ANTIVIRAL ACTIVITIES OF TUALANG HONEY AGAINST CHIKUNGUNYA VIRUS-INFECTED HUMAN FIBROBLAST-LIKE SYNOVIOCYTES (HFLS)

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by

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LIST OF SYMBOLS

amps	Amperes
bp	Base pair
cm^2	Centimetres squared
g	Gram
L	Litres
mA	Miliamperes
mg	Miligram
mL	Mililitre
mm^2	Milimetres squared
mM	Milimolar
min	Minutes
nm	Nanometre
pfu	Plaque forming unit
s	Seconds
µg	Microgram
µL	Microlitre
µM	Micromolar
V	Voltage
w/v	Weight per volume
x *g*	Times gravity units
γ	Gamma
±	Plus or minus
x	Multiplication
%	Percentage
°C	Degree Celcius

LIST OF ABBREVIATIONS

Ae.	*Aedes*
ANOVA	Analysis of variance
BHK	Baby hamster kidney
BSC	Bio-safety cabinet
Bti	*Bacillus Thuringiensis Israelensis*
C	Capsid
CDC	Centers for Disease Control and Prevention
CHIKV	Chikungunya virus
CPE	Cytopathic effect
CMC	Carboxymethyl cellulose
CO_2	Carbon dioxide
DAA	Direct-acting antiviral
DENV	Dengue virus
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E	Envelope
ECSA	East/Central/South/African
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FDA	Food and Drug Administration
HBSS	Hank's balanced salt solution
HDA	Host-directed antiviral
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
HFLS	Human fibroblast-like synoviocytes
hpi	Hour post infection
IC_{50}	Inhibitory concentration 50%
Ig	Immunoglobulin
IL	Interleukin
IOL	Indian Ocean Lineage

IVT	In vitro transcription
MNTD	Maximum non-toxic dose
MOH	Ministry of Health, Malaysia
MOI	Multiplicity of infection
nsP	Non-structural protein
ORF	Open reading frame
ORS	Outdoor residual spray
PBS	Phosphate buffer saline
PC	Positive control
RDT	Rapid diagnostic test
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SE	Standard error
SPSS	Statistical Package for the Social Sciences
TBE	Tris-borate-EDTA
USD	United States dollar
WHO	World Health Organisation

LIST OF APPENDICES

AKTIVITI ANTIVIRAL MADU TUALANG TERHADAP VIRUS CHIKUNGUNYA – FIBROBLAST MANUSIA YANG DIJANGKITI - SEAKAN SINOVIOSIT (HFLS)

ABSTRAK

Chikungunya adalah penyakit virus bawaan nyamuk yang disebabkan oleh virus chikungunya (CHIKV), disebarkan terutamanya kepada manusia oleh nyamuk *Aedes aegypti* dan *Aedes albopictus*. Gejala penyakit ini adalah demam, sakit sendi, sakit otot, ruam dan sakit kepala. Dalam kes chikungunya yang kronik, sakit sendi boleh berlanjutan selama beberapa bulan atau bertahun. Walaupun sudah terdapat vaksin yang dikenali sebagai IXCHIQ, ia masih belum digunakan secara meluas dan pilihan rawatan semasa hanya tertumpu kepada melegakan simptom. Kajian ini bertujuan untuk menyelidik aktiviti antivirus madu Tualang secara *in vitro* semasa jangkitan CHIKV di dalam sel HFLS. Potensi sifat anti-CHIKV untuk pelbagai kepekatan madu Tualang pada tempoh inkubasi yang berbeza dinilai melalui ujian pra-rawatan dan pasca-rawatan. Tambahan pula, tindakan mekanisma di sebalik aktiviti antivirus madu telah dikaji dengan lebih lanjut menggunakan ujian virusid, anti-penyerapan dan anti-penembusan. Dos maksimum tidak toksik (MNTD) madu Tualang di dalam sel HFLS ialah 50 mg/mL, namun kepekatan madu ≤ 20 mg/mL telah digunakan untuk ujian antivirus. Kesan perencatan virus dinilai menggunakan ujian plak untuk mengukur titer virus dan tindak balas rantai polimerase transkriptase terbalik kuantitatif (qRT-PCR) untuk mengukur bilangan salinan RNA virus. Madu Tualang menunjukkan aktiviti profilaktik yang minimum hingga sangat baik semasa ujian pra-rawatan, dengan pengurangan titer virus sebanyak 29.21% hingga 94.87% dan bilangan salinan RNA sebanyak 12.90% hingga 69.80%. Perencatan titer virus

yang signifikan ($p < 0.05$) dapat dilihat apabila dirawat dengan madu berkepekatan 10 dan 15 mg/mL selama 6 dan 12 jam sebelum jangkitan. Menariknya, kesan anti-CHIKV madu Tualang di dalam sel HFLS lebih ketara semasa pra-rawatan berbanding pasca-rawatan. Ujian pasca-rawatan dengan madu Tualang menghasilkan perencatan yang minimum hingga perencatan yang baik, dengan pengurangan titer virus sebanyak 6.67% hingga 72.46% dan bilangan salinan RNA sebanyak 10.87% hingga 29.21%. Perencatan paling ketara diperhatikan apabila dirawat dengan madu berkepekatan 15 mg/mL pada 4 jam selepas jangkitan (hpi), walaupun kesan ini tidak signifikan secara statistik. Secara khusus, madu Tualang menunjukkan kesan virusid kerana dapat mengurangkan titer virus sehingga 80.50%. Aktiviti virusid yang signifikan ($p < 0.05$) dapat dilihat apabila madu berkepekatan 15 dan 20 mg/mL diinkubasi dengan 2 X 10^3 pfu virus. Selanjutnya, madu Tualang menunjukkan kedua-dua kesan anti-penyerapan dan anti-penembusan, dengan kesan anti-penembusan lebih ketara, mengurangkan titer virus sehingga 90.45% berbanding 66.89% untuk anti-penyerapan. Kesimpulannya, kajian ini mencadangkan bahawa madu Tualang mempunyai aktiviti anti-CHIKV dan menghasilkan kesannya melalui tindakan mekanisma yang pelbagai. Penyelidikan lanjut diperlukan untuk mengesahkan aplikasi klinikal dan menjelaskan keseluruhan mekanisma tindakannya.

ANTIVIRAL ACTIVITIES OF TUALANG HONEY AGAINST CHIKUNGUNYA VIRUS-INFECTED HUMAN FIBROBLAST-LIKE SYNOVIOCYTES (HFLS)

ABSTRACT

Chikungunya is a mosquito-borne viral disease caused by chikungunya virus (CHIKV), primarily transmitted to humans by *Aedes aegypti* and *Aedes albopictus* mosquitoes. The illness is characterised by fever, polyarthralgia, myalgia, rash and headache. In chronic chikungunya, arthralgia can persist for months or even years. Although a vaccine called IXCHIQ exists, it is not widely used and current treatment options focus on symptom relief. This study aimed to investigate the *in vitro* antiviral activity of Tualang honey against CHIKV infection in human fibroblast-like synoviocytes (HFLS) cells. The potential anti-CHIKV activity of different concentrations of Tualang honey at various incubation periods was evaluated through pre-treatment and post-treatment assays. Additionally, the modes of action were further investigated using virucidal, anti-adsorption, and anti-entry assays. The maximum non-toxic dose (MNTD) of honey in HFLS cells was found to be 50 mg/mL although for subsequent antiviral assays ≤ 20 mg/mL concentrations were used. The viral inhibitory effect was evaluated using plaque assays to measure viral titres and quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) to quantify RNA copy numbers. Tualang honey exhibited minimal to excellent prophylactic activity during the pre-treatment assay, reducing viral titres by 29.21% to 94.87% and RNA copy numbers by 12.90% to 69.80%. Significant inhibition ($p < 0.05$) of CHIKV viral titres was observed following pre-treatment with 10 and 15 mg/mL for 6 and 12 hours before infection, respectively. Interestingly, the anti-CHIKV effects of Tualang

honey in HFLS cells were more prominent during pre-treatment compared to post-treatment. Post-treatment with Tualang honey resulted in minimal to strong inhibition, reducing CHIKV viral titres by 6.67% to 72.46% and RNA copy numbers by 10.87% to 29.21%. The greatest inhibition was observed following post-treatment with 15 mg/mL concentration at 4 hour post infection (hpi), although these effects were not statistically significant. Notably, Tualang honey exhibited a virucidal effect, with up to an 80.50% reduction in CHIKV viral titres. Significant virucidal activity ($p < 0.05$) was observed when 15 and 20 mg/mL of honey were incubated with 2 X 10^3 pfu of CHIKV. Furthermore, Tualang honey demonstrated both anti-adsorption and anti-entry effects, with the latter being more pronounced, reducing viral titres by up to 90.45% compared to 66.89% for anti-adsorption. In conclusion, this study demonstrates that Tualang honey possesses anti-CHIKV activities and exerts its effects through broad modes of action. Further research is required to confirm its clinical applicability and fully elucidate its mechanisms of action.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

CHIKV is a re-emerging mosquito-borne alphavirus, first identified in 1952 in Tanzania. Over the following 50 years, CHIKV spread among vertebrate hosts and *Aedes* (*Ae.*) *aegypti* mosquitoes, causing several outbreaks in Asia and Africa (Schmidt & Schnierle, 2022). During the 2006 outbreak in La Réunion, genetic analysis revealed that 90% of CHIKV isolates carried a mutation in the E1 glycoprotein, substituting alanine with valine at position 226 (E1-A226V). While this mutation does not affect viral replication in *Ae. aegypti*, it significantly enhances viral fitness in *Ae. albopictus*, facilitating widespread transmission by this mosquito species (Schmidt & Schnierle, 2022; Vairo et al., 2019). Unlike *Ae. aegypti*, which is restricted to tropical and subtropical regions, *Ae. albopictus* is globally endemic. Consequently, CHIKV has spread rapidly and is now reported in more than 100 countries (Schmidt & Schnierle, 2022).

People infected with CHIKV typically experience a mild and self-limiting illness, characterised by fever, skin rash, myalgia, and arthralgia which can last for weeks to months. Normally, symptoms develop 4–7 days following exposure to CHIKV (Khongwichit et al., 2021). While the majority of patients with acute symptoms recover within 5–14 days, more than 40% experience arthritic manifestations that can last for more than 3 months following the acute illness (Amaral et al., 2020). In the chronic stage of CHIKV infection, severe joint pain with or without arthritis can persist for months to years after the initial infection, significantly affecting quality of life. It has been reported that between 4.1% and 78.6% of chikungunya-

associated patients progress to a chronic stage, although the cause of this variability remains unknown (Khongwichit et al., 2021). During infection, CHIKV can infect various cell types, including monocyte-derived macrophages, human epithelial and endothelial cells, primary dermal fibroblasts, and synovial fibroblasts (Sukkaew et al., 2018). Synovial tissue is one of the primary targets of CHIKV infection and is often associated with severe joint pain and arthritis (Sukkaew et al., 2018). Human fibroblast-like synoviocytes (HFLS), the dominant cell type within synovial tissue, play a crucial role in mediating arthralgia, joint damage, and bone destruction during infection (Sukkaew et al., 2020). HFLS contribute to the development of CHIKV-induced arthritis by secreting soluble inflammatory mediators such as RANKL, IL-6, IL-8, and MCP-1, which promote the recruitment and differentiation of monocytes and macrophages into osteoclast-like cells, ultimately leading to arthritis in joint (Sukkaew et al., 2020).

Currently, no specific antiviral drugs are available to treat CHIKV infection. The goal of treatment for CHIKV infection is to alleviate symptoms through the use of nonsteroidal anti-inflammatory drugs (Patil et al., 2021). IXCHIQ, a chikungunya vaccine is currently available in the United States and approved for adults aged 18 years and above. However, this vaccine is not widely used yet (CDC, 2024a). Due to the lack of effective anti-CHIKV therapies, antiviral research based on natural compounds has been explored to find new antiviral drugs for treating chikungunya fever (Martins et al., 2020). Continuous efforts should be made to discover and develop new treatments to effectively combat CHIKV infection and address the unmet need for targeted antiviral therapies.

Honey, a natural sweetener produced by bees from the nectar and secretions of plants, has been valued for centuries not only for its culinary uses but also for its potential health benefits (Eteraf-Oskouei & Najafi, 2013). Tualang honey, a local Malaysian wild honey, is among the many types of honey that has been particularly associated with valuable medicinal properties. These medicinal properties of Tualang honey are largely attributed to its phenolic and flavonoid contents (Azman et al., 2021). Current findings indicate that Tualang honey possesses antimicrobial, anti-inflammatory, antioxidant, antimutagenic, antitumor, anticancer, anti-obesity and antidiabetic properties, as well as promoting wound healing. Many of these properties are comparable to those of Manuka honey, a well-researched monofloral honey from New Zealand and Australia (Ahmed & Othman, 2013; Kamal et al., 2021). Additionally, Tualang honey has demonstrated *in vitro* antiviral activity against CHIKV infection in Vero cells through various mechanisms, including protecting cells from CHIKV infection, direct inactivation of the virus and interfering with intracellular processes during CHIKV replication (Barkhadle et al., 2021).

1.2 Problem statement

In a previous laboratory study, Barkhadle et al. (2021) investigated the *in vitro* anti-CHIKV properties of Tualang honey using Vero cells, a non-human cell line, and demonstrated that Tualang honey exhibited promising anti-CHIKV activity through multiple mechanisms (Barkhadle et al., 2021). Numerous *in vitro* studies have also investigated the potential anti-CHIKV activities of various natural compounds such as myricetin (Muñoz et al., 2023), *Boswellia serrata* (Von Rhein et al., 2016) and *Andrographis paniculata* (Wintachai et al., 2015). However, most of these *in vitro* studies used cells that are not directly involved with the pathogenesis of CHIKV,

especially during severe and chronic infection. Many of the cells commonly used in *in vitro* antiviral screening include Vero cells (derived from African green monkey kidney) (Chan et al., 2021; Lani et al., 2016; Mandova et al., 2023) and Baby hamster kidney (BHK) cells (Muñoz et al., 2023; Oo et al., 2018; Rocha et al., 2023). Although these compounds exhibit potent anti-CHIKV properties in *in vitro* studies, it is unknown whether they are effective during CHIKV infection in human. For instance, chloroquine inhibited CHIKV infection in Vero cells (Khan et al., 2010), but clinical trials showed no therapeutic benefit in humans and even suggested potential harm (Roques et al., 2018). Therefore, while these results are encouraging, it cannot be assumed that similar effects would occur in human cells.

To date, few studies have specifically focused on investigating natural products as potential treatments for chronic CHIKV infection, despite its significant impact on public health. Addressing this gap is critical, as current treatment options are limited to symptomatic relief without targeting the underlying viral mechanisms. Human fibroblast-like synoviocytes (HFLS) play a key role in chronic CHIKV infection and could therefore serve as a model for studying chronic CHIKV infection. However, they have yet to be used in antiviral studies involving various natural products including honey. Therefore, further research investigating the potential of Tualang honey as an antiviral agent against chronic CHIKV infection in human-relevant model is warranted. Such studies could provide valuable insights into its efficacy, safety and modes of action, supporting the development of new natural treatments to address the long-term effects of CHIKV.

1.3 Research questions

i) What is the maximum non-toxic dose of Tualang honey on HFLS cells?

ii) Does Tualang honey demonstrate anti-CHIKV activity in HFLS cells following CHIKV infection?

iii) What is the mode of action of Tualang honey against CHIKV infection in HFLS cells?

1.4 Research hypothesis

i) H_o: Concentration of 20 mg/mL of Tualang honey is not the maximum non-toxic dose for HFLS cells.

H_a: Concentration of 20 mg/mL of Tualang honey is the maximum non-toxic dose for HFLS cells.

ii) H_o: Tualang honey does not demonstrate anti-CHIKV activity in HFLS cells following CHIKV infection.

H_a: Tualang honey demonstrates anti-CHIKV activity in HFLS cells following CHIKV infection.

iii) H_o: Tualang honey does not exert its modes of action by preventing CHIKV entry into HFLS cells.

H_a: Tualang honey exerts its modes of action by preventing CHIKV entry into HFLS cells.

1.5 Research objectives

General objective

To investigate the anti-CHIKV properties of Tualang honey in HFLS cells following CHIKV infection.

Specific objectives

i) To determine the maximum non-toxic dose of Tualang honey on HFLS cells.

ii) To evaluate the anti-CHIKV activity of Tualang honey in HFLS cells.

iii) To elucidate the inhibitory modes of Tualang honey in HFLS cells following CHIKV infection.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of chikungunya

Chikungunya is a viral disease caused by CHIKV, an arbovirus primarily transmitted by *Aedes* (*Ae.*) species mosquitoes. CHIKV has caused sporadic outbreaks in various regions, including Africa, Asia, the Indian Ocean islands, and southern Europe since it was first discovered (Amaral et al., 2020). Over 1.7 million suspected cases have been identified across 46 American countries since the virus first appeared in the Western Hemisphere in 2013 (Wimalasiri-yapa et al., 2019). Currently, an estimated 39% of the global population resides in countries where CHIKV is endemic and is therefore at risk of infection (Li et al., 2023).

Although rarely fatal, infection with the virus can lead to severe arthralgia that may last for months or even years. Developing low- and middle-income countries are significantly impacted by the spread of CHIKV infection due to inadequate medical infrastructure and their reliance on manual labour for economic activity (DeFilippis, 2022). Unfortunately, there are currently no approved prophylactic or therapeutic treatments available for CHIKV infection (Schwameis et al., 2016).

2.1.1 Disease burden

The disease burden of chikungunya refers to the overall impact of the disease on public health, including the incidence of infection, increased hospitalisation risks, morbidity, mortality and economic costs associated with the disease (Costa et al., 2023). As of now, there have been reports of 3.7 million suspected and laboratory-confirmed cases of chikungunya in 50 American countries and territories (de Souza et al., 2024).

Moreover, the disease can have a significant economic impact, varying greatly across healthcare systems and levels of societal development. This includes direct medical costs for treating acute symptoms (de Brito et al., 2016), as well as long-term expenses associated with chronic inflammatory conditions, which are often more prolonged and costly (Costa et al., 2023). More importantly, significant indirect financial impacts arise from both acute and chronic CHIKV infection, including work absences due to illness, reduced productivity, and frequent travel to healthcare providers (DeFilippis, 2022).

For example, in the Americas, the average direct medical cost per patient for the adult population in Colombia was USD 66.6 (USD 26.5 to 317.3). Notably, 57.4% of these expenses were associated with consultations or treatment by specialist doctors, representing the largest portion of the overall medical costs (Alvis-Zakzuk et al., 2018). In a single year on La Réunion, the medical care costs associated with CHIKV were substantial, reaching approximately USD 13.3 million. This included USD 5.38 million for medications and USD 9.15 million for hospitalisations. When considering both direct medical expenses and indirect costs, such as lost productivity, the total estimated cost reached USD 47.5 million (Costa et al., 2023).

Indirect costs were also evident during the 2017 chikungunya epidemic in Brazil, where a total of 746 days of absence and a financial impact of USD 14,490.90 due to work absenteeism were recorded. This was caused by 123 healthcare professionals being infected with chikungunya, resulting in sick leave durations ranging from 1 to 19 days (Oliveira de Andrade et al., 2022). Another example of indirect costs can be found in the Americas, where productivity losses were reported. The average

cost of lost productivity for each adult patient in Colombia was USD 81.3, with a range of USD 72.2 to 203.2 (Alvis-Zakzuk et al., 2018).

2.1.2 Epidemiology

CHIKV was first discovered in Tanzania from patient serum samples collected in 1952 in the midst of a febrile illness outbreak (Davenport et al., 2020). The name originates from a Makonde word meaning "that which bends you up," referring to the characteristic posture of individuals experiencing severe joint pain from CHIKV infection (Battisti et al., 2021). Chikungunya is endemic in India, Southeast Asia, Africa, the Pacific Islands, and Central and South America, but it occurs sporadically in Mediterranean Europe and the United States (Kumar et al., 2021). According to phylogenetic analysis of CHIKV sequences, the virus first appeared in Africa over 500 years ago (Silva & Dermody, 2017), with a common lineage classified into three distinct genotypes: Asian, East/Central/South African (ECSA), and West African (WA) (Kalyanasundram et al., 2024). These genotypes have spread globally, with the ECSA and Asian genotypes being the most prevalent (de Lima Cavalcanti et al., 2022).

In 2004, CHIKV re-emerged along the Kenyan coast, and it later spread to neighbouring islands in the Indian Ocean and South Asia, including La Réunion (Battisti et al., 2021). The viruses responsible for this outbreak evolved from the ECSA clade and gave rise to a new lineage called the Indian Ocean Lineage (IOL). This lineage was consistently linked to CHIKV outbreaks between 2005 and 2014 (Silva Jr. et al., 2018). A defining feature of the IOL genotype was the emergence of the A226V mutation in the envelope glycoprotein E1 (CHIKV 06.21), which was first identified during this outbreak (Battisti et al., 2021). This mutation, together with other particular

mutations in the E2 protein, facilitated the virus's adaptation from primarily relying on *Ae. aegypti* to also utilising the more widespread *Ae. albopictus*, enabling its spread across regions with diverse temperature conditions (Battisti et al., 2021).

After 2005, the virus spread globally driven either by travellers or local outbreaks in tropical and temperate regions, involving both mosquito species (Srivastava et al., 2020). The first identified autochthonous CHIKV case in Europe occurred in 2007 in northeast Italy, near the Adriatic coast. It was most likely brought to the continent by an infected Indian traveller (Caputo et al., 2020). In September 2010, an autochthonous CHIKV case was reported in southeastern France, with infected travellers being the likely source. That same year, CHIKV re-emerged in La Réunion and spread to Thailand, the Maldives, Indonesia, India, and Myanmar. Imported cases from Asia, India, Indonesia, and La Réunion were also reported in Brazil, the United States, Taiwan and France (Silva Jr. et al., 2018).

In December 2013, a strain from the Asian lineage appeared on the Caribbean island of Saint Martin, causing another significant outbreak. Following that, the virus has spread to over 50 countries across South America, leading to an estimated 1 million infections (Bartholomeeusen et al., 2024). The ECSA lineage was discovered in northeast Brazil in 2014 (Bartholomeeusen et al., 2024). In 2016, Argentina reported the first autochthonous transmission of chikungunya after an outbreak involving over 1000 suspected cases (Carbajo & Vezzani, 2015).

In Asia, CHIKV was initially isolated in 1958 in Bangkok, Thailand and the isolate was categorised as the Asian genotype (Khongwichit et al., 2021). CHIKV subsequently spread to several other Asian countries, including Cambodia (1961), India (1963), Philippines (1965), Vietnam (1967), Sri Lanka (1969), Indonesia (1972) and

Myanmar (1975). It also expanded to other countries such as Malaysia, Singapore, and others (de Lima Cavalcanti et al., 2022; Khongwichit et al., 2021). In Malaysia, CHIKV was first reported in 1998-1999 in Port Klang, Selangor, affecting 51 individuals (Sam & Abu Bakar, 2006). In 2006, Bagan Panchor, Perak, a second outbreak was reported in Malaysia, where more than 200 people were infected with CHIKV (Sam & Abu Bakar, 2006). This was followed by outbreaks in Kinta District, Perak (2006), Tangkak, Johor (2008), and after that, the virus spread to other Peninsular Malaysian states, where more than 2,692 cases were recorded (Apandi et al., 2010). The highest number of infections occurred in the Federal Territory of Kuala Lumpur, Johor, Malacca, and Selangor, with fewer cases in Sabah, Kelantan and Sarawak. However, in 2009, the most affected areas shifted to Sarawak, Kelantan and Kedah, with a total of 4,430 cases reported that year (Apandi et al., 2010). The outbreaks may have been caused by an increase in immigrants from endemic countries and high vector abundance (Muhammad Azami et al., 2013). Although there is no active outbreak, the risk of CHIKV re-emerging remains as it continues to circulate nationwide. To this day, the virus spreads globally, with increasing infections due to climate change, globalisation and insufficient immunity within the population (Battisti et al., 2021).

2.1.3 Virological characteristics and CHIKV genomic structure

CHIKV is a positive-sense, single-stranded RNA virus belonging to the genus *Alphavirus* of the Togaviridae family, which also includes other alphaviruses such as Venezuelan Equine Encephalitis virus (VEEV), Sindbis virus (SINV), Semliki Forest virus (SFV), and Ross River virus (RRV) (Baxter & Heise, 2018). CHIKV is primarily transmitted through the bite of infected female mosquitoes, *Ae. aegypti* and *Ae. albopictus* (Singh et al., 2018).

The genomic structure of CHIKV plays a pivotal role in its replication and virulence. Its genome is approximately 11.8 kb long and comprises two open reading frames (ORFs), ORF1 and ORF2, (Figure 2.1) which are separated by an untranslated junction (J) (Jain et al., 2017). ORF1 encodes four non-structural proteins (nsP1, nsP2, nsP3 and nsP4) while ORF2 encodes five structural proteins (envelope protein E (E1, E2, and E3), capsid (C), and 6K) that are required for the formation of new CHIKV virions. The genome has a 5' cap and a 3' poly A tail (Jain et al., 2017; Ning et al., 2024).

CHIKV has a capsid with a diameter of approximately 700 Å and exhibits a spherically shaped, T = 4 quasi-icosahedral symmetry (Singh et al., 2018). The CHIKV virion is about 70 nm in diameter. The E1 and E2 glycoproteins form heterodimers that assemble into trimeric spikes on the surface (Figure 2.2). The nucleocapsid (NC) core, which is 35 nm in diameter and consists of the capsid protein (C) and the viral genome, is located at the centre of the virion (Zhang et al., 2019).

The non-structural proteins (nsPs) of CHIKV each play a crucial role in the viral replication process. nsP1 possesses methyl transferase and guanyl transferase activity that is significant for viral mRNA capping (Feibelman et al., 2018). Similarly, nsP2 possesses nucleoside triphosphatase and RNA triphosphatase activities, both of which are essential for RNA capping (Feibelman et al., 2018). In addition, nsP2 also has helicase and protease activities essential for viral replication. Furthermore, a scaffold protein called nsP3 is crucial for the association of factors that form the replication complex. Complementing these roles, nsP4 functions as the RNA-dependent RNA polymerase, a key enzyme required for synthesising the viral RNA genome (Rajesh Kumar et al., 2021).

Equally important are the structural proteins, which play distinct roles in the viral lifecycle. Among them, the E1 protein is crucial for the fusion of the viral envelope with the host cell membrane, while the E2 glycoprotein mediates the attachment and entry of the virus into host cells (Freppel et al., 2024). Following entry, the capsid protein plays a vital role in the growth and assembly of new virions by encapsulating the viral RNA and forming the nucleocapsid core (Kiser et al., 2021). To ensure proper assembly, the E3 protein regulates the correct folding of the E2 precursor (p62) and facilitates the formation of E1-p62 heterodimers. Moreover, it prevents premature fusion of the E2-E1 heterodimers with cellular membranes, ensuring that fusion occurs only at the appropriate stage (Singh et al., 2018). Additionally, the 6K glycoprotein aids in the translocation of structural polyproteins to the endoplasmic reticulum and contributes to the cleavage of p62 into mature structural proteins E2 (Singh et al., 2018).

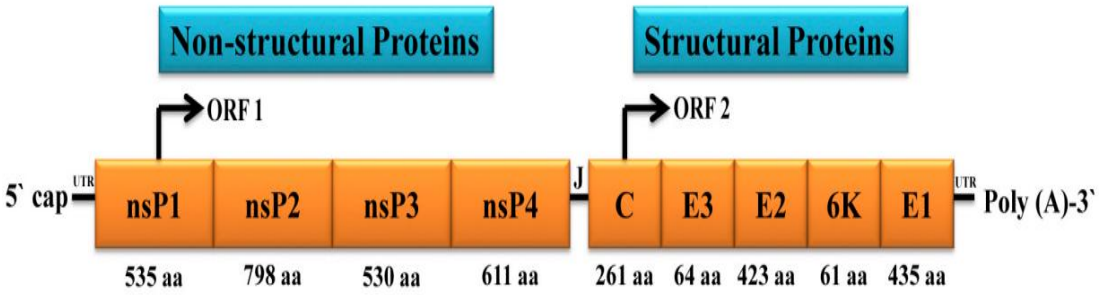

Figure 2.1 Chikungunya virus genome. Adopted from Jain et al., (2017).

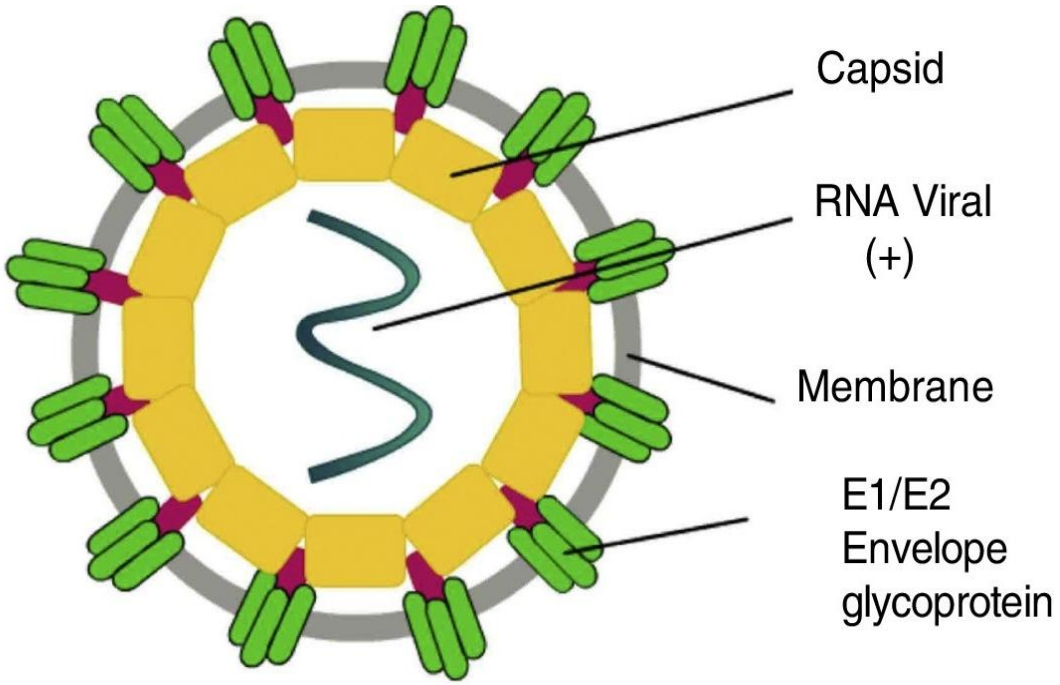

Figure 2.2 The structure of chikungunya virus. Adopted from Castillo-Macías et al., (2017).

2.1.4 Viral replication

Similar to other alphaviruses, CHIKV enters cells through an initial interaction between its viral proteins with attachment factors and specific receptors from the host cell. The surface of the CHIKV virion is composed of 80 trimeric spikes made up of E1 and E2 glycoproteins (Battisti et al., 2021). At the start of an infection, E2 glycoprotein binds to specific receptors on the surface of host cells as shown in Figure 2.3. The virus then enters the host cell through clathrin-mediated endocytosis (Constant et al., 2021). After internalisation, acidification of endosomes triggers conformational changes of the viral envelope glycoprotein E1, which results in the fusion of the viral envelope with the endosomal membrane (Silva & Dermody, 2017).

This fusion results in the release of the nucleocapsid into the cytoplasm of the host cell, where it is disassembled to release the viral RNA genome. The viral RNA genome is then translated by the host cell translation machinery generating non-structural polyprotein (PI234), which is subsequently cleaved into precursor P123 and the viral non-structural-protein nsP4 (Abdelnabi et al., 2017). P123 and nsP4 together with host proteins assemble to form the initial replication complexes (RCs) which are responsible for synthesising negative-strand RNA (Pietilä et al., 2017). The negative-strand RNA serves as a template for the synthesis of new positive-strand RNA and 26S sub-genomic RNA. Once the concentration of P123 and nsP4 reaches a threshold, the cleavage of precursor P123 into free non-structural proteins (nsPs) is triggered, further advancing viral replication (Battisti et al., 2021).

The 26S RNA, which is produced during this process, functions as the mRNA that encodes the structural viral proteins (C-pE2-6K-E1). Following translation, the capsid protein (C) dissociates from the polyprotein immediately through its

autocleavage activity. In the meantime, the pE2-6K-E1 precursor is directed to the ER lumen, where it undergoes its maturation process into pE2, 6K, and E1 (Constant et al., 2021). The pE2 and E1 then associate in the Golgi and subsequently migrate towards the plasma membrane, where pE2 is cleaved into the mature E2 and E3 proteins. Finally, the viral assembly is facilitated by the attachment of the viral nucleocapsid to the viral RNA and the recruitment of the membrane-associated envelope glycoproteins (Schwartz & Albert, 2010). The nucleocapsids then bud through the cell membrane, where they acquire the lipid bilayer envelope embedded with the virus-encoded glycoproteins, completing the formation of new viral particles (Abdelnabi et al., 2017).

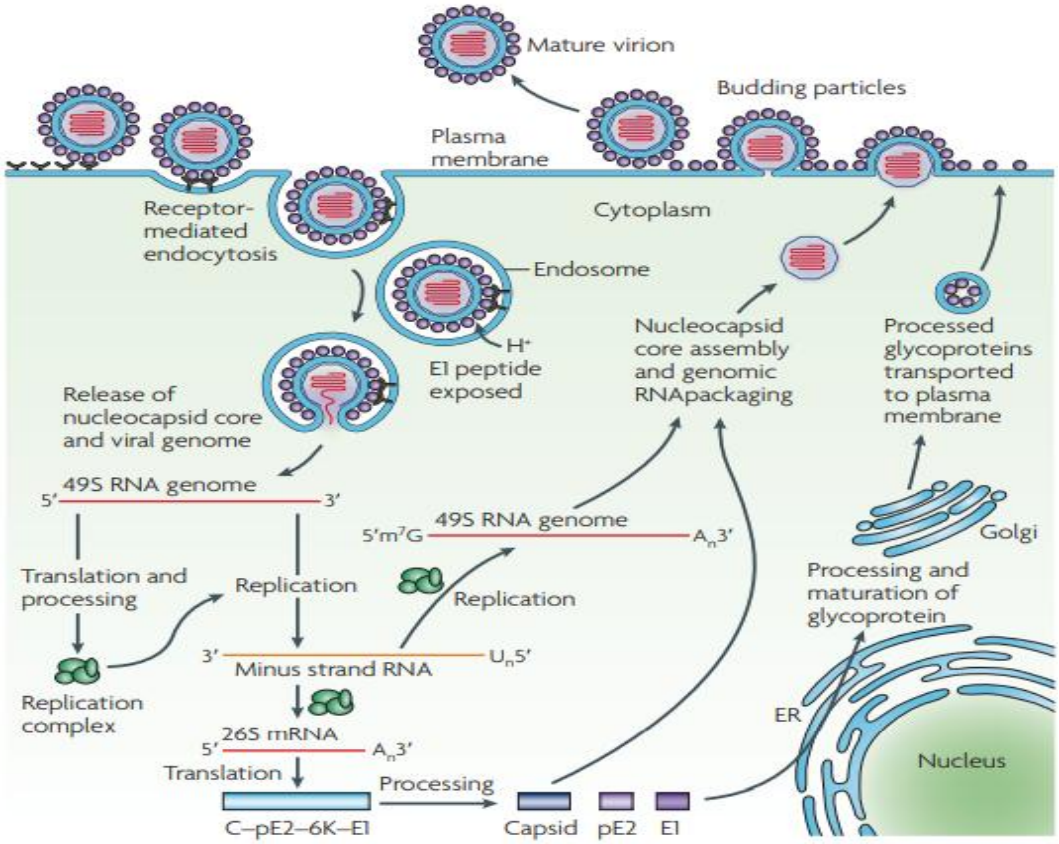

Figure 2.3 CHIKV replication processes. Adopted from Schwartz & Albert, (2010).

2.2 Clinical manifestation

Chikungunya infection can be categorised into three phases: acute, post-acute, and chronic. The acute phase is defined as the first 21 days of clinical symptoms (da Cunha & Trinta, 2017) and is divided into the viraemic phase (5 to 10 days) and the subacute post-viraemic phase (6 to 21 days). The viraemic phase is marked by a sudden onset of high fever (often >39°C) (Ng et al., 2024), along with polyarthritis/polyarthralgia, conjunctivitis, myalgias, and exanthema, which can appear either as widespread or localised. Many patients also develop vesicles, purpura, skin hyperesthesia, and pruritus (Pathak et al., 2019).

The post-viraemic phase is characterised by apyrexia, along with persistent arthritis and polyarthralgia. Additionally, symptoms such as myalgia, pruritus, fatigue, soft tissue edema, anorexia, and lymphadenopathies may also occur, though to a lesser extent (Ng et al., 2024). Joint pain is typically symmetric and progressive, affecting both large and small joints in the arms (fingers, wrists, elbows and shoulders) and the legs (knees, ankles, hips and feet) (Zaid et al., 2018). Atypical symptoms may involve the vertebral, sternoclavicular and temporomandibular joints. Notably, signs of stiffness and swelling, which suggest synovitis and typically detected through joint ultrasound, are most commonly seen in the ankles, fingers, wrists, and toes, while larger joints rarely exhibit these symptoms (Simon et al., 2015). In addition, bursitis and tenosynovitis can further contribute to the rheumatic symptoms of acute chikungunya disease (Ng et al., 2024).

The post-acute phase begins after day 21 and can last up to three months. This phase is characterised by various manifestations that prolong the initial inflammatory symptoms (acute arthritis), including tenosynovitis, bursitis, slowly resolving enthesitis,

and periarticular involvement. Additionally, non-rheumatic and systemic symptoms may also occur during this phase (Zaid et al., 2018).

The disease is classified as chronic when arthralgia lasts longer than three months. Up to 79% of people with chronic illnesses have arthralgia or arthritis, which is the main symptom of chronic chikungunya disease (Suhrbier, 2019). In addition, less commonly reported signs and symptoms include fatigue, alopecia, headache, sleep disturbances, depression, memory impairment, hearing difficulties, blurred vision, digestive issues, and skin lesions or rashes (McCarthy et al., 2022). Importantly, chronic arthralgia in chikungunya usually affects the same joints that experienced pain during the acute phase, and the associated arthropathy is generally non-erosive. Beyond these symptoms, chikungunya can also result in long-term sequelae, including chronic fatigue, depression, and other neurological disorders, which significantly impair quality of life (Suhrbier, 2019). Furthermore, factors that increase the likelihood of developing chronic chikungunya disease include older age (over 35 to 45 years for joint pain), comorbidities (such as diabetes and osteoarthritis), higher viraemia levels, and the severity of the disease during the acute phase (van Aalst et al., 2017).

It is also crucial to highlight that children are more susceptible to developing severe forms of chikungunya. In this age group, fever is the most common symptom (Tanabe et al., 2018). However, children often experience atypical and severe cases, with the disease causing hyperpigmentation, erythema, bullous skin lesions, and neurological symptoms, including seizures and encephalitis, and a range of other complications (Tanabe et al., 2018). These severe manifestations highlight the increased vulnerability of children to the more dangerous forms of the disease.

2.3 Diagnosis

Similar to other arboviral infection, chikungunya patients may have a variety of symptoms, including a high fever, headaches, skin rashes, muscle pain, joint pain, and neurological issues (Khongwichit et al., 2021). The overlapping symptoms of chikungunya and other arboviral diseases make clinical diagnosis challenging, particularly in areas where CHIKV, dengue virus (DENV), and zika virus (ZIKV) co-circulate. As a result, specific laboratory testing is crucial to accurately differentiate chikungunya from other disease-causing agents (Khongwichit et al., 2021; Silva Jr. et al., 2018).

The diagnosis of CHIKV infection in the laboratory is based on techniques such as viral isolation, molecular methods like reverse transcription (RT) and real-time PCR for detecting viral RNA, and serological tests to identify IgM and IgG antibodies including enzyme-linked immunosorbent assays (ELISA) and rapid immunochromatographic tests (da Cunha & Trinta, 2017; Khongwichit et al., 2021). The selection of a molecular or serological test primarily depends on the timing and volume of the samples available (Erin Staples et al., 2009). Virus isolation and PCR provide the highest detection rates for CHIKV within the first week of infection. In later stages (>5 days post-infection), as antibodies develop, serological assays like IgM and IgG tests become more reliable for diagnosing chikungunya (Álvarez-Argüelles et al., 2019).

Viral isolation should be carried out using samples collected within the first 7 days of infection (Erin Staples et al., 2009). This technique relies on observing cytopathic effects induced by CHIKV in cell lines such as Vero, BHK-21, and HeLa, which typically appear within three days. CHIKV isolation can be confirmed using RT-

PCR or immunofluorescence (da Cunha & Trinta, 2017). Although viral isolation is a highly specific and accurate technique, it is not commonly performed due to the lengthy process required to obtain results and the necessity of conducting the procedure in a biosafety level 3 (BSL-3) laboratory (da Cunha & Trinta, 2017).

Furthermore, CHIKV viral RNA can be identified using RT-PCR within the first seven days of infection, but its reliability decreases beyond this timeframe (Pathak et al., 2019). These assays with high specificity and sensitivity are particularly useful for early diagnosis in a variety of settings because they can be carried out outside of conventional laboratory settings and are field deployable, utilising small equipment that can run on mains or batteries and has data transmission capabilities (Bartholomeeusen et al., 2024).

During the acute or convalescent phase, antibodies (specifically IgM and IgG) can be detected using serological methods such as ELISA or rapid diagnostic tests (RDTs) (Erin Staples et al., 2009). CHIKV serum IgM typically appears as the first week of symptoms comes to a close (between days 3 and 8) (Chua et al., 2017), peaks between 3 and 5 weeks after the acute phase begins, and gradually decreases over one to three months. IgG levels, mainly IgG3 type, can usually be detected from day 4 to 10 and may persist for years (Schwameis et al., 2016). While ELISA is usually highly specific (Erin Staples et al., 2009), it can yield false positives due to cross-reactivity with other arboviruses, such as DENV and viruses from the Semliki Forest antigenic complex, including o'nyong-nyong and Mayaro viruses (Khongwichit et al., 2021). In addition to lab-based quantitative tests such as ELISA, antibody detection can be achieved through single-use, disposable RDTs designed for point-of-care use (Bartholomeeusen et al., 2024). These rapid tests are advantageous because they do not

require laboratory infrastructure and are suitable for use in remote areas, providing results within 10–20 minutes without the need for refrigeration or specialised equipment (da Cunha & Trinta, 2017).

2.4 Mode of transmission

CHIKV transmission occurs in two distinct cycles, the sylvatic cycle and the urban cycle. Urban transmission occurs in a human-mosquito-human cycle and is mainly mediated by *Ae. aegypti* or *Ae. albopictus* mosquitoes (Silva & Dermody, 2017). Historically, *Ae. aegypti* has served as the main vector for the spread of CHIKV. However, in recent outbreaks in Gabon, Europe, and La Réunion, *Ae. albopictus* emerged as a significant vector. Despite this, *Ae. aegypti* remains an important vector, as evidenced by its role in the Caribbean outbreak of 2013 (Ganesan et al., 2017).

In the sylvatic cycle, the virus circulates between nonhuman primates and mosquitoes that inhabit forested areas, with occasional transmission to humans living near these forested regions (Weaver et al., 2020). This cycle involves a range of mosquito species as vectors, including *Ae. dalzieli*, *Ae. vittatus*, *Ae. neoafricanus*, *Ae. furcifer-taylori*, *Ae. luteocephalus*, *Ae. aegypti formosus*, *Ae. africanus*, and *Culex quinquefasciatus* (Ramphal et al., 2024).

Besides vector transmission, vertical transmission of CHIKV from mother to baby has been suggested as a possible exposure route since 2005. This happens during pregnancy or childbirth when an infected mother transfers the virus to her infant (Ganesan et al., 2017). Although the occurrence of vertical transmission is still debated, studies suggest that the risk is higher if the mother is infected within four days postpartum (Ganesan et al., 2017). During the 2005–2006 outbreak in La Réunion,

about 50% of mothers who were infected around the time of delivery transmitted the virus to their infants. Among these neonates, half developed encephalopathy shortly after birth (Bartholomeeusen et al., 2024).

2.5 Pathogenesis

CHIKV infection begins when an infected mosquito bites a human, introducing the virus into the skin (Schwartz & Albert, 2010). After being introduced into the bloodstream or skin through the bite of an infected mosquito, CHIKV replicates at the site of inoculation, primarily in fibroblasts and potentially in macrophages (Silva & Dermody, 2017). In addition, local epithelial and endothelial cells are also susceptible to the virus and allow for its replication (Schwameis et al., 2016). The infection triggers an innate immune response as the host recognises CHIKV through pathogen-associated molecular patterns (PAMPs) detected by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and retinoic acid-inducible gene I-like receptors (RLRs). This activation leads to the release of inflammatory cytokines, which induce a pyrogenic reaction (Freppel et al., 2024; Schwameis et al., 2016). Among the immune responses, the type I interferon (IFN-α/β) response plays a pivotal role during the early stages of infection. This response is critical for viral clearance and controlling the spread of CHIKV (Schwameis et al., 2016). Additionally, the levels of IFN signaling and certain chemokines, such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), are associated with disease severity and viral load (IL-6 and MCP-1). Elevated IL-6 and MCP-1 levels are particularly linked to persistent arthralgia, a hallmark of chikungunya fever (Schwameis et al., 2016).