ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCES
SCHOOL OF BIOMEDICINE AND MEDICAL LABORATORY SCIENCES
DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND PARASITOLOGY

PREVALENCE OF DENGUE AND CHIKUNGUNYA IN MALARIA-SUSPECTED FEBRILE PATIENTS: MOSQUITO-BORNE DISEASES' KNOWLEDGE, PRACTICES AND DIAGNOSTIC CHALLENGES IN SELECTED DISTRICTS OF AFAR NATIONAL REGIONAL STATE, NORTHEAST ETHIOPIA

By

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Prevalence of Dengue and Chikungunya in Malaria-Suspected Febrile Patients: Mosquito-Borne Diseases' Knowledge, Practices and Diagnostic Challenges in Selected Districts of Afar National Regional State, Northeast Ethiopia

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DECLARATION

I, the undersigned, declare that the dissertation I am submitting, entitled “Prevalence of Dengue and Chikungunya in Malaria-Suspected Febrile Patients: Mosquito-borne Diseases’ Knowledge, Practices and Diagnostic Challenges in Selected Districts of Afar National Regional State, Northeast Ethiopia”, is my original work. It has never presented at this or any other university. I fully acknowledged all the resources and materials used in this dissertation.

Name: Biruk Zerfu Habtemariam

Signature: _________________

Date _____________________

Place: Addis Ababa, Ethiopia

DEDICATION

I dedicate this work to my mother with deep love and respect, whose unwavering strength, sacrifices, and encouragement made this achievement possible. This dissertation is not only an academic milestone but also a tribute to her resilience and unconditional support.

ABSTRACT

Background: Dengue virus (DENV) and chikungunya virus (CHIKV) infections are an emerging burden to rising non-malarial febrile illnesses in tropical and subtropical regions, including Ethiopia. Ethiopia has experienced several significant outbreaks of arboviral diseases over the past two decades, particularly in the eastern and northeastern regions, including in the Afar National Regional State. Given the overlap in symptoms and the risk of co-infection, it is important to assess the true burden of these diseases among malaria-suspected patients during non-outbreak periods and understand community awareness and healthcare worker (HCW) challenges to improve public health intervention and strengthen healthcare response to these diseases. Hence, this study was designed to assess the prevalence of DENV and CHIKV infection in malaria-suspected febrile patients, community knowledge, attitudes, and practices (KAP) about these arboviral diseases and healthcare workers' (HCWs) knowledge, practices and challenges in diagnosing and managing arboviral and non-malarial febrile illnesses in malaria-endemic selected districts of the Afar National Regional State, northeast Ethiopia.

Methods: Between September 2022 to March 2023, a cross-sectional study was conducted among malaria-suspected febrile patients, with axillary body temperature of ≥37.5°C, attending healthcare facilities to assess the prevalence of CHIKV and DENV infection. Socio-demographic and clinical features were collected using a structured questionnaire. Five milliliters of venous blood were collected and thick and thin blood films were prepared for the diagnosis of malaria on-site. Sera were separated from remaining blood and stored appropriately. It was tested for anti-DENV and anti-CHIKV IgM and IgG antibodies using enzyme-linked immunosorbent assay (ELISA). IgM-positive sera were pooled and screened for viral RNA using in-house reverse transcription-polymerase chain reaction (RT-PCR) and a one-step multiplex RT-PCR. Those positive pools were disaggregated and retested individually to identify specific RNA-positive patient serum. To assess community KAP and HCWs' knowledge, practices, and challenges, structured questionnaires, as well as focus group discussions (FGDs) with community members, in-depth interviews (IDIs) with frontline HCWs were conducted. Additionally, secondary data on febrile patient diagnosis and management were reviewed from public healthcare facilities to evaluate HCW diagnostic practices and trends. Quantitative data were coded and entered into EpiData 3.1, and exported and analyzed using Stata 14, with p-value < 0.05 statistically significant. Thematic analysis was applied to qualitative data.

Results: A total of 411 malaria-suspected febrile patients (55.5% female, age ranged from 5-80 years, with a mean age ± SD of 27.3± 13.9) years participated in the prevalence study. Among 368 sera tested for anti-CHIKV antibodies, 176(47.8%) were IgM positive, indicating acute CHIKV infection and 23(6.3%) were IgG positive, suggesting previous exposure. Non-married patients and those having back pain were associated with positivity for acute CHIKV infection (AOR = 2.34, 95% CI: 1.14- 4.96 and AOR = 1.78, 95% CI: 1.08-2.95), respectively. Among 410 sera tested for anti-DENV IgM, 101(24.6%) were positive, indicating acute DENV infection. Of 367 sera tested for IgG, 142 (38.7%) were positive, indicating a previous exposure. Previous DENV exposure was significantly associated with being female (AOR = 2.24; 95% CI: 1.32-3.26) and employed (AOR = 1.90; 95% CI: 1.03-8.15) participants. From all 411 febrile patients, only 44 (10.7%) were positive for *Plasmodium* infection, mainly due to *P. falciparum* (84.1%). Co-infections were observed in 15.8% of patients for acute DENV-CHIKV, 4.4% for acute DENV-malaria and 4.3% for acute CHIKV-malaria among those tested for both infections. Out of 53-pooled samples positive for one or both viruses' IgM, three were confirmed positive for DENV RNA, two of which were from DENV-CHIKV dual IgM-positive samples and the remaining one from a CHIKV IgM-positive only pooled sample by in-house RT-PCR, while two CHIKV RNA samples were identified from DENV-CHIKV dual IgM-positive pools using commercial RT-PCR methods.

In the KAP study, 296 community members (mean age 34.2 ± 10.6 years; 36.8% female) and 116 FGD participants were included. While 67.3% of the community had heard of CHIK, only 44.7% recognized it as viral, 48.7% identified *Aedes* mosquitoes as vectors, and 16.5% knew they bite during the day. The mean KAP scores were moderate, with knowledge at 63.2%, attitudes at 60% and practices at 60%. Higher education (9-12) and student status were linked to good knowledge; age 45–59 and being single were associated with positive attitudes. Among 82 HCWs surveyed (plus 10 in IDIs), 93.9% had heard of CHIKV and DENV, with the overall mean knowledge score of 13.1 ± 3.9 (62.4%), showing a moderate knowledge level, but only 36.4% demonstrated good knowledge, while 35.1% showed moderate knowledge of the diseases. Higher knowledge was significantly associated with higher educational levels. The patient record review revealed over-diagnosis of malaria infection and low diagnosis of non-malarial febrile illness, while limited differential testing capability for arbovirus infections, inadequate training for HCWs, low community involvement and low awareness of arboviral and non-malarial mosquito-borne febrile diseases were identified as challenges.

Conclusion: The seroprevalence of DENV and CHIKV infections is substantial among malaria-suspected acute febrile patients, highlighting the need for integration of arbovirus infections in regular screening, management and control of the infections in the study area. Limited awareness and diagnostic capability demand strengthening diagnostic capacity, updating fever case management and diagnosis policies, such as availing rapid test kits, serological tests (IgM tests) and affordable PCR tests for arboviral infections and scaling up HCW training and community sensitization are critical for effective arboviral and other mosquito-borne diseases control in malaria-endemic settings.

Keywords: Seroprevalence; Dengue fever; Dengue virus; Chikungunya virus; Febrile illness; Konwledge, Attitude; Practice; Afar Region

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Zerfu B, *Kassa T, Mamo G, Larrick JW, Legesse M. High seroprevalence of IgM antibodies against chikungunya among patients with acute febrile illness seeking healthcare in a malaria-endemic area in the Afar Region, Northeast Ethiopia. SAGE Open Med. 2024 Sep 9;12:20503121241276557. doi: 10.1177/20503121241276557.*

Zerfu B*, Kassa T, Mamo G, Larrick JW, Legesse M. Seroprevalence of dengue virus infection among febrile patients visiting healthcare facilities in the selected districts of Afar Region, Northeast Ethiopia. BMC Infect Dis. 2025 Jul 28;25(1):948. doi: 10.1186/s12879-025-11406-3.)*

2. Papers Presented at Conference

*High Seroprevalence of IgM Antibodies against Chikungunya Virus Infection among Patients with acute febrile illness seeking healthcare in a malaria-endemic area in the AfaRegion, Northeast Ethiopia; A Research Week Program at Aklilu Lemma Institute of Pathobiology, Addis Ababa University May 13-17, 2024 (Ginbot 5-9, 2016 E.C) (**ORAL**)*

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3. Review Publication

Zerfu B*, Kassa T, Legesse M. Epidemiology, biology, pathogenesis, clinical manifestations, and diagnosis of dengue virus infection, and its trend in Ethiopia: a comprehensive literature review. Trop Med Health. 2023 Feb 24;51(1):11. doi: 10.1186/s41182-023-00504-0.*

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LISTS OF ABBREVIATIONS

AFI - Acute febrile illness

AOR- Adjusted odd ratio

CHIK - Chikungunya

CHIKV - Chikungunya virus

DENV - Dengue virus

DF - Dengue fever

DHF - Dengue Hemorrhagic Fever

DSS - Dengue Shock Syndrome

ELISA- Enzyme linked Immunosorbent assay

EPHI - Ethiopian Public Health Institute

FGD - Focus group discussion

GBV - G Barker Virus

HCV - Hepatitis C virus

HCW- Healthcare worker

HHS - Household survey

IDI- in-depth interview

IgG- Immunoglobulin G

IgM- Immunoglobulin M

JEV - Japanese encephalitis virus

KAP - Knowledge, attitude and practices

NS - Non-structural

RDT - Rapid diagnostic test

RNA - Ribonucleic acid

RT-PCR - Reverse transcription polymerase chain reaction

SD - Standard deviation

SSA - Sub-Saharan Africa

TBEV - Tick-borne encephalitis virus

WHO - World Health Organization

WNV - West Nile virus

YFV - Yellow fever virus

ZIKV - Zika virus

CHAPTER ONE

1. INTRODUCTION

1.1. Background and Justification of the Study

The Dengue virus (DENV) and chikungunya virus (CHIKV) are arthropod-borne viruses that cause Dengue fever (DF) and chikungunya (CHIK) illnesses, respectively. These arboviruses primarily transmit to humans through the bites of infected female *Aedes aegypti* and *Ae. albopictus* mosquitoes (Brady *et al.,* 2012; WHO, 2023). Nowadays, infections of these arboviruses have become significant global health threats, mainly in the tropical and subtropical areas, with recent spread into previously unaffected regions, such as temperate regions. Climate change, globalization, urbanization and the adaptation of the *Aedes* mosquito to diverse environments are the contributing factors to this global spread (Gloria-Soria *et al.,* 2016; WHO, 2022d, 2023). To curb the expansion, the World Health Organization (WHO) has included these diseases in its prioritized list recently, aiming to facilitate action, drive investment and innovation in research and public health interventions and strengthen international collaboration and resource allocation.

The four distinct serotypes' DENV is an enveloped single-stranded RNA virus, belonging to the genus *Flavivirus* in the family Flaviviridae. It is one of the fastest-spreading mosquito-borne infections, with over 30-fold increase in incidence over the past five decades. Annually, DENV infects approximately 390 million people, with more than 100 million symptomatic cases and thousands of deaths, primarily due to severe DF like Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) (Brady *et al.,* 2012; WHO, 2023). Currently, more than 141 countries have reported active autochthonous DENV transmission, though many cases remain unreported due to weak surveillance systems in many endemic countries (Gloria-Soria *et al.,* 2016; WHO, 2023). Therefore, the number of cases and deaths due to DENV infection continues to escalate, with new records reported each year over the past two decades, leading to nearly half of the world's population being at risk(WHO, 2023).

The Togaviridae family's *alphavirus* genus includes an enveloped single-stranded RNA genome CHIKV. This virus has a huge resurgence in the last two decades, with infection has been identified in over 110 countries in Asia, Africa, Europe and the Americas (An *et al.,* 2017; Silva & Dermody, 2017). CHIKV infection is a non-fatal, though frequently it causes a debilitating joint pain, which may persist for months or years, thereby affecting patients' quality of life & straining healthcare systems (PAHO, 2011; Silva & Dermody, 2017).

Both DENV and CHIKV frequently co-circulate in the same geographical areas and their infection exhibit overlapping clinical manifestations, such as sudden fever, headache, muscle pain and joint pain, making their clinical differentiation difficult, which increases the likelihood of misdiagnosis as other febrile illnesses, particularly malaria in endemic areas (Gloria-Soria *et al.,* 2016; WHO, 2023). The accurate diagnosis of DF and CHIK typically involves serological tests that detect IgM and IgG antibodies or NS1 protein of DENV using ELISA or Rapid diagnostic tests (RDTs), viral RNA detection using molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) and viral isolation using different cell lines. Both DENV and CHIKV infection treatments remain pain relief supports, with no specific antiviral available. Severe DF patients would be managed with fluid management, while those suffering from CHIK typically receive symptomatic treatment for joint pain (WHO, 1997, 2009, 2023). Therefore, these arboviral diseases prevented through vector control is crucial, including insecticide use, environmental management and personal protection (WHO-RTTD, 2017).

In Ethiopia, vector-borne diseases like malaria and arboviruses have been public health challenges for a long time. The arboviral challenges in Ethiopia date back to the 1960s, when the yellow fever (YF) outbreak was reported in the southern region around south Omo (Serie *et al.,* 1964; Mekonnen & Kroos, 2006). During the past two decades, various outbreaks of arbovirus infections have occurred in Ethiopia. DENV infection was confirmed in Ethiopia for the first time in 2013 during a major outbreak in Dire Dawa, while the CHIKV infection was first confirmed in 2016 from an outbreak associated with non-malarial febrile illness in the Dollo Ado district of the Somali Region (CRISIS, 2021; 2021a; Sisay *et al.,* 2022; WHO, 2021). Following these confirmed outbreaks, many outbreaks of arboviral diseases, including DF and CHIK, have occurred in various regions, where malaria is endemic (Degife *et al.,* 2019; Gutu *et al.,* 2021; Mesfin *et al.,* 2022). These outbreaks have shown the risk that has been posed and is to be posed to Ethiopia, particularly in the eastern and northeastern regions, including the Afar National Regional State, which is malaria endemic area.

Given the clinical presentation and geographical overlapping, as well as the co-infection risks and possibility of misdiagnosis, assessing the true burden of these arboviral diseases and their clinical impacts among malaria-suspected febrile patients, including their co-infection with malaria, is essential in areas where both mosquito-borne diseases potentially co-exist, during non-outbreak periods. Furthermore, understanding community knowledge, attitude, practices (KAPs), as well as the awareness, diagnostic practices and challenges faced by HCWs, would be essential to provide insight for strengthening prevention and control strategies. Additionally, the lack of diagnostic capacity and infrastructure in many healthcare facilities limits healthcare professionals to the timely and accurate identification of arboviral diseases. This not only hinders the delivery of appropriate healthcare services but also compromises disease surveillance and public health preparedness.

Therefore, this study aimed to address these gaps by producing preliminary evidence by investigating the prevalence of DENV and CHIKV infections among malaria-suspected febrile patients during non-outbreak periods in selected districts of the Afar National Regional State, northeast Ethiopia. It also aimed to assess the community's KAP and HCWs' awareness practices and challenges related to diagnosing and managing arboviral diseases and other non-malarial febrile illnesses in this malaria-endemic pastoral setting. The findings will inform public health interventions, support evidence-based decision-making and strengthen diagnostic and surveillance capacities of healthcare facilities to manage these emerging infectious diseases. To our knowledge, this is the first study to assess arboviral infections among malaria-suspected febrile patients during non-outbreak periods in the Afar National Regional State of Ethiopia.

1.2. Statements of the Problem

Although DF and CHIK are increasingly emerging as significant public health threats worldwide, they have not yet been considered important public health diseases in most African countries, including Ethiopia. Additionally, DF and CHIK have an acute febrile illness presentation similar to malaria, and they often co-circulate with malaria parasites, leading to frequent misdiagnosis of these arbovirus infections, particularly in many malaria-endemic areas, such as the Afar National Regional State. To curb these arbovirus infections emergence, the WHO has recently a plan to control and prevent these infection as a neglected tropical disease, for example, the organization targeted to reduce the incidence of DF by 25%, achieve zero case-fatality rates, and increase outbreak detection and response capability by 75% by 2030 (WHO, 2020b).

Despite this target, most endemic countries for mosquito-borne diseases often neglect these viral infections due to the national and international healthcare programs historically prioritizing only malaria. As a result, DF and CHIK cases are frequently misdiagnosed as malaria and other non-malarial febrile illnesses, or left undiagnosed as unknown acute febrile illnesses. Such gaps undermine patient care and hinder the understanding of the true burden and public health impact of the diseases in endemic areas. This furthermore hampers the evidence-based implementation of support strategies proposed by the WHO, which include enhancing training, optimizing diagnostic protocols, and establishing comprehensive fever management (WHO-RTTD, 2022).

Ethiopia has experienced several significant outbreaks of arboviral diseases over the past two decades, including in the Afar National Regional State. For example, a recent large outbreak accounted for approximately 53,000 cases of dengue-like illnesses originating in Dire Dawa and spreading into the Afar National Regional State, including Amibara, Harunka and Awash Sebat city district (EPHI, 2021). However, the true burden and public health effects of DENV and CHIKV infections remain unknown, particularly during non-outbreak periods. The few existing studies are limited both geographically and in scope (Endale *et al.,* 2020; Ferede *et al.,* 2018). These significant gaps limit the implementation of public health interventions targeting these arbovirus infections.

On the other hand, in many endemic African countries, including Ethiopia, besides the overlap of clinical manifestations between DF, CHIK and malaria and low recognition, there is poor access to diagnostic facilities for non-malarial febrile illnesses, including these arbovirus infections (Omatola *et al.,* 2020). These gaps contribute to misclassification of acute febrile illnesses, improper case management practices, such as antimicrobial misuse and may fuel the emergence of drug resistance, delaying opportunities for early detection and outbreak control.

Advanced awareness and practices of the community regarding arbovirus infections play an important role in reducing exposure to the infections because the behaviors, such as water storage management, vegetation management and personal protection from mosquito bites, significantly influence transmission risk. However, in Ethiopia, awareness and community-based preventive practices, especially in arboviral outbreak-prone areas like the Afar National Regional State, are inadequate or have not yet been assessed. The gap undermines the routine vector surveillance, reduces community engagement and weakens resilience to emerging public health threat diseases.

The miss-inclusion of arboviral diseases into the lists of public health priority diseases in Ethiopia, except for YF in the southern parts of the country, results in a lack of clear guidelines and protocols for diagnosis and management and situation-based interventions. This gap results in HCWs with insufficient awareness and preparedness, even in areas with recurrent outbreaks. As a result, the HCWs have uncertainties or are unable to detect and manage arboviral cases in their healthcare facilities, as well as being ineffective in outbreak detection and control, particularly in the pastoral communities with limited healthcare infrastructure, like the Afar National Regional State.

1.3. Significance of the study

Generally, this study is pivotal for bridging gaps in the understanding of DENV and CHIKV infections, diagnosis, prevention, and control of the diseases in the Afar National Regional State and similar settings. The outcomes benefit the study areas and similar settings in Ethiopia and contribute to the global effort to mitigate the burden of arboviral infections in vulnerable regions.

In particular, this study is significant:

- By generating empirical data on the prevalence of these arboviral diseases, the findings will contribute to improving public health priorities to include these diseases and the disease management system in the region. Understanding the seroprevalence of DENV and CHIKV infections provides valuable insights into the burden of these diseases in febrile patients, enabling health authorities to assess the true impact of arboviral diseases within the current malaria-focused diagnosis framework.

- By addressing critical awareness gaps of the public about the infections and the role of involvement in disease prevention, the findings guide the development of targeted health education campaigns for empowering communities with knowledge and prevention strategies for controlling the spread of arboviral diseases.

- By assessing HCWs' awareness and practices gaps regarding arboviral diseases, and the challenges in diagnosing and managing these arboviral diseases, the finding will be crucial evidence to design knowledge and skill gap trainings for professionals to capacitate their ability to address the infections effectively, as well as for strengthening the healthcare system facilities and to supporting them.

- Finally, this study's findings inform national health policies by emphasizing the importance of including arboviral diseases as a public health priority. The generated evidence can contribute to establishing sentinel surveillance systems, improving diagnostic protocols and ensuring a comprehensive approach to vector-borne disease control in Ethiopia.

1.4. Hypothesis and Objectives of the Study

1.4.1. *Hypothesis of the Study*

- Arboviral infections such as DENV and CHIKV are prevalent among malaria-suspected febrile patients in the study area.
- The community has limited knowledge, attitudes, practices (KAPs) regarding arboviral diseases such as DF and CHIK in the study area.
- HCWs have limited awareness and practices about arboviral diseases and face limited challenges in diagnosing and managing arboviral and other non-malarial febrile diseases in the study area.

1.4.2. *General Objective*

- To generate evidence on the prevalence of DENV and CHIKV infections among malaria-suspected febrile patients and to assess the community's KAP about these arboviruses and HCWs' knowledge, practices and challenges in diagnosing and managing these arboviral diseases and other non-malarial febrile diseases in selected districts of the Afar National Regional State, Northeast Ethiopia.

1.4.3. *Specific Objectives*

1) To determine the seroprevalence of DENV and CHIKV infections among malaria-suspected febrile patients

2) To detect DENV and CHIKV RNA in IgM-positive serum samples

3) To evaluate the knowledge, attitudes, and practices (KAP) of the community members regarding CHIK and other mosquito-borne diseases

4) To explore HCWs' knowledge, practices and systemic challenges in diagnosing and managing arboviral (CHIK and DF) and other non-malarial febrile illnesses in this malaria-endemic study area

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Overview of Acute Febrile Illnesses

An acute febrile illness (AFI) is characterized by a sudden onset of fever, which is commonly defined as a body temperature of over 37.5°C and typically accompanied by other symptoms such as headache, body rash, and muscle and joint pains and last for a short period of time, about a week (Tun *et al.,* 2016). In tropical and subtropical regions of the world, fever without specific cause is common symptom reported by patients seeking medical care. In resource-constrained nations, clinicians usually face challenges due to similar clinical features of various potential etiologies (Maze *et al.,* 2018; Prasad *et al.,* 2015).

Various arboviruses, malaria, leptospirosis, parvovirus, enterovirus, group A streptococcus, rubella, measles, adenovirus, post-infectious arthritis, and rheumatologic conditions are among the most common causes of acute febrile illness (Akelew *et al.,* 2022; Crump *et al.,* 2013). It is challenging to differentiate between these different AFI etiologies based just on the clinical history and physical examination because of their non-specific presentations.

Diagnostic assays for a large number of febrile diseases are frequently expensive, complicated, difficult to obtain in areas with limited resources, and may have low sensitivity and specificity (Crump & Kirk, 2015). Furthermore, there is often limited epidemiological information on locally or regionally prevailing endemic and emerging diseases, which can complicate AFI diagnoses (Crump *et al.,* 2013; Maze *et al.,* 2018). Consequently, timely life-saving treatment and effective public health interventions may not be implemented as clinicians' decisions are often guided by syndrome-based guidelines using empirical treatment rather than knowledge of the predominant local and regional etiologic pathogens (Iroh Tam *et al.,* 2016).

In tropical areas of SSA, the predominant cause of undifferentiated fever has historically been assumed to be malaria (Iroh Tam *et al.,* 2016). Since the last 2015, there has been progress towards malaria control, including recommending all malaria-suspected cases be confirmed by parasitological testing before treatment by World Health Organization (WHO) by wide spreading more rapid diagnostic tests (RDTs) use to exclude malaria (WHO, 2015). This has led to a global decrease in malaria incidence, creating challenges for clinicians facing a growing proportion of non-malarial febrile patients and limited tests to guide diagnosis and management (Kapito-Tembo *et al.,* 2020; WHO, 2015). In SSA, the burden of non-malarial fever diseases, including arboviral diseases, is estimated to be as high as 69% in some countries (Kapito-Tembo *et al.,* 2020). However, limited resources, a lack of training to differentiate non-malarial illnesses, and a poor public understanding of seeking medical care contribute to the ineffective diagnosis and treatment of non-malarial fever cases (Hooft *et al.,* 2017). While evaluating patients with fever, especially when malaria tests are negative, HCWs often face challenges, especially undifferentiated febrile illnesses without focal symptoms (Bhargava *et al.,* 2018; Hooft *et al.,* 2017). Consequently, there is empiric over-treatment for other potential causes of AFI with both anti-malarial and antimicrobial drugs, promoting antimicrobial resistance (Laxminarayan *et al.,* 2013).

Vector-borne diseases, including arboviral diseases contribute significantly to AFI (Wu *et al.,* 2016). The interaction of climate, environment and population density with vectors and AFI-causing pathogens is complex and may affect the occurrence and distribution of the etiology of AFI. Changes in climate may affect the burden and distribution of vector-borne diseases and malaria; for example, increasing temperature provides an environment suitable for other arboviruses including DENV, CHIKV transmissions (Mordecai *et al.,* 2020). Malaria remains a primary public health concern in Ethiopia. Over 75% of the country is malaria endemic, with the most frequently identified plasmodium species are *Plasmodium falciparum* while the less frequent species are *P. vivax* (Daba *et al.,* 2023; National Malaria Control Team *et al.,* 2014; Zerfu *et al.,* 2018). In contrast, there is limited information on the etiology, incidence and risk factors of non-malarial AFI-causing diseases including Arboviral diseases in Ethiopia. The causes of non-malarial AFIs are diverse and yet to be fully determined. However, in addition to bacterial bloodstream infection, recent studies highlight the role of viral pathogens, bacterial zoonoses, disseminated tuberculosis, and cryptoccal disease, for which the antimicrobials currently recommended by the WHO for acute febrile illness may not be effective (Maze *et al.,* 2018).

2.2. Arboviral diseases

Arboviruses are a group of viruses that in a transmission cycle and amplification undertake between vertebrate hosts and blood-sucking arthropods, including mosquitoes, biting midges, sandflies and ticks (Arrigo *et al.,* 2016; Vasilakis *et al.,* 2016). Currently, more than 500 arboviruses have been identified, with more than 100 known to cause diseases in humans or animals (WHO, 2020). These viruses are classified into various groups based on their antigenic relationships, morphology and replication mechanisms. Currently, they're categorized into six families: Togaviridea, Flaviviridea, Bunyaviridea, Rhabdoviridea, Orthomyxoviridea, and Reoviridea (Figure 2.1) (Weaver & Reisen, 2010). Three families, namely Togaviridae*,* Flaviviridae and Bunyaviridae comprise viruses transmissible to humans (Vasilakis *et al.,* 2016). The involvement of human in this transmission cycle is incidental even though humans can be reservoir host for some arboviruses (Weaver & Reisen, 2010).

Flavivirus and *Alphavirus* of the families Flaviviridea and Togaviridea, respectively, are of particular clinical significance due to having human epidemic arboviruses such as Yellow fever virus (YFV), DENV, Zika virus (ZIV), and West Nile virus (WNV) (of genus Flavivirus), and CHIKV (genus Alphavirus). In recent years, these viruses have emerged as the most critical infections, with risking approximately 3.6 billion people over 141 countries (Paixão *et al.,* 2018; Shragai *et al.,* 2017), predominantly in tropical and subtropical regions, disproportionately affecting the poor populations living in these areas. There have been increasingly causes of arboviral outbreaks, including DF, CHIK, YF and Zika across numerous countries. The outbreaks have also resulted in fatalities and severely strained healthcare systems. Furthermore, certain arboviruses can cause chronic health issues, lifelong morbidity, disability and occasionally lead to stigmatization (WHO, 2020). The burden and severity of arboviral diseases are influenced by factors, including climate change, habitat destruction, urbanization, and host density. The factors affect survival and reproduction rates of the vectors, the suitability and distribution of their habitats, the intensity and timing of vector activity, and the rate of pathogenic development, survival and reproduction in the vectors. These create potential for arboviruses to cause outbreaks or pandemics (ECDC, 2021).

DENV and CHIKV are the major *Aedes* mosquito species transmitted emerging and re-emerging arboviruses of public health importance, affecting more than half of the global population in tropical and subtropical areas (Gloria-Soria *et al.,* 2016; WHO, 2022d, 2023). The transmission of arboviruses occurs with a biological transmission cycle that involves blood-feeding arthropods as vectors and the amplification of the virus within both arthropods and vertebrate hosts, including humans. Humans are considered incidental and dead-end hosts for majority of arboviruses, do not contribute to the transmission cycle (Alatoom & Payne, 2009; Weaver & Reisen, 2010).

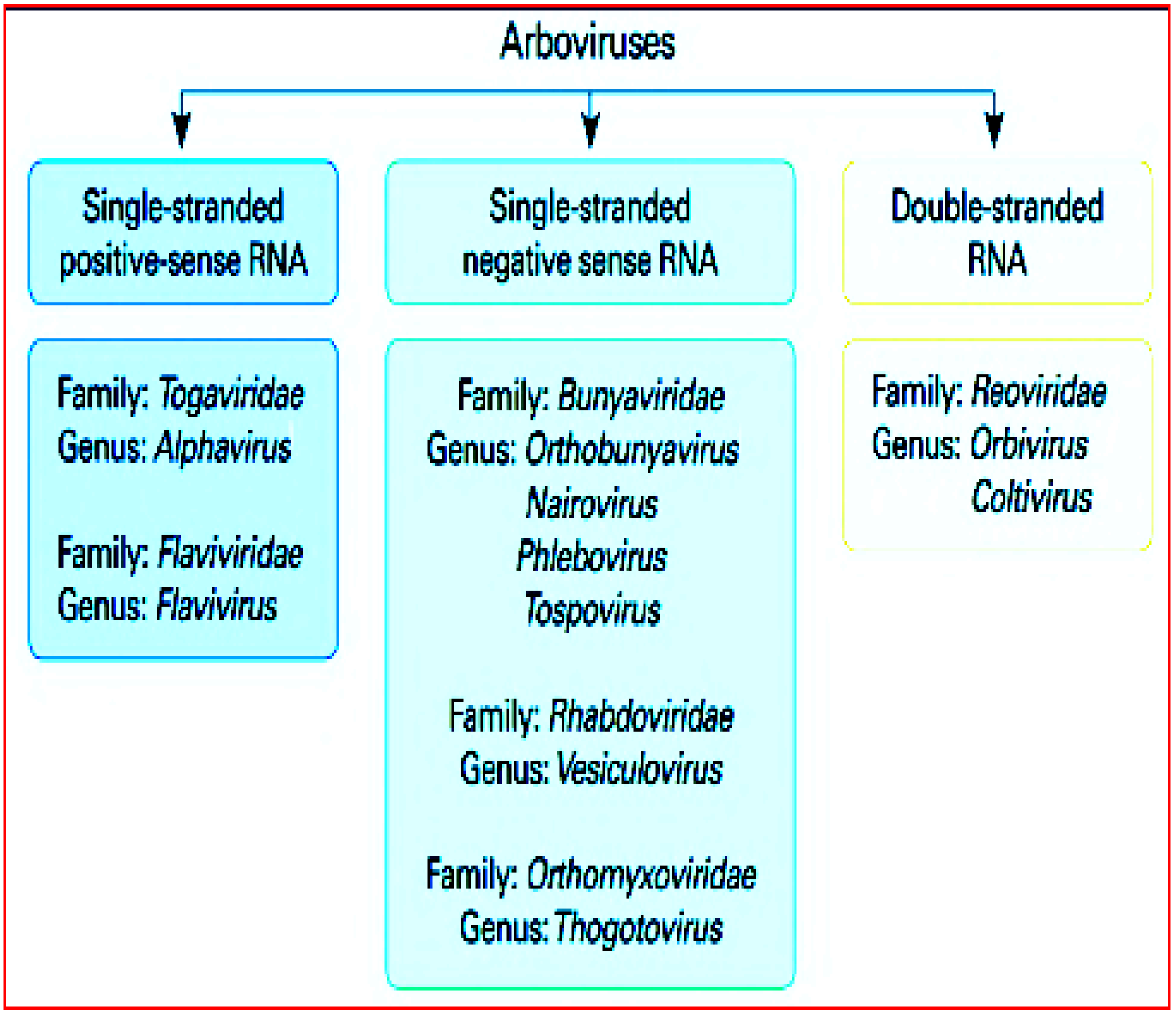

Figure 2.1: Classification of arboviruses (Weaver & Reisen, 2010)

2.3. Dengue virus

2.3.1. Taxonomy and its Relationship with other Flaviviruses

DENV belongs to the genus *Flavivirus* of the family Flaviviridae (WHO, 2023). This family comprises the genus *Flavivirus* that contains viruses like DENV, yellow fever virus (YFV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), West Nile virus (WNV) and Zika virus (ZIKV). In addition, the Flaviviridae contains genus *Hepacivirus* that contains hepatitis C virus (HCV), genus *Pestivirus* and many others, including the newly proposed genus consisting of G Barker Virus (GBV) isolates (Neyts *et al.,* 1999; Simmonds *et al.,* 2017; Wang *et al.,* 2022). Although members of the family Flaviviridae share similarities in morphology, genome organization and replication, each genus differs in antigenic and biological properties. The genus *Flavivirus* has distinct characteristics including most of the viruses in the genus are arthropod (mosquito or tick) borne and share closely related genomic organization and sequence homology, leading to antigenic cross-reactivity among members (Simmonds *et al.,* 2017).

Generally, DENV is characterized as approximately spherical morphology, with a diameter of around 50 nanometers. However, it contains two distinct morphological forms: the mature virion and the intracellular immature virion **(Figure 2.2)**. The mature virion is identified by the presence of two virus-encoded membrane-associated E and M proteins, forming a relatively smooth surface. Conversely, the intracellular immature virion spikes the surface in an asymmetric, containing an E protein and a precursor membrane (prM) protein, which will be cleaved proteolytically into the M protein during maturation. The immature virion also undergoes extensive rearrangements of its intracellular virus-encoded surface proteins upon acidification during maturation within infected cells (Newton *et al.,* 2021; Pierson & Diamond, 2012). It is also important to note that, in some instances, partially mature/immature forms of the virus may be released from infected cells (Pierson & Diamond, 2012).

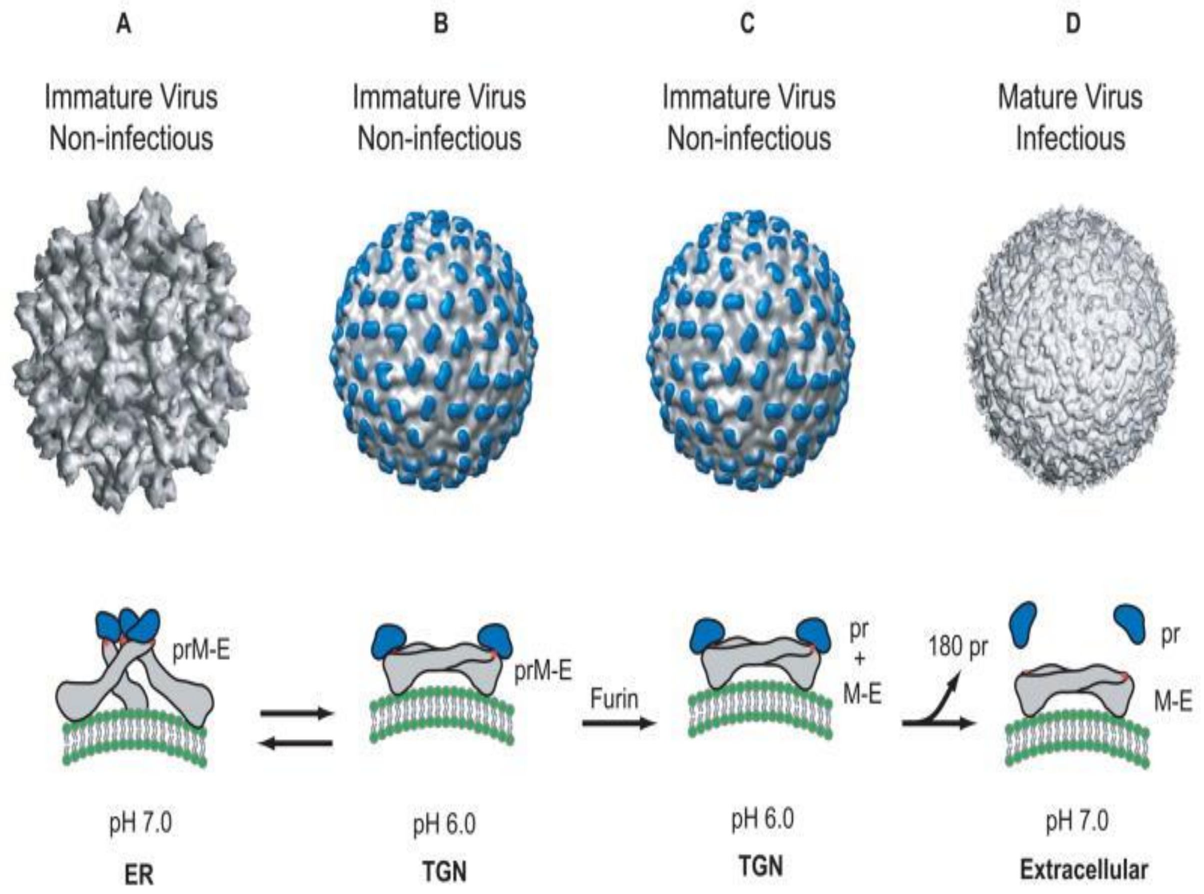

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Figure 2.2: Morphology and Structure of the dengue virion and conformational changes of surface proteins

2.3.2. Global Epidemiology of Dengue Virus

The DF was first identified and named in 1789 by Benjamin Rush, who coined the term "break-bone fever" because of the symptoms of myalgia and arthralgia. DF epidemics were occurred and recognized simultaneously in Asia, Africa, and North America in the 1780s (Dengue Virus Net, 2022). The disease is endemic in many countries of WHO regions, including Africa, the Americas, the Eastern Mediterranean, Asia, Australia and the Western Pacific (Bhatt *et al.,* 2013; Guzman *et al.,* 2010; Kalayanarooj, 2011). However, the Americas, South-East Asia and Western Pacific regions are the most seriously affected, with Asia representing ~70% of the global burden of DF disease (WHO, 2023). The lack of a unified and coordinated effort at the regional levels to initiate population-based epidemiological surveillance with clear operational goals leads to differences in burden reports within regions (Guha-Sapir & Schimmer, 2005).

Since 1940, the risk of contracting the DENV infection has increased by over 30-fold and has widespread dramatically as population movements during World War II spread around the world (Dengue Virus Net, 2022). In the late 1990s, DF was the second most important mosquito-borne disease after malaria, with approximately 40 million DF cases and hundreds of thousands of DHF cases each year (Dengue Virus Net, 2022). Currently, a half of the world populations are at risk of contracting DF disease. Annually, an estimated 390 million DF infections, of which about 100 million manifest clinical features and nearly a thousand cases develop fatal DHF/DSS, predominantly in the tropical and subtropical regions including the Americas, Asia, Australia, and Africa (Bhatt *et al.,* 2013; Ross, 2010; Stanaway *et al.,* 2016). The spreading and distribution of DF cases are attributed to the factors like globalization, population, and urbanization growths, variations in climate and environmental factors, lack of sanitary services, ineffective mosquito control, and increasing DENV surveillances and case reports. The factors may contribute to the increases mosquito populations and susceptibility to circulating serotypes and creating favorable temperature, precipitation, and humidity for the reproduction and feeding patterns of the mosquito populations and for the DENV incubation period (WHO, 2023). Besides, in recent years, the losses of enzootic amplification requirements and adaptation for replication at higher temperatures of the vector have made DENV cause the largest and the most extensive epidemic in tropical urban areas (Weaver & Reisen, 2010). DF transmission cycles are augmented with significant morbidity, mortality, and economic costs, particularly in developing countries (Harapan *et al.,* 2020).

Since 2000, a sharp rise in DF incidence, the spread of cases to new countries, and the urban-to-rural spread risk of about half of the world's population (Brady *et al.,* 2012; WHO, 2023). The DENV's limit of infectivity has now reached 129 countries with good evidence of DF cases and outbreaks, including 36 countries previously classified as dengue-free by WHO and/or the US CDC (Brady *et al.,* 2012). Annually, an estimated 390 million DENV infections could have occurred, from that estimated 67-136 million cases can manifest clinical features and thousands of case develops into severe /deaths most frequently in the population of countries in tropical and subtropical regions of the world. The tendency of increase may be due to globalization, the growth of the population and urbanization, variations in climate and environmental factors, the lack of sanitary services, ineffective mosquito control and increasing DENV surveillances and case reports (WHO, 2023).

Over the past 20 years, the number of DF cases and deaths reported to WHO has increased by more than 8-fold and 4-fold, respectively. The reported cases increased from 505,430 in 2000 to more than 2.4 million in 2010 and to more than 5.2 million cases in 2019, where the reported deaths increased from 960 in 2000 to 4,032 in 2015 (WHO, 2023). Moreover, the cases of DF have shifted from mostly affecting children 40-50 years ago to affect all age groups (Kalayanarooj, 2011). During the 2020- 2021 years, the total number of reported cases and deaths contrarily seemed to decrease. However, the data were not completed yet as well as the COVID-19 pandemic that hampered case reporting in several countries (WHO, 2023). Even though the DF case and deaths reports have been growing over wide geographical locations and in all ages of populations, the current global distribution remains highly uncertain (Brady *et al.,* 2012).

In Africa, the epidemiology of DF is poorly characterized even though the vector mosquitoes present a high burden in the neighboring Middle East and in SSA as well all serotypes of DENV circulate in 19 countries of the continent (Dengue Virus Net, 2022). In the region of Sub-Saharan Africa, DENV infection seems a significant burden for public health, with an estimated burden of about 25% (21-29%) by IgG, 10% (9-11%) by IgM and 14% (12-16%) by viral RNA tests (Eltom *et al.,* 2021). Moreover, many countries of the region, including Burkina Faso in 2016 and 2017, Côte d'Ivoire in 2017, Cape Verde in 2009, and Egypt in 2015 have experienced of DF outbreaks that were reported to Africa CDC (Africa CDC, 2023).

2.3.3. Epidemiology of Dengue Virus in Ethiopia

In Ethiopia, DF is understudied though the country has a sharply increased number of DF outbreak cases, continued transmission, and increased viral infection (Roth *et al.,* 2018). These would be reasons to recommend that Ethiopia have high potential risk factors for DENV transmission, with the following findings further substantiating the recommendation.

First, *Ae. Aegypti*, the vector transmitting DENV, has been extraordinarily identified at indoor and outdoor levels (Woyessa *et al.,* 2014). From immature stages collected from discarded containers and other artificial water containers found around houses and peri-domestic areas, about 50-84% of them were morphologically identified as *Ae. Aegypti* (Ferede *et al.,* 2018; Getachew *et al.,* 2015). Second, DF has been potentially misdiagnosed as malaria because over-diagnosis of malaria in areas of low transmission has been well documented and overestimation of malaria(≈61%) by clinical diagnosis (Woyessa, 2014). DF-like syndromes identified during the yellow fever (YF) outbreak in 1960-62 in southern Ethiopia. Moreover, various arboviruses, including Zika virus (ZIKV), West Nile virus (WNV), CHIKV, Wesselsbron, Talaguine and Sindbis viruses were serologically identified in human populations and wild animals in various areas of the country, like in Gamo Gofa and Wollega (Woyessa, 2014). That means these viruses have serological cross-reactivity and clinical presentation similarity by causing febrile illness with DENV infection, which would be evidence to conceive the circulation of DENV in Ethiopia for a long ago.

Over the past decade, DF has become an emerging public health problem in Ethiopia, with occurrences of outbreaks at various localities of the country in year-round fashions. The first outbreak of DF occurred in Dire Dawa city administration in 2013, registering 12,000 DF-related cases, and 88 of the cases were confirmed by ELISA and RT-PCR, where 50 of the cases were found positive for DENV infection (WHO Africa, 2014;Woyessa *et al.,* 2014). The next year, many outbreaks occurred in the Dire Dawa city administration, in Godey Town of the Somali Region, and the Adar district of the Afar National Regional State (Degife *et al.,* 2019; Gutu *et al.,* 2021). In the Somali National Regional State, during the same months of three series of years, which were in January, February and March of 2014, 2015 and 2016, DF outbreaks with a total of 440 cases occurred (Ahmed & Salah, 2016). In May 2017, a DF outbreak from Kabridahar Town in the Somali National Regional State was reported with a total of 101 cases, including five with severe DF and one death (Gutu *et al.,* 2021).

Similarly, the epidemiological evidence reveals that DF outbreaks were recorded from 2017-2021 in the Somali National Regional State and the Dire Dawa city administration (CRISIS, 2021; 2021a; Sisay *et al.,* 2022; WHO, 2021). Furthermore, from Jan 01 to Feb 04, 2021, Ethiopian health officials reported DF outbreaks of 160 confirmed DF cases in Warder

Woreda of Dolo Zone and 47 suspected DF cases in Dolo Ado Woreda of Liban Zone of the Somali National Regional State which are areas that had an experience of past DF outbreaks in 2017 and 2018 (WHO, 2021). Contributing factors to the outbreaks include the weakened nutritional status of the community due to prolonged drought, population displacement, poor household water handling, living with ill people and lack of formal education (CRISIS, 2021; Gutu *et al.,* 2021). Similarly, a few serological studies reported DENV infections from different localities of the country. From northwest Ethiopia, in Gondar referral hospital, 7.5% current (acute) and 13.0% past DENV infections (Akelew *et al.,* 2022) and in Metema and Humera, 19% current and 21% previous DENV infections were reported from acute febrile patients (Ferede *et al.,* 2018). In southern Ethiopia, in the Borena zone of the Oromia region, 22.9% against anti-DENV IgG and 7.9% against IgM of DENV infection were reported (Geleta, 2019).

From the confirmed DF infections, the responsible serotypes for the outbreaks were DENV1-3 serotypes (Akelew *et al.,* 2022; Sisay *et al.,* 2022; Woyessa *et al.,* 2014). Even though DENV infection would be transmitted all year round in Ethiopia, the risk of contracting it in the country is the most significant during and immediately after the rainy season, which runs from June to August (WHO, 2021). The close contact with DF patients, non-use of bed nets, and the presence of stagnant water around the village were identified as risk factors for contracting DF in Ethiopia (Degife *et al.,* 2019).

2.3.4. Serotypes and lineages of Dengue virus

Four independent but antigen-related serotypes of DENV (1-4) have been identified worldwide (Chen & Vasilakis, 2011; WHO, 2023). The serotypes exhibit a high degree of similarity in terms of genetic diversity, transmission dynamics, and epidemic potential, with the notable exception of DENV-4, which is unique genetically and antigenically from the others (Weaver & Vasilakis, 2009). All serotypes are recognized to share similar geographic distributions and associations with hosts and vectors and are known to cause a broad range of DF diseases, suggesting that, despite their genetic and antigenic distinctions, they may be classified as a single species (Murugesan & Manoharan, 2020). In addition to the four serotypes identified globally, a fifth serotype, DENV-5, was discovered in a Malaysian patient in 2007 through isolation and genetic sequence analysis and was officially recognized in 2013 (Mustafa *et al.,* 2015).

The serotypes of DENV exhibit a variety of subtypes, lineages, or genotypes, which are the result of several alterations in the viral genome (Chen & Vasilakis, 2011; Roy & Bhattacharjee, 2021). DENV-1 was categorized into five (I-V) genotypes based on the E gene sequence. Genotypes I, IV, and V were observed the most prevalent, while genotypes II and III were less active, appeared dormant (Chen & Vasilakis, 2011; Goncalvez *et al.,* 2002). In a recent update, DENV-1 was redefined into three genotypes (I, II, and III) through whole-genome sequencing (Hapuarachchi *et al.,* 2016; Ma *et al.,* 2021). However, the specific roles of different genotypes in triggering outbreaks remain poorly understood (Ma *et al.,* 2021).

DENV-2 consists of six genotypes, including Asian/American, Asian I, Asian II, Cosmopolitan, American, Sylvatic, along with 15 additional subpopulations/lineages. These variations arise from specific codons in envelope genes that confer antigenicity and lineage diversity to American strains of the Asian/American genotypes, as well as from codons in NS genes of DENV-2 that contribute to lineage diversity in the Asian I, Cosmopolitan, and Sylvatic genotypes (Waman *et al.,* 2016). DENV-3 is composed of five genotypes (I-V), with genotype III forming three distinct subpopulations: III-a, III-b, and III-c (Waman *et al.,* 2017; Wittke *et al.,* 2002). Subpopulations III-a and III-c include Asian strains, while III-b and IV include American strains. The III-b subpopulation predominantly consists of American strains, with a smaller presence of South Asian strains. Additionally, many genotype III and V strains include both Asian and American strains (Poltep *et al.,* 2021; Waman *et al.,* 2017).

Similarly, DENV-4 is characterized by five genotypes (I, IIA, IIB, III, and Sylvatic), with genotype I found in the Philippines and genotype IIA being the most common in Southeast Asia and China. Genotype IIB has been isolated in Southeast Asia and the Pacific Islands, and genotype III has only been reported in Thailand (Klungthong *et al.,* 2004; Phadungsombat *et al.,* 2018; Wittke *et al.,* 2002). The variation in genotypes and serotypes is largely attributed to the high mutation rate of the viral genome. This is accelerated by the absence of proofreading activity in the RNA-dependent RNA polymerase, resulting in an estimated one mutation per replication cycle of the DENV genome (Chen & Vasilakis, 2011). It is also noted that each serotype can elicit a lifelong immunity to the infected person only against the specific serotype (Murphy & Whitehead, 2011; Rodenhuis-Zybert *et al.,* 2010). These differentiations of genotypes and serotypes as well as eliciting of incomplete immune responses against different serotypes present considerable challenges in the development of a tetravalent vaccine.

2.3.5. Structural Components of Dengue Virus

The virion comprises three virus-encoded structural proteins and seven non-structural (NS) functional proteins as well as a host cell-derived lipid bilayer envelope membrane (Gupta *et al.,* 2012). The structural proteins include capsid C protein (12kDa), membrane M protein (8kDa), which is derived from the PrM protein (21kDa), and envelope E protein (53 kDa). The NS proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Perera & Kuhn, 2008). The virion particles are structured as follows: a layer of virus-encoded outer proteins, a host cell-derived lipid bilayer envelope membrane, and an inner spherical nucleocapsid core (Kuhn *et al.,* 2002).

On the outer surfaces, there are 180 copies of each E and M/PrM proteins, which are anchored into the lipid bilayer membrane and span across it to form an icosahedral arrangement. In mature DENV and Flavivirus, the 180 copies of the 395-residue E monomer proteins are organized into 90 head-to-tail homodimers on the outer layer. Each E monomer protein consists of three domains that are arranged as central domain I connecting immunoglobμLin-like domain III to a dimerization domain II (Rey *et al.,* 1995), a membrane-proximal stem, and a transmembrane anchor (Klein *et al.,* 2013). The domain III is thought to interact with host cell receptors for entry and contains a key epitope for binding neutralizing antibodies (Roy & Bhattacharjee, 2021). The membrane-proximal stem of each E monomer protein has two predicted amphipathic helices, one hydrophilic, and one hydrophobic, that lies against the viral membrane and span the length of domain II during fusion. At the tip of domain II, a hydrophobic fusion loop is exposed after the pr peptide is removed from PrM, which is crucial for the interaction and fusion with host membranes (Klein *et al.,* 2013).

Another protein comprising the outer surfaces of the virus is M/PrM protein. The prM protein is cleaved at position 91 by furin or furin-like proteases, resulting in the formation of the pr peptide and the M protein. The M protein consists of an N-terminal loop (the first 20 residues), a α-helical domain (MH), and two transmembrane spans (MT1 and MT2). The MH domain, a highly conserved region located between 20 and 38 amino acids downstream from the prM cleavage site, plays a crucial role in regulating prM cleavage during viral particle maturation and host cell entry (Hsieh *et al.,* 2014). This cleavage of the 'pr' peptide from prM ensures that the M protein remains transmembrane - bound within the protein E shell during the maturation process (Perera & Kuhn, 2008). Additionally, the lipid bilayer envelope membrane, which surrounds the central spherical core of the virion, is derived from the host endoplasmic reticulum (ER) membrane during the maturation process (Modis *et al.,* 2004).

During reconstructions using cryoelectron microscopy and the fitting of the known structure of the E glycoprotein, the viral surface envelope reveals an icosahedral scaffold composed of 90 E glycoprotein dimers (Kuhn *et al.,* 2002). These dimers undergo conformational changes in response to varying environmental pH levels, resulting in unique structural features for both the immature and mature forms of DENV (Christian *et al.,* 2013). In the immature form, the PrM and E proteins form 90 heterodimers that extend as 60 trimeric spikes on the particle's surface (Figure **2.2A**). Conversely, in mature particles, the E protein dimers arrange themselves into 90 homodimers, forming flat raft-shaped groups of three E protein dimers that create a 'smooth' herringbone-like pattern on the viral surface (Figure **2.2.D**). As the virus transitions through the trans-Golgi network (TGN), it predominantly experiences conformational changes in the E protein, which are influenced by pH changes that signal a structural shift from the spiky immature to the smooth mature form (Modis *et al.,* 2004). Based on the mass of the E protein and its comparison with other viruses, it has been suggested that DENV may contain three E subunits per icosahedral asymmetric unit (Kuhn *et al.,* 2002). The presence of three E-monomers in each icosahedral asymmetric unit indicates that the DENV virion lacks true T = 3 symmetry. This absence may contribute to its ability to thrive in three chemically distinct environments and may play different roles at various stages of the disease (Rodenhuis-Zybert *et al.,* 2010).

The DENV C protein, consisting of about 100 amino acid residues, plays a crucial role in encapsulation of the viral RNA, forming nucleocapsids to protect. Additionally, it facilitates the interaction with various host proteins, thereby promoting the proliferation of the virus

(Rodenhuis-Zybert *et al.,* 2010). C protein forms homodimers in solution, showing an affinity for both nucleic acids and lipid membranes. The C protein is linked to prM by an internal hydrophobic signal peptide, referred to as the anchor, which extends across the ER membrane and facilitates the translocation of prM into the ER lumen. The mature capsid is released through proteolytic processing of the capsid–anchor junction by the viral NS3 protease, which necessitates the viral NS2B cofactor (NS2B-3). In the ER lumen, the host signal peptidase cleaves the anchor-prM–prM junction (Byk *et al.,* 2016).

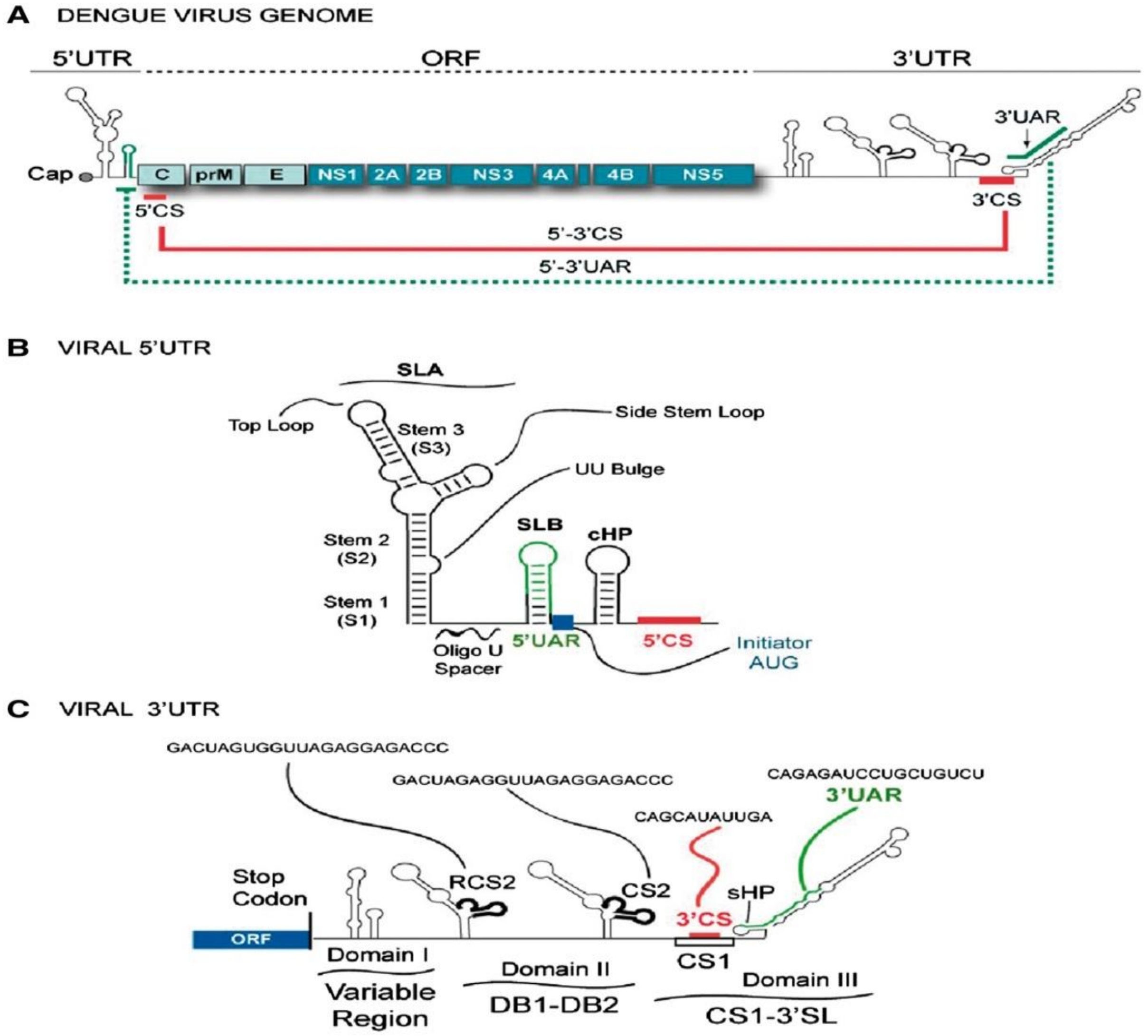
The functional proteins that are NS proteins are a sequence motif characteristic, including viral serine protease, RNA helicase and RNA-dependent RNA polymerase. They involve in controlling, coordinating and regulating various intracellular processes of viral life cycles (Ramakrishnaiah *et al.,* 2013; Simmonds *et al.,* 2017).

2.3.6. Genome of Dengue Virus

The DENV comprises about 11 kb long single-stranded positive-sense RNA molecule genome. The genome has a single open reading frame (ORF) flanked by 5′- and 3′-terminal untranslated regions (UTRs) (Figure **2.3A**). ORF encoded for a single of about 3390 residues of large polyproteins (Murugesan & Manoharan, 2020). The 5' and 3' UTRs are non-coding regions used for RNA genome maintenance. The UTRs containing conserved sequences are cis-acting elements that regulate the processes of genome amplification, translation, and packaging by altering stability, localization, and translational efficiency (Gebhard *et al.,* 2011; Guzman *et al.,* 2010; Roy & Bhattacharjee, 2021; Weaver & Vasilakis, 2009).

The 5′ UTR is an upstream region of ORF which is a short and highly structured sequences region of between 95 -101 nucleotides of the four serotypes. The region has two structural domains of distinct functions during RNA synthesis. The domains are separated by a short oligo(U) sequence that functions as a spacer to enhance viral RNA synthesis. The first domain is about 70 nucleotides sequence, predicted to fold to large stem-loop A (SLA) that is proposed to act as a promoter for the viral RNA-dependent RNA polymerase (NS5). SLA shows portions of three helical structure regions (S1, S2, and S3), a side stem-loop (SSL), and a top loop (TL) which are essential structures recognized by viral RNA-dependent RNA polymerase (NS5) during viral RNA synthesis. The S1 and S2 regions represent one of the most conserved elements, but the sequence and structure of the S3 and side stem-loop show the most variation in *flaviviruses*. The second domain of 5′ UTR is a 16-nucleotide-long sequence, which is predicted to form a short stem-loop B (SLB). The SLB is identified as a 5′ upstream AUG region (5′UAR), which is a complementary region to the counterpart 3′UAR present at the 3′ ends of the genome. The downstream of 5' UTR is positioned by ~100 nucleotides long coding sequence for C that contains a highly conserved in all four DENV serotypes 5′ complementary sequences (5′CS) to 3′CS, a stable capsid region hairpin (cHP) and RNA element that modulates DENV replication in mosquito and mammalian cells. The 5′ CS, which is 11 nucleotides long (134-UCAAUAUGCUG), mediates long-range RNA-RNA interactions between the ends of the RNA for the genome cyclization. The cyclization of the viral RNA occurs when the 5′UAR and 5′CS hybridize with the counterparts in the 3′ UTR. It is a process required for transferring the viral polymerase from the 5′ SLA to the 3′ ends to initiate genome replication (Figure **2.3B**) (Alvarez *et al.,* 2005, 2008; Gebhard *et al.,* 2011; Gritsun & Gould, 2007; Lodeiro *et al.,* 2009; Murugesan & Manoharan, 2020; Polacek *et al.,* 2009).

The 3′UTR is a relatively long nucleotide sequence region that comprises about 470, 450, 430 and 385 (shortest) nucleotides sequence for DENV-1, -2, -3 and -4, respectively. The 3`UTR lacks a poly (A) tail (polyadenylation), which is a crucial tail for stimulation and stabilization of cellular mRNAs translational initiation but ends with a conserved 3' stem-loop (3'SL). The 3′ UTRs of DENV and ZIKV have three major domains. Domain I: is a stem-loop (SL) domain that immediately follows the stop codon of NS5 and a highly variable region inside the 3′ UTR. This SL has a significant sequence and length variation that may vary from less than 50 nucleotides to greater than 120 nucleotides between the serotypes. Domain II: is a dumbbell (DB) characteristic shape and duplicate in tandem. This domain has conserved CS2 and its repeated CS2 (RCS2) sequences. Domain III: The most conserved region of the 3'UTR, containing a CS1 element, which is followed by a terminal 3'SL structure. CS1 is a structure involved in long-range RNA-RNA interaction between the end of the viral genome during cyclization (Figure **2.3C**) (Gebhard *et al.,* 2011; Murugesan & Manoharan, 2020; Polacek *et al.,* 2009).



© 2011 Viruses, 3, 1739-1756; doi: 10.3390/v3091739

Figure 2.3. Schematic representation of the DENV genome: *(A) ORFs showing structural proteins (C-prM-E) and NS proteins (NS1-NS2AB-NS3-NS4AB-NS5) and 5' and 3' UTRs. Positions of complementary sequences are indicated by solid lines for 5'-3'CS and dashed lines for 5'-3'UAR. (B) Predicted secondary structure of the 5' UTR of the genome. Structural elements of the 5' terminal region include stem loop A (SLA), stem loop B (SLB), oligo (U) track spacer, translation initiator AUG, capsid region hairpin (cHP), and 5' CS element. (C) Predicted representation of RNA elements at the 3'UTR of the genome. Predicted secondary structures of three defined domains are shown: domain I (variable region, VR), domain II (dumbbell structure, DB1 and DB2), and domain III (conserved sequences CS1 and 3'SL). In addition, the respective positions and sequences of the conserved elements corresponding to RCS2, CS2, 3'CS and 3'UAR are indicated* (Gebhard *et al.,* 2011).

2.3.7. Transmission of Dengue Virus

Transmissions of DENV infection to humans can be known to undergo two types of transmission cycles, called urban and enzootic cycles (Chen & Vasilakis, 2011). The urban transmission cycle to humans occurs from domestic/peri-domestic habitats by female mosquitoes, mainly *Ae.aegypti* species and, to a lesser extent, *Ae. Albopictus* (Waman *et al.,* 2016; WHO, 2023). Both species of mosquito that transmit DENV and many other human arboviruses share a close overlap with both current and historical distributions. However, *Ae.aegypti* has a wider distributional potential across tropical and subtropical regions, while *Ae.albopictus* has a broader distributional potential across temperate Europe and the United States (Kamal *et al.,* 2018; WHO, 2020). Morphologically, both *Ae. aegypti,* known as the YF mosquito and *Ae.albopictus,* known as the Asian tiger mosquito, are small, black mosquitoes with white stripes on their back and legs. However, *Ae. aegypti* has a silvery, lyre-shaped dorsal pattern on its scutum (Figure 2.4A), while *Ae. albopictus* is smaller, with a single, longitudinal, silvery dorsal stripe (Figure 2.4B) (Lwande *et al.,* 2020). These mosquitoes live close to people and used to lay their eggs in small container, indoors and outdoors. Female *Aedes* mosquitoes bite during the day, and even a few mosquitoes can become a significant nuisance. They become infected when they bite a person with the virus, and infected mosquitoes can then transmit the virus to others through bites. These mosquitoes prefer to bite people both day and night (CDC, 2021). Unlike many *flaviviruse*s, DENV confines to its natural vertebrate host, which includes primates as amplification and reservoir hosts.

The enzootic cycle DENV transmission involves non-human primates in sylvatic habitats using arboreal mosquitoes like *Ae.taylori* and *Ae. Fucifer* (Waman *et al.,* 2016). The urban transmission cycles are an endemic or epidemic cycle that takes place between human reservoir hosts and the mosquitoes where larval maturation occurs around domestic water containers. DENV cannot be spread directly from person to person, however, infected humans are known to carry the infection from one country to another or from one area to another during the stage when the virus circulates and reproduces in the blood system (WHO, 2023).

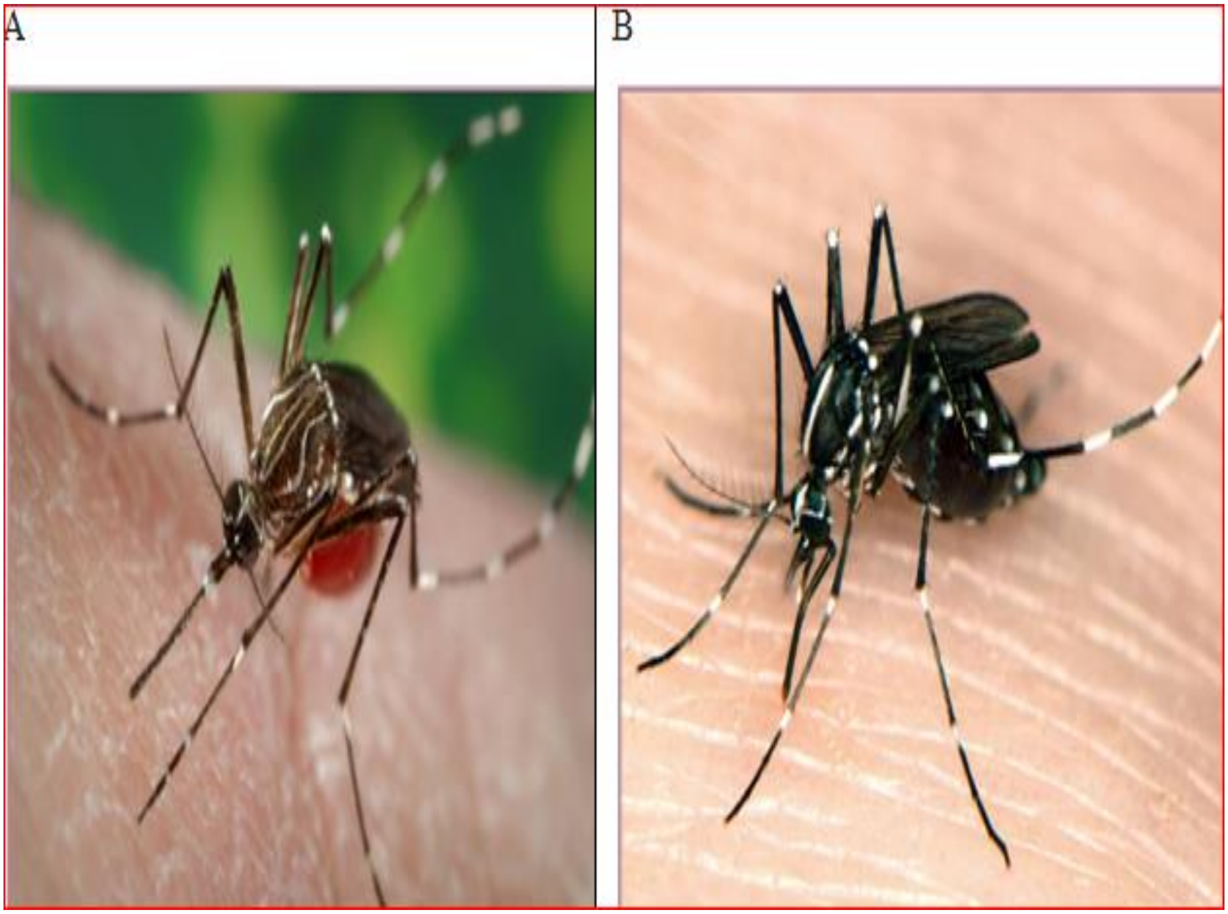


Figure 2.4: Morphological differences of *Aedes aegypti* (A) and *Aedes albopictus* (B) (Lwande *et al.,* 2020; CDC, 2021)

2.3.8. Replication of Dengue Virus

DENV replication involves a series of systematic processes within host immune cells, utilizing cellular machinery. It specifically targets various immune cells to infect, such as dendritic cells (DC), skin Langerhans cells, B cells, T cells, monocytes, macrophages, lymphocytes, and liver cells (Wan *et al.,* 2018; Zitzmann *et al.,* 2020). The subsequent stages of the infection life cycle are detailed below.

2.3.8.1. Host Cell Attachment and Entry

The virus's attachment on the surface of immune cells is the initial phase of infection to initiate host cell entry through a process called endocytosis. During this attachment phase, specific receptors on the immune cell surface, such as macrophage mannose-binding receptor

(MR) and lectin-like receptors like dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) play a crucial role in facilitating DENV infection. The Fc receptor is also involved in a mechanism known as antibody-dependent enhancement (ADE), which is a key process for subsequent infections of different DENV serotypes. During infection, the E protein binds to these receptors, initiating receptor-mediated endocytosis through clathrin-coated vesicles, which are sac-like structures called endosomes (Roy & Bhattacharjee, 2021; Seema & Jain, 2005; Wahala & Silva, 2011). The virus attaches to the cell surface, either by rolling over various surface receptors or moving diffusely as a virus-receptor complex towards pre-existing clathrin-coated cavities during the process (van der Schaar *et al.,* 2008). Alternatively, DENV can also enter target cells through a non-classical endocytic pathway, such as via dynamin (Acosta *et al.,* 2009).

After internalization, the virus particles are transported to Rab5-positive early endosomes, where they mature into Rab7-positive late endosomes. At this stage, membrane fusion exclusively occurs (van der Schaar *et al.,* 2008). The interior pH of the endosome can be lowered by proton pumps, which cause the virus to undergo a change in E protein conformation, resulting in the formation of spike-like structures. It is hypothesized that the acidic pH within the endosomes leads to the dissociation of E homodimers, causing domain II to project outward and expose the hydrophobic fusion loop peptide to the target endosome membrane (Modis *et al.,* 2004). The hydrophobic residues of the fusion loop penetrate the endosome membrane. Domain III also undergoes a shift and folds back toward the fusion loop peptide, adopting a hairpin-like conformation (White & Whittaker, 2016). Subsequently, membrane-proximal stems extend across the length of domain II. Finally, the stems are aligned across the entire length of domain II, facilitating the assembly of the TM anchor and the fusion loop, thereby completing the process of membrane merger and pore formation. This pore structure allows the virus to release its nucleocapsid into the cytoplasm (Figure 2.5) (Cuartas-López *et al.,* 2018; Rodenhuis-Zybert *et al.,* 2010).

2.3.8.2. Uncoating and translation

Prior to the viral translation and replication processes, the nucleocapsid must undergo disintegration, thereby enabling the separation of the C protein from it into the cytoplasm. Its highly basic protein that binds to the viral RNA with a high affinity but low specificity characterizes this C protein. The mechanism by which the C protein is released from the viral genome remains unknown. Nonetheless, it is hypothesized that the non-degradative steps of ubiquitination may play a crucial role in this uncoating processs (Byk *et al.,* 2016). This hypothesis is supported by certain studies indicating that the inhibition of ubiquitination prevents the uncoating of the DENV genome. These studies have demonstrated that the inhibition of the ubiquitin E1 activating enzyme stabilizes the viral genome, thereby maintaining it within endosomes or nucleocapsids during the infection process (Harapan *et al.,* 2020; Nicholls *et al.,* 2020). The uncoated viral genome is subsequently transported to the rough endoplasmic reticulum (ER), although the precise mechanism of translocation remains poorly understood, it is believed that the continuous modification of the cytoskeletal machinery aids in this translocation process (Marianneau *et al.,* 1997).

The translation of the genome RNA into viral proteins occurs at the surface of the ER membrane, subsequently initiating a crucial step in the synthesis and amplification of the genome RNA (Mazeaud *et al.,* 2018). Flavivirus including DENV genome translation initiation is dependent on canonical caps. The genome RNA, similar to cellular messenger RNAs (mRNAs), contains an m7GpppN cap structure at the 5' end but lacks a 3' poly (A) tail (Garcia-Blanco *et al.,* 2016; Polacek *et al.,* 2009). To facilitate the cyclization of genomic mRNA, which stimulates and stabilizes translation initiation, the *Flavivirus* genome lacks a poly (A) tail binding to a poly (A) binding protein (PABP) to interact with the cap-binding eukaryotic translation initiation factor 4 (eIF4F) complexes. However, the eIF4F, a key regulator of the cellular translation initiation complex for cap-dependent translation, recognizes and binds to the m7GpppN cap structure at the 5' end of mRNA, bridging it to the 40S ribosomal subunit to translate into a single polyprotein (Fernández-García *et al.,* 2021).

DENV also has relied alternatively on cap-independent cellular translation initiation to enable viral protein synthesis (Fernández-García *et al.,* 2021). During the inhibition of cap-dependent translation by targeting the cap-binding protein eIF4E, DENV replication and translation remain unaffected (Edgil *et al.,* 2006). However, the specific region in the mRNA that allows for internal initiation of cap-independent translation, known as the internal ribosome entry site (IRES), has not yet been identified in Flaviviruses (Pérard *et al.,* 2013). Nonetheless, a study has suggested that cap-independent translation may be regulated by both the 5' and 3' UTRs (Mazeaud *et al.,* 2018). The ribosomal subunit and associated factors recruit viral mRNA and scan 5' UTR until they find the start codon. The start codon selection may be facilitated by a secondary structure element called cHP, located at 14 nucleotides downstream of the start codon, which halts the 40S ribosomal subunit to ensure the correct start codon is selected (Clyde & Harris, 2006).

During the process of polyprotein translation, the signal- and stop-transfer sequences within the polyprotein guide its translocation across the ER membrane. Subsequently, the polyprotein cleavage by both viral and cellular proteases results in the production of viral proteins. Specifically, the polyprotein is cleaved at specific sites by the viral serine protease (NS2B/NS3) or by a host-derived signalase, furin or furin-like protease, which cleaves the pr/M protein into the M protein (75 amino acids) after assembly during maturation (Simmonds *et al.,* 2017). This process is followed by the arrangement of three structural proteins at the N-terminus of the polyprotein, in the following order: highly basic C protein (100 amino acids), prM protein (166 amino acids), which proteolysis yields the M protein (75 amino acids), and E protein (495 amino acids) (Ramakrishnaiah *et al.,* 2013; Simmonds *et al.,* 2017). In the meantime, *Flaviviruses* manipulate host cell gene expression at the translational level to enhance the production of viral proteins and to establish a replication-friendly cellular environment (Mazeaud *et al.,* 2018).

2.3.8.3. Genome Replication

The Flaviviridae family of viruses share a uniform structural organization and possess a positive-sense, single-stranded RNA genome. This genome serves as an mRNA template for the replication of the virus into a complete, negative-sense single-stranded RNA molecule upon entering the host cell (Simmonds *et al.,* 2017). Positive-sense RNA viruses have a limited number of viral replication proteins, typically ranging from 3 to 10 genes. However, they extensively utilize host proteins, membranes, lipids, and metabolites throughout their life cycle, including replication (Nagy & Pogany, 2012). These viruses induce significant rearrangements of intracellular membranes to create specialized microenvironments, derived from ER, mitochondria, Golgi apparatus, plasma membranes or other organelles, for the replication of viral RNA. These microenvironments are essentially organelle-like structures that facilitate replication by spatially separating the replicating RNA from ribosome and aiding in the assembly of C protein. Furthermore, these structures not only increase the concentration of components necessary for efficient replication and assembly by reducing diffusion space but also shield viral RNA from cellular nucleases and innate immunity-triggering pattern recognition receptors (Chatel-Chaix & Bartenschlager, 2014). DENV-induced microenvironment compartment of the RNA replicates is a vesicle packet (VP), formed through ER invagination. These VPs represent a continuous network within the ER where functional viral replication complexes (VRCs) are assembled, creating isolated compartments to evade detection by viral double-stranded RNA (dsRNA)-activated innate cellular immunity (Diaz & Ahlquist, 2012). Within these VPs, VRCs act as molecular factories, coordinating viral RNA replication by accommodating most of viral NS proteins (NS3, NS4B, NS5), replication intermediate dsRNA, and host factors (Gillespie *et al.,* 2010; Lescar *et al.,* 2018; Mazeaud *et al.,* 2018; Neufeldt *et al.,* 2018).

The DENV RNA molecule functions as a template for replication and actively facilitates the regulation of replication through the provision of specific signals that act as promoters, enhancers and silencers of RNA replication. These regulatory signals are predominantly located within the 5' and 3' UTRs and within the viral coding sequences (Gebhard *et al.,* 2011). The RNA-dependent RNA polymerase (RdRp) of the NS5 protein initiates the synthesis of viral RNA by binding to the SLA (structural element) present in the 5' UTR of the viral RNA molecule (Filomatori *et al.,* 2006). In *flaviviruses*, the presence of complementary sequences 5'-3' CS and cyclization sequence 5'-3' UAR, which span the 5' and 3' base pairs, is crucial for the synthesis of viral RNA. To reach the replication initiation sites located at the 3' ends of the RNA molecules, the RdRp bound to SLA utilizes long-range 5'-3' RNA-RNA interactions facilitated by the interactions between the 5'-3' RNA-RNA complexes mediated by the genome 5'-3' CS and 5'-3' UAR hybrids of the long RNA molecules (Gebhard *et al.,* 2011). The alteration in the hybridization of complementary sequences results in the cyclization of RNA, thereby exposing the 3' end of the viral genome, which serves as a template during the initiation of negative-sense RNA synthesis (Alvarez *et al.,* 2005, 2008; Zhang *et al.,* 2008). The RNA-dependent RNA polymerases, in conjunction with the viral protease/ helicase NS3, other viral non-structural proteins, and presumably host factors, catalyze the enzymatic reaction process to synthesize a negative sense RNA that will subsequently serve as a template for the amplification of positive sense genomic RNA (Ci & Shi, 2021). The synthesis of progeny viral RNA occurs through asymmetric, semi-conservative replication on replicative form (RF) templates or through dsRNA recycling, as the replication process produces 10- to 100-fold more positive-sense progeny RNA than intermediate negative-sense RNA. Consequently, the newly synthesized positive-strand RNA is required to be released from the replication compartment once the optimal RNA synthesis has been achieved (Westaway *et al.,* 2003).

2.3.8.4. Virion assemblage and liberation

Virion assembly involves encapsulation, envelopment and acquisition of a lipid envelope containing glycoproteins by budding across the intracellular membrane. The NS2A protein recruits viral genome RNA, structural proteins and proteases to virion assembly sites and orchestrates nucleocapsid and virus formation. The 3′UTR end of the viral genome that serves as a recruitment signal for packaging is linked to the cytoplasm loop of NS2A, allowing NS2A to recruit nascent RNA from the VRC to the virion assembly site. NS2A also recruits the C-prM-E polyprotein and the NS2B-NS3 protease to the virion assembly site through interactions with prM, E, and NS3, resulting in coordinated C-prM-E cleavage (Xie *et al.,* 2019).

In members of the genus *Flavivirus*, nascent RNA is assembled into virions by encapsulation, after which the envelope buds into the ER lumen. Primarily mature C proteins are encapsulated into the viral RNA to form nucleocapsids, which are subsequently loaded with prM and E proteins and conquer a lipid bilayer envelope containing glycoproteins by budding across the intracellular membrane to form virions. In DENV VPs, the opening into the cytosol allows access for metabolites (such as nucleoside triphosphates (NTPs) to the VRC and for newly replicated viral RNA to exit the VP for translation or assembly of virus particles. The replicated DENV genomes released through the VP pore can be used directly for packaging into virion particles and buds through the ER membrane in near the VP (Chatel-Chaix & Bartenschlager, 2014). The close proximity of the replication and assembly sites to the VP may reveal DENV RNA selectivity for encapsulation by shifting the balance from RNA translation to genome encapsulation. This transient regulation is C protein accumulates to sequester viral RNA for replication DENV life cycle, whereas released viral RNA is preferably used for translation during early time points after infection when low levels of structural proteins are present (Chatel-Chaix & Bartenschlager, 2014). The formed virus particles are transported to cytoplasmic vesicles via the secretory pathway before being released by exocytosis (Figure 2.5). The initial immature virion, containing 60 prM and E heterotrimers in an icosahedral arrangement on the surface virion, migrates through the Golgi network, where the acidic environment triggers cleavage of prM by the cellular furin protease, leading to infectious mature virions production (Li *et al.,* 2008; Yu *et al.,* 2008).

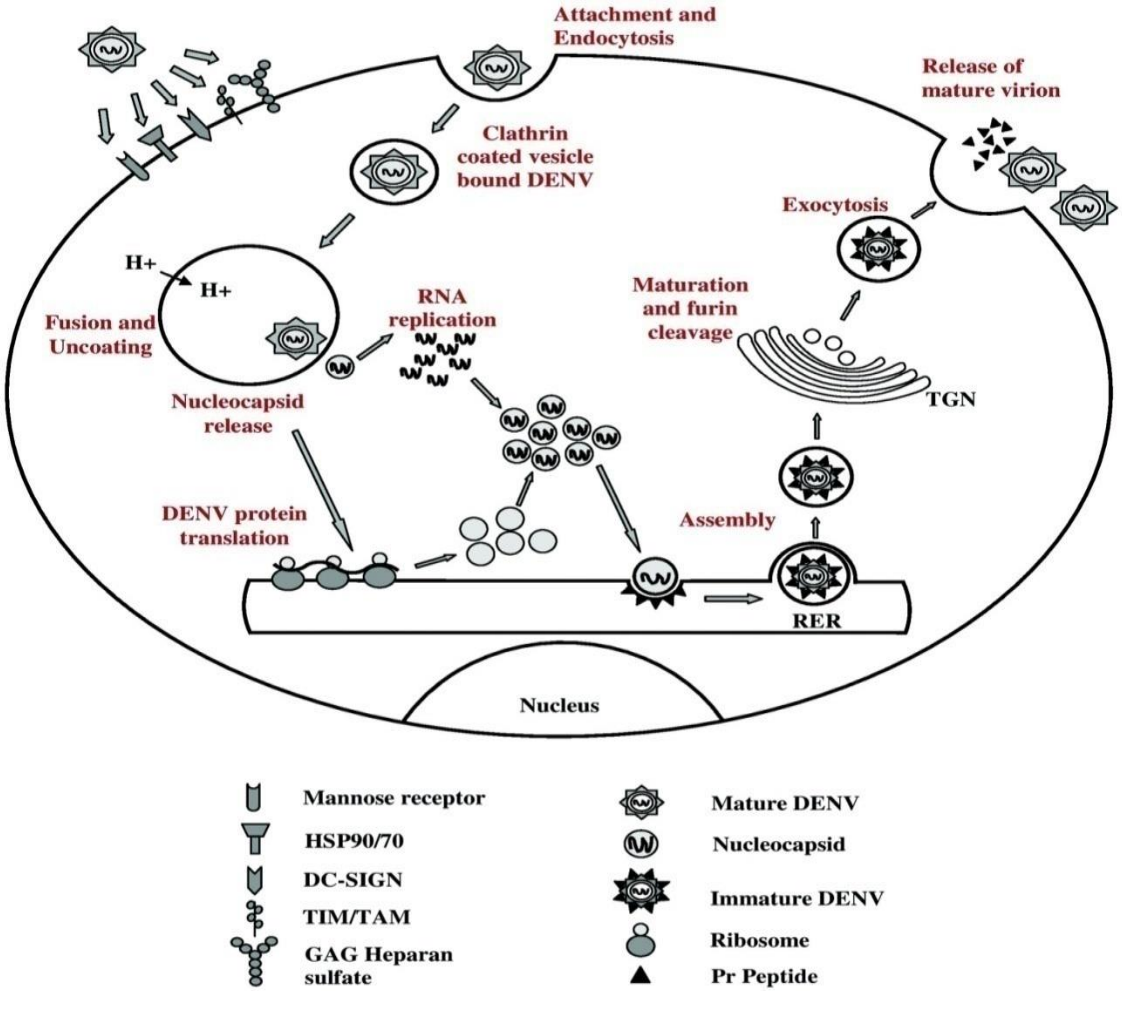


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Figure 2.5: Step-by-step processes of dengue virus entry in the host cell and its life cycle

2.3.9. Pathogenesis of Dengue Virus infection

The pathogenesis of DENV infection is complex and not fully understood. The spectrum of DENV pathogen severity varies across different serotypes, ranging from mild DF to severe DHF and DSS (Begum *et al.,* 2019). The pathogenesis is attributed to a complex interaction between the virus, host genes, and the host's immune responses (Bhatt *et al.,* 2021). The clinical features and severity of DF are influenced by various factors, including being a neonate or young child, female, having a high body mass index, genetic polymorphisms and previous DENV infection. If a patient has been previously infected with DENV-1, for example, the risk of contracting DENV-2 or DENV-3 increases severity, and co-morbidities such as diabetes and asthma can exacerbate the severity of the disease (Guzman *et al.,* 2010; WHO, 2009). In severe cases of DF, DENV can cause abnormal blood clotting, plasma leakage, and increased vascular fragility, leading to DHF. Additionally, the virus can increase capillary permeability, resulting in fluid loss and hypovolemic shock, as well as multiple organ failures in DSS (Pang *et al.,* 2017). Therefore, the pathophysiological features of severe DF may be attributed to plasma leakage and abnormal hemostasis.

The loss of plasma and its complications have been recognized for the past decade, but the specific mechanisms by which the virus expresses these effects remain unclear (Sellahewa, 2013). Conversely, the severity of DENV infection typically peaks after the virus has been cleared by the host's immune system, rather than during the peak of viral load (Green & Rothman, 2006). This finding underscores the critical role of the host's immune response in the pathogenesis of DENV infection.

The tropism of host organs and tissues for DENV is recognized as a significant factor in the pathogenesis of the disease. However, the lack of comprehensive tropism assays and suitable animal models has impeded the understanding of how DENV replicates within tissues and contributes to disease progression (Martina *et al.,* 2009). The presence of DENV (-) sense RNA or NS3/NS5 proteins in specific tissue cells suggests active replication of the virus, whereas the detection of other DENV antigens (E, prM, C, (+)-sense RNA) may indicate that DENV replication has ceased, as these antigens are absent when replication is not occurring. In such cases, cells may non-specifically uptake viral RNA and other antigens from the surrounding environment. The in vitro and through autopsy study following mosquito bites have shown that skin cells become infected with DENV, which then disseminates to the draining lymph nodes. Within these nodes, resident macrophages and various unidentified cells become infected; subsequently deliver the virus to the lymphatic and vascular systems. This process leads to the infection of bone marrow and spleen. Subsequently, Peyer's patches and lymph nodes may become infected directly from the draining lymph nodes or through bone marrow and spleen. Additionally, numerous non-lymphoid organs, including the stomach, thymus, lung, brain, gastrointestinal tract, liver, kidney, and heart, are likely to be infected (Begum *et al.,* 2019).

The immune system cells and the endothelial cell (EC) lining of blood vessels play a crucial role in DENV tropism and severe pathogenesis. The infection of DENV to host cells, including macrophages, hepatocytes, and EC, influences hemostatic and immune responses, representing a significant risk factor for severe illness development. Infected cells undergo apoptosis, with necrosis also contributing by releasing toxic products that activate coagulation and fibrinolytic systems. On the other hand, the hemopoiesis process is suppressed, leading to a decrease in blood clotting factors' levels, which affects thrombogenicity. The extent of infection and the presence of certain cytokines, such as IL6, IL8, IL10 and IL18, can modulate this effect. High viral loads, tropism for EC, and severe thrombocytopenia, resulting in thrombocytopenia, lead to increased capillary fragility, causing DHF. This is characterized by symptoms like petechiae, easy bruising, and gastrointestinal mucosal bleeding (Nachman & Rafii, 2008).

Despite the immune response exacerbating the pathogenesis, DENV infection stimulates the development of specific antibodies and cellular immune responses. Research has shown that IgM antibodies produced against DENV can cross-react with EC, platelets and plasmin, initiating a cycle that amplifies vascular permeability and coagulopathy. Enhanced IgG antibodies, which bind to heterologous viruses during secondary infections of different serotypes, and improved antigen-presenting cells (APCs) infection, contribute to the highest viral load viremia in some patients during secondary infections. The viral load can also overestimate both low and high avidity cross-reactive T cells, particularly in certain haplotypes of HLA. These cross-reactive T cells delay virus clearance, leading to high levels of pro-inflammatory cytokines and other mediators. Elevated levels of soluble factors induce changes in the CE, resulting in coagulopathy and contributing to plasma loss, which is seen in DSS (Figure 2.6).

Primary infections of DENV typically manifest as either asymptomatic or mild febrile illnesses, although they can also cause hemorrhagic fever, especially in infants born to mothers with DENV immunity. Subsequent infections with distinct DENV serotypes may result in severe clinical manifestations, including DHF and DSS (Guzman *et al.,* 2013; Mathew & Rothman, 2008). It is noted that individuals infected with DENV may develop lifelong immunity specific to the infecting serotype; however, this immunity offers only temporary and partial protection against other serotypes (Rodenhuis-Zybert *et al.,* 2010). The severity of DENV infection escalates during subsequent infections of different serotypes, attributed to the presence of weakly neutralizing antibodies from the initial infection that bind to the second serotype, thereby facilitating antibody-dependent enhancement (ADE) of infection by immune cells such as monocytes and macrophages(Wahala & Silva, 2011). The cross-reactivity of antibodies produced during the primary DENV infection can lead to the formation of infectious immune complexes, which then enter Fc-receptor-bearing cells, resulting in a prolonged duration of infection and increased viral replication within these cells (Guzman *et al.,* 2013). The severe manifestations of DF in humans are influenced by a complex interplay between viral serotypes and various host factors, including ADE, memory cross-reactive T cells, anti-DENV NS1 antibodies, and autoimmunity (Bhatt *et al.,* 2021). Therefore, the severity of DF is likely to be influenced by multiple factors, although the specific mechanisms underlying severe disease progression remain under investigation.

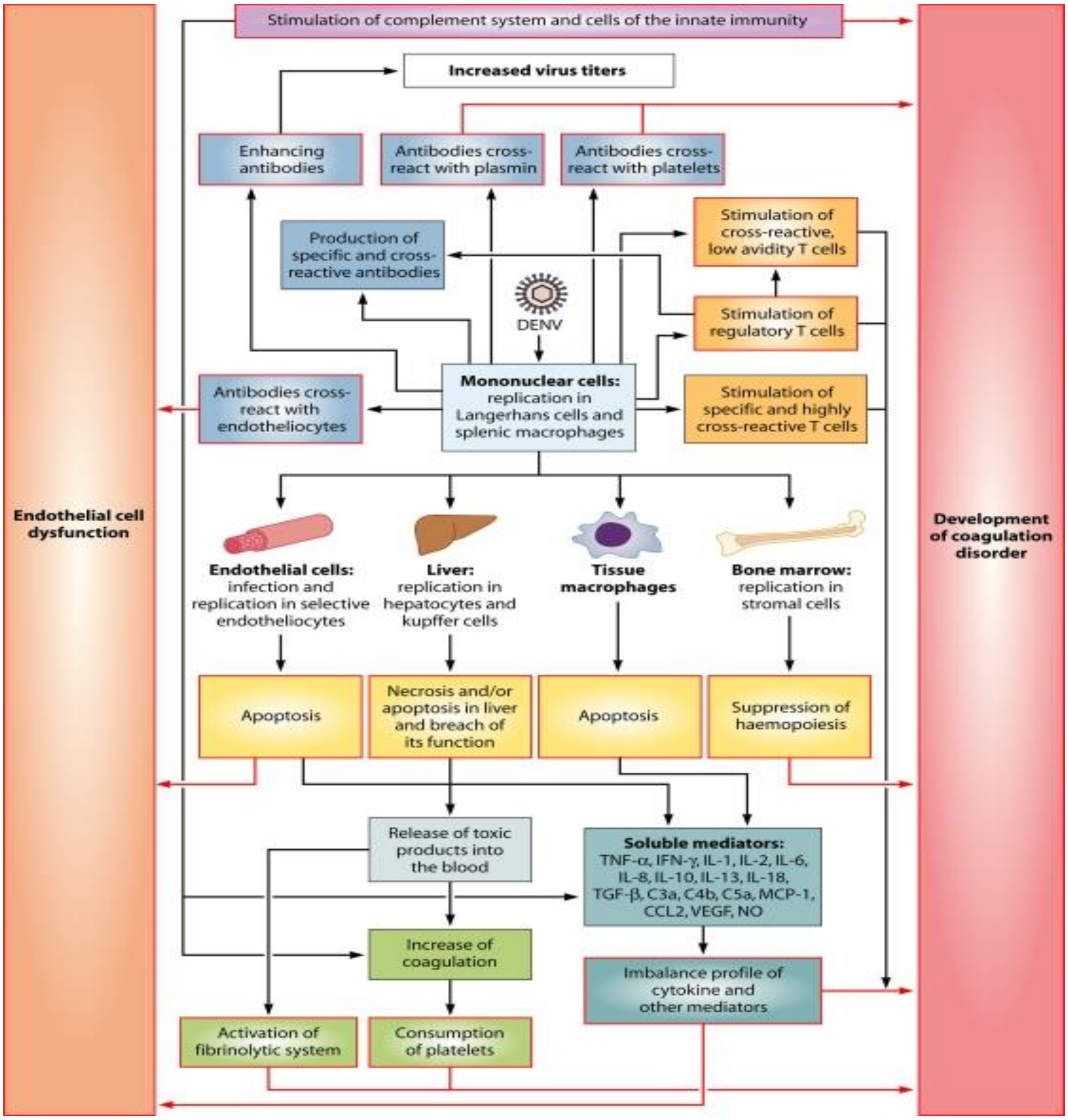

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Figure 2.6: Model for the pathogenesis of DF, DHF and DSS. *Black arrows- processes leading to the indicated event; colored boxes with white centers- pathological events. Each event will ultimately affect the EC or the hemostatic system (purple arrows)*

2.3.10.Clinical features of Dengue Virus Infection

DENV and other *flavivirus* can cause serious diseases ranging from febrile illness to fatal hemorrhagic, neurologic, and gastrointestinal symptoms (Gerold *et al.,* 2017). DENV infections, following an incubation period of 4-10 days after being bitten by an infected mosquito, may have clinical features that last 2-7 days including asymptomatic or may lead to undifferentiated fever, DF or DHF with plasma leakage that may lead to hypovolaemic shock, DSS (WHO, 2009). The clinical features of DF frequently depend on the age of the patient. Infants and young children may have an undifferentiated febrile disease, often with a maculopapular rash. Older children and adults may have mild febrile symptoms or classic debilitating disease with rapid onset of fever, severe headache, retroorbital pain, myalgia, arthralgia and gastrointestinal discomfort, often with a skin rash and sometimes minor bleeding in the form of petechiae, nosebleeds, gastrointestinal bleeding and bleeding gums. And in those with signs of bleeding, usually leukopenia and occasionally thrombocytopenia can be observed in DF (Guilarde *et al.,* 2008; Kittigul *et al.,* 2007; WHO, 1997).

In the 2000s, the DF expert groups agreed: DF is a fundamental disease that has different clinical manifestations and often has unpredictable clinical features and outcomes; DF cases reclassification into severity levels has strong potential for practical utility in clinicians' decisions about where and how intensively a patient should be monitored and treated (WHO, 2009). Consequently, in 2009 WHO categorized DF as non-severe and severe DF based on a set of clinical and/or laboratory parameters (WHO, 2009). Furthermore, the organization had split a large non-severe DF patient group for practical purposes into two categories: DF without warning signs (D-W) and DF with warning signs (D+W). Criteria for probable DF (D-W) would be living in or travelling to DF endemic area and showing fever and two of the following clinical symptoms: nausea (vomiting), skin rash, soreness, positive tourniquet test, leucopenia or any of the warning signs. The D+W patients, which require strict observation and medical intervention, can show all clinical symptoms of D-W, and abdominal pain or tenderness, persistent vomiting, clinical fluid retention, mucosal bleeding, lethargy/restlessness, liver enlargement > 2 cm, and/or laboratory findings with an increase in hematocrit (HCT) associated with a rapid decrease in platelet count. The criteria for DHF are all symptoms of DF and associated with hemorrhagic manifestations (positive tourniquet test or spontaneous bleeding), thrombocytopenia and signs of increased vascular permeability that increase hemoconcentration or fluid effusion in the chest or abdominal cavity (Figure **2.7**). As the DHF severity extends, plasma leakage occurred to leads DSS and fluid accumulation with respiratory distress, severe bleeding based on clinician assessment, or severe organ involvement including liver (AST or ALT ≥ 1000), CNS (impaired consciousness), heart and other organs(WHO, 2009). In Children, DHF commonly presents with a sudden temperature rise accompanied by facial flush and other non-specific constitutional symptoms resembling DF, such as anorexia, vomiting, headache, and muscle or bone and joint pain. DSS is a shock and deterioration occurs suddenly after a fever of 2–7 days or shortly after defervescence (during the return of fever to normal), whereas DSS is a rapid, weak pulse (≤20 mmHg) or hypotension accompanied by cold skin and dizziness in the early stages of shock. Hence, for patients who do not receive prompt and appropriate treatment, a period of profound shock can occur, in which pulse and blood pressure are undetectable, leading to death within 12 to 36 hours after the onset of shock (Figure **2.7**) (WHO, 1997, 2009).

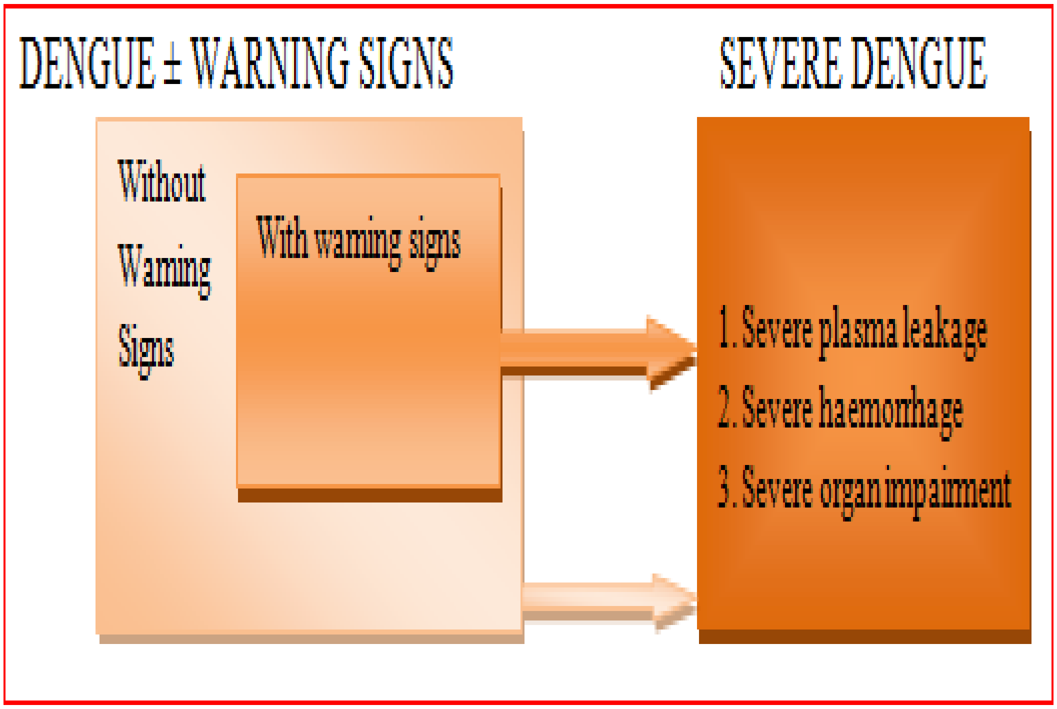

Figure 2.7: Suggested dengue case classification and levels of severity(WHO, 2009).

2.3.11.Diagnosis of Dengue Virus infection

DF should be considered in a patient typically present with acute onset of fever, headache, body aches and sometimes rash spreading from the trunk and who lives in or recently traveled to a disease-endemic area in the 2 weeks before symptom onset (CDC, 2024b). The diagnosis can be performed by detecting the virus, viral nucleic acids, antigens, anti-DENV antibodies, or combinations of the techniques (WHO, 2023). In the early stages of the disease (seven days or less after onset of illness), DENV infection can be diagnosed from serum, plasma, circulating blood cells, or tissues by detecting viral RNA with nucleic acids amplification tests, and NS1 protein using some commercial tests. And also viral isolation in mammalian or mosquito cell culture to further genotyping and lineage for virus characterization (CDC, 2024b). DENV is thermally labile; RNA detection and isolation of the virus are highly dependent on well-preserved specimens for accurate diagnosis results (Iani *et al.,* 2021). The samples awaiting shipment to the laboratory should be stored in a refrigerator or freezer. That is, for storage up to 24 hrs, samples should be stored at 4-8°C and for long-term storage samples should be frozen in a -70°C refrigerator or liquid nitrogen container (WHO, 2009).

For patients with suspected DF disease, serum specimens during acute phases (≤ 7 days after onset of illness) would be collected and diagnosed by detecting the viral RNA sequence by reverse transcription-polymerase chain reaction (RT-PCR) or NS1 protein by enzyme-linked immunosorbent assays (ELISA) or rapid point-of-care tests (WHO, 2022c). The nested RT-PCR protocol was developed using universal dengue primers targeting the C/prM region of the genome for the initial reverse transcription and amplification step, followed by a nested PCR amplification for identification of the infecting serotype-specific qualitatively (Lanciotti *et al.,* 1992). Moreover, the combination of the four serotype-specific oligonucleotide primers in a single reaction tube which utilizes one-step multiplex RT-PCR was an interesting alternative to the nested RT-PCR (Harris *et al.,* 1998). The advancement of RT-PCR into real-time(rRT-PCR) by incorporating dyes and probes (SYBR green and TaqMan) in a single step is capable of providing quantitative data (Nunes *et al.,* 2022). The presence of the virus by rRT-PCR or NS1 antigen in a single diagnostic sample is considered laboratory-confirmed dengue in patients with compatible clinical and travel histories (CDC, 2024b). Patients with more than 4 days of illness onset, DF can be diagnosed by testing serum for IgM antibodies produced against DENV using IgM antibody capture enzyme-linked immunosorbent assay

(MAC-ELISA) whereas for patients presenting within the first week after fever, testing for DENV should include detection of rRT-PCR or NS1 and IgM (CDC, 2024b).

After the acute phase of infection has subsided or after 7 days of fever onset, detection of IgM antibodies is the preferred method of diagnosis using ELISA and hemagglutination inhibition (HI) although NS1 has been reported positive up to 12 days after fever onset (CDC, 2021; WHO, 2009). If the DENV infection occurred in a person who had no previous flavivirus infections or had not been vaccinated against *flavivirus*es like YFV, JEV, and TBE, patients would develop a primary antibody response which slowly increases for a limited time long (WHO, 2022c). The IgM isotype is the primary emerging antibody and detection rate in serum is increased as follows: by days 3-5 after the disease onset, it is detected in 50% of patients, by day 5 detection increased to 80% of patients and by day 10 to 99% of patients (WHO, 2009). During DENV infection occurs in a place where other potentially cross-reactive *flavivirus*es such as ZIKV, WNV, YFV, and JEV are not a risk, a single serum sample IgM test result strongly suggests a recent DENV infection and should be presumed confirmatory for DF (CDC, 2021). The IgM level peaks at about 2 weeks after disease onset and declines to undetectable levels after 2-3 months. Contrarily, IgG started to be detected at low titers in serum, usually at the end of the first week of onset, and slowly increases thereafter and is detected after months and even years (WHO, 2009).

During secondary DENV infection or after vaccination or infection with a non-dengue flavivirus, the IgG isotype antibody titers rise rapidly; the predominant antibody isotype is detected in secondary infection with high levels in an acute phase and lasts 10 months and sometimes lifelong (WHO, 2022c). During the convalescent phase, IgM antibodies can be reliably detected but negative for viral RNA or NS1 test (CDC, 2024b). The IgM levels during early convalescence are significantly lower within secondary than primary infections and may be undetectable depending on the tests used. Hence, IgM detection is a reliable serological diagnostic test target in primary DENV infections (Chanama *et al.,* 2004). IgM/IgG antibody ratios and HI tests are used to distinguish between primary and secondary dengue infections (Falconar *et al.,* 2006). Anti-DENV IgM and IgG antibodies are useful to confirm recent or past infection because where IgM can be formed about 3-5 days after infection and reaches peak 2-4 weeks after the onset of disease, the formation time of IgG level is longer than that of IgM but IgG will stay in the body for many years (WHO, 2023). Thus, when the presence of IgM indicates a recent infection, the presence of IgG indicates a previous DENV infection.

DENV can be isolated from serum, plasma, and peripheral blood mononuclear cells and obtained from tissue autopsies (e.g. liver, lung, lymph nodes, thymus, or bone marrow), although specimens for isolation should be collected early in the infection process and during the viremia stage (usually before the 5th day) (Begum *et al.,* 2019). Cell culture is the most widely used method for isolating DENV, mainly using the mosquito cell lines C6/36 (cloned from *Ae. albopictus*) or AP61 (a cell line from *Ae. pseudoscutellaris*) routinely. However, many mammalian cell cultures such as Vero, LLCMK2, and BHK21 cannot be used very efficiently (Guzman *et al.,* 2017; Walker *et al.,* 2014).

2.3.12.Prevention and control of Dengue Virus Infection

Careful medical detection and monitoring of patients with DF can significantly reduce mortality from severe dengue (Africa CDC, 2023). Currently, there is no specific treatment for DENV infection. Symptoms of muscle pain, fatigue and fever can be relieved and reduced by treatment with acetaminophen or pain relievers such as acetaminophen. Non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and aspirin, are not recommended because these anti-inflammatory drugs have a blood losing effect and blood anticoagulants that can worsen the prognosis of diseases with a risk of hemorrhage. For severe DF, medical care by doctors and nurses familiar with the effects and course of the disease can reduce mortality from more than 20% to less than 1% by maintaining patients' fluid volume, essential for the management of severe DF (WHO, 1997, 2009, 2023). Currently, Dengvaxia® is the only DENV vaccine approved and in use in the United States for children ages 9 to 16 with laboratory-confirmed evidence of previous DENV infection and living in areas where DF is common (CDC, 2021). Control of DF/DHF relies primarily on the use of insect repellents, wearing long sleeves and long trousers, and mosquito repellent inside and outside the home. According to the Global Vector Control Response (GVCR) noted, epidemiological surveillance with case detection and control, and entomological surveillance and control would be pillars to prevent and control DENV infections (WHO, 2017).

2.4. Chikungunya Virus

2.4.1. Taxonomy and its Relationship with Alphavirus

The CHIKV which causes chikungunya (CHIK) disease is a ribonucleic acid (RNA) virus belonging to the genus *Alphavirus* of the family *Togaviridae* (WHO, 2022d). *Alphaviruses* are typically maintained in natural cycles involving transmission by an arthropod vector among susceptible vertebrate hosts (Powers *et al.,* 2001) The genus consists of roughly 30 viruses that likely diverged a few thousand years ago. Some are non-pathogenic, while others cause mild to severe diseases (Powers *et al.,* 2001). *Alphaviruses* are found worldwide and are categorized into two groups: the New World *Alphavirus*, which is predominantly responsible for diseases involving the nervous system to cause encephalitis, and the Old World *Alphavirus* or Semliki forest antigenic group, which has a predominantly rheumatic tropism to cause polyarthritis and a rash (Powers *et al.,* 2001; Weaver & Reisen, 2010). The two groups have evolved different ways of interacting with their respective hosts, in their pathogenicities, tissue and cellular tropism, cytotoxicity, and interference with virus-induced immune responses. It is important to note that most *Alphavirus* infections in humans and domesticated animals are considered ’dead ends,' meaning the virus cannot be transmitted to a new host. As a result, the pressures driving viral diversification may be linked to their true host species (Schwartz & Albert, 2010).

The Semliki Forest antigenic complex includes viruses that are significant human pathogens and are known to cause arthralgia. These viruses can lead to clinical symptoms such as fever, skin rash and polyarthritis (CHIKV is now widely distributed, ONNV is located in Sub-Saharan Africa, Mayaro virus (MAYV) in Latin America, and Ross River virus (RRV) in Australia and the Pacific island countries and territories) or can be associated with encephalitis (Powers *et al.,* 2000; Suhrbier *et al.,* 2012). Although these viruses can be dispersed globally, their geographic range is restricted by the range of their respective arthropod vectors, with Antarctica being the only exception (Krambrich, 2024).

CHIKV is a significant member of the *Alphavirus* family, specifically the arthralgia *Alphaviruses* of the Semliki Forest complex. This virus becomes a major public health concern, affecting millions of people in Africa, Asia, and parts of Europe (Flahault *et al.,* 2007; Volk *et al.,* 2010). CHIKV is transmitted by human-biting mosquitoes in urban or peri-domestic cycles, without involving wild animals as amplification or reservoir hosts (Forrester *et al.,* 2012). The *Aedes* mosquito is the specific transmitter of this virus, and it circulates through the urban epidemic cycle with a high infection rate (Silva & Dermody, 2017). Human beings are the main CHIKV reservoir hosts during epidemic periods, while monkeys, rodents, and birds sustain virus circulation in the environment in the absence of human cases (Galán-Huerta *et al.,* 2015; Inoue *et al.,* 2003) even if an extensive exploration of other zoonotic viral reservoirs has not been conducted. During the outbreak on the island of La Réunion, cases of meningo-encephalitis primarily in neonates and hemorrhagic disease were recorded, indicating important sequellae of acute CHIKV infection (Gérardin *et al.,* 2008; Paquet *et al.,* 2006; Weaver & Reisen, 2010). Unlike typical encephalogenic *alphaviruses*, which infect neurons, CHIKV seems to infect the stromal cells of the central nervous system and the lining of the choroid plexus (Schwartz & Albert, 2010).

The CHIKV is a virus with a spherical shape and an enveloped icosahedra symmetry structure, measuring about 70 nm in diameter. It contains a positive sense single-stranded single molecule of RNA genome, capsid (C) protein, a host-derived lipid bilayer, and repeating units of E1 and E2 transmembrane glycoproteins on its surface(240 heterodimers of E2/E1 arranged as trimeric spikes on its surface) (J. Tang *et al.,* 2011).

2.4.2. Global Epidemiology of Chikungunya Virus

CHIKV was first discovered in the United Republic of Tanzania in 1952 during an outbreak of severe arthritic febrile disease (Staples *et al.,* 2009). The virus was named "chikungunya" after the Makonde or Kimakonde language, translating to "That which things bend upwards," referring to the characteristic bent posture and stiff gait of individuals infected with the virus (Robinson, 1955). The Kimakonde is a language spoken by Makonde ethnic group in southeast Tanzania and northern Mozambique. The virus then spread to Asia and Africa in the second half of the 20th century, causing urban outbreaks primarily in Thailand in 1967 and India in the 1970s (Nimmannitya *et al.,* 1969; Padbidri & Gnaneswar, 1979; Powers *et al.,* 2000; Staples *et al.,* 2009). Following this, no major outbreaks were recorded except periodic minor outbreak occurrences over the next 30 years.

Since the early 2000s, there has been a significant increase in the frequency and spread of CHIK outbreaks (Staples *et al.,* 2009). In 2004, Kenya faced a large CHIK outbreak, with about 5,000 reported cases, which later spread to Comoros (Simon *et al.,* 2006). This increase in outbreaks was supposed to partly due to viral adaptations that made it easier for the disease to be transmitted by *Ae. albopictus* mosquitoes (WHO, 2022d)**.** From 2005 to 2006, the outbreak of this virus infection was badly affect La Réunion Island and other Indian Ocean Islands, with an estimated nearly half of the population infected and 237 deaths reported among a population of approximately 785,000 (Bonn, 2006; Schuffenecker *et al.,* 2006; Simon *et al.,* 2006). Genetic analysis identified that there are a link between the infections in La Réunion and the outbreak in Kenya (Kariuki Njenga *et al.,* 2008). Additionally, the outbreak spread to India, Europe, and the United States, likely due to infected travelers returning from high-risk areas (Schwartz & Albert, 2010).

In the 2000s CHIK outbreaks reemerged in various tropical and subtropical countries; for instance 50,000 cases reemerged in Kinshasa, Congo in 1999-2000 (Pastorino *et al.,* 2004) and outbreaks were documented in 24 locations across Indonesia from 2001 to 2003 (Laras *et al.,* 2005). Furthermore, an estimated 300,000 cases occurred in Indian Ocean islands and La Réunion from 2005-2007 (Schuffenecker *et al.,* 2006), with estimated 1.4-6.5 million cases in 2006-2007 in India (Mavalankar *et al.,* 2007; Saxena *et al.,* 2006). This emergence and reemergence of CHIK have attracted increased attention within the public health sector. In 2008, the National Institute of Allergy and Infectious Diseases (NIAID) in the USA designated CHIK as a category 'C' priority, underscoring its growing public health significance (Staples *et al.,* 2009). As a result, the characteristics of the virus and the disease have been studied. It is estimated that the transmission and attack rates of the virus vary in different regions, with the basic reproduction number (R0, R naught: indicating how infectious disease is contagious) ranging from 2 to 4 and attack rates of 16–55% reported in Asia, La Reunion, and the Americas, alongside seroprevalence rates between 13 and 90% (Rojas, 2018).

Currently, reports of CHIKV infection have been documented in more than 110 countries as an outbreak, under three different lineages (West African genotype, Asian genotype, and Eastern, Central, and South Africa (ECSA) genotype), and has caused significant outbreaks over several months, particularly in urban settings (WHO, 2018, 2022d). The outbreak is characterized by intermittent reemergence, with irregular intervals of 2-20 years between occurrences (An *et al.,* 2017; Powers & Logue, 2007). The difficulties related to diagnosis and reporting have resulted in underestimating the number of individuals affected by CHIKV (WHO, 2022d)**.** In the year 2024, a total of 350,000 CHIKV infection cases and more than 140 deaths were reported worldwide. These cases were reported from 21 countries across the Americas (13), Asia (6), Africa (1), and Europe (1) (ECDC, 2024).

Africa has experienced numerous CHIKV outbreaks, with increased cases since 2010, reaching the highest values ever recorded, including Democratic Republic of Congo (2011, 2019-20), Sudan (2015, 2018), Gabon (2010), Senegal (2010, 2015), Republic of Congo (2011, 2019–2020), Sierra Leone (2012-13) Kenya (2016, 2018, 2022), Ethiopia (2016, 2019, 2022) (Chinedu Eneh *et al.,* 2023; Mengesha Tsegaye *et al.,* 2019). The outbreaks were primarily caused by the *Ae. albopictus* (Asian tiger mosquito), which became the primary vector for the dissemination of CHIKV in certain African countries by 2006 and 2007 (de Lamballerie *et al.,* 2008; Leroy *et al.,* 2009).

2.4.3. Epidemiology of Chikungunya Virus in Ethiopia

In Ethiopia, there have been numerous instances of unidentified febrile illnesses, potentially cases of arboviral diseases including CHIK, even though the cases were not officially confirmed for the arboviral diseases by laboratory tests. It is believed that during the 1960-1962 Yellow Fever epidemics and its aftermath, viruses causing symptoms similar to DF and CHIK were suspected as the causative agents for the febrile illnesses that were accompanied by a rash (Mekonnen & Kroos, 2006). Since September 2013, Eastern Ethiopia has experienced recurring outbreaks of DF, particularly during the rainy season. A survey of vectors conducted in this region also revealed a high abundance of *Ae. aegypti* mosquitoes, which are responsible for transmitting both DENV and CHIKV (Ahmed & Salah, 2016; Degife *et al.,* 2019; Woyessa, 2014).

In 2016, the first cases of CHIKV were reported in Ethiopia, specifically in the Dollo Ado district of the Somali National Regional State (Mengesha Tsegaye *et al.,* 2019). Since then, significant outbreaks have occurred in the eastern regions of the country, including the Dire

Dawa City Administration, and the Afar and Somali National Regional States.These outbreaks have affected a large number of people, resulting in high morbidity rates. From July 29, 2019, to October 20, 2019, a large outbreak occurred, in Dire Dawa Administrative City, with a total of 41162-suspected cases of CHIK reported and 16 laboratory-confirmed cases. The overall attack rate of the outbreak was 12.3%, with an attack rate of 12.32% for females and 6.19% for males, and a higher attack rate in the 15 to 44 age group (50.35%). Major clinical symptoms observed during the outbreak included fever, headache, joint pain, and back pain (EPHI, 2021; Geleta *et al.,* 2020).

Another outbreak occurred in the Somali National Regional State of Ethiopia from May 19, 2019, to June 8, 2019, of which 74 CHIKV cases were identified using real-time polymerase chain reaction (RT-PCR) at the Ethiopian Public Health Institute Laboratory. Not using a bed net during daytime sleeping, the presence of open water-holding containers, the presence of *Aedes* mosquito larvae in water-holding containers in and around households, and not wearing full-body cover clothes were found to be significant risk factors for CHIKV infection (Alayu *et al.,* 2021; Takele, 2020).

The pooled seroprevalence of CHIKV infection in Ethiopia was estimated 12.35%, with the highest prevalence reported in the Southern Nations, Nationalities, and Peoples' Region (SNNPR) at 43.6%, and the lowest seroprevalence in Dire Dawa at approximately 12% (Dagnaw *et al.,* 2024; Endale, Michlmayr, *et al.,* 2020). In a hospital-based study among febrile patients in northwest Ethiopia, where malaria is endemic, 22.5% of patients tested positive for CHIKV-specific IgM, indicating recent CHIKV infection. The study also found that the monsoon and post-monsoon periods increased the rate of anti-CHIKV IgM seropositivity. The most common clinical presentations observed were fever, headache, and joint pain (Ferede *et al.,* 2021). Hence, CHIKV infection is an unrecognized public health problem among patients who had fever, headaches and joint pains in the country. Due to the heterogeneous transmission of CHIKV between and within countries and regions, more seroprevalence studies and molecular confirmations are needed for better understanding of the potential outbreaks and public health impact of the circulating endemic.

2.4.4. Genome of Chikungunya Virus

The CHIKV genome is 11.8 kb in long and encodes nonstructural proteins at the 5′ end and the structural proteins at the 3′ end. The genome has two open reading frames (ORF), flanked by 5' and 3' UTRs and separated by non-coding intergenic regions. The 5'UTR, which is 76 nucleotides in length, contains a 5' type-O N-7-methyl guanosine cap to initiate cap-dependent translation. Contrarily, the 3'UTR varies in length from about 500 to 900 nucleotides and contains a 3′ polyadenylate tail. The ORF1 encodes non-structural proteins (nsP1-4), which form the distinct components of the viral replicase complex responsible for the synthesis of virus RNA. The replication of genomic positive-sense RNA to the full-length negative-sense intermediate occurs in the membrane-bound replication complex on the plasma membrane. As the replication progresses, the proteolytic processing of non-structural protein precursors in the replicase complex facilitates its association with the negative strand and subsequent replication of positive-sense full-length genomic transcripts. Additionally, the subgenomic (26S) ORF2 transcript that encodes structural proteins (capsid, envelope glycoprotein, and 6K viral porin channel) is synthesized from a subgenomic promoter in the minus strand (Figure **2.8**) (Hakim & Aman, 2022; Kallio *et al.,* 2016; Melton *et al.,* 2002).

This nucleocapsid is surrounded by a lipid bilayer derived from the host cell, housing 240 copies of the CHIKV transmembrane glycoproteins E1 and E2. The outer shell of the mature CHIKV particle exhibits an icosahedral shape, with the E1 and E2 glycoproteins arranged in 80 spikes. These proteins play crucial roles in the early stages of infection, with E2 promoting binding to host cells, and E1 mediating membrane fusion through its hydrophobic fusion loop (Kendall *et al.,*2019). Additionally, *alphaviruses* use host cell's membrane for RNA replication, creating membrane invaginations known as spherules. Disruption of essential functions for RNA synthesis prevents the development of these spherule structures in the Semliki Forest virus replication system (Kallio *et al.,* 2016).

The CHIKV comprises three distinct genotypes, categorized based on their respective historical geographic regions: West African genotype, Asian genotype, and Eastern, Central, and South African (ECSA) genotype (Powers *et al.,* 2000). However, between 2005 and 2006 a novel lineage derived from the ECSA, termed the Indian Ocean lineage (IOL) which was identified during the epidemic that started in Kenya 2004. The first CHIKV isolates from La

Réunion epidemic demonstrated an alanine at the E1 envelope protein residue 226. Subsequent isolates exhibited a substitution at this position with valine (i.e. A226V), resulting in an enhanced affinity for the *Ae.albopictus*. This genetic variation has been associated with significant outbreaks occurring in 2005-6 and thereafter across the Indian Ocean Islands and the Indian subcontinent (Abraham *et al.,* 2016; Shragai *et al.,* 2017).

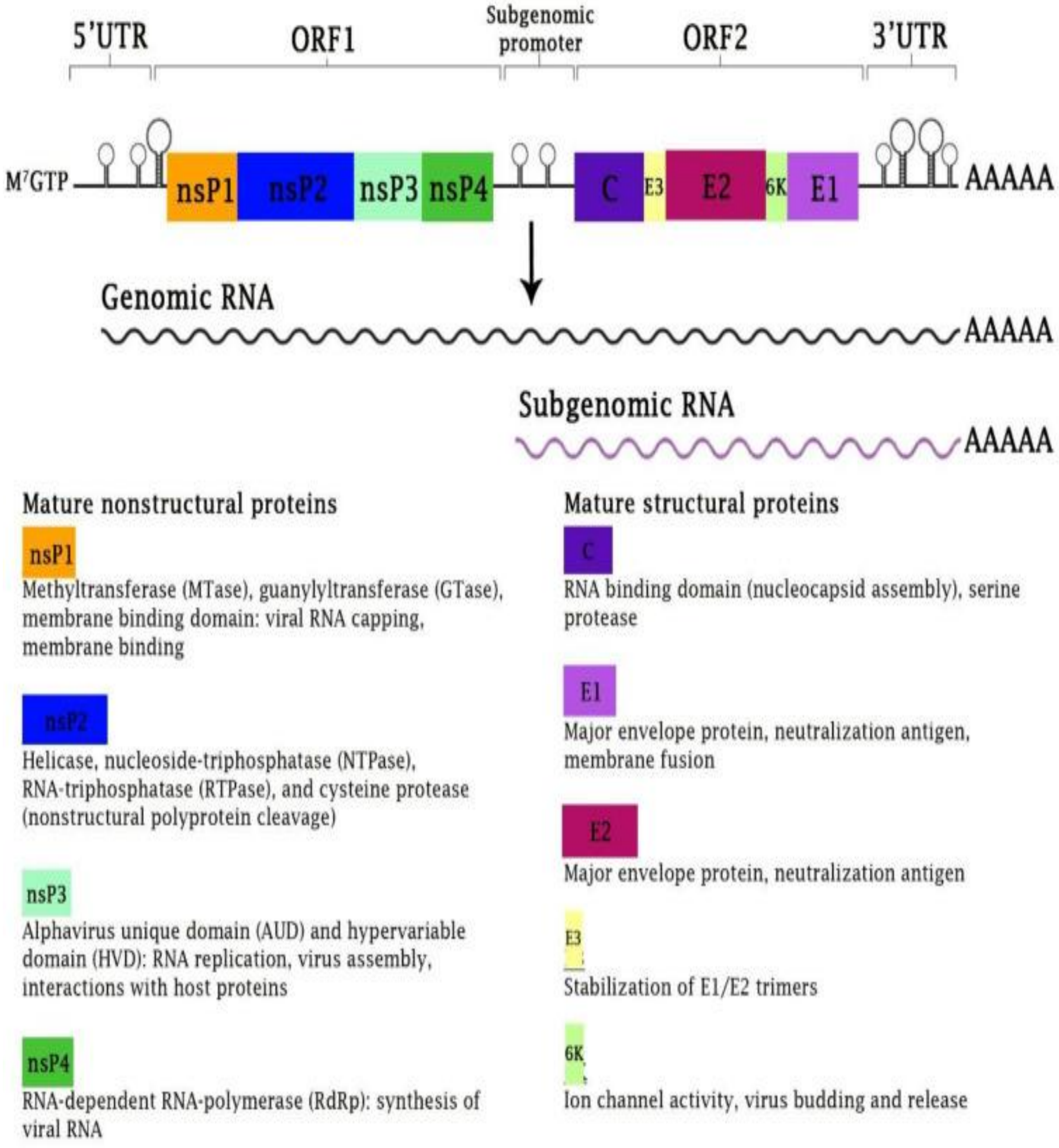

Figure 2.8: The genome organization and viral proteins of Chikungunya virus (Hakim & Aman, 2022)

2.4.5. Replication of Chikungunya Virus

2.4.5.1. Host cell receptors for Chikungunya Virus infection

The CHIKV relies on specific host cell receptors to initiate viral host cell entry and replication. These receptors include matrix-remodeling-associated protein 8 (Mxra8) and Basigin, also known as Extracellular Matrix Metalloproteinase Inducer (EMMPRIN). Both Mxra8 and Basigin play crucial roles in the entry and replication of several *alphaviruses*, including CHIKV, ONNV, RRV and MAYV. Studies have shown that inhibiting CHIKV infection with Mxra8-Fc fusion protein and anti-Mxra8 monoclonal antibodies is effective both in vitro and in vivo. Additionally, the binding of CHIKV to glycosaminoglycans (GAGs), particularly heparin/heparan sulfate (HS), is an important aspect of CHIKV entry and infection. Furthermore, other molecules such as prohibitin (PHB) and T-cell immunoglobµLin, as well as mucin domain 1 (TIM-1), are also involved in interactions with CHIKV (Constant *et al.,* 2021; Hakim & Aman, 2022).

2.4.5.2. Host Cell Entry of Chikungunya Virus

CHIKV enters target cells through a process called clathrin-mediated endocytosis. This process begins with the virus attaching to a receptor on the cell surface and surrounded by the target host cell membrane to form endosome. Membrane fusion then occurs, particularly when the virus encounters Rab5-positive endosomes. The presence of cholesterol in the target membrane speeds up this fusion process, which is facilitated by the acidification of endosomes to transform it into Rab7-positive endosomes. The acidification leads to the penetration and uncoating of the CHIKV virus (Hoornweg *et al.,* 2016). It was shown that methyl β-cyclodextrin, a drug that reduces cholesterol levels, significantly decreases CHIKV infection. Additionally, treatment with lysosomotropic drugs like bafilomycin-A1 and chloroquine, and short hairpin RNAs against dynamin-2, which inhibit endosomal acidification, has produced similar results (Bernard *et al.,* 2010; Sourisseau *et al.,* 2007). Consequently, viral entry occurs through pH-dependent clathrin-coated endocytosis to traverse cell membranes and deliver the CHIKV genome into the cytoplasm (Hakim & Aman, 2022; Sourisseau *et al.,* 2007).

Alternative pathways not involving clathrin have been shown to facilitate CHIKV entry into target cells through the process of macropinocytosis, which provides an additional route for CHIKV to enter human muscle cells (Bernard *et al.,* 2010; Izumida *et al.,* 2020; Lee *et al.,* 2019). Macropinocytosis involves the formation of large, uncoated vesicles called macropinosomes, which play a role in the non-specific uptake of material from outside the cell into the cytoplasm (Hakim & Aman, 2022). These collectively demonstrate that CHIKV utilizes multiple host receptors and entry mechanisms, allowing it to infect various cell types in different tissues.

2.4.5.3. Chikungunya Virus Replication within the Target Cells

Within a late endosome marked by the presence of Rab7, the fusion of the virus's E1 protein with the endosomal membrane is triggered due to the environment becoming acidic. This change in acidity causes the fusion peptide in E1 to insert into the endosomal membrane. Consequently, this fusion of the viral envelope and the endosomal membrane leads to the release of the nucleocapsid into the cytosol (Silva & Dermody, 2017). Subsequently, the viral nucleocapsid breaks down and releases the positive-sense genomic RNA (gRNA) into the cytoplasm. The positive-sense gRNA then functions as messenger RNA (mRNA), leading to the immediate translation of non-structural proteins (nsP), resulting in the production of an inactive precursor, P1234. This precursor is self-cleaved by the C-terminal cysteine protease region of nsP2 to produce nsP4, which acts as RNA-dependent RNA polymerase (RdRp) and forms the viral replicase complex composed of individual non-structural proteins (Rausalu *et al.,* 2016). The nsP4 then synthesizes the negative-strand RNA intermediate as a template for the synthesis of the positive-strand gRNA (Freire *et al.,* 2022). Additionally, the viral replicase complex is responsible for the production of the subgenomic viral RNAs (sgRNA) necessary to direct the synthesis of the structural proteins (Figure **2.9**) (Albulescu *et al.,* 2014).

At the plasma membrane (PM), four nsPs, the viral gRNA, and potentially host proteins come together to form viral replication compartments (spherules) containing viral double-strand RNA intermediate (dsRNA). The internalization of spherules allows the formation of large cytopathic vacuoles (CPV-1) that contain multiple spherules. Spherules at the PM or within

CPV-I are fully functional. Within these spherules, the four nsPs (nsP1-4) function to generate gRNA, antigenomic (negative-strand RNA intermediate), and sgRNA. It is believed that the replication process within these spherules protects the viral dsRNA from intracellular RNA sensors (Silva & Dermody, 2017). Subsequently, the sgRNA is translated into a structural polyprotein precursor, C-p62-6K-E1. With a serine protease domain, the capsid (C) protein can self-cleave from the rest of the structural polyproteins, releasing the C protein into the cytoplasm. Its cleavage occurs independent of the host cell machinery (Sharma *et al.,* 2018; Thomas *et al.,* 2010). Once produced, the C protein combines with the gRNA to form the icosahedral nucleocapsid core (Figure 2.8). Several capsid binding sites within the gRNA of CHIKV have been identified to facilitate selective gRNA packaging, which is crucial step in new viral production (Hakim & Aman, 2022).

The processing of the structural polyprotein and the modifications after translation, including glycosylation, occurs in the ER and Golgi apparatus (Lancaster *et al.,* 2016). Proteases from the host, such as furin protease, will separate the p62-6K-E1 precursor to generate individual structural proteins, including E1, E2, E3, and 6K, contributing to the creation of new viral particles (Yap *et al.,* 2017). The nucleocapsid core then migrates to the regions of the plasma membrane that contain the E1 and E2 proteins. E1 and E2 comes together to form groups of three heterodimers that are embedded in the viral membrane. Mature virions are released through the budding process from the infected cells (Chmielewski *et al.,* 2022). It is hypothesized that this process is affected by temperature, pH, and certain host factors such as cholesterol, actin, and tetherin (Brown *et al.,* 2018).

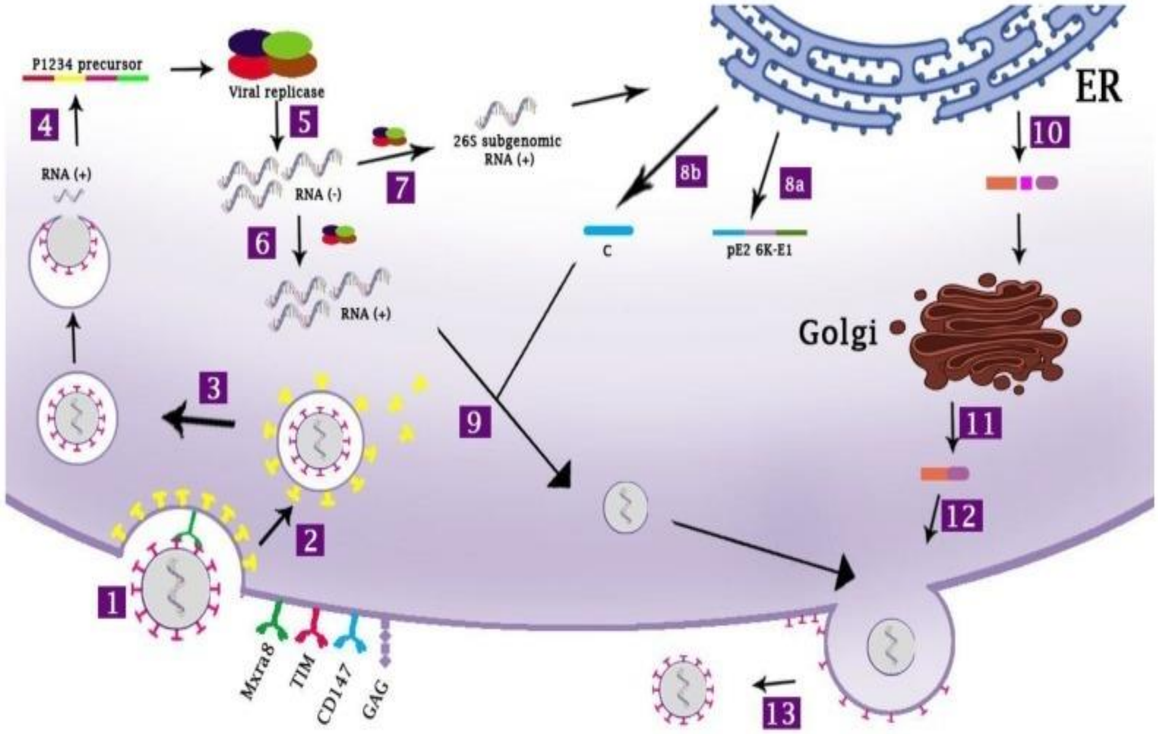

Figure 2.9: CHIKV infection life cycle: 1) *CHIKV utilizes various receptors including Mxra8, CD147, GAGs and TIM for entry into target cells. 2) Virus enters cells through clathrin-mediated endocytosis, with alternative entry pathways unaccounted. 3) Following early endosome formation, clathrin separates from the endocytic vesicle, and acidic pH of the endosome triggers fusion with viral membranes, releasing genomic RNA. 4) The genome is immediately translated into non-structural polyproteins (P1234 precursor) by ribosomes. P1234 polyprotein is then cleaved by nsP2, releasing individual non-structural proteins to form viral replicase complex; 5) This complex mediates the synthesis of negative-strand RNA. The negative strand RNA serves as a template for: 6) new positive-strand RNA synthesis and 7) 26S subgenomic RNA synthesis. The specialized replication compartments termed spherules (not depicted) facilitate the synthesis of negative-strand RNA intermediates, genomic RNA and subgenomic RNA. The subgenomic RNA is then translated into the structural polyprotein precursor C-pE2-6K-E1 in the rough endoplasmic reticulum (RER). 8b) C protein, containing a protease domain, self-cleaves, dissociates from the polyprotein, and 9) associates with genomic RNA to form icosahedral nucleocapsid core in the cytoplasm. 8a) the pE2-6K-E1 precursor moves to the RER lumen 10) for maturation, 11) leading to the formation of E1-E2 heterodimers. 12) The heterodimers are inserted into the cell membrane, forming the "virus budding microdomain".13)The assembled icosahedral nucleocapsid core migrates to this domain, and new viral particles are released extra cellularly through the budding process* (Constant *et al.,* 2021; Hakim & Aman, 2022).

2.4.6. Pathogenesis of Chikungunya Virus Infection

CHIKV infection causes a febrile inflammatory disease in humans, affecting the musculoskeletal system for a week to several months (Morrison *et al.,* 2011). The virus replicates in the skin and may spread through the blood to various organs, such as the liver, muscles, joints, lymphatic tissues, and brain (Figure **2.10**) (Lo Presti *et al.,* 2014). In tissue culture experiments about cellular tropism of CHIKV in humans, it was found that adherent cells such as skin fibroblasts, epithelial cells, and lymphoid tissues were found susceptible to CHIKV infection and replication, while macrophages have been susceptible to a lesser extent (Tang, 2012).

CHIKV is unable to replicate in lymphoid and monocytoid cell lines, including lymphocytes and monocytes, or monocyte-derived dendritic cells (Sourisseau *et al.,* 2007). In lymphoid cell lines, B and T lymphocyte cells are not susceptible to CHIKV in vitro (Solignat *et al.,* 2009; Sourisseau *et al.,* 2007). In contrast, another study conducted on arthralgia alphavirus, particularly on RRV, has shown that the virus persistently infects macrophages and exhibits significantly enhanced resistance to IFN-β-stimulated antiviral activity (Lidbury *et al.,* 2011). CHIKV is capable of replicating in human muscle satellite cells but not in differentiated myotubes (Ozden *et al.,* 2007).

Studies have also indicated that CHIKV infection triggers rapid and robust innate immune responses, which play a crucial role in clearing the virus during the acute phase of infection(B. L. Tang, 2012). Furthermore, in animal models, increased levels of immune mediators and the migration of immune cells accompany the inflammatory response into infected joints and nearby tissues. The innate and adaptive responses of the host are involved in clearing the virus and providing protection, while also potentially contributing to virus-induced immune pathogenesis (Burt *et al.,* 2017). The study of these host immune response mechanisms for CHIKV pathogenesis is crucial for developing treatments and vaccines against CHIKV infection.

Additionally, it has been observed that CHIKV exhibits high cytopathic effects in human cell cultures, leading to rapid apoptotic cell death in infected cells, which likely contributes to the virus's pathological properties (Fields, 2007; Sourisseau *et al.,* 2007). Moreover, a recent study has shown that CHIKV infection induces apoptosis in human microglial cells and results in robust viral replication. The study identified that CHIKV infection induces apoptosis in C20 microglial cells via the mitochondrial pathway, with significant alterations in cell surface marker expression, particularly CD14, which is linked with apoptosis induction (N. Kumar *et al.,* 2024). This finding has implications for understanding the virus's effects on the nervous system. In mouse models lacking the type I IFN receptor, CHIKV primarily targets muscle, joint, and skin fibroblasts, as well as the epithelial and endothelial layers of various organs (Couderc *et al.,* 2008). Newborn and young mice are highly susceptible to CHIKV infection and serve as valuable models for studying CHIKV pathogenesis (Couderc *et al.,* 2008; Ziegler *et al.,* 2008).

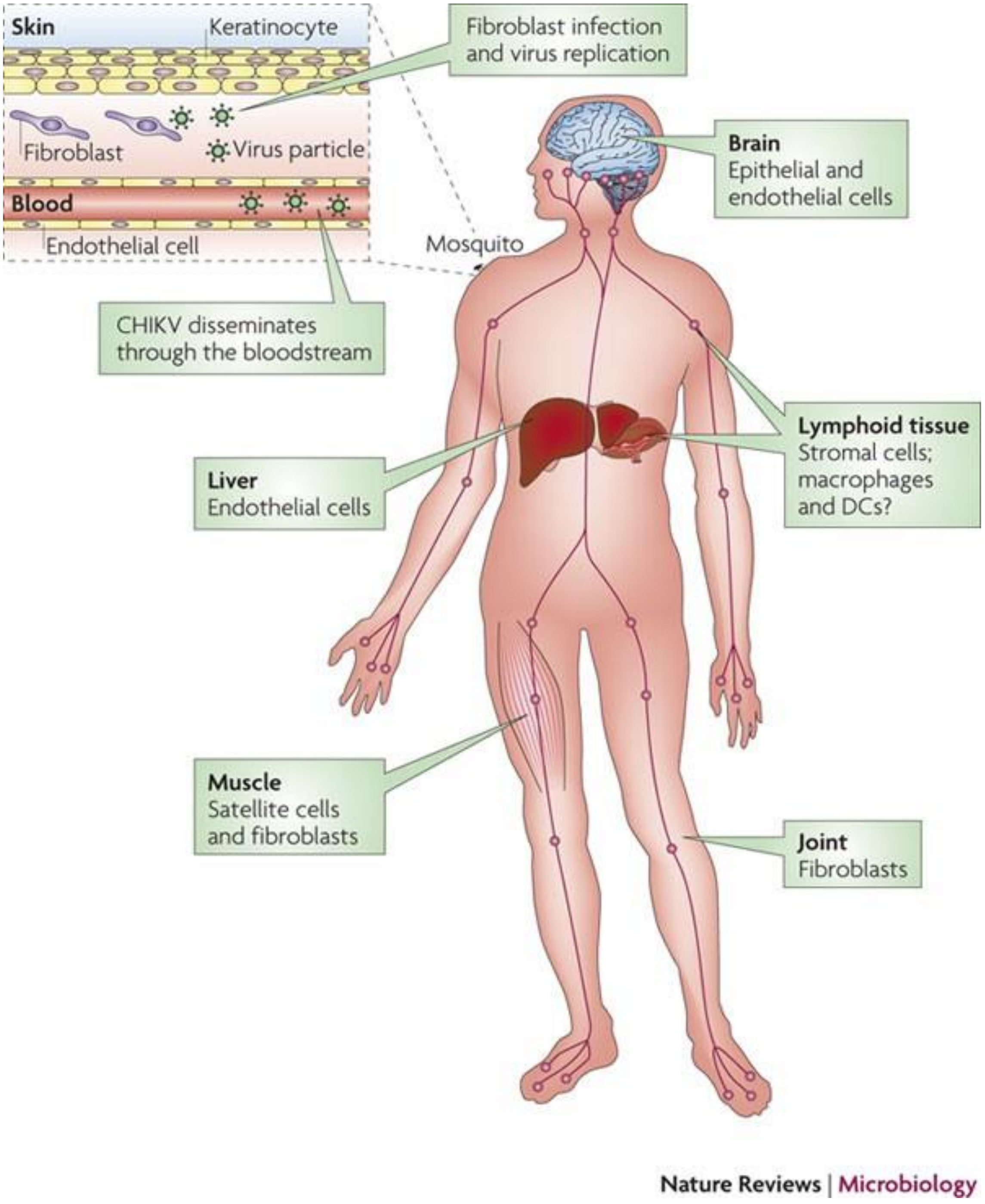

Figure 2.10: Dissemination of chikungunya virus in vertebrates: *The CHIKV is transmitted through a bite from a mosquito, such as Aedes aegypti or Aedes albopictus. After the mosquito bite, CHIKV multiplies in the skin, specifically in fibroblasts, and then spreads to the liver, muscle, joints, lymphoid tissue (including lymph nodes and spleen), and brain. The specific cells targeted in each tissue are identified* (Schwartz & Albert, 2010).

2.4.7. Clinical features of Chikungunya virus infection

After being bitten by infected mosquito with CHIKV, most people will develop symptoms after an incubation period of 2 to 4 days that may be followed by a sudden onset of clinical disease with no prodromal phase (Figure **2.11**) (Schwartz & Albert, 2010). However, not all people infected with the virus will develop symptoms. Serological surveys show that 3% to 28% of people with CHIKV antibodies have asymptomatic infections. CHIKV can cause acute, sub acute and chronic diseases. During ongoing outbreak attacks, the illness is characterized by severe symptoms with unknown reasons (Lemant *et al.,* 2008).

The clinical symptoms of CHIKV infection include non-specific flu-like symptoms and characteristic rashes with joint pain, which may last for a long time after the infection subsides. The most common features of acute illness are sudden high fever (usually over 39°C) and severe joint pain. In addition other signs and symptoms, including headache, diffuse back pain, myalgia, nausea, vomiting, polyarthritis, skin rash, and conjunctivitis may be observed (PAHO, 2011; Silva & Dermody, 2017).The death rate is not particularly high, but excessive death rates have been observed in large CHIKV outbreaks. The deregulation of innate defence mechanisms, such as cytokine inflammatory response, can be involved in the establishment of the most important clinical symptoms and persistent (chronic) disease of CHIKV infection (Caglioti *et al.,* 2013). The inflammatory response coincides with raised levels of immune mediators and infiltration of immune cells into infected joints and surrounding tissues (Figure 2.11). Animal models have provided insights into disease pathology and immune responses. Although host innate and adaptive responses have a role in viral clearance and protection, they can also contribute to virus-induced immune pathology. Understanding the mechanisms of host immune responses is essential for the development of treatments and vaccines

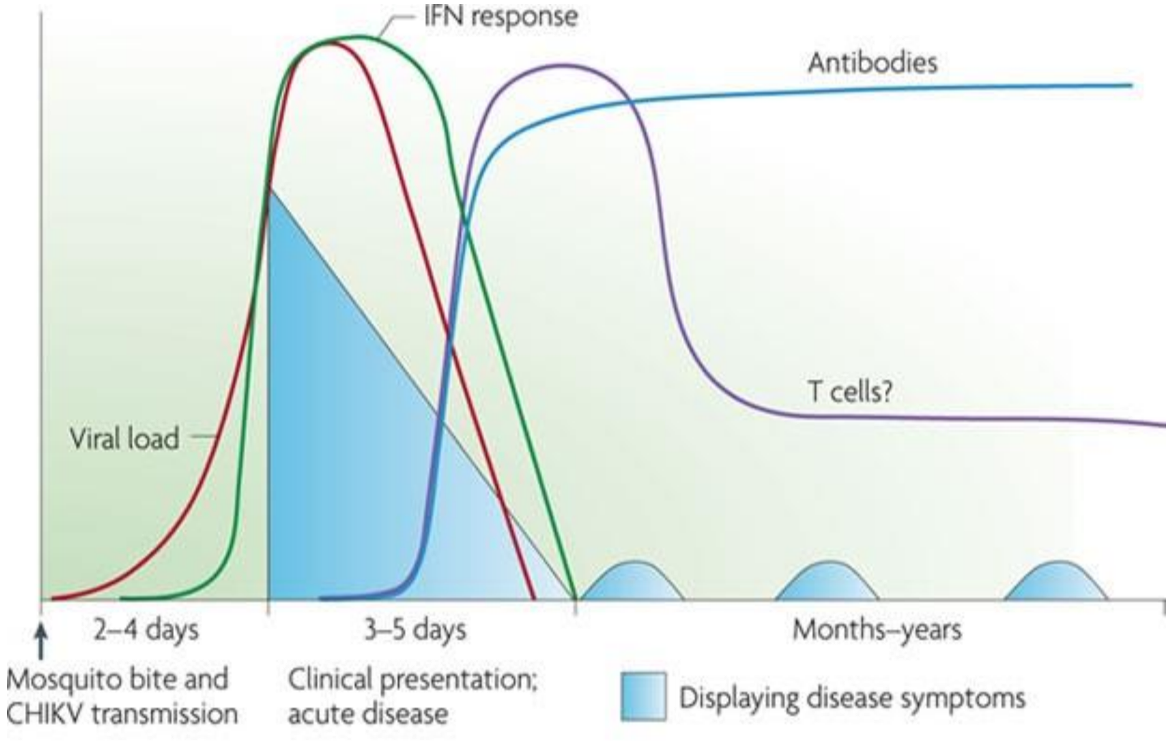

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Figure 2.11: Chikungunya virus infection clinical presentation: *After being bitten by a mosquito carrying the virus, infected individuals typically develop symptoms 2–4 days of incubation period. These symptoms may include high fever, chills, headache, and a spotted or bumpy rash. Most infected people also suffer from severe joint pain that can be very debilitating. As the disease progresses, the virus levels in the body increase, which triggers the body's innate immune response, characterized by the production of type I interferons (IFNs). It takes about 1 week for patients to eliminate the virus successfully, and only then does the body's specific immune response (T cell and antibody-mediated responses) become apparent. It's important to note that around 30% of individuals may experience long-term effects such as joint pain and, in some cases, arthritis* (Schwartz & Albert, 2010).

The acute phase of CHIK lasts 3-10 days. Fever usually lasts from a few days to a week. It can be continuous or intermittent; however, the drop in body temperature has nothing to do with worsening symptoms, fever may be related to relative bradycardia. Joint symptoms are usually symmetrical, most commonly in the hands and feet, but can also affect the more proximal joints. Swelling can also be seen and usually associated with tenosynovitis. Patients are often severely incapacitated by pain, tenderness, swelling and stiffness. Many patients are unable to perform normal tasks or go to work, and many patients are bedridden due to these symptoms. The rash usually occurs two to five days after the onset of fever in about half of patients. Usually a maculopapular rash affects the trunk and limbs, but it may also include the palms, soles of the feet, and face. The rash can also appear as diffuse erythema, which turns white under pressure. In infants, vesiculobullous lesions are usually the most common skin manifestation (PAHO, 2011). Unlike a member of *alphavirus* that causes arthritis, CHIKV have characteristics of cases of meningoencephalitis (especially in neonates) and bleeding disorders recorded during the island of La Réunion outbreak in 2005-6. The meningoencephalitis signs of neurologic involvement, mainly acute confusional state were identified (Borgherini *et al.,* 2007). The genotypes of CHIKV associated with encephalitis and both the Asian and ECSA lineages were responsible for encephalitis but not the West African lineage, and the strains associated with encephalitis have mutations in the nsP1, nsP2, and nsP3 proteins (Hopkins *et al.,* 2022). Unlike the typical encephalogenic *Alphaviruses* that infects neurons, CHIKV seems to infect the stromal cells of the central nervous system, especially the lining of the choroid plexus (Figure **2.10**) (Schwartz & Albert, 2010).

2.4.8. Diagnoses of Chikungunya Virus infection

Diagnosing CHIKV infection involves using laboratory tests such as virus isolation, molecular detection, and serological tests from serum samples collected during the stages of symptom onset. In cases with neurological symptoms, suggestive of meningoencephalitis cerebrospinal fluid (CSF) can be obtained for testing(WHO, 2022d). Virus isolation and quantitative molecular assays, such as RT-PCR, can be used to confirm CHIK cases by detecting viremia in blood within 8 days of symptom onset. Serological assays, such as ELISA or neutralization assays (e.g. PRNT, micro-neutralization, flow cytometry), can be used to identify the immune response, such as IgM or neutralizing antibodies elicited by a candidate CHIKV vaccine to establish correlates of protection or to detect CHIK cases after a few days of symptom onset (WHO, 2018).

2.4.8.1. Viral Culture for Isolation of Chikungunya Virus Infection

The current gold standard for detecting CHIKV is virus isolation and cultivation, which normally takes at least 7 days to report the results. The method is labor-intensive, time-consuming, and costly, making it unsuitable for acute infections or resource-limited areas

(Quick *et al.,* 2017). The procedure necessitates time for viral growth and subsequent identification, as well as specialized equipment and skilled laboratory personnel to carry out the process. Nevertheless, the advantage of cell culture lies in its ability to amplify the virus biologically and isolate strains from human infections. These isolates enable further characterization of viral species and serve as invaluable resources for understanding immune responses and conducting fundamental virology research with contemporary strains (Hudu *et al.,* 2016; Patramool *et al.,* 2013). Virus isolation can be carried out from pooled female *Aedes* mosquitoes, serum samples collected from acute febrile patients within 8 days of illness or CSF (Mulyatno *et al.,* 2012; Schuffenecker *et al.,* 2006; PAHO, 2011). Cold chain transportation (2-8°C or icebox) of the serum to the laboratory within 48 hours is necessary to inoculate in a susceptible cell line or baby mice for isolation.

The isolation of CHIKV and examination of viral replication and pathogenesis have utilized both mosquito and mammalian cell culture systems (Ghosh *et al.,* 2018; Sourisseau *et al.,* 2007). It has been demonstrated that in *Ae. albopictus* C6/36 mosquito cells, CHIKV establishes a persistent non-cytopathic infection, while in mammalian cells such as Vero, BHK-21, 293T and HeLa cell lines, it induces strong cytopathic effects (CPE) within three days of inoculation and apoptosis (Ghosh *et al.,* 2018; Li *et al.,* 2013). C6/36 cells permit 100-fold higher viral titers compared to Vero cells, although they may not be readily available in clinical diagnostic laboratories (Li *et al.,* 2013). Notably, recent CHIKV isolates show significantly increased infectivity in C6/36 cell lines, suggesting that the enhanced infectivity and the recent epidemic may be attributed to the evolution of the CHIKV genome beyond the E1-A226V substitution (Wikan *et al.,* 2012). Isolation can be carried out in T-25 flasks or shell vials, with shell vials being more sensitive and yielding CPE earlier (PAHO, 2011). It is crucial to conduct CHIKV isolation in biosafety level 3 (BSL-3) laboratories to minimize the risk of viral transmission. CHIKV isolation should be confirmed using either immune-fluorescence assay (IFA) with CHIKV-specific antiserum or by RT-PCR of the culture supernatant or mouse brain suspension (Natrajan *et al.,* 2019).

2.4.8.2. Molecular Testing for Chikungunya Virus Infection

Molecular testing is essential for confirming CHIKV infection by detecting its genomic RNA. Various molecular assays for CHIKV detection have been published or are commercially available (Natrajan *et al.,* 2019). Reported molecular diagnostic techniques for CHIKV include conventional RT-PCR, real-time RT-PCR (rRT-PCR), isothermal methods, and multiplex assays (Mishra *et al.,* 2011; Natrajan *et al.,* 2019; Patel *et al.,* 2016; S. Sharma *et al.,* 2010). The RT-qPCR has been widely used recently to detect various viruses, including CHIKV (Álvarez-Díaz *et al.,* 2021). They offer higher sensitivity and specificity, enabling early detection of viruses during the window period of pathogen-specific antibody/antigen detection (Falzone *et al.,* 2021). There is no molecular gold standard to evaluate reported assays, and the decision to implement a particular test depends on the relative advantages and disadvantages of the method as well as the capabilities of a given laboratory. Arboviral Diagnostic Laboratory in CDC USA, has been used RT-PCR assay showing a sensitivity of less than 1pfu (plaque-forming unit) or 50 genome copies (PAHO, 2011). Recent progress has been made with RT-PCR-based portable diagnostic devices, such as the Visby point-of-care device for SARS-CoV-2 testing (Renzoni *et al.,* 2021).

Conventional virus detection methods, such as virus isolation, anti-CHIKV IgM detection, and RT-qPCR, have limitations including being time-consuming, having a narrow detection window period, high laboratory requirements, and a risk of transmission. As a result, there is a critical need for an integrated platform for CHIKV identification that includes sample treatment and specific CHIKV target sequence amplification (Xu *et al.,* 2024). A few studies on integrated RT-qPCR equipment for field detection have been published, but they are not yet suitable for home-based self-testing, as they require separate reverse transcription and PCR steps, manual controls, and an external power supply (Lan *et al.,* 2022).

Recently, various isothermal nucleic acid amplification techniques, such as strand displacement amplification (SDA), recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP) have been proposed and developed (Zhao *et al.,* 2015). These methods have been used for virus detection, in areas with limited resources, due to their simplicity, particularly RPA and LAMP. Unlike LAMP, RPA uses viscous crowding agents to enhance performance by distributing amplicons and activating the amplification system, which contains several enzymes and other protein molecules, requiring an intermittent mixing step after 3–6 minutes of incubation (Lillis *et al.,* 2016). LAMP is capable of amplifying target sequences using 4–6 primers and only DNA polymerase with a strand displacement function at a constant temperature of around 60 °C. It has been widely employed in the diagnosis of various viruses such as ZIKV and DENV (Gaber *et al.,* 2022; Lamb *et al.,* 2020). Moreover, a single-tube one-step RT-LAMP assay also developed for CHIKV even though it may be supposed to produce false negatives due to the presence of amplification inhibitors and necessitates an additional temperature-varying step for characterization (Lopez-Jimena *et al.,* 2018). Other detection methods for LAMP amplicons, such as turbidity measurement, fluorescence dye hybridization, electrochemical luminescence, and melting curve analysis have recently been designed, but require costly equipment for fluorescence detection or additional analysis such as melting curve analysis, which restricts their use in clinical diagnosis, especially in resource-limited settings (Atceken *et al.,* 2023; Shirshikov & Bespyatykh, 2022).

2.4.8.3. Serological Test for Chikungunya Virus Infection

Serological testing is valuable for diagnosing and understanding the immune response to CHIKV infection from serum, plasma, or CSF specimens (Kashyap *et al.,* 2010; WHO, 2022d). During infection, the body produces CHIKV-specific IgM and IgG antibodies against viral antigens. The detection of these specific antibodies during the acute and convalescent phases of infection through serological testing is crucial for efficient clinical diagnosis. However, specific antibody-based assays have lower sensitivity in the first few days of infection, especially during the acute phase (Natrajan *et al.,* 2019). Therefore, it is important to consider the extended duration of antibody detection following acute infection when using serological testing.

An IgM ELISA is the most commonly used serology test for diagnosing CHIKV infection, a few days after symptom onset (Andrew *et al.,* 2022). However, this assay may exhibit cross-reactivity with other *alphaviruses* to some extent. Both commercially available and in-house ELISAs are utilized to detect antibodies against whole viral antigens, provided that performing External Quality Assessment (EQA) studies of these methods could be crucial (Natrajan *et al.,* 2019). Commercial ELISAs developed by various manufacturers have demonstrated acceptable performance; however they may cross-react with other *alphaviruses* like ONNV and MAYV (Litzba *et al.,* 2008; Prat *et al.,* 2014). The results of EQA indicate that commercial Indirect Immunofluorescence Assays (IFAs) showed the best overall sensitivity, while in-house ELISAs were more sensitive than commercial ELISAs (Jacobsen *et al.,* 2016). An IgM-capture ELISA and plaque reduction neutralization testing (PRNT) may provide improved performance for demonstrating IgM antibodies specific to CHIKV in acute and convalescent specimens. IgM capture ELISA is a rapid and versatile diagnostic method that permits the combination of multiple arboviral assays, while PRNTs are highly accurate but necessitate BSL-3 facilities (Chua *et al.,* 2017; Martin *et al.,* 2000). While PRNT is rarely conducted in clinical laboratories, it remains integral for confirming the results of IgM capture ELISAs due to observed cross-reactivity with certain members of the Semliki Forest virus sero-groups, such as ONNV and MAYV (Chua *et al.,* 2017). Neutralization assays are considered the most significant functional serological assays owing to their superior specificity and the critical role of neutralizing antibodies in providing protection. Nonetheless, these assays demand more resources than traditional ELISA assays and may require a BSL3 for the save manipulation of live CHIKV. In the absence of PRNT, other serological tests, such as hemagglutination inhibition (HI), can be used to detect recent *Alphavirus* infections (Martin *et al.,* 2000; PAHO, 2011; WHO, 2018). Furthermore, various rapid diagnostic tests have been developed and are commercially available for anti-CHIKV IgM and IgG. Nevertheless, these tests have limited sensitivity and specificity (Natrajan *et al.,* 2019; Prat *et al.,* 2014).

CHIKV induces activation of B-cells and early production of IgM antibodies during the viremia phase starting from day 2 of CHIKV infection. This leads to the identification of specific anti-CHIKV IgM antibodies in the serum, which can be detected from the beginning of the infection until approximately three months post-infection (Grivard *et al.,* 2007). However, in the first few days, their levels might be too low to be detected by most serological tests (Andrew *et al.,* 2022). Clinical evidence indicates that anti-CHIKV IgM is less commonly detected from day 4 to day 7 of the illness, leading to false-negative results (Banerjee *et al.,* 2021). Nevertheless, IgM tests can reliably identify CHIKV infection when samples are collected more than 6 days after the onset of symptoms (Natrajan *et al.,* 2019). Typically, CHIKV-specific IgM levels peak between 4 and 20 days after the onset of symptoms and may persist for 11 to 14 months (Chua *et al.,* 2017). Therefore, the detection of anti-CHIKV IgM during the acute phase of infection can only provide a preliminary diagnosis, while CHIKV-specific IgG can remain detectable long after the infection and may indicate lifelong immunity. It has been observed that IgG antibodies typically appear after IgM, but IgG antibodies targeting CHIKV E2 epitopes have been identified as early as 6 days after the onset of symptoms (Chua *et al.,* 2016).

Anti-CHIKV IgM antibody primarily targets and binds to epitopes on the CHIKV surface E1-E2 glycoproteins to neutralize the virus (Chua *et al.,* 2017). Various studies have shown that using multiple antigenic E2 peptides, preparing E1 or E2 proteins, or utilizing recombinant monoclonal E2 antibodies results in high specificity compared to whole-virus detection (Fumagalli *et al.,* 2018; Kumar *et al.,* 2012; Natrajan *et al.,* 2019; Verma *et al.,* 2016; Yathi *et al.,* 2011). While both E1 and E2 exhibit high specificities for IgM capture ELISA, E2 has been found to have higher sensitivity compared to E1 (Cho *et al.,* 2008). The employment of such reagents in capture ELISAs may help in mitigating the biohazard risk associated with the use of whole-virus preparations (Cho *et al.,* 2008; Priya *et al.,* 2014). Moreover, these assays are more specific and have the potential to resolve issues related to cross-reactivity among *alphaviruses*.

Several antigen-capture assays have also been developed for the detection of CHIKV, though they are not as commonly used as antigen-based methods for DENV (Natrajan *et al.,* 2019). An assay using monoclonal antibodies that target the E1 protein antigen has been developed to detect CHIKV infection in serum using an immune-chromatographic method (Okabayashi *et al.,* 2015). However, it has been noted that this test has more sensitivity for the ECSA lineage (89%) and less for the Asian lineage (only 33%) (Huits *et al.,* 2018). Furthermore, an E1 antigen capture ELISA showed 96% agreement with real-time RT-PCR results for acute-phase samples in India (Shukla *et al.,* 2009), while the whole CHIKV antigen test in acute-phase samples demonstrated an overall agreement of 94% with RT-PCR (Jain *et al.,* 2018). Antigen-based assays are making a progress and may provide rapid methods for confirming CHIKV diagnosis, leading to extend testing capabilities to laboratories without molecular testing capacity.

2.4.9. Transmission of Chikungunya Virus

The CHIKV is primarily transmitted by mosquitoes, notably by *Ae. aegypti*, as well as by *Ae. albopictus,* particularly in the regions of Africa. Additionally, *Ae.albopictus* and various other *Aedes* species, including *Ae. bromeliae, Ae. furcifer, Ae. taylori, Ae. luteocephalus, Ae. metallicus, Ae. opok, Ae. vittatus,* and *Aedes simpsoni* complex serve as sylvatic bridge vectors for multiple arboviruses to humans (Hanley *et al.,* 2013). Beyond mosquito-borne transmission, vertical transmission may also occur from mother to child during pregnancy, putting newborns at the highest risk of serious infections during the prenatal period (PAHO, 2011).

For efficient transmission of CHIKV and other Arboviruses, various factors, including ecological conditions, mosquito egg-laying sites existence, populations with no prior exposure to the virus, different vectors, and their adaptation to new habitats, viral genetics and mutations contribute for the transmission. Additionally, surveillance practices, CHIKV's introduction time to specific areas, vector population and behavior or adaptation, vector control measures, and lifestyle differences can influence the transmission of CHIKV (Caglioti *et al.,* 2013; Hanley *et al.,* 2013). While CHIKV infects people of all ages and genders equally, the clinical presentation can vary by age. Newborns, the elderly, and individuals with underlying health conditions are identified as having a higher risk for poor disease outcomes(PAHO, 2011).

2.4.10.Prevention and Controls of Chikungunya Virus Infection

Currently, there are no specific treatments or vaccines against CHIKV infection. However, ongoing researches are focused on developing new drugs and vaccines for CHIKV. Clinical case management involves providing analgesic care to alleviate symptoms and expedite recovery, either through non-pharmacologic or pharmacologic interventions. Non-pharmacologic interventions, such as dietary modifications, fluid intake and bed rests are crucial to boost the patient's immune response. Specifically, consuming citrus fruits, using multi-vitamin supplements and stay hydrated play a vital role in optimizing the immune system's response to the virus. Pharmacologic interventions employ drugs like non-steroidal anti-inflammatory drugs (NSAIDs) to manage symptoms like Aspirin for anti-pyretic (body temperature lowering) and analgesic (pain relieving) properties, paracetamol or acetaminophen commonly to manage fever and mild pain and ibuprofen, diclofenac and naproxen to alleviate pain to maintain a favorable risk–benefit balance (CDC, 2024a; WHO, 2022d).

To prevent CHIK cases and outbreaks, it is more essential to focus on controlling the virus infection. Strategies for prevention include administering vaccinations, managing the vector and providing extra care for infected patients. Regarding vaccines, a promising candidate, VLA1553, developed by Valneva SE, has progressed to phase three clinical trials. VLA1553 is a live-attenuated vaccine candidate for active immunization and prevention of disease caused by CHIKV. The strong immune response and the generation of sero-protective titers in almost all vaccinated participants suggest that VLA1553 is an excellent candidate for the prevention of disease caused by the virus (Schneider *et al.,* 2023). The vaccine has elicited a strong immune response in participants and led to the generation of sero-protective titers in almost all vaccinated individuals. Notably, it has received Breakthrough Designation status from the FDA of USA. The vaccine candidate has also demonstrated the ability to induce protective CHIKV-neutralizing antibody titers in 98.5% of participants 28 days after a single shot.

Furthermore, controlling the mosquitoes that spread the virus is crucial. Preventing mosquito bites and controlling the vectors are crucial for avoiding the infections. When residing in or traveling to areas where the disease is endemic, it is important to take personal protective measures, such as wearing long-sleeved clothing, using insect repellent and using mosquito nets to prevent being bitten by *Aedes* mosquitoes that carry CHIKV and other arboviruses like DENV, ZIKV and WNV. Vector control methods are generally categorized as chemical or non-chemical approaches. Both strategies can target immature and adult stages by either killing those using chemical or biological larvicides/adulticides or by eliminating the necessary habitat for these stages, such as draining marshes. To reduce the contact of adult vectors with human hosts, tropical repellents, insecticide-treated bed nets and housing improvements are used. Furthermore, public education plays a critical role in preventing CHIKV transmission (CDC, 2021;ECDC, 2024; Wilson *et al.,* 2020).

There are innovative methods such as male sterile rear and release, synthetic gene drives and the use of *Wolbachia* bacterium to reduce virus transmission by manipulating mosquito reproduction (Bourtzis & Vreysen, 2021; Johnson, 2015; Wedell *et al.,* 2019) (107). While the ultimate goal of male sterile is to introduce genetic changes, induce sterility, or reduce vector competence within the targeted mosquito population(Ritchie & Johnson, 2017), synthetic gene drives aim to alter the vector population so that they are more susceptible to pesticides or have reduced ability to transmit a virus (Wedell *et al.,* 2019). Introducing *Wolbachia* species strain into *Ae. aegypti* mosquitoes demonstrated a significant reduction in the replication and transmission potential of CHIKV or other arboviruses such as DENV, YFV and ZIKV, which all share *Ae. aegypti* as a common vector (Hoffmann *et al.,* 2011; van den Hurk *et al.,* 2012). These methods show promise in controlling virus spread without relying on chemical insecticides but also come with limitations such as high costs, organizational challenges and public opposition, especially for genetically modified mosquitoes.

2.5. Awareness and Practices regarding Arboviral diseases

The prevention and control of emerging febrile illnesses, including arboviral infections, are vital to address public health threats. The intervention strategies must leverage social determinants, including the knowledge, attitudes, and perceptions about these diseases, specifically regarding their modes of transmission, clinical manifestations, diagnostics, and preventive measures (Acharya *et al.,* 2005; Mourad *et al.,* 2022). Despite the increasing prevalence and outbreaks of arboviral diseases in Africa, many communities of the continent have low levels of awareness and information about the viruses to prevent and control the spread (Corrin *et al.,* 2017). In Tanzania, where CHIKV was first isolated, there is insufficient knowledge regarding DF and CHIK among community members and HCWs, only 61.6% and 3.2% of community members have heard about DF and CHIK, respectively, while 96.8% and 87.2% of HCWs have heard about DF and CHIK, respectively (Kajeguka *et al.,* 2017).

HCWs play a crucial role in the early diagnosis and accurate and effective management of febrile illnesses. Despite their critical role, there is a paucity of studies concerning HCWs' knowledge and practices related to febrile illnesses, arboviral infections in Ethiopia, especially within malaria-endemic areas. Nonetheless, there exists a significant gap in the literature concerning their knowledge and practices related to febrile illnesses, particularly arboviral infections in malaria-endemic areas of Ethiopia. Few studies have highlighted insufficient knowledge among the HCWs regarding mosquito-borne viral diseases, notably in regions such as Dire Dawa and Gambella (Asebe *et al.,* 2021; Endale *et al.,* 2020; Legesse *et al.,* 2018; Yusuf & Ibrahim, 2019). It's important to conduct evidence-based behavioral and social interventions to prepare communities for potential public health measures and promote risk reduction designs and implementations (WHO, 2009). However, in Ethiopia, there are a lack of data indicating or evaluating the knowledge or awareness of community members regarding arbovirus infections, despite the presence of emerging cases and vectors. In addition, the persistent challenges of the Ethiopian healthcare infrastructure scarcity complicate the diagnosis and management of non-malarial febrile illnesses. This, compounded by insufficient awareness and training among HCWs, exacerbates the under-diagnosis and mismanagement of arboviral diseases in high-risk areas such as Afar (WHO, 2023).

Identifying these knowledge gaps is crucial for the development of targeted interventions and healthcare policies aimed at enhancing health service delivery. In Ethiopia, a few studies have reported healthcare professionals and community members' knowledge about arboviruses and their vectors (Kebede *et al.,* 2023; Mohan *et al.,* 2021; Yusuf & Ibrahim, 2019). Gaps in knowledge and practice among HCWs may result in the non-targeted administration of medications, such as anti-malarial treatments, for instances of unknown febrile illnesses. Hence, raising awareness and implementing sustainable control measures within the community as well as evaluating the knowledge level of HCWs about certain illnesses is very important (WHO, 2023).

2.6. Trends in Diagnosing and Managing Non-malarial fever diseases

Acute febrile illness is a leading cause for healthcare consultations, particularly in low- and middle-income countries (LMICs), where the successes of malaria control programs have significantly curtailed malaria transmission rates (Liu *et al.,* 2012). As a result, non-malarial fever cases have been observed to increase significantly among patients previously suspected of having malaria in tropical and subtropical countries (Kapito-Tembo *et al.,* 2020; WHO, 2013). In SSA, the burden of non-malarial fever diseases, including arboviral diseases, is estimated to be as high as 69% in some countries (Kapito-Tembo *et al.,* 2020). However, limited resources, a lack of training to differentiate non-malarial illnesses, and a poor public understanding of seeking medical care contribute to the ineffective diagnosis and treatment of non-malarial fever cases (Hooft *et al.,* 2017). While evaluating patients with fever, especially when malaria tests are negative, HCWs often face challenges, especially undifferentiated febrile illnesses without focal symptoms (Bhargava *et al.,* 2018; Hooft *et al.,* 2017). This causes difficulty in identifying the pathogen and getting evidence-based treatment.

A critical challenge in managing non-malaria febrile illnesses lies in the insufficient understanding of their epidemiology and limited availability of diagnostic tools, which is crucial for informing public health strategies, allocating resources and monitoring the effects of interventions in a specific region. Furthermore, the episodic natures of febrile illnesses are often observed over a short time, typically lasting only a few months. This limitation prevents a comprehensive assessment of the disease incidence and its seasonal variations, which are vital for a complete understanding of febrile illness dynamics throughout the year (Iroh Tam *et al.,* 2016).

Furthermore, there is a slow yet growing recognition of the prevalence of viral pathogens in acute febrile illnesses, although documentation remains sparse in many LMICs. Consequently, the lack of robust epidemiological data and accurate diagnostic tools leads to the overuse of anti-malarial drugs, with administration rates reported to be as high as 99% among patients with negative malaria smears (Mwanziva *et al.,* 2008). Additionally, there is a significant incidence of overtreatment with empirical antibacterial agents, with up to 60% of patients receiving antibiotics despite lacking a confirmed diagnosis of malaria (Nadjm *et al.,* 2012).

Africa, including Ethiopia, faces a dual burden of malaria and non-malarial febrile illnesses, where malaria has traditionally been the leading cause. However, studies indicate that non-malarial febrile illnesses now constitute a significant proportion of febrile cases, with recent studies indicating a growing prevalence of arboviral diseases such as CHIK and DF (Kapito-Tembo *et al.,* 2020; WHO, 2013). In Ethiopia, non-malarial febrile cases account for a growing burden of diseases, particularly in the malaria-endemic regions like Afar (Mehari *et al.,* 2021; Zerfu *et al.,* 2023). The co-existence of malaria and non-malaria febrile illnesses including CHIK and DF complicates the diagnosis and management of these illnesses due to their overlapping clinical symptoms (Adam & Jassoy, 2021). While this approach has reduced malaria misdiagnosis, it has led to a growing recognition of non-malarial febrile illnesses, constituting a significant proportion of febrile cases in tropical and subtropical countries (Kapito-Tembo *et al.,* 2020; WHO, 2013). In SSA, including Ethiopia, studies estimated that up to 69% of febrile illnesses are non-malarial diseases (Kapito-Tembo *et al.,* 2020). Arboviral infections, including CHIKV and DENV, have been reported as common causes of non-malarial febrile fevers, affecting up to 60% of children attending health centers in the SSA (Maze *et al.,* 2018). Despite these findings, routine diagnostic tools remain unavailable for CHIK and DF in Ethiopia, leaving HCWs to rely on clinical judgment and limited laboratory support (Shimelis *et al.,* 2022). A thorough assessment of healthcare facilities' practices and identifying the gaps in diagnosing and managing febrile illnesses is crucial to contribute for evidence-based strategies for improving healthcare services in resource-limited settings like Afar National Regional State.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Study Settings and Population

The study was conducted in three selected districts of Zone three in the Afar National Regional Stateal State of Ethiopia. Afar National Regional State, which located in northeastern parts of Ethiopia, is characterized by its arid climate, high temperatures, and limited healthcare infrastructure. The region has favorable conditions for the multiplication and expansion of *Aedes* mosquitoes and the transmission of arboviral diseases. In the past two decades, the Afar National Regional State, along with neighboring regions like the Somali Region and Dire Dawa Administration, has experienced periodic outbreaks of vector-borne diseases affecting humans and animals. These outbreaks include diseases such as DF, CHIK and sandfly fever. Specifically, chikungunya and dengue fever outbreaks have been reported in the Mille, Gewane, Amibara, Harunka, and Awash Sebat City Administration districts of the Afar National Regional State, while sandfly fever was reported in Assayta, Dubti, and Afambo (EPHI, 2021; Gutu *et al.,* 2021; Mesfin *et al.,* 2022; Woyessa *et al.,* 2014). Moreover, non-malarial febrile illnesses resembling of arboviral diseases have been reported in the region (Mehari *et al.,* 2021; Zerfu *et al.,* 2018).

This region shares borders with Eritrea in the northeast and Djibouti in the east. The population of the region consists mainly of nomadic pastoralists, with some practicing agro-pastoralism along the Awash River. Additionally, the community has experienced continuous migration within the region, as well as international cross-border movements for social interactions with similar communities in Eritrea and Djibouti, as well as in search of grazing areas. The region is geographically diverse, with altitudes ranging from 116 meters below sea level to 1600 meters above sea level. The climate is characterized by arid and semi-arid conditions, with temperatures varying from 25 °C to 48 °C and mean annual rainfall, ranging below 500 mm in the semi-arid areas, to 2000 mm in higher elevations.

The Afar National Regional State is home to an estimated population of 2,033,002, with a population density of 28.2/km² and an annual population change of 2.5% from 2007 to 2022 within an estimated 72,053 km² area according to central statistic projection in 2022 (CSA, 2022). The Region comprises five administrative zones, each containing five to seven districts and city administrations and each district has various *kebeles* (the lowest administrative unit). In this study, we identified Zone 3, which is approximately 140 kilometers from the regional capital, Semera, and 260 kilometers from the national capital, Addis Ababa. The total estimated population of the zone is 319,354. The zone comprises one city administration and seven districts: Awash City Administration, Awash Fentale, Amibara, Harunka, Birumodytu, Gewane, Dulessa and Argoba special districts. This Zone has been hit by periodic flooding of the Awash River as well as is a nomadic forested area with high biodiversity, including a range of non-human primates and mosquitoes that attribute and increase the risk of vector-borne diseases, including arboviral spillover and zonotic diseases transmissions (Althouse *et al.,* 2018;EPHI, 2021). We specifically selected three districts for this study - Awash City Administration, Harunka, and Amibara (Figure 3.1) - due to their prior experience with outbreaks of DF and CHIK, and their proximity to the main international road. In these districts, there are totally five functional Health centers (HCs) and one general referral hospital. Namely, Awash Arba HC, Werer HC and Sidafage HC in Amibara District, Andido HC in Harunka District, and Awash Sebat HC in Awash Sebat City Administration and the Mohammed Akle Memorial General (MAMG) Referral Hospital in the Amibara districts but administer by the Zone.

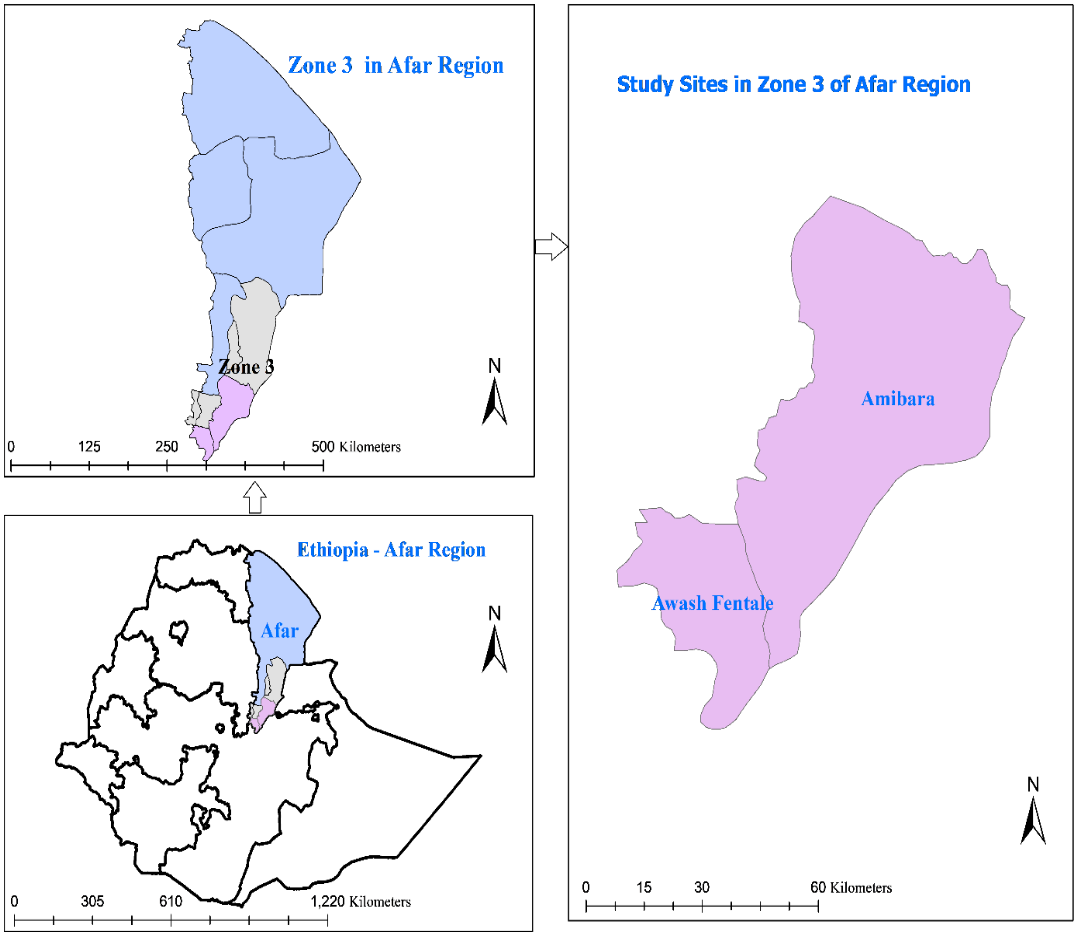

Figure 3.1: Map of the study sites in the Afar National Regional State, Northeast Ethiopia

3.2. Study Design and Period

Between September 2022 and March 2023, a health facility-based cross-sectional study design was used to assess the prevalence of selected arboviral diseases in patients with acute febrile illnesses and seeking treatment. Similarly, a cross-sectional study design was also used to assess community's KAP and HCWs' knowledge regarding these arboviral diseases, as well as HCWs' practices and challenges associated with the diagnosis and management of arboviral and non-malarial febrile diseases in this malaria endemic study area.

3.3. Sample Size Estimation

The sample size estimation to detect the selected arboviral diseases in patients with febrile illness, seeking healthcare facilities, was based on the previous report of seroprevalence of DF among non-malarias acute febrile patients, which was 25.1% for anti- IgG and 8.1% for anti-IgM in Arba Minch districts, southern Ethiopia (Eshetu *et al.,* 2020). Similarly, to estimate the sample size for CHIK, the prevalence of CHIKV-specific IgM from north Ethiopia, which was 22.5% among febrile patients in health facility-based study, was used (Ferede *et al.,* 2021). Using the formula $n = (Z\alpha /2)^2 P (1 – P)/ d^2$), where n = sample size; Z/2 = 1.96 at 95% confidence interval; P = prevalence (25.1% for DF and 22.5% for CHIK) and d = 5% desired level of precision (Sullivan, 2006), sample size calculation was prerformed. After incorporating 10% non-response rate, the minimum sample size for the seroprevalence study was 321 for DF and 294 for CHIK. Finally, 411 febrile patients were enrolled and diagnosed for malaria as a baseline and entry point for the study to identify malaria-suspected febrile patients. Then, arbovirus screenings were conducted based on a phased procurement process of funding logistics (Initial Phase for CHIKV testing, n=368) and Next Phase for DENV testing: DENV IgM =410 and IgG =367).

To assess the community's KAP level, a 50% awareness level was used to estimate the sample size due to the lack of a previous similar study in the study area. Using the sample size calculation formula indicated above, the sample size was estimated to be 384. However, only 296 community members were ultimately included for a quantitative study because of environmental conditions, as well as time and financial constraints. This final sample size of 296 provides valuable insights. At the same 95% confidence interval and 50% prevalence, a sample size of 296 increases the margin of error only slightly, approximately 5.7% compared to the original 5%, which is acceptable in many epidemiological field studies. Additionally, to obtain critical insights about the public's understanding about the mosquito-borne febrile diseases, including arboviral and malaria diseases, 116 community members were considered for focus group discussions (FGDs), organized into eight FGDs of 10-15 members of the same sex (three FGDs with females) to ensure homogeneity by grouping members of the same gender.

For the evaluation of HCWs knowledge and their practices/challenges in diagnosing and managing arboviral and other non-malarial febrile diseases, a mixed-methods approach encompassing quantitative survey and in-depth interview (IDI) among HCWs alongside a review of febrile patient records were used. For quantitative data, 88 frontline HCWs present during data collection time and a one-year acute febrile patient diagnosis and management data were considered, while 12 frontline HCWs were selected as key informants for qualitative interviews to obtain expert insights to significantly enrich the findings.

3.4. Study Participants and Sampling Technique

For the prevalence study of arboviral infections in febrile patients: Patients aged 5 years and older, presenting with acute febrile illnesses, with axial body temperature of at least 37.5°C, and willing to provide informed consent or assent, were recruited to be study participants. Study participants were recruited based on the inclusion criteria from the outpatient departments (OPDs) of the four public health centers: Awash Arba, Sidafage, Andido, and Awash Sebat HCs. Children under 5 years old, pregnant women and individuals who appeared to be anemic were excluded from the study.

Study participants and sampling of community members: community members aged 18 years or older were sampled in two selected *Kebeles,* each from Amibara and Awash Sebat City Administration districts, ensuring the sample represented the selected *Kebeles* within the districts according to their proportional sizes. The districts were purposely chosen for this study, with each two *Kebeles,* namely Awash Arba and Sidafage from Amibara and *Ragale* and *Kedeba* from Awash Sebat, were selected based on their accessibility and proximity to roads. For the quantitative study, a social mapping technique was employed to identify households within a 50-meter radius of each site (Dhimal *et al.,* 2014). This method allowed us to systematically sample up to 25 households, moving in four to six directions from the center of each site until we reached a maximum of 25 households. From the identified house, a head of household or an eligible family member was selected by lottery method. If the number of identified households fell below 25, we did not extend our search beyond six directions. Any households without eligible participants or that were closed during data collection were excluded. Additionally, households with members who had come from other districts or countries in the past year were excluded to maintain the consistency of the results within the study area, mentally not well and critically ill participants were excluded

For FGD, adult community members (aged 18 years old or older) were recruited and organized into 10 -15-member FGDs involving men and women independently. During discussions, two FGDs were conducted in the Goble *Kebele* from Awash Sebat City District, while one FGD was conducted in the Andido *Kebele* from Harunka District. Five FGDs were conducted in the Asgodum, Tiyara Bora, Sidafage, Beda'amo and Halydege *Kebeles* from Amibara District. These *kebele*s were randomly selected from other *kebele*s not included in the study,

Study participants and sampling of HCWs: Frontline HCWs in healthcare facilities were sampled for quantitative data using a convenience-sampling method based on availability and willingness to participate, as well as for IDIs data using purposeful sampling from all functional public healthcare facilities in the study area. Six public healthcare facilities located in the selected districts, namely Awash Arba, Sidafage, Andido, Werer and Awash Sebat HCs and MAMG Referral Hospital, were used for both datasets. Frontline HCWs considered in the study, included physicians, public health officers and nurses/midwives who were responsible for clinically diagnosing, notifying and managing cases in Ethiopian healthcare settings. The IDI participants were also frontline HCWs, specifically physicians or public health officers. They were chosen based on their relative higher academic qualifications, epidemiology awareness and full-time roles in the outpatient department (OPD), with at least one year of clinical service experience in the study area.

3.5. Data collection

3.5.1. Blood sample collection and processing

At each public HC, outpatient departments HCWs were trained to collect sociodemographic and clinical features using a structured questionnaire after obtaining written informed consent or assent. Trained medical laboratory personnel collected 5 ml venous blood samples using a gel-containing serum separation tube (SST). Thick and thin blood films were prepared and stained with Giemsa stain to examine malaria parasite infection on-site following the Ethiopia Ministry of Health (MOH) guidelines (MOH, 2012). The remaining blood was left to clot, and the serum was separated using centrifugation. Serum was stored at - 80 °C until tested for the arboviral infections at the laboratory of the Aklilu Lemma Institute of Pathobiology (ALIPB), currently named Aklilu Lemma Institute of Health Research (ALIHR), Addis Ababa University.

3.5.2. Data collection from community members

Data collection was conducted through a face-to-face household survey (HHS) using a structured questionnaire to obtain quantitative data while FGD was conducted to collect qualitative data. Eight FGDs (three FGDs with females) were conducted with members of same gender to ensure homogeneity. The data collections were conducted by data collectors that were selected based on their understanding of the local norms, customs and languages. The data collectors were trained on how to collect data through HHS and FGDs under the supervision of the research team. The questionnaire was translated into the national language, Amharic, and then orally into the local language, Afar language. In the study area, CHIK is known by the name *'Tilobign'* and mosquito is known by its Afar name *'kagna'ata'*. So, these local names were used during questionnaire-based data collection. After the objectives of the survey were explained and discussed with the informants to ensure their consent to participate, data collection were conducted. The data collectors were given no information on the correct answers to survey questions to minimize interviewer bias during data collection.

The HHS questionnaire was developed by reviewing studies previously conducted for arboviral diseases such as dengue and CHIK, and employed the Health Belief Model as a theoretical framework which helps to identify cues for action for the intended population (Kajeguka *et al.,* 2017; Laranjo, 2016; Phuyal *et al.,* 2022). The content, wording and cultural appropriateness were adjusted by reviewing the literature and consulting with experts as described in the Annex 3. The questionnaire contains two sections. Section 1 contains 12 questions that gather participants' sociodemographic data, knowledge about common febrile illness in the area, information about CHIK and DF, and source of information. Section 2 was the KAP section. The section consisted of 22 closed-ended items for knowledge questions that addressed the CHIK disease, etiology, common signs and symptoms, transmission modes, and prevention of CHIKV infection. The attitude items contained 15 close-ended questions with a 5-point Likert scale. The items included the community's beliefs on CHIK disease, its preventability and risks of perceptions. The final practice section contained 13 close-ended items, which consisted of questions to address preventive measures against CHIKV infection, primarily practices employed to reduce human-mosquito contacts and breeding opportunities of mosquitoes. Experts in the field, who suggested excluding questions related to the management of CHIK since the study districts did not consider it a major public health issue, except for the history of outbreaks, confirmed the questionnaire's validity. To ensure the questionnaire's reliability, a pilot study was conducted with a group of 20 participants. The pilot study was used to assess internal consistency reliability using Cronbach's alpha, and the questionnaire demonstrated adequate internal consistency reliability (Cronbach's alpha = 0.67).

Similarly, FGDs were conducted separately with men and women at different times on the same day. Guidelines addressing specific topics for discussions were prepared topic by topic, allowing adequate discussion on each topic. Key topics for discussion included awareness of prevalent febrile illnesses, particularly mosquito-borne diseases in the area. Participants explored their knowledge concerning arboviral diseases including CHIK, signs and symptoms, as well as comparisons with malaria regarding similarities and differences, transmissibility, and prevention strategies. The discussions also highlighted participants' health-seeking behaviors, and covered health intervention related CHIK and malaria, including modes of transmissions and controls measures as described in the Annex 4. The principal investigator and a trained health worker moderated the discussion. Visual aid, such as images of *Aedes* and *Anopheles* mosquitoes, were used to enhance understanding and engagement during the conversations. Responses were recorded in a notebook, translated into Amharic and then into English. Socio-demographic characteristics of the participants were recorded during the discussions.

3.5.3. Data collection from Healthcare professionals

Frontline HCWs that were present in the selected healthcare facilities at the time of data collection were considered after consultation with the heads of healthcare facilities to obtain quantitative data and IDIs for qualitative data. Participation in the study was voluntary, and verbal consent was obtained after explaining the study's purpose the participants by the data collectors/investigators.

Quantitative data survey: a self-administered a closed-ended structured questionnaire, with few open-ended questions were used. This questionnaire was developed after an extensive literature review (Kajeguka *et al.,* 2017; Saringe *et al.,* 2019). The questionnaire has four parts, as described in the annex. Part 1 was used to collect information on the facility details and participant details like socio-demographic characteristics, such as gender, age, educational level, profession/specialty and service years at the study site and elsewhere. Part 2 focused on the awareness and practices of fever-related illnesses diagnoses and management in the area including arboviral diseases (eight items). Part 3 focused on knowledge of the HCWs about major arboviral diseases particularly DF and CHIK diseases. It was composed of 21 questions split into four domains, including knowledge about: 1) severity of the illnesses (two items), 2) transmission, vectors and spread (five items), 3) signs and symptoms of the diseases (main twelve items), and 4) management and prevention (five items). Part 4 was used to collect information about the practices of HCWs to manage and prevent DF and CHIK in their work area (Annex 5). The survey was conducted in English to assistance if required, as study participant HCWs understood English. The data collectors or investigator administered the questionnaire and collected it on same day.

Qualitative data collection: HCWs were selected as key informants for the IDIs. The interviews were conducted in a private office in Amharic without translating medical terms, as the HCWs understood the terms. The investigator and data collector conducted the interview collaboratively; while one conducts the interview, the other took notes. Interview was guided by open-ended questions, which were designed to allow flexibility and for probing to gain more insight on awareness, practices and challenges related to mosquito-borne febrile illnesses including malaria and non-malaria illnesses, including arboviral diseases (DF and CHIK) and diagnostic and control practices (Annex 8. 6).

Review of febrile patient records: All six functional public healthcare facilities present in the selected study area were considered to review the practices of febrile patient diagnosis and management. However, two facilities were excluded owing to missing HIMS personnel during the data collection period or a lack of willingness to share the information. Finally, data were retrieved from the final four healthcare facilities registered for a year. One year was chosen to provide a sound database and reflection of all seasonal fluctuations throughout the year (Bohle *et al.,* 2022). The data collector, along with HIMS personnel, retrieved total number of febrile patients per month, the types of diagnoses made (including malaria rapid diagnostic tests or blood films), the results of those diagnoses, and the treatment prescribed by. In addition, the availability of supplies and reagents for diagnosing febrile illnesses, including malaria was observed in real-time stock. If present, stock card records dating back one year were reviewed, stockholders in the healthcare facilities were interviewed, and functionality of the microscopes was assessed in each facility. The reviewed data of the febrile patients' records were organized in a spreadsheet to analyze the practices of febrile patient diagnosis and management, including diagnosis conducted, etiology, and frequency of diagnosed diseases such as malaria and non-malaria febrile illnesses among patients presenting with febrile conditions at the health facilities.

3.6. Laboratory tests for arbovirus Infections

3.6.1. Serological tests for Dengue and Chikungunya viruses

For serological assays, detection of antibodies against DENV and CHIKV infections were conducted using ELISA by targeting anti-CHIKV and anti-DENV IgM and IgG antibodies. Serion ELISA Classic anti-CHIKV IgM (Cat. No. ESR148M, Germany) and IgG (Cat. No. ESR148G, Germany) as well as anti-DENV IgM (Cat. No. ESR114M, Germany) and IgG (Cat. No. ESR114G, Germany) kits were used to identify anti-viral IgM as evidence of recent or acute viral infections and to identify anti-viral IgG as evidence of previous or secondary infections of the viruses, following the manufacturer's instructions. The kit for detection anti-CHIKV IgM antibodies has indicated sensitivity of 91.7% and specificity over 99%, while the kit for anti-CHIKV IgG antibodies shows both sensitivity and specificity over 99%. For the detection of the anti-DENV IgM, the kit also has sensitivity of 91.7% and specificity over 99%, and the kit for anti-DENV IgG antibodies shows sensitivity and specificity exceeding 99% (SERION Chikungunya ELISA, 2024; SERION Dengue ELISA, 2024). The testing procedure for anti-CHIKV and -DENV IgM and IgG antibodies was similar. However, the sera were pretreated with rheumatoid factor absorbent during the preparation of specimen dilutions for the IgM antibodies assays of both viral infections.

Absorbances of the preparations were measured using Spectrophotometer at 405 nm using a micro plate reader. The qualitative interpretation of the results was based on the absorbance cut-off ranges, which were established using the mean optical density (OD) values of the standard serum (STD) samples. Samples with OD values below the lower cut-off were considered negative, while those above the upper cut-off were considered positive. Any OD value within the middle column of the cut-off range was considered borderline (equivocal)(SERION Chikungunya ELISA, 2024; SERION Dengue ELISA, 2024; Wittke *et al.,* 2002). During analysis, borderline results were interpreted as negative.

3.6.2. Molecular assays for arboviruses

Serum samples that tested positive for IgM antibodies against DENV and CHIKV were subjected to further molecular analysis at the Armauer Hansen Research Institute (AHRI) laboratory using a pooled approach method due to supply and financial constraint. The approach utilized by pooling of 2-5 serum samples into a single test, provided that the samples were collected from the same areas, positive for both anti-DENV and -CHIKV IgM, positive only for anti-DENV IgM, or positive only for anti-CHIKV IgM antibodies by ELISA. Samples which were found positive for IgG and/or negative for IgM and IgG antibodies were not analyzed because shortage of reagents.

For molecular analysis, 212 serum samples that tested positive for anti-DENV and CHIKV IgM antibodies were subjected to molecular screening in a pooling approach. The samples were pooled according to serological test result and sample collection site similarities, with 60 positive sera for both anti-DENV and CHIKV IgM antibodies were allocated four to one to form final 15 pools. Additionally, 112 positive sera only for CHIKV were pooled into 28 pools - most of which combined four serum samples each; however, two pools were formed from five samples, and one pool included two samples according to their shared similarities of collection sites. Finally, 40 samples that tested positive for DENV antibodies were combined into 12 pools using the same four-sample method, except four pools were formed from five samples while two pools were formed from two sera. All pools tested positive for viral RNA were disaggregated and tested individually.

RNA extraction: RNA extraction was performed from the pooled sera. The extraction was employed with the automated MagaBio plus RNA Purification kit II, following the manufacturer's instructions, using a Bioer NPA-32P instrument (Bior Technology, Zhejiang, China). Briefly, 300 µl of thawed and briefly vortexed sera were added to columns #1 and #7 of the 96-well pre-loaded plates. The plate was then placed into the machine, which agitated the plates every 10 seconds for 9 minutes until the extraction was completed, resulting in 70µl RNA extracts and stored at -80°C until further use.

The RNA extracted from pools of sera was tested by an in-house RT-PCR with complementary DNA (cDNA) synthesis using SSIV RT kit (ThermoFisher Scientific, Cat. No. 18090010) and then PCR amplification targeting DENV, CHIKV, ZIKV, and WNV to detect, and by commercial RT-PCR, which is a one-step TaqMan™ Arbovirus Triplex kit designed for real-time RT-PCR to detect DENV, ZIKV and CHIKV.

1) **cDNA synthesis and PCR amplification:**

Extracted RNA was converted to cDNA using SuperScript IV First-Strand Synthesis System from Thermo Fisher Scientific, following Protocols.io and Thermo Fisher Scientific (Angel & Petrova, 2021). This process uses random hexamer primers to initiate cDNA synthesis from RNA. These primers are short, randomly sequenced oligonucleotides that can bind to various regions of the RNA gemone molecule, which facilitats reverse transcription of the entire RNA molecules.

The procedure begins with annealing the primer to the template RNA. In detail, the following components were mixed in a reaction tube to anneal primer to template RNA: 1µL random hexamers (50µM), 1µL dNTP mix (10mM each), and up to 11µL template RNA, made up to 13µL with nuclease-free water. The RNA-primer mixture was heated at 65°C for 5 minutes and then immediately incubated on ice for at least 1 minute. Second, prepare the RT reaction mix by combining 4 µL 5x SSIV Buffer, 1µL 100mM DTT, 1µL RiboLock RNase Inhibitor, and 1µL SSIV RT (200U/µL). Cap the tube, mix, and briefly centrifuge the contents. Third, combine the annealed RNA and RT reaction mix by adding the RT reaction mix to the annealed RNA, then incubate at 23°C for 10 minutes, followed by incubation at 53°C for 30 minutes, and finally inactivate the reaction by incubating at 80°C for 10 minutes.

The synthesized viral cDNAs were used as templates to amplify using a quantitative PCR System (ThermoFisher) with thermal cycling and detection on the CFX96 Real-Time PCR Detection System to detect the targeted viruses. Primers and probes, which were adapted from a previous study with minor modification of the fluorophores and quenchers for the probes, were designed for specific viral RNA detections targeting the 3' UTR regions of ZIKV and DENV, the NSP2 region of CHIKV and the NS5 region of WNV genomes. The probes that were synthesized to include a fluorophore and a quencher covalently attached to the 5' and 3' ends, respectively, as detailed in Table 1 (Mishra *et al.,* 2011). Based on differences of fluorophores, DENV and ZIKV were detected in a single PCR reaction, while CHIKV and WNV were detected in a single PCR reaction.

For DENV and ZIKV detection, each primer was used at a volume of 0.4 µL, while probes were used at 0.2µL, resulting in a total volume of 2.4µL for primers and probes. The PCR reaction included 10µL of 2xMaster Mix, 2µL of template cDNA, and nuclease-free water, for a final volume of 20µL. For CHIKV and WNV, the primers were set at 0.2µL each, with probes at 0.1µL (total of 1µL primers and probes) and 10µL of 2xMaster Mix, 2µL of cDNA sample and nuclease-free water, for a final volume of 20µL.

The thermal cycling conditions were optimized, beginning with a Taq DNA polymerase activation step at 95°C for 5 min. This was followed by 40 cycles of quantitative PCR, with denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 15 seconds. Fluorescence signal intensity was detected after completion of each qPCR cycle, and data were collected using CFX Manager Software provided by Bio-Rad; samples were considered negative (not determined) if no cycle threshold (C_t) value for target virus amplification was recorded after 40 cycles. Some PCR positive products were then analyzed using gel electrophoresis using 2.5% agarose gel for DENV positives to check a visible band at 107 bp band. In addition, since no CHIKV RNA was detected using this in-house assay, the commercial TaqMan Arbovirus Triplex Kit was employed.

2) **TaqMan Real-time RT-PCR assay:**

The extracted RNA was detected using rRT-PCR using TaqMan™ Arbovirus Triplex Kit (ZIKV/DENV/CHIKV), 0.2‑mL block lyophilized reagents for multiplex real-time RT-PCR detection of DENV, CHIKV and ZIKV. The kit includes virus-specific primers and probes for those viruses and internal control peptidylprolyl isomerase A (PPIA) targets, along with other reagents (PCR master mix) for RT-PCR, in a lyophilized format.

To prepare the samples, we added 25µl of the RNA extract to the kit components, mixed the contents by flicking the tube several times or by briefly vortexing, and then centrifuged briefly to reconstitute the mixture for real-time RT-PCR. The tube strips were loaded into the real-time PCR instrument programmed with the following thermal cycling conditions: reverse transcription at 50°C for 20 minutes, RT inactivation at 95°C for 2 minutes, and followed by 40 cycles amplification for 15 seconds at 95°C and for 1 minute at 60°C. The assay detects an internal control targeting human endogenous PPIA to monitor that the extracted test specimen contains amplifiable RNA and to serve as a process control for the RT-PCR. The fluorescent signal intensity was captured using a real-time PCR instrument provided by QuantStudio5 Real-Time PCR system (ThermoFisher Scientific). Samples were considered negative (not determined) if the C_t value for target amplification is not recorded within 40 cycles.

Table 3.1: Sequences of primers and probes used for in house RT-PCR of Arbovirus infections

Primer and probe	Gene target	Primer Sequence (5’ to 3’)
DENV_3P_Probe	3’ UTR	FAM-CCCAGCGTCAATATGCTGT-MGB
DENV_3P_QF	3’ UTR	ACTAGAGGTTAGAGGAGACCCCC
DENV_3P_QRA	3’ UTR	GGCGCTCTGTGCCTGGATT
DENV_3P_QRB	3’ UTR	TGGCGTTCTGTGCCTGGAAT
ZIKA.3P.Probe	3’ UTR	HEX-GGGGAAAGCTGTGCAGCCTGT-BHQ-2
ZIKA.3P.QF	3’ UTR	ARTGTTGTCAGGCCTGCTAG
ZIKA.3P.QR	3’ UTR	CTTGRTTTCCCAGCKTCTCCT
CHIK_NSP2_Probe	NSP2	FAM-AAAAGTATCTCCAGGCGG-MGB
CHIK_NSP2_NQF	NSP2	CATCTGCACYCAAGTGTACCA
CHIK_NSP2_NQR	NSP2	GCGCATTTTGCCTTCGTAATG
WNV.NS5.Probe.	NS5	Q670-TGGGTCCCTACCGGAAGAACCACGT- BHQ-2
WNV.NS5.NQF	NS5	CATTTGCTCCGCTGTCCCTGTGAA
WNV.NS5.NQR	NS5	CCACTCTCCTCCTGCATGGATGGAC

3.7. Operational Definition

Acute (Recent) DENV or CHIKV Infection: In this study, acute infections were identified by the presence of virus-specific IgM antibodies detected through ELISA, indicating a current or recent infection that occurred within about one week before sample collection. Clinically, these infections are associated with acute febrile illness that arises 1–8 days after onset, representing the viremic phase of DENV or CHIKV infections.

Previous Infection (Past Exposure): This term refers to prior exposure to DENV or CHIKV infections, which was determined by the detection of IgG antibodies against these viruses in the study participants.

Co-infection: This term describes the simultaneous detection of two or more pathogens (such as DENV and CHIKV, or DENV/CHIKV and malaria) in a single patient sample, confirmed through serological or microscopic testing.

Non-Malarial Febrile Illness (NMFI): This refers to fever in patients who tested negative for malaria through microscopy or rapid diagnostic tests (RDT), but who show symptoms consistent with other causes, including arboviral diseases.

Knowledge (K): This reflects the understanding and awareness of community members and HCWs regarding the causes, symptoms, transmission, and prevention of mosquito-borne diseases like DF and CHIK. It was assessed using structured questions, with each correct answer earning one point.

Attitude (A): This indicates the perceptions, beliefs, and feelings of participants about the seriousness, preventability, and personal responsibility for controlling arboviral diseases. It was measured using a Likert scale to measure agreement or disagreement with various statements.

Practice (P): This refers to the actual preventive and control actions taken by participants, such as removing mosquito breeding sites, using bed nets, and wearing protective clothing. Practices were evaluated based on self-reported answers in the questionnaire.

Knowledge, Attitude, and Practice (KAP) Scores: These scores are quantitative measures based on the correct answers provided by respondents, categorized as Good (≥70%), Moderate (51–69%) and Poor (≤50%). The scores reflect the participants' overall understanding, perceptions, and preventive behaviors related to mosquito-borne diseases.

3.8. Ethical Considerations

The study obtained ethical approval from the Institutional Review Board of ALIPB, AAU (ALIPB IRERC/88/2014/22). Before conducting the study, permission obtained from Amibara, Harunka and Awash Sebat City Health Offices. Informed consent obtained from all individuals aged 18 years or above and from guardians or parents for children below 12 years and assent obtained from children aged 12 to 17 years. Malaria-positive participants were treated as per malaria treatment guidelines, and the physicians managed non-malaria cases as per the clinical based judgment. For the awareness and practices studies, verbal consent obtained from the community members and HCWs after providing a comprehensive explanation of the study's objectives, since the study posed no risks to the participants, as well as from the healthcare officers on behaves of patients for the retrospective data.

3.9. Data Management and Statistical Analysis

3.9.1. Quantitative data analysis

Data were coded and entered using EpiData 3.1 and exported to and analyzed with Stata 14 analytical software. The number and distributions of socio-demographic characteristics, clinical features and other categorical variables were tabulated to present in frequencies and percentages, while continuous variables were summarized to present in mean with SD.

Seroprevalence analysis: The prevalence of anti-DENV and anti-CHIKV antibodies was estimated by dividing the number of participants with positive test results by the total number of study participants. Univariable logistic regression analysis was conducted to identify associations between the prevalence of anti-viral IgM and IgG antibodies and demographic characteristics and clinical features. Additionally, multivariable logistic regression analysis was performed to assess the impact of independent variables, such as gender, age, occupation, and clinical features on the outcome variable while adjusting for all other variables. Odds ratios (OR) with 95% confidence intervals (CI) were used to determine the strength of the association between seropositivity and the variables, with statistical significance set at p-value < 0.05.

Community KAP analysis: To determine the KAPs score of each participant, one point was given for every correct answer, while zero points were given for incorrect answers and "not sure" responses (Phuyal *et al.,* 2022). The knowledge and practice questions were scored with 0 for wrong or not-sure answers and 1 for correct answers. The attitude questions had a scale response of 1 for Strongly Agree, 2 for Agree, 3 for "not sure," 4 for Disagree, and 5 for Strongly Disagree, which was then dichotomized into 1 for positive (1 and 2) and 0 for negative (3, 4, and 5) answers. However, for the specific question "Do you agree that health service stakeholders are the only entity responsible for reducing larvae and adult mosquitoes in your home?" the responses of 1, 2, and 3 were scored as 0, while 4 and 5 were scored as 1. The total score for each KAP domain was calculated by adding the number of correct or positive responses to questions in the questionnaire. The maximum cumulative score (MCS) for each domain was 22 for knowledge, 15 for attitude and 13 for practice. After a total score for each KAP domain, the participant's mean score was calculated as the score obtained divided by MCS for each domain. The mean correct or positive responses of each KAP score (score obtained/MCS ×100) were calculated for each participant. The overall mean correct or positive response rate would be the proportion of participants who achieved a summed score equal to or greater than the mean of each KAP domain and their levels were determined to be good (score $\geq$ 70%), fair (score 50.1-69.9%) and poor (score $\leq$ 50%) (Mallhi *et al.,* 2018; Nepal, 2021). Respondents who had never heard of CHIK were excluded from the KAP score analysis. The Shapiro-Wilk W test was used to identify the normality of the KAP data. The knowledge and practice domains were found to be normally distributed ($p > 0.05$) while the attitude domain was found not normally distributed ($p < 0.001$). However, skewness (0.0001) and kurtosis (0.0709) values of attitude were within acceptable limits, between -2 and +2, for deciding the use of parametric analysis to confirm or reject research hypotheses (George & Mallery, 2010). A one-way analysis of variance (ANOVA) or independent sample t-test was performed to determine the factors affecting knowledge, using the mean knowledge score as dependent variable and sex, age, gender, level of education, profession, work experience, districts and health institutes as independent variables. Multiple linear regression analysis was conducted to study the association between the KAP scores and sociodemographic factors. Beta coefficient (β), at 95% confidence interval (CI), was used to determine the strength of the association between the scores and variables. Pearson's correlation coefficient (r) was analyzed to determine if there is a relationship between the KAP domains. The statistical significance of the study was established at p-value < 0.05.

HCW Knowledge and practice analysis: Similarly, the responses of the participant were scored as follow: each correct answer was scored "1", while incorrect answers and "don't know," or "not sure" responses were scored "0" (Phuyal *et al.,* 2022). The knowledge about DF and CHIK) was assessed through a series of 21-item questions, where each correct response contributed one point to the overall score. Cumulative knowledge scores (CKS) were calculated for each participant by summing the correct responses across all 21 items, as well as for each 4 knowledge domains. The total percent knowledge score was derived by multiplying CKS by 100, allowing an evaluation against the maximum possible score within each domain and overall. The mean knowledge scores were categorized into three levels: good (≥ 14.7; 70%), moderate (10.6-14.6; 51-69%), and poor (≤ 9.5; 50%), which was adopted from previous studies (Jawed *et al.,* 2018; Mallhi *et al.,* 2018). To assess factors influencing the knowledge levels, an ANOVA or an independent sample t-test was used. In these analyses, the mean knowledge score was served as dependent variable, while sociodemographic factors were independent variables. A p-value < 0.05 was considered statistically significant.

3.9.2. Qualitative data analysis

The qualitative data complemented the quantitative data. A conventional content analysis approach was employed for qualitative data analysis(Hsieh & Shannon, 2005). The results of FGDs and IDIs were transcribed verbatim into English, and categorized in themes and sub-themes. After identifying the themes, thematic analyse were conducted. The themes of FGDs and IDIs were relevant supplementary insights for the goals and objectives of the study.

CHAPTER FOUR

4. RESULTS

4.1. Seroprevalence of Chikungunya Virus Infection among Malaria-suspected

4.1.1. Sociodemographic features of participants of CHIKV infection study

For the seroprevalence study of CHIKV infection, 368 febrile patients (55.4% females, age ranged from 5 to 80, mean ±SD = 27.3 ±14.0 years old) were involved. Most of the participants of the study were in the age range between 21-30 years old (37.2%), without formal education (45.7%), married (60.6%) and residents of the Amibara district (59.4%). Most of the patients were from Awash Arba HC (44.8%), followed by Awash Sebat HC (34.2%)(**Table 4.1**).

Table 4.1: Socio-demographic characteristics of participants involved in the seroprevalence study of CHIKV infection in the selected districts in the Afar National Regional State, Northeast Ethiopia, from September 2022 to March 2023 (N = 368)

General characteristics		Number (%)
Sex	Male	164 (44.6)
	Female	204 (55.4)
Age (years)	5-10	35 (9.5)
	11-20	94(25.5)
	21-30	137(37.2)
	31-40	49(13.3)
	41-50	28(7.6)
	51 and above	25(6.8)
Educational status	No-formal education	168(45.7)
	Grade 1-8th	118(32.1)
	Grade 9-12th	62(16.8)
	College and above	20(5.4)
Resident	Urban	198(53.8)
	Rural	170(46.2)
Occupational status	Agro/pastoralist	41(11.1)
	Government/private worker	129(35.1)

	House wife	104(28.3)
	Students	94(25.5)
Marital status	Married	223(60.6)
	None-married	88(23.9)
	Children under 18 years	57(15.5)
Religion	Muslim	226(61.4)
	Christians	142(38.6)
Ethnicity	Afar	177(48.9)
	Others	185(51.1)
Districts	Amibara	201(54.6)
	Awash Sebat	126(34.1)
	Harunka	41(11.1)
Health institutes	Awash Arba	165(44.8)
	Awash Sebat	126(34.2)
	Sidafage	36(9.8)
	Andido	41(11.1)

4.1.2. Reported clinical features of participants involved in this study

. The most frequently reported clinical features were headache (90.7%), joint pain (71.2%) and back pain (50.3%), with symptom duration ranging from one to eight days, and a mean duration of 2.9 ± 1.4 days. Most (93.7%) of the study participants experienced the illness for five or fewer days (Table 4.2).

Table 4.2: Clinical features reported by febrile patients participated in CHIK study in selected districts of the Afar National Regional State, Northeast Ethiopia (N =368)

Duration and clinical features		Number (%)
Duration of the illness	≤ 5 days	345(93.7)
	> 5 days	23(6.3)
Headache		334(90.7)
Joint pain		262(71.2)
Back pain		185(50.3)
Chills		167(45.4)
Nausea		130(35.3)
Persisting vomit		46(12.5)
Diarrhea		49(13.3)

Malaise	81(22.0)
Muscle pain	81(22.0)
Sore throat	42(11.4)
Abdominal pain/tenderness	72(19.6)
Rash	6(1.6)
Calf pain	13(3.5)
Cough	70(19.0)
Difficulty in breathing	8(2.2)
Pain behind the eyes	4(1.1)

4.1.3. Seroprevalence of CHIKV infection in febrile patients

Out of 368 serum samples tested for the presence of anti-CHIKV IgM and IgG antibodies using ELISA, 189 (51.4%; 95%CI: 46.2-56.6%), sera were tested positive for anti-CHIKV IgM and/or IgG antibodies. Notably, 176 (47.8%; 95% CI: 42.7-52.9%) of the sera were positive for anti-CHIKV IgM antibodies, indicating a high level of recent or acute CHIKV infection. The borderlines for anti-CHIKV IgM antibodies were 19.0%, while the remaining (33.2%) were negative for the IgM antibodies. In contrast, only 23 (6.3%) of the tested sera were found to be positive for anti-CHIKV IgG antibodies, with 2.7% of tested sera were positive for both anti-CHIKV IgM and IgG antibodies (Figure 4.1A).

4.1.4. Seropositivity of anti-CHIKV IgM and sociodemographic/ clinical features

The logistic regression analyses of sociodemographic and clinical features showed that seropositivity of anti-CHIKV IgM antibodies was slightly higher in females (49.0%) than in males (46.3%). The lowest seropositivity rate for anti-CHIKV IgM antibodies was found among individuals aged 51 and above (COR = 0.187, 95% CI: 0.059-0.587). However, this difference was not statistically significant in the multivariable logistic regression analyses. The multivariable logistic regression analysis indicated that the odds of non-married study participants being seropositive were about four times higher than those of married participants (AOR = 3.908; 95% CI = 1.298-11.763). Additionally, the odds of participants tested positive for anti-CHIKV IgM antibodies were significantly higher for Awash Sebat (AOR = 1.976; 95% CI = 1.031-3.787), Sidafage (AOR = 4. 870; 95% CI: 1.961-12.095) and Andido HCs (AOR = 2.624; 95% CI: 1.142-6.031) compared to those from Awash Arba HC. Furthermore, the odds for participants who reported back pain to be seropositive for anti-CHIKV IgM antibodies were about twice as high as those without back pain (AOR = 1.712; 95% CI: 1.031-2.844) (Table 4.3).

Table 4.3: Seropositivity of anti-CHIKV IgM antibodies and associated sociodemographic and clinical features in the selected districts in the Afar National Regional State, Northeast Ethiopia, from September 2022 to March 2023 (N = 368)

Variable		N° tested	N° +ve (%)	COR (95%CI)	AOR (95%CI)
Sex	Male	164	76(46.3)	1	1
	Female	204	100(49.0)	1.113(0.738-1.681)	1.527(0.885-2.636)
Age (years)	5-10	35	22(62.9)	1	1
	11-20	94	52(55.3)	0.732(0.330-1.624	0.809(0.315-2.074)
	21-30	137	63(46.0)	0.503(0.234-1.079)	0.827(0.285-2.397)
	31-40	49	22(44.9)	0.481(0.198-1.169)	0.627(0.177-2.222)
	41-50	28	11(39.3)	0.382(0.137-1.063)	0.427(0.109-1.672)
	51 and more	25	6(24.0)	**0.187(0.059-0.587)**	0.271(0.060-1.222)
Educational status	No formal education	168	80(47.6)	1	1
	Grade 1-8th	118	58(49.2)	1.063(0.664-1.703)	0.540(0.263-1.111)
	Grade 9-12th	62	30(48.4)	1.031(0.576-1.847)	0.818(0.348-1920)
	College & above	20	8(40.0)	0.733(0.285-1.886)	0.223(0.117-1.200)
Resident	Urban	198	94(47.5)	1	1
	Rural	170	82(48.2)	1.031(0.684-1.554)	0.971(0.519-1.816)
Occupational status	Agro/pastoralist	41	21(51.2)	1	1
	Government/private worker	129	62(48.1)	0.881(0.436-1.780)	1.028(0.427-2.476)
	House wife	104	42(40.4)	0.645(0.312-1.334)	0.602(0.237-1.528)
	Students	94	51(54.3)	1.130(0.542-2.354)	0.922(0.304-2.800)
Marital status	Married	223	90(40.4)	1	1
	Non-married	88	56(63.4)	**2.586(1.553-4.307)**	**3.908(1.298-11.763)**
	Children under 18 years	57	30(52.6)	1.642(0.915-2.946)	1.941(0.909-4.146)
Districts^a	Amibara	201	88(43.8)	1	
	Awash Sebat	126	63(50.0)	1.284(0.821-2.007)	
	Harunka	41	25(61.0)	**2.006(1.010-3.986)**	

Health Institute	Awash Arba HC	165	63(38.2)	1	1
	Awash Sebat HC	126	63(50.0)	**1.619(1.012-2.590)**	**1.976(1.031-3.787)**
	Sidafage HC	36	25(69.4)	**3.68(1.694-7.992)**	**4.87(1.961-12.095)**
	Andido HC	41	25(61.0)	**2.53(1.254-5.103)**	**2.624(1.142-6.031)**
Illness duration	≤ 5 days	345	165(47.8)	1	1
	>5 days	23	11(47.8)	1.00(0.430-2.328)	0.781(0.282-2.168)
Joint pain	No	106	43(40.6)	1	1
	Yes	262	133(50.8)	1.510(0.956-2.386)	1.488(0.878-2.522)
Headache	No	34	18(52.9)	1	1
	Yes	334	158(47.3)	0.798(0.393-1.618)	0.921(0.414-2.052)
Back pain	No	183	83(45.4)	1	1
	Yes	185	93(50.3)	1.218(0.809-1.834)	**1.712(1.031-2.844)**
Chills	No	201	89(44.3)	1	1
	Yes	167	87(52.1)	1.368(0.906-2.066)	1.121(0.661-1.905)
Nausea	No	238	112(47.1)	1	
	Yes	130	64(49.2)	1.091(0.711-1.673)	
vomiting	No	322	154(47.8)	1	1
	Yes	46	22(47.8)	1.00(0.539-1.856)	1.056(0.660-1.690)
Malaise	No	287	142(49.5)	1	1
	Yes	81	34(42.0)	0.739(0.449-1.216)	0.800(0.444-1.438)
Muscle pain	No	287	136(47.4)	1	1
	Yes	81	40(49.4)	1.083(0.661-1.774)	0.980(0.537-1.786)
Rash	No	362	171(47.2)	1	1
	Yes	6	5(83.3)	5.584(0.646-48.278)	10.124(0.954-107.405)

COR, crude odds ratio, AOR, adjusted odd ratio, CI, confidence interval, ^{e}xcluded from multiple logistic regression model due to collinearity

4.1.5. Prevalence of malaria infection and sociodemographic/ clinical features

From 411 malaria-screened patients, 44 (10.7%), participants were diagnosed with *Plasmodium* parasite infections. Specifically, *P.falciparum* was responsible the highest proportion (84.1%), while *P. vivax* accounted for 11.4 % (Figure 4.1B). The prevalence of *P. falciparum* was 11.0% in males and 7.3% in females. *P. falciparum* was detected in all age groups, with relatively higher rates in the age groups of 31-40 years (14.3%) and 21-30 years (12.4%) age groups. *P. vivax* was found to be similar in both males and females, while it was detected only among patients aged 20 years or younger. On the other hand, mixed infections were only found among males; with a prevalence of 1.2%, (data are not presented).

Logistic regression analysis indicated that malaria infection had no differences among socio-demographic features of the patients. However, in a univariable logistic regression analysis, febrile patients found in Awash Sebat HC had approximately 0.2 times lower odds of testing positive for malaria compared to those from Awash Arba HC (COR = 0.209, 95% CI: 0.079-0.551). On the other hand, a multivariable logistic regression analysis showed that febrile patients from Andido HC had odds of *plasmodium* infection that were also about 0.2 times lower in comparison to their counterpart from Awash Arba HC (AOR = 0.162, 95% CI: 0.028-0.945)(Table 4.4).

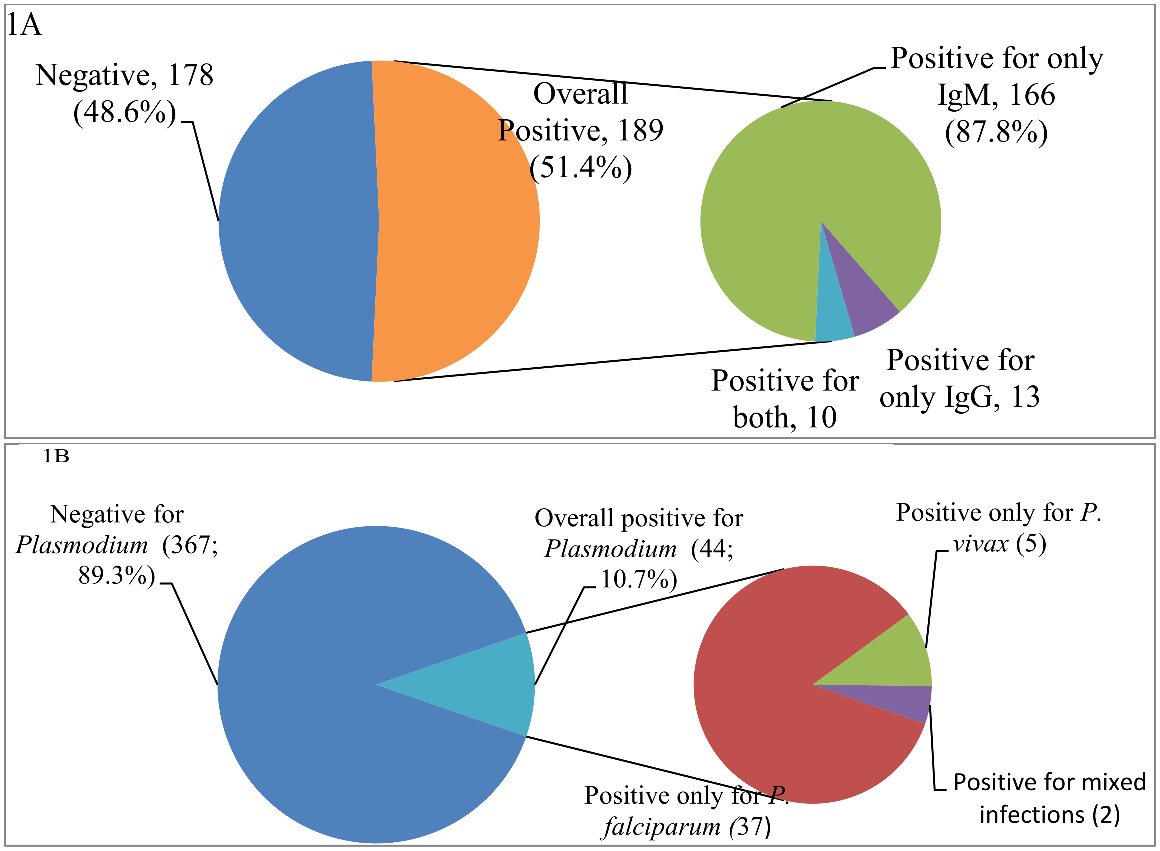

Figure 4.1: Prevalence of anti-CHIKV antibodies (1A) and *Plasmodium* infections (1B)

Table 4.4: Prevalence of malaria and associated sociodemographic/ clinical features in febrile patients in the Afar National Regional State, Northeast Ethiopia, from September 2022 to March 2023 (N =411)

Variables		N° tested	N° +ve (%)	COR(95%CI)	AOR(95%CI)
Sex	Male	183	24(13.1)	1	1
	Female	228	20(8.8)	0.637(0.339-1.194)	0.493(0.193-1.256)
Age (years)	5-10	40	3(7.5)	1	1
	11-20	106	10(9.3)	1.285(0.335-4.930)	2.517(0.380-16.688)
	21-30	148	18(12.2)	1.708(0.477-6.115)	1.895(0.242-14.821)
	31-40	57	9(15.8)	2.312(0.585-9.148)	3.730(0.353-39.358)
	41-50	34	3(8.8)	1.193(0.225-6.340)	1.553(0.113-21.285)
	51 and more	26	1(3.8)	0.493(0.048-5.017)	0.615(0.028-13.695)
Education status	Illiterate	191	20(10.5)	1	1
	Grade 1-8th	127	11(8.7)	0.811(0.374-1.756)	1.625(0.488-5.402)
	Grade 9-12th	70	9(12.9)	1.261(0.545-2.920)	1.823(0.489-6.793)
	College & above	23	4(17.4)	1.800(0.557-5.820)	3.084(0.581-16.366)
Resident	Urban	207	18(8.7)	1	1
	Rural	204	26(12.7)	1.534(0.813-2.894)	1.041(0.408-2.655)
Occupation	Agro/pastoralist	41	3(7.3)	1	1
	Government/private worker	148	19(12.8)	1.866(0.524-6.645)	2.107(0.434-10.222)
	House wife	114	12(12.5)	1.490(0.398-5.572)	2.342(0.444-12.367)
	Students	108	10(9.3)	1.292(0.337-4.953)	0.418(0.054-3.244)
Marital status	Married	249	25(10.0)	1	1
	Non-married	98	9(9.2)	0.906(0.407-2.017)	1.691(0.486-5.887)
	Children under 18	64	10(15.6)	1.659(0.752-3.661)	
Health Institute	Awash Arba HC	200	33(16.5)	1	1
	Awash Sebat HC	126	5(4.0)	**0.209(0.079-0.551)**	0.337(0.085-1.339)
	Sidafage HC	44	4(9.1)	0.506(0.169-1.510)	0.904(0.219-3.734)
	Andido HC	41	2(4.9)	0.259(0.060-1.128)	**0.162(0.028-0.945)**
Duration of illness	≤ 5 days	386	44(11.4)	1	
	>5 days	25	0(0.0)	Omitted	Omitted
Headache	No	39	1(2.6)	1	1
	Yes	372	43(11.6)	4.967(0.665-37.10)	3.262(0.336-31.701)
Joint pain	No	117	20(17.1)	1	1
	Yes	294	24(8.2)	**0.431(0.228-0.815)**	**0.297(0.123-0.681)**
General back pain	No	206	27(13.1)	1	1
	Yes	205	17(8.3)	0.599(0.316-1.137)	0.479(0.203-1.126)
Chills	No	229	38(16.6)	1	1
	Yes	182	6(3.3)	**0.171(0.071-0.415)**	**0.161(0.054-0.487)**
Nausea	No	266	35(13.2)	1	1

	Yes	145	9(6.2)	**0.437(0.204-0.936)**	0.549(0.238-1.267)
Vomiting	No	359	33(9.2)	1	
	Yes	52	11(21.1)	**2.650(1.245-5.643)**	0.81(0.31-2.13)
Diarrhea	No	357	39(10.9)	1	1
	Yes	54	5(9.3)	0.832(0.313-2.213)	1.079(0.303-3.846)
Malaise	No	320	37(11.6)	1	1
	Yes	91	7(7.7)	0.637(0.274-1.481)	0.371(0.112-1.228)
Muscle pain	No	325	38(11.7)	1	1
	Yes	86	6(7.0)	0.566(0.231-1.388)	2.308(0.700-7.608)
Sore throat	No	365	43(11.8)	1	1
	Yes	46	1(2.2)	0.166(0.022-1.238)	0.276(0.030-2.557)

COR, crude odds ratio, AOR, adjusted odd ratio, CI, confidence interval

4.2. Seroprevalence of DENV and Co-infection with CHIK and Malaria

4.2.1. *Sociodemographic features of participants involved in the study*

In this study, 411 febrile patients (55.5% females, age ranged from 5 to 80, with mean ± SD = 27.3±13.9 years old) were involved. Most of the participants were between 21-30 years old (37.2%), without formal education (46.5%), married (60.6%), and residents of the Amibara district (59.4%)(Table 4.5).

Table 4.5: Socio-demographic characteristics of participants involved in the seroprevalence study of DENV infection in the selected districts in the Afar National Regional State, Northeast Ethiopia, from September 2022 to March 2023 (N = 411)

General characteristics		Number (%)
Sex	Male	183 (44.5)
	Female	228 (55.5)
Age (years)	5-10	40(9.7)
	11-20	106(25.8)
	21-30	148(36.0)
	31-40	57(13.9)
	41-50	34(8.3)
	51 and above	26(6.3)
Education status	No formal education	191(46.5)
	Grade 1-8th	127(30.9)
	Grade 9-12th	70(17.0)
	College and above	23(5.6)
Resident	Urban	207(50.4)
	Rural	204(49.6)
Occupational status	Agro/pastoralist	41(10.0)

	Government/private worker	148(36.0)
	Housewife	114(27.7)
	Students	108(26.3)
Marital status	Married	249(60.6)
	Non-married	98(23.8)
	Children under 18 years old	64(15.6)
Religion	Muslim	251(61.1)
	Christians	160(38.9)
Ethnicity	Afar	199(48.4)
	Others	212(51.6)
Districts	Amibara	244(59.4)
	Awash Sebat	126(30.7)
	Harunka	41(10.0)
Health institutes	Awash Arba	200(48.7)
	Awash Sebat	126(30.7)
	Sidafage	44(10.7)
	Andido	41(10.0)

4.2.2. Clinical features reported by participants/parents involved in the study

Along measured fever, participants or parents reported other various clinical symptoms, with frequently, reported symptoms were headache (90.5%), joint pain (71.5%), and back pain (49.9%). The participants reported the duration of the illness, which ranged from one to 8 days, with an average ± SD range of 2.9 ± 1.4 days before seeking medical attention. Most participants (93.9%) experienced the illness for 5 days or less (Table 4.6). Notably, none of the respondents mentioned any additional clinical symptoms typically associated with severe DF, such as mucosal bleeding, bloody vomit or stool, bleeding gums or nose, or petechiae.

Table 4.6: Clinical features reported by febrile patients participated in the study in selected districts of the Afar National Regional State, Northeast Ethiopia (N = 411)

		Number (%)
Duration of the illness	≤ 5 days	386(93.9)
	> 5 days	25(6.1)
Headache		372(90.5)
Joint pain		294(71.5)
Back pain		205(49.9)
Chills		182(44.3)
Nausea		145(35.3)
Diarrhea		54(13.1)

Malaise	91(22.1)
Muscle pain	86(20.9)
Sore throat	46(11.2)
Abdominal pain/tenderness	83(20.2)
Persisting vomits	52(12.6)
Calf pain	18(4.4)
Rash	6(1.5)
Cough	81(19.7)
Difficulty in breathing	10(2.4)
Pain behind the eyes	6(1.5)

4.2.3. Seroprevalence of anti-Dengue virus antibodies

In this study, a total of 410 patient sera were examined for anti-DENV IgM antibodies, with one serum was excluded due to an unsuitable for serological test. Additionally, 367 of these sera were tested for anti-DENV IgG antibodies due to shortage of reagents for the ELISA IgG assays. To visually represent the distribution of ELISA absorbance values, scatter plots were generated for both anti-DENV IgM and IgG assays after subtraction of substrate blank. These scatter plots display individual OD readings substrate blank subtraction, assisting the visual assessment of serological result patterns. The average upper and lower OD cut-off values for IgM kits were approximately 0.517 and 0.357, respectively, while upper and lower cut-off values for the IgG kits were 0.550 and 0.395, respectively. These thresholds were employed to categorize results into positive, borderline and negative (Figures 4.2 and 4.3).

Of those tested, a quarter (24.6%; 95% CI: 20.4-28.8%)) of sera tested positive for anti-DENV IgM antibodies, indicating recent or acute DENV infection, while 21.0% of the sera were found to be borderline for anti-DENV IgM antibodies(Figures 4.4A) On the other hand, among 367 sera tested for anti-DENV IgG antibodies, 38.7% (95% CI: 33.7-43.7%) tested positive, suggesting previous exposure to DENV infection, while 20.4% showed borderlines for anti-DENV IgG antibodies(Figures 4.4B). Among 367 sera tested for both anti-DENV IgM and IgG antibodies, 196 (53.4%; 95% CI: 48.3-58.5%) sera tested positive for anti-DENV IgM and/ or IgG antibodies, of which 36(9.8%) sera found positive for both anti-DENV IgM and IgG antibodies ((Figures 4.4C). The seroprevalence of anti-DENV IgM antibodies was comparable between males and females (25.1% vs. 24.2%), while females exhibited a higher positivity for anti-DENV IgG antibodies (45.0 vs. 32.3%)($p < 0.05$) (Table 4.7).

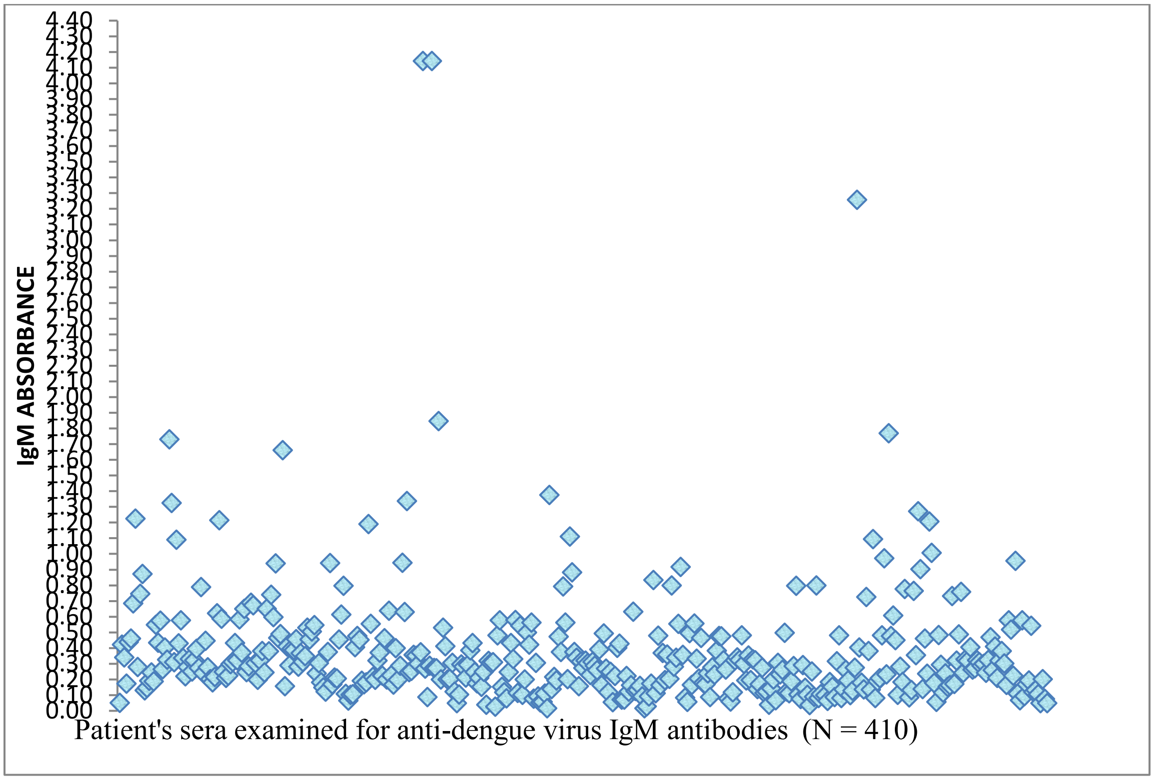

Figure 4.2: Scatter plot of anti-DENV IgM ELISA absorbance values

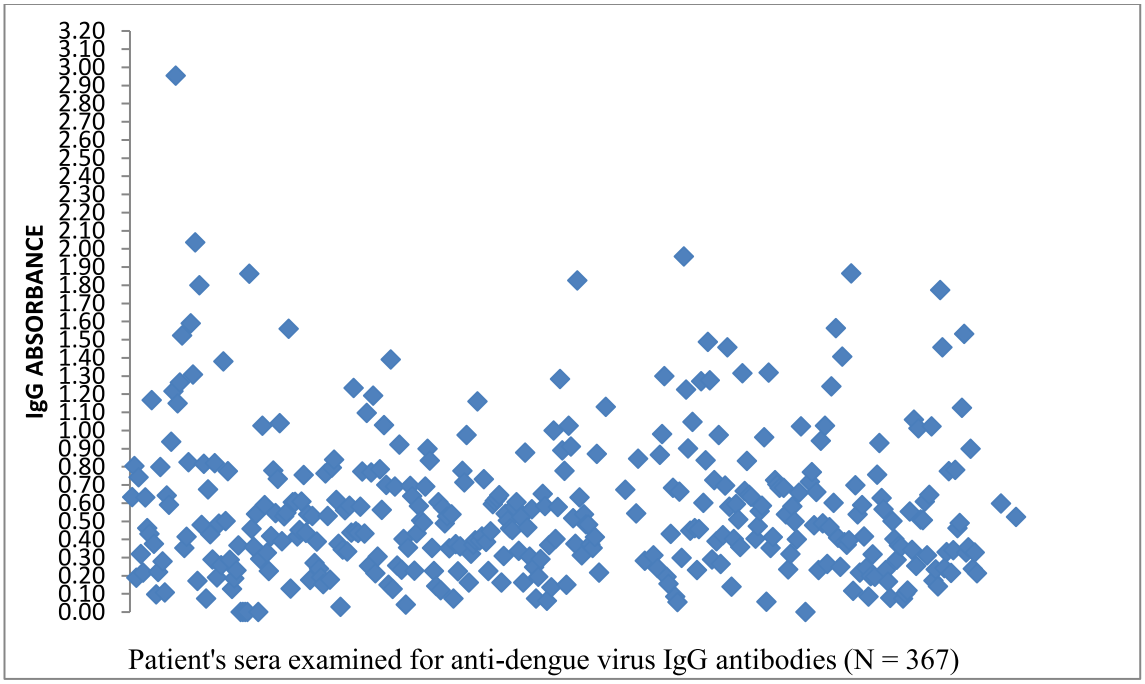

Figure 4.3: Scatter plot of anti-DENV IgG ELISA absorbance values

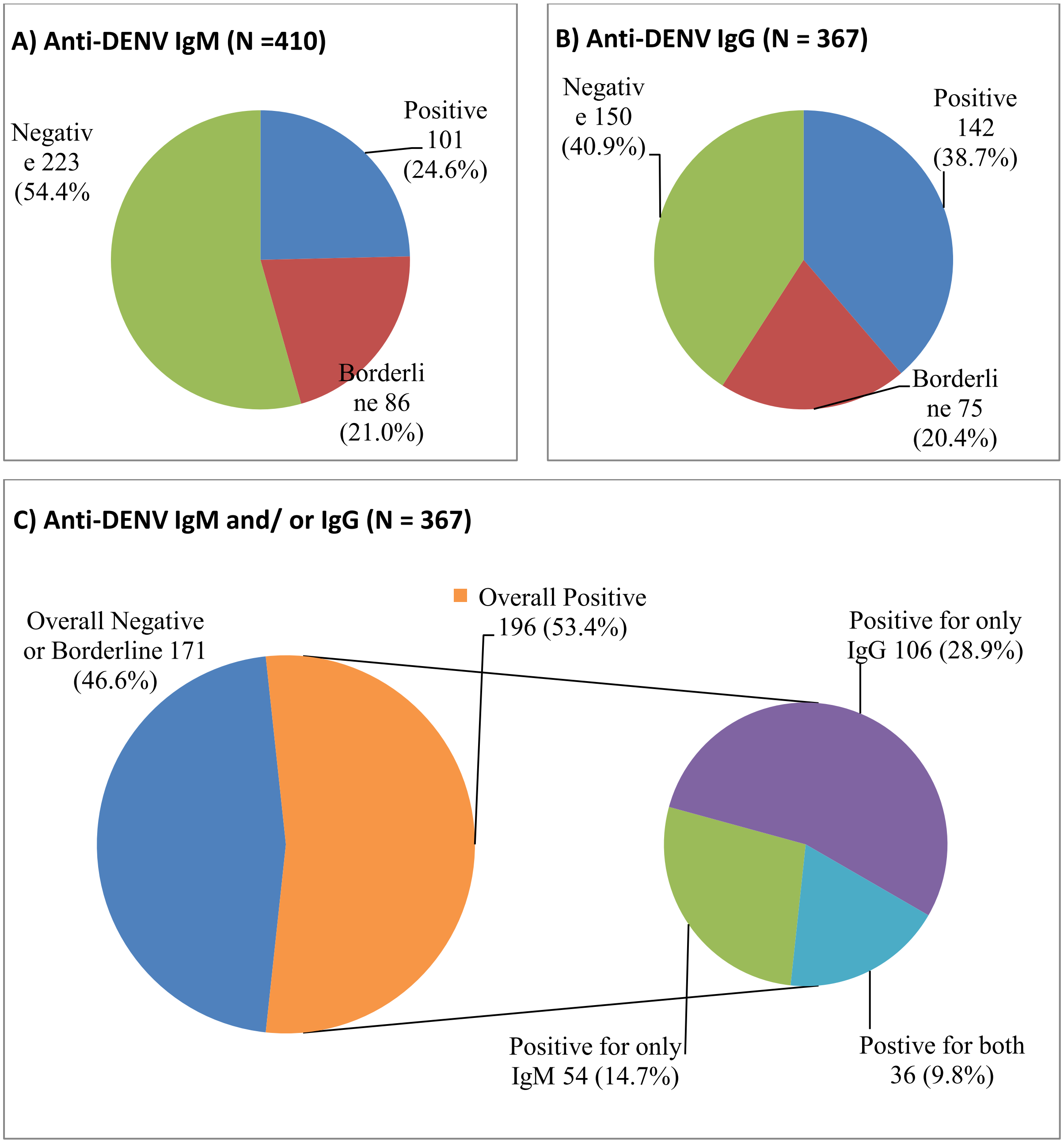

Figure 4.4: Prevalence of anti-DENV antibodies: (A) Anti-DENV IgM (N =410), (B) Anti-DENV IgG (N = 367) and (C) Anti-DENV IgM and IgG (N = 367)

Table 4.7: Distribution of seroprevalence of anti-DENV antibodies in sociodemographic features among febrile patients in selected districts in the Afar National Regional State, Northeast Ethiopia, from September 2022 to March 2023

		Anti-Dengue IgM (N = 410)			Anti-Dengue IgG (N = 367)		
Variable		N° tested	N° +ve (%)	X^2; p value	N° tested	N° +ve (%)	X^2; p value
Sex	Male	183	46(25.1)	0.045; 0.832	167	54(32.3)	**6.123;0.013**
	Female	227	55(24.2)		200	90(45.0)	
Age (years)	5-10	40	5(12.5)	4.950; 0.422	37	21(56.8)	10.045; 0.074
	11-20	105	28(26.7)		99	35(35.4)	
	21-30	148	36(24.3)		129	42(32.6)	
	31-40	57	18(31.6)		48	23(47.9)	
	41-50	34	8(23.5)		30	14(46.7)	
	51 and above	26	6(23.1)		24	9(37.5)	
Educational status	No formal education	191	46(24.1)	3.484; 0.323	175	67(38.3)	0.336; 0.953
	Grade 1-8th	126	32(25.4)		109	45(41.3)	
	Grade 9-12th	70	14(20.0)		66	25(37.9)	
	College and above	23	9(39.1)		17	7(41.2)	
Resident	Urban	206	49(23.8)	0.160; 0.689	192	82(42.7)	2.035; 0.154
	Rural	204	52(25.5)		175	62(35.4)	
Occupational status	Agro/pastoralist	41	11(26.8)	1.048; 0.790	33	8(24.4)	5.147; 0.161
	Government/private worker	148	40(27.0)		134	59(44.0)	
	Housewife	114	26(22.8)		101	36(35.6)	
	Students	107	24(22.4)		99	41(41.4)	
Marital status	Married	248	66(26.6)	0.923; 0.630	218	80(36.7)	5.228; 0.073
	Non-married	98	21(21.4)		90	44(48.9)	
	Children under 18	64	15(23.4)		59	21(35.6)	
Religion	Muslim	251	54(21.5)	3.394; 0.065	222	85(38.3)	0.212; 0.645
	Christians	159	47(29.6)		145	59(40.7)	
Ethnicity	Afar	199	44(22.1)	1.326; 0.249	170	61(35.9)	1.495; 0.221
	Others	211	57(27.0)		197	83(42.1)	
Districts	Amibara	244	64(26.2)	2.545; 0.280	225	85(37.8)	1.217; 0.544
	Awash Sebat	125	31(24.8)		121	52(43.0)	
	Harunka	41	6(14.6)		21	7(33.3)	
Health institutes	Awash Arba	200	58(29.0)	7.130; 0.068	200	75(37.5)	1.275; 0.735
	Awash Sebat	125	31(24.8)		121	52(42.9)	
	Sidafage	44	6(13.6)		25	10(40.0)	
	Andido	41	6(14.6)		21	7(33.3)	

4.2.4. Risk factors Linked to recent and previous DENV infection

Both univariable and multivariable logistic regression analyses revealed no significant difference in the positivity rates for anti-DENV IgM antibodies between females and males. However, the odds of anti-DENV IgG antibodies positivity were significantly higher in females, with COR of 1.71 (95% CI: 1.12-2.62), and AOR of 2.24 (95% CI: 1.32-3.26). In a univariable regression analysis, age demonstrated significant association with anti-DENV IgM antibodies, particularly the odds of positivity in participants aged 31-40 years was more than three times than odds of positivity in the reference age group (children aged 5-10 years) (COR = 3.23; 95% CI: 1.08-9.62). This association, however, lost significance in the multivariable analysis (Table 4.8).

Study sites/location influenced the odds of positivity for anti-DENV IgM antibodies; participants from Sidafage and Andido HCs displayed lower odds of positivity compared to those from Awash Arba HC, with AORs of 0.25 (95% CI: 0.09-0.71) and 0.35 (95% CI: 0.12-0.97), respectively. Occupational status was also found a factor affecting positivity of anti-DENV IgG antibodies, with government and private sectors employees displayed higher odds of positivity compared to pastoralist and agro-pastoralists, with COR of 2.46 (95% CI: 1.03-5.84) and AOR of 1.90 (95% CI: 1.03-8.15). Furthermore, specific symptoms were linked to positivity of anti-DENV IgG antibodies, with patients reporting muscle pain exhibiting higher odds of positivity (AOR = 2.26; 95% CI: 1.21-4.191) (Table 4.8).

Table 4.8: Seropositivity of anti-DENVantibodies and associated sociodemographic and clinical features among febrile patients in the selected districts in the Afar National Regional State, Northeast Ethiopia, from September 2022 to March 2023

Variable		Anti-DENV IgM		Anti-DENV IgG	
		COR (95%CI)	AOR (95%CI)	COR (95%CI)	AOR (95%CI)
Sex	Male	1	1	1	1
	Female	0.95(0.61-1.50)	1.14(0.66-1.99)	**1.71(1.12-2.62)**	**2.24(1.32-3.26)**
Age (years)	5-10	1	1	1	1
	11-20	2.54(0.91-7.15	2.56(0.82-7.99)	0.42(0.19-0.90)	0.42(0.17-1.05)
	21-30	2.25(0.82-6.17)	1.88(0.53-6.64)	0.37(0.17-0.78)	0.44(0.15-1.26)
	31-40	**3.23(1.08-9.62)**	2.25(0.54-9.48)	0.70(0.29-1.66)	0.94(0.26-3.40)
	41-50	2.15(0.63-7.35)	1.48(0.31-7.06)	0.67(0.25-1.75)	0.72(0.18-2.79)
	51 and above	2.10(0.57-7.76)	1.42(0.27-7.45)	0.46(0.16-1.31)	0.63(0.15-2.72)

Education status	No formal education	1	1	1	1
	Grade 1-8th	1.07(0.64-1.81)	1.26(0.62-2.58)	1.13(0.70-1.85)	0.95(0.46-1.94)
	Grade 9-12th	0.79(0.40-1.54)	0.62(0.26-1.47)	0.98(0.55-1.76)	1.01(0.45-2.25)
	College or above	2.03(0.82-4.99)	1.87(0.65-5.38)	1.13(0.41-3.11)	0.88(0.27-2.83)
Resident	Urban	1	1	1	1
	Rural	1.10(0.70-1.72)	1.53(0.82-2.84)	0.74(0.48-1.12)	0.82(0.45-1.53)
Occupational status	Agro/pastoralist	1	1	1	1
	Gov./private worker	1.01(0.46-2.20)	1.12(0.45-2.78)	**2.46(1.03-5.84)**	**1.90(1.03-8.15)**
	Housewife	0.81(0.35-1.82)	0.73(0.28-1.94)	1.73(0.71-4.23)	1.65(0.55-4.91)
	Students	0.79(0.34-1.80)	1.170.36-3.79)	2.21(0.91-5.38)	2.13(0.62-7.29)
Marital status	Married	1	1	1	1
	Non-married	0.76(0.44-1.34)	0.63(0.29- 1.38)	**1.75(1.06-2.88)**	2.02(0.69- 5.95)
	Children under 18	0.86(0.45-1.64)		1.01(0.55-1.85)	1.14(0.52-2.50)
Religion	Muslim	1	1	1	1
	Christians	1.53(0.97-2.41)	1.59(0.77-3.28)	1.11(0.72-1.70)	0.85(0.43-1.66)
Ethnicity	Afar	1	1	1	1
	Others	1.30 (0.83-2.50)	0.94(0.45-1.96)	1.30(0.85-1.98)	1.07(0.53-2.15)
Districts[a]	Amibara	1		1	1
	Awash Sebat	0.93(0.56-1.20)		1.24(0.79-1.95)	
	Harunka	0.48(0.19-3.99)		0.82(0.31-2.12)	
Health care institute	Awash Arba	1	1	1	
	Awash Sebat	0.81(0.48-1.34)	0.85(0.44-1.66)	1.26(0.79-1.99)	0.98(0.53-1.81)
	Sidafage	**0.39(0.15-0.96)**	**0.24(0.08-0.69)**	1.11(0.47-2.60)	1.02(0.38-2.72)
	Andido	0.42(0.17-1.05)	**0.35(0.12-0.97)**	0.83(0.32-2.16)	0.66(0.21-2.01)
Illness duration	≤ 5 days	1	1	1	1
	>5 days	1.20(0.48-2.97)	1.36(0.49-3.79)	1.76(0.73-4.26)	2.10(0.76-5.79)
Joint pain	No	1	1	1	1
	Yes	1.18(0.71-1.97)	1.24(0.69-2.22)	0.90(0.57-1.43)	0.78(0.46-1.33)
Headache	No	1	1	1	1
	Yes	0.81(0.39-1.70)	0.89(0.39-2.03)	0.78(0.39-1.53)	0.76(0.35-1.68)
Back pain	No	1	1	1	1
	Yes	0.92(0.59-1.45)	0.84(0.50-1.41)	0.89(0.58-1.35)	1.04(0.63-1.71)
Chills	No	1	1	1	1
	Yes	0.91(0.58-1.43)	1.16(0.65-2.06)	0.95(0.62-1.45)	0.68(0.40-1.16)
Nausea	No	1	1	1	1
	Yes	0.85(0.53-1.37)	0.77(0.45-1.33)	1.42(0.92-2.19)	1.41(0.87-2.30)

Malaise	No	1	1	1	1
	Yes	0.99(0.57-1.70)	0.85(0.46-1.57)	0.61(0.36-1.04)	0.59(0.32-1.09)
Muscle pain	No	1	1	1	1
	Yes	1.24(0.73-2.12)	1.29(0.69-2.43)	1.57(0.94-2.62)	**2.26(1.21-4.19)**
Rash	No	1	1	1	1
	Yes	3.12(0.62-15.72)	2.73(0.45-16.51)	1.56(0.31-7.84)	2.46(0.34-17.53)

'COR' crude odds ratio, 'AOR' adjusted odd ratio, 'CI' confidence interval, '^{a}' excluded from multiple logistic regression model due to collinearity

4.2.5. Prevalence of DENV, CHIKV and malaria co-infections

The seroprevalence of co-infections with acute DENV infection, acute CHIKV infection and/or malaria parasites was examined among participants who were tested for these infections. Out of 367 febrile patients diagnosed with acute DENV, acute CHIKV, and *plasmodium* infections, 40.3% of individuals had one of the three infections, 18.5% were found to be co-infected with two infections, and 1.9% of individuals were co-positive for all three infections.

Of 367 sera tested for anti-DENV and anti-CHIKV IgM antibodies, 58 samples (15.8%; (95% CI: 12.0-19.5%)) were co-positive for anti-DENV and anti-CHIKV IgM antibodies, indicating recent or acute co-infections of both viruses. similarly, of 410 patients diagnosed for both acute DENV and *Plasmodium* infections, 18 patients(4.4%; 95% CI: 2.4-6.4%) were co-positive for both anti-DENV IgM antibodies and *Plasmodium* infection, while among 368 study participants tested for both acute CHIKV infection and malaria parasites, 16 patients (4.3%) were co-positive for anti-CHIKV IgM and *Plasmodium* infections (Table 4.9).

Multivariable logistic regression analyses were carried out to identify potential risk factors associated with DENV-CHIKV co-infections. The analysis revealed no significant difference in the co-positivity rates across various sociodemographic and clinical features. However, the odds of co-positive for anti-DENV-CHIKV IgM antibodies were notably higher among rural residents than among urban residents, with an AOR of 2.23 (95% CI: 1.01-5.34). Additionally, a joint pain was associated with increased co-positivity, showing that patients with this symptoms had over 2.5 times odds of testing co-positive for anti-DENV-CHIKV IgM antibodies than those without the symptom (AOR = 2.28; 95% CI: 1.14-5.83) (Table

4.10). Due to the low number of positive results, logistic regression analysis to identify risk factors for DENV-malaria and CHIKV-malaria co-infections was not performed.

Table 4.9: Prevalence of co-infections of DF, malaria and CHIK among febrile patients in the Afar National Regional State, Northeast Ethiopia, from September 2022 to March 2023

Cases diagnosed	Results	Number (%)
Dengue-chikungunya co-infections (N = 367)	Positive for dengue or chikungunya IgM	151(41.1)
	Positive for both dengue & chikungunya IgM	57(15.8)
	Negative for both dengue & chikungunya IgM	158(43.1)
Dengue-malaria co-infection (N = 410)	Positive for either malaria & dengue IgM	106(25.8)
	Positive for both malaria & dengue IgM	18(4.4)
	Positive for both *falciparum* & dengue IgM	15(3.7)
	Positive for both only *vivax* & dengue IgM	4(1.0)
	Negative for both dengue IgM & malaria	283(69.0)
Malaria-chikungunya co-infection (N = 368)	Positive for both malaria & chikungunya IgM	16(4.3)
	Positive for either malaria or chikungunya IgM	183(49.7)
	Negative for both malaria & chikungunya IgM	169(45.9)
Dengue-malaria-chikungunya co-infection (N = 367)	Co-infection with all three infections	7(1.9)
	Co-infection with either of the two infections	68(18.5)
	Positive for only one infections	148(40.3)
	Negative for all infections	144 (39.2)

Table 4.10: Seropositivity for dual anti-DENV-CHIKV IgM antibodies, associated sociodemographic and clinical features in the selected districts in the Afar National Regional State, Northeast Ethiopia, from September 2022 to March 2023

Variable		N° tested	N° +ve (%)	COR (95%CI)	AOR (95%CI)
Sex	Male	164	22(13.4)	1	1
	Female	203	36(17.7)	0.97(0.64-1.47)	1.66(0.81-3.40)
Age (years)	5-10	35	3(8.6)	1	1
	11-20	93	21(22.6)	0.72(0.32-1.66)	2.29(0.53-9.94)
	21-30	137	19(13.9)	0.59(0.27-1.29)	1.22(0.21-7.24)
	31-40	49	9(18.4)	0.66(0.27-1.66)	1.69(0.24-11.86)
	41-50	28	3(10.7)	0.40(0.14-1.11)	0.79(0.09-7.16)
	51 and above	25	3(12.0)	**0.26(0.09-0.76**	0.83(0.09-7.56)
Educational status	No formal education	168	26(15.5)	1	1
	Grade 1-8th	117	18(15.4)	1.17(0.73-1.89)	1.19(0.46-3.04)
	Grade 9-12th	62	9(14.5)	1.02(0.57-1.83)	0.87(0.28-2.71)
	College and above	20	5(25.0)	0.79(0.31-1.99)	2.40(0.59-9.76)

Resident	Urban	197	27(13.7)	1	1
	Rural	170	31(18.2)	1.00(0.67-1.52)	**2.23(1.01-5.34)**
Occupational status	Agro/pastoralist	41	7(17.1)	1	1
	Government/private worker	129	18(13.9)	0.92(0.45-1.88)	0.85(0.28-2.59)
	Housewife	104	16(15.4)	0.62(0.29-1.28)	0.68(0.21-2.26)
	Students	93	17(18.3)	1.01(0.48-2.15)	1.41(0.33-6.02)
Marital status	Married	222	36(16.2)	1	1
	Non-married	88	15(17.1)	**2.18(1.29-3.**68)	0.62(0.16- 2.39)
	Children under 18	57	7(12.3)	1.53(0.85-2.78)	0.53(0.17-1.61)
Religion	Muslim	226	35(15.5)	1	1
	Christians	141	23(16.3)	1.13(0.74-1.74)	0.97(0.40-2.37)
Ethnicity	Afar	177	27(15.2)	1	1
	Others	190	31(16.3)	0.95(0.63-1.43)	1.26(0.50-3.15)
Healthcare institute	Awash Arba	165	30(18.2)	1	1
	Awash Sebat	125	20(16.0)	1.35(0.85-2.16)	0.98(0.43-2.26)
	Sidafage	36	4(11.1)	**2.19(1.01-4.74)**	0.32(0.09-1.15)
	Andido	41	4(9.8)	1.86(0.91-3.80)	0.37(0.11-1.26)
Illness on-set duration	≤ 5 days	344	55(16.0)	1	1
	>5 days	23	3(13.0)	1.19(0.50-2.82)	0.78(0.20-3.09)
Joint pain	No	105	9(8.6)	1	1
	Yes	262	49(18.7)	1.22(0.78-1.94)	**2.58(1.14-5.83)**
Headache	No	34	7(20.6)	1	1
	Yes	333	51(15.3)	0.92(0.45-1.88)	0.76(0.27-2.11)
Back pain	No	182	30(16.5)	1	1
	Yes	185	28(15.1)	1.08(0.71-1.63)	0.87(0.46-1.67)
Chills	No	200	34(17.0)	1	1
	Yes	167	24(14.4)	1.36(0.91-2.07)	0.90(0.43-1.88)
Nausea	No	237	40(16.9)	1	1
	Yes	130	18(13.8)	1.05(0.68-1.61)	0.68(0.34-1.36)
Malaise	No	287	48(16.7)	1	1
	Yes	80	10(12.5)	0.74(0.45-1.22)	0.65(0.29-1.48)
Muscle pain	No	286	41(14.3)	1	1
	Yes	81	17(21.0)	0.93(0.56-1.53)	1.61(0.76-3.41)
Rash	No	361	56(15.5)	1	1
	Yes	6	2(33.3)	1(emplty	2.93(0.37-23.22)

4.3. Molecular Detection of Viral RNA

To confirm the presence of viral RNA among seropositive serum for IgM antibodies, 53 pooled serum samples were prepared based on similarities in collection sites and IgM seropositivity for DENV and/or CHIKV, and tested using both in-house and commercial RT-PCR assays as described in Figure 4.3 & 4.4.

Among these 53 pools, three pools were identified as positive for DENV RNA, while two pools tested positive for CHIKV RNA. Specifically, from 15 pools prepared from dual-positive for DENV and CHIKV IgM-positive sera, two pools tested positive for DENV RNA using in-house RT-PCR assay, as well as two positive pools for CHIKV RNA using the commercial real-time RT-PCR assay. Both DENV and CHIKV RNA positive pool samples were from either Awash Arba HC or Awash Sebat HC, with DENV RNA positive pooled sera from Awash Arba was co-positive for CHIKV RNA by each test. The DENV RNA amplicons displayed the expected 107 base pair band size when visualized on 2.5% agarose gel electrophoresis.

Out of 28 pools prepared from sera positive only for anti-CHIKV IgM antibodies, one pool was tested positive for DENV RNA with in-house RT-PCR, while no viral RNA was detected in 10 pools prepared from anti-DENV IgM-only positive sera. No RNA of ZIKV and WNV were detected in any of the tested pools (Figure 4.5 & 4.6).

However, upon subsequent individual testing of all serum samples comprising the RNA-positive pools, no viral RNA was detected in any single sample.

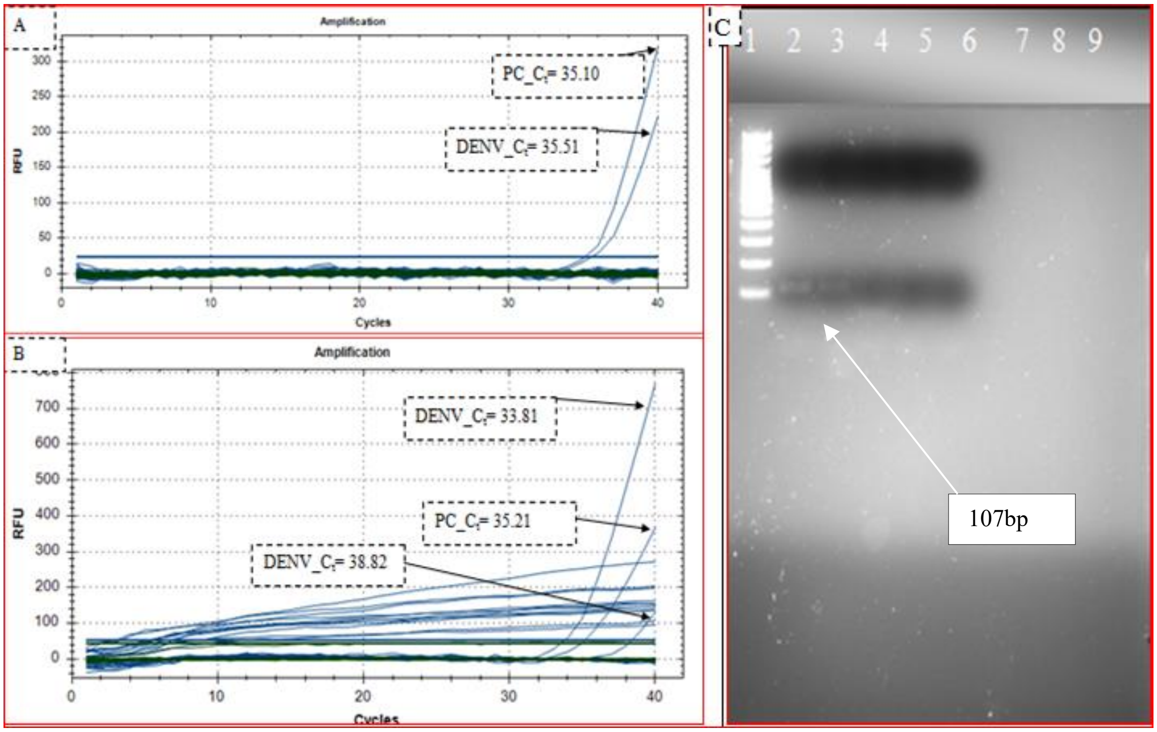

Figure 4.5: An in-house RT-PCR amplification for DENV in pool serum samples: (A-B) Curves plots of DENV; (C) Agarose gel Electrophoresis of validation of in-house RT-PCR in pool clinical samples. Lane 1: 100-bp molecular marker; lane 2: DENV with Ct 33.81; lane 3: DENV with Ct 38.82; lanes 4 and 5: samples without curves; lane 6: no template control

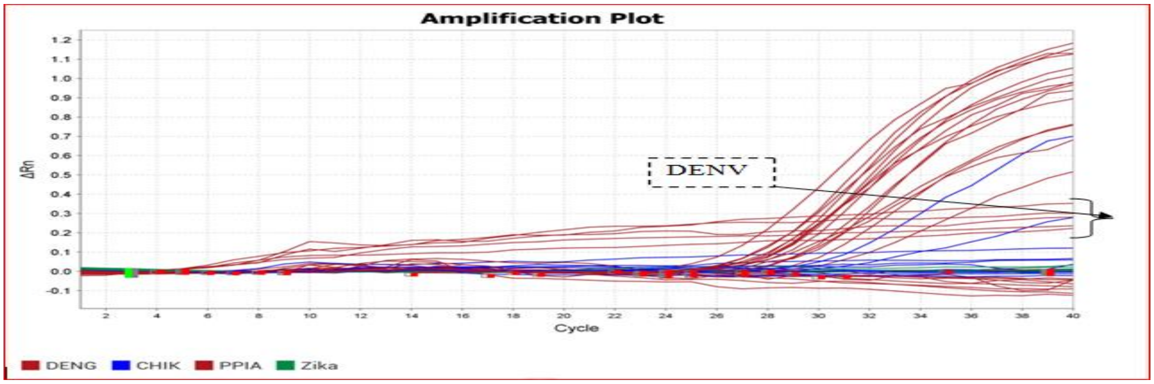

Figure 4.6: Pools of samples analysis using a one-step multiplex RT-PCR amplification for the detection of DENV, ZIKV and CHIKV

4.4. Community Knowledge, Attitude and Practices about Chikungunya

4.4.1. General Sociodemographic features of the study participants

In the quantitative study, 296 community members (63.2% males, age ranging from 19 to 80 years with a mean (±SD) of 34.2 (±10.6) years) participated. Most of the participants (81.4%) were married, and almost half (48.3%) were between the age of 30-44 years. In the qualitative study, 116 participants (63.8% males, age ranging from 18-80 years) participated. Three of the FGDs comprised only females. The majority of the participants (41.4%) were in the age range between 30-44 years, and only a small proportion of participants (11.2%) had formal education (Table 4.11).

Table 4.11: Sociodemographic characteristics of the study participants involved in the KAP study, selected districts of Afar National Regional State, Northeast Ethiopia

General characteristics		Number of participants (%)
Household survey participants (N= 296)		
Sex	Male	187 (63.2)
	Female	109 (36.8)
Age groups (years)	18-29	109 (36.8)
	30-44	143 (48.3)
	45-59	35 (11.8)
	60 or above	9 (3.0)
Religion	Muslim	179 (60.5)
	Orthodox	102 (34.5)
	Protestant	15 (5.0)
Ethnicity	Afar	125 (42.2)
	Oromo	27 (9.1)
	Amhara	58 (19.6)
	Others	86 (29.0)
Marital status	Married	241(81.4)
	Single	55 (18.6)
Education status	Illiterate	92 (31.1)
	Grade 1-8th	74 (25.0)
	Grade 9-12th	78 (26.3)
	College or above	52 (17.6)
Occupation	Agro/pastoralist	70 (23.7)
	Government/private worker	186 (63.1)
	Housewife	30 (10.2)
	Students	9 (3.0)

Districts	Amibara	157 (53.0)
	Awash Sebat	139 (47.0)
Kebeles	Awash Arba	124 (41.9)
	Sidafage	32 (10.8)
	Regale	78 (26.3)
	Kedeba	62 (21.0)
Focus Group Discussion participants(N= 116)		
Sex	Male	74 (63.8)
	Female	42 (36.2)
Age group (years)	18-29	42 (36.2)
	30-44	48 (41.4)
	45-59	14 (12.1)
	60 or above	12 (10.3)
Education status	Illiterate	103 (88.8)
	Primary and above	13(11.2)

4.4.2. Community Awareness about common febrile and mosquito-borne illnesses

Majority of the study participants (91.2%) reported malaria as the most prevalent mosquito-borne febrile illness in the study area. Typhoid was mentioned as the second most frequent febrile illness (68.9%), followed by typhus (44.6%). About 39.9% of participants said that CHIK is a cause of febrile illness in the area. Of the total number of community members who participated, 199 (67.3%) reported that they heard about CHIK disease locally called *Tilobign*. Among those who had heard about CHIK, only 2 (2.0%) were also aware of dengue fever. About half (49.3%) of the participants reported that they obtained information about CHIK at a health facility during a previous outbreak. Others heard about CHIK through *“Dagu*" (information-sharing system by the local people) (34.2%), while very few obtained the information through media and meetings (Table 4.12).

During the FGD, it was observed that the participants were familiar with malaria as a common febrile illness and a disease transmitted by mosquitoes. However, some participants believed that malaria could also be contracted through physical contact (sleeping with a patient who has a fever). The majority of the participants identified *Anopheles* mosquito, which they called *'kagna'ata'*, as the primary vector of malaria. It was generally agreed upon that CHIK is a newly emerged disease, potentially originating from Eastern Ethiopia, Dire Dewa city, and is locally known as *'tilobign'*. However, the participants had no adequate information on how it can be transmitted.

Table 4.12: Participants 'knowledge about frequent febrile illnesses and information about CHIK & DF in selected districts of Afar National Regional State, Northeast Ethiopia (N =296)

		No. Respondents (%)
What are/is Common febrile illness in this area?	Malaria	270 (91.2)
	Typhoid	204 (68.9)
	Typhus	132 (44.6)
	Dengue	0
	Chikungunya	118 (39.9)
	Non-specific disease like cough, pneumonia, etc	67 (22.6)
Have you heard about CHIK and DEN diseases?	Yes	199 (67.2)
	No	97 (32.8)
About which diseases did you hear?	Only CHIK	197 (99.0)
	Both CHIK and DEN	2 (1.0)
Source of information	Media	22 (11.1)
	'*Dagu*'*	68 (34.2)
	Teacher/Education	3 (1.5)
	Seminar/meeting	8 (4.0)
	In health facility during outbreaks	98 (49.3)

CHIK = Chikungunya, DEN = Dengue, *traditional information sharing system of the Afar community

4.4.3. Community Knowledge regarding chikungunya virus and prevention

This study unveiled both quantitative and qualitative data revealed comparable results. The quantitative analysis revealed an overall mean knowledge score regarding CHIKV of 13.9 (SD: ±3.0), which ranged from 5 to 20 out of a possible 22 points. The overall mean correct response rate of study participants was found to be 63.2% (13.9/22*100), indicating a moderate level of knowledge. Within the knowledge assessment, the correctness of responses varied significantly; the lowest rates of correct answer among those who were asked that specific question was 16.5% for the question "When does *Aedes* mosquito bite?", while the highest was 90.9% for "Does CHIK cause joint pain?".

Furthermore, the findings indicated that 44.7% of the participants correctly identified CHIK as a viral disease, and 56.8% knew that not all mosquitoes transmit CHIK. About 48.7% of the respondents were able to recognize the *Aedes* mosquito, while only 16.5% knew that it bites during daytime. Most of the participants knew that CHIK is not spread by food and water (74.4%), ticks (73.9%), and flies (72.9%). However, 40.7% of the study participants believed that person-to-person contact could transmit CHIK. More than half of the participants (57.3%) recommended using pesticide sprays to control mosquitoes and prevent CHIK. Additionally, a large number of participants were able to identify the common symptoms of CHIK such as fever (89.9%), headache (89.5%), joint pain (90.9%), and muscle pain (75.4%)(Table 4.13).

During the FGDs, it was noticed that most participants were aware of the common signs and symptoms of CHIK, which include fever, joint pain, headache, and back pain. However, their understanding of how CHIKV spreads and the role of the *Aedes* mosquito was limited. While some participants thought that the disease was caused by Allah (God), others believed that it could be spread through personal contact or bed sharing with CHIK patients. Interestingly, the FGD participants gave some accurate suggestions on how to protect and control mosquitoes, such as using bed nets, clearing stagnant water, fumigating, and using indoor chemical insecticide spray. The summary of the FGD participants' knowledge about CHIK and mosquitoes are highlighted as follows:

A 40-year-old man shared his experience of bringing his 9-year-old daughter to a health centre due to fever and joint pain. At the centre, he observed many patients with similar symptoms to his daughter. He was told for the first time that it was an outbreak of a disease called CHIK, locally known as "Tilobign." However, he did not hear how it transmits to humans or whether it would affect him or his other family members.

A 30-year-old woman shared her knowledge about two types of mosquitoes. She explained that the Anopheles mosquito is a carrier of malaria, locally known as 'Danaso'. This mosquito usually bites at night and can be prevented by using bed nets. Anopheles mosquitoes breed on plants during the rainy season. However, she had no information about the Aedes mosquito. Another 35-year-old woman described the Aedes mosquito, which can be recognized by its stripes that resemble military uniforms and is locally known as 'kagna'ata' similar to anopheles but differs in color. This mosquito is known for its painful bite and bites during the daytime. It breeds in the forest and is difficult to protect. She believed that this mosquito is transmitting diseases such as malaria, HIV, and TB, but she had no information whether it transmits CHIK. A 20-year-old man who holds a bachelor's degree in business management explained that CHIK is a febrile illness similar to malaria, but it is characterized *by joint pain. While it can affect people of all ages, it is more common in adults. CHIK is a newly emerging disease, originated from animals and is transmitted through personal contact.*

Table 4.13: knowledge about CHIK etiology, transmission, signs, symptoms and preventions in selected Districts of Afar National Regional State, Northeast Ethiopia (N = 199)

Knowledge aspect questions used in the study		Number of Respondents (%)
Is CHIK caused by virus?	**Yes**	89 (44.7)
	No	86 (43.2)
	Not sure	24 (12.1)
Can all mosquitoes transmit CHIK virus?	Yes	76 (38.2)
	No	113 (56.8)
	Not sure	10 (5.0)
Can you identify *Aedes* mosquito?	**Yes**	97 (48.7)
	No	102 (51.3)
When does *Aedes* mosquito bite?	**Day time**	16 (16.5)
	Night time	44 (45.4)
	Any time	37(38.0)
Do flies transmit CHIK?	Yes	39 (19.6)
	No	145 (72.9)
	Not sure	15 (7.5)
Do ticks transmit CHIK?	Yes	35 (17.6)
	No	147(73.9)
	Not sure	17 (8.5)
Do person-to-person contacts transmit CHIK?	Yes	81 (40.7)
	No	110 (55.3)
	Not sure	8 (4.0)
Is CHIK transmitted through food and water?	Yes	46 (23.1)
	No	148 (74.4)
	Not sure	5(2.5)
Do mosquitoes breed in stagnant water?	**Yes**	166 (83.4)
	No	31 (15.6)
	Not sure	2 (1.0)
Do window screen and bed net reduce mosquitoes?	**Yes**	167 (83.9)
	No	16 (16.1)
	Not sure	0 (0.0)
Do insecticide sprays reduce mosquitoes and prevent CHIK?	**Yes**	114 (57.3)
	No	79 (39.7)
	Not sure	6 (3.0)
Do tightly covering water containers reduce mosquito?	**Yes**	146 (73.4)
	No	52 (26.1)

	Not sure	1 (0.5)
Do mosquitoes repellent prevent mosquitoes' bites?	**Yes**	125 (62.8)
	No	66 (33.2)
	Not sure	8 (4.0)
Is the rainy season when CHIK present?	**Yes**	128 (64.3)
	No	64 (32.2)
	Not sure	7 (3.5)
Does CHIKV affect all age groups	**Yes**	141 (70.8)
	No	57 (28.6)
	Not sure	1(0.5)
Does CHIK cause fever?	**Yes**	179 (89.9)
	No	20 (10.1)
Does CHIK cause joint pain?	**Yes**	181 (90.9)
	No	18 (9.1)
Does CHIK cause headache?	**Yes**	178 (89.5)
	No	21 (10.5)
Does CHIK cause muscle pain?	**Yes**	150 (75.4)
	No	49 (24.6)
Does CHIK cause bone pain?	**Yes**	73 (36.7)
	No	126 (63.3)
Does CHIK cause nausea?	**Yes**	94 (47.2)
	No	105 (52.8)
Does CHIK cause rash?	**Yes**	55 (27.6)
	No	144 (72.4)
Overall knowledge score	Mean (± SD)= 13.9 (±3.0); Range = 5-20	

Note: Those with **bolds** are correct answer responses

4.4.4. Attitudes or perceived risk towards Chikungunya

In the quantitative study, the community exhibited an overall mean attitude score of 9.0 (SD: ±3.1), with score ranging from zero to 14 out of a possible 15 points. The overall mean positive attitude was found to be 60.0% (9.0/15*100), indicating a moderate positive attitude towards CHIK disease. Among the attitude-related inquiries, the question with the lowest positive response rates (23.7%) was "Do you agree that health service stakeholders are the only entity responsible to reduce larvae and adult mosquitoes in your home?". Conversely, the highest positive response rates (73.9%) was "Do you agree that everyone with sign and symptoms of CHIK need to consult immediately community health services?". Additionally, notable proportion of participants expressed strong agreement or agreement that CHIK can be prevented (68.8%) and that every individual plays a critical role in prevention CHIK (70.3%).

However, less than half of the participants (47.3%) strongly agreed/agreed that water accumulation around the house in discarded tires, broken pots, and bottles serves as breeding places for mosquitoes, such as *Aedes*. In contrast, 64.8% of participants strongly agreed or agreed that controlling mosquito-breeding places constitutes an effective strategy for preventing CHIKV infection (Table 4.14).

During FGDs, a few participants articulated their believed and perception that CHIK causes a serious disease with origins potentially linked to Dire Dewa city or super natural and could potentially be fatal. The perception of FGD participants regarding the risk associated with CHIK and mosquitoes are summarized as follows: *An 18-year-old high school student correctly identified CHIK as a new disease that originates from Dire Dawa city, and is transmitted to humans through the bites of Aedes mosquitoes. In Amibara village, a 28-year-old woman mentioned that there was a risk of CHIK in Awash Arba and Awash Sebat cities 2-3 years ago. However, at present, the disease does not exist in the community. She said that I do not know how it spreads, and she suggested that Allah causes it.*

Table 4.14: Participants' attitude and perceived risks towards CHIK and transmitting vectors in selected Districts of Afar National Regional State, Northeast Ethiopia (N = 199)

Attitude aspect questions	Number of Response of the participants (%)				
	S/ agree	**Agree**	Not Sure	Disagree	S/disagree
Do you agree that CHIK is a serious illness?	29 (14.6)	61 (30.7)	54 (27.1)	50 (25.1)	5 (2.5)
Do you agree CHIK is preventable?	41(20.6)	96(48.2)	40(20.1)	22 (11.1)	0 (0.0)
Do you agree that water around house in discarded tires, broken pots and bottles are breeding place for mosquitoes like *Aedes*?	35 (17.6)	59 (29.7)	101 (50.7)	4 (2.0)	0 (0.0)
Do you agree that controlling mosquitoes breeding places are good strategy to prevent CHIK?	34 (17.1)	95 (47.7)	57 (28.6)	13 (6.5)	0
Do you agree that communities should actively participate in controlling the CHIK vector?	31 (15.6)	85 (42.7)	56 (28.1)	27 (13.6)	0
Do you agree that everyone has a chance to be infected by CHIK?	43 (21.6)	88 (44.2)	36 (18.1)	31 (15.6)	1 (0.5)
Do you agree that everyone with sign and symptoms of CHIK need to	42 (21.1)	105 (52.8)	38 (19.1)	13 (6.5)	1 (0.5)

consult immediately community health services?					
Do you agree that everyone is a key in preventing CHIK?	36(18.1)	104(52.3)	44 (22.1)	15 (7.5)	0
Do you agree that all CHIK patients have a chance for a full recovery?	37(18.6)	99(49.7)	39 (19.6)	22 (11.1)	2 (1.0)
Do you agree that health service stakeholders are the only entity responsible to reduce larvae and adult mosquitoes in your home? **	26(13.1)	77(38.9)	48 (24.2)	44 (22.2)	3 (1.5)
Do you agree that your area is CHIK high-risk area?	31(15.6)	81 40.7)	49 (24.6)	36 (18.1)	2 (1.0)
Do you agree that the communities are capable of preventing CHIK?	39 (19.6)	89 (44.7)	43 (21.6)	27 (13.6)	1 (0.5)
Do you agree that you are capable of preventing CHIK	32 (16.1)	99 (49.7)	40 (20.1)	28 (14.1)	0
Do you agree that government actions are needed to prevent CHIK?	28 (14.1)	99 (49.8)	35 (17.6)	33 (16.6)	4 (2.0)
Do you agree that you are responsible to prevent spread of mosquito in your home?	33 (16.7)	99 (50.0)	41 (20.7)	23 (22.1)	3 (1.5)
Overall attitude score	Mean (± SD)= 9.0(±3.1); Range = 0-14				

S = Strongly; Answers with **bolds** are correct answer responses; **positive responses were S/disagree or disagree

4.4.5. Practices Regarding Chikungunya virus infection prevention and Control

During the HHS based study and FGDs, several measures were discussed to reduce mosquito exposure. The overall mean practice score was 7.8 (SD: ± 2.5), ranging 1-13 and resulting 60.0% (7.8/13*100) overall mean correct response rates for CHIK practice for prevention and control. Cleaning of garbage/trash to reduce mosquito breeding sites (88.4%), draining ponds or degrading muddy/wet areas (81.4%), cutting down bushes (78.9%), disposing of water-holding containers such as tires, plastic, bottles, or broken pots (73.9%), and covering water containers around the home (70.3%) were commonly practiced measures to reduce mosquitoes. However, the measures attempted to avoid mosquito bites were relatively low, with 51.3% of respondents practicing using fans, 46.7% wearing long clothes when working in the bush, farm, and forest, and 36.2% using bed nets when sleeping during the day to protect mosquito bites (Table 4.15).

During the FGDs, it was understood that the participants did not take appropriate preventive measures to protect themselves from daytime mosquito bites that can cause CHIK. The main reason was the lack of awareness about the risk of daytime mosquito bites and the fact that they did not consider this mosquito as a vector of CHIK. Additionally, some participants reported that the CHIK outbreaks in the area did not concern them.

Table 4.15: Participants' practice related to preventive and control measures against CHIK in selected districts of Afar National Regional State, Northeast Ethiopia (N= 199)

Practice aspect questions		Number of responses (%)
Do you drain ponds or degrade muddy/wet areas to reduce mosquitoes?	**Yes**	162 (81.4)
	No	37 (18.6)
Do you cut down bushes too short to reduce mosquitoes?	**Yes**	157 (78.9)
	No	42 (21.1)
Do you clean garbage/trash to reduce mosquito-breeding sites?	**Yes**	176 (88.4)
	No	23 (11.6)
Do you dispose water holding containers like tires, plastic, bottles or broken pots?	**Yes**	147 (73.9)
	No	52 (26.1)
Do you cover water containers around in the home?	**Yes**	140 (70.3)
	No	59 (29.7)
Do you change the water of plant containers in the house every week?	**Yes**	68 (34.2)
	No	131 (65.8)
Do you check waste/garbage that can block water	**Yes**	118 (59.3)

flow around the home?	No	81 (40.7)
Do you participate in any of anti-chikungunya campaigns in the community?	**Yes**	79 (39.7)
	No	120 (60.3)
Do you check and clean drains/gutters/roofs before the rainy season?	**Yes**	105 (52.8)
	No	94 (47.2)
Do you empty frequently water filled containers and drain water around the house?	**Yes**	138 (69.3)
	No	61 (30.7)
Do you use fan to prevent mosquito biting	**Yes**	102 (51.3)
	No	97 (48.7)
Do you cover body with long sleeve clothes when working in the bush, farm, and forest	**Yes**	93 (46.7)
	No	106 (53.3)
Using bed net when sleeping during the day	**Yes**	72 (36.2)
	No	127 (63.8)
Overall Practice score	Mean (± SD)= 7.8 (±2.5); Range = 1-13	

Note: Those with **bolds** are correct answer responses

4.4.6. Correlation between KAP domains of the community

A Pearson correlation coefficient was used to evaluate the linear association among the three KAP domains. The three variables showed positive correlations ($p < 0.05$). The highest correlation was observed between knowledge and attitude scores, with $r(199) = 0.388$ and $p < 0.001$(Table 4.16).

Table 4.16: Correlations between knowledge, attitudes and practices relating to Chikungunya in the selected districts of the Afar National Regional State, Northeast Ethiopia

Variable	Observation	R	p value*
Knowledge- attitudes	199	0.388	<0.001
Knowledge-practices	199	0.202	0.004
Attitudes- practices	199	0.342	<0.001

*Correlation is significant at the 0.05 level

4.4.7. The KAP Scores and Associated with Sociodemographic factors

In the quantitative study, the mean scores for KAP were found to be 13.9±3.0 (range 5-20), 9.0±3.1 (range 0-14), and 7.8±2.5 (range 1-13), respectively. A multiple linear regression analysis was conducted to examine the relationship between KAP scores and sociodemographic characteristics. Educational status significantly influenced knowledge, with participants in 9-12th grade (14.6 ± 3.9) showing a 1.6-point increase in scores ($p = 0.020$), and those with a college education or above (14.8 ± 3.0) showing a 1.8-point increase ($p =$

0.016) as in comparison with the illiterate, indicating a positive impact of education on knowledge scores. Additionally, being a student (17.3 ± 1.4) was associated with a 3.5-point increase in knowledge scores (p = 0.009) in comparison with agro/pastoralist participants, suggesting the value of study. Marital status was found to be significant for attitude, with being single (10.2 ± 2.4) showing a 1.5-point increase in scores (p = 0.033) compared to married participants, indicating a positive influence on attitude scores. Moreover, participants aged 45-59 years demonstrated a 1.6-point increase the in attitude scores (9.68 ± 2.67) when compared with the 18-29 age group (p = 0.040). Individuals with the educational levels of 9-12th showed a 1.4-point increase in attitude score (9.73 ± 2.93) compared to illiterate participants (p = 0.038). However, no significant association was found between practices and sociodemographic characteristics (Table 4.17).

Table 4.17: The mean of KAPs scores and multiple linear regression analysis of KAP scores among sociodemographic factors in selected Districts of Afar National Regional State, Northeast Ethiopia(N = 199)

Variables		Knowledge score			Attitude score			Practice score		
		Mean(SD)	β value	p value	Mean(SD)	β value	p value	Mean(SD)	β value	p value
Sex	Male	13.93(3.08)	1		9.12 (3.03)	1		7.71 (2.56)	1	
	Female	13.97(3.00)	0.27	0.569	8.81 (3.28)	-0.03	0.99	8.06 (2.33)	0.74	0.067
Age (years)	18-29	14.35(2.63)	1		9.04 (2.82)	1		7.84 (2.21)	1	
	30-44	13.88(3.17)	0.15	0.750	8.83 (3.43)	0.39	0.443	7.69 (2.72)	0.03	0.945
	45-59	12.56(3.40)	-0.77	0.284	9.68 (2.67)	1.57	0.040	8.32 (2.19)	0.84	0.164
	60+	13.25(3.53)	0.23	0.837	9.15 (3.80)	1.08	0.374	7.63(3.42)	0.02	0.984
Ethnicity	Afar	13.79(2.99)	1		8.70 (2.90)	1		7.16 (2.52)	1	
	Non-Afar	13.93(3.09)	-0.90	0.113	9.22 (3.23)	-0.3	0.378	8.22 (2.38)	0.35	0.458
Religion	Muslim	13.54(2.99)	1		8.79 (3.19)	1		7.42 (2.52)	1	
	Christian	14.35(3.08)	0.72	0.196	9.37(2.99)	0.44	0.456	8.41(2.32)	0.52	0.263
Marital status	Married	13.61(3.12)	1		8.71 (3.22)	1		7.69 (2.54)	1	
	Single	14.88(2.54)	0.53	0.350	10.21 (2.38)	1.46	0.015	8.33 (2.34)	0.61	0.202
Educati	Illiterate	12.33(2.90)	1		7.98 (2.85)	1		7.05 (2.57)	1	

onal status	Grade 1-8	13.46(2.87)	0.84	0.210	9.10 (3.06)	0.93	0.192	8.04 (2.39)	0.53	0.345
	Grade 9-12	14.62(3.90)	1.60	0.016	9.73 (2.93)	1.45	0.038	8.02 (2.55)	0.61	0.267
	College or above	14.77(3.00)	1.78	0.012	8.93 (3.47)	0.59	0.433	8.07 (2.33)	0.65	0.277
Occupational status	Agro/pastoralist	12.37(2.99)	1		7.37 (4.00)	1		7.00 (2.65)	1	
	Gov./private worker	14.07(2.96)	0.88	0.223	9.34 (2.91)	1.38	0.074	8.13 (2.48)	0.34	0.572
	Housewife	12.95(3.15)	0.02	0.980	8.52 (2.83)	1.13	0.270	7.05 (1.93)	-0.87	0.282
	Students	17.29(1.38)	3.49	0.009	9.14 (3.29)	0.74	0.599	6.0 (1.63)	-1.87	0.095
Districts	Amibara	14.28(3.11)	1		9.04 (3.07)	1		8.04 (2.59)	1	
	Awash Sebat	13.56(2.97)	-0.66	0.145	9.02 (3.17)	0.29	0.578	7.66 (2.39)	-0.06	0.874

4.5. HCWS' Knowledge, Practices and Challenges in Diagnosing and Managing Arboviral and Non-Malarial Febrile Illnesses

4.5.1. Sociodemographic characteristics of HCWs participated in the study

From 88 frontline HCWs identified for the quantitative survey from six healthcare facilities, 82 (54.9% females, age ranged from 20-54 with a mean of 29.3±7.3 years old) completed the self-administered questionnaire (response rate: 93.2%). Most participants (59.8%) were between 20-29 years old, have a bachelor's degree (48.8%) and worked as nurses or midwives (70.7%). For the qualitative component, 12 HCWs were initially identified for IDI, but two withdrew voluntarily. Finally, 10 IDI participants, with 7 males, 5 holding a master's or doctoral degree, 4 working as physicians, and 5 having 3-5 years of clinical experience participated in the study (Table 4.18).

Table 4.18: General characteristics of HCWs participated in the study and fever-related illness diagnosis and management in the selected districts of the Afar National Regional State, Northeast Ethiopia

General characteristics		Number of respondents (%)	
		Quantitative(N = 82)	IDI (N = 10)
Sex	Male	37(45.1)	7 (70.0)
	Female	45(54.9)	3(30.0)

Age (years)	Mean ± SD (range)	29.3 ±7.3 (20-54)	
	20-29	49 (59.8)	
	30-44	28(34.1)	
	45 and above	5(6.1)	
Education status	Masters /Doctoral level	7(8.5)	5(50.0)
	Bachelors of Science	40(48.8)	5(50.0)
	Diploma/ level IV	35(42.7)	
Professional specialty	Physicians	5(6.1)	4(40.0)
	Public health officer	19(23.2)	5(50.0)
	Nurse /midwifery	58(70.7)	1(10.0)
Service years	2 or less than 2 years	23(28.0)	3(30.0)
	3- 5 years	24(29.3)	5(50.0)
	6- 10 years	18(22.0)	0
	More than 10 years	17(20.7)	2(20.0)
Clinical Work experience other areas	No	60(73.2)	7(70.0)
	Yes	22(26.8)	3(30.0)
Health facility	Awash Arba H/C	12(14.6)	2(20.0)
	Werer H/C	15(18.3)	1(10.0)
	MAMG Referral Hospital	24(29.3)	4(40.0)
	Andido H/C	11(13.4)	1(10.0)
	Sidafage H/C	8(9.8)	2(20.0)
	Awash Sebat H/C	12(14.6)	0
Types of facility	Hospital	22(26.8)	4(40.0)
	Health centre	60(73.2)	6(60.0)
Of the fever-related illnesses, which disease/s is/are typically diagnosed and treated in this area?	Malaria	78(95.1)	
	Typhoid fever	39(47.6)	
	Typhus fever	14(17.1)	
	Other non-specific like cough, pneumonia, etc	4(4.9)	
Are there high numbers of febrile illnesses with unknown causes or negative for malaria, typhoid and typhus?	Yes	58(70.7)	
	No	17(20.7)	
	Rare	7(8.5)	
What do you do for those febrile patients?	Refer to other hospital	2(3.4)	
	Provide treatment/ antibiotics for bacterial infections	40(69.0)	
	Provide supportive treatment/antiviral for viral infections	13(22.4)	
	Left untreated by providing anti-pain	3(5.2)	

Have you heard about chikungunya/dengue diseases?	Yes	77(93.9)	
	No	5(6.1)	
If ‘Yes’, about which viral diseases have you heard?	Both	77 (100.0)	
	Dengue fever	0	
	Chikungunya	0	
What is/are source/s of information	Media	41(53.2)	
	Teacher/Book	18(23.4)	
	Seminar/meeting	7(9.1)	
	Diagnosed with during previous outbreak	11(14.3)	

MAMG - Mohammed Akle Memorial General; H/C – Health Center

4.5.2. HCWs’ Knowledge about DF and CHIK Diseases

Among 82 frontline HCWs, 93.9% had prior information about both CHIK and DF diseases. Their sources of information were media (53.2%), educational settings (23.4%), personal experiences of the illnesses during outbreaks (14.3%), and meetings and workshops for a few participants (Table 4.18).

Of the HCWs who had prior information about those arboviral diseases, the mean overall knowledge score was 13.1 ± 3.9 out of 21 items (62.4%), indicating a moderate level of knowledge. Among these HCWs, only 36.4% of HCWs demonstrated good knowledge scores about these diseases, while the rest showed moderate (35.1%) and poor knowledge (28.6%). Of the four knowledge domains, the domain regarding "transmission, vector and spread" had the lowest knowledge score (46.0%), while the other domains had moderate knowledge scores, each scoring 60.0% (Table 4.19).

During linear regression or ANOVA analyses of the mean overall knowledge score over sociodemographic factors of the participants, HCWs with master’s or doctoral degrees, as well as those who are physicians in occupation, demonstrated significantly higher mean knowledge scores ($p < 0.05$). However, other demographic factors showed no significant differences (Table 4.20).

Table 4.19: Healthcare workers' knowledge about arboviral diseases in selected districts of the Afar National Regional State, Ethiopia (No̲ = 77)

Variables		No̲ of respondents (%)	MKS
Knowledge on agent and seriousness of illness [% Score (obtained mean score/total score): 60.0%**]**			1.2 ± 0.8
Do the infections of chikungunya and dengue viruses cause serious/sever illness?	**Yes**	35(45.4)	0.5± 0.5
	No	24(31.2)	
	Not sure	18(23.4	
Do chikungunya and dengue infection affect people of all age groups?	**Yes**	54(70.1)	0.7 ± 0.5
	No	10(13.0)	
	Not sure	13(16.9)	
Knowledge on transmission, vector and spread[% Score (obtained mean score/total score): 46.0%]			2.3 ± 1.7
How do chikungunya and dengue viruses transmit to humans?	**Mosquito bites**	56(72.7)	0.7 ± 0.4
	Tick bites	2(2.6)	
	House fly/contamination	3(3.9)	
	Air droplet	4(5.2)	
	Do not know	12(15.6)	
Which specific mosquito can transmit chikungunya and dengue viruses?	*Anopheles*	9(16.1)	
	Aedes	33(58.9)	0.4 ± 0.5
	Both	8(14.3)	
	No sure	6(10.7)	
Aedes mosquito bites humans preferably.	At any time	13(23.2)	
	At day time	12(21.4)	0.2 ± 0.4
	At night time	27(48.2)	
	Not sure	4(7.1)	
What is the typical breeding site for that mosquito?	**Water storage container / stagnant water**	38(67.9)	0.5 ± 0.5
	Dirty water	7(12.5)	
	Garbage and mud	4(7.1)	
	Not sure	7(12.5)	
Which seasons are common for transmissions of chikungunya and dengue viruses?	Dry season	12(15.6)	
	Rainy season	39(50.6)	0.5 ± 0.5
	At any seasons	6(7.8)	
	Not sure	20(26.0)	
Knowledge about major signs and symptoms [% Score (obtained mean score/total score): 60.0%]			7.2 ± 2.4
Is chikungunya cause high Fever	**Yes**	70(93.3)	0.9 ± 0.3
Is chikungunya cause severe Joint pain	**Yes**	70(93.3)	0.9 ± 0.3
Is chikungunya cause Headache	**Yes**	63(84.0)	0.8 ± 0.4

Is chikungunya cause Skin rash	**Yes**	20(26.7)	0.3 ± 0.4
Is chikungunya cause Joint swelling	**Yes**	20(26.7)	0.3 ± 0.4
Is Dengue cause a high Fever	**Yes**	71(92.2)	0.9±0.3
Is Dengue cause a severe Headache	**Yes**	64(83.1)	0.8±0.4
Is Dengue cause a Muscle Pain	**Yes**	54(70.1)	0.7±0.5
Is Dengue cause a Joint Pain	**Yes**	71(92.2)	0.9±0.3
Is dengue cause a pain behind eye	**Yes**	22(28.6)	0.3±0.4
Is dengue cause a Rash	**Yes**	20(26.0)	0.3±0.4
Is dengue cause bleeding gum, nose or petechial	**Yes**	12(15.6)	0.2±0.4
Knowledge on treatment and prevention [% Score (obtained mean score/total score**): 60.0%]**			2.4± 1.0
Are chikungunya and dengue preventable diseases?	**Yes**	59(76.6)	0.8 ± 0.4
	No	10(13.0)	
	Am not sure	8(10.4)	
Are there any laboratory tools to diagnosis patients suspected of chikungunya and dengue?	**Yes**	33(42.9)	0.4 ± 0.5
	No	24(31.2)	
	Do not know	20(26.0)	
Are there specific drugs available for chikungunya and dengue treatment other than anti-pain?	Yes	17(22.1)	
	No	48(62.3)	0.6 ± 0.5
	Am not sure	12(15.6)	
Are there available vaccines for chikungunya and dengue viruses' infections prevention?	Yes	10(13.0)	
	No	46(59.7)	0.6 ± 0.5
	Am not sure	21(27.3)	
The overall mean knowledge scores [% Score (obtained mean score/21**): 62.4%]**			13.1± 3.9

Bold - represents the correct answer; MKS – mean knowledge score

Table 4.20: Healthcare workers' mean knowledge score and associated demographics features in selected districts of the Afar National Regional State, Ethiopia (N = 77)

Variables		Nǫ of respondents	Knowledge score	
			Mean (SD) out of 21	P value
All HCWs		77	13.1(3.9)	
Sex	Male	34	13.6(3.5)	0.3188*
	Female	43	12.7(4.2)	
Age (in years)	18-29	47	13.1(4.0)	0.7994**
	30-44	25	113.3(3.4)	
	45 and above	5	12.0(5.4)	
Education status	Masters /Doctoral	7	15.4(4.4)	**0.0103****
	BSc level	37	14.0(3.6)	
	Diploma/ level IV	33	11.7(3.7)	
Professional specialty	Physicians	5	16.6(4.2)	**0.0054****
	Public health officer	18	14.8(3.1)	
	Nurse /midwifery	54	12.2(3.8)	

Clinical Service years	2 or less than 2 years	22	13.0(4.0)	0.7255**
	3- 5 years	24	12.4(4.1)	
	6- 10 years	15	13.6(3.8)	
	More than 10 years	16	13.7(3.6)	
Clinical services in other areas	No	57	13.3(4.2)	0.3768*
	Yes	20	12.4(2.8)	
Districts	Amibara	56	12.8(4.0)	0.1374**
	Harunka	11	12.6(3.4)	
	Awash Sebat	10	15.4(3.2)	
Health facility	Awash Arba H/C	12	11.0(4.6)	0.1135**
	Werer H/C	15	14.2(3.3)	
	MAMG Referral Hospital	22	13.1(3.7)	
	Andido H/C	11	12.6(3.4)	
	Sidafage H/C	7	11.8(4.6)	
	Awash Sebat H/C	10	15.4(3.12)	

*t test **one way ANOVA

4.5.3. HCWs' Practices in the management and prevention of DF and CHIK

More than half (55.8%) of the frontline HCWs who participated in the quantitative survey indicated that informing the population about these diseases is the most effective strategy for improving the management of CHIK and DF cases. Their management can also be improved by training medical staff and periodic sensitization of HCWs, as indicated by 42.9% and 28.6% of participants, respectively. Furthermore, HCWs also recommended preventive measures against arboviral infections, including using bed nets during sleeping (75.4%), wearing long-sleeved clothes in high-risk areas (40.0%) and applying insect repellents (20.0%) were effective protecting against mosquito bites. Notably, a significant professional training gap was found among HCWs, with only 7.8% of participants having received training on diagnosing, managing and preventing arboviral diseases, including DF and CHIK (Table 4.21).

Participants of IDI also underscored this knowledge gap, revealing that the HCWs possess a limited understanding of arboviral diseases, which have not yet been designated as significant public health threats. One public health officer mentioned, *"Our knowledge about diseases like chikungunya is limited to what we learned in medical school and there have been no refresher courses or training on arboviral diseases.* Similarly, a physician stated, *"We need* *regular updates and training on diseases like chikungunya and dengue, especially for diagnosing and managing outbreaks."*

Table 4.21: Management & prevention improvements of arboviral diseases among healthcare workers in selected districts of the Afar National Regional State, Ethiopia (N = 77)

Variables			No of respondents (%)	MPS
How the CHIK and DF case management can be improved?	By training medical staff	Yes	33(42.9)	0.4 ± 0.5
		No	44(57.1)	
	By informing the population	Yes	43(55.8)	0.6 ± 0.5
		No	34(44.2)	
	By periodic sensitization of healthcare workers	Yes	22(28.6)	0.3 ± 0.4
		No	55(71.4)	
The practical advices for individuals to prevent themselves from CHIKV and DENV infections	To wear long sleeve clothes at risk area.	Yes	26 (33.8)	0.3 ± 0.5
		No	51(66.2)	
	To use repellents	Yes	13(16.9)	0.2 ± 0.4
		No	64(83.1)	
	To use bed net during sleeping daytime	Yes	49(63.6)	0.6 ± 0.5
		No	28(36.4)	
Have you ever been trained for disease like dengue and chikungunya case diagnosis, management and prevention?		Yes	6(7.8)	
		No	71(92.2)	

4.5.4. HCWs' Practices and Experiences in Diagnosing and Managing Acute Febrile Illnesses

A retrospective analysis of 33,398 febrile patient records revealed that only 13,638 (40.8%) of cases were laboratory-confirmed malaria and received appropriate anti-malarial treatment. In contrast, 59.2% of the cases were managed as non-malarial febrile illnesses without appropriate confirmatory diagnosis. The records demonstrated a significant rise in cases of non-malarial febrile illness during the months of August and September, which corresponds with seasonal patterns of wet and hot in this malaria-endemic area (Figure 4.7). The quantitative report of HCWs aligns with this data, wherein a majority (95.1%) mentioned malaria as the predominantly assumed cause of febrile presentation. However, 70.7% of HCWs reported that they frequently encountered non-malarial febrile illnesses of unknown origin, which were often treated with antibiotics (69.0%), antivirals (22.4%), while a small fraction (3.4%) referred patients to tertiary care facilities for further evaluation (Table 4.18).

Upon reviewing the diagnostic resources available for acute febrile illnesses in the healthcare facilities, it was noted that malaria-testing reagents, including Giemsa stain for blood film microscopy and absolute methanol for thin blood film fixation, were the only resources consistently available throughout the year. Functional microscopes were also consistently available, strengthening the availability of malaria testing over the previous year. Notably, MAMG Hospital offered additional diagnostics services, including total and differential blood count, as well as Widal and Weil-flex serological tests, at a comparatively higher frequency than the other healthcare facilities.

IDI participants also estimated that frontline HCWs faced a weekly average of 35 to 100 (median: 72.5) febrile cases. Malaria remained the primary diagnosis established for these febrile patients using blood film microscopy. For patients with negative malaria tests or in instances where diagnostic tools were unavailable, the IDIs participants indicated that clinical assessments were often utilized to identify alternative febrile infections, facilitating the provision of appropriate treatment with antibiotics, antivirals or anti-pain medication accordingly. Participants reported a decline incidence of malaria but expressed concern over the rising incidence of non-malaria febrile illnesses of unknown origin. Diagnoses for these cases were commonly based on symptomatic differential, with management protocol frequently involving antibiotics such as ciprofloxacin or Amoxicillin for conditions like typhus, typhoid, pneumonia or urinary tract infections (UTI). In certain instances, anti-malarial drugs were administered due to suspected false negative results from laboratory tests or fever-reducing medications like paracetamol and diclofenac were prescribed.
A physician explained:*"I primarily request laboratory tests for malaria diagnosis. If negative, I rely on clinical symptoms. As malaria if the cause is 48 hours of fever and intermittent, typhoid if the fever is above 40°C and persistent or with a step-ladder pattern (intermittent), pneumonia if fever with cough and rapid breathing and urinary tract infection if fever with fever and painful urination is based on my previous knowledge of these diseases from medical school."* Another physician also noted, *"I use clinical management of febrile cases when the RDT/microscopy is negative and the patient has a high fever for 48 hours and intermittent as malaria, considering the endemicity of malaria in this area. Otherwise, I offer broad-spectrum antibiotics such as ciprofloxacin, assuming the bacterial infections like typhoid fever."*

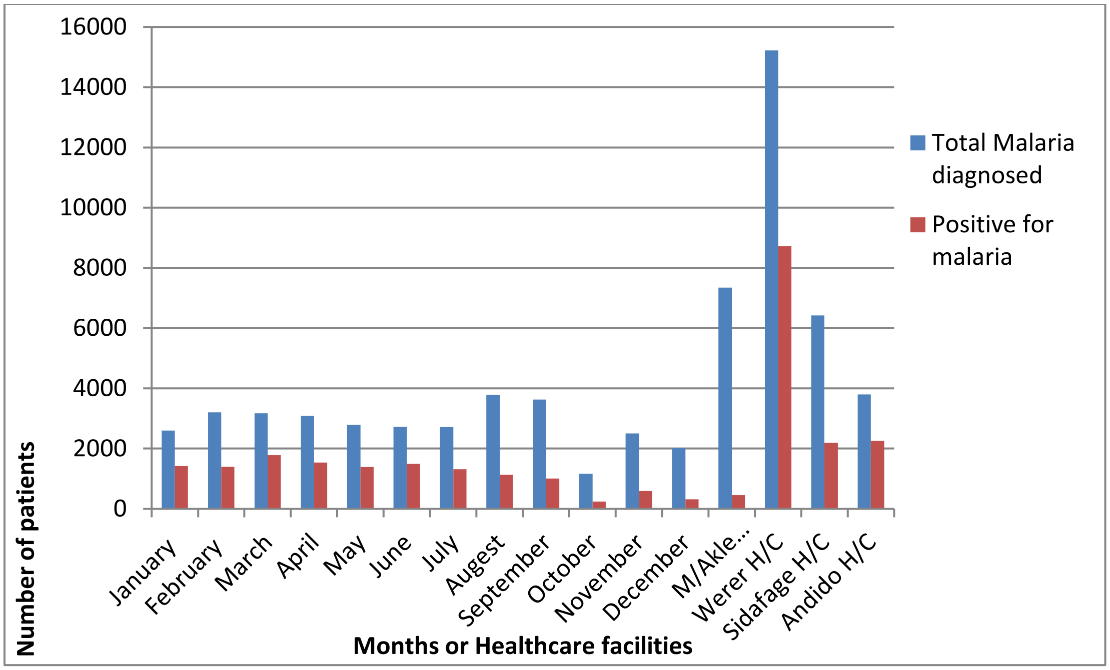

Figure 4.7: Aecord review about febrile patient diagnosis and management from January 11, 2021 to January 10, 2022 in selected districts of Afar National Regional State, Ethiopia.

4.5.5. HCWs' challenges in diagnosing and managing arboviral and non-malarial febrile illnesses

This study identified several significant challenges faced by HCWs in diagnosing and managing arboviral and non-malarial febrile illnesses in the study area. The key obstacles include lack of laboratory infrastructure and training specifically related to non-malarial febrile illnesses, as well as low community awareness and misconceptions about arboviral diseases and non-malarial febrile illnesses. The IDI participants highlighted that despite the suitable climate and environmental conditions for the transmission and spread of various mosquito borne non-malaria febrile illnesses, such as arboviral diseases like DF and CHIK, the healthcare facilities lack the necessary infrastructure, equipment, trained staff, supplies and reliable laboratory testing for these non-malaria febrile cases. Furthermore, clinicians' awareness of arboviral diseases is limited to their time during education in medical school or college.

IDI participants highlighted the need for better infrastructure and training. A physician noted "*Our health facilities are not equipped to diagnose diseases like chikungunya or dengue. We* *need proper tools and training to handle these emerging illnesses."* Moreover, a public health officer noted, *"I have noticed an increase in non-malarial fever-related diseases like chikungunya and dengue fevers cases, including their outbreaks, but neither my health hospital nor others nearby can have diagnostic tools for these diseases except few serological tests such as the Widal for typhoid and Weil-Felix for typhus fevers. However, these test tools are not currently recommended for differential diagnosis for continuous positivity due to previous infections or cross-reactivity with other various infections. In this area no laboratory and preventive skills and facilities for dengue and chikungunya diseases, though large outbreaks of these diseases have existed in this area and its surroundings for the past four to five years. We, the HCWs, do not have the professional skills to treat these diseases, except for what I learned at the university."*

In addition, IDIs participants emphasized low community awareness about non-malarial febrile illnesses, with most people assuming any fever is malaria and self-medicating with anti-malarial drugs. These patients would come to the healthcare facilities when symptoms persist and become severe. A public health officer shared, *"I noticed that in many cases, community members with fever, including children with fever, usually do not go to the health centre; instead, they buy malaria drugs from the pharmacy and take it for themselves or give it to the child with fever. Only if the symptoms persist, they will come or bring their children to the health centre."*

CHAPTER FIVE

5. DISCUSSION

This dissertation/work assessed the prevalence and public health importance of CHIKV and DENV infections among malaria-suspected febrile patients in the malaria-endemic area in the Afar National Regional State, northeastern Ethiopia. The findings would compel evidence that arboviral diseases are under-diagnosed, despite being a public health concern in the study areas of the region. The infections of these arboviruses have demonstrated a higher prevalence than malaria infection among febrile patients, indicating a shift in the etiology of febrile illnesses from those traditionally attributed to *Plasmodium* infections. The findings also identified that, despite the moderate awareness of mosquito-borne diseases in the study area, there are substantial gaps in the community and HCWs regarding knowledge and preventive practices of arboviral diseases and non-malarial febrile illnesses. The gaps of the KAP highlight the influence of education on effective preventive behavior, but the limited diagnostic tools have challenged clinicians by overlapping symptoms of arbovirus infections and other endemic diseases, such as malaria.

These findings underscore the urgent need to integrate routine diagnosis and surveillance of arboviral diseases, health education and vector control into regional public health strategies. The subsequent discussion sections delve into each study in detail, examining the epidemiological findings, contextualizing them within regional and global evidence, and offering targeted recommendations.

5.1. Chikungunya Virus Infection

The seroprevalence of CHIKV infection among malaria-suspected febrile patients during non-outbreak periods was found to be significant in the study areas of the Afar National Regional State. More than half (51.4%) of sera febrile patients diagnosed for anti-CHIKV antibodies were found to be positive for anti-CHIKV IgM and/or IgG antibodies. This finding revealed that CHIK disease is widespread in the area, as one of the causes of febrile illness. The finding is higher than the findings of studies conducted among febrile patients at Metema and Humera Hospitals in northern Ethiopia (23%) (Ferede *et al.,* 2021). It is also higher than studies conducted among patients with clinical features suggestive of arboviral infections referred to Kassala Hospital, Eastern Sudan (43.7%) (N. Mohamed *et al.,* 2019), attending hospitals in

West Bengal, India (36.9%) (Chattopadhyay *et al.,* 2016), and attending emergency services at the Jorge Cristo Sahium Hospital in Villa del Rosario, Colombia(29.9%) (Carrillo-Hernández *et al.,* 2018). Contrarily, it is lower than the study report conducted immediately after the end of the 2014 outbreak in HIV-infected people followed up in clinical cohorts at the University hospitals of Guadeloupe and Martinique in France's Caribbean islands(61.0%) (Curlier *et al.,* 2021). This difference may be due to changing climate and weather patterns that facilitate the spread of vector-borne diseases (Fouque & Reeder, 2019), as well as differences in diagnostic methods and study participants. For example, the study in Brazil used specific and sensitive molecular diagnostic techniques, while study conducted in Caribbean islands were recruited HIV-positive adult patients and diagnosed only through anti-CHIKV IgG antibodies.

In this study, nearly half (47.8%) of the participants had anti-CHIKV IgM antibodies, indicating acute CHIKV infection, while anti-CHIKV IgG was only 6.3%, suggesting a low number of previous positive CHIKV infections. The discrepancy between IgM and IgG would be the likelihood of recent emergence or early-stage transmission dynamics of CHIKV infection in this study area. This seroprevalence of acute CHIKV infection is higher than the findings of previous studies in Ethiopia(22.5%) (Ferede *et al.,* 2021) and Tanzania (12.9%) (Kajeguka *et al.,* 2016). Hence, CHIKV has been caused a wave of AFI in this study area that likely misdiagnosed etiology of fever. The overlapping of CHIK clinical feature presentation with malaria makes it easy to be misdiagnosed in tropical countries, particularly countries with limited laboratory facilities. The misdiagnosis can lead to the miss-implementation of the necessary prevention and control interventions for CHIKV and other arbovirus infections, ultimately contributing to the spread of the disease (Irekeola *et al.,* 2022). Despite various outbreaks of CHIKV infection being reported in this study area and the surrounding areas, CHIKV and other arbovirus infections have not been considered as a major public health problem in the study area or the country at large (Mehari *et al.,* 2021; Zerfu *et al.,* 2018). However, the findings of this study highlight CHIKV infection as a significant public health concern, particularly among acute febrile patients in this study area.

This study identified back pain was significantly associated with a high seroprevalence of acute CHIKV infection, consistent with previous reports from Tanzania and Ethiopia (Ferede *et al.,* 2021; Kajeguka *et al.,* 2016). Back pain provides a valuable specific clinical predictor for CHIK where tests are limited like in this study area. However, patients with joint pain and chills were less likely to test positive for malaria infections. Several previous studies have indicated that joint pain is among the most common clinical symptoms of CHIKV infection (Chang *et al.,* 2018; Manimunda *et al.,* 2010). The findings support the need for further studies to standardize clinical indicators for the diagnosis of CHIKV infection in countries like Ethiopia, where both malaria and CHIK are widespread but adequate laboratory diagnostic tools are often lacking.

The rates of acute CHIKV infection were similar between males and females in this study area. This finding is contrasted with the results of studies conducted in Northern Ethiopia, and Anyigba, Nigeria, which showed that males had twice as high a seroprevalence of anti-CHIKV IgM compared to females (Ferede *et al.,* 2021; Omatola *et al.,* 2020). Furthermore, this study found a lower seropositivity rate for anti-CHIKV IgM antibodies among individuals aged 51 and above. This result aligns with a recent study conducted in India, which reported a higher prevalence among younger individuals (Badoni *et al.,* 2023). However, it differs from the findings of a study conducted in Nigeria, which showed a higher anti-CHIKV IgM antibody in older patients (Omatola *et al.,* 2020). The difference in the findings may imply that individuals in the age group below 50 years are more exposed to day-biting mosquitoes because of movement to forest areas for various activities, such as attending animals, in the case of Afar pastoralists. Previous studies have shown a negative association between age and antibody responses to mosquito saliva. The biomarkers for exposure to *Aedes* mosquito saliva to predict disease risk have shown a negative association between age and antibody responses to *Ae. aegypti* salivary gland extract (SGE) (Parker *et al.,* 2023). Additionally, single individuals were more likely to test positive for anti-CHIKV IgM compared to those who were married. This result is supported by a report from Mato Grosso, Brazil, which stated that being single is a risk factor for CHIKV infection. However, in contrast to this study's findings, married individuals, either currently or previously (divorced or widowed), were associated with an increased risk of arboviral diseases, including CHIK and dengue (Power *et al.,* 2022). The linkage to non-married (often-active workers) individuals in this study may reflect occupational or behavioral exposure to vectors as most adolescents in this Afar region dwell outdoor for animal grazzing.

Despite the similar prevalence of anti-CHIKV IgM antibodies between males and females and lower prevalence among older individuals in this study, other studies have highlighted that women and older individuals are at a higher risk of experiencing chronic symptoms, such as chronic joint pain, swelling and headache after contracting the disease (Silva & Dermody, 2017; Zingman *et al.,* 2017). Therefore, it is crucial to pay attention to females and the elderly when implementing prevention and control interventions.

In the present study, only 10.7% of malaria-suspected participants were confirmed to have malaria parasites, despite the region being known as a malaria-endemic area of the country (National Malaria Control Team *et al.,* 2014). The true prevalence of malaria infection may be higher than this finding, as the study only included febrile patients with an axillary body temperature of 37.5 °C or higher. It is possible that the study missed malaria patients who presented during typical clinical paroxysmal cycles, such as the cold and shivering stages, when body temperature can drop below 37.5 °C. These findings highlight that malaria remains a significant public health problem and that efforts to eliminate the disease are far from achieving. Furthermore, the results indicate that the prevalence of recent or acute CHIKV infection is higher than that of malaria infection among those febrile patients suspected of having malaria. This discrepancy in case prevalence may be attributed to the study's criteria of including only those with an axillary body temperature of 37.5 °C or higher or diagnostic methods which was employed only microscopic report.. Or it could be related to various ongoing preventive measures, such as the use of insecticide-treated bed nets, residual spraying, and the adoption of artemisinin-based combination therapies administered by the Ministry of Health (MOH) to eliminate and control the spread of the disease (MOH, 2012).

Among the study participants diagnosed with malaria, 84.6% exhibited *P. falciparum* infection, 10.3% had *P. vivax* and 5.1% had mixed infections. This finding is in agreement with studies reporting a higher incidence of *P. falciparum* infection among febrile patients attending public health institutes in Ethiopia (Belay *et al.,* 2021; Negatu *et al.,* 2022). The prevalence of malaria cases in this area should not be ignored as low and insignificant, as it can contribute to the ongoing transmission of the disease and potentially cause future outbreaks in the community. *P. falciparum*, the most severe form of malaria, is widely distributed in the study area and its surroundings, similar to previously reported studies

(Mehari *et al.,* 2021; Zerfu *et al.,* 2018). The results call attention to further strengthening malaria control and prevention programs in the study area and its surroundings. This study also found that co-infection with malaria and CHIKV infections was occurred in 4.3% of study participants, indicating that these independent mosquito-borne microorganisms coexist in the area. This may potentially exacerbate the illness and make it difficult to differentiate clinically between malaria and arboviral diseases, including CHIK. Furthermore, patients with this co-infection may be at risk of developing severe malaria or severe CHIK with complications, as reported in cases of malaria and dengue co-infection (Kotepui *et al.,* 2020).

5.2. Dengue Virus Infection

The outbreaks of DENV infection in the tropics and sub-tropics, particularly in SSA, pose a significant public health challenge (Aniakwaa-Bonsu *et al.,* 2021; Goswami *et al.,* 2018). Understanding of the global or local prevalence of DENV infection and its localized incidence, particularly as a neglected disease during non-outbreak time, is of paramount importance. Thus, this study also examined the seroprevalence of DENV infection among acute febrile patients suspected of malaria, as well as recognizing associated risk sociodemographic and clinical features.

The findings of the study indicate the prevalence of anti-DENV IgM and IgG antibodies among acute febrile patients at 24.6% and 38.7%, respectively. These elevated IgM & IgG may indicate an ongoing well-established DENV transmission. Notably, the seroprevalence observed in this study is higher than similar studies across Africa, including Ethiopia, where DENV circulation has increasingly been documented. A study in Southern Ethiopia reported prevalence of 7.9% for anti-DENV IgM and 22.9% for IgG antibodies among febrile patients visiting health institutes from July to August 2016 in Borena (Geleta, 2019). Another study indicated seropositivity rates of 25.1% for anti-DENV IgG and 8.1% for anti-DENV IgM among non-malarial febrile patients from May to August 2016 in Arba Minch *(Eshetu et al.,* 2020). Additionally, data from Metema and Humera hospitals in Northern Ethiopia, collected from March 2016 to May 2017, showed the overall IgM and IgG prevalence rates of 19% and 21%, respectively (Ferede *et al.,* 2018). A recent meta-analysis estimated a pooled prevalence of 9% for IgM and 21% for IgG across Ethiopia (Nigussie *et al.,* 2024), while another analysis estimated a pooled prevalence of 24.8% for IgG and 10.8% for IgM among febrile populations in Africa (Simo *et al.,* 2019). This higher seroprevalence evidence of recent and previous exposure implies that DF has been circulating undetected in the region for a considerable period, likely due to inadequate recognition. These findings, with evidence of CHIKV infection in this study, affirm that DF and CHIK continue to be prevalent yet neglected, primarily due to limited surveillance and diagnostic capabilities during non-outbreak periods. During such situations, a prompt call to action is recommended to enhance awareness among HCWs & engage researchers and health policy makers (Simo *et al.,* 2019).

Additionally, the study reveals that the seroprevalence of anti-DENV IgM antibodies does not correlate with sociodemographic features, such as gender, age, occupation or educational background, nor with clinical features. In contrast, the seroprevalence of anti-DENV IgG was significantly higher among female patients and those reporting symptoms of nausea and muscle pain. This study aligns with a study conducted in Burkina Faso, which also found no clinical presentation associated with positive anti-DENV IgM antibodies (Ouédraogo *et al.,* 2024). This finding contrasts with the WHO reports, which indentify fever, body pain, headache, nausea, and rash as common symptoms associated with acute or recent DF (WHO, 2023). Another study noted that rash and retro-orbital pain were associated with DF during outbreak periods, while rash and gastrointestinal symptoms, including nausea and vomiting, were prevalent in febrile patients with DF during a non-outbreak scenario (Lim *et al.,* 2019). The absence of severe DF symptoms in this study such as mucosal bleeding, persistent vomiting or hemorrhagic manifestations in our study, may account for the discrepancy between the serological findings and the associated clinical features. However, the discrepancy between serological findings and associated clinical features suggests that clinical presentation-based diagnosis is challenging for DF because of the nonspecific nature of symptoms.

In this study finding, patients visited Sidafage HC exhibited lower anti-DENV IgM antibodies positivity, while the government and private sectors employed showed higher anti-DENV IgG antibodies positivity. The findings may imply that individuals residing near Sidafage HC, which is the HC situated away from the international road leading to Djibouti, may be at a lower risk of DENV transmission, due to the travel related DENV infection. Similarly, the actively working community members might have higher exposure to *Aedes* mosquitoes due to spending most of their daytime at outdoors, which increases the risk of mosquito bites. This is due to the fact that these mosquitoes are recognized for their daytime feeding behavior when there are favorable environmental conditions that promote their breeding and abundance (Socha *et al.,* 2022). However, further entomological studies are necessary to establish a conclusive hypothesis that could aid in implementing effective vector control measures.

Furthermore, this study identified that a quarter (25.1%) were classified as borderline for anti-DENV IgM and/ or IgG antibodies among sera examined for both antibodies, with 21.2% borderline for anti-DENV IgM and 20.2% borderline for anti-DENV IgG antibodies of participants, indicating early-stage infections, low-level antibody responses, or cross-reactivity with other *flaviviruse*s (Vilibic-Cavlek *et al.,* 2022). The presence highlights diagnostic uncertainty that potentially lead to the underestimation of true DENV exposure. These may be a factor in Ethiopia for under-recognizing arbovirus infection in national public health priorities, except outbreak reports, leading to gaps in early detection and response efforts (EPHI, 2021; Zerfu *et al.,* 2023). It underscores the critical need for continuous surveillance and molecular testing to accurately assess the burden of DF in the study area and the country as a whole. To do so, it is crucial to incorporate arbovirus infections into national public health priorities, thereby strengthening routine surveillance and developing effective control strategies. Integrating control and management systems for both malaria and DENV vectors, alongside applying a One-Health approach, is essential. Furthermore, community-based interventions are vital in mitigating risks of exposure through the elimination of stagnant water sources, improving waste management and promoting the use of bed nets.

Educational intervention focusing stakeholders, including HCWs, are crucial to raise awareness regarding clinical suspicion and prevention practices related to arboviral diseases (Socha *et al.,* 2022). Furthermore, situation-based training for HCWs and equipping healthcare facilities with diagnostic tools for differential diagnosis of arboviral diseases, malaria and other febrile illnesses is imperative. This will ensure timely and accurate diagnoses, thereby enhancing public health responses.

5.3. Dengue, Chikungunya and Malaria co-infections

Furthermore, the co-infections of DENV, CHIKV and *Plasmodium* parasites were assessed in this study. Of the assessed febrile patients, 18.5% patients were found with dual co-infections, while 1.9% co-positive for all three pathogens among malaria-suspected acute febrile patients. These findings revealed that, besides the co-circulation, the co-infections of these mosquito-borne viruses and *Plasmodium* parasites are significant in this study area. The findings show an exposure to multiple infectious diseases simultaneously from single mosquito or multiple bites is substantial in this study area, leading to misdiagnosis without confirmatory diagnosis & to amplify disease severity (e.g., worse thrombocytopenia in DENV + Malaria). These co-infections among febrile patients emphasized the epidemiological and clinical challenges arising due to overlapping clinical presentations, leading to misdiagnosis in the absence of confirmatory diagnostics.

The seroprevalence of acute DENV-CHIKV co-infection was 15.8%, providing further evidence of overlapping arboviral circulation in this study area. In contrast, the lower co-infections involving DENV-*Plasmodium* parasite (4.4%) and CHIKV-*Plasmodium* parasite (4.3%) were found in this study, which are still epidemiologically important, especially in malaria-endemic settings, where non-malarial febrile illnesses are frequently overlooked. The co-infection findings of this study align with trends in endemic areas, particularly in India, which recently identified that DENV-CHIKV co-infection is the most frequently observed finding in endemic areas(Bala *et al.,* 2025). Furthermore, these arboviral co-infections are significantly higher than a study conducted employing serological methods in neighboring Sudan (1.9%) (Mohamed *et al.,* 2024).

The DENV-CHIKV co-infection showed almost an equal distribution among socio-demographic factors; however, rural populations exhibit significantly higher prevalence of DENV-CHIKV co-infection than the urban populations. On the other hand, joint pain was found to be a significantly associated clinical symptom in co-infected cases, revealing that joint pain is the overlapping clinical presentation of DENV, CHIKV and their co-infections. Similarly, a previous study has reported this overlap by identifying arthralgia and thrombocytopenia as common features of DENV-CHIKV co-infection cases (Kaur *et al.,* 2018). Generally, the increased prevalence of DENV and CHIKV infections, as well as their co-infection, presents an important public health concern, which warrants the implementation of strict control measures (Kaur *et al.,* 2018).

The prevalence of DENV-malaria co-infection in this study is lower than other studies report, which range from 6.6% in neighboring Sudan (Alsedig *et al.,* 2023), 7.1% in French Guiana (Epelboin *et al.,* 2012), 7.4% in India (Mohapatra *et al.,* 2012), to 8.3% in Brazil (Magalhães *et al.,* 2012). In contrast, the co-infection exceeds a recent report of 2.2% in Burkina Faso (Ouédraogo *et al.,* 2024). These variations may be due to differences in sample size and geographic factors.

In this study, the malaria infection among malaria-suspected febrile patients was found to be low (10.7%), which is aligned with the declining national trend in malaria prevalence due to expanded control efforts. However, the simultaneous existence of cases and outbreaks with other febrile illnesses, including arbovirus infections, may hinder these control efforts and complicate the epidemiological landscape management. Because, the serological data from this study indicate considerable rates of acute (24.6%) and previous (38.7%) DENV infections, acute CHIKV infection (47.8%) and low rate of previous CHIKV infection (6.3%), indicating a long-standing endemicity of DENV infection with a considerable burden, as well as a recent aggressive emergence of CHIKV infection, which have been potentially misdiagnosed as malaria, other non-malarial febrile illnesses or left undiagnosed as unknown febrile illnesses in the study area.

The potential misclassification of febrile illnesses as malaria or malaria-only may lead to delays in accurate diagnosis and treatment, ultimately affecting patient outcomes of patients through increased morbidity and mortality (Ward, 2006). Furthermore, Ethiopia, including other Horn African countries, is estimated to face a moderate risk of concurrent malaria and other vector-borne disease outbreaks (WHO, 2024). This needs a transition of malaria-centered diagnosis and management approach to a more comprehensive diagnostic algorithm that routinely incorporates arboviral testing, as well as reinforcing integrated vector control strategies. Similarly, public health interventions, including community sensitization and HCW training, are crucial to enhance case management and prevent future outbreaks (Gebremariam *et al.,* 2023). The approach is essential for effectively addressing the issue posed by these arbovirus infection, co-infections, as well as their co-infections with malaria parasites, particularly in pastoral regions of Ethiopia, such as in the Afar.

5.4. Molecular Findings of Dengue and Chikungunya Virus Infection

Molecular diagnosis of pooled sera in this study provided evidence of active or recent circulation of DENV and CHIKV infections among febrile patients in the Afar National Regional State. Two pools with dual DENV and CHIKV IgM-positive sera and one pool with CHIKV IgM-only sera tested positive for DENV RNA, while both pools with CHIKV RNA-positive results were also found in dual IgM-positive sera. This viral RNA detection depended on the assay employed. The identified DENV RNA was detected only by in-house RT-PCR, while CHIKV RNA was detected only by the commercial RT-PCR, revealing the variability in assay sensitivity and specificity for the target. Hence, selecting appropriate and verified molecular diagnostic tools is essential for arboviral detection.

A pooled testing strategy is a commonly used effective strategy in testing when resources are limited to reduce the cost of screening a large number of individuals for infectious diseases (Mahmoud *et al.,* 2021). If the pool tests negative, all individuals within it would be diagnosed as negative. If the pool tests positive, retesting is needed to decode the positive individuals from the negative individuals (Bilder & Tebbs, 2012). However, an unexpected and significant finding in this study was that no viral RNA was detected when these positive pools were disaggregated and tested individually, which necessitates the cautious interpretation of the results. Arboviral infections, including DENV and CHIKV, have a marked viraemia period (usually 4-7 days following the initiation of symptoms) during which viral RNA can be detected in serum (WHO, 2009). However, individual sera may have viral RNA, which is below detection threshold levels, while pooling effectively concentrates viral targets above this threshold. Hence, pooling has also inadvertently served as an RNA concentrator, while many samples with low viral levels were pooled together and allowing detection concentration that was not detectable in the individual samples (Iani *et al.,* 2021). Therefore, this finding may be due to low viral RNA copies (degraded, early or post-viremia) in individual sera (below detection limit) and become detectable when multiple low-level sera combined. Besides, it may be due to PCR inhibition in some individual sera that was diluted out when samples were pooled (Schrader *et al., 2012*, Eckhart *et al.* 2000). Less likely, may be due to minor cross-contamination, though the gel electrophoresis showed band and negative controls were clean, reducing this likelihood.

In this study, DENV RNA was detected in CHIKV IgM-only pools, revealing the possibility of co-infection or assay cross-reactivity, but this could not be confirmed with individual testing. Moreover, DENV RNA was not detected in DENV IgM-only pools, indicating most sera were potentially collected after the end of the viremia phase (WHO, 2022c). RNA is typically present and highly detectable during the initial stage of symptoms, while serological markers (IgM) are detectable for weeks or months following acute infection (WHO, 2009). However, most of these study participants had a reported onset of symptoms less than five days, yet it comes with its limitations, with recall bias. The detection of viral RNA is limited near symptom resolution, during which antibody levels rise and viral titers decrease (Falzone *et al.,* 2021).

Detection of high viral IgM seropositivity and low molecular findings in this study suggests that most samples might be collected after the viremic period, degradation of RNA or RNA drops below detection levels (Grivard *et al.,* 2007). Sample handling and processing, including frequent freeze-thaw cycles, suboptimal storage, and delay in sample processing, may cause RNA degradation, leading to false-negative results (Iani *et al.,* 2021;Grivard *et al.,* 2007). It was made to reduce laboratory contamination by employing strict precautions and consistent assay procedures; however, it could not be completely ruled out. However, no RNA was detected in the controls and reproducible results in RT-PCR and electrophoresis support the validity of these findings.

In these study findings, despite the molecular data being limited by a lack of confirmation from individual samples, the results offer the first molecular evidence of concurrent DENV and CHIKV circulation in the Afar National Regional State during a non-outbreak period. Furthermore, challenges in detecting arboviral RNA in settings where patients present at different periods of symptoms onset highlight the importance of combining serological and molecular diagnostics to improve case detection, as suggested in other outbreak investigations

(Grivard *et al.,* 2007; WHO, 2009;WHO, 2022c). The findings of the study further suggest that early sample collection, particularly within 5-7 days of symptom onset would be important to optimize the effectiveness of molecular assays, as well as in resource-limited, malaria-endemic areas where arboviral infections may be under-diagnosed, adopting a combined diagnostic approach (serology + PCR) could enhance case identification and patient management.

5.5. Community's Knowledge, Attitude and Practices

This study assessed the KAPs of the community members towards CHIK in the Afar National Regional State, Northeast Ethiopia, using quantitative and qualitative study methods. Both quantitative and qualitative findings revealed similar levels of awareness and understanding about arboviral disease in the study area. Less than 50% of FGDs and about 67.2% of HHS participants had heard of CHIK. The finding is lower than studies conducted in Sudan and Bangladesh, where over 90% of respondents had information about CHIK (Bilal Mohamed Hassan *et al.,* 2020; Or Rashid *et al.,* 2018). The main sources of information about CHIK were health institutes that diagnosed the disease during outbreak events (49.3%) and the *"Dagu"* information-sharing system (34.2%). However, a few respondents had learned about CHIK through the media, seminars or meetings. Typically, people gain information about newly emerging diseases like arboviral diseases from media sources such as TV and radio, as well as health education activities through campaigns and mass media (C. Yboa & J. Labrague, 2013; Nasir *et al.,* 2015; Shuaib *et al.,* 2010). Low awareness in this study area would be due to information source, reliance on outbreak experience for knowledge, which may be memory fade during non-outbreak periods. Health information sharing through media sources and health professional campaigns is crucial to improve households' understanding of the community about the diseases' features, transmissions, and control and prevention strategies (Nyangau *et al.,* 2023). However, the findings of the study showed that the community members had professional-assisted health intervention gaps in this study area.

In both HHS and FGDs findings, most of the study participants believed that malaria was the most common febrile illness and mosquito-borne disease in the study area. About 91.2% of respondents from HHS believed that malaria was the primary cause of febrile disease, followed by typhoid and typhus, with 68.9% and 44.6% of respondents considering them as causes, respectively. However, the community members had limited knowledge about non-malaria febrile illnesses, including arbovirus diseases in the study area, with most fever cases believed to be caused by malaria infection. It is important to note that there have been reports of febrile illness cases and outbreaks caused by arboviral diseases such as CHIK and DF in the study area and surrounding regions (EPHI, 2021; D. Geleta *et al.,* 2020; Gutu *et al.,* 2021). In this dissertation study, the seroprevalence of CHIK (about 50%) and DF (about 25%) was higher than that of malaria (about 10%) among healthcare-seeking febrile patients in the study area. The lack of awareness and consideration of febrile illnesses other than malaria and emerging arbovirus infections can lead to significant but hidden public health issues in the area and the country in general.

Analyses of KAPs of HHS participants who heard about CHIK showed that most participants had satisfactory knowledge of CHIK, with 63.2% scoring a mean or above-mean overall correct knowledge response rate. The result has various agreement results with previous studies, reporting correct knowledge response rates of 40-85% in different countries (Hussain *et al.,* 2018; Nazer Ali *et al.,* 2018; Nyangau *et al.,* 2023). However, a study conducted in Tanzania, where CHIKV was first isolated, reported low, with only 3.2% of community members having heard about the disease and only 12.8% of healthcare workers had heard about (Kajeguka *et al.,* 2017). However, a lack of sufficient knowledge about the etiology, transmission, prevention and control of CHIK was found in these FGDs and HHS study participants. Some FGD participants believed CHIK was a new disease, with a few suggesting it came from a neighboring city, while others reported that Allah (God) introduced it. Of HHS participants, only 44.7% knew a virus causes CHIK, while only 16.5% knew the *Aedes* mosquito, which is a daytime biter, is responsible for transmitting the virus. On the bright side, most participants correctly identified the common symptoms of CHIK, such as fever, joint pain, headache and muscle pain. This finding contrasts findings of previous studies. In India, for example, most people (85%) knew CHIKV is transmitted through mosquito bites but had inadequate knowledge of disease symptoms, with only 48% reporting joint pain and fever being the most common symptoms (Hussain *et al.,* 2018). Other studies, including those in Bangladesh (50%), Sudan (87%), and India (90.2%), found that study participants correctly answered that CHIKV is transmitted from mosquitoes to humans, specifically by *Aedes* mosquitoes (Akhter *et al.,* 2022; Bilal Mohamed Hassan *et al.,* 2020; Satish Patil, 2013). This finding showed that community members had awareness and knowledge variation and uncertainty about CHIK in the study area. Health education about CHIK and other arboviral diseases helps the community to better understand the disease, its transmission, and preventive and control measures (Kajeguka *et al.,* 2017). Therefore, health interventions to advance the community's understanding of the disease and the transmitting vector are vital to reduce any future outbreaks of infections like CHIKV infections.

An analysis of the attitudes of participants in the HHS study showed that they had a moderate positive attitude towards CHIK, with 60.0% of the participants scoring a mean or above a mean overall correct attitude response. The high attitude score observed might be a result of the focus on the perceived risk and responsibility of the participants in individual and collective mosquito prevention and control. It has been reported before that risk perception influences emotional, behavioral, and social reactions (Mbanzulu *et al.,* 2022). This finding is similar to a study conducted in France, where the participants had a moderate level of attitude and perceived risk of contracting mosquito-borne diseases (Raude *et al.,* 2012). Most participants (73.9%) in this study believed that anyone with symptoms of CHIK should seek immediate help from community health services. However, only a few participants (45.3%) perceived CHIK as a serious illness. It is important to correct this misconception because joint pain and swelling can worsen and persist for months or even years, depending on the viral load and individual health status (Tsetsarkin *et al.,* 2007).

In this study, while most participants believed that CHIK could be prevented, addressing personal and general community activities, as well as government action, was considered crucial. Mosquito breeding control was identified as an effective strategy, although there was some lack of awareness regarding specific breeding places of mosquitoes, as well as the responsible person to reduce larvae and adult mosquitoes. Previous studies showed that most study participants in the Indian Ocean Islands believed CHIK was a controllable disease (Raude & Setbon, 2009), while in another study conducted in India, less than half of the participants believed they were personally responsible for mitigating CHIK (Vaidya & Sawant, 2013). In La Réunion, about 55% of the study participants from the general public believed that public authorities were capable of doing everything in their power to stop the spread of CHIK and 54% did not believe that individually they had any control over the disease (Setbon & Raude, 2009). Meanwhile, management directives from health authorities were perceived as ineffective by 60.4% of health professionals to control (Fenétrier *et al.,* 2013). Despite moderate overall attitudes about CHIK in these study findings, many community members had uncertain attitudes and risk perceptions about CHIK disease and its vector due to an inefficient understanding of the disease, the risk of contracting the virus, the vector and its breeding characteristics. Therefore, it is important to educate the community to develop a comprehensive understanding of preventing mosquito vectors.

The overall practices of the community members in controlling and preventing CHIK were found to be fair (60.0%) in this study area. The participants reported that they frequently practiced in draining ponds or degraded muddy/wet areas, cutting down bushes too short, cleaning garbage or trash to reduce mosquito breeding sites, disposing of water-holding containers like tires, plastic, bottles, or broken pots, and covering water containers around the home. However, the participants reported less frequently using of fan, or a bed net, and covering their bodies with clothes when working in the bush, farm and forest. These findings align with a study conducted in Tanzania, which also reported the frequent draining of stagnant water and clearing of bushes around houses, with a small proportion of participants using bed nets (Kajeguka *et al.,* 2017). However, the findings differ from a study conducted in Bangladesh, which reported that only about half of the participants practiced regular cleaning of their household water storage to prevent breeding, and almost all were found to use bed nets regularly to prevent mosquito bites (Mobin *et al.,* 2022). Although the participants of this study performed moderately well in eliminating mosquito-breeding areas and reducing mosquito-resting sites, the community had practice gaps related to proper personal prevention of mosquito bites and control of mosquito-borne diseases like CHIK.

In a multiple linear regression analysis, having higher educational levels had a significant influence on knowledge scores. This finding was consistent with previous studies, which indicated that education had a strong positive correlation with high knowledge scores towards arbovirus diseases (Ghani *et al.,* 2019; Wong *et al.,* 2015). People with higher levels of education are more likely to participate in health awareness campaigns and educational initiatives, and have access to more information about the diseases (Ahmed *et al.,* 2022). The study also found that marital status was a significant factor for attitude scores. However, the finding of this study contrasts with other findings of studies focusing on DF. Those studies reported a positive correlation between attitude and educational level (Al-Dubai *et al.,* 2013; Phuyal *et al.,* 2022). However, no significant association was found between preventive practices and sociodemographic characteristics. In contrast to this study's findings, other studies have reported that education was an independent predictor for practice scores, indicating the role of education in changing people's attitudes (Dhimal *et al.,* 2014). It is important that education and dedicated interventions promote knowledge in order to improve attitudes towards preventive practices.

In correlation analysis, the participants of this study had a strong positive correlation between the KAPs. Although there was a scarcity of similar studies to compare, the study's findings were like previous studies conducted on related arboviruses, such as dengue fever, which also reported a similar positive correlation of KAP (Ahmed *et al.,* 2022). Some studies have found a link between knowledge and a positive attitude, as well as between a positive attitude and effective preventative practices toward arbovirus disease (Honório *et al.,* 2009; Wong *et al.,* 2015). This study's findings revealed that the awareness or information the community acquires would be converted or linked to community attitudes and practices, which are useful habits.

5.6. HCWs' knowledge and practices and challenges in diagnosing and managing

The assessment of HCWs' knowledge, practices and systemic challenges in diagnosing and managing arboviral diseases was conducted, with particular focus on DF and CHIK, as well as other non-malarial febrile illnesses, using mixed quantitative and qualitative studies among HCWs and retrospective analyses of febrile data, in the Afar National Regional State, Northeast Ethiopia. The findings of this study provided critical insights into the knowledge deficits and systemic challenges encountered by HCWs in the diagnosis and management of arboviral diseases and other acute febrile illnesses in malaria-endemic regions.

The quantitative analysis revealed that most HCWs (93.9%) had prior information about DF and CHIK diseases, mostly relying on informal information sources, such as the media. The participants demonstrated a moderate overall knowledge score about these arboviral diseases, while only 36.4% of participants demonstrated good knowledge assessment results. This result is consistent with studies conducted in other malaria-endemic regions, which showed that HCWs have a limited understanding of CHIK and DF (Kajeguka *et al.,* 2017; Mallhi *et al.,* 2018;Yusuf & Ibrahim, 2019; Saringe *et al.,* 2019). The knowledge gap can be attributed to the lack of specific training on arboviral diseases and the absence of integration of such diseases into the routine public health priority list. The recent arboviral diseases as an outbreak in Ethiopia and other SSA countries, have complicated the management of febrile patients (Cunha & Trinta, 2017; EPHI, 2021). The limited training and preparedness of HCWs to manage these emerging arboviral diseases is a critical barrier in the malaria-endemic areas, including in this study area.

Moreover, this study identified that the knowledge gaps of HCWs were particularly pronounced for transmission dynamics, the vector and its control domain of the arboviral diseases. Inconsistent with this finding, studies have reported in Africa that HCWs have insufficient knowledge regarding the epidemiology and clinical presentation of these emerging arboviral diseases (Bangoura *et al.,* 2023). The result highlighted that these domains are critical to be urgently improved in the frontline HCWs for a comprehensive understanding to be able to practice in diagnosing, managing and controlling these viral infections. The qualitative findings also complemented that the HCWs' knowledge of CHIK and DF diseases was limited to what they learned during their medical education, with little to no opportunities for continuing professional development. A comprehensive understanding of the causes, transmission routes, management, and control of diseases is crucial for effective disease prevention and control (Yeboaa *et al.,* 2023).

The findings of this study showed that HCWs with higher educational levels, such as master's or doctoral degrees, and those working as physicians, demonstrated better knowledge scores, highlighting the importance of advanced training in improving HCWs' understanding of emerging infectious diseases. Although physicians and public health officers have limited representation in this study, training programs that are comprehensive and provided for a longer duration for these health professionals, like physicians, may contribute to knowledge differences among participants. Consistent with this finding, a previous study linked higher education levels with improved knowledge of infectious diseases (WHO, 2022a). Similarly, a study conducted in Cameroon reported that medical doctors with relatively higher educational levels among healthcare professionals showed significantly higher knowledge scores than other medical professionals (Nana-Ndjangwo *et al.,* 2021). Despite differences in educational qualifications among healthcare workers, it is important that all HCWs, including nurses, need to receive appropriate training, as they are often the first responders during medical emergencies and disease outbreaks.

In these study findings, malaria disease is the most commonly diagnosed febrile illness, while neglecting non-malarial febrile illnesses, which is a growing burden. As the IDI participants suggested, the healthcare facilities can encounter about 35 to 100 febrile cases per week, while malaria parasites are the most commonly laboratory-based diagnosed disease for these cases. However, the medical record reviews revealed that among malaria-suspected patients who visited healthcare facilities, only 40.8% of them were confirmed malaria and received appropriate anti-malaria treatment, while the rest 59.2% of the cases were attributed to non-malarial febrile illnesses. These high but unconfirmed non-malarial cases would be a significant public health problem, particularly in diagnostic infrastructure for emerging non-malarial febrile diseases, including arboviral diseases, which remain underdeveloped (Ashenafi *et al.,* 2024).

Non-malarial febrile illnesses can be caused by various etiologies, although not yet fully understood. However, traditionally, certain diseases such as RVF, DF, CHIK, leptospirosis, tick-borne relapsing fever, and Q-fever have been associated with these non-malarial cases in Africa, including Ethiopia (Chipwaza *et al.,* 2014; Mediannikov & Raoult, 2012). The IDI participants suggested that the practices of differential diagnosis of the non-malarial febrile cases largely rely on clinical presentations, such as fever patterns, cough and urinary symptoms, to distinguish the case as typhoid, pneumonia or urinary tract infections (UTIs). Even though such judgments of patients with clinical presentations are a widespread practice in various low-resource countries in Africa, including Ethiopia, they often lead to misdiagnosis and mismanagement of the febrile cases (Shimelis *et al.,* 2022). Additionally, diagnosing febrile cases based only on clinical features has an inherent challenge due to the nonspecific nature of febrile illnesses (Shimelis *et al.,* 2022).

The healthcare professionals who participated in this study emphasized that the HCWs have considerable limitations in diagnosing arboviral diseases like CHIK and DF due to the absence of reliable diagnostic tools in this malaria-endemic area. Additionally, the resident community lacks awareness regarding non-malarial febrile illnesses, with febrile symptom cases usually understood as due to malaria, which leads to self-medication with anti-malaria medications. This would delay seeking appropriate care, which would further complicate the disease management outcome. Hence, the health education for the community is a key intervention to improve health-seeking behavior in malaria-endemic areas to manage non-malarial febrile illnesses (Kajeguka *et al.,* 2017; Saringe *et al.,* 2019).

Non-malarial illnesses, including arboviral diseases, brucellosis, typhoid and typhus fever, are increasingly recognized as major contributors to acute febrile illnesses (Mehari *et al.,* 2021; Zerfu *et al.,* 2023, 2024). However, the IDI participants noted that these non-malarial febrile cases have usually been treated empirically without laboratory confirmation with antibiotics or anti-malarial, due to a lack of diagnostic tools. The absence of appropriate diagnostic tools and trained personnel has been a critical challenge for diagnosing and managing the rising number of febrile cases of unknown origin (Elven *et al.,* 2020; Koliopoulos *et al.,* 2024). The gap prevents the ability of accurate identification of each etiology, particularly in low-resource settings (Ashenafi *et al.,* 2024; Shimelis *et al.,* 2022). These identified challenges of limited diagnostic facilities can result in inappropriate treatments, including the overuse of antibiotics or anti-malarial drugs, which contribute to the development of antimicrobial resistance. Therefore, capacity-building programs, as well as an improved laboratory infrastructure, need to be implemented to address the diagnostic gap.

5.7. Limitations of the Study

The studies have several limitations that affect the findings. First, the study is a cross-sectional design, limiting the ability to define the timing and direction of infection. It can only measure prevalence and not concurrent or sequential infection, and it cannot establish temporal causality. Second, while pooling sera to facilitate molecular detection is convenient, it is likely to dilute low viral loads and be less sensitive, resulting in more false negatives. These will tend to underestimate the number of active DENV or CHIKV infections. Third, the in-house RT-PCR platform was not externally verified, leaving its sensitivity and specificity unknown. This undermines the reliability of the molecular positivity for DENV as well as negatives for CHIKV and vice versa. Fourth, the Serological tests, particularly IgM ELISA tests, are prone to cross-reactivity with flavivirus, which inflates DENV or CHIKV IgM seroprevalence. Virus-specific confirmation cannot be ascertained in the absence of PRNT. Fifth, frequent freeze-thaw cycles, suboptimal storage, and delays in processing may degrade RNA, leading to false-negative results. This prevents the opportunity to correlate RNA detection with clinical and demographic data. Furthermore, molecular testing may have been performed after the viremic period in most patients, particularly those with high IgM positivity, which could have resulted in low RNA detection rates, despite serological evidence of recent infection. Additionally, the study faced a limitation due to available laboratory and logistical capabilities, restricting the extent of molecular analysis and it was a limited subset of patients based on reagent availability. The KAP surveys may have social desirability bias; respondents might have given answers they thought the interviewers wanted to hear, potentially overestimating knowledge and positive practices. Therefore, actual knowledge and practice gaps in the community could be even larger than reported. The study was conducted in selected Afar districts with limited geographic and seasonal coverage, so the results cannot reflect the seasonality or the arboviral epidemiology of the entire region.

5.8. Strength of the Study

This study is among the first comprehensive investigations to assess the seroprevalence and molecular evidence of DENV and CHIKV infections in malaria-suspected febrile patients in the Afar National Regional State, a previously understudied area of Ethiopia. It employed a multi-dimensional approach, combining serological, molecular, and community-level (KAP and HCW) data, thereby providing a holistic picture of the burden and determinants of arboviral infections in the context of malaria-endemic settings. The detection of viral RNA in pooled sera, despite resource limitations, confirmed active transmission and validated the reliability of serological findings. The study's community-based component enriched understanding of local awareness and behaviors toward mosquito-borne diseases, essential for designing targeted vector control and health education interventions. The inclusion of HCWs' knowledge and diagnostic practices allowed identification of critical gaps in surveillance and clinical management, directly informing public health training and policy.

CHAPTER SIX

6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

This dissertation presents evidence of the circulation of DENV and CHIKV among malaria-suspected febrile patients in targeted districts of Afar National Regional State, Ethiopia. CHIKV IgM (47.8%) and DENV IgM (24.6%) seroprevalence indicates a high recent arboviral infection rate. In addition, a notable proportion of malaria infection (10.7%) as well as co-infections of DENV-CHIKV, DENV-malaria and CHIKV-malaria.

Despite the failure to confirm the positivity of RNA at the single serum sample level, DENV and CHIKV RNA detection in pooled sera indicates that these arboviruses have an ongoing transmission in the Afar National Regional State of Ethiopia. The findings of the study call for improved and validated molecular diagnostic capacity and systematic arboviral surveillance in the Afar National Regional State to address these overlapping burdens of arboviral as well as malaria diseases.

Furthermore, the community surveys found low levels of awareness and inconsistent practice of CHIKV infection prevention. The HCWs Survey found excessive use of malaria diagnostic methods, empirical treatment of fevers of uncertain etiology, and insufficient resources and capacity to respond to arboviral disease.

In conclusion, this dissertation highlights the need to extend diagnosis and surveillance approaches from malaria to emergent arboviral diseases in high-risk areas such as the pastoral Afar National Regional State.

6.2. Recommendations

Based on the findings of this study, the following recommendations are forwarded for different stakeholders.

- **To the Ministry of Health and Regional Health Bureaus:**
  - Develop or revise guidelines to integrate arboviral differential diagnosis into AFI diagnostic algorithms in this area and other similar regions.
- **For Healthcare Facilities and Healthcare Professionals**
  - Provide targeted HCWs' education in the arboviral infection's clinical presentation, diagnosis, and treatment.
- **On Research and Surveillance for Scholars**
  - Set longitudinal surveys to monitor seasonal and demographic arboviral disease trends in Afar and comparable areas.
  - Standardize and validate platforms of RT-PCR for CHIKV and DENV in terms of sensitivity and specificity, primarily targeting multiplex assays.
  - Utilize a One Health strategy in surveillance to integrate environmental, entomological, and clinical information on vector-borne diseases.
- **For Community Engagement**
  - Public education programs need to be implemented to increase the awareness of mosquito-borne diseases, their symptoms and prevention targeting the rural communities.

7. REFERENCES

Abraham, R., Manakkadan, A., Mudaliar, P., Joseph, I., Sivakumar, K. C., et al (2016). Correlation of phylogenetic clade diversification and in vitro infectivity differences among Cosmopolitan genotype strains of Chikungunya virus. *Infection, Genetics and Evolution*, *37*, 174–184.

Acharya, A., Goswami, K., Srinath, S., & Goswami, A. (2005). Awareness about dengue syndrome and related preventive practices amongst residents of an urban resettlement colony of south Delhi. *J Vect Borne Dis*, *42*(3), 122–127.

Acosta, E. G., Castilla, V., & Damonte, E. B. (2009). Alternative infectious entry pathways for dengue virus serotypes into mammalian cells. *Cellu Microbiol*, *11*(10), 1533–1549.

Adam, A., & Jassoy, C. (2021). Epidemiology and Laboratory Diagnostics of Dengue, Yellow Fever, Zika, and Chikungunya Virus Infections in Africa. *Pathogens (Basel, Switzerland)*, *10*(10), 1324.

Africa CDC. (2023). *Dengue Fever, Diseases*. Africa Centres for Disease Control and Prevention. https://africacdc.org/disease/dengue-fever/

AFRO. (2014, July 24). *Ethiopia Steps Up Actions for Dengue Prevention and Control*. African Region World Health organization https:/ /www.afro.who.int/news/ethiopia-steps-actions-dengue-prevention-and-control

Ahmed, A. E., Almarhabi, M. A., Shami, M. O., Alhazemi, A. A., Alsharif, H. M., et al. (2022). Knowledge, Attitudes, and Practices of the Population in Jazan Region, Saudi Arabia Regarding Dengue Fever and Its Prevention Measures: A Community-Based Cross-Sectional Study. *International Journal of Environmental Research and Public Health*, *19*(24), 16812.

Akelew, Y., Pareyn, M., Lemma, M., Negash, M., Bewket, G., et al. (2022). Aetiologies of acute undifferentiated febrile illness at the emergency ward of the University of Gondar Hospital, Ethiopia. *Trop Medi Inter Health*, *27*(3), 271–279.

Akhter, F., Begum, N., & Jahan, H. (2022). Knowledge, Practice, and Awareness about Preventive Measures of Chikungunya among Traders Community in Mitford Region of Dhaka City. *Asia J Medi Health*, 46–56.

Alatoom, A., & Payne, D. (2009). An Overview of Arboviruses and Bunyaviruses. *Lab Med*, *40*(4), 237–240.

Alayu, M., Teshome, T., Amare, H., Kinde, S., Belay, D., et al. (2021). Risk Factors for Chikungunya Virus Outbreak in Somali Region of Ethiopia, 2019: Unmatched Case-Control Study. *Adv Viro*, *2021*, 1–7.

Albulescu, I. C., Tas, A., Scholte, F. E. M., Snijder, E. J., & Van Hemert, M. J. (2014). An in vitro assay to study chikungunya virus RNA synthesis and the mode of action of inhibitors. *J Gen Viro*, *95*(12), 2683–2692.

Al-Dubai, S. A. R., Ganasegeran, K., Mohanad Rahman, A., Alshagga, M. A., & Saif-Ali, R. (2013). Factors affecting dengue fever knowledge, attitudes and practices among selected urban, semi-urban and rural communities in Malaysia. *The Southeast Asian Journal of Tropical Medicine and Public Health*, *44*(1), 37–49.

Alsedig, K., Eldigail, M. H., Elduma, A. H., Elaagip, A., Altahir, O., et al. (2023). Prevalence of malaria and dengue co-infections among febrile patients during dengue transmission season in Kassala, eastern Sudan. *PLOS Negl Trop Di*, *17*(10), e0011660.

Althouse, B. M., Guerbois, M., Cummings, D. A. T., Diop, O. M., Faye, O., et al. (2018). Role of monkeys in the sylvatic cycle of chikungunya virus in Senegal. *Nature Communications*, *9*(1), 1046.

Alvarez, D. E., Filomatori, C. V., & Gamarnik, A. V. (2008). Functional analysis of dengue virus cyclization sequences located at the 5’ and 3’UTRs. *Virology*, *375*(1), 223–235.

Alvarez, D. E., Lodeiro, M. F., Ludueña, S. J., Pietrasanta, L. I., & Gamarnik, A. V. (2005). Long-range RNA-RNA interactions circularize the dengue virus genome. *Journal of Virology*, *79*(11), 6631–6643.

Álvarez-Díaz, D. A., Valencia-Álvarez, E., Rivera, J. A., Rengifo, A. C., Usme-Ciro, J. A., et al. (2021). An updated RT-qPCR assay for the simultaneous detection and quantification of chikungunya, dengue and zika viruses. *Infection, Genetics and Evolution*, *93*, 104967.

An, W., Ge, N., Cao, Y., Sun, J., & Jin, X. (2017). Recent progress on chikungunya virus research. *Virologica Sinica*, *32*(6), 441–453.

Andrew, A., Navien, T. N., Yeoh, T. S., Citartan, M., Mangantig, E., et al. (2022). Diagnostic accuracy of serological tests for the diagnosis of Chikungunya virus infection: A systematic review and meta-analysis. *PLoS Neglected Tropical Diseases*, *16*(2), e0010152.

Angel, R., & Petrova, E. (2021). *cDNA synthesis using SuperScript^{TM} IV v2*.

Aniakwaa-Bonsu, E., Amoako-Sakyi, D., Dankwa, K., Prah, J. K., & Nuvor, S. V. (2021). Seroprevalence of Dengue Viral Infection among Adults Attending the University of Cape Coast Hospital. *Adv Infect Dis*, *11*(01), 60–72.

Arrigo, N. C., Weaver, S. C., & Calisher, C. H. (2016). The Taxonomy of Arboviruses. In *Arboviruses: Molecular Biology, Evolution and Control* (pp. 9–30).

Asebe, G., Mamo, G., Wieland, B., Medhin, G., Tilahun, G., et al. (2021). Community awareness and experiences of health workers concerning mosquito-borne viral diseases in selected districts of Gambella Region, Southwestern Ethiopia. *Infection Ecology & Epidemiology*, *11*(1), 1988453.

Ashenafi, A., Sule, O., Peter, T., Mashate, S., Otieno, O., et al. (2024). Diagnostics for detection and surveillance of priority epidemic-prone diseases in Africa: An assessment of testing capacity and laboratory strengthening needs. *Frontiers in Public Health*, *12*, 1438334.

Atceken, N., Munzer Alseed, M., Dabbagh, S. R., Yetisen, A. K., & Tasoglu, S. (2023). Point-of-Care Diagnostic Platforms for Loop-Mediated Isothermal Amplification. *Advanced Engineering Materials*, *25*(8), 2201174.

Badoni, G., Gupta, P. K., Gupta, P., Kaistha, N., Mathuria, Y. P., et al. (2023). Dengue-chikungunya infection in the tertiary care hospital of northern India: Cross-sectional latent class cluster analysis in viral infection. *Heliyon*, *9*(3), e14019.

Bala, A., Singh, K., Chhabra, A., Sidhu, S. K., & Oberoi, L. (2025). Incidence of dengue, chikungunya, malaria, typhoid fever, scrub typhus, and leptospirosis in patients presenting with acute febrile illness at a tertiary care hospital, Amritsar. *Journal of Vector Borne Diseases*.

Banerjee, T., Patel, T., Pashchenko, O., Elliott, R., & Santra, S. (2021). Rapid Detection and One-Step Differentiation of Cross-Reactivity Between Zika and Dengue Virus Using Functional Magnetic Nanosensors. *ACS Applied Bio Materials*, *4*(5), 3786–3795.

Bangoura, S. T., Hounmenou, C. G., Sidibé, S., Camara, S. C., Mbaye, A., et al. (2023). Exploratory analysis of the knowledge, attitudes and perceptions of healthcare workers about arboviruses in the context of surveillance in the Republic of Guinea. *PLoS Neglected Tropical Diseases*, *17*(12), e0011814.

Begum, F., Das, S., Mukherjee, D., Mal, S., & Ray, U. (2019). Insight into the Tropism of Dengue Virus in Humans. *Viruses*, *11*(12), E1136.

Belay, B., Gelana, T., & Gebresilassie, A. (2021). Malaria prevalence, knowledge, attitude, and practice among febrile patients attending Chagni health center, Northwest Ethiopia: A cross-sectional study. *Tropical Diseases, Travel Medicine and Vaccines*, *7*(1), 20.

Bernard, E., Solignat, M., Gay, B., Chazal, N., Higgs, S., et al. (2010). Endocytosis of Chikungunya Virus into Mammalian Cells: Role of Clathrin and Early Endosomal Compartments. *PLoS ONE*, *5*(7), e11479.

Bhargava, A., Ralph, R., Chatterjee, B., & Bottieau, E. (2018). Assessment and initial management of acute undifferentiated fever in tropical and subtropical regions. *BMJ*, k4766.

Bhatt, P., Sabeena, S. P., Varma, M., & Arunkumar, G. (2021). Current Understanding of the Pathogenesis of Dengue Virus Infection. *Current Microbiology*, *78*(1), 17–32.

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., et al. (2013). The global distribution and burden of dengue. *Nature*, *496*(7446), 504–507.

Bilal Mohamed Hassan, M., Ibrahim Osman Ahmed, M., Dhaif Allah Awad Alharbi, S., Abdull Ah Mohammed Albeshi, U., et al. (2020). Knowledge attitude and practice toward chikungunya infection among kassala community in sudan 2018-2019. *American Journal of Nursing Science*, *9*(6), 417.

Bilder, C. R., & Tebbs, J. M. (2012). Pooled-testing procedures for screening high volume clinical specimens in heterogeneous populations. *Statistics in Medicine*, *31*(27), 3261-8.

Bohle, L. F., Abdallah, A.-K., Galli, F., Canavan, R., & Molesworth, K. (2022). Knowledge, attitudes and practices towards malaria diagnostics among healthcare providers and healthcare-seekers in Kondoa district, Tanzania: A multi-methodological situation analysis. *Malaria Journal*, *21*(1), 224.

Bonn, D. (2006). How did chikungunya reach the Indian Ocean? *The Lancet Infectious Diseases*, *6*(9), 543.

Borgherini, G., Poubeau, P., Staikowsky, F., Lory, M., Moullec, N. L., et al. (2007). Outbreak of Chikungunya on Reunion Island: Early Clinical and Laboratory Features in 157 Adult Patients. *Clinical Infectious Diseases*, *44*(11), 1401–1407.

Bourtzis, K., & Vreysen, M. J. B. (2021). Sterile Insect Technique (SIT) and Its Applications. *Insects*, *12*(7), 638.

Brady, O. J., Gething, P. W., Bhatt, S., Messina, J. P., Brownstein, J. S., et al. (2012). Refining the global spatial limits of dengue virus transmission by evidence-based consensus. *PLoS Neglected Tropical Diseases*, *6*(8), e1760.

Brown, R., Wan, J., & Kielian, M. (2018). The Alphavirus Exit Pathway: What We Know and What We Wish We Knew. *Viruses*, *10*(2), 89.

Burt, F. J., Chen, W., Miner, J. J., Lenschow, D. J., Merits, A., et al. (2017). Chikungunya virus: An update on the biology and pathogenesis of this emerging pathogen. *The Lancet Infectious Diseases*, *17*(4), e107–e117.

Byk, L. A., Iglesias, N. G., De Maio, F. A., Gebhard, L. G., Rossi, M., et al. (2016). Dengue Virus Genome Uncoating Requires Ubiquitination. *mBio*, *7*(3), e00804-16.

C. Yboa, B., & J. Labrague, L. (2013). Dengue Knowledge and Preventive Practices among Rural Residents in Samar Province, Philippines. *American Journal of Public Health Research*, *1*(2), 47–52.

Caglioti, C., Lalle, E., Castilletti, C., Carletti, F., Capobianchi, M. R., et al. (2013). Chikungunya virus infection: An overview. *The New Microbiologica*, *36*(3), 211–227.

Carrillo-Hernández, M. Y., Ruiz-Saenz, J., Villamizar, L. J., Gómez-Rangel, S. Y., & Martínez-Gutierrez, M. (2018). Co-circulation and simultaneous co-infection of dengue, chikungunya, and zika viruses in patients with febrile syndrome at the Colombian-Venezuelan border. *BMC Infectious Diseases*, *18*(1), 61.

CDC. (2021a, June 25). Dengue; prevention. *Centres for Disease Control and Prevention*. https://www.cdc.gov/dengue/prevention/dengue-vaccine.html

CDC. (2024a). *Chikungunya Virus.* Centers for Disease Control and Prevention. https://www.cdc.gov/chikungunya/prevention/index.html

CDC. (2024b, May 14). *Dengue: Serologic Tests for Dengue Virus*. Centres for Disease Control and Prevention. https://www.cdc.gov/dengue/hcp/diagnosis-testing/serologic-tests-for-dengue-virus.html

Chanama, S., Anantapreecha, S., A-nuegoonpipat, A., Sa-gnasang, A., Kurane, I., et al. (2004). Analysis of specific IgM responses in secondary dengue virus infections: Levels and positive rates in comparison with primary infections. *Journal of Clinical Virology: The Official Publication of the Pan American Society for Clinical Virology*, *31*(3), 185–189.

Chang, A. Y., Encinales, L., Porras, A., Pacheco, N., Reid, St. P., et al. (2018). Frequency of Chronic Joint Pain Following Chikungunya Virus Infection: A Colombian Cohort Study. *Arthritis & Rheumatology*, *70*(4), 578–584.

Chatel-Chaix, L., & Bartenschlager, R. (2014). Dengue virus- and hepatitis C virus-induced replication and assembly compartments: The enemy inside--caught in the web. *Journal of Virology*, *88*(11), 5907–5911.

Chattopadhyay, S., Mukherjee, R., Nandi, A., & Bhattacharya, N. (2016). Chikungunya virus infection in West Bengal, India. *Indian Journal of Medical Microbiology*, *34*(2), 213–215.

Chen, R., & Vasilakis, N. (2011). Dengue—Quo tu et quo vadis? *Viruses*, *3*(9), 1562–1608.

Chinedu Eneh, S., Uwishema, O., Nazir, A., El Jurdi, E., Faith Olanrewaju, O., et al. (2023). Chikungunya outbreak in Africa: A review of the literature. *Annals of Medicine & Surgery*, *85*(7), 3545–3552.

Chipwaza, B., Mugasa, J. P., Mayumana, I., Amuri, M., Makungu, C., et al. (2014). Community knowledge and attitudes and health workers’ practices regarding non-malaria febrile illnesses in eastern Tanzania. *PLoS Neglected Tropical Diseases*, *8*(5), e2896.

Chmielewski, D., Schmid, M. F., Simmons, G., Jin, J., & Chiu, W. (2022). Chikungunya virus assembly and budding visualized in situ using cryogenic electron tomography. *Nature Microbiology*, *7*(8), 1270–1279.

Cho, B., Jeon, B.-Y., Kim, J., Noh, J., Kim, J., et al. (2008). Expression and evaluation of Chikungunya virus E1 and E2 envelope proteins for serodiagnosis of Chikungunya virus infection. *Yonsei Medical Journal*, *49*(5), 828–835.

Christian, E. A., Kahle, K. M., Mattia, K., Puffer, B. A., Pfaff, J. M., et al. (2013). Atomic-level functional model of dengue virus Envelope protein infectivity. *Proceedings of the National Academy of Sciences*, *110*(46), 18662–18667.

Chua, C.-L., Sam, I.-C., Chiam, C.-W., & Chan, Y.-F. (2017). The neutralizing role of IgM during early Chikungunya virus infection. *PloS One*, *12*(2), e0171989.

Chua, C.-L., Sam, I.-C., Merits, A., & Chan, Y.-F. (2016). Antigenic Variation of East/Central/South African and Asian Chikungunya Virus Genotypes in Neutralization by Immune Sera. *PLoS Neglected Tropical Diseases*, *10*(8), e0004960.

Ci, Y., & Shi, L. (2021). Compartmentalized replication organelle of flavivirus at the ER and the factors involved. *Cellular and Molecular Life Sciences*, *78*(11), 4939-54.

Clyde, K., & Harris, E. (2006). RNA secondary structure in the coding region of dengue virus type 2 directs translation start codon selection and is required for viral replication. *Journal of Virology*, *80*(5), 2170–2182.

Constant, L. E. C., Rajsfus, B. F., Carneiro, P. H., Sisnande, T., Mohana-Borges, R., et al. (2021). Overview on Chikungunya Virus Infection: From Epidemiology to State-of-the-Art Experimental Models. *Frontiers in Microbiology*, *12*, 744164.

Corrin, T., Waddell, L., Greig, J., Young, I., Hierlihy, C., et al. (2017). Risk perceptions, attitudes, and knowledge of chikungunya among the public and health professionals: A systematic review. *Tropical Medicine and Health*, *45*(1), 21.

Couderc, T., Chrétien, F., Schilte, C., Disson, O., Brigitte, M., et al. (2008). A mouse model for Chikungunya: Young age and inefficient type-I interferon signaling are risk factors for severe disease. *PLoS Pathogens*, *4*(2), e29.

CRISIS. (2021, February 9). Ethiopia: Dengue fever outbreak reported in Somali Region in 2021. *GardaWorld*. https://crisis24.garda.com/alerts/2021/02/ethiopia-dengue-fever-outbreak-reported-in-somali-region-in-2021

Crump, J. A., & Kirk, M. D. (2015). Estimating the Burden of Febrile Illnesses. *PLoS Neglected Tropical Diseases*, *9*(12), e0004040.

Crump, J. A., Morrissey, A. B., Nicholson, W. L., Massung, R. F., Stoddard, R. A., et al. (2013). Etiology of severe non-malaria febrile illness in Northern Tanzania: A prospective cohort study. *PLoS Neglected Tropical Diseases*, *7*(7), e2324.

CSA. (2022). Population Size by Sex, Area and Density by Region, Zone and Wereda. Ethiopian Statistical Service. http://www.statsethiopia.gov.et/wp-content/uploads/2022/07/Population-Size-by-Sex-Zone-and-Wereda-July-2022.pdf

Cuartas-López, A. M., Hernández-Cuellar, C. E., & Gallego-Gómez, J. C. (2018). Disentangling the role of PI3K/Akt, Rho GTPase and the actin cytoskeleton on dengue virus infection. *Virus Research*, *256*, 153–165.

Cunha, R. V. D., & Trinta, K. S. (2017). Chikungunya virus: Clinical aspects and treatment - A Review. *Memórias Do Instituto Oswaldo Cruz*, *112*(8), 523–531.

Curlier, E., Fagour, L., Herrmann-Storck, C., Staelen, A., Vingadassalom, I., et al. (2021). Seroprevalence of chikungunya virus infection among HIV-infected adults in French Caribbean Islands of Martinique and Guadeloupe in 2015: A cross-sectional study. *PLoS Neglected Tropical Diseases*, *15*(4), e0009267.

Daba, C., Atamo, A., Debela, S. A., Kebede, E., Woretaw, L.,et al. (2023). A Retrospective Study on the Burden of Malaria in Northeastern Ethiopia from 2015 to 2020: Implications for Pandemic Preparedness. *Infection and Drug Resistance*, *Volume 16*, 821–828.

Dagnaw, G. G., Gessese, A. T., Kinde, M. Z., Bitew, A. B., Dejene, H., et al. (2024). *Seroprevalence and Risk Factors of Chikungunya in Ethiopia: A Systematic Review and Meta-Analysis*.

de Lamballerie, X., Leroy, E., Charrel, R. N., Ttsetsarkin, K., Higgs, S., et al. (2008). Chikungunya virus adapts to tiger mosquito via evolutionary convergence: A sign of things to come? *Virology Journal*, *5*, 33.

Degife, L. H., Worku, Y., Belay, D., Bekele, A., & Hailemariam, Z. (2019). Factors associated with dengue fever outbreak in Dire Dawa administration city, October, 2015, Ethiopia—Case control study. *BMC Public Health*, *19*(1), 650.

Dengue Virus Net. (2022). Dengue Epidemiology. *Dengue Virus Net*. http://www.denguevirusnet.com/epidemiology.html

Dhimal, M., Aryal, K. K., Dhimal, M. L., Gautam, I., Singh, S. P., et al. (2014). Knowledge, Attitude and Practice Regarding Dengue Fever among the Healthy Population of Highland and Lowland Communities in Central Nepal. *PLoS ONE*, *9*(7), e102028.

Diaz, A., & Ahlquist, P. (2012). Role of host reticulon proteins in rearranging membranes for positive-strand RNA virus replication. *Current Opinion in Microbiology*, *15*(4), 519–524.

ECDC. (2021, March 4). *Factsheet about dengue*. European Centre for Disease Prevention and Control. https://www.ecdc.europa.eu/en/dengue-fever/facts

ECDC. (2024, February 8). *Countries/territories reporting Chikungunya cases since July 2023, and as of June 2024* [An agency of the European Union]. European Centre for Disease Prevention and Control. https://www.ecdc.europa.eu/en/chikungunya-monthly

Eckhart, L., Bach, J., Ban, J. and Tschachler, E. (2000) Melanin binds reversibly to thermostable DNA polymerase and inhibits its activity. *Biochem Biophys Res Commun* **271**, 726–730.

Edgil, D., Polacek, C., & Harris, E. (2006). Dengue Virus Utilizes a Novel Strategy for Translation Initiation When Cap-Dependent Translation Is Inhibited. *Journal of Virology*, *80*(6), 2976–2986.

Eltom, K., Enan, K., El Hussein, A. R. M., & Elkhidir, I. M. (2021). Dengue Virus Infection in Sub-Saharan Africa Between 2010 and 2020: A Systematic Review and Meta-Analysis. *Frontiers in Cellular and Infection Microbiology*, *11*, 678945.

Elven, J., Dahal, P., Ashley, E. A., Thomas, N. V., Shrestha, P., et al. (2020). Non-malarial febrile illness: A systematic review of published aetiological studies and case reports from Africa, 1980–2015. *BMC Medicine*, *18*(1), 279.

Endale, A., Medhin, G., Hilo, A. A., Abegaz, W. E., & Legesse, M. (2020). Knowledge, Attitude, and Practice of Jinka University Students About Yellow Fever, Ethiopia: A Cross-Sectional Study. *Risk Management and Healthcare Policy*, *13*, 1225-36.

Endale, A., Michlmayr, D., Abegaz, W. E., Asebe, G., Larrick, J. W., et al. (2020). Community-based sero-prevalence of chikungunya and yellow fever in the South Omo Valley of Southern Ethiopia. *PLOS Neglected Tropical Diseases*, *14*(9), e0008549.

Epelboin, L., Hanf, M., Dussart, P., Ouar-Epelboin, S., Djossou, F., et al. (2012). Is dengue and malaria co-infection more severe than single infections? A retrospective matched-pair study in French Guiana. *Malaria Journal*, *11*(1), 142.

EPHI. (2021, October). *Arboviral Disease Vectors Surveillance and Control Guideline. Public Health Entomology Research Team*. Ethiopia Public Health Institute, Addis Ababa Ethiopia. https://ephi.gov.et/wp-content/uploads/2021/12/ Arboviral-Disease-Vectors-Surveillance-and-Control-Guideline.pdf

Eshetu, D., Shimelis, T., Nigussie, E., Shumie, G., Chali, W., et al. (2020). Seropositivity to dengue and associated risk factors among non-malarias acute febrile patients in Arba Minch districts, southern Ethiopia. *BMC Infectious Diseases*, *20*(1), 639.

Falconar, A. K. I., de Plata, E., & Romero-Vivas, C. M. E. (2006). Altered enzyme-linked immunosorbent assay immunoglobulin M (IgM)/IgG optical density ratios can correctly classify all primary or secondary dengue virus infections 1 day after the onset of symptoms, when all of the viruses can be isolated. *Clinical and Vaccine Immunology: CVI*, *13*(9), 1044–1051.

Falzone, L., Gattuso, G., Tsatsakis, A., Spandidos, D., & Libra, M. (2021). Current and innovative methods for the diagnosis of COVID‑19 infection (Review). *International Journal of Molecular Medicine*, *47*(6), 100.

Fenétrier, E., Sissoko, D., Vernazza-Licht, N., Bley, D., Gaüzère, B.-A., et al. (2013). Retour d’expérience des médecins généralistes deux ans après l’épidémie de chikungunya de 2005–2006 à La Réunion. *Bulletin de la Société de pathologie exotique*, *106*(3), 193–200.

Ferede, G., Tiruneh, M., Abate, E., Kassa, W. J., Wondimeneh, Y., et al. (2018). Distribution and larval breeding habitats of Aedes mosquito species in residential areas of northwest Ethiopia. *Epidemiology and Health*, *40*, e2018015.

Ferede, G., Tiruneh, M., Abate, E., Wondimeneh, Y., Damtie, D., et al. (2018). A serologic study of dengue in northwest Ethiopia: Suggesting preventive and control measures. *PLoS Neglected Tropical Diseases*, *12*(5), e0006430.

Ferede, G., Tiruneh, M., Abate, E., Wondimeneh, Y., Gadisa, E., et al. (2021). Evidence of chikungunya virus infection among febrile patients in northwest Ethiopia. *International Journal of Infectious Diseases: IJID: Official Publication of the International Society for Infectious Diseases*, *104*, 183–188.

Fernández-García, L., Angulo, J., Ramos, H., Barrera, A., Pino, K., et al. (2021). The Internal Ribosome Entry Site of Dengue Virus mRNA Is Active When Cap-Dependent Translation Initiation Is Inhibited. *Journal of Virology*, *95*(5), e01998-20.

Fields, B. N. (2007). *Fields’ virology* (Vol. 1). Lippincott Williams & Wilkins.

Filomatori, C. V., Lodeiro, M. F., Alvarez, D. E., Samsa, M. M., Pietrasanta, L., et al. (2006). A 5′ RNA element promotes dengue virus RNA synthesis on a circular genome. *Genes & Development*, *20*(16), 2238–2249.

Flahault, A., Aumont, G., Boisson, V., de Lamballerile, X., Favier, F., et al. (2007). [Chikungunya, La Réunion and Mayotte, 2005-2006: An epidemic without a story?]. *Sante Publique (Vandoeuvre-Les-Nancy, France)*, *19 Suppl 3*, S165-195.

Forrester, N. L., Palacios, G., Tesh, R. B., Savji, N., Guzman, H., et al. (2012). Genome-Scale Phylogeny of the Alphavirus Genus Suggests a Marine Origin. *Journal of Virology*, *86*(5), 2729–2738.

Fouque, F., & Reeder, J. C. (2019). Impact of past and on-going changes on climate and weather on vector-borne diseases transmission: A look at the evidence. *Infectious Diseases of Poverty*, *8*(1), 51.

Freire, M. C. L. C., Basso, L. G. M., Mendes, L. F. S., Mesquita, N. C. M. R., Mottin, M., et al. (2022). Characterization of the RNA-dependent RNA polymerase from Chikungunya virus and discovery of a novel ligand as a potential drug candidate. *Scientific Reports*, *12*(1), 10601.

Fumagalli, M. J., de Souza, W. M., Espósito, D. L. A., Silva, A., Romeiro, M. F., et al. (2018). Enzyme-linked immunosorbent assay using recombinant envelope protein 2 antigen for diagnosis of Chikungunya virus. *Virology Journal*, *15*(1), 112.

Gaber, M., Ahmad, A. A., El-Kady, A. M., Tolba, M., Suzuki, Y., et al. (2022). Dengue fever as a reemerging disease in upper Egypt: Diagnosis, vector surveillance and genetic diversity using RT-LAMP assay. *PloS One*, *17*(5), e0265760.

Galán-Huerta, K. A., Rivas-Estilla, A. M., Fernández-Salas, I., Farfan-Ale, J. A., & Ramos-Jiménez, J. (2015). Chikungunya virus: A general overview. *Medicina Universitaria*, *17*(68), 175–183.

Garcia-Blanco, M. A., Vasudevan, S. G., Bradrick, S. S., & Nicchitta, C. (2016). Flavivirus RNA transactions from viral entry to genome replication. *Antiviral Research*, *134*, 244–249.

Gebhard, L. G., Filomatori, C. V., & Gamarnik, A. V. (2011). Functional RNA Elements in the Dengue Virus Genome. *Viruses*, *3*(9), 1739–1756.

Gebremariam, T. T., Schallig, H. D. F. H., Kurmane, Z. M., & Danquah, J. B. (2023). Increasing prevalence of malaria and acute dengue virus coinfection in Africa: A meta-analysis and meta-regression of cross-sectional studies. *Malaria Journal*, *22*(1), 300.

Geleta, D., Tesfaye, N., & Ayigegn, H. (2020). Epidemiological Description of Chikungunya Virus Outbreak in Dire Dawa Administrative City, Western Ethiopia, 2019. *International Journal of Clinical and Experimental Medical Sciences*, *6*(3), 41.

Geleta, E. N. (2019). Serological evidence of dengue fever and its associated factors in health facilities in the Borena Zone, South Ethiopia. *Research and Reports in Tropical Medicine*, *10*, 129–136.

George, D., & Mallery, P. (2010). *SPSS for Windows step by step: A simple guide and reference, 17.0 update* (10. ed). Allyn & Bacon.

Gérardin, P., Barau, G., Michault, A., Bintner, M., Randrianaivo, H., et al. (2008). Multidisciplinary Prospective Study of Mother-to-Child Chikungunya Virus Infections on the Island of La Réunion. *PLoS Medicine*, *5*(3), e60.

Gerold, G., Bruening, J., Weigel, B., & Pietschmann, T. (2017). Protein Interactions during the Flavivirus and Hepacivirus Life Cycle. *Molecular & Cellular Proteomics*, *16*(4), S75–S91.

Getachew, D., Tekie, H., Gebre-Michael, T., Balkew, M., & Mesfin, A. (2015). Breeding Sites of Aedes aegypti: Potential Dengue Vectors in Dire Dawa, East Ethiopia. *Interdisciplinary Perspectives on Infectious Diseases*, *2015*, 706276.

Ghani, N., Shohaimi, S., Hee, A., Chee, H.-Y., Emmanuel, O., et al. (2019). Comparison of Knowledge, Attitude, and Practice among Communities Living in Hotspot and Non-Hotspot Areas of Dengue in Selangor, Malaysia. *Tropical Medicine and Infectious Disease*, *4*(1), 37.

Ghosh, A., Alladi, P. A., Narayanappa, G., Vasanthapuram, R., & Desai, A. (2018). The time course analysis of morphological changes induced by Chikungunya virus replication in mammalian and mosquito cells. *Acta Virologica*, *62*(4), 360–373.

Gillespie, L. K., Hoenen, A., Morgan, G., & Mackenzie, J. M. (2010). The endoplasmic reticulum provides the membrane platform for biogenesis of the flavivirus replication complex. *Journal of Virology*, *84*(20), 10438–10447.

Gloria-Soria, A., Ayala, D., Bheecarry, A., Calderon-Arguedas, O., Chadee, D. D., et al. (2016). Global genetic diversity of Aedes aegypti. *Molecular Ecology*, *25*(21), 5377-95.

Goncalvez, A. P., Escalante, A. A., Pujol, F. H., Ludert, J. E., Tovar, D., et al. (2002). Diversity and Evolution of the Envelope Gene of Dengue Virus Type 1. *Virology*, *303*(1), 110–119.

Goswami, L., Runumi, C., & Rasul, E. S. (2018). Seroprevalence of Dengue Infection in a Tertiary Care Hospital in Assam. *International Journal of Medical and Dental Sciences*, *7*(1), 1582.

Green, S., & Rothman, A. (2006). Immunopathological mechanisms in dengue and dengue hemorrhagic fever. *Current Opinion in Infectious Diseases*, *19*(5), 429-36.

Gritsun, T. S., & Gould, E. A. (2007). Origin and evolution of flavivirus 5'UTRs and panhandles: Trans-terminal duplications? *Virology*, *366*(1), 8–15.

Grivard, P., Le Roux, K., Laurent, P., Fianu, A., Perrau, J., et al. (2007). Molecular and serological diagnosis of Chikungunya virus infection. *Pathologie-Biologie*, *55*(10), 490–494.

Guha-Sapir, D., & Schimmer, B. (2005). Dengue fever: New paradigms for a changing epidemiology. *Emerging Themes in Epidemiology*, *2*(g), 1.

Guilarde, A. O., Turchi, M. D., Siqueira, J. B., Feres, V. C. R., Rocha, B., et al. (2008). Dengue and dengue hemorrhagic fever among adults: Clinical outcomes related to viremia, serotypes, and antibody response. *The Journal of Infectious Diseases*, *197*(6), 817–824.

Gupta, N., Srivastava, S., Jain, A., & Chaturvedi, U. C. (2012). Dengue in India. *The Indian Journal of Medical Research*, *136*(3), 373–390.

Gutu, M. A., Bekele, A., Seid, Y., Mohammed, Y., Gemechu, F., et al. (2021a). Another dengue fever outbreak in Eastern Ethiopia-An emerging public health threat. *PLoS Neglected Tropical Diseases*, *15*(1), e0008992.

Guzman, M. G., Alvarez, M., & Halstead, S. B. (2013). Secondary infection as a risk factor for dengue hemorrhagic fever/dengue shock syndrome: An historical perspective and role of antibody-dependent enhancement of infection. *Archives of Virology*, *158*(7), 1445–1459.

Guzman, M. G., Fuentes, O., Martinez, E., & Perez, A. B. (2017). Dengue. In *International Encyclopedia of Public Health* (pp. 233–257). Elsevier.

Guzman, M. G., Halstead, S. B., Artsob, H., Buchy, P., Farrar, J., et al. (2010). Dengue: A continuing global threat. *Nature Reviews. Microbiology*, *8*(12 Suppl), S7-16.

Hakim, M. S., & Aman, A. T. (2022). Understanding the Biology and Immune Pathogenesis of Chikungunya Virus Infection for Diagnostic and Vaccine Development. *Viruses*, *15*(1), 48.

Hanley, K. A., Monath, T. P., Weaver, S. C., Rossi, S. L., Richman, R. L., et al. (2013). Fever versus fever: The role of host and vector susceptibility and interspecific competition in shaping the current and future distributions of the sylvatic cycles of dengue virus and yellow fever virus. *Infection, Genetics and Evolution*, *19*, 292–311.

Hapuarachchi, H. C., Koo, C., Kek, R., Xu, H., Lai, Y. L., et al. (2016). Intra-epidemic evolutionary dynamics of a Dengue virus type 1 population reveal mutant spectra that correlate with disease transmission. *Scientific Reports*, *6*(1), 22592.

Harapan, H., Michie, A., Sasmono, R. T., & Imrie, A. (2020). Dengue: A Minireview. *Viruses*, *12*(8), E829.

Harris, E., Roberts, T. G., Smith, L., Selle, J., Kramer, L. D., et al. (1998). Typing of dengue viruses in clinical specimens and mosquitoes by single-tube multiplex reverse transcriptase PCR. *Journal of Clinical Microbiology*, *36*(9), 2634–2639.

Hoffmann, A. A., Montgomery, B. L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P. H., et al. (2011). Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. *Nature*, *476*(7361), 454–457.

Honório, N. A., Nogueira, R. M. R., Codeço, C. T., Carvalho, M. S., Cruz, O. G., et al. (2009). Spatial Evaluation and Modeling of Dengue Seroprevalence and Vector Density in Rio de Janeiro, Brazil. *PLoS Neglected Tropical Diseases*, *3*(11), e545.

Hooft, A. M., Ripp, K., Ndenga, B., Mutuku, F., Vu, D.,et al. (2017). Principles, practices and knowledge of clinicians when assessing febrile children: A qualitative study in Kenya. *Malaria Journal*, *16*(1), 381.

Hoornweg, T. E., Van Duijl-Richter, M. K. S., Ayala Nuñez, N. V., Albulescu, I. C., Van Hemert, M. J., et al. (2016). Dynamics of Chikungunya Virus Cell Entry Unraveled by Single-Virus Tracking in Living Cells. *Journal of Virology*, *90*(9), 4745–4756.

Hopkins, H. K., Traverse, E. M., & Barr, K. L. (2022). Chikungunya Encephalitis: An Inconsistently Reported Headache and Cause of Death in Patients with Pre-Existing Conditions. *Current Tropical Medicine Reports*, *9*(3), 73–91.

Hsieh, H.-F., & Shannon, S. E. (2005). Three Approaches to Qualitative Content Analysis. *Qualitative Health Research*, *15*(9), 1277–1288.

Hsieh, S.-C., Wu, Y.-C., Zou, G., Nerurkar, V. R., Shi, P.-Y., et al. (2014). Highly conserved residues in the helical domain of dengue virus type 1 precursor membrane protein are involved in assembly, precursor membrane (prM) protein cleavage, and entry. *The Journal of Biological Chemistry*, *289*(48), 33149–33160.

Hudu, S. A., Alshrari, A. S., Syahida, A., & Sekawi, Z. (2016). Cell Culture, Technology: Enhancing the Culture of Diagnosing Human Diseases. *Journal of Clinical and Diagnostic Research: JCDR*, *10*(3), DE01-05.

Huits, R., Okabayashi, T., Cnops, L., Barbé, B., Van Den Berg, R., et al. (2018). Diagnostic accuracy of a rapid E1-antigen test for chikungunya virus infection in a reference setting. *Clinical Microbiology and Infection*, *24*(1), 78–81.

Hussain, M. S., Ganjoo, G., Akhtar, M., & Najmi, A. K. (2018). Knowledge, awareness and preventive practices regarding chikungunya fever after a major outbreak. *International Journal of Infectious Diseases*, *73*, 268.

Iani, F. C. de M., Caetano, A. C. B., Cocovich, J. C. W., Amâncio, F. F., Pereira, M. A., et al. (2021). Dengue diagnostics: Serious inaccuracies are likely to occur if pre-analytical conditions are not strictly followed. *Memorias Do Instituto Oswaldo Cruz*, *115*, e200287.

Inoue, S., Morita, K., Matias, R. R., Tuplano, J. V., Resuello, R. R. G., et al. (2003). Distribution of three arbovirus antibodies among monkeys (Macaca fascicularis) in the Philippines. *Journal of Medical Primatology*, *32*(2), 89–94.

Irekeola, A. A., Engku Nur Syafirah, E. a. R., Islam, M. A., & Shueb, R. H. (2022). Global prevalence of dengue and chikungunya coinfection: A systematic review and meta-analysis of 43,341 participants. *Acta Tropica*, *231*, 106408.

Iroh Tam, P.-Y., Obaro, S. K., & Storch, G. (2016). Challenges in the Etiology and Diagnosis of Acute Febrile Illness in Children in Low- and Middle-Income Countries. *Journal of the Pediatric Infectious Diseases Society*, *5*(2), 190–205.

Izumida, M., Hayashi, H., Tanaka, A., & Kubo, Y. (2020). Cathepsin B Protease Facilitates Chikungunya Virus Envelope Protein-Mediated Infection Via Endocytosis or Macropinocytosis. *Viruses*, *12*(7), 722.

Jacobsen, S., Patel, P., Schmidt-Chanasit, J., Leparc-Goffart, I., Teichmann, A., et al. (2016). External quality assessment studies for laboratory performance of molecular and serological diagnosis of Chikungunya virus infection. *Journal of Clinical Virology: The Official Publication of the Pan American Society for Clinical Virology*, *76*, 55–65.

Jain, J., Okabayashi, T., Kaur, N., Nakayama, E., Shioda, T., et al. (2018). Evaluation of an immunochromatography rapid diagnosis kit for detection of chikungunya virus antigen in India, a dengue-endemic country. *Virology Journal*, *15*(1), 84.

Jawed, S., Khaliq, S., Abid, M., Shaikh, J. R., Salam, O., et al. (2018). Knowledge of Chikungunya Fever Among Medical Students of Jinnah Sindh Medical University, Karachi, Pakistan. *Cureus*.3179

Johnson, K. N. (2015). The Impact of Wolbachia on Virus Infection in Mosquitoes. *Viruses*, *7*(11), 5705–5717.

Kajeguka, D. C., Desrochers, R. E., Mwangi, R., Mgabo, M. R., Alifrangis, M., et al. (2017). Knowledge and practice regarding dengue and chikungunya: A cross-sectional study among Healthcare workers and community in Northern Tanzania. *Tropical Medicine & International Health*, *22*(5), 583–593.

Kajeguka, D. C., Kaaya, R. D., Mwakalinga, S., Ndossi, R., Ndaro, A., et al. (2016). Prevalence of dengue and chikungunya virus infections in north-eastern Tanzania: A cross sectional study among participants presenting with malaria-like symptoms. *BMC Infectious Diseases*, *16*(1), 183.

Kalayanarooj, S. (2011). Clinical Manifestations and Management of Dengue/DHF/DSS. *Tropical Medicine and Health*, *39*(4 Suppl), 83–87.

Kallio, K., Hellström, K., Jokitalo, E., & Ahola, T. (2016). RNA Replication and Membrane Modification Require the Same Functions of Alphavirus Nonstructural Proteins. *Journal of Virology*, *90*(3), 1687–1692.

Kamal, M., Kenawy, M. A., Rady, M. H., Khaled, A. S., & Samy, A. M. (2018). Mapping the global potential distributions of two arboviral vectors Aedes aegypti and Ae. Albopictus under changing climate. *PLOS ONE*, *13*(12), e0210122.

Kapito-Tembo, A., Mathanga, D., Bauleni, A., Nyirenda, O., Pensulo, P., et al. (2020). Prevalence and Clinical Management of Non-malarial Febrile Illnesses among Outpatients in the Era of Universal Malaria Testing in Malawi. *The American Journal of Tropical Medicine and Hygiene*, *103*(2), 887–893.

Kariuki Njenga, M., Nderitu, L., Ledermann, J. P., Ndirangu, A., Logue, C. H., et al. (2008). Tracking epidemic Chikungunya virus into the Indian Ocean from East Africa. *The Journal of General Virology*, *89*(Pt 11), 2754–2760.

Kashyap, R. S., Morey, S. H., Chandak, N. H., Purohit, H. J., Taori, G. M., et al. (2010). Detection of viral antigen, IgM and IgG antibodies in cerebrospinal fluid of Chikungunya patients with neurological complications. *Cerebrospinal Fluid Research*, *7*, 12.

Kaur, M., Singh, K., Sidhu, S. K., Devi, P., Kaur, M., et al. (2018). Coinfection of chikungunya and dengue viruses: A serological study from North Western region of Punjab, India. *Journal of Laboratory Physicians*, *10*(4), 443–447.

Kebede, T., Tesema, B., Mesfin, A., & Getachew, D. (2023). A Community-Level Knowledge, Attitude, and Practice about Dengue Fever and the Identification of Mosquito Breeding Containers in Dire Dawa City of Ethiopia: A Cross-Sectional Study. *Canadian Journal of Infectious Diseases and Medical Microbiology*, *2023*, 1–15.

Kendall, C., Khalid, H., Müller, M., Banda, D. H., Kohl, A., et al. (2019). Structural and phenotypic analysis of Chikungunya virus RNA replication elements. *Nucleic Acids Research*, *47*(17), 9296–9312.

Kittigul, L., Pitakarnjanakul, P., Sujirarat, D., & Siripanichgon, K. (2007). The differences of clinical manifestations and laboratory findings in children and adults with dengue virus infection. *Journal of Clinical Virology: The Official Publication of the Pan American Society for Clinical Virology*, *39*(2), 76–81.

Klein, D. E., Choi, J. L., & Harrison, S. C. (2013). Structure of a dengue virus envelope protein late-stage fusion intermediate. *Journal of Virology*, *87*(4), 2287–2293.

Klungthong, C., Zhang, C., Mammen, M. P., Ubol, S., & Holmes, E. C. (2004). The molecular epidemiology of dengue virus serotype 4 in Bangkok, Thailand. *Virology*, *329*(1), 168–179.

Koliopoulos, P., Kayange, N., Jensen, C., Gröndahl, B., Eichmann, J., et al. (2024). Challenges in Diagnosing and Treating Acutely Febrile Children with Suspected Malaria at Health Care Facilities in the Lake Mwanza Region of Tanzania. *The American Journal of Tropical Medicine and Hygiene*, *110*(2), 202–208.

Kotepui, M., Kotepui, K. U., Milanez, G. D. J., & Masangkay, F. R. (2020). Prevalence of and risk factors for severe malaria caused by Plasmodium and dengue virus co-infection: A systematic review and meta-analysis. *Infectious Diseases of Poverty*, *9*(1), 134.

Krambrich, J. (2024). *A Mosquito's Guide to Viral Emergence: Unveiling Mosquito-Borne Virus Transmission Dynamics, Vector Competence, and Genetic Evolution*. Acta Universitatis Upsaliensis.

Kuhn, R. J., Zhang, W., Rossmann, M. G., Pletnev, S. V., Corver, J., et al. (2002). Structure of dengue virus: Implications for flavivirus organization, maturation, and fusion. *Cell*, *108*(5), 717–725.

Kumar, J. S., Khan, M., Gupta, G., Bhoopati, M., Lakshmana Rao, et al. (2012). Production, characterization, and application of monoclonal antibodies specific to recombinant (E2) structural protein in antigen-capture ELISA for clinical diagnosis of Chikungunya virus. *Viral Immunology*, *25*(2), 153–160.

Kumar, N., Santhoshkumar, R., & Venkataswamy, M. M. (2024). Chikungunya virus infection in human microglial C20 cells induces mitochondria-mediated apoptosis. *Frontiers in Cellular and Infection Microbiology*, *14*, 1380736.

Lamb, L. E., Bartolone, S. N., & Chancellor, M. B. (2020). Detection of Zika Virus Using Reverse Transcription-Loop-Mediated Isothermal Amplification (RT-LAMP). In G. Kobinger & T. Racine (Eds.), *Zika Virus* (Vol. 2142, pp. 137–146.

Lan, Z., Guo, Y., Wang, K., Zhang, Y., Chen, Y., et al. (2022). Hundreds-Dollar-Level Multiplex Integrated RT-qPCR Quantitative System for Field Detection. *Biosensors*, *12*(9), 706.

Lancaster, C., Pristatsky, P., Hoang, V. M., Casimiro, D. R., Schwartz, R. M., et al. (2016). Characterization of N -glycosylation profiles from mammalian and insect cell derived chikungunya VLP. *Journal of Chromatography B*, *1032*, 218–223.

Lanciotti, R. S., Calisher, C. H., Gubler, D. J., Chang, G. J., & Vorndam, A. V. (1992). Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *Journal of Clinical Microbiology*, *30*(3), 545–551.

Laranjo, L. (2016). Social Media and Health Behavior Change. In *Participatory Health Through Social Media* (pp. 83–111). Elsevier.

Laras, K., Sukri, N. C., Larasati, R. P., Bangs, M. J., Kosim, R., et al. (2005). Tracking the re-emergence of epidemic chikungunya virus in Indonesia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *99*(2), 128–141.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K. M., Wertheim, H. F. L., et al. (2013). Antibiotic resistance-the need for global solutions. *The Lancet. Infectious Diseases*, *13*(12), 1057–1098.

Lee, C. H., Regina, Mohamed Hussain, K., & Chu, J. J. H. (2019). Macropinocytosis dependent entry of Chikungunya virus into human muscle cells. *PLOS Neglected Tropical Diseases*, *13*(8), e0007610.

Legesse, M., Endale, A., Erku, W., Tilahun, G., & Medhin, G. (2018). Community knowledge, attitudes and practices on Yellow fever in South Omo area, Southern Ethiopia. *PLOS Neglected Tropical Diseases*, *12*(4), e0006409.

Lemant, J., Boisson, V., Winer, A., Thibault, L., André, H., et al. (2008). Serious acute chikungunya virus infection requiring intensive care during the Reunion Island outbreak in 2005-2006. *Critical Care Medicine*, *36*(9), 2536–2541.

Leroy, E. M., Nkoghe, D., Ollomo, B., Nze-Nkogue, C., Becquart, P., et al. (2009). Concurrent Chikungunya and Dengue Virus Infections during Simultaneous Outbreaks, Gabon, 2007. *Emerging Infectious Diseases*, *15*(4), 591–593.

Lescar, J., Soh, S., Lee, L. T., Vasudevan, S. G., Kang, C., et al. (2018). The Dengue Virus Replication Complex: From RNA Replication to Protein-Protein Interactions to Evasion of Innate Immunity. *Advances in Experimental Medicine and Biology*, *1062*, 115–129.

Li, L., Lok, S.-M., Yu, I.-M., Zhang, Y., Kuhn, R. J., et al. (2008). The flavivirus precursor membrane-envelope protein complex: Structure and maturation. *Science (New York, N.Y.)*, *319*(5871), 1830–1834.

Li, Y.-G., Siripanyaphinyo, U., Tumkosit, U., Noranate, N., A-nuegoonpipat, A., et al. (2013). Chikungunya virus induces a more moderate cytopathic effect in mosquito cells than in mammalian cells. *Intervirology*, *56*(1), 6–12.

Lidbury, B. A., Rulli, N. E., Musso, C. M., Cossetto, S. B., Zaid, A., et al. (2011). Identification and characterization of a ross river virus variant that grows persistently in macrophages, shows altered disease kinetics in a mouse model, and exhibits resistance to type I interferon. *Journal of Virology*, *85*(11), 5651–5663.

Lillis, L., Siverson, J., Lee, A., Cantera, J., Parker, M., et al. (2016). Factors influencing Recombinase polymerase amplification (RPA) assay outcomes at point of care. *Molecular and Cellular Probes*, *30*(2), 74–78.

Lim, J. K., Seydou, Y., Carabali, M., Barro, A., Dahourou, D. L., et al. (2019). Clinical and epidemiologic characteristics associated with dengue during and outside the 2016 outbreak identified in health facility-based surveillance in Ouagadougou, Burkina Faso. *PLOS Neglected Tropical Diseases*, *13*(12), e0007882.

Litzba, N., Schuffenecker, I., Zeller, H., Drosten, C., Emmerich, P., et al. (2008). Evaluation of the first commercial chikungunya virus indirect immunofluorescence test. *Journal of Virological Methods*, *149*(1), 175–179.

Liu, L., Johnson, H. L., Cousens, S., Perin, J., Scott, S., et al. (2012). Global, regional, and national causes of child mortality: An updated systematic analysis for 2010 with time trends since 2000. *The Lancet*, *379*(9832), 2151–2161.

Lo Presti, A., Lai, A., Cella, E., Zehender, G., & Ciccozzi, M. (2014). Chikungunya virus, epidemiology, clinics and phylogenesis: A review. *Asian Pacific Journal of Tropical Medicine*, *7*(12), 925–932.

Lodeiro, M. F., Filomatori, C. V., & Gamarnik, A. V. (2009). Structural and Functional Studies of the Promoter Element for Dengue Virus RNA Replication. *Journal of Virology*, *83*(2), 993–1008.

Lopez-Jimena, B., Wehner, S., Harold, G., Bakheit, M., Frischmann, S., et al. (2018). Development of a single-tube one-step RT-LAMP assay to detect the Chikungunya virus genome. *PLoS Neglected Tropical Diseases*, *12*(5), e0006448.

Lwande OW, Obanda V, Lindström A, Ahlm C, Evander M. (2020). Globe-Trotting Aedes aegypti and Aedes albopictus: Risk Factors for Arbovirus Pandemics. Vector Borne Zoonotic Dis. 20(2):71-81.

Ma, M., Wu, S., He, Z., Yuan, L., Bai, Z., et al. (2021). New genotype invasion of dengue virus serotype 1 drove massive outbreak in Guangzhou, China. *Parasites & Vectors*, *14*(1), 126.

Magalhães, B. M. L., Alexandre, M. A. A., Siqueira, A. M., Melo, G. C., Gimaque, J. B. L., et al. (2012). Clinical Profile of Concurrent Dengue Fever and Plasmodium vivax Malaria in the Brazilian Amazon: Case Series of 11 Hospitalized Patients. *The American Journal of Tropical Medicine and Hygiene*, *87*(6), 1119–1124.

Mahmoud, S. A., Ibrahim, E., Thakre, B., Teddy, J. G., Raheja, P., et al. (2021). Evaluation of pooling of samples for testing SARS-CoV- 2 for mass screening of COVID-19. *BMC Infectious Diseases*, *21*(1), 360.

Mallhi, T. H., Khan, Y. H., Tanveer, N., Bukhsh, A., Khan, A. H., et al. (2018). Awareness and knowledge of Chikungunya infection following its outbreak in Pakistan among health care students and professionals: A nationwide survey. *PeerJ*, *6*, e5481.

Manimunda, S. P., Vijayachari, P., Uppoor, R., Sugunan, A. P., Singh, S. S., et al. (2010). Clinical progression of chikungunya fever during acute and chronic arthritic stages and the changes in joint morphology as revealed by imaging. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *104*(6), 392–399.

Marianneau, P., Cardona, A., Edelman, L., Deubel, V., & Desprès, P. (1997). Dengue virus replication in human hepatoma cells activates NF-kappaB which in turn induces apoptotic cell death. *Journal of Virology*, *71*(4), 3244–3249.

Martin, D. A., Muth, D. A., Brown, T., Johnson, A. J., Karabatsos, N., et al. (2000). Standardization of Immunoglobulin M Capture Enzyme-Linked Immunosorbent Assays for Routine Diagnosis of Arboviral Infections. *Journal of Clinical Microbiology*, *38*(5), 1823–1826.

Martina, B. E. E., Koraka, P., & Osterhaus, A. D. M. E. (2009). Dengue Virus Pathogenesis: An Integrated View. *Clinical Microbiology Reviews*, *22*(4), 564-81.

Mathew, A., & Rothman, A. L. (2008). Understanding the contribution of cellular immunity to dengue disease pathogenesis. *Immunological Reviews*, *225*, 300–313.

Mavalankar, D., Shastri, P., & Raman, P. (2007). Chikungunya epidemic in India: A major public-health disaster. *The Lancet Infectious Diseases*, *7*(5), 306–307.

Maze, M. J., Bassat, Q., Feasey, N. A., Mandomando, I., Musicha, P., et al. (2018). The epidemiology of febrile illness in sub-Saharan Africa: Implications for diagnosis and management. *Clinical Microbiology and Infection*, *24*(8), 808–814.

Mazeaud, C., Freppel, W., & Chatel-Chaix, L. (2018). The Multiples Fates of the Flavivirus RNA Genome During Pathogenesis. *Frontiers in Genetics*, *9*, 595.

Mbanzulu, K. M., Mboera, L. E. G., Wumba, R., Zanga, J. K., Luzolo, F. K., et al. (2022). Community Knowledge, Attitude, and Practices Regarding Mosquitoes and Mosquito-Borne Viral Diseases in Kinshasa, Democratic Republic of the Congo. *Epidemiologia*, *4*(1), 1–17.

Mediannikov, O., & Raoult, D. (2012). Acute febrile illness in Africa. *International Journal of Infectious Diseases*, *16*, e62–e63.

Mehari, S., Zerfu, B., & Desta, K. (2021). Prevalence and risk factors of human brucellosis and malaria among patients with fever in malaria-endemic areas, attending health institutes in Awra and Gulina district, Afar National Regional State, Ethiopia. *BMC Infectious Diseases*, *21*(1), 942.

Mekonnen, M., & Kroos, H. (2006). *Yellow fever and other arboviral diseases," In epidemiology and ecology of health and disease in Ethiopia.* (Y. Berhane, D. Haile Mariam, & H. Kroos, Eds.; 1st ed.). Shama Books.

Melton, J. V., Ewart, G. D., Weir, R. C., Board, P. G., Lee, E., et al. (2002). Alphavirus 6K proteins form ion channels. *The Journal of Biological Chemistry*, *277*(49), 46923–46931.

Mengesha Tsegaye, M., Tayachew, A., Belay, D., Alemu, A., & Beyene, B. (2019). A35 The first laboratory confirmation of chikungunya outbreak in Ethiopia. *Virus Evolution*, *5*(Supplement_1), vez002.034.

Mesfin, Z., Ali, A., Abagero, A., & Asefa, Z. (2022). Dengue Fever Outbreak Investigation in Werder Town, Dollo Zone, Somali Region, Ethiopia. *Infection and Drug Resistance*, *15*, 7207–7217.

Mishra, B., Sharma, M., Pujhari, S. K., Ratho, R. K., Gopal, D. S., et al. (2011). Utility of multiplex reverse transcriptase-polymerase chain reaction for diagnosis and serotypic characterization of dengue and chikungunya viruses in clinical samples. *Diagnostic Microbiology and Infectious Disease*, *71*(2), 118–125.

Mobin, M., Khan, M., Anjum, H., Rahman, H., Marzan, M., et al. (2022). Knowledge, Attitudes, and Practices in Relation to Mosquito-Borne Diseases in Bangladesh. *International Journal of Environmental Research and Public Health*, *19*(14), 8258.

Modis, Y., Ogata, S., Clements, D., & Harrison, S. C. (2004). Structure of the dengue virus envelope protein after membrane fusion. *Nature*, *427*(6972), 313–319.

MOH. (2012). *National Malaria Guidelines. Addis Ababa: Ethiopia*. Federal Democratic Republic of Ethiopia Ministry of Health.

Mohamed, N., Magzoub, M., Mohamed, R. E. H., Aleanizy, F. S., Alqahtani, F. Y., et al. (2019). Prevalence and identification of arthropod-transmitted viruses in Kassala state, Eastern Sudan. *Libyan Journal of Medicine*, *14*(1), 1564511.

Mohamed, N. S., Siddig, E. E., Muvunyi, C. M., Musa, A. O., Elduma, A., et al. (2024). Undetected circulation of major arboviruses in West Sudan: Urging for institutionalizing multisectoral one health strategy for the preparedness, prevention, and control of zoonotic arboviral diseases. *BMC Research Notes*, *17*(1), 386.

Mohammed Yusuf, A., & Abdurashid Ibrahim, N. (2019). Knowledge, attitude and practice towards dengue fever prevention and associated factors among public health sector health-care professionals: In Dire Dawa, eastern Ethiopia. *Risk Management and Healthcare Policy*, *12*, 91–104.

Mohan, A., Fakhor, H., Nimavat, N., Wara, U. U., Lal, P. M., et al. (2021). Dengue and COVID-19: A risk of coepidemic in Ethiopia. *Journal of Medical Virology*, *93*(10), 5680–5681.

Mohapatra, M. K., Patra, P., & Agrawala, R. (2012). Manifestation and outcome of concurrent malaria and dengue infection. *Journal of Vector Borne Diseases*, *49*(4), 262–265.

Mordecai, E. A., Ryan, S. J., Caldwell, J. M., Shah, M. M., & LaBeaud, A. D. (2020). Climate change could shift disease burden from malaria to arboviruses in Africa. *The Lancet. Planetary Health*, *4*(9), e416–e423.

Morrison, T. E., Oko, L., Montgomery, S. A., Whitmore, A. C., Lotstein, A. R., et al. (2011). A Mouse Model of Chikungunya Virus–Induced Musculoskeletal Inflammatory Disease. *The American Journal of Pathology*, *178*(1), 32–40.

Mourad, O., Makhani, L., & Chen, L. H. (2022). Chikungunya: An Emerging Public Health Concern. *Current Infectious Disease Reports*, *24*(12), 217–228.

Mulyatno, K. C., Susilowati, H., Yamanaka, A., Soegijanto, S., & Konishi, E. (2012). Primary isolation and phylogenetic studies of Chikungunya virus from Surabaya, Indonesia. *Japanese Journal of Infectious Diseases*, *65*(1), 92–94.

Murphy, B. R., & Whitehead, S. S. (2011). Immune response to dengue virus and prospects for a vaccine. *Annual Review of Immunology*, *29*, 587–619.

Murugesan, A., & Manoharan, M. (2020). Dengue Virus. In *Emerging and Reemerging Viral Pathogens* (pp. 281–359). Elsevier.

Mustafa, M. S., Rasotgi, V., Jain, S., & Gupta, V. (2015). Discovery of fifth serotype of dengue virus (DENV-5): A new public health dilemma in dengue control. *Medical Journal, Armed Forces India*, *71*(1), 67–70.

Mwanziva, C., Shekalaghe, S., Ndaro, A., Mengerink, B., Megiroo, S., et al. (2008). Overuse of artemisinin-combination therapy in Mto wa Mbu (river of mosquitoes), an area misinterpreted as high endemic for malaria. *Malaria Journal*, *7*(1), 232.

Nachman, R. L., & Rafii, S. (2008). Platelets, petechiae, and preservation of the vascular wall. *The New England Journal of Medicine*, *359*(12), 1261–1270.

Nadjm, B., Mtove, G., Amos, B., Walker, N. F., Diefendal, H., et al. (2012). Severe febrile illness in adult hospital admissions in Tanzania: A prospective study in an area of high malaria transmission. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *106*(11), 688–695.

Nagy, P. D., & Pogany, J. (2012). The dependence of viral RNA replication on co-opted host factors. *Nature Reviews Microbiology*, *10*(2), 137–149.

Nana-Ndjangwo, S. M., Mony, R., Bamou, R., Ango'o, G. B., Tchangou, D. P. W., et al. (2021). Knowledge of Healthcare Workers Regarding Dengue and Chikungunya in Some Health Facilities of the City of Yaoundé (Cameroon) and Its Neighbourhood. *Open Journal of Clinical Diagnostics*, *11*(03), 77–91.

Nasir, B., Fuad, M., Abdelqader, M., Baobaid, M., Elnajeh, M., et al. (2015). Knowledge, attitude and practice of dengue fever and heath education programme among students of Alam Shah science school, Cheras, Malaysia. *Malaysian Journal of Public Health Medicine*, *15*, 69–74.

National Malaria Control Team, Ethiopian Public Health Institute, World Health Organization, Addis Ababa University, & the INFORM Project. (2014, March). *An epidemiological profile of malaria in Ethiopia. A report prepared for the Federal Ministry of Health, Ethiopia, the Roll Back Malaria Partnership and the Department for International Development, UK*.

Natrajan, M. S., Rojas, A., & Waggoner, J. J. (2019). Beyond Fever and Pain: Diagnostic Methods for Chikungunya Virus. *Journal of Clinical Microbiology*, *57*(6), e00350-19.

Nazer Ali, A., Jia Hui, C., Prajapati, S. K., & Zulfikar Ahmed, N. (2018). Knowledge and awareness regarding Chikungunya: A cross-sectional study among healthcare students in a private university, Malaysia. *MOJ Bioequivalence & Bioavailability*, *5*(6).

Negatu, G. A., Abebe, G. A., & Yalew, W. G. (2022). Prevalence of Malaria and Associated Factors among Malaria-Suspected Patients Attending Hamusit Health Center, Northwest Ethiopia: A Cross-Sectional Study. *Journal of Parasitology Research*, *2022*, 1–7.

Nepal, H. P. (2021). Knowledge of chikungunya and mosquito avoidance and control practices among newly admitted students at an offshore medical University in the Caribbean. *Caribbean Medical Journal*. 06.003

Neufeldt, C. J., Cortese, M., Acosta, E. G., & Bartenschlager, R. (2018). Rewiring cellular networks by members of the Flaviviridae family. *Nature Reviews. Microbiology*, *16*(3), 125–142.

Newton, N. D., Hardy, J. M., Modhiran, N., Hugo, L. E., Amarilla, A. A., et al. (2021). The structure of an infectious immature flavivirus redefines viral architecture and maturation. *Science Advances*, *7*(20), eabe4507.

Neyts, J., Leyssen, P., & De Clercq, E. (1999). Infections with flaviviridae. *Verhandelingen - Koninklijke Academie Voor Geneeskunde Van Belgie*, *61*(6), 661–697; discussion 697-699.

Nicholls, C. M. R., Sevvana, M., & Kuhn, R. J. (2020). Structure-guided paradigm shifts in flavivirus assembly and maturation mechanisms. *Advances in Virus Research*, *108*, 33–83.

Nigussie, E., Atlaw, D., Negash, G., Gezahegn, H., Baressa, G., et al. (2024). A dengue virus infection in Ethiopia: A systematic review and meta-analysis. *BMC Infectious Diseases*, *24*(1), 297.

Nimmannitya, S., Halstead, S. B., Cohen, S. N., & Margiotta, M. R. (1969). Dengue and chikungunya virus infection in man in Thailand, 1962-1964. I. Observations on hospitalized patients with hemorrhagic fever. *The American Journal of Tropical Medicine and Hygiene*, *18*(6), 954–971.

Nunes, P. C. G., Lima, M. R. Q., & Dos Santos, F. B. (2022). Molecular Diagnosis of Dengue. *Methods in Molecular Biology (Clifton, N.J.)*, *2409*, 157–171.

Nyangau, P. N., Nzuma, J. M., Irungu, P., Junglen, S., & Kassie, M. (2023). Health education impact on knowledge and management of arboviral diseases in Kenya: Evidence from randomised control trials. *Global Public Health*, *18*(1), 2274436.

Okabayashi, T., Sasaki, T., Masrinoul, P., Chantawat, N., Yoksan, S., et al. (2015). Detection of Chikungunya Virus Antigen by a Novel Rapid Immunochromatographic Test. *Journal of Clinical Microbiology*, *53*(2), 382–388.

Oliveira Ferreira, D. T., Atanaka, M., Espinosa, M. M., Schuler-Faccini, L., Herrero Da Silva, J., et al. (2020). Sociodemographic and sanitary profile of chikungunya virus infection in medium-sized municipality in Mato Grosso, from January to March 2018, Brasil. *Población y Salud En Mesoamérica*.

Omatola, C. A., Onoja, B. A., Fassan, P. K., Osaruyi, S. A., Iyeh, M., et al. (2020). Seroprevalence of chikungunya virus infection in five hospitals within Anyigba, Kogi State of Nigeria. *The Brazilian Journal of Infectious Diseases: An Official Publication of the Brazilian Society of Infectious Diseases*, *24*(1), 1–6.

Or Rashid, M. H., Or Rashid, M. H., Sultana, H., & Zzaman, M. T. (2018). Knowledge and Awareness Regarding Chikungunya among Urban Community People of Selected Area of Dhaka City Bangladesh. *Journal of Infectious Diseases & Therapy*, *06*(01).

Ouédraogo, J. C. R. P., Ilboudo, S., Compaoré, T. R., Bado, P., Nitiéma, M., et al. (2024). Determinants and prevalence of symptomatic dengue fever among adults in the Central Region of Burkina Faso: A hospital-based cross-sectional study. *BMC Infectious Diseases*, *24*(1), 22.

Ozden, S., Huerre, M., Riviere, J.-P., Coffey, L. L., Afonso, P. V., et al. (2007). Human muscle satellite cells as targets of Chikungunya virus infection. *PloS One*, *2*(6), e527.

Padbidri, V. S., & Gnaneswar, T. T. (1979). Epidemiological investigations of chikungunya epidemic at Barsi, Maharashtra state, India. *Journal of Hygiene, Epidemiology, Microbiology, and Immunology*, *23*(4), 445–451.

PAHO. (2011). *Preparedness and Response for Chikungunya Virus: Introduction in the Americas*. Pan American Health Organization, Washington, D.C. https://iris.paho.org/bitstream/handle/10665.2/4009/chikungunyavirus.pdf

Paixão, E. S., Teixeira, M. G., & Rodrigues, L. C. (2018). Zika, chikungunya and dengue: The causes and threats of new and re-emerging arboviral diseases. *BMJ Global Health*, *3*(Suppl 1), e000530.

Pang, X., Zhang, R., & Cheng, G. (2017). Progress towards understanding the pathogenesis of dengue hemorrhagic fever. *Virologica Sinica*, *32*(1), 16–22.

Paquet, C., Quatresous, I., Solet, J.-L., Sissoko, D., Renault, P., et al. (2006). Chikungunya outbreak in Reunion: Epidemiology and surveillance, 2005 to early January 2006. *Euro Surveillance: Bulletin Europeen Sur Les Maladies Transmissibles = European Communicable Disease Bulletin*, *11*(2), E060202.3.

Parker, D. M., Medina, C., Bohl, J., Lon, C., Chea, S., et al. (2023). Determinants of exposure to Aedes mosquitoes: A comprehensive geospatial analysis in peri-urban Cambodia. *Acta Tropica*, *239*, 106829.

Pastorino, B., Muyembe-Tamfum, J. J., Bessaud, M., Tock, F., Tolou, H., et al. (2004). Epidemic resurgence of Chikungunya virus in democratic Republic of the Congo: Identification of a new central African strain. *Journal of Medical Virology*, *74*(2), 277–282.

Patel, P., Abd El Wahed, A., Faye, O., Prüger, P., Kaiser, M., T et al. (2016). A Field-Deployable Reverse Transcription Recombinase Polymerase Amplification Assay for Rapid Detection of the Chikungunya Virus. *PLoS Neglected Tropical Diseases*, *10*(9), e0004953.

Patramool, S., Bernard, E., Hamel, R., Natthanej, L., Chazal, N., et al. (2013). Isolation of infectious chikungunya virus and dengue virus using anionic polymer-coated magnetic beads. *Journal of Virological Methods*, *193*(1), 55–61.

Pérard, J., Leyrat, C., Baudin, F., Drouet, E., & Jamin, M. (2013). Structure of the full-length HCV IRES in solution. *Nature Communications*, *4*(1), 1612.

Perera, R., & Kuhn, R. J. (2008). Structural proteomics of dengue virus. *Current Opinion in Microbiology*, *11*(4), 369–377.

Phadungsombat, J., Lin, M. Y.-C., Srimark, N., Yamanaka, A., Nakayama, E. E., et al. (2018). Emergence of genotype Cosmopolitan of dengue virus type 2 and genotype III of dengue virus type 3 in Thailand. *PLOS ONE*, *13*(11), e0207220.

Phuyal, P., Kramer, I. M., Kuch, U., Magdeburg, A., Groneberg, D. A., et al. (2022). The knowledge, attitude and practice of community people on dengue fever in Central Nepal: A cross-sectional study. *BMC Infectious Diseases*, *22*(1), 454.

Pierson, T. C., & Diamond, M. S. (2012). Degrees of maturity: The complex structure and biology of flaviviruses. *Current Opinion in Virology*, *2*(2), 168–175.

Polacek, C., Friebe, P., & Harris, E. (2009). Poly(A)-binding protein binds to the non-polyadenylated 3' untranslated region of dengue virus and modulates translation efficiency. *The Journal of General Virology*, *90*(Pt 3), 687–692.

Poltep, K., Phadungsombat, J., Nakayama, E. E., Kosoltanapiwat, N., Hanboonkunupakarn, B., et al. (2021). Genetic Diversity of Dengue Virus in Clinical Specimens from Bangkok, Thailand, during 2018–2020: Co-Circulation of All Four Serotypes with Multiple Genotypes and/or Clades. *Tropical Medicine and Infectious Disease*, *6*(3), 162.

Power, G. M., Vaughan, A. M., Qiao, L., Sanchez Clemente, N., Pescarini, J. M., et al. (2022). Socioeconomic risk markers of arthropod-borne virus (arbovirus) infections: A systematic literature review and meta-analysis. *BMJ Global Health*, *7*(4), e007735.

Powers, A. M., Brault, A. C., Shirako, Y., Strauss, E. G., Kang, W., et al. (2001). Evolutionary relationships and systematics of the alphaviruses. *Journal of Virology*, *75*(21), 10118–10131.

Powers, A. M., Brault, A. C., Tesh, R. B., & Weaver, S. C. (2000). Re-emergence of chikungunya and o'nyong-nyong viruses: Evidence for distinct geographical lineages and distant evolutionary relationships. *Microbiology*, *81*(2), 471–479.

Powers, A. M., & Logue, C. H. (2007). Changing patterns of chikungunya virus: Re-emergence of a zoonotic arbovirus. *Journal of General Virology*, *88*(9), 2363-77.

Prasad, N., Murdoch, D. R., Reyburn, H., & Crump, J. A. (2015). Etiology of Severe Febrile Illness in Low- and Middle-Income Countries: A Systematic Review. *PloS One*, *10*(6), e0127962.

Prat, C. M., Flusin, O., Panella, A., Tenebray, B., Lanciotti, R., et al. (2014). Evaluation of commercially available serologic diagnostic tests for chikungunya virus. *Emerging Infectious Diseases*, *20*(12), 2129–2132.

Priya, R., Khan, M., Rao, M. K., & Parida, M. (2014). Cloning, expression and evaluation of diagnostic potential of recombinant capsid protein based IgM ELISA for chikungunya virus. *Journal of Virological Methods*, *203*, 15–22.

Quick, J., Grubaugh, N. D., Pullan, S. T., Claro, I. M., Smith, A. D., et al. (2017). Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. *Nature Protocols*, *12*(6), 1261-76.

Ramakrishnaiah, V., Thumann, C., Fofana, I., Habersetzer, F., Pan, Q., et al. (2013). Exosome-mediated transmission of hepatitis C virus between human hepatoma Huh7.5 cells. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(32), 13109–13113.

Raude, J., Chinfatt, K., Huang, P., Betansedi, C. O., Katumba, K., V et al. (2012). Public perceptions and behaviours related to the risk of infection with Aedes mosquito-borne diseases: A cross-sectional study in Southeastern France. *BMJ Open*, *2*(6), e002094.

Raude, J., & Setbon, M. (2009). The role of environmental and individual factors in the social epidemiology of chikungunya disease on Mayotte Island. *Health & Place*, *15*(3), 689–699.

Rausalu, K., Utt, A., Quirin, T., Varghese, F. S., Žusinaite, E., D et al. (2016). Chikungunya virus infectivity, RNA replication and non-structural polyprotein processing depend on the nsP2 protease's active site cysteine residue. *Scientific Reports*, *6*(1), 37124.

Renzoni, A., Perez, F., Ngo Nsoga, M. T., Yerly, S., Boehm, E., et al. (2021). Analytical Evaluation of Visby Medical RT-PCR Portable Device for Rapid Detection of SARS-CoV-2. *Diagnostics*, *11*(5), 813.

Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C., & Harrison, S. C. (1995). The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. *Nature*, *375*(6529), 291–298.

Ritchie, S. A., & Johnson, B. J. (2017). Advances in Vector Control Science: Rear-and-Release Strategies Show Promise… but Don't Forget the Basics. *The Journal of Infectious Diseases*, *215*(suppl_2), S103–S108.

Robinson, M. C. (1955). An epidemic of virus disease in Southern Province, Tanganyika territory, in 1952–1953. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *49*(1), 28–32.

Rodenhuis-Zybert, I. A., Wilschut, J., & Smit, J. M. (2010). Dengue virus life cycle: Viral and host factors modulating infectivity. *Cellular and Molecular Life Sciences*, *67*(16), 2773–2786.

Ross, T. M. (2010). Dengue virus. *Clinics in Laboratory Medicine*, *30*(1), 149–160.

Roth, G. A., Abate, D., Abate, K. H., Abay, S. M., Abbafati, C., et al. (2018). Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: A systematic analysis for the Global Burden of Disease Study 2017. *The Lancet*, *392*(10159), 1736–1788.

Roy, S. K., & Bhattacharjee, S. (2021). Dengue virus: Epidemiology, biology, and disease aetiology. *Canadian Journal of Microbiology*, *67*(10), 687–702.

Saringe, S., Kajeguka, D. C., Kagirwa, D. D., Mgabo, M. R., & Emidi, B. (2019). Healthcare workers knowledge and diagnostic practices: A need for dengue and chikungunya training in Moshi Municipality, Kilimanjaro Tanzania. *BMC Research Notes*, *12*(1), 43.

Satish Patil, S. (2013). A study of the outbreak of Chikungunya fever. *Journal of Clinical and Diagnostic Research*.

Saxena, S. K., Singh, M., Mishra, N., & Lakshmi, V. (2006). Resurgence of chikungunya virus in India: An emerging threat. *Weekly Releases (1997–2007)*, *11*(32).

Schneider, M., Narciso-Abraham, M., Hadl, S., McMahon, R., Toepfer, S., et al. (2023). Safety and immunogenicity of a single-shot live-attenuated chikungunya vaccine: A double-blind, multicentre, randomised, placebo-controlled, phase 3 trial. *The Lancet*, *401*(10394), 2138–2147.

Schrader C., Schielke A., Ellerbroek L., Johne R. (2012). PCR inhibitors – occurrence, properties and removal, *Journal of Applied Microbiology*, 113(5), 1014–1026

Schuffenecker, I., Iteman, I., Michault, A., Murri, S., Frangeul, L., et al. (2006). Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLoS Medicine*, *3*(7), e263.

Schwartz, O., & Albert, M. L. (2010). Biology and pathogenesis of chikungunya virus. *Nature Reviews Microbiology*, *8*(7), 491–500.

Seema, null, & Jain, S. K. (2005). Molecular mechanism of pathogenesis of dengue virus: Entry and fusion with target cell. *Indian Journal of Clinical Biochemistry: IJCB*, *20*(2), 92–103.

Sellahewa, K. H. (2013). Pathogenesis of Dengue Haemorrhagic Fever and Its Impact on Case Management. *ISRN Infectious Diseases*, *2013*, 1–6.

Serie, C., Andral, L., Lindrec, A., & Neri, P. (1964). [EPIDEMIC OF YELLOW FEVER IN ETHIOPIA (1960-1962). PRELIMINARY STUDY]. *Bulletin of the World Health Organization*, *30*, 299–319.

SERION Chikungunya ELISA. (2024). *SERION ELISA classic Chikungunya Virus IgG/IgM; Enzyme-immunoassay for determination of human antibodies for in vitro diagnostic us*.

SERION Dengue ELISA. (2024). *Instructions—Dengue Virus IgG/IgM, Dengue Virus superior IgM. Enzyme-immunoassay for determination of human antibodies for in vitro diagnostic use. English (Version V 114.5)*.

Setbon, M., & Raude, J. (2009). Le chikungunya à la Réunion: Facteurs sociaux, environnementaux et comportementaux en situation épidémique: *Population*, *Vol. 63*(3), 555–583.

Sharma, R., Kesari, P., Kumar, P., & Tomar, S. (2018). Structure-function insights into chikungunya virus capsid protein: Small molecules targeting capsid hydrophobic pocket. *Virology*, *515*, 223–234.

Sharma, S., Dash, P. K., Santhosh, S. R., Shukla, J., Parida, M., et al. (2010). Development of a Quantitative Competitive Reverse Transcription Polymerase Chain Reaction (QC-RT–PCR) for Detection and Quantitation of Chikungunya Virus. *Molecular Biotechnology*, *45*(1), 49–55.

Shimelis, T., Vaz Nery, S., Tadesse, B. T., Bartlett, A. W., Belay, F. W., et al. (2022). Clinical management and outcomes of acute febrile illness in children attending a tertiary hospital in southern Ethiopia. *BMC Infectious Diseases*, *22*(1), 434.

Shirshikov, F. V., & Bespyatykh, J. A. (2022). Loop-Mediated Isothermal Amplification: From Theory to Practice. *Russian Journal of Bioorganic Chemistry*, *48*(6), 1159-74.

Shragai, T., Tesla, B., Murdock, C., & Harrington, L. C. (2017). Zika and chikungunya: Mosquito-borne viruses in a changing world. *Annals of the New York Academy of Sciences*, *1399*(1), 61–77.

Shuaib, F., Todd, D., Campbell-Stennett, D., Ehiri, J., & Jolly, P. E. (2010). Knowledge, attitudes and practices regarding dengue infection in Westmoreland, Jamaica. *The West Indian Medical Journal*, *59*(2), 139–146.

Shukla, J., Khan, M., Tiwari, M., Sannarangaiah, S., Sharma, S., et al. (2009). Development and evaluation of antigen capture ELISA for early clinical diagnosis of chikungunya. *Diagnostic Microbiology and Infectious Disease*, *65*(2), 142-49.

Silva, L. A., & Dermody, T. S. (2017). Chikungunya virus: Epidemiology, replication, disease mechanisms, and prospective intervention strategies. *The Journal of Clinical Investigation*, *127*(3), 737–749.

Simmonds, P., Becher, P., Bukh, J., Gould, E. A., Meyers, G., *et al.,* & Ictv Report Consortium, null. (2017). ICTV Virus Taxonomy Profile: Flaviviridae. *The Journal of General Virology*, *98*(1), 2–3.

Simo, F. B. N., Bigna, J. J., Kenmoe, S., Ndangang, M. S., Temfack, E., et al. (2019). Dengue virus infection in people residing in Africa: A systematic review and meta-analysis of prevalence studies. *Scientific Reports*, *9*(1), 13626.

Simon, F., Tolou, H., & Jeandel, P. (2006). Chikungunya, l'épidémie que l'on n'attendait pas. *La Revue de Médecine Interne*, *27*(6), 437–441.

Sisay, C., Waldetensai, A., Seyoum, M., Tayachew, A., Wossen, M., et al. (2022). Detection of serotype 1-Dengue fever outbreak in Dire Dawa city, Eastern Ethiopia. *Ethiopian Journal of Public Health and Nutrition*, *5*(1), 49–54.

Socha, W., Kwasnik, M., Larska, M., Rola, J., & Rozek, W. (2022). Vector-Borne Viral Diseases as a Current Threat for Human and Animal Health—One Health Perspective. *Journal of Clinical Medicine*, *11*(11), 3026.

Solignat, M., Gay, B., Higgs, S., Briant, L., & Devaux, C. (2009). Replication cycle of chikungunya: A re-emerging arbovirus. *Virology*, *393*(2), 183–197.

Sourisseau, M., Schilte, C., Casartelli, N., Trouillet, C., Guivel-Benhassine, F., et al. (2007). Characterization of reemerging chikungunya virus. *PLoS Pathogens*, *3*(6), e89.

Stanaway, J. D., Shepard, D. S., Undurraga, E. A., Halasa, Y. A., Coffeng, L. E., et al. (2016). The global burden of dengue: An analysis from the Global Burden of Disease Study 2013. *The Lancet. Infectious Diseases*, *16*(6), 712–723.

Staples, J. E., Breiman, R. F., & Powers, A. M. (2009). Chikungunya Fever: An Epidemiological Review of a Re-Emerging Infectious Disease. *Clinical Infectious Diseases*, *49*(6), 942–948.

Suhrbier, A., Jaffar-Bandjee, M.-C., & Gasque, P. (2012). Arthritogenic alphaviruses—An overview. *Nature Reviews Rheumatology*, *8*(7), 420–429.

Sullivan, L. M. (2006). Estimation from samples. *Circulation*, *114*(5), 445–449.

Takele, D. (2020). Factors associated with Chikungunya fever virus outbreak in Ethiopia, June 2016. *International Journal of Infectious Diseases*, *101*, 246.

Tang, B. L. (2012). The cell biology of Chikungunya virus infection: Cell biology of CHIKV infection. *Cellular Microbiology*, *14*(9), 1354–1363.

Tang, J., Jose, J., Chipman, P., Zhang, W., Kuhn, R. J., et al. (2011). Molecular links between the E2 envelope glycoprotein and nucleocapsid core in Sindbis virus. *Journal of Molecular Biology*, *414*(3), 442–459.

Thomas, S., Rai, J., John, L., Günther, S., Drosten, C., et al. (2010). Functional dissection of the alphavirus capsid protease: Sequence requirements for activity. *Virology Journal*, *7*(1), 327.

Tsetsarkin, K. A., Vanlandingham, D. L., McGee, C. E., & Higgs, S. (2007). A Single Mutation in Chikungunya Virus Affects Vector Specificity and Epidemic Potential. *PLoS Pathogens*, *3*(12), e201.

Tun, Z. M., Moorthy, M., Linster, M., Su, Y. C., Coker, R. J., et al. (2016). Characteristics of acute febrile illness and determinants of illness recovery among adults presenting to Singapore primary care clinics. *BMC Infectious Diseases*, *16*(1), 612.

Vaidya, V., & Sawant, S. (2013). A KAP Study in Pune City Involving School Children as a Strategy for effective Vector Control in Chikungunya. *Indian Journal of Public Health Research & Development*, *4*(4), 238.

van den Hurk, A. F., Hall-Mendelin, S., Pyke, A. T., Frentiu, F. D., McElroy, K., et al. (2012). Impact of Wolbachia on infection with chikungunya and yellow fever viruses in the mosquito vector Aedes aegypti. *PLoS Neglected Tropical Diseases*, *6*(11), e1892.

van der Schaar, H. M., Rust, M. J., Chen, C., van der Ende-Metselaar, H., Wilschut, J., et al. (2008). Dissecting the cell entry pathway of dengue virus by single-particle tracking in living cells. *PLoS Pathogens*, *4*(12), e1000244.

Vasilakis, N., Lambert, A., MacLachlan, N. J., & Brault, A. C. (2016). Genomic Organization of Arboviral Families. In *Arboviruses: Molecular Biology, Evolution and Control* (pp. 31–44). Caister Academic Press.

Verma, A., Chandele, A., Nayak, K., Kaja, M. K., Arulandu, A., et al. (2016). High yield expression and purification of Chikungunya virus E2 recombinant protein and its evaluation for serodiagnosis. *Journal of Virological Methods*, *235*, 73–79.

Vilibic-Cavlek, T., Ferenc, T., Vujica Ferenc, M., Bogdanic, M., Potocnik-Hunjadi, T., et al. (2022). Cross-Reactive Antibodies in Tick-Borne Encephalitis: Case Report and Literature Review. *Antibodies*, *11*(4), 72

Volk, S. M., Chen, R., Tsetsarkin, K. A., Adams, A. P., Garcia, T. I., et al. (2010). Genome-Scale Phylogenetic Analyses of Chikungunya Virus Reveal Independent Emergences of Recent Epidemics and Various Evolutionary Rates. *Journal of Virology*, *84*(13), 6497–6504.

Wahala, W. M. P. B., & Silva, A. M. de. (2011). The human antibody response to dengue virus infection. *Viruses*, *3*(12), 2374–2395.

Walker, T., Jeffries, C. L., Mansfield, K. L., & Johnson, N. (2014). Mosquito cell lines: History, isolation, availability and application to assess the threat of arboviral transmission in the United Kingdom. *Parasites & Vectors*, *7*(1), 382.

Waman, V. P., Kale, M. M., & Kulkarni-Kale, U. (2017). Genetic diversity and evolution of dengue virus serotype 3: A comparative genomics study. *Infection, Genetics and Evolution*, *49*, 234–240.

Waman, V. P., Kolekar, P., Ramtirthkar, M. R., Kale, M. M., & Kulkarni-Kale, U. (2016). Analysis of genotype diversity and evolution of Dengue virus serotype 2 using complete genomes. *PeerJ*, *4*, e2326.

Wan, S.-W., Wu-Hsieh, B. A., Lin, Y.-S., Chen, W.-Y., Huang, Y., et al. (2018). The monocyte-macrophage-mast cell axis in dengue pathogenesis. *Journal of Biomedical Science*, *25*(1), 77.

Wang, R., Wang, X., Zhang, L., Feng, G., Liu, M., et al. (2022). The epidemiology and disease burden of children hospitalized for viral infections within the family Flaviviridae in China: A national cross-sectional study. *PLoS Neglected Tropical Diseases*, *16*(7), e0010562.

Ward, D. I. (2006). A case of fatal Plasmodium falciparum malaria complicated by acute dengue fever in East Timor. *The American Journal of Tropical Medicine and Hygiene*, *75*(1), 182–185.

Weaver, S. C., & Reisen, W. K. (2010). Present and future arboviral threats. *Antiviral Research*, *85*(2), 328–345.

Weaver, S. C., & Vasilakis, N. (2009). Molecular evolution of dengue viruses: Contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. *Infection, Genetics and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases*, *9*(4), 523–540.

Wedell, N., Price, T. a. R., & Lindholm, A. K. (2019). Gene drive: Progress and prospects. *Proceedings. Biological Sciences*, *286*(1917), 20192709.

Westaway, E. G., Mackenzie, J. M., & Khromykh, A. A. (2003). Kunjin RNA replication and applications of Kunjin replicons. *Advances in Virus Research*, *59*, 99–140.

White, J. M., & Whittaker, G. R. (2016). Fusion of Enveloped Viruses in Endosomes. *Traffic (Copenhagen, Denmark)*, *17*(6), 593–614.

WHO. (1997). *Dengue haemorrhagic fever Diagnosis, treatment, prevention and control, 2nd edition*. http://apps.who.int/iris/bitstream/ handle/10665/41988/ 9241545003

WHO. (2009). *Dengue guidelines for diagnosis, treatment, prevention and control: New edition*. World Health Organization. https://apps.who.int/iris/handle/10665/44188

WHO. (2013). *WHO informal consultation on fever management in peripheral health care settings: A global review of evidence and practice*. World Health Organization. https://iris.who.int/handle/10665/95116

WHO. (2015). *Guidelines for the treatment of malaria* (3rd ed). World Health Organization. https://iris.who.int/handle/10665/162441

WHO. (2017). *Global vector control response 2017-2030. UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases*. World Health Organization. https://apps.who.int/iris/handle/10665/259205

WHO. (2018). *WHO consultation on Chikungunya vaccine evaluation; Research and Diagnostics Blueprint* [Meeting Report (DRAFT)]. World Health Organisation. www.who.int/chikungunya-vaccines-workshop-29-november-2018

WHO. (2020). *Vector-borne diseases; key facts*. World Health Organisation. https:// www.who.int/news-room/fact-sheets/detail/vector-borne-diseases

WHO. (2020b). Ending the neglect to attain the Sustainable Development Goals: a road map for neglected tropical diseases 2021–2030. .World Health Organization; 2020. Available from: www.eliminateschisto.org/WHO_Revised-Draft-NTD-Roadmap2021-2030-23Apr2020

WHO. (2021, February 4). WHO supports Ethiopia’s response to a dengue fever outbreak in Somali Region. *World Health Organization*. https://www.afro.who.int/countries/ethiopia/news/who-supports-ethiopias-response-dengue-fever-outbreak-somali-region

WHO. (2022a). *Surveillance and Control of Arboviral Diseases in the WHO African Region: Assessment of Country Capacities* (1st ed). World Health Organization.

WHO. (2022b). *Surveillance and Control of Arboviral Diseases in the WHO African Region: Assessment of Country Capacities; WHO Special Program for Research Training Tropical Diseases* (1st ed). World Health Organization.

WHO. (2022c, July 14). *Laboratory testing for Zika virus and dengue virus infections; Interim guidance*. World Health Organization. https://www.who.int/publications/i/item/WHO-ZIKV_DENV-LAB-2022.1

WHO. (2022d, December 8). *Chikungunya. Key facts*. World Health Organisation. https://www.who.int/news-room/fact-sheets/detail/chikungunya

WHO. (2023, March 17). *Dengue and severe dengue. Fact Sheet*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue

WHO. (2024, October 31). *Disease Outbreak News; Malaria in Ethiopia*. World Healht oragnization. http://www.who.int/emergencies/disease-outbreak-news/item/2024-DON542

Wikan, N., Sakoonwatanyoo, P., Ubol, S., Yoksan, S., & Smith, D. R. (2012). Chikungunya virus infection of cell lines: Analysis of the East, Central and South African lineage. *PloS One*, *7*(1), e31102.

Wilson, A. L., Courtenay, O., Kelly-Hope, L. A., Scott, T. W., Takken, W., et al. (2020). The importance of vector control for the control and elimination of vector-borne diseases. *PLoS Neglected Tropical Diseases*, *14*(1), e0007831.

Wittke, V., Robb, T. E., Thu, H. M., Nisalak, A., Nimmannitya, S., et al. (2002). Extinction and rapid emergence of strains of dengue 3 virus during an interepidemic period. *Virology*, *301*(1), 148–156.

Wong, L. P., Shakir, S. M. M., Atefi, N., & AbuBakar, S. (2015). Factors Affecting Dengue Prevention Practices: Nationwide Survey of the Malaysian Public. *PLOS ONE*, *10*(4), e0122890.

Woyessa, A. (2014). The emergence of dengue fever in Ethiopia. *The Ethiopian Journal of Health Development*, *28*(3).

Woyessa, A. B., Mengesha, M., Kassa, W., Kifle, E., Wondabeku, M., et al. (2014). The first acute febrile illness investigation associated with dengue fever in Ethiopia, 2013: A descriptive analysis. *The Ethiopian Journal of Health Development*, *28*(3).

Woyessa, A. B., Omballa, V., Wang, D., Lambert, A., Waiboci, L., et al. (2014). An outbreak of acute febrile illness caused by Sandfly Fever Sicilian Virus in the Afar National Regional State of Ethiopia, 2011. *The American Journal of Tropical Medicine and Hygiene*, *91*(6), 1250–1253.

Wu, X., Lu, Y., Zhou, S., Chen, L., & Xu, B. (2016). Impact of climate change on human infectious diseases: Empirical evidence and human adaptation. *Environment International*, *86*, 14–23.

Xie, X., Zou, J., Zhang, X., Zhou, Y., Routh, A. L., et al. (2019). Dengue NS2A Protein Orchestrates Virus Assembly. *Cell Host & Microbe*, *26*(5), 606-622.e8.

Xu, C., Chen, Y., Zhu, G., Wu, H., Jiang, Q., et al. (2024). A Portable, Integrated, Sample-In Result-Out Nucleic Acid Diagnostic Device for Rapid and Sensitive Chikungunya Virus Detection. *Micromachines*, *15*(5), 663.

Yap, M. L., Klose, T., Urakami, A., Hasan, S. S., Akahata, W., et al. (2017). Structural studies of Chikungunya virus maturation. *Proceedings of the National Academy of Sciences*, *114*(52), 13703–13707.

Yathi, K. K., Joseph, J. M., Bhasker, S., Kumar, R., & Chinnamma, M. (2011). Recombinant CHIK virus E1 coat protein of 11 KDa with antigenic domains for the detection of Chikungunya. *Journal of Immunological Methods*, *372*(1–2), 171–176.

Yeboaa, C., Odoi, H., Owusu Ntim, R., Boakye, Y. D., Kwakye-Nuako, G., et al. (2023). Diversity and antibiograms of bacteria isolated from cutaneous leishmaniasis wounds in the Nkwanta South District of Ghana. *Archives of Microbiology*, *205*(2), 74.

Yu, I.-M., Zhang, W., Holdaway, H. A., Li, L., Kostyuchenko, V. A., et al. (2008). Structure of the immature dengue virus at low pH primes proteolytic maturation. *Science (New York, N.Y.)*, *319*(5871), 1834–1837.

Yusuf, A. M., & Ibrahim, N. A. (2019). Knowledge, attitude and practice towards dengue fever prevention and associated factors among public health sector health-care professionals: In Dire Dawa, eastern Ethiopia. *Risk Management and Healthcare Policy*, *Volume 12*, 91–104.

Zerfu, B., Kassa, T., & Legesse, M. (2023). Epidemiology, biology, pathogenesis, clinical manifestations, and diagnosis of dengue virus infection, and its trend in Ethiopia: A comprehensive literature review. *Tropical Medicine and Health*, *51*(1), 11.

Zerfu, B., Kassa, T., Mamo, G., Larrick, J. W., & Legesse, M. (2024). High seroprevalence of IgM antibodies against chikungunya among patients with acute febrile illness seeking healthcare in a malaria-endemic area in the Afar National Regional State, Northeast Ethiopia. *SAGE Open Medicine*, *12*, 20503121241276557.

Zerfu, B., Medhin, G., Mamo, G., Getahun, G., Tschopp, R., et al. (2018). Community-based prevalence of typhoid fever, typhus, brucellosis and malaria among symptomatic individuals in Afar National Regional State, Ethiopia. *PLOS Neglected Tropical Diseases*, *12*(10), e0006749.

Zhang, B., Dong, H., Stein, D. A., Iversen, P. L., & Shi, P.-Y. (2008). West Nile virus genome cyclization and RNA replication require two pairs of long-distance RNA interactions. *Virology*, *373*(1), 1–13.

Zhao, Y., Chen, F., Li, Q., Wang, L., & Fan, C. (2015). Isothermal Amplification of Nucleic Acids. *Chemical Reviews*, *115*(22), 12491–12545.

Ziegler, S. A., Lu, L., da Rosa, A. P. A. T., Xiao, S.-Y., & Tesh, R. B. (2008). An animal model for studying the pathogenesis of chikungunya virus infection. *The American Journal of Tropical Medicine and Hygiene*, *79*(1), 133–139.

Zingman, M. A., Paulino, A. T., & Payano, M. P. (2017). Clinical manifestations of chikungunya among university professors and staff in Santo Domingo, the Dominican Republic. *Revista Panamericana de Salud Pública*, *41*, 1.

Zitzmann, C., Schmid, B., Ruggieri, A., Perelson, A. S., Binder, M., et al. (2020). A Coupled Mathematical Model of the Intracellular Replication of Dengue Virus and the Host Cell Immune Response to Infection. *Frontiers in Microbiology*, *11*, 725.

8. ANNEXES

Annex 8.1: Information sheet and consent form_Amharic version

ለጥናቱ ተሳታፊዎች የሚሰጥ መግለጫ

የጥናቱ ርዕስ: - Prevalence of Dengue and Chikungunya in Malaria-Suspected Febrile Patients: Mosquito-Borne Diseases’ Knowledge, Practices and Diagnostic Challenges in Selected Districts of Afar National Regional State, Northeast Ethiopia
የጥናቱ ተመራማሪዎች

ብሩክ ዘርፉ **(**የ’PhD’ ተማሪ፣ ሞባይል፡ ***251-911-99-37-99***፣ ኢ-ሜይል፡ Biruk.zerfu@aau.edu.et)

ፕ/ር መንግሰቱ ለገሴ **(**የጥናቱ ዋና አስተባባሪ፣ ሞባይል፡ ***0911345908*)**

አድራሻ፡ አክሊሉ ለማ ፓቶባዮሎጂ መካነ ጥናት

አዲስ አበባ ዩኒቨርሲቲ፣ አዲስ አበባ

ስልክ፡ ***251 -11-276-30-91* (**ቢሮ**)**

የፖስታ ሳጥን ቁጥር፡ ***1176*** አዲስ አበባ፣ ኢትዮጵያ

ለምርምሩ ሥራ የገንዘብ ድጋፍ ያደርገዉ ድርጅት፡ አዲስ አበባ ዩኒቨርሲቲ፣ የጥናትና ምርምር ቢሮ

በመጀመሪያ በጥናቱ ላይ ለመሳተፍ ፍቃደኛ ስለሆኑ ልባዊ ምስጋናዬን አቀርባለው። ስለ□ህ እባ□ ን □ህንን የተሳታኝ- ች መረጃ ከልብ ዕንዲያዳምጡ በትህትና እንጠይቃለን፡፡ ስለጥናቱ ያለዎትን ጥያቄ በማንኛውም ጊዜ መጠየቅ ይችላሉ፡፡

መ□ቢ□

አብዛኛቹ ትኩሳት አምጪ በሽታ- ች ህመምና ምልክቶች ተመሳሳይነት ዐላቸዉ ፡፡ ስለዚህ በትክክል ለመመርመርና ለማከም ዐዳጋች ይሆናሉ፡፡ በተጨማሪም የህሙማንና የሙአች ቁጥር ኢዲጨምር ይሆናል፡፡ በዚህ ምክኒያት ክስካሁን ድረስ አብዛኛቹ ትኩሳት አምጪ በሽታ- ች ለመከላከልም ሆነ ለማከም ስርጭቱ በግልጽ አይታወቅም። በትንኝ ንክሻ የሚተላለፉቫይረስ(Arbovirus) በሽታዎች አደገኛ እና ገዳይ የሆኑ በቫይረስ አማካኝነት የሚከሰቱ ትኩሳት አምጪ በሽታዎች ናቸዉ። በበሽታዎቹ የተያዘ ሰዉ ከፍተነኛ የሆነ ትኩሳት፤ ማንቀጥቀጥ፤ የምግብፍላጎት መቀነስ፤ ማቅለሽለሽ፤ ማስመለስ፤ የጡንቻመዛል፤ የሰዉነትና የዓይን ቀለም ቢጫ መምሰል፤ የተለያዪ የሰዉነት ክፍሎች መድማት፤ የአንጎል ቁስለት እና የመሳሰሉ ምልክቶች ልያሳይይችላል። አገራችን ኢትዮጵያ ለበሽታዉ ተጋለጭ ከሆኑ አገሮች ዉሰጥ አንዷ ስትሆን ባለፉት ከሁለት እስከ አምስት ዓመታት ዉሰጥ ከነዚህ በሽታዎች ዉሰጥ የቢጫ ወባ በሽታ በደቡብ ኦሞ ዞን እና ችኩንጉያ በድሬድዋ አካባቢዎች በርካታ ሰዎች የታመሙና ጥቂት የማይባሉ ሰዎች ደግሞ በበሽታዉ ምክንያት መሞታቸዉ ተረጋግጧል። ይሁን እነ፯ የበሽታዉ መጠነ-ስርጭትና ተያያዥ አጋላጭ መንስኤዎች በአፋር ዞኖችና ዋርዳዎች የማይታወቅ በመሆኑ ለዚሁ መልስ ይሆን ዘንድ በአፋር ክልል በተመረጡ ወረዳዎች ዉስጥ የሚገኙ ጤና ተቋማት የሚገኙ ትኩሳት ካለባቸዉ ህሙማን በትንኝ ንክሻ አማካኝነት የሚተላለፉ የቨይረስበሽታዎች (DENGUE FEVER እና CHICKUNGUNYA) ስርጭት ማጥናት ይሆናል። የጥናቱ አስፈላጊነትም ታምኖበት ከአዲስ አበባ ዩኒቨርሲቲ አክሊሉ ለማ ፓቶ ባዮሎ መካነ ጥናት ኢንስቲቱት ጥናቲና ምርምር ስነ-ምግባር ኮሚቴ ፈቃድ አግኝቱአል።

ስለዚህ ዕርሶም በጥናቱ ላይ እንዲሳተፉ ፈቃድዎን በትትና እንጠይቃለን። በምርምሩ ለመሳተፍ ከመወሰንዎ በፊት የሚከተለዉን ዝርዝር መረ□ ማለትም ስለጥናቱ አላማ፤ የጥናቱ ተሳታፊዎች የሚሰጡት ናሙና፤ የሚደረገዉ ምርመራ፤ በጥናቱ ሲሳተፉ ሊኖሩ የሚችሉ ችግሮች፤ የጥናቱ ጥቅም፤ የሚስጥር አጠባበቅ በተመለከተ በማንበብ ወይም እንዲነበብሎት በማድረግና ጥያቄ ካሎት በመጠየቅ በቂ ግንዛቤ እንዲወስዱ ይጠየቃሉ። **በዚህ ጥናት መሳተፍ ሙሉ በሙሉ በፈቃደኝነት ላይ የተመሰረተ ሲሆን ምንም አይነት ምክንያት መስጠት ሳያስፈልጎት በጥናቱ የሚኖሮትን ተሳትፎ በማንኛዉም ጊዜ ማቆም ይችላሉ፡፡ በጥናቱ ባለመሳተፎ ምክንያት ከጤና ተቋአም ሊያገኙ የሚችሉትን የጤና አገልግሎት አሁንም ሆነ ወደፊት ያለምንም አድል- □ሰ□ቫል።**

የጥናቱ አላማ

በአፋር ክልል በተመረጡ ወረዳዎች ዉስጥ የሚገኙ ጤና ተቋማት የሚገኙ ትኩሳት ካለባቸዉ ህሙማን በትንኝ ንክሻ አማካኝነት የሚተላለፉ የቨይረስ በሪታዎች (DENGUE FEVER እና CHICKUNGUNYA) ስርጭት ማጥናት ይሆናል።

የጥናቱ ተሳታኝ- ች

በዚህ ጥናት የሚሳተፉት በአፋር ክልል በተመረጡ ወረዳዎች ዉስጥ የሚገኙ ጤና ተቋማት የሚገኙ ትኩሳት ያለባቸዉና አድሜአቸዉ 5 አመትና ከዚያ μላይ የሆኑ ናቸዉ፡፡

የናሙና አሰባሰብ ሂደት:

ናሙና ለመስጠት ፍቃደኛ ከሆኑልን ክንዶ ላይ ከለዉ ደም ስር አስፈላጊዉን ቅድመ-ዝግጅት ከተደረገ ቡሀላ በሰለጠነ ጤና ባለሞያ 3-5 ምሊሊትር የሆነ የደም ናሙና ይወሰዳል፡፡ ናሙናዉ በምንወስድበት ወቅት የሚሰማዉ ህመም ዕደማንኛዉም ላቦራቶሪ ደም ሲወሰድ የሚሰማ ጥቂት ህመም ስሜት ነዉ፡፡
ከናሙናዉ በእስላይድ ላይ ለወባ ምርመራ ካዘጋ□ን ቡሀላ ሰረሙ ተለይቶ ታይፎድን በዋይዳልና፤ ታይፈስን በዌልፍሌክስ ክ□ሁ ጤና ተቁአም ምርመራ ይደረጋል። ቀሪዉ ናሙና በትንኝ ንክሻ አማካኝነት የሚተላለፉ የቨይረስ በሪታዎች እኪመረመር በማቀዝቀዣ ዉስጥ ይቀመጣል።
በምርመራዉ ወቅት ከላይ የተጠቀሱት በሪታ- ች ከተገኘ በሀኪም አማከኝነት ተገቢዉን እ□ምና እንዲሰጦት ይደረጋል፤ ካልተገኘ ግን እደአስፈላጊነቱ በአኪሙ ዉሳኔ ምርመራና እክምና ያደርጋሉ።

የጥናቱ ተሳታኝ- ች ጥቅም

ተሳታኝ- ች በጥናቱ በመሳተፍ ምንም አይነት ቀ□ተኛ የገንዘብ ጥቅም አያገኙም፡፡ ነገር ግን ተሳታኝ- ች የናሙናዉ ምርመራ ውጤት ህመም ከዐለበት ተገቢውን ህክምና□እን□ታከሙ □□ረፉል፡፡

ከጥናቱ ሊመጡ የሚችሉ የጕንዮሽ ጉዳቶች

ልምድ ያለዉ የሰለጠነ የጤና ባለሞያ ከእርሶ አግባብ ባለዉ መልኩ ናሙና ይወሰዳል። ስለዚህ ናሙና ከመስጠትና በምርምሩ በመሳተፍ ጋር በተያያዘ ችግሮች ይከሰታል ተብሎ አይጠበቅም። ሆኖም ደም በሚወሰድበት ጊዜ መጠነኛ ህመም ሊሰማዎት ይችላል። በማንኛዉም መልኩ በምርምሩ በመሳተፎዎ ምክንያት ችግር ቢከሰት አስፈላጊዉን የህክምና እርዳታ ያለምንም ወጪ ይደረግሎታል።

ሚስጥር አጠባበቅ

ከእርሶ የተሰበሰበ **ማንኛዉም መረጃ በሚ**ስጥር ይያዛል። የስምምነትና የመጠየቂያቅጾች ጥናቱ ከተጠናቀቀ ከአንድ አመት **በኁሀላ** የሚቃጠል:: የምርመራና የላቦራቶሪ ዉጤቶም ከሀኪሞዎና ከተመራማሪዎች ዉጭ **ለሌላ ሰዉ ተላል**ፎ አይሰጥም።

1. □ተሳታፊዎች የስምምነት ቅ□/ □17 አመት በላይ ለሆኑ ሰዎች/

□ተሳተኝ ስም ---□ክትሜ--------- □ታ-------
□ሚስ□ር ቁ□ር--------------- የጥናቱ ቦታ/የጤና ተቋም ስም--------------------------------

እኔ ስሜ ከላይ የተጠቀሰዉ ግለሰብ ስለ በትንኝ ንክሻ አማካኝነት የሚተላለፉ የቨይረስ በሪታዎች ስርጭት በሚመለከት ስለሚደረገዉ ጥናትና ምርምር በቂ መረጃ የተሰጠኝ መሆኑን አረጋግጣለዉ። በጥናቱ ተሳታፊ ለመሆን ከተስማማዉ **3-5**ሚ.ሊ የሚሆን የደም ናሙና እንደምሰጥ ተገል□ልኛል። ተመራማሪዎቹ በምርምሩ በመሳተፍ ምክንያት ሊከሰት የሚችል ችግር እንደልለ አረጋግጠዉልኛል። በተጨማሪም የኔ የላብራቶሪና የሀኪም ምርመራ ዉጤት ለምርምር ብቻ እንደሚዉልና ከኔ ጋር ግንኙነት ያላቸዉ ማንኛዉም መረጃዎች በሚስጥር እንደሚያዝ ተገል□ልኛል። በምርምሩ መሳተፍ ሙሉ በሙሉ በፈቃደኝነት ላይ የተመሰረተ እንደሆነና አስፈላጊ ከሆነም ምንም አይነት ምክንያት መስጠት ሳያስፈልገኝ በማንኝዉም ጊዜ የጥናቱ ተሳታፊነቴን ማቋአረጥ እንደምችል ተረድቻለዉ።

በጥናቱ ባለመሳተፌ ምክነያት ከጤና ተሸሙ የሚገኘዉ የጤና አገልግሎት አሁንም ሆነ ወደፊት እንደማይስተጉአጉል ተገንዝቤያለዉ። አስፈላጊዉ መረጃ ሁሉ ግልጽና በሚገባኝ ሽንሽ እንደተሰጠኝ አረጋግጣለዉ። በመጨረሮም የተሰጠኝን መረጃ በቂ ጊዜ ተሰጥቶኝ በሚገባ ከአሰብኩበት ቡሀላ በምርምሩ ለመሳተፍ መስማማቴን በፊርማዬ አረጋግጣለዉ።

የተሳታፊ ስም ------------------------------ ፊርማ-------------------- ቀን-------------------

የተሳታፍዉን ስምምነት የተቀበለዉ ሀኪም ስም -------------------- ፊርማ ------ ቀን---------

በስምምነት ወቅት የነበሩ እማኝ ስም ------------------ፊርማ --------------ቀን ---------

2. የወላጅ ወይም ያሳዳጊ ፈካደኝነት ቅፅ /ከ12አመት እድሜ በታች ያሉ ታዳጊዎች ብቻ/

የአሳዳጊው/ የወላጅ ስም------------------------------ያላቸዉ ግንኙነት----------------
□ተሳተኝ ስም ---□ክትሜ--------- □ታ-------
□ሚስ□ር ቁ□ር --------------- የጥናቱ ቦታ/ቀበሌ ስም--------------------------------

እኔ ስሜ ከላይ የተጠቀሰዉ **አሳ□ይ/ወለ□** ስለ በትንኝ ንክሻ አማካኝነት የሚተላለፉ የቨይረስ በሪታዎች ስርጭት በሚመለከት ስለሚደረገዉ ጥናትና **ምርምር** በቂ መረጃ የተሰጠኝ መሆኑን አረጋግጣለዉ። በጥናቱ ተሳታፊ ለመሆን ከተስማማዉ **ከተሳተፊዉ ልጅ ከ3-5** ሚ.ሊ የሚሆን የደም ናሙና እንደሚሰድ ተገል□ልኛል። ተመራማሪዎቹ በምርምሩ በመሳተፍ ምክንያት ሊከሰት የሚችል ችግር እንደልለ አረጋግጠዉልኛል። በተጨማሪም የላብራቶሪና የሀኪም ምርመራ ዉጤት

ለምርምር ብቻ እንደሚዉልና ከኛ ጋር ግንኙነት ያላቸዉ ማንኛዉም መረጃዎች በሚስጥር እንደሚያዝ ተገል□ልኛል፡፡ በምርምሩ መሳተፍ ሙሉ በሙሉ በፈቃደኝነት ላይ የተመሰረተ እንደሆነና አስፈላጊ ከሆነም ምንም አይነት ምክንያት መስጠት ሳያስፈልገኝ በማንኛዉም ጊዜ የጥናቱ ተሳታፊነቴን ማቋረጥ እንደምችል ተረድቻለዉ፡፡

በጥናቱ ባለመሳተፌ ምክነያት ከጤና ተሸሙ የሚገኘዉ የጤና አገልግሎት አሁንም ሆነ ወደፊት እንደማይስተጉአጉል ተገንዝቤያለዉ፡፡ አስፈላጊዉ መረጃ ሁሉ ግልጽና በሚገባኝ ሸነሸ እንደተሰጠኝ አረጋግጣለዉ፡፡ በመጨረሮም የተሰጠኝን መረጃ በቂ ጊዜ ተሰጥቶኝ በሚገባ ከአሰብኩበት ቡሀላ **ልጄ** በምርምሩ የመሳተፍ **ፍቃደኝነት** መስጠቴን በፊርማዬ አረጋግጣለዉ፡፡

የአሳዳጊው/ የወላጅ ስም-------------------- ኘርማ--------- ቀን -----------
የአጥኒዉ ስም---------------------------------- ኘርማ--------- ቀን --------------
□መስ□ር ስም--------------------------------- ኘርማ------ ቀን -------------

3. □ፈቃደኝነት ማረጋገጫ/ ከ12-17 ለሆኑ ታዳጊዎች/

የጥናቱ ተሳታኝ ስም-------------------- የአሳዳጊው/ የወላጅ ስም------------------
□ሚስ□ር ቁ□ር --------------- የጥናቱ ቦቫሬቀበሌ ስም--------------------------------

□እኛ ስማችን ከላይ የተጠቀሰዉ **ትኩሳት ከአለባቸዉ ሰ- ች** በትንኝ ንክሻ አማካኝነት የሚተላለፉ የቨይረስ በሪታዎች **ስርጭት** ስለ ማ□ናት መሆኑን **ተነግሮናል/ተገልጾልናል::** በጥናቱ ተሳታፊ ለመሆን ከተስማማን ከ**3-5**ሚሊ ሊትር የሚሆን የደም ናሙና እንደምንሰጥ ተገል□ልናል፡፡ ተመራማሪዎቹ በምርምሩ በመሳተፍ ምክንያት ሊከሰት የሚችል ችግር እንደሌለ አረጋግጠዉልናል፡፡ በተጨማሪም የላብራቶሪና የሀኪም ምርመራ ዉጤት ለምርምር ብቻ እንደሚዉልና ከኛ ጋር ግንኙነት ያላቸዉ ማንኛዉም መረጃዎች በሚስጥር እንደሚያዝ ተገል□ልናል፡፡ በምርምሩ መሳተፍ ሙሉ በሙሉ በፈቃደኝነት ላይ የተመሰረተ እንደሆነና አስፈላጊ ከሆነም ምንም አይነት ምክንያት መስጠት ሳያስፈልገን በማንኛዉም ጊዜ የጥናቱ ተሳታፊነታችንን ማቋረጥ እንደምንችል ተረድተናል፡፡

በጥናቱ ባለመሳተፋችን ምክነያት ከጤና ተሸሙ የሚገኘዉ የጤና አገልግሎት አሁንም ሆነ ወደፊት እንደማይስተጉአጉል ተገንዝበናል፡፡ አስፈላጊዉ መረጃ ሁሉ ግልጽና በሚገባን ሸንሸ እንደተሰጠን እናረጋግጣለን፡፡ በመጨረሮም የተሰጠንን መረጃ በቂ ጊዜ ተሰጥቶን በሚገባ ከአሰብንበት ቡሀላ በምርምሩ ለመሳተፍ መስማማታችንን በፊርማችን እናረጋግጣለን፡፡

የጥናቱ ተሳታኝ ስም--------------------ኘርማ---------------ቀን -----------
የአሳዳጊው/ የወላጅ ስም------------------ኘርማ--------------ቀን ------------
የአጥኒዉ ስም-----------------------------ኘርማ-------------ቀን ---------
□መስ□ር ስም------------------------------ኘርማ---------------ቀን -----------

Annex 8.2: Questionnaire for febrile patients attending healthcares institutes

Addis Ababa University

Aklilu Lema Institute of Pathobiology

Questionnaire used for demographic and clinical manifestations of febrile patients attending health institutes towards Arboviral diseases in selected districts,Afar National Regional State; Ethiopia, 2022

- **NOTICE**

Inclusion: Febrile patients with the age of 5 years old or older than 5 years that are attending health facility to get management/treatment for their illnesses will be included

Exclusion: Pregnant women and apparently anemic patients will be excluded

Code ____Date ___/__/_____ District_______ Kebele ______Health institute ______

I. Socio-demographic data

Name of the patient……………………………….. ……………

1. Sex 1) Male 2) Female
2. Age -------
3. Educational status: A. Illiterate B. 1^0 school(1-8)
   A. High school (9 – 12) D. higher institute (college or university)
4. Resident? A. Urban B. Semi urban C. Rural
5. Occupation? A. Pastoralist B. Agro pastoralist C. labor worker
   D. Government employee E. No job F. House wife G. Student H. Other specify…
6. Religion? 1) Muslim 2) Orthodox 3) Protestant 4) others………..
7. Ethnicity: 1) Afar 2) others, specify ………………..
8. Marital status A. Married B. Divorced C. widowed D. non married
9. Duration of residence in the area _______________ (years)
10. Do you have any travel history? A) Yes B) No
11. If yes, to which area or country and when? _____________ ; __________

II. Clinical data

1. Measured axial body temperature__________________ (^{o} c)
2. Duration of this febrile illness onset? ________________ (in days)
3. Other Clinical signs and symptoms:

1) Diarrhoea
2) Maculopapular rash
3) Joint pain
4) Headache
5) Sore throat
12. Malaise
13. Muscle pain
14. Calf pain
15. Back pain
16. Cough
17. Chills
18. Nausea
19. Pain behind eyes, conjunctivitis
20. Light bleeding (gums, nose, petechiae, bruising or positive tourniquet test)
21. Persisting vomiting
22. Abdominal pain/tenderness
23. Heavy Bleeding: (bloody vomit , bloody stool, mucosal bleeding
24. Difficulty in breathing
25. Other_________

4. Diagnosis/treatment history for the current illness 1. Yes 2. No
5. If **#4 Yes**, when ……………. (In days)
6. If **#4 yes**, which of the following providers/facility provide the treatment?

1) Health facility 2) Traditional healers 3) Religious 4) Other (specify) ______________

7. If **#6 (1)**, what was the diagnosis? ________
8. Was/is any family or other person sick from the same disease in the area in last 30 days?
   1. Yes 2. No
9. If **#8 yes**, how many persons do you think sick? ______
10. History of chronic diseases like HIV, MD etc 1. Yes 2. No
11. If **#10 yes**, indicate the disease _______________________________

III. Laboratory Results BF Result _______

Annex 8.3: Questionnaire for community Knowledge, Attitude and Practice

Addis Ababa University

Aklilu Lemma Institute of Pathobiology

<u>Questionnaire used for assessing Knowledge, Attitude and Practice of community towards chikungunya and mosquito-borne diseases in selected districts of Afar National Regional State; Ethiopia, 2022</u>

Code __________Date ___/____/_____ District______ Kebele ____________

Dear study participants, please kindly provide the following information

Participants' Socio-demographic Data and general awareness on febrile illness			
No.	Questions	Response/value level	Skip
101	Sex	1) Male 2) Female	
102	Age	______(yrs)	
103	Place of birth		
104	Religion	1) Muslim 2) Orthodox 3) Protestant 4) others, specify …………	
105	Ethnicity	1) Afar 2) Oromo 3) Amhara 4) Others ___	
106	Marital status	1) Married 2) widowed 3) divorced 4) single	
107	Educational status	1) Illiterate 2) Primary school (1-8) 3) High school (9-12) 4) TVET/ university 5) Others ………..	
108	Occupational status	1) Pastoralist 2) Agro-pastoralist 3) labor work 4) house wife 5) student 6) other……..	
109	Common cause of fever (febrile illness) in this area	1) Malaria 2) Typhoid 3) Typhus 4) Dengue fever 5) Chikungunya fever 6) others……	
110	Have you heard about Dengue and Chikungunya fever?	1) Yes 2) No	If no, stop here
111	If #109 is 'Yes', from Where did you hear?	1) Media 2) Colleagues 3) Teacher/ Education 4) Seminar/ meeting 5) others…….	
112	About which diseases did you hear?	1) Only about Dengue fever 2) Only about Chikungunya fever 3) About both	
Participants' Knowledge about Chikungunya fever			

Questions/ statement		Responses	
201	Is chikungunya (CHIK) caused by a virus	1. Yes 2. No 3. Not sure	
202	Can all mosquitoes transmit CHIK disease?	1. Yes 2. No 3. Not sure	
203	Can you identify Aedes Mosquito?	1. Yes 2. No	
204	If #503 is ‘Yes’, When does this Aedes Mosquito most likely to feed/bite?	1) Day time 2) Night time 3) At any time	
205	Do flies transmit CHIK?	1. Yes 2. No 3.Not sure	
206	Do ticks transmit CHIK?	1. Yes 2. No 3. Not sure	
207	Do person-to-person contacts can transmit CHIK disease?	1. Yes 2. No 3. Not sure	
208	Is CHIK transmitted through food and water?	1. Yes 2. No 3. Not sure	
209	Do mosquitoes breed in stagnant water?	1. Yes 2. No 3. Not sure	
210	Do window screen and bed net reduce mosquitoes?	1. Yes 2. No 3. Not sure	
211	Do insecticide sprays reduce mosquitoes and prevent CHIK?	1. Yes 2. No 3. Not sure	
212	Does tightly covering water container reduce mosquitoes?	1. Yes 2. No 3. Not sure	
213	Do mosquitoes repellant prevent mosquito bites?	1. Yes 2. No 3. Not sure	
214	Is the rainy season the only season when CHIK present?	1. Yes 2. No 3. Not sure	
215	Does CHIK affect Infants, Children and Adults?	1. Yes 2. No 3. Not sure	
2	What is/ are the major signs and symptoms of CHIK? (Circle as many as possible) 01) Fever 02) Joint pain 03) Headache 04) Muscle pain 05) Bone pain 06) Nausea 07) Rash		
Participants’ attitude about Chikungunya fever			

Questions/ statement		Responses	
301	Do you agree CHIK is a killer/serious disease?	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	

302	Do you agree CHIK is preventable?	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	
303	Do you agree water around house in discarded tires, broken pots and bottles are breeding place for mosquitoes like Aedes?	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	
304	Do you agree controlling mosquitoes breeding places are good strategy to prevent CHIK?	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	
305	Do you agree communities should actively participate in controlling the CHIK vector?	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	
306	Do you agree everyone has a chance to be infected by CHIK?	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	
307	Do you agree every one with sign and symptoms of CHIK need to consult immediately community health services?	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	
308	Do you agree everyone is a key in preventing CHIK?	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	
309	Do you agree all CHIK patients have a chance for a full recovery?	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	
310	Do you agree health service stakeholders are the only entity responsible to reduce larvae and adult mosquitoes in your home?	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	
311	Do you agree your neighborhood is CHIK high risk area?	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	
312	Do you agree community members are capable of preventing CHIK?	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	
313	Do you agree you are capable of preventing CHIK	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	
314	Do you agree government actions are needed to prevent CHIK?	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	
315	Do you agree you are responsible to prevent spread of mosquito larvae in your home?	1) Strongly Agree 2) Agree 3) Not sure 4) Disagree 5) Strongly disagree	
Participants’ practices about Chikungunya fever			

Questions/ statement		Responses	
401	Do you drain ponds or degrade muddy/wet areas to reduce mosquitoes	1) Yes 2) No	
402	Do you cut down bushes too short to reduce mosquitoes	1) Yes 2) No	
403	Do you clean of garbage/trash to reduce mosquito breeding sites	1) Yes 2) No	
404	Do you dispose water holding containers like tires, plastic, bottles or broken pots	1) Yes 2) No	
405	Do you use fan to prevent mosquito biting	1) Yes 2) No	
406	Do you cover body with clothes when working in the bush, farm, and forest	1) Yes 2) No	
407	Do you cover water containers around in the home	1) Yes 2) No	
408	Do you change the water of plant containers in the house every week	1)Yes 2) No	
409	Checking waste/garbage that can block water flow around the home	1) Yes 2) No	
410	Participating in any of anti-chikungunya campaigns in the community	1) Yes 2) No	
411	Checking and cleaning drains/gutters/roofs before the rainy season	1) Yes 2) No	
412	Using bed net when sleeping during the day	1) Yes 2) No	
413	Cleaning frequently water filled containers and draining around the house.	1) Yes 2) No	

Thank you for your response!

Annex 8.4: Focus group discussion Guide

Addis Ababa University

Aklilu Lemma Institute of Pathobiology

Focus group discussion Guide used for assessing experience and awareness of community towards mosquito-borne chikungunya disease in districts of Afar National Regional State; Ethiopia, 2023

District______________ Kebele ______________ Total no. Group ______

Gender of group members' _________Date _____/_____/_______

SECTION A: Experience and awareness of the community about febrile illnesses and arbovirus disease

1. What do you think the common febrile illnesses in this area? (PROBE for specific type of illnesses)
2. What do you think the common Mosquito borne diseases in this area? (PROBE for specific type of illnesses)
3. Did you heard or diagnosed Chikungunya ("Tilobign")? For those answered "Yes", what are the common sign and symptoms? (PROBE if they list the following sign and symptoms of CHIK?

---

1) Fever
2) Joint pain
3) Headache
4) Muscle pain
5) Bone pain
6) Nausea
7) Rash
8) Stomach pain

---

4. Are there any differences of sign and symptoms of malaria and CHIKF diseases? (count the numbers of respondent) A. Yes _____ B. No _____ C. Do not know _______
5. If For those Responded **"Yes"**, PROBE to list the difference
6. Does CHIKF affect all age groups? (If YES, PROBE for specific age group)
7. Do you think CHIKF is a killer/serious disease? (If YES, PROBE for specific reason or examples of illnesses. If NO PROBE reasons for the none- killer/serious)
8. Do you think CHIKF is a public health problem in this area? (count the numbers of respondent) A. Yes _______ B. No ________ C. Do not know ________
9. For those responded "Yes", since when do you think it is a public health problem?

10. From where do you think CHIK came here?
11. Do you think CHIKF is a treatable/manageable disease? (If YES, PROBE for specific ways of treatment. If NO PROBE reasons for why?)

SECTION B: Mode of transmission and controls of CHIK

12. Do you think CHIKF is a transmissible disease? (count the numbers of respondent)

  A. Yes _______
  B. No ________
  C. Do not know ________

13. For those Responded "Yes", what are the modes of transmission? (PROBE for listing modes of transmission)

14. For those responded "**Mosquito Bite",** do malaria mosquito similar with CHIK mosquito? (count the numbers of respondent)

  A. Yes _______ B. No ________ C. Do not know ________

15. For those Responded "No", how do they differ? (PROBE for specific reason?

16. Then Show the Atlas picture if they identify Aedes and Anopheles mosquitoes?

  A. Aedes(N) Correct _______ wrong _______
  B. Anopheles(N) correct ________wrong ______
  C. Do not know ________

17. When do the Aedes bite? (Probe if day, night or any time)

18. Where do they bite? (PROBE if in door, outdoor, at forest----)

19. Where do they reproduce? (PROBE if stagnant water, water in tire, etc)

20. Do you think human can protect them from biting? (count the numbers of respondent)_ A. Yes _______ B. No ______ C. Do not know ________

21. For those Responded "Yes", How? (PROBE for reasons)
22. What do you think actions should be done to reduce cases illnesses due to mosquito bites? (PROBE at different levels; at the community and country level?

Annex 8.5: Questionnaire for HCWs knowledge and practices

Addis Ababa University

Aklilu Lema Institute of Pathobiology

Questionnaire to assess awareness and practices of healthcare workers about acute febrile illness diagnosis and management and their knowledge and prevention of arboviral diseases (chikungunya & dengue fever) in malaria-endemic areas in the Afar National Regional State, Northeast Ethiopia, 2022

Code ____________Date _____/_____/______Woreda: ________

Dear study participants, please kindly provide the following information.

Part I: Participants' Socio-demographic Data

Q. No.	**Questions**	**Value level**	**Skip to**
	Facility Details		
	Name of facility	_________________	
	Types of health setting	1. Hospital 2. Health Center 3. Health post 4. Clinic 5. Others specify______	
	Participant details		
101	Sex	1. Male 2. Female	
102	Age	________yrs	
103	Level of education	1. GP/Specialist 2. MPh/MSc 3. BSc 4. Diploma / level IV. 5. Others (specify) _________	
103	Profession/specialty	1. Physician/MD 2. Public H/HO 3. Nurse/ Midwife 4. Others (specify)_______	
104	Total service years	__________yrs	
105	Service years in the study site	__________ yrs	
106	Do you have previous work experience and place (s) if any	1) No 2) Yes	If 1, skip to #108
107	If #106 is Yes	______yrs, name place(s)____________	

Part II: Awareness and practices on febrile illnesses diagnoses and management, including arboviral diseases, in the area			
Q. No	**Questions**	**Responses**	**Skip**

201	Which of these fever-related illnesses are mostly diagnosed and treated in this area?	1) Malaria 2) Typhoid fever 3) Typhus fever 4) other ………	
202	Are there a high number of febrile illnesses with unknown causes or non-malaria febrile illness?	1) Yes 2) No 3) Rare	
203	If **#202** is '**Yes**' What do you do for those febrile patients?	1) Refer to other hospital 2) Provide treatment/antibiotic for bacterial infections 3) Provide supportive treatment/antiviral for viral infections 4) other ……………	
204	Have you heard about dengue (DENV) and chikungunya (CHIKV) viruses?	1) Yes Both 2) Yes only CHIKV 3) Yes only DENV 4) No	**If No, stop**
205	If **#204** is **'Yes'** or (**1, 2 or 3**), Where did you hear from?	1) Media 3) Teacher/Education 2) Books 4) Seminar/training 5) others……	
206	How do you diagnosis DENV and/or CHIKV infectons suspect cases at this health facility?	1) Lab based 2) Based on signs/symptoms 3) Do not diagnosis 4) other………	**If 2-4, skip to #208**
207	If **#206** is '**lab based**', what are/is diagnostic method?	_________________	
Part III: knowledge about chikungunya disease			
Q.No	**Questions**	**Responses**	
Domain 1. Knowledge on agent and seriousness of the illness			
208	What is the causative agent of chikungunya disease?	1) Parasite 2) Virus 3) Bacteria 4) Do not know	
214	Does chikungunya cause a serious/sever illness?	1. Yes 2. No 3. Not sure	
215	Does chikungunya affect people of all age groups?	1. Yes 2. No 3. Not sure	
Domain 2. Knowledge on transmission, vector and spread			
209	How are dengue and chikungunya diseases transmitted to humans?	1) mosquito bites 4) Air droplet 2) tick bites 5) Do not know	**If 2-5,**

		3) house fly/contamination	skip **to #211**
210	If your answer for #209 is '1', which specific mosquitoes do you think are/are vectors for these infections?	1) *Anopheles* 4) Not sure 2) *Aedes* 3) Both	
211	During which time does that mosquito bites humans preferably?	1) At any time 3) At night time 2) At day time 4) Not sure	
212	What is the typical breeding site for those mosquitoes?	1) Water storage container/stagnant water 2) Dirty water 3) Garbage and mud 4) Not sure	
213	During which season are dengue and chikungunya transmissions most common?	1) Dry season 3) At any seasons 2) Rainy season 4) Not sure	
Domain 3: knowledge about sign and symptoms			
216	What are the common signs and symptoms of dengue fever? (**circle on multiple answers as possible**)	2) Do not know 3) Fever 4) Headache 5) Joint pain 6) Muscle pain 7) Pain behind eye 8) Rash 5) Vomiting 6) Back pain 7) Bleeding on gums, nose or petechial 8) Others___	
217	What are common signs and symptoms of chikungunya fever? (**circle on multiple as answers possible**)	1) Do not know 2) Fever 3) Joint pain 4) Headache 5) Muscle pain 6) Abdomen pain 7) Skin rash 8) Nausea 9) Joint swelling 10) Others___	
Domain 4: knowledge about prevention and management			
218	Do you think there any laboratory tools to diagnosis patients suspected for dengue or chikungunya?	1) Yes 2) No 3) Do not know	**If 2&3, skip to #220**
219	If **#218 is 'Yes'**, what are/is the laboratory diagnostic method used to confirm? (**circle on multiple as answers possible**)	1) PCR 3) ELISA 2) Rapid test kit 4) other …	
220	Is chikungunya a preventable disease?	1) Yes 2) No 3) Not sure	
221	Are there any specific drugs available for dengue and chikungunya treatment?	1) Yes 2) No 3) Not sure	

222	Are there any available vaccines for dengue and chikungunya prevention?	1) Yes 2) No 3) Not sure	
Part IV. Practices to diagnose and prevent and control chikungunya			
223	How do you advice patients to prevent them against Dengue and Chikungunya infections? (**circle on multiple answers as possible**)	1) To wear long clothes at risk area 2) To use of repellents 3) To use of bed net during sleeping day time 4) Don't know	
224	How dengue and chikungunya cases management can be improved? (**circle on multiple answers as possible**)	1) Train medical staff 2) Informing the population 3) Periodic sensitization of health workers 4) No idea	
225	Have you ever been trained for diseases cases like dengue and chikungunya diagnosis, management and management?	1. Yes 2. No	**If** No, **end here**
226	If **Q225 yes**, which body/ organization provided the training?	1) MOH 2) NGO 3) Other ………	
227	If **Q225 yes**, when and how frequents?	1) One training ever ………….. 2) Every year 3) Twice a year 4) Other………………………..	

Thank you for your response!

Annex 8.6: Guideline Used for in-depth interview with health workers

Addis Ababa University
Aklilu Lema Institute of Pathobiology

Topic guide for in-depth interview (IDI) Guideline to assess awareness and knowledge of Healthcare worker towards dengue and chikungunya diseases in selected Districts, Afar National Regional State; Ethiopia, 2022

Date: _ /___/__H/ facility: _ (Hospital) _ (H/C) __other_____OPD name: _________

SECTION A: Background information

First I will ask you few questions about you and your work.

No.	Question	Answer
1	Sex of the health care worker	1. Male 2. Female
2	What is your educational qualification?	1) Doctor 3. BSc 4. Diploma / level IV. 2) MPh/MSc 5. Others (specify) _______
3	What is Profession?	1. Physician/MD 3. Nurse/Midwife 2. Public H/HO 4. Others specify______
4	What is your total experience in this health care work?	____ years
5	How long have you worked at this facility as a health care worker?	______ years
6	How many patients come on average per week at this health facility with symptoms of fever?	____ patients per week

SECTION B: Experience of healthcare workers and awareness on malaria and non-malaria febrile illnesses in the community

1. Do you think people in this community have febrile illnesses other than malaria (none-malaria)? (If YES, PROBE for specific type of illnesses. If NO PROBE reasons for the low)
2. What is the incidence of malaria? Is it decreasing or increasing in cases? What are the reasons for?
3. Do you think the patients or parents/guardians who come or bring their children to this health facility have knowledge (awareness) on non-malaria febrile illnesses? If No, what are the reasons for the luck/ inadequate of knowledge (PROBE)?
4. Do people in this community take their children to the health facility when they have a fever? Do they bring them when they are sick or very sick? Ask if all of them are coming to the medical facility for treatment or are they looking for alternative treatments. PROBE

If yes, which alternative treatment and which option is commonly used first, followed by which option and why?

SECTION C: Febrile illness diagnoses and managements

5. What happens if a febrile patient is come or brought to your health care facility? PROBE how they attain final diagnosis and treatment.
6. What tests do you use to diagnose a patient with fever? See if they test for malaria, ask which tests they use (mRDT or microscopy), and ask about other clinical tests they perform and what illnesses they have.
7. If mRDT/microscopy is not available at health facility, do they use clinical diagnosis what make them to think the patient has malaria, typhoid fever, urinary tract infection, pneumonia etc? How clinically do febrile illnesses are differentiated?
8. If a febrile patient has shown a negative result for mRDT/microscopy, do you examine for other febrile illnesses? Ask which drugs which are commonly prescribed.
9. Do you think this health facility can manage to offer diagnosis and treatment of non-malaria febrile illnesses? If Yes the available laboratory tests and for which diseases?
10. Does the health facility have adequate infrastructure, staff, equipment, supplies and reliable laboratory testing for arboviruses like dengue fever, chikungunya fever? PROBE for challenges to manage the illnesses due to the mentioned limitations.
11. Do you think you have adequate skills to manage such diseases? If YES, PROBE for any medical training offered / attended and how often?

SECTION D: control and prevention of non-malaria febrile illnesses & arboviruses

12. What do you think should be done to reduce cases of non-malaria febrile illnesses like dengue fever and chikungunya? PROBE at different levels; at the community and country level?
13. What do you think should be done to improve the care and management of non-malaria febrile illnesses like dengue fever and chikungunya in public health facilities in the country? PROBE: the role of health facility, district, Government (Ministry of Health) etc.