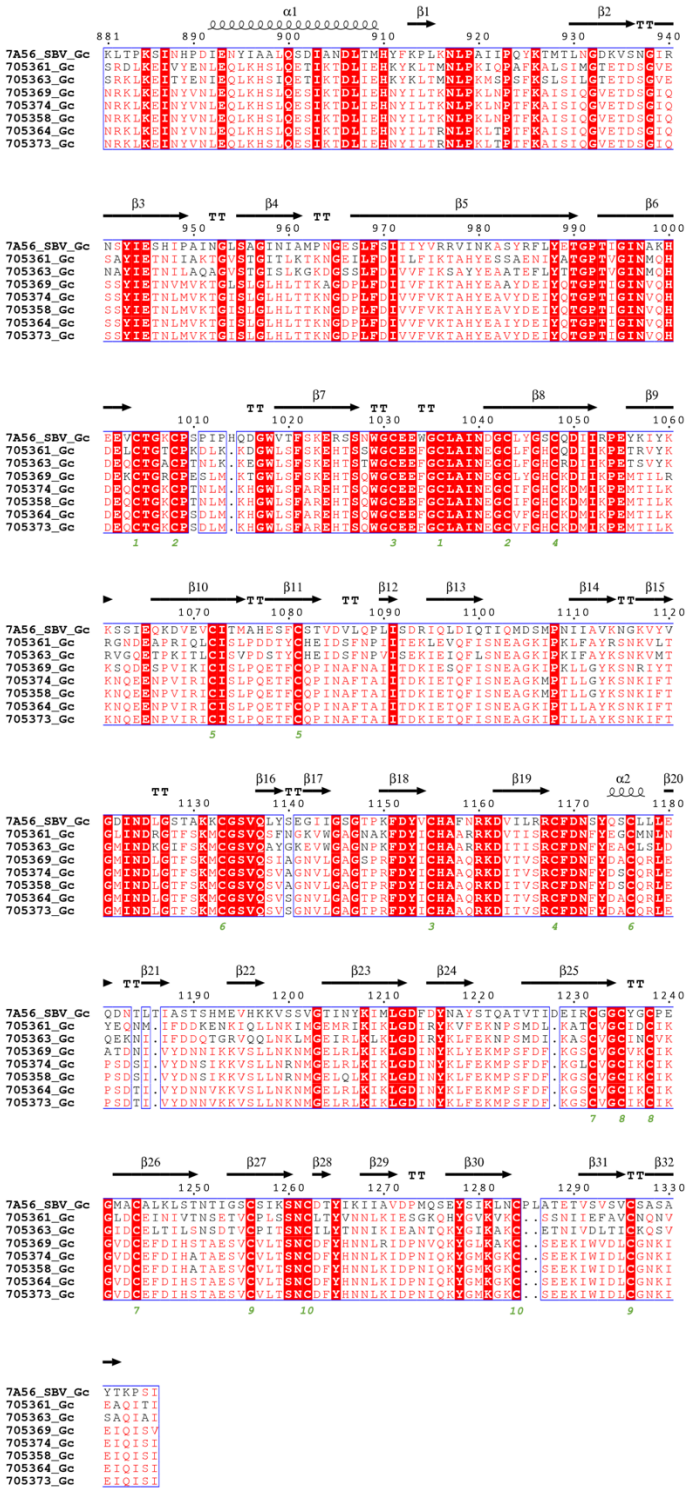
Supplementary Figure 2. Cytopathic effects were observed on Vero cells inoculated with tissues from mice displaying signs of disease in 2017*. A. Cytopathic effect from heart tissue. B. Cytopathic effect from liver tissue. C. Cytopathic effect from spleen tissue and D. Cytopathic effect from spleen tissue.*



Supplementary Figure 3. *Linear range for the multiplex rRT-PCR assay for four novel bunyaviruses: Aguas (A-B), Aruza (C-D), and Matusagaratí/Madrid (E-F). Four concentrations (8.0, 6.0, 4.0, and 2.0 log_{10} copies/µL of eluate) were run in quadruplicate on a single run of the assay. All curves and data points are displayed.*



Supplementary Figure 4. *Multiple amino acid sequence alignment of orthobunyaviral Gc glycoproteins.*



Supplementary Figure 5. *Multiple amino acid sequence alignment of orthobunyaviral Gc glycoproteins.*

G. Appendix to additional work published during the DPhil

1. de Souza WM*, Buss LF*, Candido DDS*, **Carrera JP***, *et al*. Epidemiological and clinical characteristics of the COVID-19 epidemic in Brazil. Nat Hum Behav. 2020 Aug;4(8):856-865. doi: 10.1038/s41562-020-0928-4.

2. de Lima STS, de Souza WM, Cavalcante JW, da Silva Candido D, Fumagalli MJ, **Carrera JP**, *et al*. Fatal Outcome of Chikungunya Virus Infection in Brazil. Clin Infect Dis. 2021 Oct 5;73(7):e2436-e2443. doi: 10.1093/cid/ciaa1038.

3. Franco D, Gonzalez C, Abrego LE, **Carrera JP**, *et al*. Early Transmission Dynamics, Spread, and Genomic Characterization of SARS-CoV-2 in Panama. Emerg Infect Dis. 2021 Feb;27(2):612-615. doi: 10.3201/eid2702.203767.

4. Caicedo EY, Charniga K, Rueda A, Dorigatti I, Mendez Y, Hamlet A, **Carrera JP**, *et al*. The epidemiology of Mayaro virus in the Americas: A systematic review and key parameter estimates for outbreak modelling. PLoS Negl Trop Dis. 2021 Jun 3;15(6):e0009418. doi: 10.1371/journal.pntd.0009418.

5. de Souza WM, Fumagalli MJ, **Carrera JP**, *et al*. Paramyxoviruses from neotropical bats suggest a novel genus and nephrotropism. Infect Genet Evol. 2021 Nov;95:105041. doi: 10.1016/j.meegid.2021.105041.

6. Sloyer KE, Santos M, Rivera E, Reeves LE, **Carrera JP**, *et al*. Evaluating sampling strategies for enzootic Venezuelan equine encephalitis virus vectors in Florida and Panama. PLoS Negl Trop Dis. 2022 Apr 13;16(4):e0010329. doi: 10.1371/journal.pntd.0010329.

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