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TRANSLATIONAL FRONTIERS FOR CHIKUNGUNYA VIRUS: NEW WORLD EPIDEMIC, NEW STRAINS, NEW THERAPEUTIC TARGETS

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TRANSLATIONAL FRONTIERS FOR CHIKUNGUNYA VIRUS: NEW WORLD EPIDEMIC, NEW STRAINS, NEW THERAPEUTIC TARGETS

by

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Dedication

I dedicate this dissertation to: my family, who provided me a loving, stable, and supportive environment (even through the early war-years with my sisters) and the voluminous numbers of MDs and PhDs among my nuclear family and cousins, which gave me the drive that only an inferiority complex can inspire; and especially my parents for pushing me to be the best I can be and ensuring my education was the best possible; my past (and current) teachers and professors, for giving me the knowledge and skills to be successful in graduate school and in life; my friends, for easing the tensions wrought by my rigorous education; and finally, my fiancé and the love of my life Eric, for his love, support, and tireless endeavors to keep me grounded and sane, even though my increasing and persistent neuroticism drove him crazy. All of these people have filled my life with more love than I know how to handle, which ultimately made all of this possible.

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Chikungunya virus (CHIKV) infection results in chikungunya fever (CHIKF), marked by sudden onset of high fever, rash, and debilitating polyarthritis and polyarthralgia, which can last for months to years. Despite the severe morbidity and impact on disability adjusted life-years, an FDA-approved CHIKV vaccine or therapeutic remains elusive. This project addresses two aspects of therapeutic development: surveillance and drug target development. First, an outbreak of CHIKF in La Romana, Dominican Republic is described. During this outbreak, several parameters of established CHIKV epidemiology were challenged, such as the percent of patients presenting with arthritis as well as demographic distribution of disease. Overall, CHIKF was underdiagnosed in La Romana, likely due to attenuated CHIKV-disease phenotype. Related to this, the virulence of strains from distinct CHIKV lineages was compared in the lethal A129 CHIKF mouse model. Isolates from the Caribbean and Mexico were attenuated compared to the West African, East/Central/South African, and Indian Ocean isolates, and even compared to other Asian isolates to which strains in the Caribbean are most closely related. Surveillance is an

important aspect of developing appropriate trials for future vaccines and therapeutics, and together these data suggest that the incidence of CHIKF in the New World is likely underestimated, and surveillance practices need to be adjusted to include identification of an attenuated disease phenotype. Finally, the development of novel antiviral drug targets was explored. As the envelope proteins of alphaviruses are replete with structure-maintaining disulfide bonds, inhibitors to the host enzymes responsible for disulfide bond formation—protein disulfide isomerase (PDI) family chaperones—were utilized as a tool to potentiate PDI as an anti-CHIKV (and anti-alphavirus) drug target. PDI-inhibitors PACMA31 and 16F16, as well as inhibitors EN460 and auranofin to PDI regulatory proteins endoplasmic reticulum oxidoreductin-1 (ERO-1) and thioredoxin reductase (TRX-R), respectively, all greatly reduced CHIKV replication *in vitro*; 16F16 likely caused misfolding of the envelope proteins and subsequent production of non-functional virus particles, while auranofin likely selectively induced apoptosis in infected cells. Auranofin modestly reduced viral replication and significantly reduced footpad swelling in infected C57Bl/6 mice. In all, this project addressed two important translational avenues of CHIKV research.

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List of Abbreviations

[UTMB	University of Texas Medical Branch
GSBS	Graduate School of Biomedical Science
TDC	Thesis and Dissertation Coordinator
CHIKV	Chikungunya virus
CHIKF	Chikungunya fever
VEEV	Venezuelan Equine Encephalitis Virus
RRV	Ross River virus
SINV	Sindbis Virus
SFV	Semliki Forrest Virus
MAYV	Mayaro virus
EEEV	Eastern equine encephalitis virus
ZIKV	Zika virus
DENV	Dengue virus
DENF	Dengue Fever
ER	Endoplasmic reticulum
PDI	Protein disulfide isomerase
HSP90	Heat-shock protein 90
DR	Dominican Republic
ASN	Asian
ECSA	East, Central, and South African
IOL	Indian Ocean lineage
CRBN	Caribbean
WA	West African
RT-qPCR	Real Time- quantitative Polymerase Chain Reaction
ELISA	Enzyme-linked immunosorbent assay
DTT	Dithioreitol
BME	Beta-mercapto ethanol

CHAPTER I. AN INTRODUCTION TO CHIKUNGUNYA VIRUS

Alphaviruses are arthropod-borne, positive sense, single-stranded RNA viruses in the family *Togaviridae* genus *Alphavirus*. Some important alphaviruses in terms of human disease include Venezuelan and eastern equine encephalitis (VEEV and EEEV), Sindbis (SINV), chikungunya (CHIKV), Semliki Forest (SFV), Mayaro (MAYV), and Ross River (RRV) viruses. These are all considered zoonoses, circulating in nature with occasional crossover to human populations via a mosquito vector. CHIKV arguably causes the most morbidity of these generally neglected pathogens, causing explosive epidemics of chikungunya fever (CHIKF).

CHIKV has infected many millions of individuals since its initial discovery, and continues to spread throughout the world. CHIKF precipitates many health and financial complications, and as such represents an important human pathogen. Although some work has been done to describe alphavirus biology and CHIKV pathophysiology, translational research regarding CHIKV remains limited.

CHIKV EVOLUTION AND EPIDEMIOLOGY

Epidemiological history of CHIKV

The word "chikungunya" is derived from the Makonde verb *kungunyala*, meaning "to become contorted or fold up," reflecting the origins of the discovery of CHIKV. The virus was first isolated from a patient from the Makonde plateau in then-Tanganyika, located between Tanzania and Mozambique, in 1952 during an outbreak of febrile illness accompanied by debilitating joint symptoms affecting the plateau and surrounding areas (1, 2). While this was the first time the virus and associated disease were formally described, some researchers suspect that CHIKV may have circulated in Asia as early as the 1700s (3). Regardless, more CHIKF outbreaks were subsequently noted throughout Africa after initial reports from Tanganyika were published in 1955; outbreaks appeared to occur cyclically, occurring every 4-9 years (4).

Autochthonous, or locally acquired, CHIKF outside of Africa was first described in Bangkok, Thailand in 1958 (5), and subsequent outbreaks were recognized in other areas of Asia through the 1960s and 1970s (6-9). Despite continuing reports of CHIKF outbreaks through the 1990s (10-12), the virus receded into relative obscurity until the 2000s. Then, in 2005, CHIKV re-emerged in areas around the Indian Ocean off the Eastern coast of Southern Africa. The 2005-2006 CHIKF outbreak on the French island of La Reunion is particularly well documented, and the virus quickly spread to India and even Southern Italy and France (13-17). Since then, CHIKF epidemics have been reported regularly throughout Asia and some areas in Europe, in part due to increased surveillance practices (18-20). CHIKV is classified as an Old World alphavirus, as it was generally limited to the Eastern hemisphere until late 2013, when the first outbreak in the New World (Western hemisphere) began on the French island of St. Martin in the Caribbean (21). While this introduction precipitated subsequent CHIKF outbreaks throughout the Caribbean and South and Central America (22-26), a concurrent introduction of a distinct CHIKV strain was made in Brazil in 2014 (27), causing several outbreaks in Brazil and Bolivia (Pan American Health Organization [PAHO]). Currently, CHIKV has caused over 1.9 million suspected cases of CHIKF in the Americas and the Caribbean (PAHO). Locally acquired cases have been reported in the United States in Texas and Florida, suggesting that CHIKF outbreaks in the United States are imminent.

CHIKV Strain evolution and nomenclature

Over this time period, several genetically distinct CHIKV lineages have arisen as shown through phylogenetic analyses based on both gene-specific (28) and whole genome sequences (29, 30). Generally, CHIKV strains are classified according to three lineages: West African (WA); East, Central, and South African (ECSA); and Asian (ASN). Another distinct clade arising during the Indian Ocean outbreak, evolving from the broader ECSA lineage, is alternately referenced as ECSA lineage or as its own distinct lineage, the Indian Ocean lineage (IOL). The

most important lineages for human health considerations are the ASN, IOL, and the broader ECSA lineages. These lineages are almost solely responsible for all major CHIKV outbreaks over the last 15 years. For example, A CHIKV strain from the ASN lineage is responsible for the majority of CHIKF outbreaks in the Americas (31), while the strain introduced in Brazil in 2014 is part of the ECSA lineage (27). The CHIKV strain responsible for most 2005-2011 outbreaks beginning in La Reunion is from the IOL lineage (30). Strains from all 3 of these lineages continue to arise in Asia and Africa (32-34). Literature regarding WA CHIKV strain outbreaks is scarce, particularly in contemporary literature, but this may not reflect the absence of this lineage but rather the remoteness of areas in which WA strains might circulate. The 5' and 3' untranslated regions (UTRs) found at the ends of the genome vary widely between CHIKV lineages and species, and are usually left out of phylogenetic analyses (35). However, it is proposed that CHIK UTRs have a profound effect on CHIKV evolution, particularly by influencing the fitness of CHIKV strains in mosquitos (35, 36). Another source of genetic variation is the envelope proteins of the virus, while the non-structural proteins are tightly conserved with the exception of non-structural protein (nsP) 3 (37, 38).

CHIKV transmission cycles

CHIKV has two transmission cycles: a sylvatic or enzootic cycle, which is maintained in nature; and an urban, or epidemic, cycle, which occurs in explosive outbreaks when a competent vector species introduces the virus into a naïve human population (Ill. 1, CHIKV infectious cycle)(39). The sylvatic cycle occurs primarily in Africa, where CHIKV is thought to be maintained in a cycle between non-human primates and sylvatic *Aedes* mosquito species. The dominant sylvatic vector of CHIKV remains unknown, as CHIKV has been isolated from many different African *Ae.* species pools, including *Ae. furcifer/taylori*, *Ae. africanus*, and *Ae. dalzieli*, among others (4, 40, 41). However, *Ae. furcifer* is strongly suspected to be the primary sylvatic

vector in several locations. Similarly, a single primate reservoir has yet to be positively incriminated, as replicating virus, RNA, and/or CHIKV-specific antibodies have been isolated from a variety of non-human primate genera, including *Cercopithecus* (vervet monkeys) and *Papio* (baboons) (41). While many studies regarding sylvatic CHIKV cycling focus on CHIKV-enzootic areas in Africa, replicating CHIKV has also been isolated from non-human primate sera in Malaysia (42). Further, sequencing revealed that the four viruses isolated from Malaysian primates are genetically similar to CHIKV strains implicated in two separate outbreaks in Malaysia, one in 1998 and one in 2006. This is further corroborated by evidence of neutralizing antibodies in Malaysian macaques (43). IgM analyses of rhesus macaques revealed that over 10% tested CHIKV positive, while IgG analysis revealed almost 60% were CHIKV-positive in the Philippines (44). Together, the evidence suggests that sylvatic cycles may potentially occur outside of Africa, although this remains unconfirmed. It is currently unknown whether New World non-human primates can participate in sylvatic CHIKV transmission cycles.

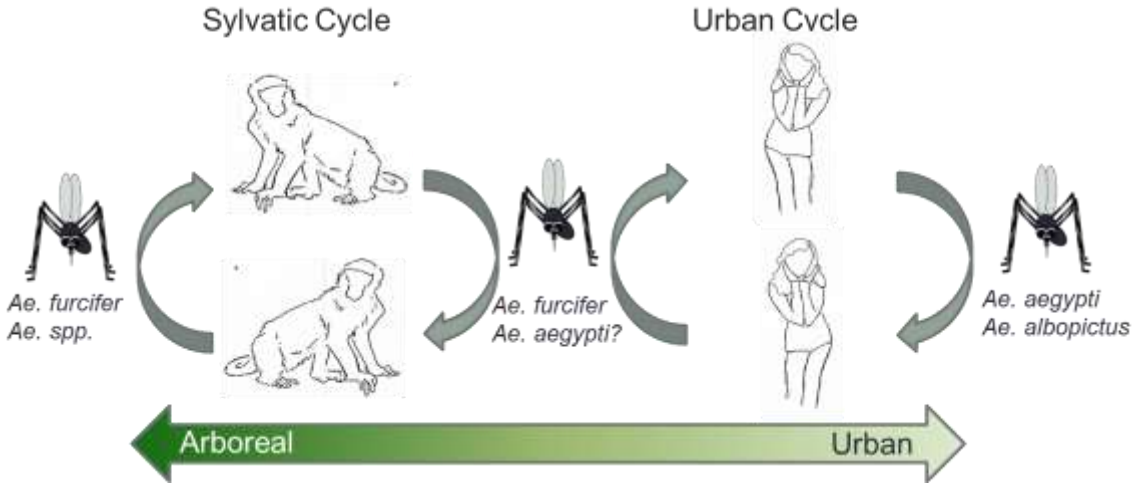

Illustration 1. CHIKV transmission cycles.

CHIKV has two transmission cycles, a sylvatic cycle and an urban cycle. The sylvatic is maintained in nature, where CHIKV is thought to cycle between non-human primates through arboreal Aedes species. When a bridge vector transmits CHIKV to a human in an urban center, then an urban or epidemic cycle begins, with the virus cycling between humans through Ae. aegypti or Ae. albopictus. Illustrations by Rose and Erika Langsjoen.

The urban cycle, which results in CHIKF outbreaks, begins when a human contracts CHIKV from a sylvatic mosquito or a transitional vector species and subsequently exposes urban mosquitos to the virus. Humans become viremic for 4-10 days after infection with CHIKV (45), exposing subsequent vectors to the virus; once the newly infected vector feeds on another host, the urban cycle is continued. The primary urban vector of CHIKV is *Ae. aegypti*, as shown both in the field and the laboratory setting. Where mosquitos have been collected around urban outbreak areas, such as in Chiapas, Mexico and Senegal, both CHIKV RNA and replicating virus has been isolated from pooled specimens of *Ae. aegypti* (4, 46). To provide evidence of transmission from a mosquito, two things must happen: the mosquito must be able to infected by CHIKV, with CHIKV disseminating to the salivary glands; and the mosquito, once infected, must be able to transmit the virus to a host on which it feeds. *Ae. aegypti* can do both, as it has been found that CHIKV inoculated into mosquitos through artificial blood feeds (47) cannot only

readily be isolated from salivary glands, but infected *Ae. aegypti* mosquitos have been found to transmit virus to mammalian hosts during subsequent live feeds (48). Lastly, *Ae. aegypti* collected in rural and forest settings have also tested positive for CHIKV (both replicating virus and RNA), indicating it may serve an additional role as a bridge vector between the sylvatic and urban cycles (40). Interestingly, CHIKV has also been isolated from male mosquito pools (49) as well as larvae raised from eggs laid by infected females (50), indicating potential transovarial transmission. However, several other urban *Ae.* species have demonstrated potential vector competency. The most well-known example is *Ae. albopictus*, a secondary vector of CHIKV which is readily infected by the virus, but traditionally is less capable of transmission (48, 51). However, the overall vector competence of *Ae. albopictus* increases drastically dependent on several mutations in the CHIKV envelope proteins. Specifically, an alanine→valine change at amino acid position 226 in the E1 protein is primarily responsible for enhanced vector competency of *Ae. albopictus* (52), in conjunction with several other supporting mutations in the E2 protein (53, 54). These mutations have only been identified in CHIKV isolates stemming from the Indian Ocean outbreak, i.e. IOL CHIKV strains, and do not appear to enhance the vector competency of *Ae. albopictus* for other CHIKV lineages. Secondly, laboratory transmission studies have shown *Ae. polynesiensis* to be susceptible to CHIKV infection and to capably transmit to subsequent mammalian hosts (55, 56). While this vector may typically be considered arboreal or peri-urban, it may potentially play a role in precipitating outbreaks of CHIKF in areas of Polynesia where *Ae. aegypti* isn't currently found (55).

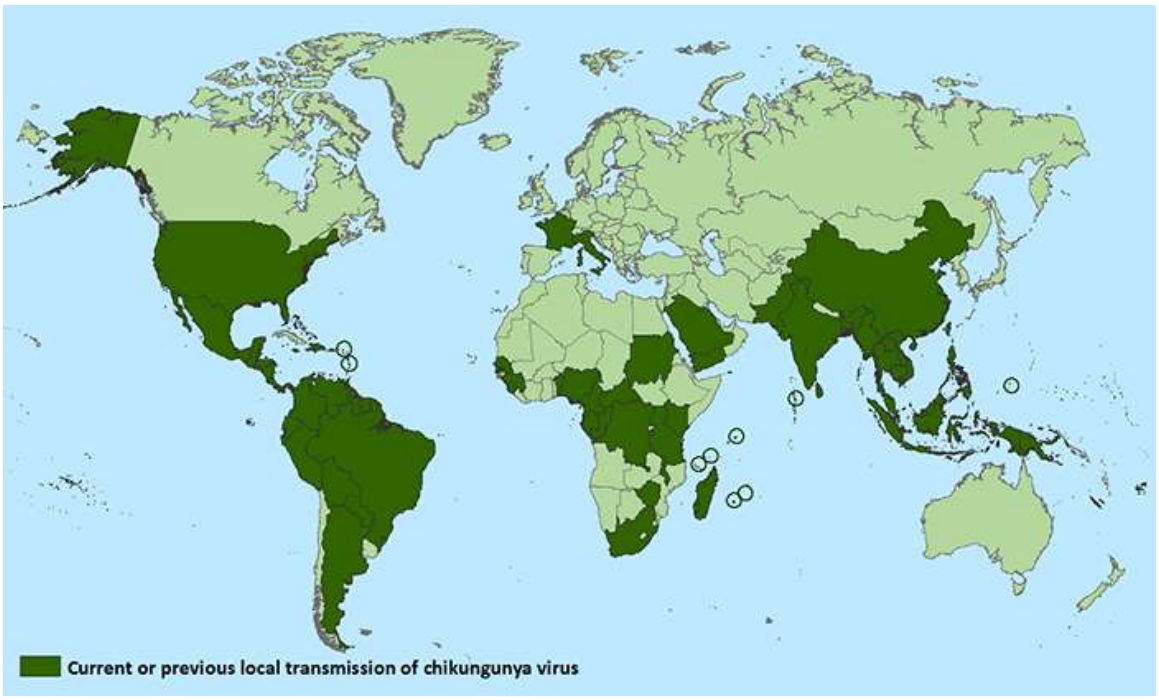

Illustration 2. Global reach of CHIKV.

In recent years, CHIKV has spread from isolated areas in Asia and Africa to the Americas. Figure courtesy of the Centers for Disease Control and Prevention (http://www.cdc.gov/chikungunya/geo/).

CLINICAL, PUBLIC HEALTH, AND FINANCIAL OUTCOMES OF CHIKF

Diagnosis of CHIKF

Alphavirus infection can result in one of two primary disease outcomes: the first is an encephalitic disease typified by the equine encephalitis viruses; the second is a severe polyarthritis and polyarthralgia typified by CHIKV. There are a variety of methods to diagnose CHIKF. Serological tests, namely enzyme-linked immunosorbent assays (ELISAs) measuring CHIKV-specific IgM antibodies, are commonly employed in areas with access to the appropriate equipment. There are several commercially available CHIKV-IgM ELISAs with reasonable specificity, although sensitivity varies (57-59). ELISAs, however, only diagnose a recent infection with CHIKV and may not necessarily indicate acute or active CHIKV infection, since IgM seroconversion does not occur until viremia has declined or resolved completely.

Polymerase chain reaction (PCR), either conventional or quantitative, using CHIKV-specific primers can detect CHIKV RNA in the serum which indicates an active infection (60-63). While both IgM ELISA and PCR are great diagnostic options and are used for many research studies and by some clinics, many areas to which CHIKV is endemic cannot necessarily afford these diagnostic tests, as equipment and consumables may be prohibitively expensive, or they simply do not have facilities nearby (64). Therefore, CHIKF is most commonly diagnosed using clinical case definitions only (21). This can be problematic, however, because CHIKV co-circulates with dengue virus (DENV), as well as other fever-causing agents. Dengue fever (DENF) is almost clinically indistinguishable from CHIKF, leading to frequent misdiagnosis (65, 66).

Acute CHIKF

CHIKF, though rarely fatal, is a devastating disease in terms of morbidity. After a brief incubation period lasting an average of 2-4 days, there is an abrupt onset of febrile disease; signs and symptoms include fever, rash, myalgia, and severe polyarthritis and polyarthralgia. Other commonly noted symptoms include retroocular pain or headache, or gastrointestinal symptoms in younger patient populations. Painful joint symptoms are most common and are considered a hallmark of CHIKF, occurring in 87-97% of cases. CHIKV arthritis is described as rheumatoid-like, primarily affecting distal joints with diminishing symptomology in larger proximal joints. Tenosynovitis is also common with CHIKV infection. The acute stage of illness is defined by a high average viremia of 10^9 genome copies/mL, which lasts up to 10 days, as well as lymphopenia corresponding to the level of viremia (13, 16, 45, 67). As viremia wanes, usually near 4 days after symptom onset, CHIKV-specific IgM antibodies arise. Curiously, CHIKV-infection often produces a prolonged IgM antibody response, usually lasting about 3 months. Several atypical manifestations of CHIKV, which lead to lethality, including a lethal meningo-encephalitis disease course and a severe myocarditis, have been described (68-71); however, these generally remain rare. IgG antibodies arise around a week after infection and naïve

infection results in life-long immunity to subsequent CHIKV infection (72). It was previously thought that nearly all individuals who contract CHIKV exhibit CHIKF symptoms with very few asymptomatic cases, although a prospective study examining a recent ASN CHIKV strain outbreak found that there were between 4-5 subclinical infections for every 1 symptomatic infection, dependent on age group (73). The general clinical progression is illustrated in Ill. 3.

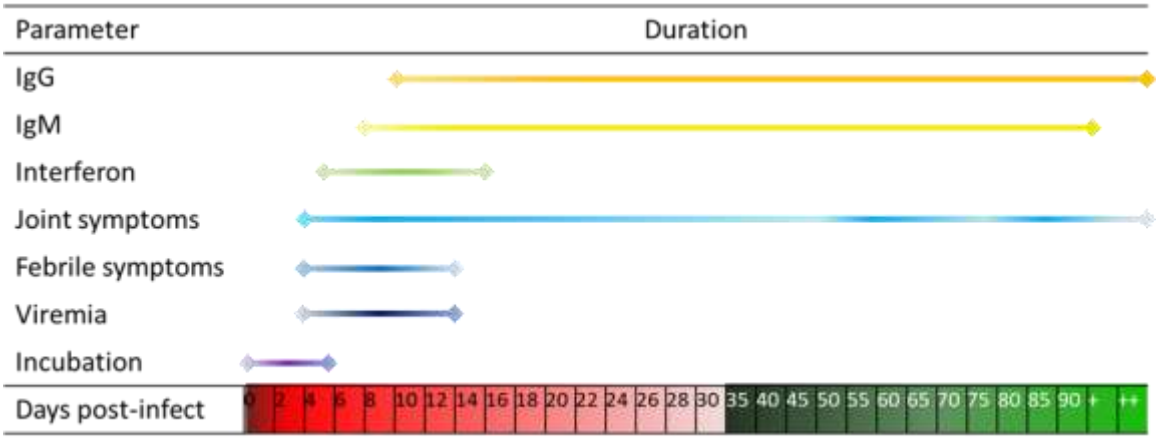

Illustration 3. Duration of symptoms and relevant immune responses after exposure to CHIKV.

Days in red indicate acute-convalescent stage, while days in green represent post-convalescence. After an incubation of 4-6 days, flu-like symptoms rise in conjunction with viremia. Joint symptoms also begin at that time, and may be sustained for long periods or recur at intervals. IgM titers rise around 8-12days after initial exposure (4-8 days after onset of symptoms), and IgG levels begin to rise shortly thereafter at about 12-16 days after initial exposure. A typically short-lived but intense interferon response begins shortly after viremia level rises, and prolonged interferon response may indicate a potential for chronic or recurring arthritis.

Chronic CHIKF

Although febrile symptoms resolve within a few days and lingering joint symptoms usually resolve completely within one month of disease onset, up to 70% of CHIKF patients develop chronic or recurring joint symptoms (74). These symptoms can last anywhere from a few months to years after initial disease convalescence. The pathogenesis of chronic and recurring CHIKF arthritis and arthralgia remains a mystery, with very few predictive markers for disease

chronicity. Some physicians and scientists postulate that preexisting joint conditions predispose patients to chronic CHIKF symptoms, as many patients with chronic joint symptoms have underlying medical problems ranging from asthma to arthritis and diabetes (74-76). However, the number of patients specifically with previous joint maladies, such as osteoarthritis, varies widely between epidemics, ranging between 0-51% (75-79). Another hypothesis is that chronic CHIKF arthritis is aggravated rheumatoid arthritis (RA), with CHIKV infection exacerbating subclinical/mild RA or activating latent RA (80). However, only 12% of chronic CHIKF patients test positive for rheumatoid factor and even fewer (6%) test positive for anti-cyclic citrullinated protein antibodies, both of which indicate that the majority of patients tested negative for the major diagnostic tests for RA in that particular study (81). Still other virologists and rheumatologists insist that chronic CHIKF arthritis constitutes seronegative RA (82, 83). This language is misleading, however, as seronegative RA represents a heterogeneous pool of idiopathic arthritides loosely defined by generalized immune dysfunction leading to polyarthritis and joint destruction, many with distinct clinical features and likely distinct etiologies despite similar clinical outcomes (84-87). Moreover, while CHIKV does elicit prolonged joint pain and inflammation, symptoms usually resolve and outright joint destruction is rarely observed. Because many aspects of CHIKV arthritis do not align with general diagnostic criteria for rheumatoid arthritis, especially since the role of the immune system in human CHIKF arthritis hasn't been entirely elucidated, many scientists describe CHIKF arthritis as rheumatoid-like or a rheumatoid arthritis mimic (88).

Nevertheless, chronic rheumatoid-like arthritis following CHIKV infection has been a topic of scientific interest, and some empirical studies have been performed in the pursuit of elucidating a pathophysiological mechanism. For example, it has been proposed that the polarity of the immune response to initial infection may predict the development of chronic symptoms, with a mixed TH1/TH2 response predicting completely resolving symptoms and a prolonged pro-inflammatory TH1-polarized response indicating potentially chronic symptoms (Ill. 4) (89, 90).

Interestingly, increased eotaxin levels seem to correlate to completely resolving symptoms (91). It is unclear whether viral presence is causative, but CHIKV RNA has been detected in macrophages isolated from affected joints of chronic CHIKF patients (77, 92). In general, it has been observed that women are more prone to develop chronic joint symptoms, but the effect of age on chronic joint symptom development is variable and dependent on the specific outbreak (77, 90, 91). Similarly, reports on the effect of viral load on chronic joint symptom development are inconsistent (77, 91). However, this may be due to the fact that the overwhelming majority of CHIKV epidemiology studies employee quantitative real-time PCR to quantify viral load, which does not necessarily reflect infectious viral load and is extremely sensitive to differences in primer/probe and reference standard design. Interestingly, it has also been observed that duration of IgM response correlates to both increased severity and duration of CHIKF-related joint symptoms (75, 93). Whether these data indicate the persistence of virus in host tissue, or persistence of a specific immune trigger or molecular mimicry, has yet to be determined.

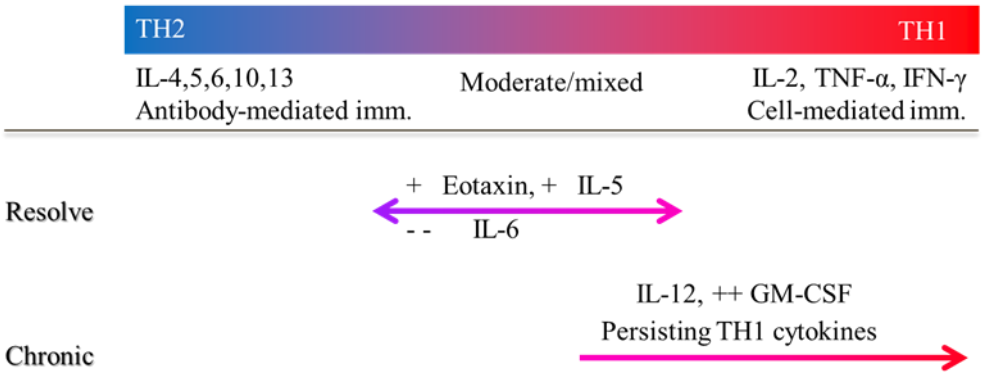

Illustration 4. Potential immune predictors of chronic or recurring CHIKV arthritis.

A sustained inflammatory TH-1 polarized cytokine response piquing cell-mediate immunity may indicate chronic or recurring arthritis, while a moderate or mixed response relying on antibody-mediated immunity may predict a completely resolving case.

Societal Consequences of CHIKF

Taking into account missed work days due to severe symptoms, hospitalizations, and cost of treatment, CHIKV is a considerable economic burden in epidemic and endemic areas. For

example, it is estimated that the 2005-2006 CHIKF epidemic on La Reunion island cost €43.9 million (approx. 60.4 million USD, circa 2005) in terms of healthcare costs associated with infection (94). This estimate excludes the cost of long-term treatment, such as for persistent rheumatic symptoms. Another study following a cohort of gendarmes from the Reunion epidemic found that a significant portion of those infected with CHIKV continued to seek medical care up to 30 months after the acute infection resolved (95). Similarly high costs associated with CHIKV disease are well documented throughout India, where most medical expenses are out-of-pocket and thus present a significant financial challenge to families affected by the disease (96-98). Calculation of disability adjusted life-years (DALYs) is a common way to measure the effect of a non-lethal disease, such as CHIKF, on a population; the calculation takes into account both years of life lost to mortality, as well as to physical and financial disability, so that the impact of both lethal and non-lethal diseases can be compared for a given population (99). As one might expect, CHIKF outbreaks precipitate severe losses in DALYs through medical expenses and years lived with physical disability (94, 96, 97, 100). Although estimates vary by outbreak and population, to compare DENV and CHIKV in Colombia: estimates suggest that the DALYs lost per 100,000 citizens due to CHIKF were as high as 300 in some areas of Colombia in 2014 (101, 102), whereas DENF is estimated to cause close to 100 years lost per 100,000 citizens in Colombia during epidemic years (103).

Although CHIKF is not typically fatal, the morbidity and associated costs incurred by CHIKF is damaging to both individuals and populations, making the problem of CHIKV spread a translational research priority.

THE BASICS: ALPHAVIRUS MOLECULAR BIOLOGY AND REPLICATION

Alphavirus genome organization and replication

Despite translational research deficits, much has been elucidated regarding the basic biology of alphaviruses, which can be applied to all alphaviruses including CHIKV. Similar to flaviviruses, alphaviruses have a positive sense, single-stranded RNA genome. The typical alphavirus genome is approximately 11kb in length and encodes 9 proteins: 4 non-structural, nsP1-4; and 5 structural, capsid, E3, E2, TF/6K, and E1. The genome encompasses two open reading frames, one genomic-length and a second under control of a separate sub-genomic promoter. Because the genome is capped and poly-adenylated, translation of the alphavirus genome begins once viral RNA enters the cytosol. The non-structural proteins then transcribe anti-sense RNA, from which full-length sense transcripts as well as subgenomic transcripts are produced (Figure 5).The non-structural proteins are expressed from full-length genomic RNA and are primarily involved in replication of the viral genome and subversion of host processes, including pathogen recognition and normal homeostatic functions. All 4 non-structural proteins are translated as one poly-protein. This poly-protein is cleaved post-translationally at different steps during the RNA transcription process by nsP2, beginning with cleavage of nsP4, followed by nsP1, and then finally nsP2 and 3 (104-106), although these cleavage events may not be strictly necessary for the replication complex to function (107). The existence of an opal stop-codon near the C-terminus of nsP3 in all but a few ONNV and SFV isolates skews the translation bias to translation of an incomplete nsP123 polyprotein, with fewer copies of the full nsP1234 polyprotein (108, 109); the role of this opal stop codon remains unclear, but it has been suggested that it may have a role in alphavirus pathogenesis (110).

Alphavirus Non-structural Protein Function

The specific roles of the various non-structural proteins and genomic elements are described in Table 1. nsP1 exhibits both guanyl- and methyltransferase activities, which are required for viral RNA capping and subsequent association of both genomic and subgenomic viral RNAs with host ribosomes (111, 112). nsP2 possesses a myriad of proposed enzymatic

functions, although the main 3 are: helicase, nucleotide triphosphatase (NTPase), and cysteine protease. The

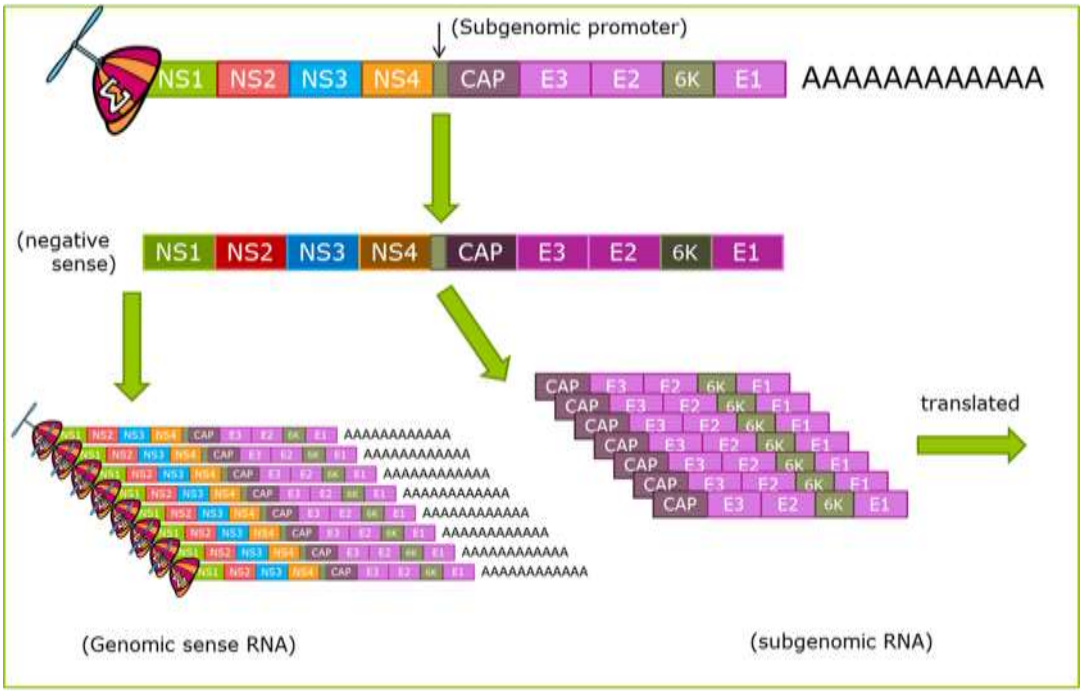

Illustration 5. Alphavirus replication.

Immediately upon entry into the cytosol, the alphavirus genome acts as mRNA and the non-structural proteins are translated. These proteins, in conjunction with cis-acting elements within the genome, transcribe negative sense RNA from which new genomic-sense RNAs, as well as sub-genomic RNAs, are transcribed. Genomic-sense RNAs are packaged into new virions, while the subgenomic RNAs are translated into the structural poly-protein.

helicase domain of nsP2 is responsible for unwinding secondary RNA structure, allowing the RNA-dependent RNA polymerase (RdRp) access to the RNA (113, 114), and is intimately associated with the protein's NTP-ase activity (115). The protease domain functions independently of the helicase function, and is essential for processing of the non-structural polyprotein into its independent constituents (116, 117). Finally, the NTPase function is vital to numerous processes, including aiding in RNA capping by nsP1 (118). nsP2 of some alphaviruses including CHIKV also plays a role in shutting off host functions, particularly synthesis of

macromolecules (119, 120), as well as potentially in the synthesis of subgenomes as a transcription factor (although this particular role has not been confirmed)(121). For CHIKV, nsP2 also plays a role in antagonizing the unfolded protein response (122). nsP3, although indispensable for alphavirus negative-sense genome and sub-genome production, has been substantially less well characterized in terms of function than the other non-structural proteins. Three domains have been identified, and some functions have been inferred from structural motifs: a macrodomain, which has been confirmed in VEEV and CHIKV to bind nucleic acid and poly-ADP-ribose, as well as exhibit ADP-specific phosphatase activity (123, 124); an alphavirus-unique domain, mutation of which results in defects in RNA synthesis and polyprotein processing (125, 126); and a hyper-variable domain, which for many alphaviruses, including CHIKV, seems to facilitate virus/host protein interactions (127-129) and may affect cellular tropism (37, 38). nsP4 is the viral RdRp, which transcribes both anti-sense and positive sense RNA (both genomic and subgenomic)(121).

Table 1. Alphavirus non-structural protein functions.

Protein	Size	Function	Reference(s)
nsP1	60KDa	RNA capping	111,112
nsP2	90KDa	Cysteine protease, NTPase, helicase, host synthesis shut-off, subgenome transcription	113-122
nsP3	60KDa	Host-virus interactions, virus-specific roles	37, 38, 123-128
nsP4	70KDa	RNA-dependent RNA polymerase	120

Alphavirus Untranslated Regions

Two other important non-structural elements in the alphavirus genome are the 5' and 3' UTRs flanking the translated regions of the alphavirus genome. While neither of these results in synthesized product, RNA-structural elements found in the UTRs play important roles

in alphavirus replication. Switching 5' UTRs between alphaviruses or completely ablating the 5' UTR has a dramatic impact on negative-strand synthesis, likely owing to a structural mismatch between corresponding conserved sequence elements (CSE) in the 5' and 3' UTRs (130, 131). Additionally, the 5' UTR aids in translation by modulating the binding of host translational factors, namely eIF4E and eIF4F, to viral RNA, potentially through secondary RNA structure (132, 133). Lastly, the 5' UTR confers immunity from certain interferon-stimulated gene products (ISGs), which preferentially recognizes the 5' cap methylation strategy employed by viruses that produce their own RNA caps (134); again, this is likely through secondary structural elements (134). The 3' UTR is the larger of the two UTRs, and varies significantly by both species and strain of alphavirus, although there is a 19-20 nucleotide (NT) conserved sequence element (CSE) immediately preceding the poly-A tail (135), which can be found across the *Alphavirus* genus. This particular CSE acts as the promoter for negative strand synthesis (136, 137), a necessary intermediate for full length RNA synthesis.

These non-structural elements are all essential for efficient alphavirus replication, and therefore deserve mention here. However, the scope of this work does not include discussion of non-structural proteins and limited discussion of the 3' UTR. For a detailed discussion of these elements, these features have been reviewed by several experts in the field (35, 138).

STRUCTURE OF ALPHAVIRUS VIRION

Alphavirus Structural Proteins

The alphavirus structural proteins are vital to infectivity and expression thereof is regulated independently from the structural proteins. A unique feature of the alphavirus genome is the sub-genomic promoter, which controls expression of subgenomic mRNA from genomic length anti-sense RNA (139, 140). The alphavirus subgenomic promoter is the basis for the differential expression of structural and nonstructural genes, resulting in many fold more

structural proteins than non-structural proteins (141). This difference in expression level is important for efficient packaging of viral RNA into functional virions, as many more structural proteins are needed per virion than genome.

There are 5 viral protein components to the virion (Table 2), as well as viral RNA and potentially host factors (Ill. 6). The viral capsid protein, as the name suggests, is the primary structural protein in the core nucleocapsid (142-145). The capsid is enveloped in a lipid membrane derived from the host cell membrane, in which the viral envelope proteins are embedded. E3, a small secreted accessory protein, serves an important role in the transport of the E2/E1 heterodimer to the cell membrane by protecting the E2/E1 heterodimer from acid-induced conformational changes in the ER and Golgi (146). However, switching E3 proteins between different alphavirus complexes (VEE, EEE, WEE, SFV) is poorly tolerated and results in attenuated replication and malformed virus particles, indicating that E3 also possesses complex-specific roles in assembly (147). E2 primarily mediates binding, both to heparan sulfate and currently unidentified viral receptors on the host cell surface which are likely unique to a given species of alphavirus (148). Additionally, 33 amino acids near the carboxy terminal of E2 interact with the nucleocapsid core, thus playing an important role in virion stability and structure (149). In the study of alphavirus proteins, uncleaved E3/E2 protein is referenced as pE2 or p62 (in the case of SFV). Cleavage of E3 from pE2/E1 is essential for subsequent viral entry and fusion processes, as it has been observed that SINV particles containing pE2 can bind heparan sulfate, but not enter host cells (150). Despite the importance of pE2 cleavage, E3 is occasionally found in the alphavirus virion along with the E2/E1 heterodimer (151). The alphavirus E1 protein is structurally homologous to the flavivirus E protein and functions similarly, mediating fusion of the viral envelope with the endosome membrane after the virion enters the cell via receptor-mediated endocytosis (152, 153). 6k is another small protein (6kDa), which is present only in small quantities between 7-30 copies on the surface of the assembled virion, and has been

described as a viroporin. Specifically, 6K appears to form cation-specific ion channels which aid in release of the assembled virion from the cell (154-156). Transframe, or TF, is the result of a -1 NT frameshift upstream of 6k, resulting in a newly described and little studied structural protein; it appears to function similarly to 6K, but this has yet to be confirmed (157). Following the observation that the efficiency of SINV infection improves over the disease course in cell culture (158), some studies have revealed that there are two varieties of SINV virion, heavy and light, which demonstrate differential infectivity based on the encapsidation of host ribosomal proteins along with viral RNA and capsid protein (159).

Table 2. Alphavirus structural protein functions

Protein/element	Approx. Size	Function	Reference(s)
Capsid	30KDa	Nucleocapsid core structure	141-144
E3	7KDa	Folding, transport of E2/E1	145, 146,171, 179
E2	52KDa	Receptor binding	147, 148, 170
6K	6KDa	Membrane modification	1553-155
Transframe	8KDa	Ion channel	156
E1	49KDa	Membrane fusion	151,152,169,174,175

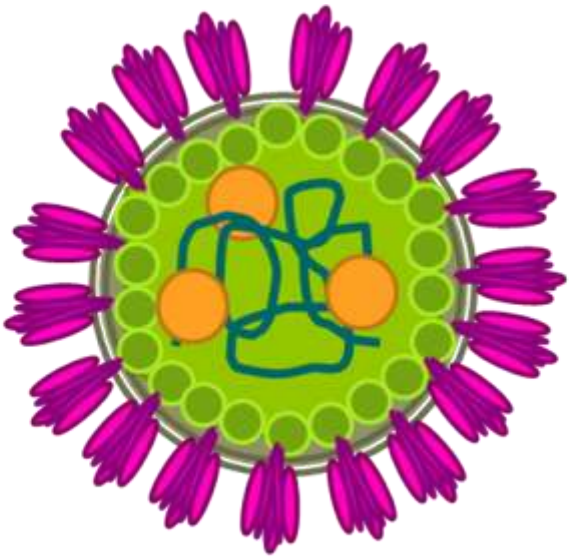

Illustration 6. Alphavirus virion structure.

One viral genome copy (blue), potentially with host ribosomal factors (orange), are encapsidated in viral capsid protein. The nucleocapsid assembly is anchored to a host-derived lipid membrane by several amino acid residues near the carboxy terminal of E2 (purple). E2 is dimerized with E1 (pink), and these dimers form trimeric spikes embedded in the lipid bilayer by transmembrane helices.

Virion structure and maturation

Together, these individual components are assembled into a highly structured virion, often used as a model for symmetric viral particles (Figure 7). The capsid protein self-assembles into an icosahedral structure with a T=4 symmetry (160), indicating that 240 copies of capsid are assembled into each virion (142-144, 152). E2/E1 heterodimers are assembled into heterotrimers, of which there are 80 embedded in the host-derived lipid bilayer. The lipid bilayer is anchored to the capsid by the E2 protein, creating a tight 40-70nm virion, depending on alphavirus species (151, 161, 162).

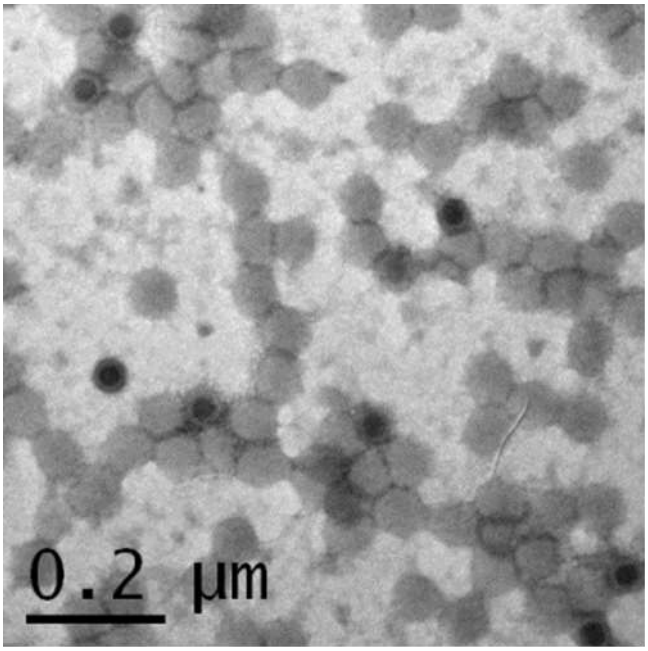

Illustration 7. Transmission EM image of CHIKV.

Negative stain of CHIKV particles demonstrating tightly structure icosahedral virions.

Like the non-structural proteins, the alphavirus structural proteins are expressed from a single transcript encoding a poly-protein, which is processed both nascently and post-translationally (Figure 8). The capsid is autocleaved upon translation, revealing an endoplasmic reticulum (ER) retention signal at the N-terminal of E3, directing the rest of the polyprotein into the ER immediately upon translation for processing (163). 6k is cleaved at both N and C-terminals by ER signalases (164, 165), upon which E1 is further processed and then dimerized with pE2 (166, 167). The entire pE2/E1 assembly is then transported to the Golgi, where E3 is finally cleaved by furin (168). E3 then re-associates with the mature E1/E2 heterodimer, after which the complex is transported to the cell membrane where the completed virion is assembled and E3 is finally dissociated from the E2/E1 monomeric spike (169).

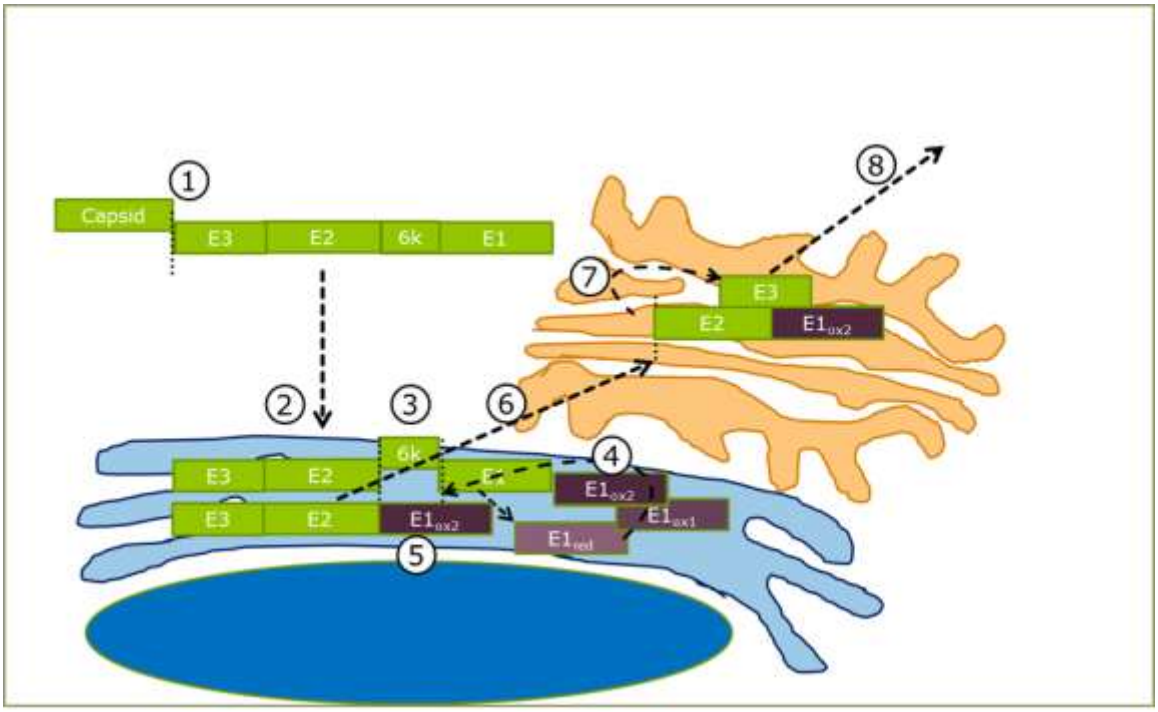

Illustration 8. Alphavirus structural protein processing.

1) capsid is autocleaved from the polyprotein, revealing 2) a ER translocation signal at the N-terminal of E3, directing the rest of the polyprotein into the ER. 3) 6K is cleaved by signalases, and E3 4) goes through 3 defined oxidative intermediates before 5) dimerizing with E2. The entire assembly 6) moves to the Golgi, where 7) E3 is cleaved by furin. E3 reassociates with the E2/E1 heterodimer, and the mature E2/E1 heterodimer is 8) transported to the cell membrane.

Envelope Protein Structure and Folding

Contributing to the tight virion structure are many important post-translational modifications, particularly within the envelope proteins, including glycosylation, palmitoylation, and disulfide bond isomerization. There are numerous disulfide bond-forming cysteine residues throughout the alphavirus envelope proteins, among which 16 in E1 (170), 12 in E2 (171), and 2 in E3 (172) are highly conserved across the alphavirus genus (Ill. 8) (162). Together, these disulfide bonds are important for both the folding and native structure maintenance of the envelope proteins.

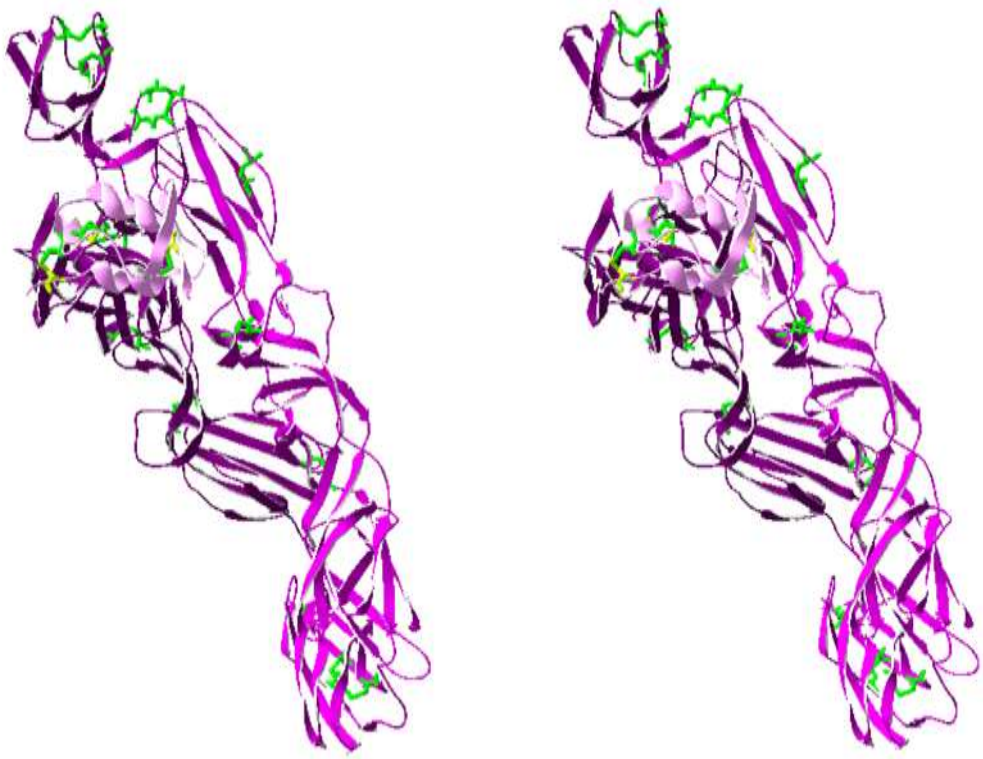

Illustration 9. X-ray crystallograph of uncleaved CHIKV pE2/E1 in stereo.

Stereo-image of CHIKV envelope proteins E1 (pink), E2 (dark purple), and E3 (light purple) show extensive disulfide bonding (green). Modified from the structure solved by Voss et al (2010; PDB ID 3N40).

Exogenous reducing agents, which reduce cysteines participating in disulfide bonds, have played a critical role in identifying and characterizing the role of disulfide bonds in alphavirus envelope folding and structure. Kaluza and Pauli first noted the importance of disulfide bonds when investigating the effects of treating purified SFV particles with ß-mercaptoethanol (BMC) (173). Not only did treated particles demonstrate differential electrophoretic mobility under reducing- and non-reducing conditions, but glycosylation patterns and hemagglutination activity were also affected when particles were treated with BMC. Together, their data indicate that reducing disulfides altered E1 and E2 conformation, sometimes enough to alter antibody binding. Anthony and colleagues made similar findings, noting that SINV virions treated with another reducing agent, dithiothreitol (DTT), were more sensitive to

protease activity (174). Additionally, they noted that exposure to low-pH conditions prior to DTT treatment not only enhanced DTT-induced protease sensitivity, but also induced conformational changes in the SINV envelope as seen by EM, which correlated with decreased infectivity of treated virions in tissue culture. Interestingly, because the effect of DTT treatment was only visually apparent when added in acidic conditions, it was suggested that exposure of disulfide bonds was conformation-dependent. These findings were further corroborated when Mulvey and Brown compared the electrophoretic mobility of the SINV envelope proteins in reducing and non-reducing conditions. Importantly, they also identified three distinct sulfide-mediated oxidative intermediates through pulse-chase analysis—named E_{α}, E_{β}, and E_{γ}—before E1 reaches its metastable native state E_{ε} (170). It was later found that the sensitivity of disulfide bonds within the SINV E1 protein to DTT decreases with each intermediate E1 species, until finally the E2/E1 heterodimer is completely resistant to the effects of DTT (175). This same study found that association of pE2 with E1 is highly sensitive to reducing agents and fails to dimerize in the presence of DTT, although are resistant post-dimerization. Later, the functional role of disulfides in SINV E1 was further characterized by Phinney and Brown, who subjected purified E1 protein to various proteolytic and reducing conditions and analyzed samples by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) analysis. They found that E1 is divided into two clear domains by distinct disulfide groupings, with implications in the mechanism of membrane fusion (176). It is abundantly clear that disulfide bonds play a critical role in the folding, structure, and function of the alphavirus E1 protein. However, it has been shown that cysteine residues in the E2 protein are important for RRV and SINV virion assembly and infectivity. Mutating these residues alters virion morphology and negatively impacts viral replication *in vitro* in a virus-specific manner (171).

Envelope Proteins and ER Chaperones

Because it has long been known that the alphavirus envelope proteins are transported through the ER and Golgi, paired with the importance of disulfide bonds in envelope protein structure, Molinari and Helenius also investigated potential associations of SFV with ER chaperones. Through pulse-chase analysis, paired with cross-linking and pull-down assays, they discovered that not only do p62 and E1 fold separately, but also that protein disulfide isomerase (PDI), as well as ERp57, interact transiently with both E1 and p62 by forming mixed disulfides (177). ERp57 was universally associated with calnexin and calreticulin whereas PDI was not, which is unsurprising given that ERp57 requires these as co-factors for binding glycosylated substrate (178, 179). In addition, it has been suggested that E3 may possess thioreductase activity similar to PDI and ERp57. Firstly, similar to early application of DTT, replacement of the E3 protein with the ER translocation signal results in failure of SFV p62 to dimerize with E1 (180). Secondly, mutating cysteine residues that participate in disulfide bonds in the E3 protein of SINV results in attenuation of replication *in vitro* or completely unviable virus (172). This, in conjunction with the presence of either a thioredoxin-like Cys-X-X-Cys or a Cys-X-X-Pro-Pro-X-Cys motif in all alphavirus E3 proteins, makes an intriguing argument for E3 acting as a protein disulfide isomerase specifically catalyzing the formation and shuffling of disulfide bonds within the pE2 and E2 proteins. In light of these results, thioreductases most likely play an important role in alphavirus replication by virtue of forming and isomerizing disulfide bonds within the envelope proteins.

In the study of protein biology, “structure equals function” is an important paradigm. As demonstrated by previous research cited here, altering the structure of an alphavirus virion, either in its entirety or through its individual constituents, will adversely affect virion stability and viability, as well as the viral processes for which constituent proteins are responsible. Chief

among these functions are viral attachment and entry, abrogation of which would effectively eliminate the ability of the virus to replicate within host cells.

CHIKV IN TRANSLATION: ANIMAL MODELS, VACCINES, AND THERAPEUTICS

While scientific interest in CHIKV has increased substantially since its debut in La Reunion, FDA-approved vaccines and therapeutics remain elusive. Palliation remains the only treatment option for CHIKF, with alleviation of symptoms being the primary goal of treatment. Fever-reducing and anti-inflammatory drugs are commonly administered to control high fevers, with the World Health Organization (WHO) recommending acetaminophen or NSAIDs, as well as ice on affected joints. Additionally, the only disease prevention strategy recommended by the WHO is to limit exposure to vector mosquitos (181). Pharmaceutical disease prevention and/or virus-targeted treatment would greatly enhance physicians' ability to manage CHIKF patients, and although no FDA-approved products have emerged, there have been endeavors to generate vaccines and antivirals.

Animal Models of CHIKF

In order to develop vaccines and therapeutics, appropriate animals models are essential. There are several models of CHIKF in animals, although murine models are the most common. CHIKV-mediated disease has been described in several strains of mice, including C57Bl/6 (182, 183), CD-1 (184), various ages of and knock-outs on these backgrounds (183, 185, 186), as well as varying ages of the IFN-α receptor knockout on the 129 background (A129) (187, 188). The most popular among these are C57Bl/6 mice of different ages and the A129 mouse. In general, several parameters may be used to evaluate the efficacy of a drug or vaccine in murine models of CHIKV infection: viral titers in blood and tissue, histopathology of organs and muscle, development of CHIKV-specific antibodies, and "clinical" signs including weight loss, hunched posture, lack of grooming, and footpad swelling (182-188). The last sign may, regardless of

strain, be measured in mice which were inoculated with CHIKV in the footpad (189), even in the absence of detectable replicating virus (personal observation). However, mice only develop footpad swelling in the inoculated foot and is not observed in contra- or ipsilateral limbs, so the utility of this measure is debatable. All measurable outcomes are affected by mouse age, with younger mice being more susceptible to CHIKV infection (182, 183, 188). Advances in technology have also allowed researchers to easily track temperature of mice through telemetric microchips (190), as well as track dissemination of virus labeled with fluorescent protein or bioluminescence enzymes using *in-vivo* imaging systems (IVIS) (191). Quantification of virus is commonly measured by either quantitative real-time PCR or plaque assay, and antibodies are commonly quantified by plaque reduction neutralization (PRNT) (Figure 10).

In addition to other outcome measures, the A129 model of CHIKF is a universally lethal model of CHIKF (187, 188). Regardless of injection route, CHIKV is able to disseminate throughout the A129 mouse, even reaching high titers in brain tissue (187). Depending on the specific strain/isolate used and the age of mice, time-to-death ranges between 2 and 7 days, with most A129 mice succumbing to infection within 3-5 days (187, 188). Because of this, prevention or delay of death is a useful and commonly used tool for evaluating new drugs and vaccines. However, the stringency of this model, owing to the critical immune deficiency in interferon (IFN) signaling, may obscure the true potential of various candidate drugs which may otherwise be sufficient in an IFN-competent host. In short, negative hits in this model may deserve another look in a second model.

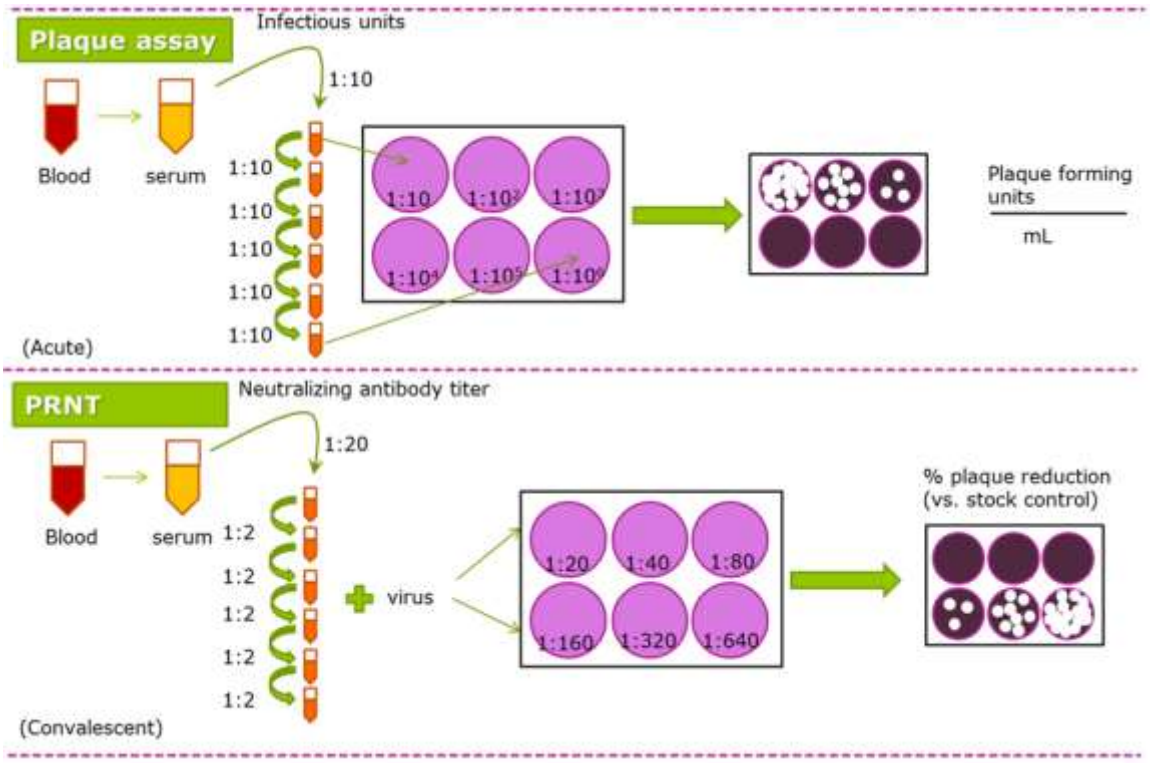

Illustration 10. Plaque and plaque reduction neutralization assays.

Plaque assays evaluate the amount of infectious virus in an acute sample, either from patient, animal, or tissue culture. Sample is diluted by 10-fold dilutions, and each dilution is plated on a monolayer of cells. When covered with a viscous material, punctate areas where cells have sloughed off (plaques) and are visible when sample is fixed and stained. Plaque-forming units are calculated, under the assumption that one virus=one plaque. Conversely, plaque reduction neutralization tests evaluate the neutralizing antibody titer in a convalescent (post-acute) sample, in which virus in no longer present. Serum containing antibodies is diluted 10-fold, and then incubated with stock virus, allowing neutralizing antibodies to bind to virus. Samples are then plated on confluent cell monolayers, and are incubated with a viscous material until plaques form. Values are determined based on which dilution achieves a specified percent plaque reduction (50%, 80%) compared to a control plate with untreated virus.

Murine models are often used for initial vaccine and therapeutic testing, but a disease model which more accurately reflects human disease may be found in one of several non-human primate models, predominately cynomolgus macaques (*Macaca fascicularis*) (192, 193) or rhesus macaques (*Macaca mulatta*) (194). These models more closely mimic human disease, with NHPs developing viremia, intermittent hypothermia, localized temperature increases in swollen joints,

rash, and lymphopenia (193, 194). Pathogenesis and transmission studies have been performed in NHPs (193, 195), as well as vaccine studies (192, 196, 197). Ultimately, before any therapeutic or vaccine can realistically be moved to clinical trials, they must show efficacy in an NHP model. Other models for CHIKV infection exist, including a hamster model (198); however, these models are rarely used, especially in a translational context, due to poor recapitulation of human disease in combination with either poor model characterization or lack of readily available model-specific reagents such as antibodies.

CHIKV Vaccines

No vaccines to date have been FDA-approved for human use to prevent CHIKF. However, several candidates have surfaced over the years. Most prominently, in response to the outbreaks in the 1950s and 60s, the United States Army developed a live-attenuated CHIKV vaccine, officially dubbed 181/clone 25 and colloquially referred to as "CHIKV army," or "CHIKV army vaccine" (197). The vaccine was developed by plaque-passaging the ASN CHIKV 15561 strain in MRC-5 cells 18 times, resulting in two attenuating mutations in the E2 protein (199). These mutations enhance the ability of the virus to bind heparan sulfate, reducing the ability of virus to disseminate *in vivo*; this allows the virus to elicit an immune response with limited systemic effects. The vaccine was tested in humans and made it to phase II clinical trials (200). However, the US Army dropped the project for a variety of reasons. The vaccine did cause joint symptoms in a small number of trial participants, and perhaps the perceived need for a vaccine decreased as major CHIKF epidemics receded (201). The 181/clone 25 virus remains useful, however; an infectious clone for the strain was created and the vaccine strain continues to be used as a positive control in contemporary CHIKV vaccine experiments and is a useful tool for studying CHIKV pathogenesis and drug screening *in vitro* at a level 2 biosafety level due to its unimpeded *in vitro* replication (whereas wild type CHIKV is studied in biosafety level 3) (189, 199).

Many other approaches to a CHIKV vaccine have been developed since 2005, in response to the Indian Ocean CHIKF outbreak. Several platforms have been utilized toward this end. Since live-attenuated viruses generally elicit stronger immune responses in those with robust immune systems (202, 203), this has likewise been a popular strategy for CHIKV vaccine development. Prominent in this group is the CHIKV-IRES vaccine strain (189). This strain was generated by ablating the subgenomic promoter of the IOL CHIKV-LR OPY2006 strain and inserting an internal ribosomal entry site (IRES) in its place, placing the structural protein translational under control of the IRES element directly from genomic RNA. In effect, this decreases envelope protein expression (189). The vaccine fully protected both mice and cynomolgus macaques against CHIKV infection (191, 192). This same attenuation strategy has been successfully applied to several other alphaviruses, including VEEV and MAYV (204-206). While the CHIKV-IRES vaccine successfully protects both mice and cynomolgus macaques from CHIKV challenge, it has yet to undergo trials in humans.

Many other attempts have been made to develop an ideal CHIKV vaccine, some of which have moved to phase II clinical trails. The most salient attempts are discussed here, but the others have been reviewed expertly elsewhere (207).

CHIKV Antivirals

FDA-approved CHIKV antivirals likewise have yet to emerge. Like vaccines, there are different approaches to antiviral development. Instead of priming the immune system, though, antivirals target the various aspects of the viral replication cycle: entry, RNA replication, protein translation, and assembly/egress (Figure 11). Interfering with RNA replication is the most common approach, with a wide variety of mechanisms to exploit. A popular target of this approach is nsP2 due to its wide array of functions. Bassetto and colleagues rationally designed an nsP2 inhibitor using an *in silico* structure-based screen, which yielded a compound thought to bind the protease active site of nsP2 (208). The compounds from this screen were subjected to

cytopathic effect (CPE) assays, which evaluates the ability of a drug to inhibit ablation of the cell monolayer due to CHIKV-induced cell death; one compound was found to limit CPE with a 50% effective concentration (EC_{50}) of 5μM (208). A second structure-based screen revealed arylalkylidene as a potential chemical scaffold for developing nsP2 targeted therapeutics, resulting in the identification of 5 compounds with an EC_{50} below 5 (209). Another potential compound was discovered when a group used *in vitro* compound library screens paired with a novel nsP2-specific phenotypic assay in yeast (210). Although the compound identified by this group had a relatively high EC_{50} of 31 μM, this screen provides a valuable tool for identifying nsP2 inhibitors.

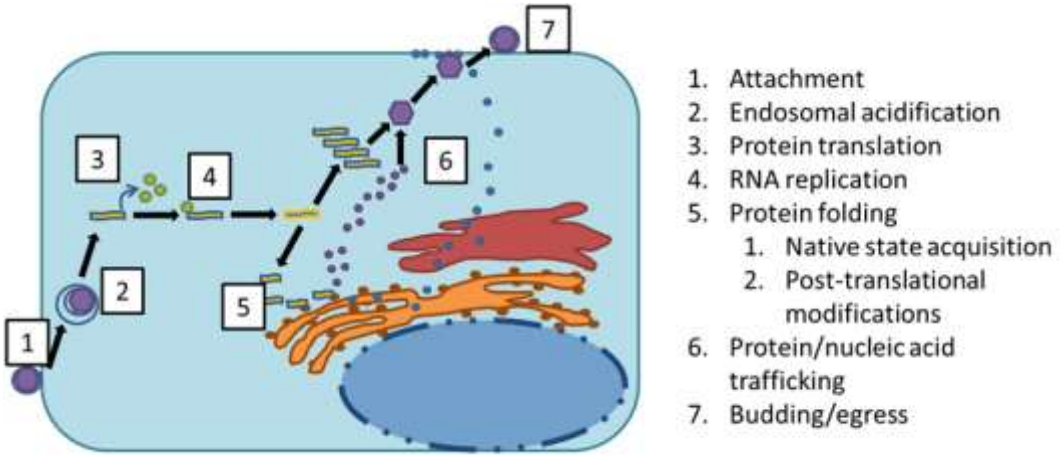

Illustration 11. Potential processes to target for antiviral agents against CHIKV replication.

As with other viruses, nucleoside analogues are another popular choice for CHIKV inhibition. Although some nucleoside analogues have multiple antiviral mechanisms, all share at least one mechanism of action: nucleoside analogues mimic nucleotides, and become misincorporated into the viral genome when a virus replicates. Due to the lack of proof-reading mechanism in viral RdRps, these misincorporations are not corrected and result in truncated and

non-functioning viral genomes (211). Several have been tried against CHIKV, including ribavirin and 6-azauridine. Ribavirin, a guanasine analog, has generally performed unconvincingly *in vitro*, reducing virus titers by modest amounts (<2 log $TCID_{50}$) at mM concentrations when singularly applied (212). Other studies have found ribavirin to greatly reduce viral replication *in vitro* (213), while other studies have found that ribavirin performs better when applied with exogenous type-1 IFN (212), and has also been observed to expedite resolution of joint symptoms in small cohorts of patients (214). Still other studies in rhesus macaques found ribavirin to ineffective at reducing viremia or alleviating disease (215). These results remain unverified by proper clinical trials, however, and the literature regarding ribavirin treatment for CHIKV remains inconsistent. 6-azauridine is much more effective than ribavirin, with modest virus reduction (<2 log $TCID_{50}$) at 1.6 μM (212), but has never been tested in CHIKF patients.

Finally, the recently developed favipiravir (T-705) likely functions by inhibiting the ability of the RdRp to incorporate ATP and GTP, although the precise mechanism by which this leads to impaired viral replication is unclear (216). Favipiravir has shown efficacy against many different isolates of CHIKV *in vitro*, as well as reduced mortality in AG129 mice (type-1 and type-2 IFN knock-out) (217). Favipiravir continues to be a compound of interest in the CHIKV antiviral field.

Researchers have also targeted the viral entry process, as preventing progeny virus from entering subsequent cells would effectively control replication and system spread. Arbidol (umifenovir) is an antiviral already approved for the treatment of respiratory viruses, including influenza, in Russia and China (218). Arbidol itself was found to inhibit CHIKV with an EC_{50} of 12 μM (219), while a several derivatives were developed to improve the selectivity index against CHIKV (220). In general, arbidol is hypothesized to affect virus binding to the host membrane (219), although it may also affect entry processes down-stream of adsorption, such as with fusion with the endosomal membrane (221). Chloroquine is another broad-spectrum drug used for other

indications, namely malaria. However, it was found that chloroquine inhibits CHIKV replication, among other viruses, in a dose-dependent manner *in vitro* (222). The proposed mechanism is that chloroquine interferes with the acidification of the endosome, an important event which activates the E1 fusion protein (223, 224). However, clinical trials in small cohorts failed to show efficacy in a human disease setting (225), and this was later confirmed by other parties (226).

Similar to other entry inhibitors, but generally classified as their own genre of antiviral, are potential monoclonal antibody treatments. Instead of using a small molecule inhibitor to bind small pockets of a protein, monoclonal antibodies bind epitopes of a protein and thus neutralize its function. This is a primary adaptive immune defense against alphavirus infection, and is often used as a correlate of protection in vaccine studies. Therefore, purified monoclonal antibodies against CHIKV represent an alluring therapeutic option. Several antibodies produced by B-cells that have been isolated from CHIKV patients have been tested both *in vitro* and in animal models. Two monoclonal antibodies, 5F10 and 8B10, isolated from a recovered CHIKF patient, greatly reduced plaque production by CHIKV in a PRNT assay with cross-strain efficiency (227). Efficacy in AG129 mice was also observed, with prophylactic treatment protecting 100% of mice and post-exposure treatment delaying time to death significantly, by 10 days (227). MAb C9, similarly isolated from a convalescent CHIKF patient, neutralized CHIKV *in vitro* as well offered protection from footpad swelling and viremia *in vivo* in C57Bl/6 mice, when given prophylactically or therapeutically (228).

Protein synthesis is another major aspect of viral replication, since RNA viruses in general must rely on their own enzymes to replicate their RNA, in addition to producing their own specific structural proteins to ensure progeny virus can infect the next cell. Several extremely successful attempts have been made in this area, including small interfering RNA (siRNA) therapeutics and harringtonine. siRNA therapeutics represent a range of strategies, from free oligonucleotides or short-hairpin RNA (shRNA)-encoded plasmids to strategically vectored

therapeutics using adenoviruses or self-assembling lipids or peptides. Some siRNA work is described here, although the nuances of siRNA therapeutic delivery are outside the scope of this work. While siRNA can target any number of nucleotide sequences in any of the CHIKV genes, the end result is obstruction of the translation of viral transcripts. Parasher et al. describe two siRNA sequences, one targeted to E2 and one to nsP1, which not only significantly decreased CHIKV titers by 2.5-6 log RNA copies *in vitro*, but also decreased CHIKV replication *in vivo* in both Swiss Webster and C57Bl/6 mice (applied separately and together) (229). Another lab also described silencing sequences against nsP1 and E1, although they combined these treatments into one shRNA-encoded plasmid. Similarly, this group found that this plasmid inhibited CHIKV replication in cell culture as well as in suckling mice (230). While siRNA therapy has proven successful in some clinical trials of respiratory syncytial virus (231-233), no RNAi-based therapeutics have made it to clinical trials for the treatment of CHIKF. Homoharringtonine, on the other hand, has been approved by the FDA for treatment of certain types of cancer (234). This compound is a natural alkaloid derived from the Japanese plum yew, a conifer native to Japan, which has also been shown to potentially inhibit CHIKV replication, especially strains carrying the A226V E1 substitution (235).

There are also inhibitors under investigation for which targets have yet to be identified; these inhibitors are not discussed here, however.

Table 3. Anti-CHIKV drugs with *in vitro* efficacy.

Target	Example(S)	Notes	Reference(s)
Viral entry	Chloroquine	Chloroquine clinical trials resulted in conflicting results.	222-226
	Arbidol	Approved for use in China and Russia	218-221
Genome replication	nsP2 inhibitors	Identified primarily through *in silico* screening methods.	208-2010
	Nucleoside analogues	Poorly reproducible results *in vitro*. Ribavirin appeared to be effective in	211-215

		small cohort of CHIKF patients.	
Protein translation	harringtonine	Homoharringtonine already FDA approved for treatment of chronic myeloid leukemia	234-235
	siRNA, shRNA	Various forms and delivery systems	229,230
Virion	mAb therapy	Various neutralizing antibody therapies have shown promise in CHIKV animal models.	227,228

Host-Targeted Antivirals

Of note, there are two distinct classes of antivirals which accomplish the same tasks of different inhibition strategies: virus-targeted antivirals, and host-targeted antivirals. Host-targeted antivirals are coming into vogue in the field of antiviral development, given the distinct advantages these targets have over traditional viral targets. Firstly, including host proteins enormously expands the repertoire of drugable targets, whereas constraining efforts to viral proteins limits drugable targets to just a handful of proteins, at best. Targeting host proteins is also an indirect method of targeting viral processes, which means that the rapidly-changing genetic landscape of the virus does not affect the binding of therapeutics to their targets, generating fewer antiviral escape mutants (Figure 12) (236, 237). For instance, the CHIKV protein-targeted monoclonal antibodies are well-known for selecting resistance to treatment (238, 239), and escape mutants have been characterized for protease and polymerase inhibitors for hepatitis C virus (240) as well as for HIV reverse-transcriptase (241), protease inhibitors (242) and RNAi-based therapies (243). Further, viruses from diverse genera rely on the same host processes, giving host-targeted therapies more potential for broad-spectrum applications (244).

One process for which many RNA viruses are nearly completely dependent on the host is protein folding. RNA viruses in particular tend to be small and genomic real-estate for protein-

coding transcripts is limited. Protein folding occurs nascently and immediately post-translation, and protein native structure is ultimately affected by various post-translational modifications such as glycosylation, palmitoylation, disulfide bond isomerization, etc. These different folding steps are carried out by a spectrum of different chaperones and enzymes, so in many cases it is more economic for a virus, particularly small RNA viruses, to simply use host chaperone proteins for viral protein folding [although some viral proteins serve limited chaperone functions, such as the DENV prE (245)]. Among host-targeted strategies, this is a simultaneously alluring and contentious method. On the one hand, protein folding is such a tightly constrained process that viruses simply cannot adapt to a different method of achieving the same goal (246); one cannot alter the need for a chaperone without altering protein structure, and since protein function is dependent on structure, it is therefore nearly impossible to escape the requirement of molecular chaperones and maintain protein function (247). This is also the main criticism of the strategy, as the same can be argued for host proteins. There are fundamental concerns about toxicity when host proteins are considered as targets, and proper protein folding is one of the most basic needs of a cell.

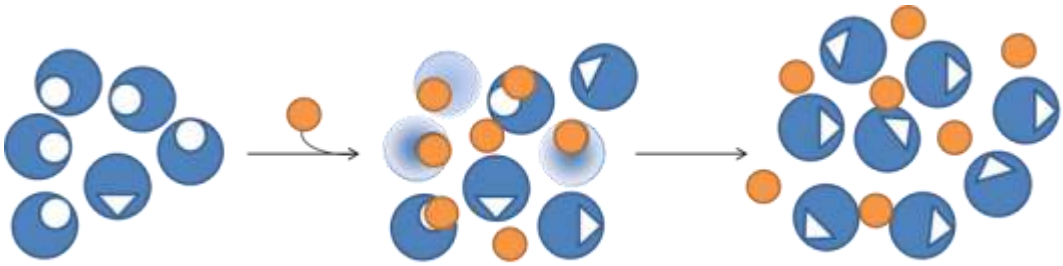

Illustration 12. Development of antiviral escape mutants.

A viral protein-targeted inhibitor is applied to a culture of virus. The antiviral applies a positive selective pressure for viruses that express a protein with an altered binding pocket. After several rounds of replication, the virus with the altered binding pocket becomes dominant and the antiviral is no longer effective.

However, inhibitors of chaperone proteins are no strangers to clinics. Heat-shock proteins (Hsp) are the most well-known group of chaperone proteins, and Hsp90 is a very good example of the utility of chaperone protein inhibitors to treat a wide variety of diseases. Highlighting its unequivocal importance, Hsp90 is evolutionarily conserved across all kingdoms of life (248) and has maintained its primary function as an ATP-ase driven chaperone protein (249, 250); however, Hsp90 also contributes to many different disease states, most prominently by its over-expression in cancer cells to control the immense protein folding burden imposed by the rapid division thereof. Many distinct inhibitors of Hsp90 exist, although they can broadly be classified into two categories: natural chemical product and derivatives, such geldanamycin or radicicol and their derivatives; and synthetic chemical and peptide products. While most geldanamycin derivatives have been dropped from clinical trials for various reasons and radicicol and derivatives have not been developed to the point of clinical trials, one geldanamycin derivative, IPI-504, is still under clinical investigation in at least 12 phase I, II, or III clinical trials for the treatment of various cancers, including solid lung, colon, and breast tumors, as well as hematologic malignancies (clinicaltrials.gov). Ongoing Hsp90 clinical studies aim to investigate synthetic Hsp90 inhibitors. These compounds include: CNF2024/BIIB021, MPC-3100, Debio 0932, NVP-AUY922, SNX-5422, STA-9090, KW-2478, AT13387, XL-888, and PU-H71 (251). Together, these compounds comprise over 100 trials at various stages and for a diverse array of applications, mainly for the treatment of treatment-resistant cancers (clinicaltrials.gov). Although side effect data are not available for all Hsp-90 inhibitors yet, comprehensive review of tolerability results of IPI-504 reveal that the most commonly reported side effects are benign slowing of the heart rate and mild gastro-intestinal symptoms including diarrhea, nausea, and vomiting (252, 253). However, these latter symptoms are very responsive to anti-emetics (253). In general, Hsp-90, a very highly evolutionarily conserved chaperone protein, has proven to be a valuable target for treating various malignancies with good tolerability, providing critical evidence that inhibiting chaperones is tenable in the clinic.

Like other viruses, CHIKV also relies on Hsp90 for efficient replication. Rathore et al. showed that not only do the Hsp90 inhibitors geldanamycin, HS-10, and SNX-2112 reduce CHIKV replication in HEK293 cells, but also reduce CHIKV titers *in vivo* and prevented footpad pathology; however, despite using the lethal A129 animal model, it remains unclear if the compounds delay or prevent death (128). Upon further investigation, it was found that nsP3 and nsP4 associate with Hsp90, suggesting the Hsp90 may exert its effects through the viral replication complex (128). Interestingly, Hsp90 inhibitors also appear to decrease the expression of nsP2 and Hsp90 appears to act through signaling downstream of mTOR phosphorylation, suggesting that the role of Hsp90 during infection may be broader than originally hypothesized (254). However, the potential for other chaperones to be targeted as potential CHIKV antivirals remains largely unexplored.

Purpose of project

Several major gaps have become apparent in the study of CHIKV: proper surveillance in epidemic areas, potential variation in the virulence of natural CHIKV isolates, and development of novel chaperone proteins as potential drug targets. Therefore, the goals set forth by this work are as follows:

1. Describe the CHIKV epidemic in the Dominican Republic, particularly the clinical symptoms of Dominican CHIKF in an attempt to refine case-definitions of CHIKF in the Americas;
2. Describe the virulence differences between CHIKV lineages in a murine model so as to provide evidence of differential pathogenesis and virulence between CHIKV lineages;
3. Explore the potential of disrupting the redox-pathways responsible for the formation and isomerization of disulfide bonds as a novel therapeutic strategy.

Together, these results will aid in CHIKV surveillance by not only refining case definitions, thereby influencing surveillance practices, but also by aiding in predicting the severity of an epidemic based on empirical evidence of lineage-specific differences in virulence. Furthermore, the project will also aid in selection of novel antiviral drug targets to exploit for not only the treatment of CHIKV, but for all alphaviruses and potentially any virus that relies on disulfide bonds within its external structures.

CHAPTER II. CHIKV IN THE AMERICAS: THE CHIKF EPIDEMIC IN LA ROMANA, DOMINICAN REPUBLIC 2014

INTRODUCTION

Among New World CHIKF outbreaks, the Dominican Republic has had the most suspected cases, with over 500,000 since 2014 [PAHO data] (Fig. 1). Here is described an outbreak in the southeastern city of La Romana. In February, 2014, the Dominican Republic's first suspected cases were reported in Nigua, a town in San Cristóbal Province, southwest of the capital (255). Suspected cases were defined by the Dominican Ministerio de Salud Pública (MSP) by acute febrile syndrome and polyarthralgia, though many patients also presented cervical, supraclavicular, and inguinal lymphadenopathy and facial, vulval and scrotal edema. It was hypothesized that CHIKV entered the country through Bajos de Haina, a port city located 2 km from Nigua. On April 3, 2014, the U.S. Centers for Disease Control and Prevention (CDC) confirmed CHIKV infection by detecting CHIKV-specific IgM antibodies in a patient's blood. Nationally, cases continued to rise, peaking between mid-July and mid-August with up to 45,000 new cases each week. After administering brief questionnaires in major cities, the MSP estimated attack rates ranging from 40% of the population-at-risk in Higüey to 81% in Azua de Compostela. In La Romana, up to 89% of households interviewed were suspected to have been affected by CHIKV as of August, 2014 (255). Clinicians and patients reported a high fever and arthralgia in the wrists and ankles, the latter which lasted up to six months in middle-aged and elderly female patients. Nevertheless, little

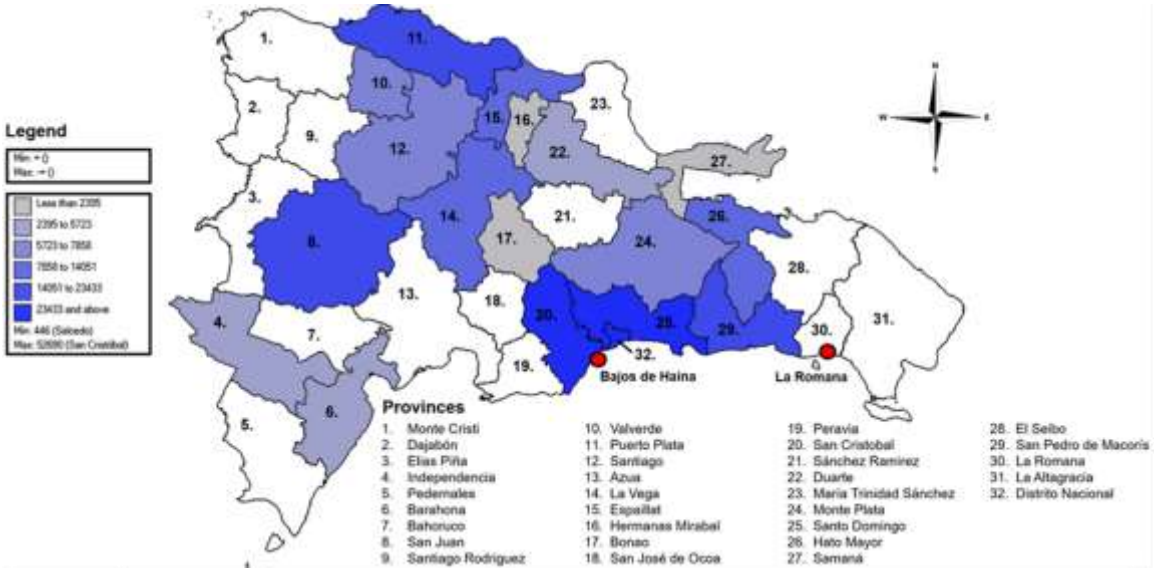

Figure 1. Distribution of total suspected cases of CHIKF reported in the Dominican Republic by province.

A suspected case was defined by the presence of sudden fever and arthralgia. The color scheme classifies provinces by number of total suspected CHIKF cases, determined by summing the number of cases reported in MSP DIGEPI weekly bulletins and Chikungunya Outbreak Bulletins for each epidemiological week between February 16, 2014 and June 6, 2015. No exact case numbers were reported to MSP DIGEPI for provinces shaded in white. Number 32, Distrito Nacional, represents the national district, which does not pertain to a province. The city of La Romana and the port of Bajos de Haina, where the outbreak is suspected to have started, are highlighted in red. Map created using Epi Info™ 7.1.5 software licensed by the Centers for Disease Control and Prevention (http://wwwn.cdc.gov/epiinfo/7/). MSP DIGEPI weekly bulletins publicly available through Minesterio de Salud Publica (http://digepisalud.gob.do/).

published information describes the rate of CHIKV seropositivity or CHIKF symptoms in the La Romana population.

Currently, the University of Texas Medical Branch has a preexisting collaboration with several clinics in La Romana, DR, which kindly provided discarded blood samples

used for complete blood count tests, from which we isolated serum to probe for CHIKV RNA and anti-CHIKV IgM antibodies. Here, I describe the outbreak of CHIKF in La Romana, Dominican Republic, by matching CHIKV diagnostic results with patient data, providing some clinical and demographic data regarding CHIKF patients in La Romana. From this, we can add to the body of literature describing disease caused by Asian CHIKV strains introduced into the Caribbean circa 2013.

METHODS

Patient data and serum collection

Between June and August, 2014, serum was collected from discarded diagnostic blood samples of febrile patients attended by the departments of emergency medicine at a public hospital, Hospital Dr. Francisco A. Gonzalvo (HFG), and a private hospital, Hospital el Buen Samaritano (HBS). The criteria for serum collection in HBS included patients for whom a complete blood count (CBC) was performed. At HFG, patients were sampled among a pool of suspected CHIKF cases. A retrospective chart review was performed to collect patient data in both sampling locations. For HBS patients who were attended in the emergency room, patient age, gender, symptoms, and CBC results were collected retrospectively wherever possible. For HBS patients who were admitted into the hospital, a physical examination and medical history were recorded, including a history of the present illness and clinical and familial antecedents. For HFG patients, age, gender, and symptoms were recorded wherever available. All patient data were assigned institution-specific identification numbers corresponding to those assigned to serum samples to ensure patient anonymity. Neither patient names nor clinic-assigned

laboratory numbers were used, and patient age was used in lieu of birthdate, eliminating all potential identifying information from the study without compromising data integrity.

All patient data were deidentified and handled under University of Texas Medical Branch Institutional Review Board Protocol #15-0265.

Quantitative RT-PCR

RNA was first isolated from serum samples using the ZR-96 Viral RNA Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Quantitative reverse transcription PCR (RT-qPCR) was then performed in triplicate using the TaqMan RNA-C_t 1-step Kit (Applied Biosystems, San Francisco, CA, USA) and previously described primers and probe (63) on QuantStudio 6 Flex Real-Time PCR system (ThermoFisher Scientific, Waltham, MA).

CHIKV IgM ELISA

Serum samples were screened for anti-CHIKV IgM antibodies by enzyme-linked immunosorbent assay (ELISA) as previously described (57) using the CHIKjj Detect MAC-ELISA kit (InBios, Inc., Seattle, WA), which was validated by the CDC (256). All samples were tested in duplicate and any inconclusive samples were retested.

Sequencing and phylogenetics

Ten serum samples with low Ct values obtained during the CHIKV RT-qPCRs were directly submitted for Illumina HiSeq sequencing, without passaging, as previously described (31). Viral genomes were assembled using the Abyss software as previously described (257). Assembled contigs were checked using bowtie2 to align reads to the contigs followed by visualization using the integrative genomics viewer (258). Genomic

sequences were manually aligned with sequences representing all three genotypes downloaded from Genbank using Se-Al (http://tree.bio.ed.ac.uk/software/seal/), and non-coding sequences were removed from the alignment, resulting in a common length of 11,241 nt. The final data set comprised of 85 complete open reading frame sequences from 26 countries isolated during 1953–2014. A Bayesian maximum clade credibility (MCC) phylogeny was inferred using the GTR+G4 nucleotide substitution model with BEAST version 1.8.2 (259).

Statistics

Odds ratios were calculated for different demographic and symptom variables from 2x2 contingency tables, followed by a two-tailed Fisher's exact analysis to test for significance. Contingency tables were constructed as follows: rows were composed of diagnostic outcome, CHIKV positive patients in the first row followed by CHIKV negative patients in the second; columns were constructed of absence (second column) and presence (first column) of specific outcome or demographic quality. Contingency tables for RNA vs. IgM positivity data were constructed similarly, with RNA positive individuals in the first row and IgM positive individuals in the second.

RESULTS

Finalized sample pool

A total of 264 serum samples was collected between July and August, 2014. Of those, 103 samples (39.0%) were positive for CHIKV RNA by RT-qPCR, 99 samples (37.5%) were positive for acute CHIKV antibodies by IgM ELISA, and 2 samples were positive for both (0.76%; Table 1). In total, 204/263 (77.3%) patient samples tested

positive for recent CHIKV infection. Viremia was sustained up to 7 days post-fever onset, with no correlation between C_T value and days of symptom evolution. CHIKV IgM antibodies were detectable from 1 day post-symptom onset, although values lower than 3 days are likely attributable to a past infection, and there was no correlation between OD value and days from symptom onset (Fig. 2).

Table 4. Diagnostic outcomes for serum samples collected from patients in La Romana, Dominican Republic between July 2014 and August 2014

Parameter	Value
Total samples	264
Samples CHIKV positive (percent)	77.3
by RT-qPCR (percent)	39.0
by IgM ELISA (percent)	37.5
By both RTq-PCR and IgM ELISA (percent)	0.76

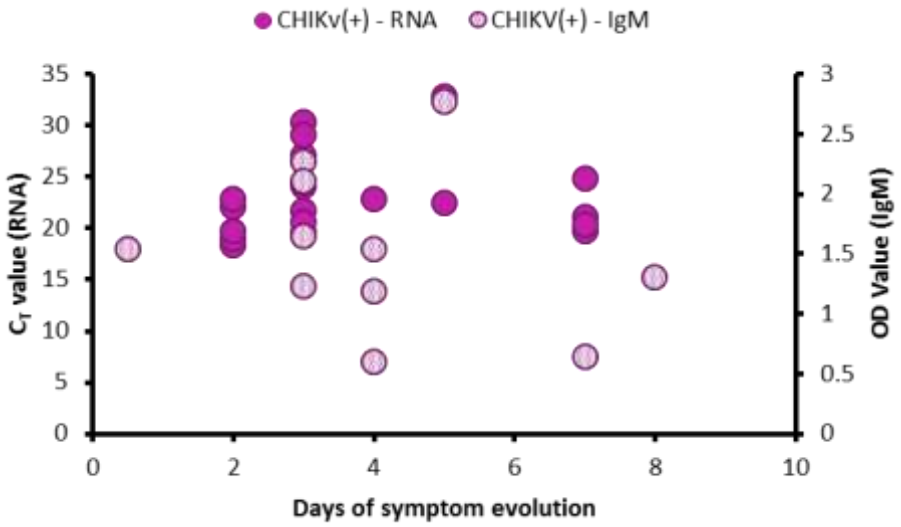

Fig. 2. Days of symptom evolution and CHIKV-RNA/IgM positivity.

Where data regarding symptom onset were available, days of fever evolution were compared to CHIKV-RNA (left X-axis, closed circles) and IgM (right X-axis, semi-closed circles). There appeared to be no correlation between RNA and IgM positivity vs. days of symptom evolution.

Circulating strain data

Using an Illumina HiSeq platform, complete genome sequences were determined directly from RNA isolated from the sera of 10 individuals. The overall alignment rate of the reads varied widely among samples, ranging from 7–88%, with a mean of approximately 20%. Nucleotide and amino acid identity amongst the consensus sequences from these individuals was >99.9%. The MCC phylogeny (Fig. 3) clearly showed that the Dominican Republic sequences clustered within the Asian lineage together with other Caribbean sequences isolated in 2014 during the outbreak (11, 14). Given the high sequence identity between DR isolates, only two consensus sequences were used to build the phylogenetic tree in order to increase clarity in discerning phylogenetic relationships. The most closely related Asian sequences circulated between 2012 and 2013 in Micronesia, the Philippines and China. Analysis of intra-host variation within the sequences suggested that there are few minority variants within the population. Interestingly, 7 of the 10 sequences contained a variant at position 9368 (i.e. amino acid residue 279 in E2) with frequencies of 1.14-3.44% among sequences. This was also previously observed in sequences obtained from an outbreak in Trinidad and Tobago suggesting this variant is being maintained during the outbreak. Two more independent variants were observed in strain N469 at position 10978 (i.e. residue 332 in E1); and strain N594 at position 6686 (i.e. residue 344 in nsp4). SNP variation within the UTR sequences were not considered due to the complexity of the NGS output read alignment in these regions.

Although the *A. aegypti* mosquito is thought to be the main vector of Asian lineage CHIKV strains, both Illumina-derived sequences as well as 7 additional samples

analyzed by Sanger sequencing (GenBank accession nos. pending) were examined for mutations known to adapt CHIKV to transmission by *A. albopictus* mosquitoes. None of the envelope protein substitutions previously reported to increase fitness in *A. albopictus* mosquitoes were observed in these sequences. This finding is further supported by the findings in Mexico (23) and Trinidad (31).

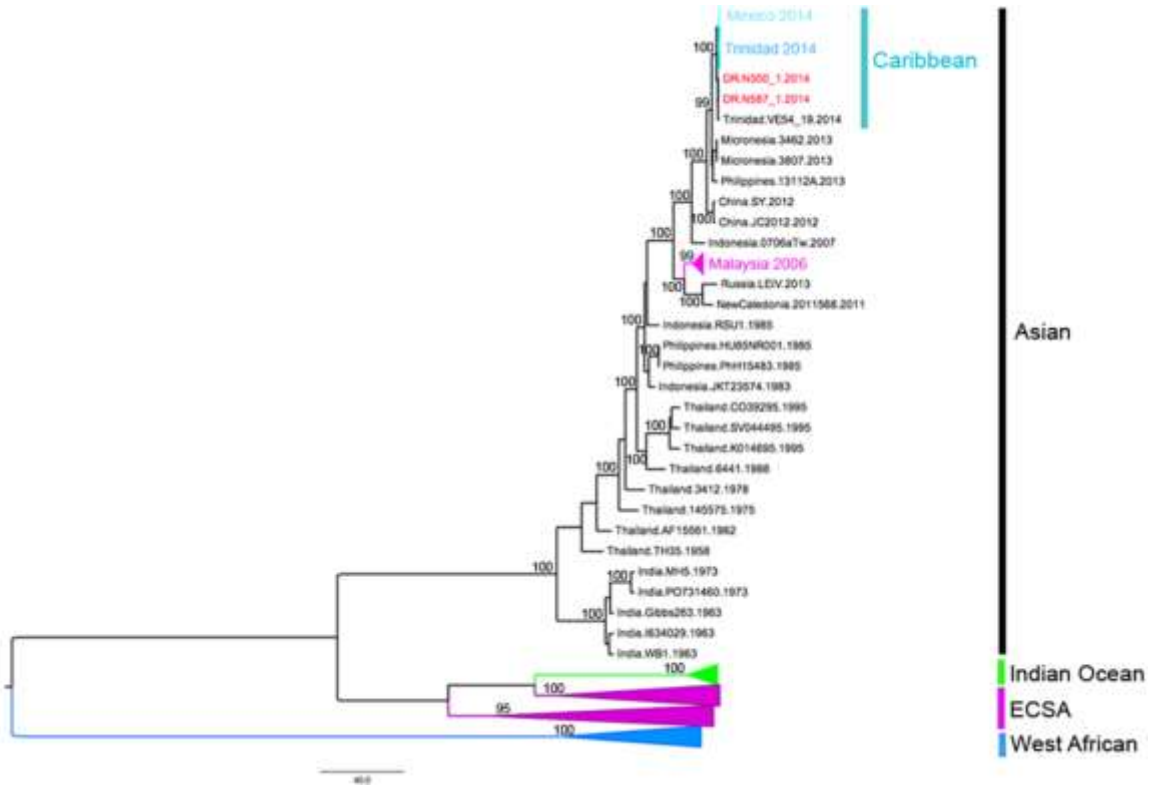

Fig. 3. Maximum clade credibility (MCC) phylogeny based on the complete coding region of 85 CHIKV sequences.

The three major CHIKV genotypes are labeled. Sequences generated from this study (Genbank accession numbers are labeled in red. Nodes with clade credibilities ≥ 95% are labeled accordingly).

Patient Demographic Data

After samples underwent diagnostic testing, de-identified patient data were analyzed for demographic characteristics such as gender, age, and hospital admission, and co-morbidities were documented (Table 5). In total, demographic data were available

for 153 CHIKF(+) (CHIKV RNA or IgM antibody positive) and 45 CHIKF(-) (both RNA and IgM negative) patients. Slightly more male than female samples were represented for both CHIKF(+) and CHIKF(-) patient groups, although gender did not appear to predispose individuals to CHIKV infection (odds ratio= 0.94, p=1.00). A broad age range of both CHIKF(+) and CHIKF(-) patients was represented, between 13 days and 82 years of age, with the majority of cases involving children between the ages of 6 and 14 years (Fig. 4). Age did not appear to affect the severity of disease, as hospitalizations were proportionate across age groups. The mean time from fever onset to clinic visit ranged from a few hours to 15 days, with an average of 4.3 days for CHIKF(+) patients and 3.9 days for CHIKF(-) patients. These data therefore suggests that most CHIKF(+) patients visited the clinic sometime between the decline of viremia and the rise of CHIKV-specific IgM antibodies. Patients in the CHIKF(-) group had a higher percentage of hospitalizations, with 19.7% of CHIKF(+) patients and 27.6% of CHIKF(-) patients requiring admission, although this difference was not significant (odds ratio= 0.64, p=0.13). Several preexisting conditions were noted, although none seemed to affect the odds of CHIKV infection. The most prominent of these were pregnancy of greater than 12 weeks, hypertension, and diabetes (type 1 and type 2). Interestingly, 2 cases of sickle-cell anemia were reported as well as one tuberculosis (TB) case in the CHIKF(+) group. Additionally, one CHIKF(+) patient visited the clinic for symptoms associated with a kidney stone.

Table 5. Demographic data for CHIKF(+) and CHIKF(-) patient samples.

Characteristic	CHIKV+ (CHIKV-) Values	Odds ratio (Fisher's exact)
Male:Female (ratio)	14:11 (59:41)	0.94 (p=1.000)
Mean years, ± STD	20.9±19.9 (18.3±18.48)	NA
Time from onset to hospital visit, mean days ± STD	4.3±2.6 (3.9±1.5)	NA
Hospitalized (percent)	19.7% (27.6%)	0.64 (p=0.13)
Preexisting conditions (# cases)		
Pregnancy (12+ weeks)	8 (1)	2.05 (p=0.69)
Hypertension	5 (1)	1.23 (p=1.00)
Diabetes	4 (0)	Incalculable (p=0.58)
Sickle cell anemia	1 (1)	0.23 (p=0.35)
Tuberculosis	1 (0)	Incalculable (p=1.00)

Symptoms, clinical diagnoses, and blood findings

In addition to demographic data, specific sign and symptom data were matched to samples (Table 6). These data were available for 82 CHIKF(+) patients and 20 CHIKF(-) patients. The most common condition of both CHIKF(+) and CHIKF(-) patients was fever (86.9% and 60%, respectively). CHIKF positivity greatly increased the odds of developing fever (odds ratio=4.42, p=0.0096). The average fever was 39.1°C for CHIKF(+) patients, while the average fever for CHIKF(-) patients was slightly lower at 38.7°C. Reports of arthralgia and myalgia were surprisingly low for CHIKF(+) patients relative to past outbreaks (16, 45, 67, 74), with 20.6% and 17.2% of patients reporting arthralgia and myalgia, respectively. Despite the low frequency of these particular signs

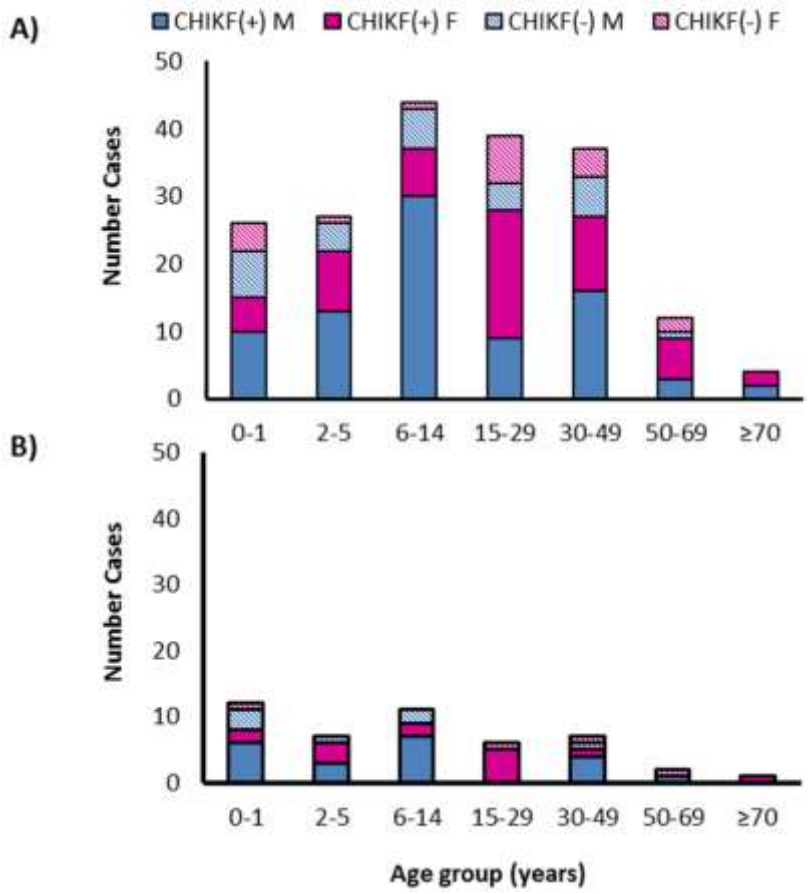

Figure 4. Age and gender distribution of patient sample pool.

A) The age and gender distribution of all patients included in the study. The majority of patients were under the age of 50 for both CHIKF(+) and CHIKF(-) groups. B) The age and gender distribution of patients who were hospitalized. CHIKV infection did not significantly affect the odds of being hospitalized (p=0.13).

and symptoms, they may still be considered of diagnostic value, as arthralgia and myalgia appeared to be CHIKV-specific, with 0 patients in the CHIKF(-) group exhibiting arthralgia or myalgia, despite a lack of statistical significance (p=0.10 and p=0.18, respectively). Headache, on the other hand, was less common among CHIKF(+) patients than CHIKF(-) patients (odds ratio= 0.31, p=0.12). A novel feature of CHIKF patients during this outbreak was enophthalmos, a uni- or bilateral posterior displacement of the

eye, which was reported in 15.5% of CHIKF(+) patients and only 5% of CHIKF(-) patients. Although these frequencies were not significantly different, CHIKV infection did appear to increase the odds of presenting with enophthalmos (odds ratio= 3.58, p=0.29). Other clinical symptoms associated in the past with CHIKV infection, malaise and rash, were not significantly associated with CHIKV infection in this study. RNA positivity significantly increased the odds of presenting with fever (odds ratio= 6.29, p=0.004) compared to IgM positivity; no other statistically significant effects of RNA vs. IgM positivity were found (Fig. 5).

Table 6. Signs and symptoms recorded for CHIKV positive (and CHIKV negative) patients for whom complete blood counts were ordered between July 2014 and August 2014

Symptom	Percent cases	Odds ratio (Fisher's exact)
Fever	86.9 (60.0)	2.37 (p=0.15)
Average (°C ± STD)	39.1±0.7 (38.7±0.6)	NA
Arthralgia*	20.6 (0.0)	Incalculable (p=0.10)
Myalgia*	17.2 (0.0)	Incalculable (p=0.18)
Headache*	15.5 (35.7)	0.31 (p=0.12)
Enophthalmos	15.5 (5.0)	3.58 (p=0.29)
Malaise/fatigue	11.9 (10)	1.25 (p=1.00)
Rash	0 (0)	Incalculable
Dehydration	33.3 (25)	1.56 (p=0.56)
Gastrointestinal[A]	16.7 (35.0)	0.38 (p=0.12)
Neurological signs[B]	1.2 (5.0)	0.48 (p=0.99)
Respiratory[C]	26.2 (40.0)	0.8 (p=0.78)

*Patient data for children under 3 years of age not included
[A]Nausea, diarrhea, vomiting [B]Delirium, dizziness, convulsions [C]pneumonia, dyspnea, ronchus, difficulty breathing, rhinorrhea
Values in percent unless otherwise noted.

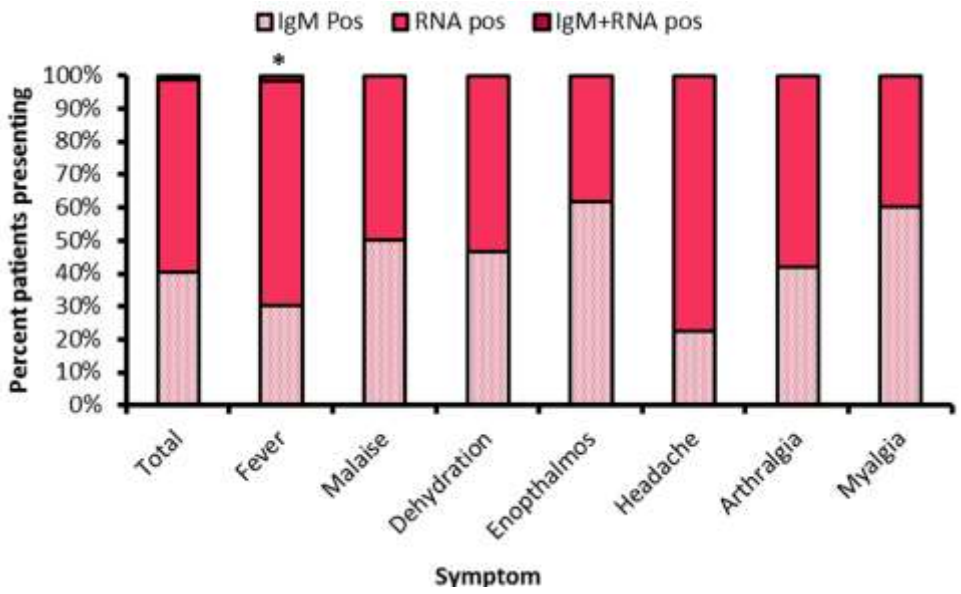

Figure 5. Percent CHIKV RNA and anti-CHIKV IgM positivity across symptom presentation.

The number of patients presenting with a specific symptom were divided into percentage testing positive for CHIKV RNA, CHIKV IgM, or both. RNA positivity increased the odds of presenting with fever compared to IgM (odds ratio= 6.29, p=0.004), suggesting fever correlated with acute viremia.

Other signs and symptoms less commonly associated with CHIKV infection can be broadly categorized as gastrointestinal, respiratory, and neurological in nature. A sizeable percentage of CHIKF(+) patients presented with gastrointestinal signs and symptoms, which included diarrhea, nausea, and gastroenteritis, although CHIKF was not significantly associated with an increased odds of presenting with one of these (odds ratio=0.38, p=0.12). Similarly, a surprising 26.2% of CHIKF(+) patients presented with respiratory signs and symptoms, although again CHIKV infection did not significantly affect the odds of having respiratory disease (odds ratio=0.8, p=0.78). Consistent with the literature, most CHIKF(+) patients exhibiting gastrointestinal signs and symptoms were below the age of 15; patients exhibiting respiratory signs and symptoms were also largely under the age of 15 (Fig. 6) (16, 45, 67, 260). RNA or IgM positivity did not statistically alter the odds of developing respiratory or gastrointestinal symptoms for any age (Fig. 7).

One CHIKF(+) patient presented with severe neurological signs, which included lapses in consciousness, meningeal inflammation, and convulsions. This patient was ultimately diagnosed with unspecified meningitis. Another CHIKF(-) patient was diagnosed with meningitis, although this patient presented with different signs and symptoms, which included photophobia, sonophobia, head and neck pain, and Brudzinski signs.

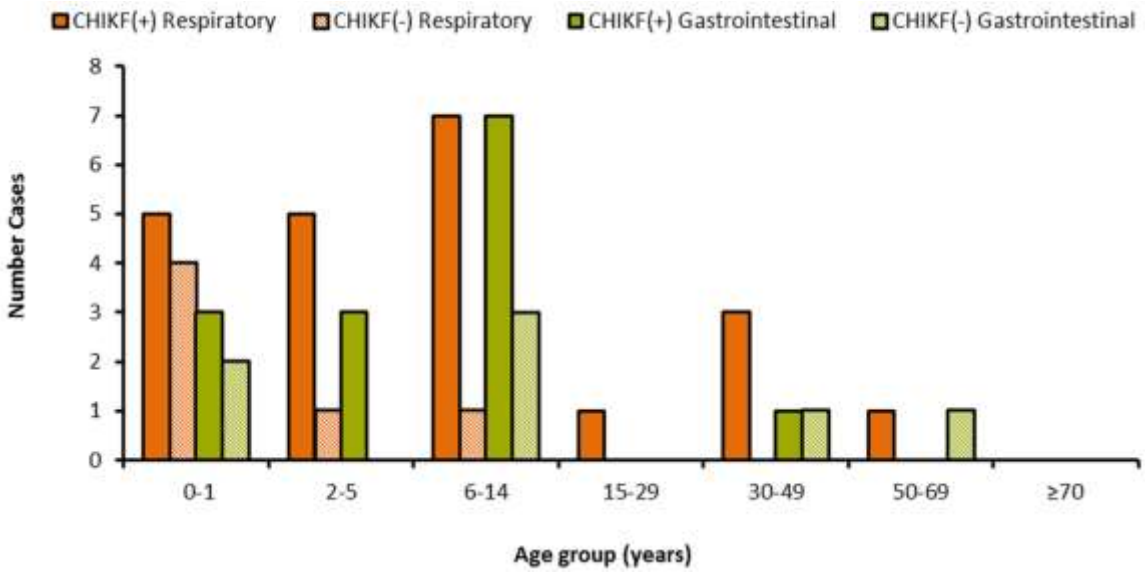

Fig. 6. Distribution of respiratory and gastrointestinal symptoms by age.

Children and young adults below the age of 15 were the primary demographic groups presenting with respiratory and gastrointestinal symptoms for both CHIKF(+) and CHIKF(-) patients, although CHIKV infection did not significantly affect the odds of developing respiratory or gastrointestinal symptoms.

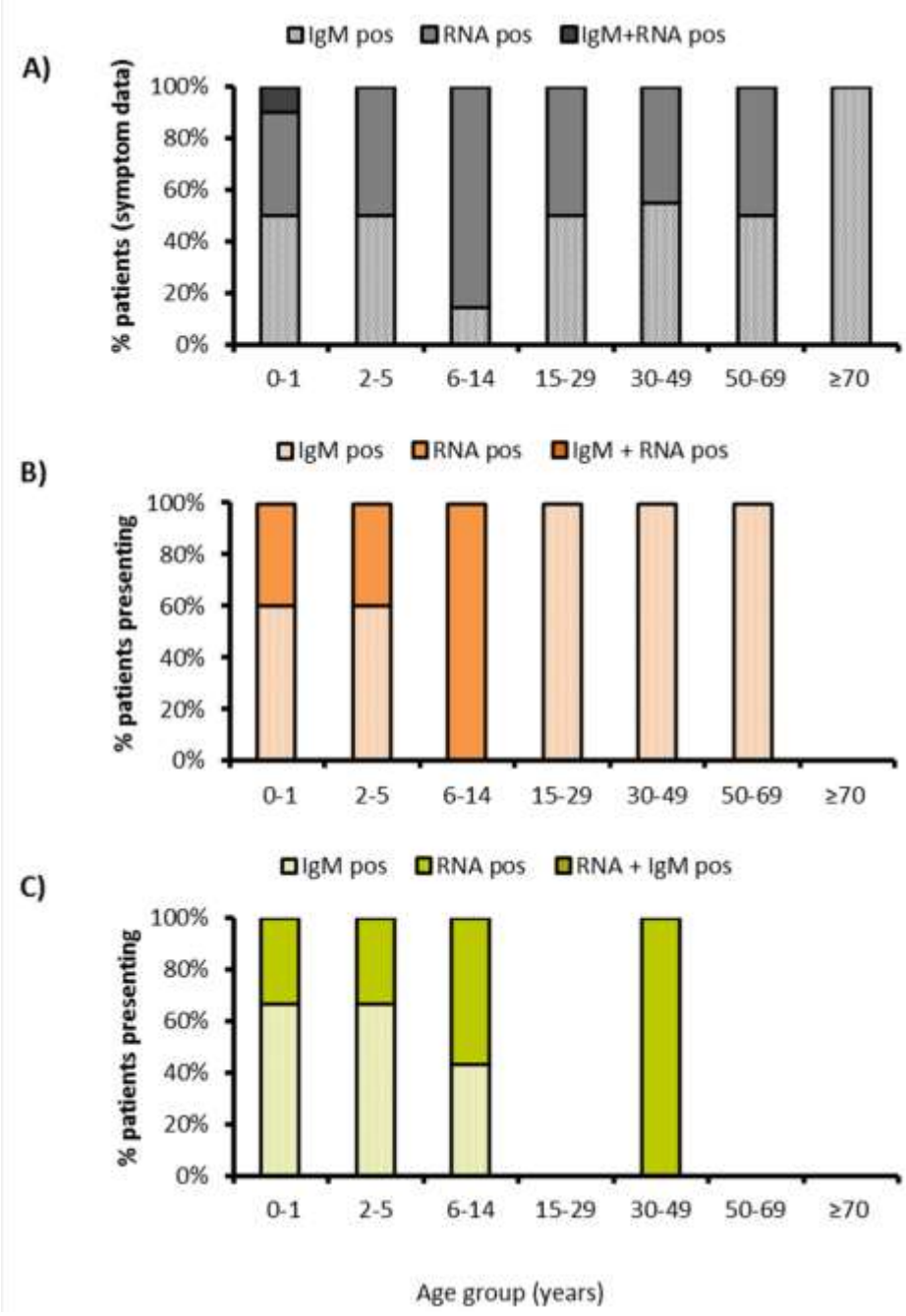

Fig. 7. Percent CHIKV RNA and anti-CHIKV IgM positivity among patients presenting with respiratory and gastrointestinal symptoms.

A) Percentage of CHIKV RNA positive, CHIKV IgM positive, and dual positive among total CHIKF(+) patients for whom symptom data were available. B) Distribution of CHIKV RNA, IgM, or dual positivity among patients presenting with respiratory symptoms. No statistically significant effects of RNA or IgM positivity on respiratory symptomology were found. C) Distribution of CHIKV RNA, IgM, or dual positivity among patients presenting with gastrointestinal symptoms. No statistically significant effects of RNA or IgM positivity on gastrointestinal symptomology were found.

Given the observation of respiratory symptoms exhibited by CHIKF(+) patients during the sampling period, host and CHIKV sequence reads were removed from deep sequencing data and the presence of other viruses in 8/10 of the samples submitted for next-gen sequencing was examined. RNA from viruses known to cause respiratory symptoms was not found in any of the samples.

Initial diagnoses were made largely based on clinical presentation (Fig. 8). Only 5% of CHIKF(+) cases were clinically diagnosed as such, likely due to the conspicuous absence of joint symptoms normally associated with CHIKV and possibly lack of knowledge about CHIKF by some health care providers. CHIKF(+) patients were more likely to be diagnosed with idiopathic febrile syndrome, dengue fever, or pneumonia. No CHIKF(-) patients were misdiagnosed with CHIKF. Diagnoses classified under "other" included meningitis, kidney stone, pregnancy, and trauma. Some diagnoses for "generalized febrile syndrome" were inferred from the prescription of fever reducing agents, namely metamizole. Not surprisingly, among CHIKF(+) individuals, RNA positivity significantly increased the odds of being diagnosed with generalized febrile syndrome over IgM positivity (odds ratio=4.770, $p=0.002$), while IgM positivity significantly increased the odds of being diagnosed with one of the indications classified as "other" over RNA positivity (odds ratio=0.0462, $p<0.001$; Fig. 20). No other significant differences in diagnosis vs. RNA or IgM positivity were found.

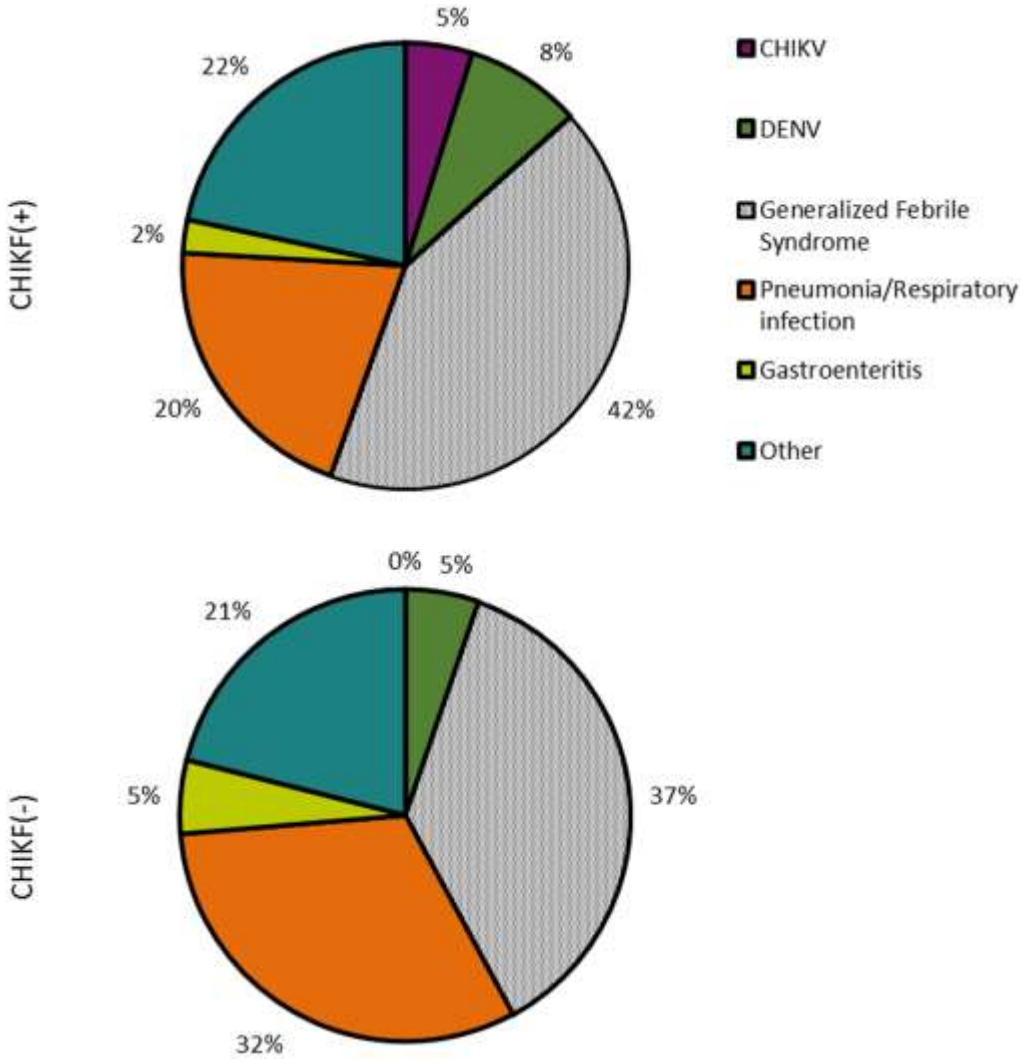

Fig. 8. Clinical diagnoses made for CHIKF(+) and CHIKF(-) patients.

Clinical diagnoses were made by physicians on the basis of sign and symptom presentation. The most common diagnosis for patients in both groups was undifferentiated febrile illness. Most notably, while CHIKF(+) patients were more likely to be diagnosed with something other than CHIKF, no CHIKF(-) patients were misdiagnosed with CHIKF.

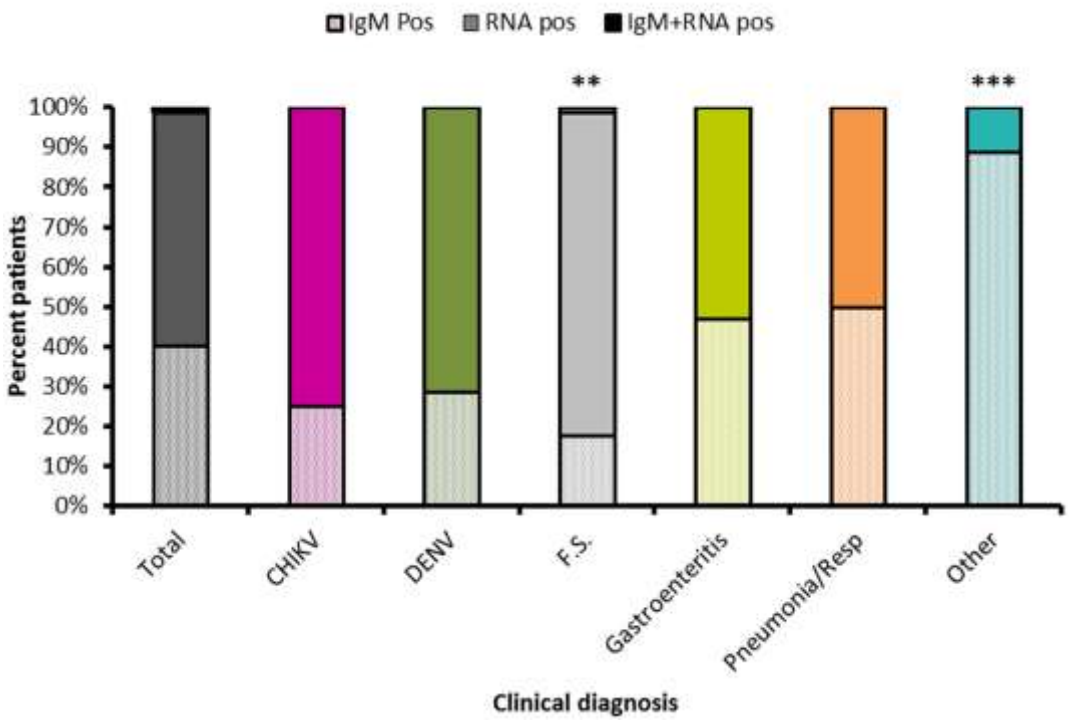

Fig. 9. Percent anti-CHIKV IgM and RNA positivity among patients given various clinical diagnoses.

The number of patients given a specific diagnosis were divided into percentage testing positive for CHIKV RNA, CHIKV IgM antibodies, and dually positive patients. While a correct diagnosis of CHIKV was not significantly associated with any class of positivity, RNA positivity significantly increased the odds of being diagnosed with undifferentiated febrile illness (odds ratio=4.770, p=0.002) while IgM positivity significantly increased the odds of being diagnosed with an indication classified under "other" (odds ratio=0.0462, p<0.001).

White blood cell (WBC) counts were largely unremarkable for both CHIKF(+) and CHIKF(-) groups, with the average for most age groups falling within the reference ranges albeit with large variation. In general, for CHIKF(+) patients: complete WBC ranged between 1.04-28.5x 10^3 cells/µL; neutrophils ranged between 0.21-25.14x10^3 cells/µL; lymphocytes ranged between 0.15-9.59x10^3 cells/µL; and platelets ranged between 22-883x10^3 cells/µL. CHIKF(-) patients showed similar ranges to CHIKF(+) patients: complete WBC ranged between 1.05-16.3x10^3 cells/µL; neutrophils ranged

between 0.84-12.714x10^3 cells/µL; lymphocytes ranged between 0.15-9.03x10^3 cells/µL; and platelets ranged between 103-487x10^3 cells/µL. Median and interquartile ranges for RNA(+) CHIKF patients and CHIKF(-) individuals are shown in table 7, as RNA(+) individuals have actively replicating virus in their blood and are therefore more likely than RNA(-) but IgM(+) acute patients to exhibit abnormal values. While most values fell within normal range for complete WBC, neutrophils, and platelets, many CHIKF(+) patients presented with varying degrees of lymphopenia when compared to reference values, especially those positive for CHIKV RNA (Fig. 10). Overall, CHIKV infection significantly increased the odds of developing lymphopenia (odds ratio=2.77, p=0.01) compared to CHIKF(-) groups, while RNA positivity greatly increased the odds of presenting with lymphopenia compared to IgM positivity (odds ratio=5.62, p<0.001) . However, basal lymphocyte counts are highly specific to individuals, so it is possible that some of the deviant values fell within normal limits for some patients. Pediatric (age<21) reference values were derived from standard hematology references (261), while adult reference values were provided by the laboratory at the Fundación Hospital General el Buen Samaritano.

Table 7. Median values and interquartile range (Median, IQR) for white blood cell (WBC) and differential counts for complete WBC, neutrophils, lymphocytes, and platelets by CHIKF positivity and age group .

Age	CHIKV +/-	WBC	Neutrophil	Lymphocyte	Platelets
ALL	+	6.2, 3.4	4.2, 3.0	1.2, 1.2	205, 96
	-	8.2, 5.2	4.1, 3.4	2.4, 2.2	250, 120
0-1	+	6.6, 1.0	4.28, 1.6	2.0, 1.2	265.5, 62.8
	-	10.2, 3.3	5.1, 2.8	4.5, 2.3	300, 133
2-5	+	9.1, 3.0	6.9, 3.1	1.9, 1.5	260.5, 73
	-	8.3, 1.9	3.3, 2.8	3.3, 0.5	341, 105
6-14	+	6.3, 3.4	4.6, 3.0	1.2, 0.7	213.5, 64.8
	-	7.5, 9.7	4.8, 8.6	2.3, 0.4	205, 120
15-29	+	4.5, 2.0	3.4, 1.7	0.7, 0.1	146.5, 41.5
	-	6.3, 0.5	3.6, 1.8	2.3, 0.4	234, 62
30-49	+	4.8, 1.8	3.2, 1.2	0.7, 0.1	152, 29
	-	9.3, 6.5	5.0, 3.1	1.8, 0.6	234, 52
50-69	+	4.8, 0.65	3.3, 0.3	6.24, 3.3	171, 24.5
	-	9.45, 3.7	6.5, 5.1	2.5, 1.2	255.5, 59.5

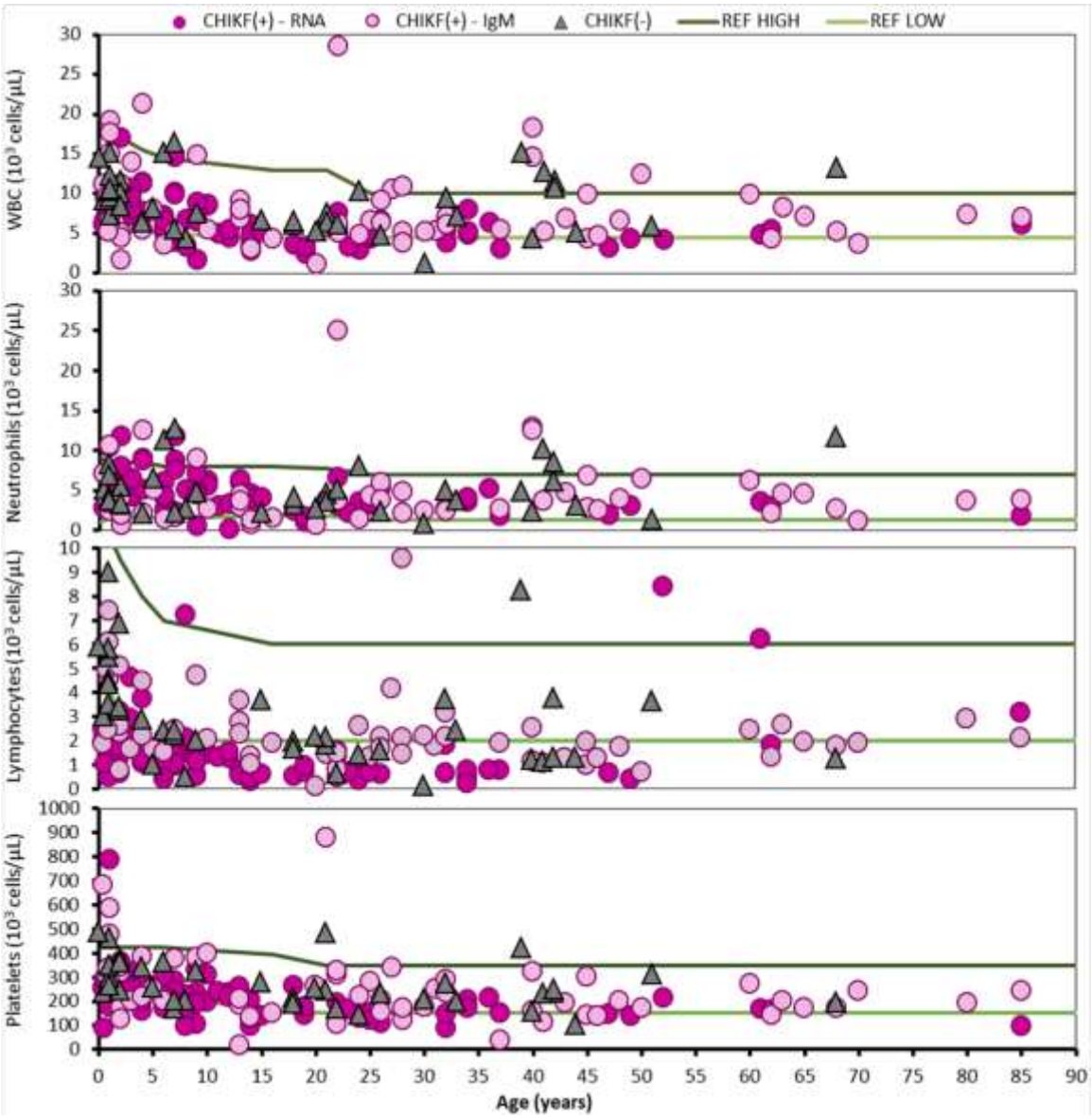

Fig. 10. White blood cell values for CHIKF(+) and CHIKF(-) patients by age.

Patients positive for CHIKV RNA (CHIKF(+) – RNA) are represented by solid purple circles, patients positive for CHIKV IgM antibodies (CHIKF(+) – IgM) are represented by semi-open purple circles, and patients negative for both CHIKV RNA and IgM antibodies (CHIKF(-)) are represented by grey triangles; green lines indicate suggested low and high reference values. Results are largely unremarkable with the exception of lymphocyte counts, for which the odds of having lymphopenia were significantly increased by CHIKV positivity by either RNA or IgM antibodies (odds ratio=2.77, p=0.01). Further, RNA positivity significantly increased the odds of presenting with lymphopenia compared to IgM positivity (odds ratio=5.62, p<0.001).

DISCUSSION

CHIKV infection was detected in 204 patients in La Romana, Dominican Republic by either RT-qPCR or IgM ELISA, and CHIKF-associated disease was described for patients; while only IgM antibodies were detected in 99 samples, CHIKV RNA was isolated from 105 samples, providing samples from which to isolate and sequence whole virus. It is important to adequately characterize the molecular and clinical aspects of the CHIKV strain responsible for CHIKF outbreaks, which can cause financial harm to affected populations and severely affect quality of life. For example, it is estimated that the 2005-2006 CHIKF epidemic on La Réunion island cost €43.9 million (approx. $60.4 million USD at the time) for healthcare costs associated with infection (94). This estimate excludes the cost of long-term treatment, typically for persistent arthralgia. Another study following a cohort of patients from the Réunion CHIKV epidemic from 2006 found that a significant portion of those infected with CHIKV continued to seek medical care up to 30 months after the acute infection resolved (95). Similarly high costs are well documented through India, where medical expenses are out-of-pocket and thus present a significant financial challenge to families affected by the disease (96-98). In Tolima, Colombia, 44.3% of patients with laboratory-confirmed CHIKV infection continued to suffer from polyarthralgia 7 months after their initial diagnosis, indicating that CHIKV infection in the Americas may also result in chronic joint symptoms (262).

CHIKV is transmitted primarily by the mosquito vector *Aedes aegypti*, although mutations in the envelope proteins of IOL CHIKV strains have been linked to CHIKV adaptation to the additional vector species *Aedes albopictus (54)*. Both mosquito species

are present in the Dominican Republic. Adaptation of this emergent CHIKV strain to *A. albopictus* mosquitoes can have major consequences as it could place temperate regions of the eastern United States and millions of naive persons at risk for infection. None of the previously described IOL mutations were found among La Romana CHIKV isolates. However, this does not necessarily preclude the possibility of these defined mutations or novel adaptive mutations arising in the future, thus strains from continuing outbreaks in the Americas should be monitored closely. Of particular interest is the detection of the minority variant at residue 279 in E2 which was shown to also exist in Trinidad. This mutation would result in a Glycine to Glutamate change and this may have an impact on viral fitness or transmissibility. If this variant is currently being selected for, further work is necessary to determine the effects of this adaptation.

Reports describing CHIKF outbreaks in the Caribbean and South and Central America suggest that arthritis and arthralgia/joint pain are major symptoms. In Trinidad and Tobago, 83.3% of confirmed patients complained of joint pain, while arthralgia was reported in 96% of patients in Colombia (26, 31). In this study, although arthritis was not reported by any physician, there was relatively little arthralgia associated with CHIKV infection, with 20.6% of patients reporting joint pain. Furthermore, other symptoms heretofore thought to be typical of CHIKF—rash, headache, and myalgia—were nearly absent in La Romana. Colombia reported that 64% of CHIKF cases exhibited rash, 57% exhibited headache, and 24% exhibited myalgia, as opposed to the outbreak in La Romana, where none of the patients evaluated exhibited rash, only 15.5% reported headache, and only 17.2% reported myalgia. These discrepancies may be due to the sampling practices, as previous Caribbean outbreak studies have included only suspected

cases of CHIKF and dengue while this study broadly evaluated all patients for whom a CBC test was ordered, regardless of clinical diagnosis. As such, cases of CHIKF were described which would not have otherwise been recognized as such without the characteristic joint symptoms and rash. In fact, many of the patients studied visited the clinics for other indications ranging from kidney stones to meningitis, and CHIKF was only confirmed incidentally. My data suggest that CHIKF should not be excluded as a potential diagnosis for febrile patients presenting without joint pain or rash, and that CHIKF may be under-diagnosed in the Dominican Republic and elsewhere.

Historically, joint symptoms have been a hallmark of CHIKF. Outbreak descriptions from La Réunion and Italy, for example, which examined febrile patients with and without joint pain, found that 96.1% and 97% of laboratory-confirmed cases reported joint pain. Similarly, an outbreak in Singapore caused by an Asian-lineage CHIKV strain found that 87.6% of patients reported arthralgia. Furthermore, rash, headache, and myalgia were also more prominent than in this study, with 38-52% of patients reporting skin rash, 40-51% reporting headache, and 46-60% reporting myalgia in Old World (e.g., Asian, African, European) outbreaks (67, 79, 263). The reasons behind these discrepancies are unknown, but may include environmental factors such as diet, human genetic factors, as well as CHIKV lineage and strain-specific variation in pathogenesis. The diagnostic value of differential CBC analysis has been a focus of debate among CHIKV researchers, as some epidemics include a large majority of patients presenting with profound lymphopenia (67) while other studies show that changes in lymphocyte counts may not be CHIKV-specific compared to other indications, namely dengue hemorrhagic fever (264). This study shows that, in La Romana, CHIKV infection

greatly increased the odds of presenting with lymphopenia given reference values derived from a combination of sources, especially during the acute phase of infection marked by viremia, when compared to CHIKF(-) patients. This would suggest that, in La Romana, lymphopenia may potentially be used to distinguish CHIKF from other febrile illnesses. A more specific study comparing WBC results in dengue virus and CHIKV infected individuals would be needed to further substantiate this claim, however.

Interestingly, in La Romana a larger percentage of children under the age of 14 were diagnosed as CHIKF(+) than previously reported. Over 50% of cases detected were children below the age of 14 and 42% were aged 10 or below. This is in stark comparison to past outbreaks, such as in La Réunion where only 5-14% of patients were between 0-9 years of age and in Italy where only 6% of patients were between 0-19 years old (67, 263). It is unclear whether this demographic difference is due to cultural differences, such as financial constraints forcing the decision to treat a child instead an adult from a family with multiple febrile members, or a genuine increase in CHIKV infection in children in La Romana due to differences in exposure to mosquitoes. As previously described, gastrointestinal CHIKF symptoms such as nausea, vomiting, and diarrhea are more common among younger patients, as well as respiratory symptoms such as productive cough, dyspnea, and pneumonia. However, an overall larger percentage of patients presented with respiratory symptoms in La Romana than most CHIKF outbreaks. Although CHIKV is known to affect the cardiopulmonary system, causing heart palpitations, dyspnea, chest pain, and rarely death due to cardiac complications, it has not been shown to directly cause overt respiratory pathology (69-71). Most likely, the respiratory illness associated with CHIKF in La Romana is either an exacerbation of

existing respiratory conditions, such as asthma, or co-infection with a respiratory pathogen.

Respiratory co-infection in CHIKF cases confirmed by pathogen isolation has been documented in past outbreaks. In the La Réunion Island from 2005-2006, Lemant and colleagues reported two patients over the age of 60 with laboratory-confirmed CHIKF who were initially diagnosed with pneumonia caused by *Streptococcus pneumoniae* and *Candida albicans* infection, respectively (69). Additionally, in a sample of cases from a 2006-2007 outbreak in Pondicherry and Karaikal, India, 87% of laboratory-confirmed CHIKF patients were co-infected with respiratory syncytial virus (RSV) (265). Of these individuals, 9% were also infected with adenoviruses. Just 4% of CHIKF cases were mono-infected with CHIKV. Curiously, only CHIKV(+) cases were co-infected with multiple respiratory viruses. In this study, five patients received a diagnosis of bronchopneumonia, 4 of pneumonia, and 4 of acute respiratory infection. However, it is unclear whether these diagnoses were confirmed with x-ray imaging. Five of these patients tested positive for CHIKV RNA, while 8 tested negative for RNA but positive for anti-CHIKV IgM. The lack of CHIKV RNA and therefore replicating virus in over half of the CHIKF(+) respiratory diagnoses, paired with the concurrently higher percentage of CHIKF(-) patients diagnosed with respiratory indications, suggests that CHIKV and respiratory co-infection is likely but not certain. Respiratory infections inflict a high burden of disease among Dominican children, and in 2002 accounted for 5% of all pediatric deaths (266). My retrospective chart review did not permit us to track the morbidity and mortality of patients following initial diagnosis and hospital admission. Nevertheless, respiratory failure and death in laboratory-confirmed CHIKF cases were

features in the outbreaks in La Réunion, France, and in southern India (69, 265, 267, 268), where co-infection with respiratory pathogens was hypothesized to contribute to the elevated morbidity and mortality. Understanding how respiratory co-infections and underlying respiratory conditions may have contributed to morbidity and mortality in the 2014 Dominican Republic outbreak and in potential future outbreaks should be a research priority.

In all, this study provided valuable clinical insights into the CHIKF outbreak in La Romana, DR with potential to broadly extrapolate to other areas in the Americas where CHIKV is now circulating.

Strengths and Limitations

This study succeeded in identifying unique clinical and laboratory factors that were present during the outbreak of CHIKF in the Dominican Republic in 2014. These data have demonstrated new clinical characteristics that will be used by the Ministry of Public Health to strengthen surveillance efforts for febrile syndromes. However, as an outbreak investigation this study had several limitations to generalizability. The study had an inherent selection bias in that samples came from two hospitals in La Romana, which is not necessarily representative of a random sample of the Dominican population and may represent sicker patients than the general infected population; further, the study only represented one town in the DR. The selection was further biased by the fact that RT-qPCR and IGM ELISA for CHIKV were run on all patients subjected to a CBC test. Reporting data, therefore, were sparse and inconsistent, and included patients who may or may not have experienced febrile symptoms. Since historical and physical exam data were not standardized during the period of study, extracting them from clinical records

created an information bias that included the possibility of various measurement errors (vital signs, interview information, etc.)

CHAPTER III. CHIKUNGUNYA VIRUS STRAINS SHOW LINEAGE-SPECIFIC VARIATION IN VIRULENCE IN LETHAL CHIKF MODEL

INTRODUCTION

The pathogenesis and virulence differences among CHIKV lineages have not been formally investigated, although some comparisons have been made based on murine models. One study comparing a single ASN strain to a single ECSA strain, both isolated in Malaysia, found that when inoculated into the brains of suckling mice, the ASN strain causes higher mortality and higher upregulation of pro-apoptotic genes, whereas the ECSA strain produces lower mortality and higher up-regulation of anti-viral and anti-apoptotic genes (269). Conversely, when the IOL isolate LR2006 OPY 1 and the Caribbean/ASN isolate CNR20235 are inoculated into adult mice, LR2006 OPY1-infected mice show greater joint pathology in terms of immune infiltrate, while CNR20235-inoculated mice show milder joint pathology and exhibit lower levels of inflammatory cytokines. Further, the same study found differing natural killer (NK) cell response to viral infection between the two strains. Together, these studies suggest that CHIKV strains from different lineages may induce differential murine cytokine and cell-signaling responses, although it's difficult to determine whether this is a strain- or lineage-dependent phenomenon as neither study investigated multiple strains from the same lineage (270). Nevertheless, differential responses to infection, if the murine model accurately represents human infection, would likely result in varying pathologies. Some researchers have suggested that ASN strains generally cause milder disease in humans, although these claims are based on anecdotal evidence rather than structured scientific inquiry. Therefore, further investigation is required to delineate lineage-specific virulence and pathogenesis differences among CHIKV strains.

This project aimed to explore the variation in virulence between all four CHIKV lineages in A129 mice. In total, 16 strains were tested, focusing on viremia, weight loss, and survival.

MATERIALS AND METHODS

CHIKV strains

CHIKV strains 15561, LR 2006 OPY1, and CAR 256 were all rescued from infectious clones as previously described (54, 271, 272). CHIKV isolates TA0006 and HIII0044 were derived directly from patient and mosquito samples, respectively. All other CHIKV strains were provided by the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA).

Mice

4-6 week old *IFNAR*$^{-/-}$ A129 mice were obtained from a colony maintained at the University of Texas Medical Branch under pathogen-free conditions (UTMB, Galveston, TX). Animals were monitored daily for signs of disease, including ruffled fur, hunched posture, lethargy, signs of dehydration, and significant weight loss. Any animal found moribund (significant lethargy, tremors, dehydration, and/or 20% weight loss) were humanely euthanized by CO_2. Animal experiments were performed in the Galveston National Laboratory animal biosafety level 3 facility, under the supervision of the animal resource center at UTMB and following approved IACUC protocol 0608096B.

Plaque assay

Vero cells were grown to 90-100% confluency in either 12- or 6-well plates. Virus was diluted in DMEM supplemented with 5% FBS and from a series of 10-fold dilutions, 100uL was plated per 12-well and 200 uL was plated per 6-well plate. After 30 minutes incubation, an overlay composed of DMEM and 0.2% agarose was added and incubated for 24-48 hours. Plates were

then fixed with 10% formaldehyde for at least one hour before staining with crystal violet, and plaques counted.

Statistics

Isolates were pooled into their respective lineages for all analyses. Kaplan-Meier survival curves were generated and analyzed in SigmaPlot (Systat, San Jose, CA). Viremia and weight data were analyzed using one-way ANOVA and repeated-measures ANOVA, respectively, using SPSS Statistics software (IBM Corp, Armonk, NY)

RESULTS

A129 mice, age 4-6 weeks, were inoculated with 10µL containing approximately $1x10^4$ PFU of CHIKV in the left rear footpad (n=5 or 6 per isolate). All mice were monitored daily for signs of disease, and any mice which met designated criteria were humanely euthanized. These deaths were recorded as occurring on the following day. CHIKV strains were selected to represent all major lineages identified in CHIKV phylogenetic trees and on availability, and passage history was investigated (Table 7). Most strains were passaged 6 times or fewer, 4 times in human cells and up to 2 times in insect cells, which have been suggested to induce lower mutation rates in RNA viruses ASN strains and isolates were divided into those isolated from Old World outbreaks and those isolated from American outbreaks (ASN and CRBN, respectively). All mice succumbed to WA strain infection by 4 days post-infection (DPI), while mice infected with IOL and ECSA succumbed by days 4 and 5, respectively. Mice infected with ASN isolates all succumbed to infection by day 5, while mice infected with isolates from the American subset of ASN strains (Caribbean, CRBN) survived up to day 8. Log-rank tests revealed that WA and CRBN survival were significantly different than ASN, IOL, and ECSA, although no differences were observed between the latter three (Log-rank analysis of survival, $p<<0.005$ for WA vs. IOL/ECSA/ASN/CRBN and CRBN vs. IOL/ECSA/ASN).

While no differences were observed for weight change (repeated measures ANOVA, p=0.32), WA isolate-infected mice became significantly more viremic than ECSA and IOL infected mice 1 DPI, and WA-infected mice also became significantly more viremic than ECSA. IOL, and CRBN infected mice 2 DPI. No WA-infected mouse serum was available for plaque assay after 3 DPI, and no other differences were observed between any other groups.

Table 8. CHIKV strain data

Strain/Isolate	Lineage	Year	Location	Source	Passage History	Backtiter (PFU/100µL)
YO111213	CRBN	2014	French Guiana	Human	C6/36-2, Vero-2	$1.09x10^4$
YO123223	CRBN	2014	Guadeloupe	Human	C6/36-2, Vero-2	$1.90x10^4$
TA0006	CRBN	2015	Mexico	Human	Vero-2	$3.00x10^4$
HIII0044	CRBN	2015	Mexico	Mosquito	Vero-2	$1.55x10^5$
R99659	CRBN	2014	British Virgin Islands	Human	C6/36-2, Vero-2	$8.50x10^3$
15561	ASN	1962	Thailand	Human	Unk,*Vero-2	$2.50x10^4$
LR	IOL	2006	La Reunion	Human	Vero-3, SM-1, *Vero 1	$1.40x10^4$
SL07	IOL	2007	Sri Lanka	Human	Vero-2, C6/36-1	$2.1x10^4$
Bianchi	IOL	2007	Italy	Human	Unk, *Vero-2	$1.6x10^4$
CAR256	ECSA		Central African Republic	Mosquito	SM-1, C6/36-1, *Vero-1	$3.4x10^4$
LSF-5	ECSA	1960	Congo	Human	SM-1, Vero-2, C6/36-1	$3.4x10^4$
ROSS	ECSA	1953	Tanzania	Human	SM-16, Vero-2, C6/36-1	$3.4x10^4$
SAH2123	ECSA	1976	South Africa	Human	Mosquito-1. SM-2, Vero-1, C6/36-1	$7.8x10^4$
SH2830	WA	1966	Senegal	Human	SM-3, Vero-2, C6/36-1	$3.8x10^4$
SV-0444-95	ASN	1995	Thailand	Human	MK2-1, Vero-1, C6/36-1, Vero-1	$1.1x10^4$
37997	WA	1983	Senegal	Mosquito	Vero-3	$5x10^4$

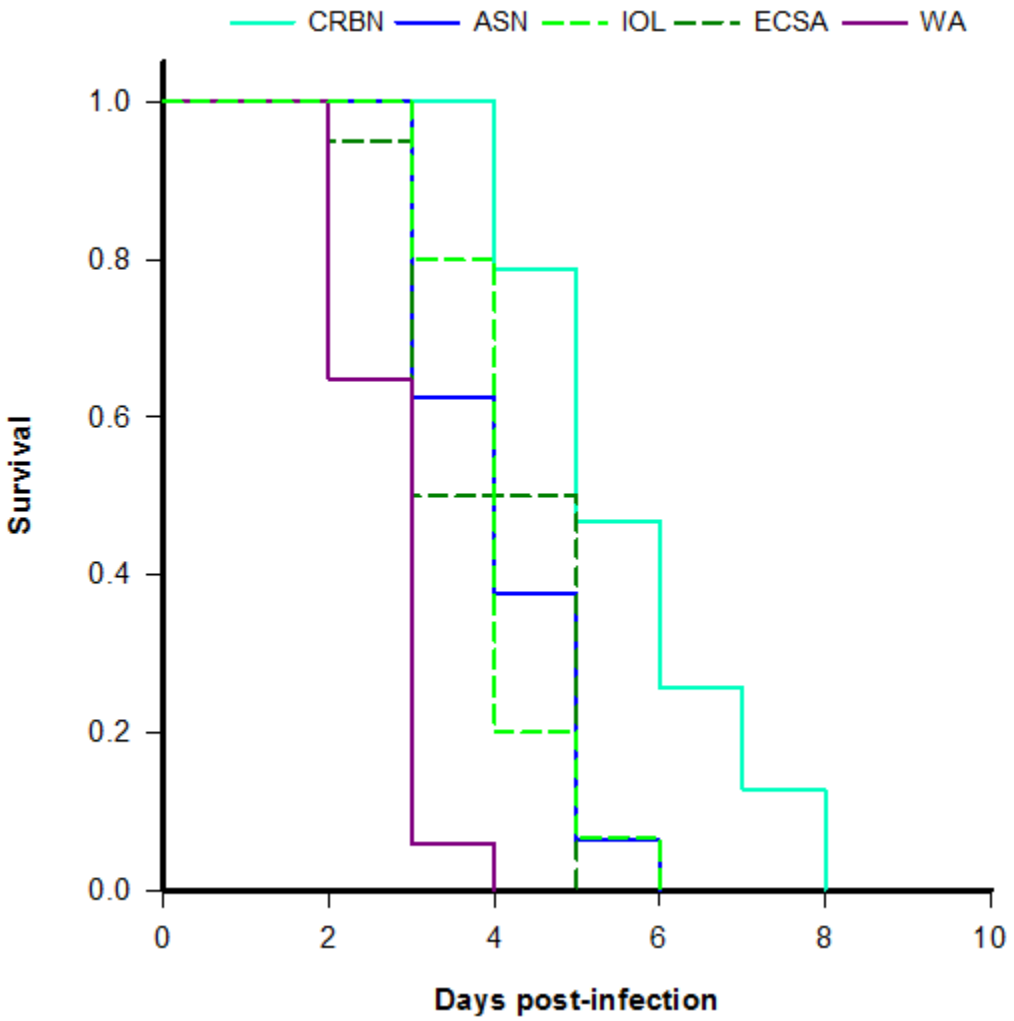

Fig. 11. Survival of CHIKV-infected A129 differs by lineage.

A129 mice were infected with approximately 10^4 PFU CHIKV isolate from one of 5 genetic CHIKV lineages (n=5 or 6 per group) and survival was assessed, with euthanasia counting as a death on the following day. Isolates were pooled into their respective groups, and survival curves were analyzed by Kaplan-Meier survival analysis using log-rank test in SigmaPlot with Holm-Sidak method of multiple pair-wise comparisons. WA strains induced a significant left-shift in the survival curve compared to all other lineages, while CRBN-strain infected mice survived significantly longer than all other lineages ($p<<0.005$ for all); survival was not significantly different between ECSA, IOL, and ASN-infected mice.

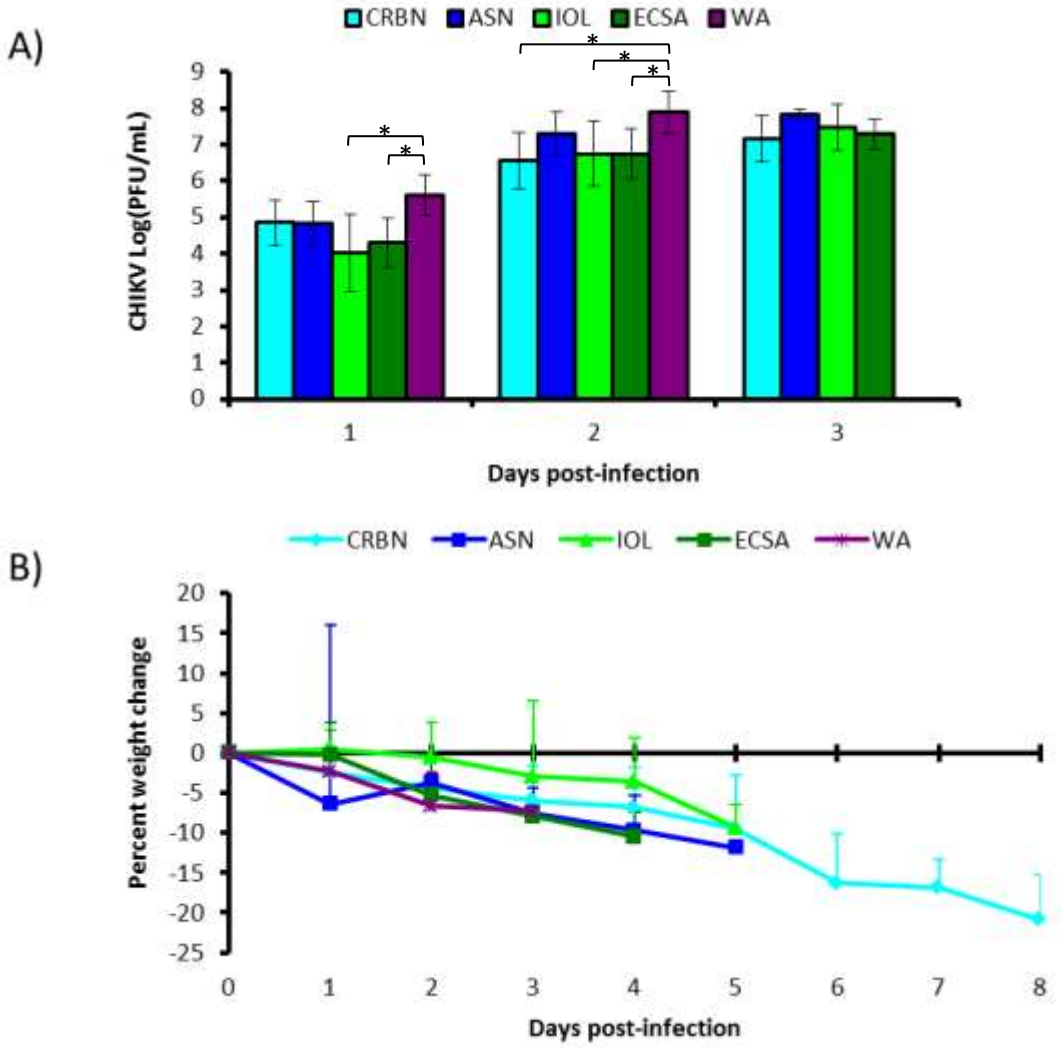

Fig. 12. Comparison of viremia and weight change between A129 mice infected with CHIKV isolates from different lineages.

A129 mice were infected with approximately 10^4 PFU CHIKV isolate from one of 5 genetic CHIKV lineages and blood was taken on days 1-3 from alternating mice to assess viremia (A) and weight was monitored daily (B); data was pooled by lineage for analysis. A129 mice infected with WA CHIKV-isolates had significantly higher viremia than those infected with ECSA and IOL isolates 1 day post-infection, and ECSA, IOL, and CRBN 2 days post-infection (ANOVA, $p<0.05$). Weight change was not significantly different (Repeated measures ANOVA, $p=0.32$)

Discussion

In general, CHIKV strains and isolates share a high nucleotide sequence identity: envelope protein genes can share over 99.8% nucleotide sequence identity between

isolates in the same clade, while isolates from different lineages can diverge by 4.4-15.5% (14). Despite nucleotide sequence divergence, however, the envelope proteins generally share between 95-99.9% amino acid identities. On the other hand, even between closely related isolates, nsP3 may diverge more than 6% in amino acid sequence identity (14). Even though this diversity is relatively small, particularly in the amino acid sequences, other groups have observed notable differences in pathogenesis and virulence between ECSA and ASN lineage isolates as noted above (269, 270). Indeed, certain amino acids substitutions even between ECSA and IOL lineages can impact *in vitro* measures of virulence, such as plaque size, cytopathic effect, and replication kinetics (273).

Here, I show that genetically distinct clades of CHIKV isolates vary in virulence in A129 mice. Notably, I have included WA strains, and divided ECSA and ASN strains into relevant sub-lineages for further analysis. This is the first time a large-scale comparison of the virulence of all CHIKV genotypes has been made, adding considerably to the research that aims to elucidate mechanisms of CHIKV emergence and severity of CHIKF outbreaks. WA strains appear to be more virulent, with mice universally succumbing to infection within 4 days, while 50% of mice survive to day 5 when infected with a CRBN strain. Further, WA isolates induce significantly higher levels of viremia than ECSA, IOL, and CRBN isolates, further suggesting enhanced replicative ability compared to these lineages. CRBN strains in general appear to be more attenuated than WA, ECSA, and IOL strains based on these results, although viremia induced by these isolates are similar to ECSA, IOL, and ASN isolates. This latter result is not surprising, as Simmons et al describe high levels of viremia in blood donations made in Puerto Rico

during the CRBN CHIKF epidemic in 2014, suggesting that patients were asymptomatic despite high levels of viremia (274); while CRBN strains were lethal in mice, CHIKV is universally lethal in A129 mice and infection with a CRBN isolate significantly extended survival compared to other lineages with no apparent decrease in viremia. These data also generally agree with *in vitro* studies examining the role of the 3' UTR in the epidemic potential of CHIKV strains. Chen et al. found that IOL strains replicate to higher titers than ASN strains *in vitro* in a mosquito cell line, and that altering the 3' UTR in the ASN strain tested resulted in altered replication kinetics in mosquito cells (although switching the IOL strain UTR for the ASN UTR had no effect on IOL strain replication) (36). Indeed, a duplication in the 3' UTR of CRBN isolates appears to be responsible for enhanced replication of these viruses in tissue culture (275). Given the high amino-acid identity but high nucleotide sequence diversity in the untranslated regions of the CHIKV (and other alphavirus) genomes, in conjunction with stark contrasts in virulence between lineages and isolates, it is likely that the 3' UTR plays a key role in pathogenesis and virulence. Although this putative variance in virulence needs to be further investigated in other disease models with more representatives of each CHIKV lineage, identifying specific factors which enhance or detract from pathogenesis is an important research goal in the field of CHIKV research.

While it has been speculated that ASN strains may cause less severe disease in humans, these observations are anecdotal and remain empirically unconfirmed (73). This is the first time a large-scale *in vivo* comparison of the virulence of all CHIKV genotypes has been made, adding considerably to research that aims to elucidate mechanisms of CHIKV emergence and severity of CHIKF outbreaks. Specifically, my virulence data

could provide researchers with a more thorough background upon which to base future virulence and emergence studies. Paired with observations made during the DR CHIKF outbreak, as well as evidence of sub-clinical cases of ASN-strain CHIKF in the Philippines (73) from which the emergence of CHIKV in the Caribbean is thought to have originated (32), these data further support the hypothesis that ASN CHIKV isolates, especially CRBN isolates, inflict less severe disease despite a high outbreak potential.

CHAPTER IV. HOST PROTEIN DISULFIDE ISOMERASE: A POTENTIAL PAN-ALPHAVIRUS ANTIVIRAL DRUG TARGET.

INTRODUCTION

Given the necessity of disulfide bonds to the structure and function of all alphavirus envelope proteins, prevention of the formation and isomerization of these bonds offers a potential pan-alphavirus approach for antiviral development. While reducing-agents such as DTT and BME are impractical for clinical purposes due to their nonselective activity, disulfide bond formation and isomerization is mediated entirely by a group of redox enzymes containing a thioredoxin (TRX)-like domain, defined by a Cys-X-X-Cys motif in the active site. This group is broadly called protein disulfide isomerases (PDIs), after their vital function in forming and isomerizing disulfide bonds.

The PDI family consists of 20 enzymes, which share considerable structural and functional similarity. In general, PDI enzymes are composed of two primary domains, “a” domains and “b” domains, configured in various numbers of each. For example, PDI (P4HB) is composed of two a domains, a and a’, and two b domains, b and b’ (276, 277). The a domain is the catalytic domain, sometimes described as a TRX-like domain, and is chiefly responsible for the enzymatic activity of the proteins (278). The a domains tend to be conserved. Conversely, the b domains tend to be divergent, sharing less than 16.5% nucleotide sequence identity, even within the same protein (279). The primary role of b domains within PDI family members is high-affinity binding of small peptide substrates, conferring specificity to the PDI in question (280). There also appear to be some redundancies among PDI-coding genes, with some paralogous PDIs as revealed through sequence alignment in Ensembl (for example, TMX3, PDIA5, TXNDC5, and DNAJC10 are paralogous; geneensembl.org). Together, PDI-family enzymes are critical for mediating redox reactions within the ER, folding un-folded proteins, and re-folding or sequestering misfolded proteins. In fact, P4HB was the first enzyme to be credited as a

chaperone protein (281). Underscoring the indispensable roles of PDIs in the cell, a viable knock-out mouse has yet to be developed for any PDI enzyme.

PDI is a target of interest in many disease states, especially since over-expression of PDI can be either pathologic or protective. On the one hand, over-expression of PDI in myocardio-pathologies has been shown to be protective, as PDI is hypothesized to decrease oxidative damage and prevent apoptosis of cardiomyocytes during hypoxic events (282, 283). On the other hand, PDI is over-expressed in many different types of tumors, and has been shown to aid in the proliferation, survival, and invasion of ovarian cancer (284), melanoma (285), and glioma (286). In light of the potential role of PDI in the pathophysiology of these cancers, several groups have tried to inhibit PDI as a means to prevent cancer cell growth. PACMA31, a compound shown to specifically bind the P4HB active site, inhibited ovarian cancer cell growth both *in vitro* in an ovarian cancer cell line and *in vivo* in a xenograft model of ovarian cancer (284). These results were observed with very little toxicity, shown both by clinical observations of mice as well as histopathological analysis of kidney and liver, at doses as high as 200mg/kg (284). Several commercially available inhibitors are available, which inhibit PDI or other enzymes which regulate their function by maintaining the oxidative state of PDI. One such compound, 16F16, is commercially available and can bind multiple isoforms of PDI (287). EN460 is a commercially available compound which selectively binds ERO-1 (288), an enzyme which oxidizes PDI (288), while auranofin is an FDA-approved, commercially available anti-rheumatic compound that inhibits thioredoxin reductase (TRX-R) (289), which reduces PDI .

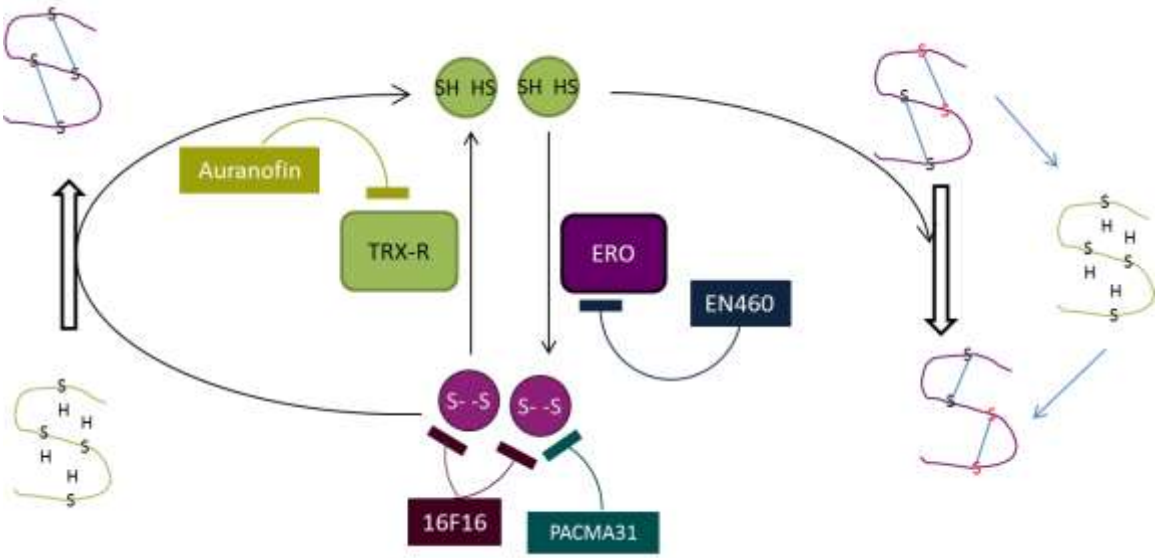

Illustration 13. PDI mediated disulfide bonding and related inhibitors.

PDI cycles between oxidized and reduced forms, with the oxidized form contributing to disulfide bond formation and the reduced form isomerizing existing disulfide bonds. While PDI cycles naturally between these two forms, ER enzymes thioredoxin reductase (TRX-R) and ER oxidoreductins (EROs) also contribute to the reduction and oxidation of PDI, respectively, in response to changes in overall oxidative state in the ER (Marzano et al 2007) (290).

Inhibition of PDI as an antiviral strategy, although still an emerging concept, has been investigated for the treatment of other viral diseases. The entry process for HIV has been well described, and it has been shown that entry of HIV into the cell is dependent on the oxidation of cysteine residues in the GP120 protein (291, 292). These discoveries were made in part because it was found that addition of bacitracin, a known inhibitor of PDI, as well as anti-PDI antibodies, to HIV-infected cell cultures inhibited replication of HIV; additionally, exogenous reducing agents had a similar effect (293). A group later identified juniferdin and derivatives as potential inhibitors of HIV, as these compounds were shown to inhibit HIV gp120 reduction (293).

Research in the HIV field supports the notion that targeting the thioredoxin system is a viable option for antiviral therapeutics. Given the importance of disulfide bonds to the architecture of the alphavirus virion, and known interactions with various PDI isoforms, PDI and its regulatory proteins may collectively represent a potential pan-alphavirus drug target. Here, the

effects of PACMA31, 16F16, EN460, and auranofin on CHIKV replication were explored, with the aim to develop PDI as a target for broadly acting antivirals against alphavirus infection.

MATERIALS AND METHODS

PDI inhibitors

16F16 was obtained from Sigma-Aldrich (St. Louis, MO). EN460 was obtained from EMD Millipore (Darmstadt, Germany). PACMA31 and auranofin were obtained from Bio-Techne (Pittsburgh, PA). For *in vitro* use, compounds were suspended in Hybri-Max DMSO (Sigma-Aldrich, St. Louis, MO) to a 10mM stock; dilutions were prepared in 96-well plates, first round of dilutions in DMSO followed by 2 rounds of 1:10 dilutions in antibiotic-free medium to a final DMSO concentration of 1%. Compound was suspended in PBS to 10% DMSO for pharmacokinetic experiments and toxicity experiments, while compounds were suspended directly into 0.4% methyl cellulose in DMEM for *in vivo* efficacy.

Virus stocks

CHIKV strains SL07 and CHIKV-181/clone 25 (attenuated vaccine candidate), and VEEV strain ZPC738 (VEEV-ZPC) were rescued from respective infectious clones as previously described (199, 271, 272, 294). ZIKV strain FSS1302 and CHIKV strain YO123223 were provided by the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA, Galveston, TX). Virus stocks were generated by passaging virus 1-2 times in either African green monkey kidney cells (Vero) or *Aedes albopictus* $C_7$10 cells.

Tissue culture

African green monkey (Vero) cells, human embryonic kidney (HEK-293) cells, and *Aedes albopictus* ($C_7$10) were obtained from ATCC (American Type Cell Culture, Bethesda, MD). Vero cells were maintained in DMEM supplemented with 1% penicillin/streptomycin (P/S) and 5%

fetal bovine serum. HEK293 cells were maintained in DMEM supplemented with 1% P/S, 10% FBS, and 1% non-essential amino acids and sodium pyruvate. $C_7$10 cells were maintained in DMEM supplemented with 10% FBS, 1% P/S, and 1% tryptose phosphate broth.

Cytotoxicity

Cytotoxicity was assessed using AlamarBlue (ThermoFisher Scientific, Waltham, MA) assays, following manufacturer's instructions. Briefly: 25-50K HEK293 cells were seeded in each well of a 96-well plate using dye-free medium and incubated over-night. Medium was removed, and 100uL of compound-treated, dye-free medium was transferred from dilution plate to cells at various time points. 8-hours after the last time point, 10uL of AlamarBlue reagent was added to each well and incubated for 4 hours at 37ºC. Absorbance readings were taken at 570 and 600 nm on a VersaMAX plate reader (Molecular Devices, Sunnyvale, CA), and cell numbers calculated from a standard plate and the formulas provided by the company.

Plaque assay

Vero cells were grown to 90-100% confluency in either 12- or 6-well plates. Virus was diluted in DMEM supplemented with 5% FBS and from a series of 10-fold dilutions, 100uL was plated per 12-well and 200 µL was plated per 6-well plate. After 30 minutes incubation, an overlay composed of DMEM and 0.2% agarose was added and incubated for 24-48 hours. Plates were then fixed with 10% formaldehyde for at least one hour before staining with crystal violet, and plaques counted.

Transmission EM

Medium was removed from CHIKV-infected HEK293 cells and flasks were immediately fixed for 2 hours in PFGPA.1 (2.5% formaldehyde, 0.1% glutaraldehyde, 0.03% picric acid (trinitrophenol), 0.03% CaCl2, 0.05 M cacodylate buffer pH 7.3–7.4). Fixative was then removed, and 0.1 M cacodylate buffer added. Flasks were stored at 4C for less than 24 hours before cells

were scraped, pelleted, and processed as previously described (295). Ultrathin sections were cut on Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo grove, IL), placed on copper grids, stained with lead citrate and examined on a Philips 201 electron microscope at 60 KV.

RNA extraction

Cells were washed and incubated with Trizol (LifeTechnologies, Carlsbad, CA). Chloroform was added to trizol samples, and RNA was extracted from aqueous phase using Zymo Direct-zol with DNase-I extraction kit RNA (Zymo Research, Irving, CA) per manufacturer's instructions.

RT-qPCR

RT-qPCR was performed on QuantStudio 6 Flex Real-Time PCR system (ThermoFisher Scientific, Waltham, MA) using the Taqman RNA-to-C_t one-step kit following manufacturer's instructions (LifeTechnologies, Carlsbad, CA), using the following primers/probes targeting the E1 gene: forward, TGGAGCTTCTGTCTGTCACC; reverse, ACGTACGGAGACGGGATAAC; and probe, 56-FAM-TCGCTTGATTAATCACGTGCGAG-3BHQ_1. RNA used to calculate standard curve were generated from plasmid containing full EILV/CHIKV chimera DNA using SP6 transcription kit per manufacturer's instructions (Ambion, Foster City, CA).

siRNA

A custom siRNA plate was ordered from Dharmacon (Dharmacon, Lafayette, CO), and HEK293 cells were transfected with 50nM siRNA using Dharmafect1 reagent (Dharmafect, Lafayette, CO) according to manufacturer's instructions. Cells were incubated with siRNA for 48 hours before infection with CHIKV-181/clone 25. Supernatant was collected 24 hours post-infection. Simultaneously, cell lysates were collected using RIPA buffer (ThermoFisher Scientific, Waltham, MA) according to manufacturer's instructions.

Western blot

Protein samples from cell lysates were quantified using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). 30ug of protein from each sample were incubated with Bolt LDS sample buffer, Bolt reducing agent, and water according to manufacturer recommendations, and 40uL of sample was run on a Bolt 10-well 4%-12% Bis-Tris gel at 200V. Transfer to nitrocellulose was performed using the iBlot semi-dry transfer system. Blotting was performed using the iBind system with iBind FD solution. Bolt, iBlot, and iBind systems by LifeTechnologies (LifeTechnologies, Carlsbad, CA). Relative band density was determined using ImageJ software.

Antibodies

Primary polyclonal rabbit anti-PDI antibodies were purchased from Abnova (Abnova, Taipei, Taiwan), as well as mouse anti-GAPDH. Secondary chicken anti-rabbit IgG/Alexa Fluor 488 conjugated and goat anti-mouse IgG/Alexa Fluor 647 conjugated antibodies were ordered from ThermoFisher (ThermoFisher Scientific, Waltham, MA)

Mice

Gestation day-18 CD-1 mice were obtained from Charles River (Charles River, Wilmington, MA), and pups were allowed to grow to 15 days of age after birth. 3-week old C57Bl/6 mice were obtained from Charles River and allowed to acclimate for 1 week (Charles River, Wilmington, MA). Animals were housed in either the ABSL-2 or ABSL-3 facilities in the Galveston National Laboratory. All animal experiments were performed in accordance with the UTMB Institutional Animal Care and Use Committee protocol #0209068B, and under the supervision of the Animal Resource Center and in strict adherence to standards of care set thereforth.

Statistics

EC_{50}, CC_{50}, and therapeutic indices were calculated using non-linear curve fitting and dose-response functions in Graphpad Prism. Normalcy was evaluated using Q-Q plots, and p-values

were calculated using either ANOVA, Kruskal-Wallis (K-W), or repeated measures ANOVA in SPSS statistics (IBM Corp, Armonk, NY).

RESULTS

PDI inhibitors inhibit CHIKV replication in vitro in dose-dependent fashion with varying levels of cytotoxicity.

Cytotoxicity is critical for interpreting dose-response data, and therefore was assessed. To develop cytotoxicity curves (Figure 12), HEK293 cell viability was assessed using AlamarBlue assay after 8, 12, 24, or 48 hour incubation with varying concentrations of PACMA31, 16F16, EN460, or auranofin. 16F16 and auranofin were the most toxic compounds, with 50% toxicity observed at concentrations at concentrations 12 μM or below after only 8 hours of treatment. Conversely, PACMA31 and EN460 were less toxic, although toxicity appeared to increase after 8 hours of treatment.

To develop dose-response curves (Figure 13), HEK293 cells were plated on 96-well plates and infected with 0.01 MOI Caribbean CHIKV isolate YO123223, chosen for its relevance to the ongoing CHIKF outbreaks in the Americas. One hour post-infection, cells were treated with concentrations between 0.1-100uM PACMA31, 16F16, or EN460, concentrations between 0.01-30uM Auranofin, or 1% DMSO. Supernatant was collected 8, 12, 24, and 48 hours post-infection (HPI) and viral titers were determined by plaque assay. All treatments resulted in a significant decrease in viral replication at higher doses at 12, 24, and 48 HPI, although only EN460 produced significant results at 8 hours post-infection (HPI) (Kruskal-Wallis, $p<0.05$).

Therapeutic indices (TIs) were calculated by dividing the cytotoxic-concentration$_{50}$ (CC_{50}) values by the effective-concentration$_{50}$ (EC_{50}), both of which were determined by sigmoidal-curve regression and point-of-inflection analysis based on cytotoxicity and dose-response curves, respectively (Table 8). The CC_{50} represents the concentration at which 50% of

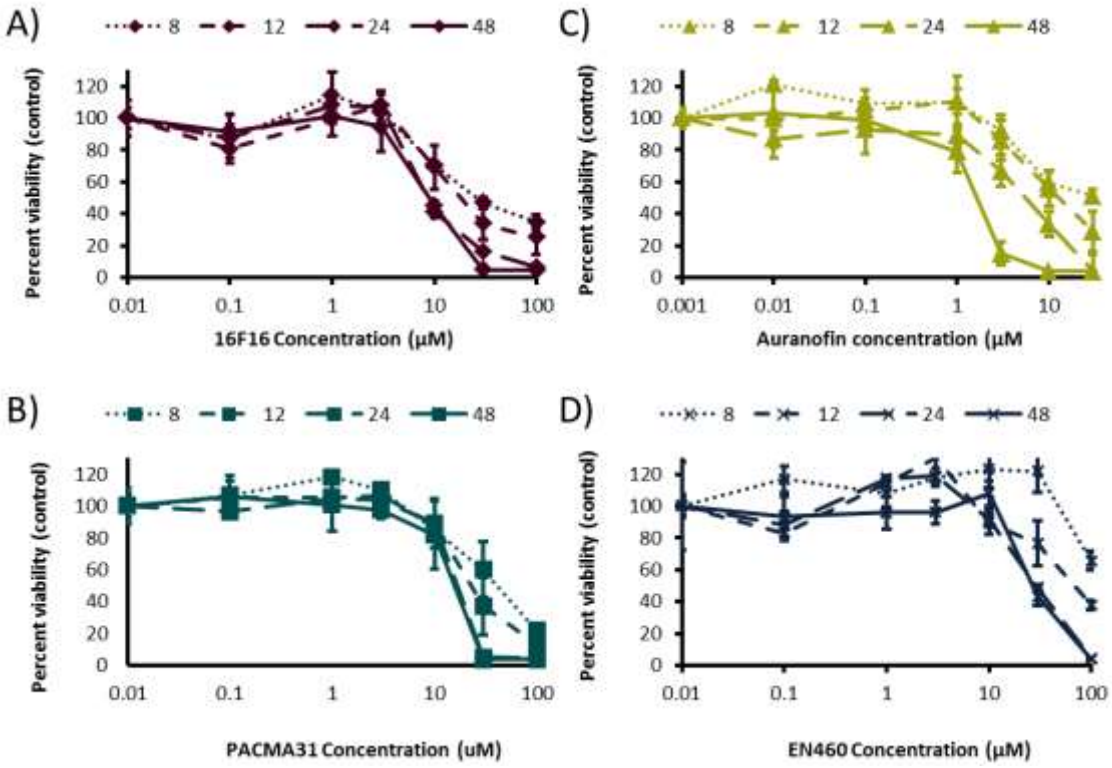

Fig. 13. Cytotoxicity of TRX- and related inhibitors

Cytotoxicity curves for A) PDI-inhibitor 16F16 B) PDI-inhibitor PACMA31 C) TRX-R inhibitor auranofin and D) ERO-1 inhibitor EN460. HEK293 cells were treated with log/half log concentrations of compound and incubated for 48, 24, 12, and 8 hours before treatment with AlamarBlue to assess percent cell viability.

the compound's overall cytotoxicity is observed, while the EC_{50} represents the concentration at which the compound shows 50% of its overall efficacy. Therefore, the TI value weighs a compound's efficacy against its toxicity, with higher values indicating high efficacy and/or low toxicity. In general, TIs were highest at the 8-hour time point for all compounds, with the exception of auranofin, which exhibited its highest TI after 12 hours. Compounds were generally most efficacious at concentrations higher than the CC_{50}, although therapeutic effect (i.e., decrease in CHIKV replication) was observed at concentrations below the CC_{50}. While most compounds produced near-significant decreases in viral replication at the EC_{50} for time points later than 8

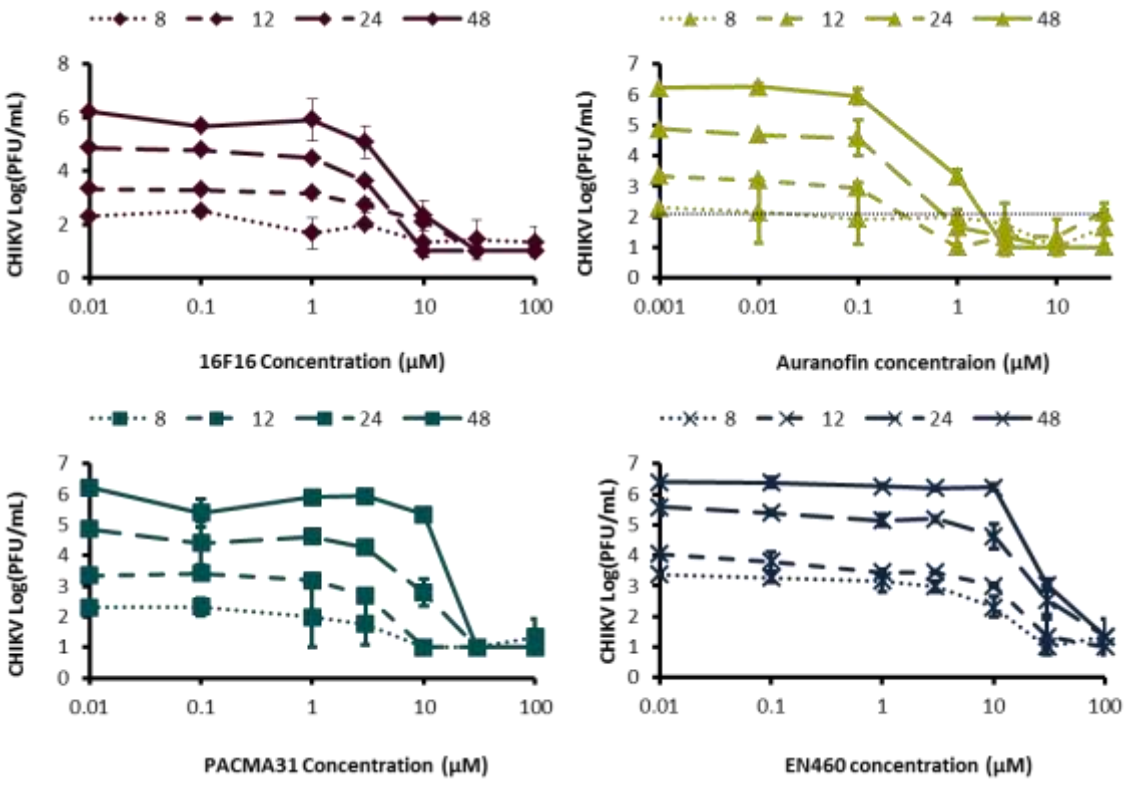

Fig. 14. Dose-response curves for TRX- and related inhibitors against CHIKV.

Dose-response curves for A) PDI-inhibitor 16F16 B) **P**DI-inhibitor PACMA31 C) TRX-R inhibitor auranofin and D) ERO-1 inhibitor EN460. HEK293 cells were infected with Caribbean CHIKV isolate YO123223 (MOI=0.01) one hour before being treated with log/half log concentrations of compound and supernatant was collected at 8, 12, 24, and 48 HPI. Viral titers were assessed by plaque assay. All curves produced after 12 HPI resulted in significant, dose-dependent reduction

HPI (Kruskal-Wallis, pair-wise comparison with Bonferroni post-hoc, $p<0.08$), PACMA31 generally failed to produce even near-significant results at the EC_{50} except at the 48 hour time point, although the therapeutic index was approximately 1. In general, the best performing compound was auranofin, which produced significant decreases in viral replication at the EC_{50} while maintaining the highest TI of 104.5 at 12 HPI. 16F16 produced significant decreases in viral replication, but these decreases were observed at concentrations higher than the CC_{50}. of CHIKV replication, while EN460 produced significant results after 8 HPI (Kruskal-Wallis, $p<0.05$).

Table 9. CC_{50}, EC_{50}, and TI values for inhibitors against CHIKV.

Treatment	Measure	8 HPI	12 HPI	24 HPI	48 HPI
16F16	CC_{50} (μM)	12.2	12	8.405	8.9
	EC_{50} (μM)	1.0	9	8.7*	6.6
	TI	12.2	1.33	0.97	1.35
PACMA31	CC_{50} (μM)	57.9	22.4	13.4	12.2
	EC_{50} (μM)	2.8	3.9	10.3	12.1
	TI	20.68	5.74	1.30	1.00
EN460	CC_{50} (μM)	94.1	40.2	32.5	31.1
	EC_{50} (μM)	10.2	15.	22.5	27.0
	TI	9.23	2.64	1.44	1.15
Auranofin	CC_{50} (μM)	5.85	10.45	7.5	1.6
	EC_{50} (μM)	3.2	0.1*	0.4	1.0
	TI	1.83	104.5	18.75	1.6

Kruskal-Wallis, pair-wise comparison with Bonferroni post-hoc *$p<0.05$

PDI inhibitors inhibit VEEV and ZIKV replication in-vitro

To assess the spectrum for which thioredoxin-pathway inhibitors are effective, dose-response curves were established for 16F16 and auranofin against Zika virus (ZIKV), a flavivirus whose E protein shares structural homology with the alphavirus E1 protein (including disulfide bonds), and VEEV, a distantly related alphavirus, using the same method as the CHIKV dose-response curves. 16F16 and auranofin were chosen since they exhibited the most drastic effect on CHIKV replication. ZIKV replication was significantly decreased in a dose-dependent manner by both auranofin and 16F16 after 24 and 48 hours (Kruskal-Wallis, $p<0.05$), while VEEV replication was significantly inhibited in a dose-dependent manner by both 16F16 and auranofin at all time points (Kruskal-Wallis, $p<0.05$) (Figure 14). Accordingly, EC_{50} values were calculated for VEEV and ZIKV dose-response curves (Table 9). Although 16F16 significantly inhibited VEEV replication, the TI for most time points was less than 1, with the exception of the 12-hour time point which was only slightly above 1, and treatment at the EC_{50} did not result in significant

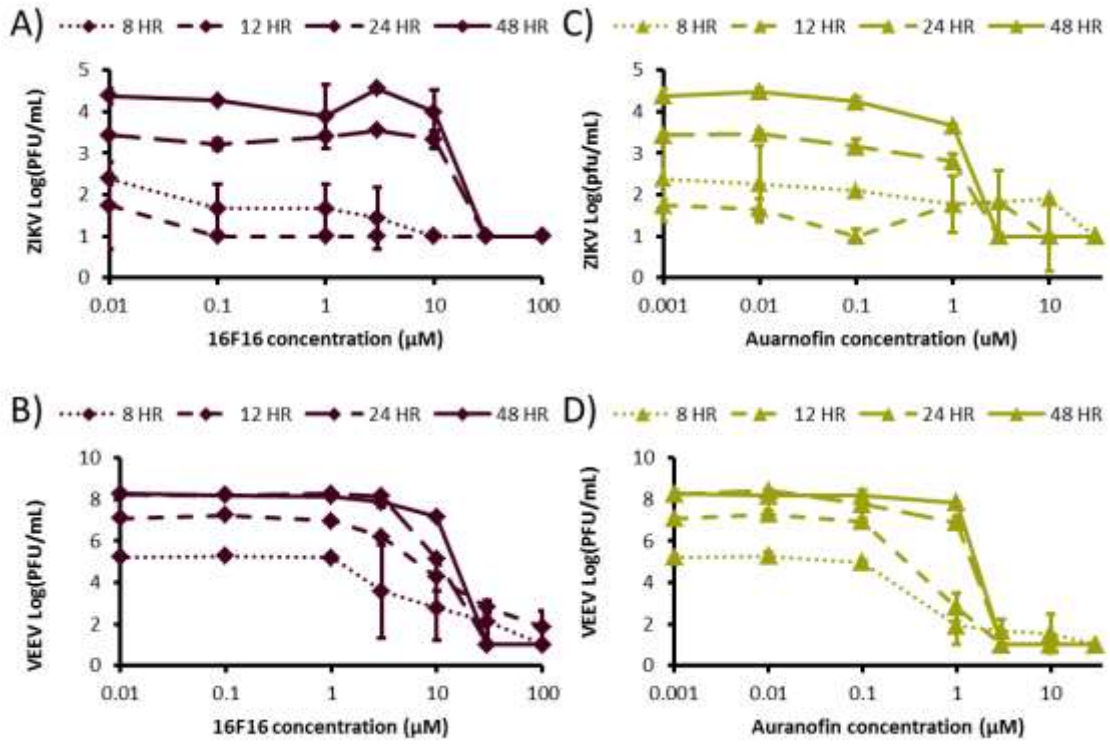

Fig. 15. Dose-response curves for TRX- and related inhibitors against VEEV and ZIKV

Dose-response curves for A,B) PDI-inhibitor 16F16 B) PDI-inhibitor C,D) TRX-R inhibitor auranofin against ZIKV (A,C) and VEEV(B,D). HEK293 cells were infected with VEEV-ZPC or ZIKV FSS13302 (MOI=0.01) one-hour before being treated with log/half log concentrations of compound and supernatant was collected at 8, 12, 24, and 48 HPI. Viral titers were assessed by plaque assay. All curves for VEEV resulted in significant reduction of virus, while both 16F16 and auranofin resulted in dose-dependent significant inhibition of replication after 24 and 48 hours (Kruskal-Wallis, $p<0.05$).

reduction in viral replication. Similar to CHIKV, auranofin had relatively high TIs ranging between 1.45-16. Although significant reduction in titers was not achieved at the EC_{50} treatment levels, significant levels of reduction were achieved at concentrations one-half log higher than the EC_{50} (Kruskal-Wallis, pair-wise comparison with Bonferroni post-hoc, $p<0.05$). ZIKV established highly irregular curve shapes in response to 16F16 treatment, preventing the reliable calculation of an EC_{50} at any time point. Treatment with auranofin also resulted in irregular curves at 8 and 12 HPI; however, significant reductions in ZIKV replication were achieved at

concentrations near the EC_{50} at both 24 and 48 HPI. This is a particularly significant finding at the 24 hour time point, where the EC_{50} fell below the CC_{50}.

Table 10. EC_{50} and TI values for inhibitors against VEEV and ZIKV

		EC_{50} (μM)/TI			
Treatment	**Virus**	**8 HPI**	**12 HPI**	**24 HPI**	**48 HPI**
16F16	VEEV	17.71/0.71	9.625/1.25	10.69/0.79	12.95/0.69
	ZIKV	Irreg. curve	Irreg. curve	Irreg. curve	Irreg. curve
Auranofin	VEEV	0.39/15	0.65/16	1.075/6.98	1.1/1.45
	ZIKV	Irreg. curve	Irreg. curve	1.1*/6.82	1.1*/1.45

Kruskal-Wallis, pair-wise comparison with Bonferroni post-hoc *$p<0.05$

PDI inhibitors alter CHIKV genome:PFU ratios

Treatment of CHIKV-, VEEV-, and ZIKV-infected cells resulted in significant reduction in viral titers. Because all of these viruses rely on disulfide bonds within their respective structural proteins, and these inhibitors act on proteins related to disulfide bonding, the effect these inhibitors have on CHIKV genome:PFU ratio was also investigated as a surrogate for total-particle:infectious-particle ratio; if these inhibitors affect the envelope proteins, one would expect to see little to no effect on viral genome (total particle) production in treated cells, but rather a decrease in infectious particles (PFU). To this end, HEK293-cells were treated with 10μM PACMA31 or 16F16, 1μM auranofin, or 1% DMSO for 1 hour prior to infection with a 2 PFU/cell MOI CHIKV-YO123223 (EN460 was excluded from these experiments, as it had very little affect at early time points in dose-response experiments). To limit viral replication to one replication cycle, supernatant was collected at 8 HPI along with cellular RNA; each supernatant sample was analyzed for both viral RNA by RT-qPCR and infectious particles by plaque assay, while cell lysates was analyzed for viral RNA by RT-qPCR. The concentration of compound was chosen such that both defective and infectious particles would expect to be observed, while limiting cellular toxicity associated with treatment. While all treated groups resulted in fewer infectious particles and similar total particle counts compared to DMSO controls, 16F16 and

auranofin treatment resulted in a statistically significant increase in genome:PFU ratio (Kruskal-Wallis and pairwise comparison with Bonferroni correction, $p<0.05$; Fig. 15A). While treatment with 16F16 and PACMA31 resulted in similar levels of viral RNA in cell lysates, auranofin treated cells had significantly less intracellular viral RNA (Kruskal-Wallis and pairwise comparison with Bonferroni correction, $p<0.05$; 15B).

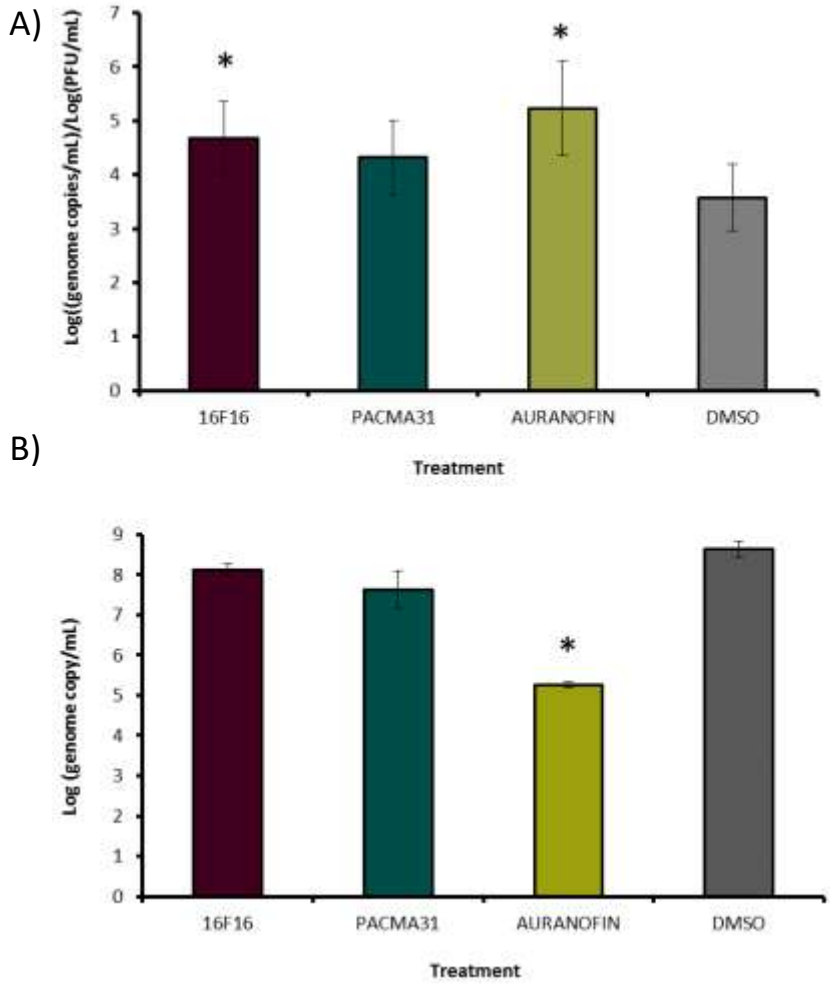

Fig. 16. Effect of inhibitors on genome:PFU ratio and intracellular RNA copies.

HEK293-cells were treated with 10µM PACMA31 or 16F16, 1µM auranofin, or 1% DMSO for 1 hour prior to infection with a 2 PFU/mL MOI CHIKV-YO123223. Supernatant was collected at 8 HPI along with cellular RNA; each supernatant sample was analyzed for both viral RNA by RT-qPCR and infectious particles by plaque assay (A), while cell lysates was analyzed for viral RNA by RT-qPCR (B). 16F16 and auranofin treatment resulted in significantly increased genome:PFU ratios vs. DMSO, while auranofin treatment also resulted in significantly less intracellular viral RNA vs. DMSO (Kruskal-Wallis and pairwise comparison with Bonferroni correction, $p<0.05$)

CHIKV grown in presence of PDI inhibitors exhibit altered virion structure

To confirm that the altered genome:PFU ratio observed in 16F16-treated cells was due to structural changes in the CHIKV virion, thin-section electron microscopy was employed to visualize CHIKV virions produced in the presence of 16F16 and DMSO. To reduce costs, PACMA31 and auranofin were excluded from this study, as they failed to show altered PFU:genome ratios and resulted in decreased intracellular RNA, respectively, strongly indicating a different mechanism of action than altered virus particle production. HEK293 cells were treated for one hour with 10μM 16F16 or 1% DMSO before infection with CHIKV 181/clone 25 (MOI=500). Cells were incubated for 12 hours, and then were fixed for 2 hours before fixative was exchanged for buffer. Cells were pelleted, processed, and sections were mounted on copper grids for visualization on a Philips electron microscope. Budding events were captured in each treatment group; however, virion morphology appeared to be altered in the 16F16-treated cells (Fig. 16). While the DMSO-treated group showed a typical tight, icosahedral morphology approximately 50-60nm in size, virions in the 16F16-treated group appeared larger (75-85nm) and pleomorphic with the membrane only loosely associating with a smaller nucleocapsid-like core.

Anti-TMX2 and anti-PDILT siRNA pools result in significant inhibition of CHIKV replication.

In order to ascertain which PDI members are most crucial to CHIKV replication, PDI mRNA was knocked-down using siRNA pools to individual PDI family members, as well as siRNA to 3 isoforms of HSP90 as controls. GAPD siRNA was also utilized as a knock-down control, in addition to a standardized non-targeting pool to assess transfection reagent toxicity and for use as a general negative control. HEK293 cells were transfected with 50nM siRNA for 48 hours prior to infection with CHIKV-181/clone 25 (MOI=0.01) or mock-infection. siRNA-transfection media was replaced after infection, and supernatant and cell lysates were collected 24

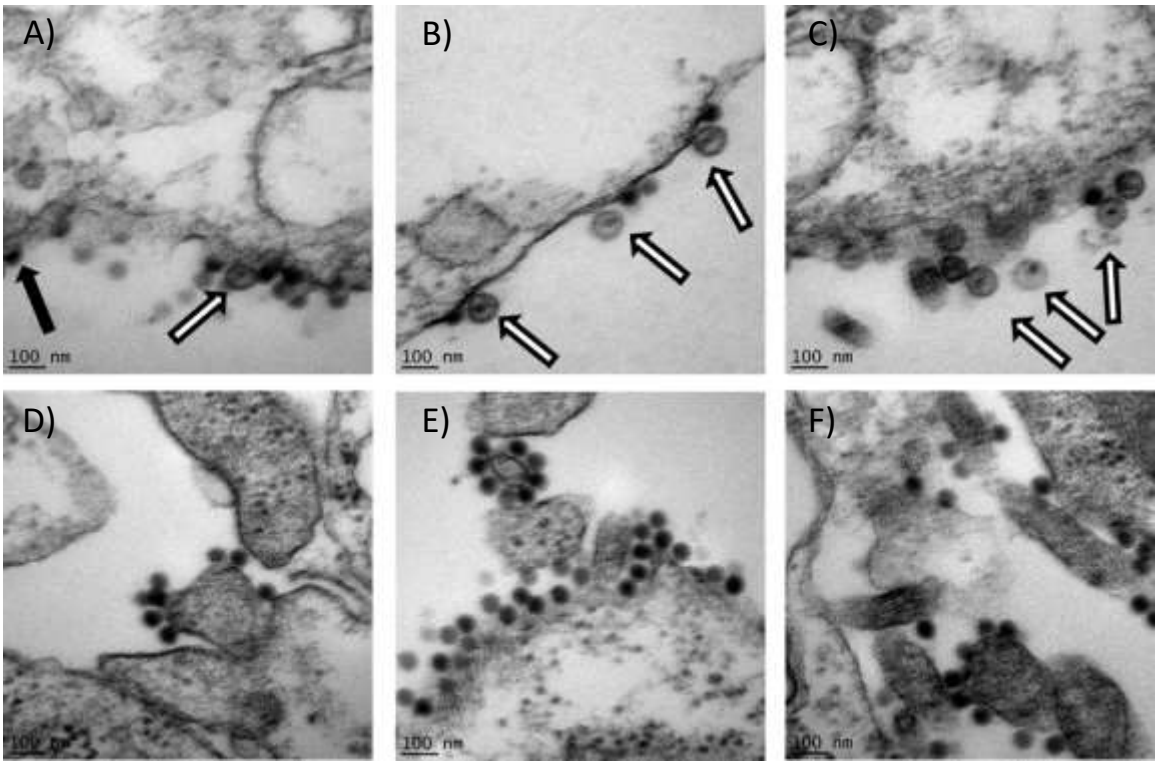

Fig. 17. Effect of 16F16 treatment on CHIKV virion structure.

HEK293 cells were treated with 10μM 16F16 (top panels, A-C) or DMSO (D-F) for one hour prior to infection with CHIKV 181/clone 25. Cells were incubated for 12 hours post-infection, and then fixed for 2 hours in PFGPA.1 buffer before storage in cacodylate buffer until further processing of sections and visualization at 60KV. Enlarged, pleomorphic virions were observed in 16F16-treated samples compared to the highly structured virions observed in DMSO-treated groups.

HPI to determine viral titer and protein knock-down, respectively. Cell viability was also assessed, both for mock-infected and infected groups. Approximately 80% protein knock-down

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viral titer and protein knock-down, respectively. Cell viability was also assessed, both for mock-infected and infected groups. Approximately 80% protein knock-down was achieved through siRNA treatment (example given in Figure 17), with mock-infected groups showing slightly higher PDI expression than infected groups, although GAPD siRNA did not perform as well as PDI siRNA. Anti-PDILT and anti-TMX2 siRNA pools significantly reduced CHIKV replication (ANOVA with Dunnet's post-hoc, $p<<0.05$ and $p=.001$, respectively; Figure 18A); however, the anti-PDILT siRNA pool was severely toxic to the cells, with less 30% of cells surviving transfection. Knock-down of TMX2 resulted in 83% viability in uninfected HEK293 cells and about 60% in infected cells (Figure 18B).

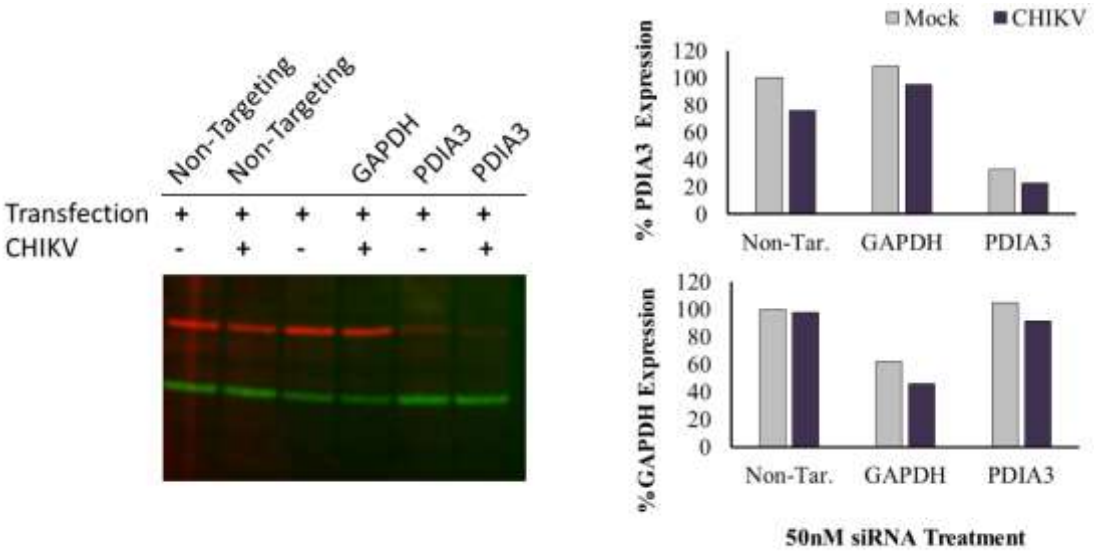


Fig 18. siRNA knock-down of ERp57 and GAPDH.

As a control for assessing siRNA knock-down, PDIA3 (ERp57) was used as an example siRNA pool and GAPDH as a control. Cells were transfected with 50nM siRNA for 72 hours, and then cell lysates were collected. Whole protein-fraction was run on a gel and stained with unconjugated primary antibodies to GAPDH and ERp57 and then Alexa Fluor-conjugated secondary antibodies. siRNA transfection resulted in greater than 60% knock-down for ERp57 in both infected and uninfected groups, while GAPDH siRNA resulted in milder knock-down of approximately 40-50%.

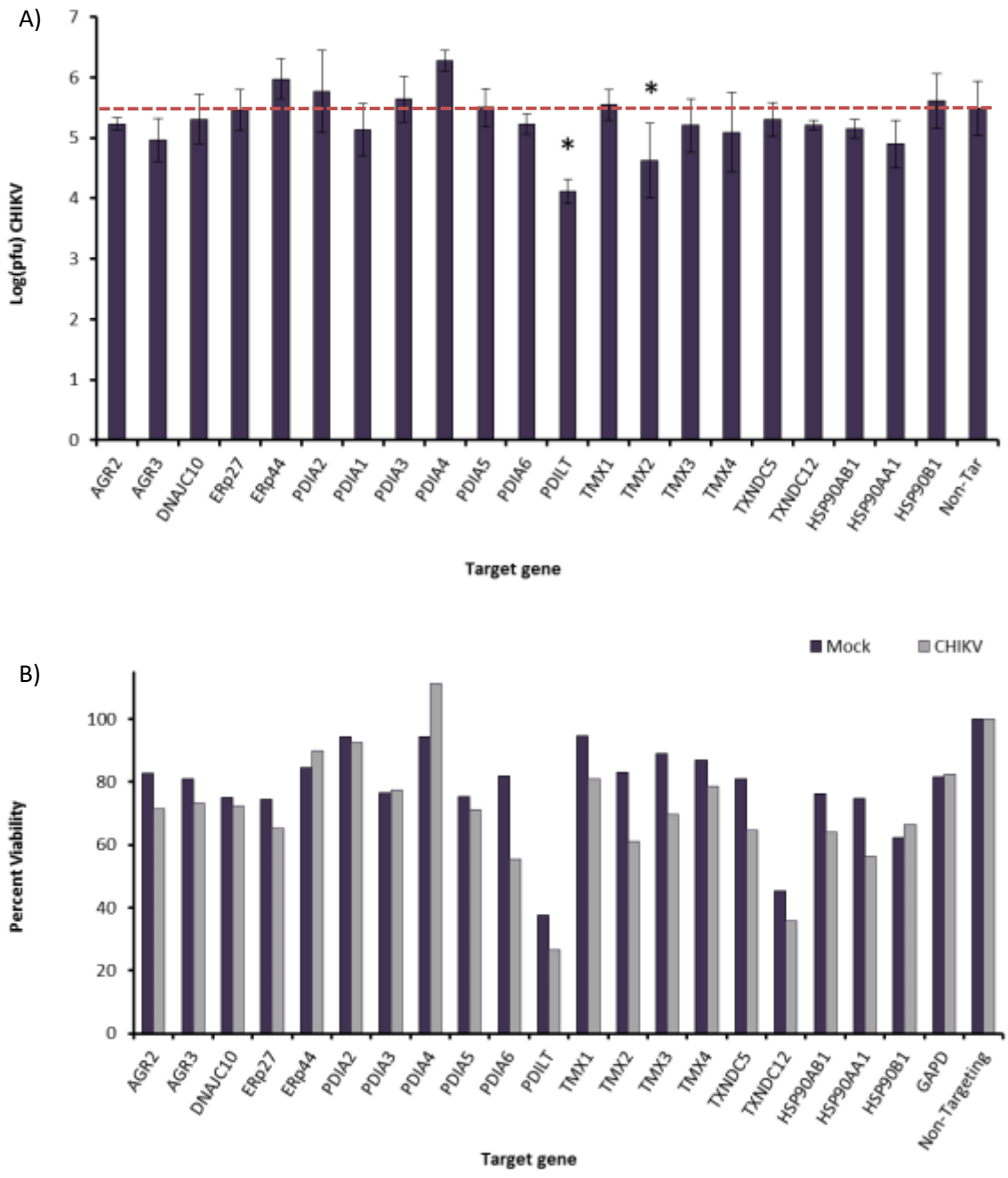


Fig 19. siRNA screen: efficacy and cytotoxicity.

HEK293 cells were transfected with 50nM siRNA for 48 hours prior to infection with CHIKV-181/clone 25 (MOI=0.01) or mock-infection. siRNA-transfection media was replaced after infection, and supernatant was collected 24 HPI to determine viral titer (A). Cell viability was also assessed, both for mock-infected and infected groups (B).

PDI inhibitors are safe to use in young mice and modestly reduce footpad swelling and CHIKV titers in adult mice.

To test the tolerability of PDI inhibitors, various doses of 16F16 and PACMA31, which currently have little to no safety or tolerability data at doses required for efficacy experiments, along with vehicle control, were administered subcutaneously to 15-16 day old CD-1 mice (n=3/group) for 3 days and mice were monitored for clinical health and weight gain. This particular mouse strain serves as a model for some CHIKV experiments (184), and young mice offer an extremely sensitive model to test the toxicity of compounds. Groups included were 10mg/kg, 50 mg/kg, and 200 mg/kg once daily, as well as a 200mg/kg AM + 50mg/kg PM group (figure 19). Most animals gained weight normally, but a few animals either lost weight or gained weight more slowly than other animals. This was associated with starting weight, with animals below 7g responding poorly to handling and/or DMSO treatment (Linear regression, r^2=.546; ANOVA, $p<<0.05$). Animals showed no clinical signs of distress, such as hunched posture or ruffled fur.

Once the safety of PDI inhibitors in young mice was established, the efficacy of PDI and PDI regulator inhibitors *in vivo* was assessed. For this purpose, PACMA31 and auranofin were employed, owing to their use in animals (and humans) previously which provided a precedent for safe dosing regimens. 4-week old C57Bl/6 mice were orally dosed with either 200mg/kg PACMA31(n=10), 20mg/kg auranofin (n=10), or vehicle (n=15) twice daily beginning one hour prior to infection, at least 8 hours apart, for days 0-2. This particular model was selected as adult C57Bl/6 develop viremia more reliably than adult CD-1 mice, and efficacy dosing with auranofin required an oral route (which is impractical in young mice due to their size); dosing regimen was selected based on highest tolerated dose in PACMA31 tolerability experiments and published usage of auranofin (296). Mice were either inoculated with 10uL PBS (n=5) or 10uL PBS containing 10^4pfu CHIKV SL-07 (n=30) and weight, clinical features, and footpad height were monitored daily for 7 days. Blood was drawn retroorbitally from alternating groups of mice (n=5)

for the first 3 days post-infection to assess viremia. CHIKV SL-07 was used instead of CHIKV YO123-223 due to an observed greater virulence in A129 mice. The experiment was carried out until footpad swelling resolved on day 13 (Figure 20). All mice lost weight over the first three days, most likely due to stress or vehicle, as uninfected vehicle controls also lost weight and no statistically significant differences were observed. Weight generally recovered once oral dosing ceased. Auranofin appeared to provide some protection from footpad swelling, delaying the development of swelling over the dosing period; however, mice appeared unprotected from the major peak in footpad swelling beginning on day 5. Taken together over the course of the study, the footpad heights of all infected mice were significantly larger than uninfected controls (one-way repeated measures ANOVA with Bonferroni post-hoc, $p<<0.005$), while footpad heights of auranofin-treated mice were significantly different than PACMA31 and vehicle treated groups ($p=0.026$ and $p=0.005$, respectively). A minor decrease in viremia was observed in compound treated groups vs vehicle controls on 1 DPI, while mixed results were observed on day 2. By day 3, only two mice in the auranofin group showed detectable levels of viremia, all mice had detectable viremia levels in the PACMA31 group, and all but one mouse had detectable viremia in the vehicle control group. The difference in viremia on day 3 between PACMA31 and auranofin groups was statistically significant, but the difference between auranofin and vehicle control was not. No mice showed any clinical signs of distress, such as hunched posture or ruffled fur, at any point during the experiment.

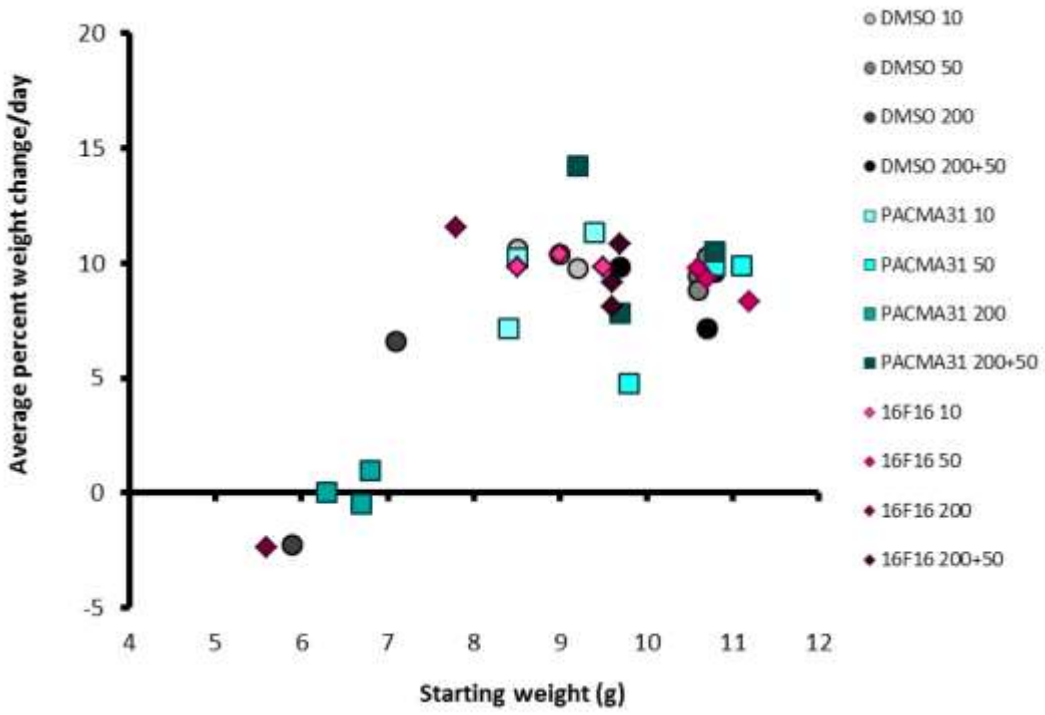


Fig 20. Tolerability of PACMA31 and 16F16 in 15 day old CD-1 mice.

Various doses of 16F16 and PACMA31, along with vehicle control, were administered subcutaneously to 15-16 day old CD-1 mice (n=3/group) for 3 days and mice were monitored for weight gain. Groups included were 10mg/kg, 50 mg/kg, and 200 mg/kg once daily, as well as a 200mg/kg AM + 50mg/kg PM group. Weight loss was associated with starting weight, with animals below 7g responding poorly to handling and/or DMSO treatment (Linear regression, r^2=.546; ANOVA, $p<<0.05$).

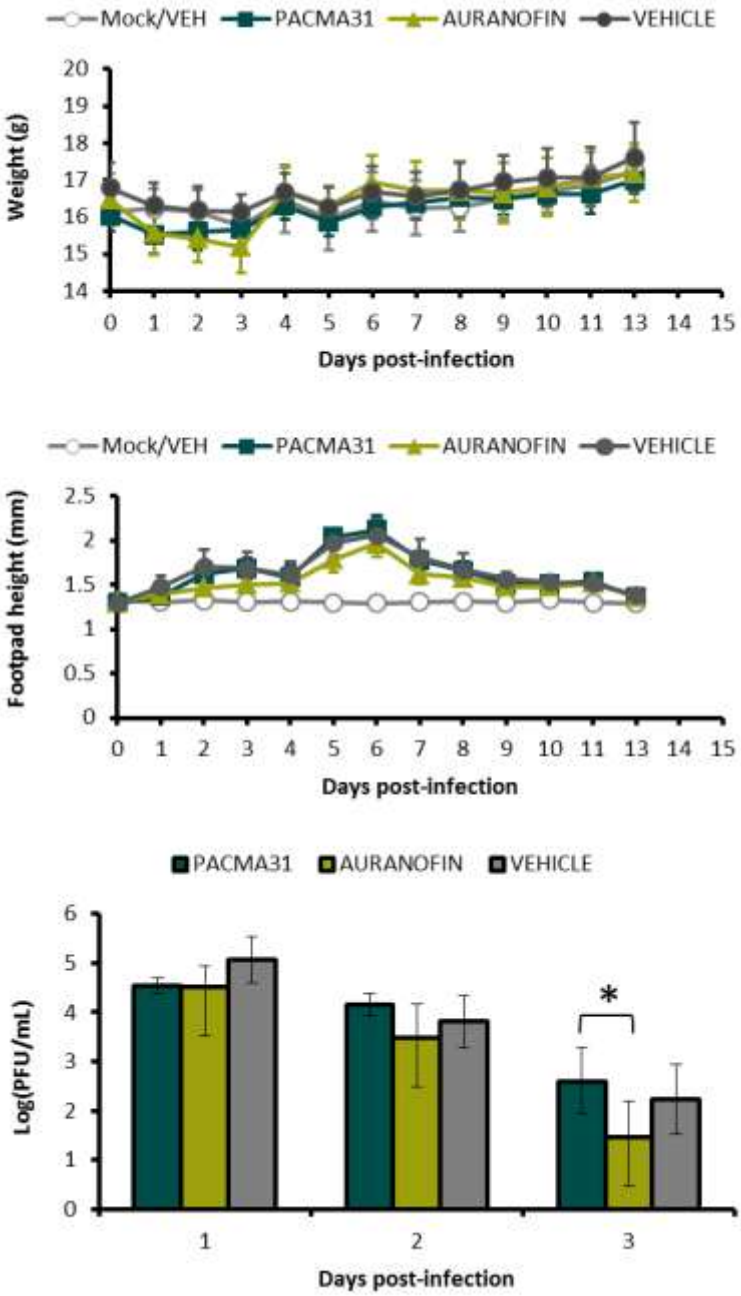


Fig 21. Auranofin protects mice from footpad swelling, viremia.

4-week old C57Bl/6 mice were orally dosed with either 200mg/kg PACMA31 (n=10), 20mg/kg auranofin (n=10), or vehicle (n=15) twice daily beginning one hour prior to infection, at least 8 hours apart, for days 0-2. Mice were either inoculated with 10uL PBS (n=5) or 10uL PBS containing 10^4pfu CHIKV SL-07 (n=30) and weight (A) and footpad height (B) were monitored. Viremia was assessed on days 1-3 post-infection (n=5/group/day) (C).

DISCUSSION

The bulk of FDA-approved antiviral drugs are indicated for the treatment of HIV, hepatitis C and B viruses, and influenza. These generally can be categorized into three classes, immune modulators (such as pegylated interferon; PEG-IFN), nucleoside/nucleotide inhibitors, or antivirals targeted to specific viral proteins (297). Drugs in the latter class, which are rationally designed and optimized for a specific active site pocket of a viral-protein, have little capacity to be repurposed for the treatment of other viral diseases. Furthermore, drugs tailored to a fit a specific binding pocket impart a strong selective pressure for viruses with mutant binding pockets. Oseltamivir, for instance, is an anti-influenza drug that was rationally designed to bind the influenza virus (IFV) neuraminidase and therefore prevent propagation of progeny viruses by preventing IFV egress from host cells (298). The drug has not been reported to show efficacy against any other viruses, and IFV is notorious for developing resistance to oseltamivir, as well as other neuraminidase inhibitors (299). Indeed, although severe decreases in fitness are normally associated with viral escape mutants (300, 301), secondary mutations in the IFV neuraminidase restored robust fitness to later isolates of a mutant strain (302). Nucleos(t)ide analogues and immune modulators are not immune from this phenomenon, either. For example, HCV ribavirin/PEG-IFN-resistance is well documented among patients without a sustained virological response, and is actually of great interest in the field of evolutionary virology due to the improved-fidelity, low genetic diversity of ribavirin escape mutants (303-306).

On the other hand, host-targeted therapies not only limit the development of escape mutants by targeting a protein with little to no chance of altering viral binding sites, but also open the possibility of broad-spectrum potential. Protein folding in particular is such a tightly constrained process, that multiple viruses are strictly required to use the same enzymes with an even lower chance of developing mutants which bypass the process (246). Here, I show that inhibiting TRX-R, a multifunctional ER enzyme, as well as its substrate PDI, can have a profound

effect on CHIKV replication *in vitro*. All compounds tested inhibited CHIKV replication, although the most effective at the least toxic levels was auranofin. Additionally, 16F16 and auranofin also reduced VEEV and ZIKV replication *in vitro,* suggesting broad-spectrum potential of this antiviral approach. Indeed, auranofin is an FDA-approved drug often prescribed for the treatment of rheumatoid arthritis; interestingly, it was also noted that an HIV patient undergoing rheumatoid-arthritis treatment with auranofin also showed improved CD4 T-cell counts and drastically reduced opportunistic infections (307). Other gold-containing anti-rheumatic compounds—which are generally thought to bind sulfur-rich peptides and pockets through gold-thiol interactions (308)— have been found to inhibit HIV *in vitro* as well (309). Various mechanisms have been attributed to the efficacy of gold-containing anti-rheumatic compounds such as auranofin against HIV. First, that auranofin selectively induces apoptosis in central memory T-cells, which serve as latent-HIV reservoirs, as opposed to their naïve precursors (310). Second, that gold-compounds also interfere with reverse-transcriptase function (309) as well as in NF-κB signaling, an important activator of HIV replication in latently infected cells (311). Most intriguingly, gold-containing anti-rheumatics alter HIV envelope protein interactions, thereby preventing fusion (312). Regardless of mechanism, however, auranofin has been shown to reduce latent HIV reservoirs in NHPs, and to rejuvenate the immune response to HIV infection thereby imparting anti-retroviral independent control of HIV infection (313, 314). A clinical trial for targeted assessment of the effect of oral auranofin on latent HIV populations in humans was submitted to clinicaltrials.gov in 2014, although the study was withdrawn early in 2015 for undisclosed reasons (clinicaltrials.gov).

Inhibition of PDI, TRX-R, and ERO-1 reduces replication of alphavirus, HIV, human papilloma virus (315), and to a limited extent ZIKV, *in vitro,* providing evidence that this strategy is broadly effective against many viral species across different families. The hypothesized mechanism behind the inhibition of alphavirus replication by 16F16, PACMA31, auranofin, and

EN460 is that inhibiting PDI or its regulators deregulates disulfide bond formation and isomerization within the alphavirus envelope proteins. The natural consequence of this would be reduced infectivity of progeny virus produced in the presence of these inhibitors. Results from the genome:PFU assay reveal that similar amounts of CHIKV RNA may be extracted from the media of auranofin and 16F16-treated HEK293 cells, but that infectious titers are reduced, resulting in an increased genome:PFU ratio. Using genome copies as a surrogate for total virus particles, this suggests that treatment of CHIKV-infected cells with PDI or TRX-R inhibitors results in an increased production of non-infectious particles. The intracellular viral RNA results support this hypothesis for 16F16, as 16F16 did not affect levels of intracellular viral RNA. Auranofin treatment, on the other hand, resulted in significantly fewer viral RNA transcripts in cells than 16F16, PACMA31, or DMSO-treated cells. In fact, when comparing RT-qPCR results from supernatant and cell lysate samples, it was found that auranofin-treated cells had fewer viral RNA copies inside the cell than outside. One potential explanation is that, rather than or perhaps in addition to effects on disulfide bonding in the CHIKV envelope proteins, auranofin induces apoptosis in virus-infected cells. This would cause infected cells to release viral RNA into the supernatant while leaving uninfected cells in-tact, thereby skewing the intracellular:extracellular viral RNA ratio. This mechanism would be in line with the proposed mechanism of auranofin against HIV (310).

To corroborate the 16F16 genome:PFU results, electron microscopy revealed that larger, pleomorphic particles with small nucleocapsids are produced in cells treated with the PDI inhibitor 16F16 in larger proportions than vehicle-treated cells. The results from this electron microscopy study are similar to those of Snyder et al and Anthony et al, who performed EM studies to examine the structural effects of mutating cysteine residues in the E2 proteins of SINV and RRV, as well as adding DTT to purified virus. Anthony et al found that in the presence of DTT, SINV virions go through a process of disassembly, with nucleocapsid detaching from the

membrane before eventually being released from the virion entirely (174). When mutating conserved cysteine residues involved in disulfide bonding, production of large, pleomorphic SINV particles with multiple nucleocapsids were observed, whereas large numbers of similarly-sized but dysmorphic RRV particles were observed (171). Together, these studies suggest that not only do disulfide bonds and the enzymes responsible for them maintain virion stability, but they may also mediate nucleocapsid interaction. This study may also implicate PDI has a mediator of either nucleocapsid-RNA interactions or capsid-envelope interactions, as while I did observe large pleomorphic particles, they did not contain multiple nucleocapsids but rather single small nucleocapsids. Additional investigation using immunogold staining of sections would provide further clarification of these observations by indicating how envelope proteins localize in the cell and associate with forming nucleocapsids.

The siRNA screen found that anti-PDILT and anti-TMX2 siRNA pools significantly reduced CHIKV replication, two minimally characterized members of the PDI family. PDILT knock-down, however, resulted in less than 40% cell viability in uninfected cells, and less than that in infected cells. Moreover, PDILT—protein disulfide isomerase-like of the testis—is a mammal-specific protein which shares approximately 27% amino acid sequence identity with P4HB, prolyl-4-hydroxylase or the "original" protein disulfide isomerase, and is only found to be expressed in the testis, shown through experiments with mice and human cell lines (316). Therefore, it is unlikely that anti-PDILT siRNA actually knocked-down expression of PDILT in human embryonic kidney cells; this was not one of the random PDI proteins selected for knock-down confirmation, however, so this remains unconfirmed. Being that it has only been found to be expressed in testicular tissue, it is more likely that the anti-PDILT siRNA produced off-target effects in HEK293 cells, which are also likely responsible for the observed toxicity. TMX2 shares significant sequence and predicted structural similarity with TMX, TMX3, and TMX4 (317), and is a membrane-bound TRX which is closely associated with the mitochondria-associated

membrane of the ER (318). However, little else is known about TMX2. The role of TMX2 in the replication of CHIKV, as well as its normal cellular functions, should be investigated further. However, ERp57 and P4HB are likely important players in the formation and isomerization of disulfide bonds in the envelope proteins, despite the respective siRNA pools failing to significantly inhibit CHIKV replication. Previous studies utilizing pull-down assays suggest the PDIs P4HB and ERp57 interact with SFV envelope proteins (170, 319). Furthermore, while PACMA31 is thought to be P4HB-specific and 16F16 is thought to bind more promiscuously to both P4HB and ERp57 (284, 287), both compounds reduced CHIKV titers *in vitro*. Due to the overlap in structure and function between PDI family members (278), it is likely that redundancies in the TRX system allowed the virus to maintain its replicative abilities, despite siRNA mediated knock-down of ERp57 and P4HB. Future experiments should further investigate the potential for combined siRNA pools to inhibit CHIKV replication.

The main criticism of targeting host-proteins, especially chaperone proteins, is that this strategy would likely result in severe host toxicity. Although little is known about PDI inhibitors in humans, HSP90 inhibitors are well-tolerated, as indicated by the numerous past and current clinical trials being conducted (clinicaltrials.gov)(251). Additionally, PACMA31 was found to be well-tolerated in mice, even at high doses of 200mg/kg (284). Here, I showed that PACMA31 and 16F16 are not only safe to use in mice, but young mice, although this is dependent on size: treated mice weighing 7g grams and above gained weight normally, while treated mice below 7g lost weight. It is unclear whether this toxicity was due to vehicle or simply handling of the animals, as DMSO-treated animals below 7g also lost weight. Furthermore, auranofin, a host-targeted drug, is FDA approved for use in humans and is routinely prescribed for rheumatoid arthritis treatment (296). Although auranofin is not a PDI inhibitor per se, it inhibits one of the proteins responsible for regulating the function of PDIs, namely by maintaining concentrations of reduced forms of PDI which function to isomerize, rather than form, disulfide bonds (320). While auranofin

treatment is associated with some side effects, namely diarrhea, nausea, and rash, they are not severe enough to prevent long-term use of the drug (321); for antiviral purposes, the drug would likely only be used for a brief time period.

Lastly, auranofin significantly reduced footpad swelling and viral titers in this study. In light of the PFU:genome results, this may not be due to effects on virion structure, but rather through cellular- and immune-response to infection by potential induction of apoptosis in infected cells (although the *in vivo* mechanism remains unexplored). Although modest, the efficacy of auranofin *in vivo* is promising: not only is auranofin already FDA-approved, but CHIKV arthritis is hypothesized to be immune-mediated at least in some capacity (90, 91, 185, 322). Additionally, other disease modifying antirheumatics, namely methotrexate and sulfasalazine, were shown to have limited efficacy in controlling persistent CHIKV arthritis in a small clinical study (323). Although the mechanism of auranofin action seemingly deviated from the proposed mechanism of altering progeny virus virion structure, the results offer promising prospects none-the-less. On the other hand, PACMA31, while slightly decreasing viral load on the first day of infection, did not produce significant decreases in viremia or footpad swelling. In this study, 16F16 showed higher efficacy than PACMA31 *in vitro* and was shown to be safe in two-week old mice; however, its pharmacokinetic properties have not been explored, nor have safe dosing regimens and routes *in vivo*. This made 16F16 an impractical choice for *in vivo* testing in this study, but future experiments should explore the use of other PDI-inhibitors *in vivo* to develop appropriate pharmacokinetic and tolerability profiles, such that antiviral testing may occur.

In all, this study provided evidence that inhibiting PDI impacts CHIKV replication *in vitro*, potentially by limiting infectivity of progeny viruses through alteration of envelope and ultimate virion structure. In addition, treatment with the antirheumatic drug auranofin showed both *in vitro* and *in vivo* efficacy in limiting viral replication and symptoms, although through a

different mechanism than PDI inhibitors. Together, these data merit further investigation of these drug targets as potential broad-spectrum antiviral therapeutics.

CHAPTER V. CONCLUDING REMARKS AND FUTURE WORK

While interest in CHIKV research is revitalized with every new reemergence of CHIKF, in recent years, outbreaks in La Reunion and the Caribbean have demonstrated that much about pathogenesis and epidemiology remains unknown. Additionally, progress toward developing antivirals for CHIKV is slow, with few investigations into novel drug targets that can broadly inhibit CHIKV as well as other alphaviruses.

Here, I first described the clinical features of a CHIKF outbreak in the DR. Many aspects of this outbreak challenged previous assertions about CHIKF epidemiology, two of which have the potential to alter surveillance practices, especially in the Americas. Firstly, children and young adults under the age of 21 were disproportionately affected by CHIKF during the DR outbreak, which corroborated passing observations made during the epidemic in Colombia, but which contradicted past Old World outbreaks perpetrated by IOL strains that disproportionately affected older age groups. Secondly, it was found that joint symptoms were reported far less often than in past outbreaks, indicating that current diagnostic criteria may need to be revised for CHIKF disease caused by CRBN strains. An interesting note from this outbreak was the prevalence of potential respiratory co-infections, the significance of which remains unknown.

In general, it might be hypothesized the CRBN CHIKV strains cause less severe disease than strains arising from other lineages. This hypothesis is in part supported by the work performed in Chapter III, which compared lineage virulence in A129 mice. The data from that study suggested that WA CHIKV strains may be the most virulent of any other lineage, but are especially more virulent than CRBN strains. Additionally, ASN isolates appeared to be more virulent than their CRBN counterparts, which supports the idea that CRBN strains appear to be attenuated, despite the large scale of the continuing outbreaks in the Caribbean and Central America.

However, both of these projects leave many questions left unanswered and suffered several limitations. First and foremost, the use of "convenience" samples limited my ability to construct a finely controlled clinical study of CHIKF symptomology. The DR CHIKF outbreak study described in Chapter II therefore suffered several significant biases, including information biases which shifted patient data availability to those who were admitted to hospitals, inconsistent interview and documenting practices which introduced even more information biases toward those physicians who were perhaps more thorough, and finally, we were forced to choose between utilizing samples from two clinics with different inclusion criteria or significantly reducing our sample number. Ideally, a prospective study in an epidemic area would be constructed to utilize a variety of clinical settings in different hospitals, e.g. emergency, urgent care, and primary care physician appointments and follow-ups, as well as sample from the general public, to gain a clearer and more relevant picture of the clinical aspects of CRBN-CHIKV infection. Along with this, interviewing practices of physicians would need to be strictly standardized to ensure all patients are interviewed similarly. However, one interesting aspect of this study is that we had candid access to diagnoses made by clinicians which were not affected by the expectation of finding CHIKF patients; in this sense, our study is more useful in evaluating the rate of misdiagnosis of CHIKF. This would not have been possible in a carefully planned study with standardized interviews/questionnaires for physicians and patients, which would bias the study toward CHIKF diagnoses.

Furthermore, the insights gleaned from the lineage virulence study generated many more hypotheses than were answered. While it provided some evidence to substantiate claims that CRBN lineage CHIKF outbreaks are milder than those caused by other lineages, especially the IOL, these studies were performed in only one immunocompromised murine model with no investigation into underlying mechanisms of pathogenesis. While there is some evidence to suggest that the CHIKV 3' UTR may act as a prominent virulence factor in CHIKV pathogenesis (35, 36), targeted studies including all CHIKV lineages have not been performed. Firstly, the

results from my study would need to be validated in a second, immunocompetent mouse model, as use of the A129 model eliminates the type-1 interferon response to infection; the biases the study to attribute enhanced virulence to CHIKV subspecies with baseline advantages in replication kinetics, and removes IFN sensitivity as a virulence factor. For example, any of the C57Bl/6 models could be used, including the B and T cell deficient $RAG^{-/-}$ or the 2-week old wild-type models, both of which show signs of CHIKV persistence in joint and muscle tissue (183, 185, 186). Secondly, other variables would need to be assessed *in vivo*, including potential differences in immune signaling, tissue tropism, and composition of immune infiltrate. This could be accomplished utilizing immunocompetent animals in conjunction with methods such as bioplex arrays to quantify relevant cytokines and different staining techniques utilizing histological specimens. Finally, controlled manipulation of potential virulence factors—including 3' UTR length and sequence—would need to be performed utilizing representative CHIKF strains from all lineages to assess the features belying observed differences in virulence and pathogenesis. After all of this is accomplished, the results would need to be verified in an NHP model of disease, and more targeted epidemiological approaches aiming to evaluate the severity of CHIKF in humans in relation to causative strain would need to be developed and executed.

Regardless of which CHIKV strain is responsible for a specific outbreak, antiviral development is a research imperative in the field of translational CHIKV work. Antivirals may reduce the time to convalescence, and in the case of CHIKV, potentially be used to prevent or treat chronic CHIKF joint symptoms. The work in Chapter IV addressed this need by exploring the potential of small-molecule inhibitors to PDIs, ERO-1, and TRX-R, all host proteins, to inhibit CHIKV replication *in vitro* and *in vivo* by interrupting enzymatic disulfide bond formation and isomerization in the CHIKV envelope proteins. 16F16, a PDI inhibitor, significantly inhibited CHIKV replication *in vitro* in a dose-dependent manner, potentially through altering virion assembly and structure, which was in line with the hypothesized mechanism of action. Auranofin also inhibited CHIKV replication *in vitro* with a more sensible therapeutic index, although the

mechanism differed from hypothesized mechanisms; auranofin, rather than altering envelope protein folding, may selectively induce apoptosis in infected cells, thereby limiting propagation of CHIKV. Auranofin appeared to show some efficacy *in vivo* as well, though the PDI inhibitor PACMA31 had no effect on either viral replication or footpad swelling in mice. A specific PDI family member responsible for enhancing CHIKV replication was not identified, though TMX2 may represent a potential target for PDI inhibitors developed for the specific purpose of inhibiting CHIKV replication.

There were some limitations to this study, however. Firstly, the siRNA experiments using combinatorial siRNA pools were never performed due to time constraints. This left a very large gap in our knowledge regarding which specific PDIs contribute substantially to CHIKV replication. I was also unable to use a young murine model of CHIKV infection for my due to the impracticality of orally dosing animals of such a small size with the gavage needles at my disposal, which led to a discrepancy in animal models used for safety and efficacy training. Using the second model became a necessity after it became clear that the smallest gavage needle size available to me was dangerous for the young mice, and was not the original experimental plan. The electron microscopy experiments, while useful, were not optimal since immunogold staining was not employed. Essentially, while the altered particles I observed were likely CHIKV virions, this cannot be proven irrefutably without antibody staining. Finally, while I utilized ZIKV to explore the potential spectrum of PDI inhibitor use, the experiments were not optimal for exploring this aspect of the project. While the ZIKV strain used is more relevant due to its relative genetic proximity to currently circulating ZIKV strains, the replication kinetics of the strain used were not ideal; the experiment would need to be extended by several days in order to adequately assess the effect of PDI inhibitors on ZIKV replication. Additionally, we had aimed to explore the effects of PDI inhibitors on the replication of a non-enveloped RNA virus—namely encephalomyocarditis virus, a picornavirus—but due to time constraints, these experiments were never performed. Because of this, we are unable to say that these PDI inhibitors strictly prevented

envelope protein folding, as we cannot say whether efficacy was observed against a non-enveloped virus.

Much still needs to be explored in the field of PDI and disulfide bonding in relation to CHIKV pathogenesis and antiviral development. Disulfide bonds play a demonstrably important role in CHIKV virion structure, and although I provided evidence here that inhibiting PDI can inhibit CHIKV replication, this was done near the CC_{50} concentration of the inhibitors used. Firstly, as more small-molecule inhibitors of PDI continue to be identified, these need to be tested against CHIKV to discover an inhibitor with an improved TI. This process would be aided by the identification of specific PDI targets, through CRISPR-CAS9, siRNA, or combination screens. Furthermore, while I used the C57Bl/6 model of infection, several parameters regarding *in vivo* testing need to be explored. For example, a larger study utilizing more mice would increase the power of statistical analyses used. Secondly, performing these experiments in the more stringent A129 model would provide survival data; because the model is so stringent, success—an increase in overall survival, or even a delay in time to death— seen in this model would provide very strong evidence of the potential drugability of PDI as an antiviral target. Since 16F16 also showed limited success in reducing VEEV replication, the use of other alphavirus models such as the universally lethal CD-1 model of VEEV infection (205, 206), would also provide vital information regarding the ability of PDI inhibitors to prevent or delay death following alphavirus infection in general. While my experiments showed *in vitro* efficacy of PDI inhibitors against CHIKV, the same efficacy was not observed *in vivo*. While this does not necessarily preclude the possibility of drugging PDI, further *in vivo* experiments need to be performed with optimized drugs and in a more stringent model before this class of targets can be confirmed or rejected.

The efficacy of auranofin was a surprising and potentially more interesting discovery. Auranofin is already FDA-approved for the treatment of rheumatoid arthritis, making the translational opportunities for this avenue of antiviral research more achievable. Similar to the PDI targets, the use of auranofin needs to be further explored in different animal models of

CHIKV-disease. Likewise, since it also showed efficacy against VEEV *in vitro*, it would also be possible to use the CD-1 VEEV model of infection in addition to the various CHIKV models. However, because auranofin is primarily used as an anti-inflammatory, the goal for auranofin use may not be limited to suppression of viral replication. Even though auranofin does reduce CHIKV replication *in vitro*, auranofin may be used to target and prevent the pathology of CHIKV infection rather than preventing viral replication *in vivo*. For example, this would mean that increasing survival in the A129 model of CHIKV-infection would not necessarily be the goal of auranofin use; preventing long-term joint and muscle destruction in the $RAG^{-/-}$ mouse model may also be considered a success. Further, auranofin is only one of many gold-based anti-inflammatories on the market and is considered to be less tolerable than others currently available (308). Exploring the use of other gold-based compounds in reducing CHIKV replication or alleviating/preventing CHIKV-induced joint pathology would be prudent.

Overall, despite the limitations of the various studies in this work, the successes of this project provide a foundation for future work in these fields. Specifically, expansion of CHIKF diagnostic criteria in the Americas could be implemented in future out-break areas to confirm the seeming attenuation of CRBN strains. Related to this attenuation, much needs to be done in the area of discerning genetic determinants of virulence using the classification of lineages as a starting point. Finally, this project provided a rationale for the further investigation of novel host-targets, namely PDI, as potential broad-spectrum antiviral drug targets, along with further investigation of repurposing FDA-approved gold-based therapeutics as either antiviral drugs or therapies for treating CHIKV-associated arthropathy.

In all, this projected has provided a firm foundation for the development and continuation of several translational avenues of CHIKV research, particularly where epidemiology and drug development are concerned.

Appendix 1. Mayaro virus infection protects against disease induced by subsequent CHIKV challenge in adult inbred mouse model

To address the impact of sequential alphavirus infections and test the hypothesis that MAYV infection protects against subsequent CHIKV infection, 3-week old C57Bl/6 mice were infected with either PBS, CHIKV vaccine strain 181/clone25 (army), VEEV vaccine strain TC-83, yellow fever virus (YFV) vaccine strain 17D, or wild type MAYV strain BeAr and then subsequently challenged with Caribbean CHIKV isolate YO123223 eight weeks later and monitored for signs of CHIKV disease. Although naïve infection with MAYV caused significant morbidity initially, MAYV mice were protected against footpad swelling and viremia caused by CHIKV challenge.

METHODS

Mice

C57Bl/6J mice were bred from colonies house at the University of Texas Medical Branch. Animals were housed in either the ABSL-2 or ABSL-3 facilities in the Galveston National Laboratory. All animal experiments were performed in accordance with the UTMB Institutional Animal Care and Use Committee protocol #0209068B, and under the supervision of the Animal Resource Center and in strict adherence to standards of care set thereforth.

Viruses

MAYV 505411, VEEV vaccine strain TC-83, and CHIKV vaccine strain 181/clone 25 viruses were rescued from infectious clones and passaged 1-2 times on Vero cells. Yellow fever virus vaccine strain 17D was kindly provided by the Barrett lab at the University of Texas Medical Branch. CHIKV YO123223 challenge strain is a low passage isolate provided by the WRCEVA.

Tissue culture

Vero cells were maintained on Gibco DMEM supplemented with 2-5% FBS and gentamicin. African green monkey (Vero) cells were obtained from ATCC (American Type Cell Culture, Bethesda, MD), and were maintained in DMEM supplemented with 1% penicillin/streptomycin (P/S) and 5% fetal bovine serum.

Plaque assay

Vero cells were grown to 90-100% confluency in either 12- or 6-well plates. Virus was diluted in DMEM supplemented with 5% FBS and from a series of 10-fold dilutions, 100uL was plated per 12-well and 200 μL was plated per 6-well plate. After 30 minutes incubation, an overlay composed of DMEM and 0.2% agarose was added and incubated for 24-48 hours. Plates were then fixed with 10% formaldehyde for at least one hour before staining with crystal violet, and plaques counted.

Statistics

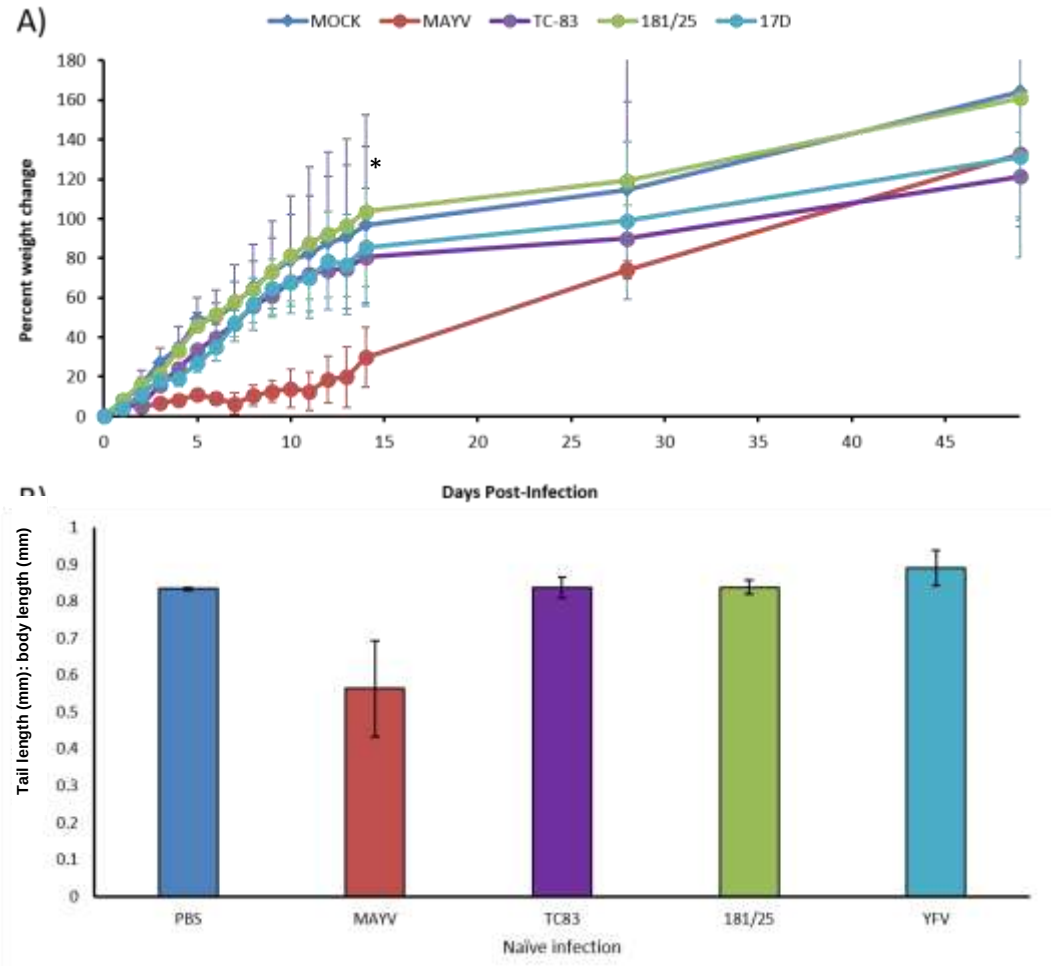
One-way ANOVAs with Tukey's post-hoc were performed to test statistical significance. All statistics were performed using SPSS software.

RESULTS

Naïve infection with wild type MAYV causes significant morbidity in 3-week old C57Bl/6J mice.

Weanling C57Bl/6J mice were inoculated with either PBS, $3.5x10^3$ pfu MAYV 505411, or $5x10^4$ pfu CHIKV army, VEEV TC-83, or 17D in the subcutaneous space near the neck. PBS, VEEV TC-83, and YFV 17D were all used as negative controls to ensure that: a) disease in the absence of any viral exposure is compared (PBS); B) to compare the extent of cross-protection between antigenically distinct alphaviruses (VEEV TC-83); and C) ensure that results are not an artifact prolonged heightened antiviral response from prior viral-induced immune provocation

(YFV 17D). CHIKV 181/25 was used as a positive control. Mice were monitored daily for days 1-14, then weekly for days 14-49. All groups proceeded to develop normally with the exception of MAYV-infected mice, which showed significantly reduced weight gain (figure 1A) and exhibited clinical signs of disease, including ruffled fur, lethargy, and hunched posture on days 6-10 post-infection. While MAYV mice gained weight to levels similar to vaccine-inoculated animals by day 49, the tails of MAYV-infected mice were significantly shorter (ANOVA with Bonferroni post-hoc, $p<0.05$; Fig 1B.).

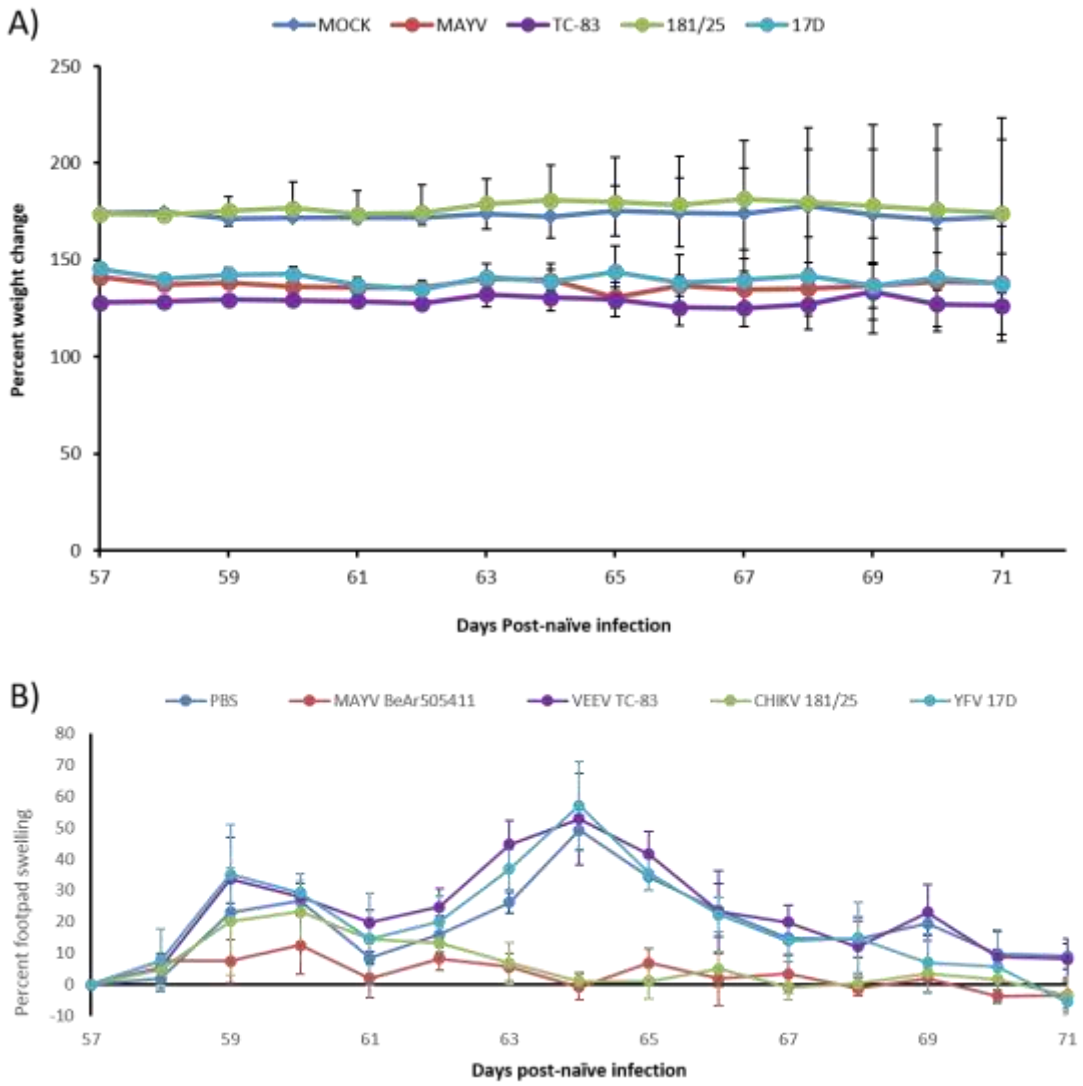


Appendix 1 Fig 1. Naïve infection with MAYV causes significant morbidity and stunted tail growth.

C57Bl/6 mice were challenged with 10^4 pfu/ffu of one of several viruses, and weight (A) was monitored to 49 days post-infection, when body:tail ratios were measured (B). MAYV infection caused significant decrease in weight gain, as well as stunted tail growth.

Previous infection with MAYV protects C57Bl/6 mice from CHIKV-induced footpad swelling and viremia.

Fifty seven days after initial infection, mice were challenged with 10^4pfu CHIKV YO123223, in the left rear footpad . Weight and footpad heights were monitored daily for 2 weeks, and serum was collected on days 2 and 4 post-infection to assess viremia via plaque assay. While weights did not change significantly (figure 2A), two peaks in footpad swelling were observed between days 2-4 and 5-10. MAYV infected mice were protected for both peaks (One way ANOVA, $p<0.1$ and $p<0.05$, respectively), while CHIKV 181/25 inoculated mice were protected for the second peak (one way ANOVA, $p<0.05$) (figure 2B). No mice developed viremia, likely due to the combination of mouse age, CHIKV strain, and CHIKV dose.



Appendix 1 Fig 2. Infection with MAYV prevents CHIKV-induced footpad swelling

57 days post-naïve challenge, mice were challenge with 10^4PFU CHIKV YO123223 in the left rear footpad, and mice were monitored for 14 days post-infection. Although weights were generally unaffected (A), footpad swelling was significantly reduced in MAYV naïve infection group.

DISCUSSION

MAYV is a highly neglected disease, lacking in surveillance, pathogenesis, and animal model data. Here, I describe for the first time the clinical manifestation of MAYV infection in weanling C57Bl/6 mice. Mice infected with MAYV became critically ill for 5 days, as exhibited by dramatically decreased weight gain and overt clinical symptoms. Further characterization of

the pathogenesis of MAYV in weanling mice could yield a useful disease model, potentiating future MAYV research heretofore impeded by the lack of a small animal model. MAYV-induced viremia, organ viral load, histopathology, and footpad pathology are all variables which should be considered for future research but which were outside the scope of this study. Curiously, all MAYV-infected mice exhibited varying degrees of stunted tail growth. This has not been described for any alphavirus animal model, although Luciferase-tagged CHIKV has been visualized in the tail of CHIKV-infected NOD/SCID mice via IVIS (S2). Although it is currently unclear how MAYV infection contributed to stunted tail growth or whether this is a universal phenomenon among arthritogenic alphavirus young mouse models, this is an interesting avenue of research which could potentially yield insights into long term joint pathology following alphavirus infection.

More importantly, MAYV infection appears to protect against morbidity caused by subsequent CHIKV infection. All mice exhibited footpad swelling greater than 10% which did not return to normal footpad height during the course of the study. For PBS, YFV, and VEEV inoculated mice, swelling was biphasic with the highest footpad heights recorded during the second peak. Although minor footpad swelling was observed, MAYV mice exhibited reduced footpad swelling during the first peak and did not appear to produce a second peak in swelling at all. This is in contrast to the mice inoculated with the CHIKV vaccine developed by the US army, a very well characterized vaccine which has been shown to be highly protective in various CHIKV disease models, which failed to protect at all against the first phase of swelling but prevented a second peak in swelling. Although all vaccine strains used—VEEV TC-83, YFV 17D, and CHIKV army—are all live-attenuated and replication competent, future work should utilize wild-type flavi- and alphaviruses to investigate the full potential of cross-protective abilities.

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Vita

Rose Langsjoen was born on August 31st, 1987 to Hans Langsjoen and Karen Rasmusson. After attending primary school in the Galveston Island school district and graduating from Ball High School in 2006, Rose enrolled at Gustavus Adolphus College in St. Peter, MN that same year. Rose graduated *cum laude* with her bachelor of arts in biology and a minor (with honors) in religion in 2010, and then taught at Huntington Learning Center in Chanhassen, MN while taking extra courses at the University of Minnesota until May 2011. Rose then matriculated in the first class of the then-new Human Pathophysiology and Translational Medicine program at the University of Texas Medical Branch, Galveston the summer of 2011, and joined Scott Weaver's lab group the summer of 2012. Since then, Rose has investigated a variety of topics related to alphavirus research, ranging from vaccine and antiviral development, epidemiological studies of CHIKV outbreaks, to characterization of novel viruses and pathogenesis of Chikungunya.

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Gustavus Adolphus College, Saint Peter, MN

Teaching

GUSTAVUS ADOLPHUS COLLEGE

a. Teaching positions

Teaching assistant, cell and molecular biology laboratory, 2009-2010
Teaching assistant, organismal biology laboratory, 2008-2010
Academic assistant, German, 2009-2010

b. Lectures/Presentations

Guest lecturer, organismal biology: "class insecta", April 2009

UTMB

a. Teaching positions

Student Coordinator, Translational Research Seminar Series, 2015-2016
Teaching assistant for Principles of Translational Science, Fall 2012

b. Students/Mentees

Maria Kastis, Ball High School Bench Tutorials, August 2014 – May 2015
Accepted to University of Texas Austin, 2015

Accepted, School of Natural Sciences at UT Austin, 2015
Kevin Le, High School Summer Research Program, Summer 2014
Accepted to University of Texas, Austin 2015
Matthew Schnizlein, SURP, Summer 2014

"Best Infectious Disease Poster"; Institute for Human Infections and Immunity, 2014

Publications

Langsjoen RM, Auguste AJ, Rossi SL, Kastis M, Schnizlein MK, Le KC, Penate HN, Watowich SJ, Weaver SC. Oxidative folding pathways offer anti-chikungunya virus drug targets with broad spectrum potential. To be submitted to Antiviral Res.

*Langsjoen RM, Roy CJ, Erasmus JH, Weaver SC, Rossi SL. Chikungunya virus strains show lineage-specific variation in virulence and cross-protective ability in murine and non-human primate models. To be submitted to mBio.

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Díaz-González EE, Kautz TF, Dorantes-Delgado A, Malo-García IR, Laguna-Aguilar M, Langsjoen RM, Chen R, Auguste DI, Sánchez-Casas RM, Danis-Lozano R, Weaver SC and Fernández-Salas I. First report of Aedes aegypti transmission of chikungunya virus in the Americas. A J Trop Med and Hyg;93(6):1325-9.

Kautz TF, Diaz-Gonzalez EE, Erasmuss J, Malo-García IR, Langsjoen RM, Patterson EI, Auguste DI, Forrester NL, Sánchez-Casas RM, Hernández Avila M, Apulche-Arande CM, Weaver SC, Fernández-Salas I. Chikungunya Virus Identified as the Etiological Agent of an Outbreak of Febrile Illness in Chiapas Mexico, 2014. Emerging Infectious Diseases; 21(11):2070-3.

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