UNIVERSITY OF NAIROBI
Department of Medical Microbiology

Emerging & Re-emerging Arboviral Infections in Patients with Acute Febrile Illness in Two Counties in Kenya

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This thesis is submitted for the award of a Degree in Doctor of Philosophy in Infectious Diseases at the University of Nairobi

Declaration

I declare that this thesis is my original work and has not been previously presented in this or any other university for a similar or any other degree award.

Signed:  Date: **11 Nov 2022**

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This thesis has been submitted for examination with our approval as university supervisors

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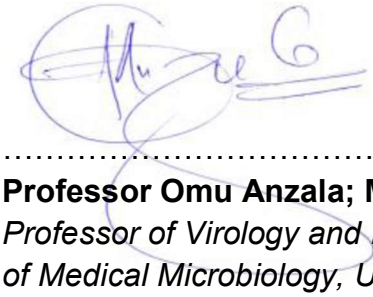

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Acronyms

AFI	Acute Febrile Illness
BLAST	Basic Local Alignment Search Tool
CMC	Carboxymethylcellulose
CCHFV	Crimean-Congo Hemorrhagic Fever virus
CHIKV	Chikungunya virus
CI	Confidence interval
DENV	Dengue virus
DMEM	Dulbecco's Modified Eagle Medium
ELISA	Enzyme-Linked Immunosorbent Assays
ERI	Emerging and Remerging Infections
FBS	Fetal Bovine Serum
HIV	Human immunodeficiency virus
HPGV	Human pegivirus
HTS	High throughput sequencing
ICTV	International Committee on Taxonomy of Viruses
IFA	Immunofluorescence Assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IQR	Interquartile Range
KSHC	Kibera South Health Centre
NS	Non-structural
ONNV	Onyong-nyong virus
OR	Odds Ratio
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PFU	Plaque-forming Unit
PRNT	Plaque Reduction Neutralization Tests
RFV	Rift Valley Fever Virus
RNA	Ribonucleic Acid
SARS	Severe Acute Respiratory Syndrome
SINV	Sindbis virus
TBEV	Tick-borne Encephalitis Virus
UH	University of Helsinki
UMC	Ushirika Medical Clinic
UoN	University of Nairobi
WHO	World Health Organization
WNV	West Nile virus
χ^2	Chi-square test
YFV	Yellow Fever Virus
ZIKV	Zika virus

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ABSTRACT

Background

Covid-19 has demonstrated the impact that emerging infections can have on our health, social and economic wellbeing. About three-quarters of emerging infections have a zoonotic origin, and are transmitted to humans by arthropod vectors and vertebrate hosts such as bats and rodents. The incidence of arthropod-borne viruses has increased in recent decades mainly due to globalization, international travel and climate change. They have the potential to emerge and re-emerge in most parts of the globe posing a substantial threat to public health. Diagnosis of emerging pathogens remains a challenge due to the non-specific clinical presentation and lack of point-of-care diagnostic assays in many regions where these infections are frequent. This means that emerging infections are often not identified at all, or in time to contain an outbreak before it spreads to other areas.

Objectives

To determine the prevalence of emerging and re-emerging arboviral infections in patients with acute febrile illness in Kibera informal settlement and Taita-Taveta County.

Methods

This was a cross-sectional study on patients with acute febrile illness presenting at selected health facilities in Taita-Taveta County (3 health facilities) and Kibera informal settlement, Nairobi (3 health facilities). A clinician-administered questionnaire to collect demographic and clinical information was used. Blood samples were analyzed for infection or exposure to arboviruses as well as the presence of other viral pathogens using ELISA and immunofluorescence assays to screen for IgG and IgM antibodies against flaviviruses and alphaviruses which are the commonest arboviruses in the country. Where sufficient sample volume was available, antibody-positive samples were analyzed using plaque reduction neutralizing tests (PRNT). Reverse-transcription PCR with degenerate group-consensus primers was used to screen for alphavirus and flavivirus nucleic acid. Samples that were PCR-positive were further analyzed using Sanger sequencing to identify the specific virus. High-throughput sequencing using Illumina MiSeq™ was used to analyze PCR-positive samples as well as 110 randomly

selected samples (in 22 pools of 5 samples each) and screened for novel or unexpected viral pathogens in the samples.

Both clinical and laboratory data were analyzed using IBM SPSS Statistics 22. Data on prevalence of arboviruses were summarized using frequencies and percentages. Associations were tested using logistic regression. A p-value of ≤ 0.05 was considered significant.

Findings

A total of 557 samples from individual patients were analyzed; 326 serum samples from Taita-Taveta and 231 plasma samples from Kibera. The prevalence of current flavivirus infection (IgM antibodies, NS1 antigen or PCR positive) was 1.5 % (2.1% in Taita-Taveta and 0.4% in Nairobi). Previous exposure to flaviviruses (IgG antibody) was 9% (13% in Taita-Taveta and 3% in Nairobi). Four patients (0.2%) had alphavirus IgM antibodies on both ELISA and PRNT. Of all samples, 49 (8.8%) were IgG positive on IFA; 22 (6.7%) in Taita-Taveta and 27 (11.7%) in Kibera. Most of the samples tested on alphavirus PRNT had higher titers (2 to 8-fold) against ONNV than CHIKV and a few with higher titers for CHIKV than for ONNV. Five samples (1%) tested positive for flavivirus and only one for alphavirus on PCR. Dengue virus was isolated from two samples in culture. Three complete coding sequences and one dengue *env* gene sequence were retrieved. All dengue sequences were identified as dengue type 2 and formed a single monophyletic cluster that was closely related to other dengue sequences recovered from coastal Kenya between 2014 and 2017. Human pegiviruses, HIV, Enterovirus A and Torquetenoviruses were also detected in sample pools that were analyzed using Illumina MiSeq™ high-throughput sequencing.

Conclusion

Arboviruses are associated with acute febrile illness even during inter-epidemic periods. Exposure to both alphaviruses and flaviviruses is moderate in both Taita-Taveta and Nairobi which are geographically disparate areas in the country. Emerging and re-emerging disease surveillance systems in Kenya should include arboviruses even in areas where arbovirus outbreaks have not yet been reported.

1.0 INTRODUCTION

Emerging and remerging infectious diseases (EIDs) are a major threat to public health and the economy globally. EIDs have been recorded throughout history and they continue to occur today (Morens and Fauci, 2013, Devaux, 2012). During this first quarter of the 21st century, coronaviruses have been the most significant emerging viruses, causing three distinct outbreaks over the last 20 years. In December 2019, SARS-CoV-2 emerged in Wuhan China and has since spread to all countries globally causing more than 535 million reported cases and over 6.3 million deaths (a mortality rate of about 1.2%) as of June 2022. Covid-19 is likely to be the most important emerging disease of the 21st century, akin to what HIV-AIDS was in the 20th century with persistent transmission globally for years (Keni et al., 2020, Ritchie et al., 2021). In May 2022, monkeypox virus which has been endemic in central and western Africa was reported for the first time in several countries in Europe and began to spread across the world causing further strain on the world, which was just beginning to surmount Covid-19 (Bragazzi et al., 2022).

Like Covid-19 and monkeypox, most emerging and re-emerging infections have a zoonotic origin, transmitted to humans through various interactions with animals. Viral infections make up a large portion of EIDs; they form about 15% of known human pathogens but cause nearly 40% of all emerging and re-emerging infections (Woolhouse and Gowtage-Sequeria, 2005, Morens and Fauci, 2013). EIDs are usually caused by flaviviruses, filoviruses, bunyaviruses, arenaviruses, alphaviruses, orthomyxoviruses paramyxoviruses, reoviruses, coronaviruses and poxviruses (Hui and Zumla, 2015, Bragazzi et al., 2022). Most of these viruses are transmitted by arthropods (mosquitoes, ticks and sandflies) or small mammals, mainly rodents and bats (Woolhouse et al., 2005, Jones et al., 2008).

Arthropod-borne (arbo) viruses pose a significant public health threat and have spread rapidly over the last few decades to all inhabited continents in the world. Previously, arboviral infections were more restricted to certain geographical areas where their hosts or vectors were plentiful, mainly in the tropics and sub-tropics. However, these restrictions have been broken several times as viruses and their vectors spread further from the equator mainly due to climate change, international travel and globalization.

Several factors fuel the emergence and re-emergence of infectious diseases. These include climate change, agricultural practices, population increase, globalization, international travel and trade, urbanization, inefficient public health systems and pathogen evolution (Woolhouse and Gowtage-Sequeria, 2005, Taylor et al., 2001, Institute of Medicine, 1992, Zanetti and Zappa, 2010). Some arboviruses such as Dengue (DENV), Zika (ZIKV), yellow fever (YFV) and chikungunya (CHIKV) do not necessarily require non-human reservoirs or amplifier hosts to cause outbreaks in urban areas and are maintained by, among other factors, urban-adapted anthropophilic *Aedes aegypti* and *Ae. Albopictus* (Wilke et al., 2021, Snyder et al., 2017, Sacchetto et al., 2020b, Messina et al., 2019).

Several studies have reported arboviral infection in Kenya including DENV, CHIKV, YFV, West Nile virus (WNV), Onyong-nyong virus (ONNV), Crimean-Congo hemorrhagic fever virus (CCHFV), Rift Valley Fever virus (RVFV), and others. The studies show that exposure to arboviruses in Kenya is common and frequent and the incidence varies with location, time of the year and study population (LaBeaud et al., 2015, Sutherland et al., 2011, Mease et al., 2011, San Juan et al., 2002, Johnson et al., 1996, Kuzmin et al., 2010, Nyaruaba et al., 2019, Tigoi et al., 2015, Ochieng et al., 2015).

Arboviruses typically present with non-specific symptoms such as fever, joint pain, muscle pain, headache and rash. They are part of the vast number of pathogens that cause acute febrile illness (AFI) whose diagnosis is challenging. Most studies on the etiology of AFIs have focused on pathogens that are common or expected in a given locality, mainly due to limitations in diagnostic assays and the vast number of potential etiological agents. Many cases of acute febrile illness remain undiagnosed (Rhee et al., 2019). It is therefore very likely that some unidentified pathogens may be a cause of acute febrile illness (AFI) in Kenya (Nyaruaba et al., 2019, Hin et al., 2021, Rhee et al., 2019). Although there are numerous tests that can detect some of the common causes of AFI, most of these assays are not readily available at the point of care in developing countries where the burden of AFI is heaviest. In addition, serological tests which are a common test for diagnosis of viruses are faced with the challenge of cross-reactivity within genera of arboviruses, making it difficult to identify specific viruses causing AFI or

detect con-infection with viruses in the same genus (Kerkhof et al., 2020). Early detection of such pathogens would not only help in clinical care but also the containment of potential outbreaks before they spiral out of control. To facilitate detection of novel or unknown etiological agents of acute febrile illness, high throughput sequencing is increasingly being utilized, with improvements to make it more sensitive and affordable (Huang et al., 2019, Piantadosi and Kanjilal, 2020).

The aim of this study was to determine the prevalence and genetic characteristics of emerging and re-emerging arboviral infections in patients with acute febrile illness in Kibera slums and Taita Taveta County, using serological assays, PCR and high throughput sequencing. This study was part of a larger project on zoonotic and arboviral infections in Kenya, studying humans, bats, rodents, mosquitoes and the environment, to understand the transmission dynamics of zoonotic viruses and inform prevention strategies such as early warning systems (Forbes et al., 2021).

2.0 Justification and Rationale

Emerging and remerging infections have occurred throughout the history of mankind, occasionally leading to devastating effects. The threat of EIDs on global health remains a significant one, for which the world must be prepared. Many EIDs present with a non-specific acute febrile illness which is a common presentation for many other infections in the tropics. Currently, most AFIs in the developing world are investigated for only a few pathogens due to the unavailability of appropriate diagnostics and the vast number of potential etiological pathogens. In these circumstances, most clinicians tend to test for pathogens known to cause fever in a given area such as malaria, brucellosis and salmonellosis. Consequently, emerging or re-emerging viruses are likely to go undetected thus missing an opportunity to contain a nascent outbreak before it spirals out of control.

Emergence and re-emergence of arboviral infections is a phenomenon that is unlikely to go away. In order to blunt the effects of disease emergence, given the unpredictable nature, we need to be prepared to respond promptly and appropriately to any outbreak. This requires surveillance of EIDs across different ecological zones and the availability and continual improvement of diagnostic tests and therapeutics, among other strategies. Since viruses have the ability to spread to new areas, we also need to investigate for novel or unexpected pathogens.

In this study we assessed EIDs occurring in two regions in Kenya and explored the use of high-throughput sequencing to identify unknown or unexpected etiological agents. The two areas selected, one urban and one rural are representative of the diverse conditions in the country and give a good picture of arboviruses circulation in different parts of the country. We used serological assays and PCR to detect specific arboviral pathogens. Although ELISA and immunofluorescence assays have limited specificity in delineating species within some genera such as flaviviruses and alphaviruses, they are very sensitive in the detection of broad categories or families of arboviruses. We also used PCR based on group-consensus primers targeting common arbovirus families rather than using pathogen-specific primers, to increase the range of pathogens detected. Additionally, we employed Illumina MiSeq™ high throughput sequencing and an in-house bioinformatics pipeline which offers the advantage of unbiased screening of

pathogens including novel and unexpected pathogens which is not inherent in serological and PCR assays.

The information provided by this study is expected to inform the diagnosis of febrile illness and surveillance of emerging and re-emerging infections in the country.

3.0 Research Questions

1. What is the prevalence of infection with and previous exposure to arboviruses in patients with acute febrile illness (AFI) in Kibera informal settlement, Nairobi and Taita Taveta County?
2. Are there any emerging or novel pathogens associated with acute febrile illness in the study areas?
3. What are the phylogenetic properties of emerging and re-emerging viral pathogens in the study population?

4.0 Study Objectives

4.1 Main Objective

To determine the prevalence of emerging and re-emerging arboviral infections in patients with acute febrile illness in Kibera informal settlement and Taita Taveta County

4.2 Specific Objectives

1. To determine the prevalence of infection and previous exposure to arboviruses in patients with acute febrile illness (AFI) in Kibera informal settlement, Nairobi and Taita Taveta County
2. To identify emerging or novel pathogens associated with AFI in the study areas
3. To determine the phylogenetic properties of emerging and re-emerging viral pathogens in the study population

5.0 LITERATURE REVIEW

5.1 Background

Emerging infections are infections that have been identified in humans recently while re-emerging infections are infections whose incidence had initially declined but are now posing a significant threat to global health through increased transmission, spreading to new geographical areas or new populations (Morens and Fauci, 2013). EIDs have occurred numerous times in recorded history. Notable examples include the plague of Athens in the 5th century which killed a quarter of the population of Athens at the time, the bubonic plague which emerged in Europe in the 12th century killing nearly half of the population and smallpox which emerged in the 16th century in the Americas causing over 20 million deaths (Dobson and Carper, 1996). In the last half-century, we have witnessed the emergence of several diseases such as Ebola, Marburg, HIV/AIDS, Zika, Severe Acute Respiratory Syndrome (SARS) and the ongoing Covid-19 (Morens and Fauci, 2013, Keni et al., 2020). More than 70% of all emerging infections are zoonotic, transmitted to humans directly or through arthropod vectors and small mammals such as bats and rodents (Woolhouse et al., 2005, Jones et al., 2008, Morens and Fauci, 2013).

Arboviral infections are viruses transmitted from animals to humans and among humans by arthropods – mosquitoes, ticks, midges and phlebotomine sandflies (Liang et al., 2015). More than a hundred arboviruses are known to cause disease in humans and animals and most of these belong to five categories - *Flaviviridae*, *Togaviridae*, *Bunyavirales*, *Rhabdoviridae* and *Reoviridae* (Liang et al., 2015). See *Table 1*. Most arboviruses have an envelope and RNA genome. These viruses typically (but not always) undergo three transmission cycles. The first is a sylvatic (enzootic) cycle where viruses are transmitted in the jungle among wild animals. Humans visiting the forest for various economic or recreational activities may also get infected. An epizootic cycle follows where viruses are transmitted among domestic animals and/or humans, especially in rural areas near forests with reservoir hosts. The infection may then spillover to the third cycle – the epidemic/urban cycle where transmission is among humans and infection can be sustained or endemic without the need for an animal host acting as a reservoir (Weaver et al., 2020, Sacchetto et al., 2020a, Valentine et al., 2019) (*Figure 1*).

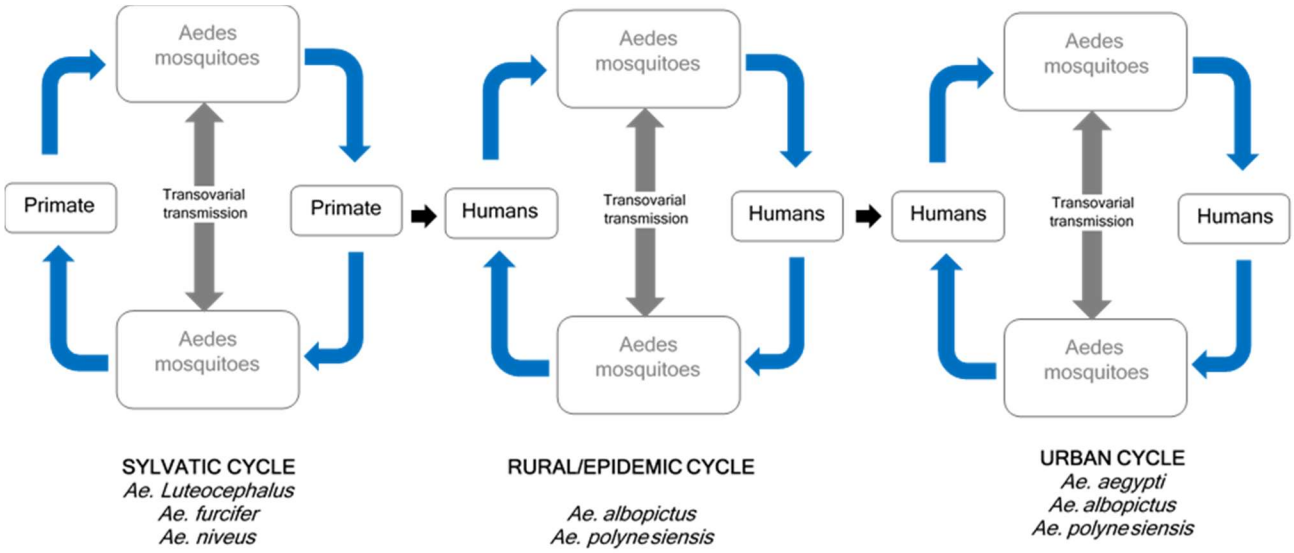

Figure 1. Dengue Virus Transmission cycles

Flaviviridae

Flaviviruses belong to the *Flaviviridae* family which has four genera – *Flavivirus, Hepacivirus, Pegivirus* and *Pestivirus* (ICTV, 2022, Simmonds et al., 2017). The last three genera are not arthropod-borne. *Flaviviruses* (the genus) have a 9-13 kb positive sense, single-stranded RNA genome that codes for a large polyprotein that is cleaved into ten distinct proteins - 3 structural (capsid, premembrane and envelope) and 7 non-structural proteins (NS1, 2a, 2b, 3, 4a, 4b, and 5) (Bidet and Garcia-Blanco, 2018, Bollati et al., 2010) (Figure 2). The NS1 antigen is notable for its role in the replication and pathogenesis of DENV and is also a target in diagnostic tests. DENV NS1 has 70% sequence homology among all four DENV types and 40-50% similarity with other flaviviruses (Songprakhon et al., 2020). DENV NS1 antigen is detectable in blood before DENV antibodies and has high specificity for DENV underpinning its utility as a complementary test to DENV antibody assays (Matheus et al., 2016, Kassim et al., 2011, Chong et al., 2020).

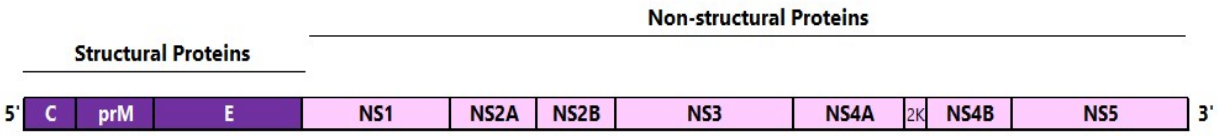

Figure 2. Flavivirus genome organization

The most common viruses in the flavivirus genus globally are DENV, YFV, ZIKV, WNV, Tick-borne encephalitis virus (TBEV) and Japanese encephalitis virus (JEV). Although the primary route of transmission is through arthropod vectors, other sources of infection have also been reported for some of the viruses. These include blood transfusion (DENV, YFV, WNV, ZIKV), and sexual intercourse (ZIKV) (WHO, 2020, Blitvich et al., 2020, Desgraupes et al., 2021, Gregory et al., 2017, Petersen and Busch, 2010).

Dengue virus is the most significant arbovirus globally as it causes the highest number of infections of any arbovirus annually. The incidence of dengue has increased steadily in the last 30 years. It is estimated that dengue caused 23 million cases and 17,000 deaths in 1990 which had increased to 100 million cases and 40,000 deaths by 2017. Currently, 3.9 billion people in 129 countries are at risk of infection with dengue (Zeng et al., 2021, Bhatt et al., 2013). DENV is mainly transmitted by *Aedes (Stegomyia) aegypti*. It has four distinct serotypes (DENV1-4). Infection with one DENV type elicits lifelong immunity against that specific serotype but no cross-protection against other dengue serotypes. Subsequent infection with a different serotype heightens the risk of severe dengue which is mediated by antibody-dependent enhancement (St. John and Rathore, 2019).

Although first detected over 70 years ago, Zika virus attracted very little attention until very recently, with 180 publications on PUBMED by 2014 which rose to over 10,000 by 2021. Zika virus was first detected in monkeys in *Ziika* forest in Uganda in 1947, then reported infrequently (less than 20 human cases) in parts of Africa and Asia till 2007 when it spread to Micronesia. By 2013, it had spread to French Polynesia and in 2015 to Brazil where it infected hundreds of thousands and then spread to the rest of the Americas by the end of 2016 (Heukelbach et al., 2016, WHO, 2020).

YFV is thought to have originated in Africa and spread to the Americas and Europe through the trans-Atlantic slave trade in the 17th century (Barrett and Monath, 2003, Nyaruaba et al., 2019, Bryant et al., 2007). Asia had reported no cases of YFV until 2016 when a traveller returning from Angola was diagnosed with the disease. This was the first report of YFV in Asia and it put billions of people at risk of infection as YFV vectors are plentiful in the region (Wasserman et al., 2016). YFV is transmitted by *Aedes* and *Haemagogus* mosquitoes and it infects humans and other primates. Ninety percent of YFV cases occur in Africa where up to 170,000 severe cases and 60,000 deaths occur per annum. Most yellow fever cases only develop an acute disease that lasts three to four days but a small proportion (15%) advance to the toxic phase whose fatality rate is up to 50% (Braack et al., 2018, WHO, 2019).

Another flavivirus, tick-borne encephalitis virus (TBEV) is mainly transmitted by several genera of ixodid ticks. A few cases have been also reported after ingestion of raw milk from infected animals (Dumpis et al., 1999, Imhoff et al., 2015). TBEV usually causes an acute febrile infection and in about one-third of the patients, it may cause a biphasic illness where the second phase is accompanied by fever and meningoencephalitis (Bogovic and Strle, 2015). TBEV has mainly been restricted to Asia and Europe (Mansfield et al., 2009, Lindquist and Vapalahti, 2008); a study in Kenya reported seroprevalence of 69%; this could be due to flavivirus cross-reactivity (Sutherland et al., 2011). TBEV is in the same serocomplex with several tick-borne flaviviruses. These include Kyasanur forest disease virus found in India, Omsk hemorrhagic fever virus reported in Russia, Powassan virus reported in the United states, Canada and Russia, Alkhurma hemorrhagic fever virus found in the Middle East, and Langat virus found in South East Asia and Russia (Mansfield et al., 2009).

Recently, Jingmen virus group, a novel group of tick-borne flaviviruses with a segmented genome has been described. This includes Alongshan virus and Jingmen tick virus which were reported in ticks in southern Finland (Kuivanen et al., 2019) and in both ticks and febrile patients in China (Wang et al., 2019, Jia et al., 2019). The genome is organized in four segments and two of these are similar to NS3 and NS5 genes of *Flavivirus* genus (Kuivanen et al., 2019).

Togaviridae

Alphaviruses are the only genus in the *Togaviridae* family. They are enveloped with a ~11 kb positive-sense RNA genome which codes for nine viral proteins – four non-structural (nsP1, P2, P3 and P4) and five structural proteins (capsid protease (CP), 6K, E1, E2, and E3) (Jose et al., 2009, Tomar and Aggarwal, 2017). Alphaviruses are classified into seven serocomplexes based on antigenic properties and into two sub-groups based on their place of origin or dominance. The Old-world (Eurasian-African-Australasian) sub-group, which includes CHIKV, ONNV, Sindbis (SINV) and Ross River virus (RRV), among others, usually causes fever, rash and arthralgia. The New-world (American) sub-group, which includes Western equine encephalitis virus (WEEV), Eastern (EEEV) and Venezuelan (VEEV) equine encephalitis viruses, usually causes encephalitis (Azar et al., 2020, Chen et al., 2018, ICTV, 2022). The seven antigenic groups are Semliki forest complex (e.g. CHIKV, ONNV, Mayaro and Ross River viruses); Western equine encephalitis complex (e.g. WEEV, SINV and Whataroa virus); Venezuelan equine encephalitis complex (e.g. VEEV, Mucambo and Rio Negro virus); Barmah forest virus, Eastern EEV complex, Middleburg virus complex, Ndumu virus complex and unclassified viruses (e.g. Eilat and Mwinilunga viruses) (Powers et al., 2001). Viruses in each serogroup are closely related antigenically and frequently cross-react on antibody assays (Hassing et al., 2010).

Alphaviruses are transmitted by arthropods and infect various vertebrate hosts such as birds, swine, horses and humans. The main vectors are *Aedes* and *Culex* mosquitoes with a smaller number transmitted by *Anopheles* and *Haemagogus* mosquitoes (Braack et al., 2018).

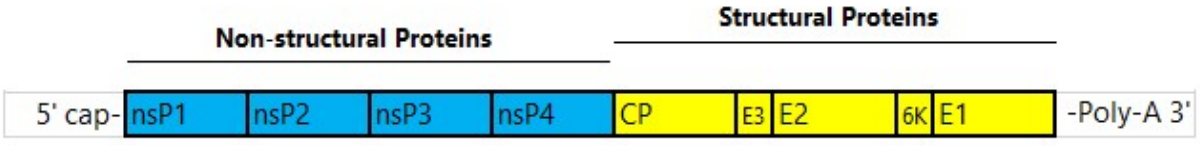

Figure 3. Alphavirus Genome Organization

Chikungunya virus is an alphavirus in the *Togaviridae* family and is transmitted by mosquitoes in the *Aedes genus*, mainly *Ae. aegypti* and *Ae. Albopictus (Stegomyia*

albopicta). CHIKV was first detected in southern Tanzania in 1953 in a dengue-like outbreak (Silva et al., 2018). It was initially restricted to Africa but has since spread to other parts of the world with reports in India, Asia, Europe and the Americas (Kariuki Njenga et al., 2008, Vega-Rua et al., 2015). The re-emergence of CHIKV has been driven, in part, by a substitution mutation (E1-A226V) which adapted the virus to thrive in *Ae. albopictus* (Maljkovic Berry et al., 2019, Tsetsarkin et al., 2011). *Ae. albopictus* as a vector has a wider geographic distribution and has been reported further from the equator than *Ae. Aegypti* (Kraemer et al., 2015). CHIKV causes a self-limiting non-specific febrile illness that may be accompanied by a rash, myalgia and arthralgia. CHIKV may also cause severe and chronic musculoskeletal and joint pain in some patients (Lemant et al., 2008, Silva et al., 2018).

Bunyavirales

Bunyaviruses make up a large number of viruses in the order *Bunyavirales* and are spread across twelve families. They have a highly diverse range of vertebrate and invertebrate hosts. Five of the twelve bunyavirus families may be transmitted to humans by arthropods - mosquitoes, ticks and phlebotomine sandflies as well as through direct contact. The three arthroborne families are *Phenuiviridae* (includes RFV), *Nairoviridae* (includes CCHFV) and *Peribunyaviridae* (includes Bunyamwera, Batai and Guaroa viruses). Two other bunyavirus families – *Arenaviridae* and *Hantaviridae* are primarily rodent-borne viruses (Evans, 2013, ICTV, 2022, Balasuriya et al., 2016).

Bunyaviridae are enveloped with a three-segment, negative-sense or ambi-sense, single-stranded genome which ranges from 11 – 19 kb. The three genomic segments are L, M and S for large, medium and small segments respectively. The L segment codes for the RNA polymerase, the M segment codes for a polyprotein that comprises of Gc and Gn glycoproteins and, in some bunyavirus families, a non-structural protein (NSm). S segment codes for the nucleoprotein (N) and in some families a non-structural protein (NSs) (Wuerth and Weber, 2016, Balasuriya et al., 2016).

Rift Valley fever virus is a zoonotic phlebovirus that has been restricted to Africa and Arabian Peninsula. RFV is transmitted by *Aedes* mosquitoes especially following unusually heavy rains (Evans, 2013). It causes severe disease in livestock with significant economic losses. In humans, it is usually asymptomatic but may cause a

mild, non-specific illness although some cases may develop a severe form that presents with neurologic, ocular or hemorrhagic symptoms (WHO, 2018, Javelle et al., 2020).

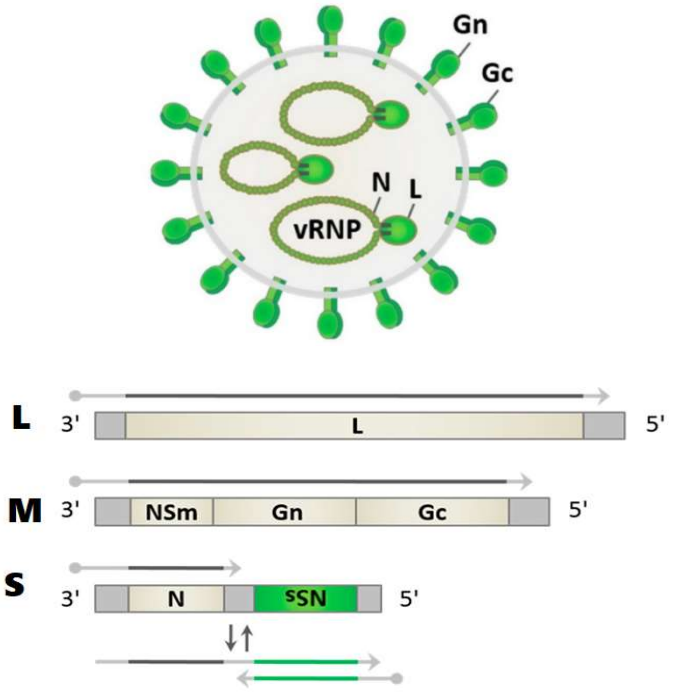

Figure 4. Phlebovirus virion and genome organization.
L and M segments are fully anti-sense while the S segment is ambi-sense, with two genes that have opposite polarities. Adapted from Wuerth and Weber used under the Creative Commons Attribution (CC-BY 4.0) license (Wuerth and Weber, 2016)

Crimean-Congo hemorrhagic fever is a tick-borne infection that is widely distributed across the world. It is an emerging disease reported in Europe, Asia, the Middle East and Africa (Messina et al., 2015, Evans, 2013). CCHFV causes a non-specific febrile illness and occasionally presents with meningism that may be accompanied by hemorrhagic, cardiovascular, renal or hepatic symptoms. A third of the cases are fatal (Shayan et al., 2015, WHO, 2013)

Several emerging bunyaviruses have been reported across the globe in recent years such Ngari virus in Kenya in 2001, a phlebovirus in China in 2013, Sandfly-fever Sicilian virus in Ethiopia in 2014, among others (Li, 2013, Ulrich et al., 2002, Woyessa et al., 2014, Bowen et al., 2001, Evans, 2013).

Table 1. Selected arboviruses that infect humans

Order/ Family	Genus	Species (Examples)	Vectors	Common Hosts	Clinical Manifestations
Amarillovirales					
Flaviviridae	*Flavivirus*	Dengue	Mosquitoes	Humans, NHPs	Fever, arthralgia, rash, hemorrhage
		Yellow fever	Mosquitoes	Humans, NHPs	Fever, rash, hepatitis, hemorrhage
		West Nile	Mosquitoes	Humans, birds, horses, dogs	Fever, encephalitis
		Zika	Mosquitoes	Humans, NHPs	Fever, arthralgia, rash
		Japanese Encephalitis Virus	Mosquitoes	Humans, pigs, horses, cattle	Fever, encephalitis
		Tick-borne EV		Humans, rodents, dogs, ruminants, birds	Fever, encephalitis
Martellivirales					
Togaviridae	*Alphavirus*	Chikungunya	Mosquitoes	Humans, NHPs	Fever, arthralgia, rash
		Onyong-nyong	Mosquitoes	Humans, NHPs	Fever, arthralgia, rash, lymphadenitis
		Sindbis	Mosquitoes	Humans, birds	Fever, arthralgia, rash
		Mayaro	Mosquitoes	Humans, NHPs	Fever, arthralgia, rash
		Semliki Forest	Mosquitoes	Humans, birds, rodents, horses	Fever, arthralgia, rash
		Eastern EEV	Mosquitoes	Humans, horses, donkeys, birds	Fever, encephalitis
		Western EEV	Mosquitoes	Humans, horses, donkeys, birds	Fever, encephalitis
		Venezuelan EEV	Mosquitoes	Humans, horses, donkeys, rodents	Fever, encephalitis
Bunyavirales					
Phenuiviridae	*Phlebovirus*	Rift Valley fever	Mosquitoes	Humans, cattle, sheep, goats	Fever, hemorrhage, encephalitis
		Sicilian phlebovirus	Sandflies	Humans	Fever
		Toscana phlebovirus	Sandflies	Humans	Fever, encephalitis
	Uukuvirus	Uukuniemi	Ticks	Humans	
Peribunyaviridae	*Orthobunyavirus*	Bunyamwera	Mosquitoes	Humans, birds, cattle	Fever, arthralgia, rash, encephalitis
		Bwamba virus	Mosquitoes	Humans	
Nairoviridae	*Orthonairovirus*	Crimean-Congo HFV	Ticks		Hemorrhagic fever
		Dugbe virus	Ticks, midges, mosquitoes	Humans, Cattle	Fever, thrombocytopenia
Reovirales					
Reoviridae	*Seadornavirus*	Banna virus	Mosquitoes	Humans, cattle, pig	Fever, encephalitis
	Seadornavirus	Kadipiro virus	Mosquitoes, ticks	Humans, pig, cattle	Fever, encephalitis
	Coltivirus	Colorado tick fever	Ticks	Humans, rodents, deer	Fever, rash, encephalitis hepatosplenomegaly,
Mononegavirales					
Rhabdoviridae	*Tibrovirus*	Bas-Congo virus	Midges	Humans, cattle	
		Kamese virus	Mosquitoes	Humans	
		Mossuril virus	Mosquitoes	Humans	

NHPs = non-human primates, EEV = equine encephalitis virus, HFV = Hemorrhagic fever virus,

5.2 Factors associated with emergence of arboviral diseases

Emergence and re-emergence of diseases is influenced by various factors. These factors concern the human (host), the pathogen as well as the environment. Host factors are both physiological and behavioral; these include 1) increased global travel and trade which eases transmission of infectious agents, 2) socio-economic activities such as hunting, logging and agriculture which increase the interactions between man and animals 3) immunosuppression in some individuals (Woolhouse and Gowtage-Sequeria, 2005, Taylor et al., 2001, Liang et al., 2015).

In addition to host factors, pathogen evolution also fuels emergence of arboviral diseases. For instance, a mutation in CHIKV allowed it to infect *Ae. albopictus* which is anthropophagic and more widely distributed than the primary urban cycle vector, *Ae. aegypti* fueling emergence of Chikungunya in other parts of the world outside Africa where the virus had been restricted to for years (Maljkovic Berry et al., 2019, Tsetsarkin et al., 2011).

The environment is another determinant of disease emergence. Climate change is expanding the geographical areas habitable by disease vectors such as mosquitoes. The global temperature increase has also bolstered the capacity of disease vectors (Watts et al., 2018). The increasing need for food for growing human populations has also increased land use and reduced land cover fueling climate change. These factors have exposed more people to vector-borne diseases such as the emergence of WNV in many parts of Europe and the emergence of DENV and CHIKV in several parts of the world. Urbanization has also made it much easier to have sustained human-to-human transmission of arboviruses in cities (Andriopoulos et al., 2013, Ogden et al., 2014, Medlock and Leach, 2015, Liang et al., 2015)

5.3 Clinical Presentation of Arboviral Diseases

Arboviral disease may present with one or more of these: 1) subclinical infection or a mild febrile disease, 2) central nervous system with meningitis or encephalitis, 3) fever, rash and arthralgia, 4) hemorrhagic fever or 5) congenital syndrome (WHO, 2020). Arboviruses tend to cause unpredictable sporadic outbreaks in humans, and may rely on reservoir hosts for survival between epidemics, and with few exceptions such as YFV and DENV, they have no specific treatment or vaccines.

In addition, symptoms of arboviral diseases overlap with other illnesses caused by parasites, fungi and bacteria. The most common presentation is an acute febrile illness which makes diagnosis by clinical manifestations alone very difficult. The only way to confirm the diagnosis is through laboratory methods and even so, some agents like flaviviruses cross-react on antibody tests (Su et al., 2015, Schwartz et al., 2000). There is also a broad range of differentials such as leptospirosis, rickettsia, group A streptococcus, brucellosis, parvovirus, enterovirus, Q fever, measles, adenovirus, post-infectious arthritis, rheumatologic diseases and Covid-19 (WHO, 2020).

5.4 Acute febrile illness

Acute febrile illness presents with fever of sudden onset accompanied by other non-specific symptoms such as headache, muscle aches, arthralgia, malaise, anorexia, nausea and vomiting. It is a common presentation in the tropical and sub-tropical regions and is caused by diverse pathogens, including viruses.

Due to the diversity of pathogens presenting with similar symptoms, accurate diagnosis of AFI is limited and in many cases, the etiology remains unknown. In places with a high incidence of malaria, many cases of AFI may be wrongly treated for malaria even when laboratory testing is negative (Mueller et al., 2014, Onchiri et al., 2015). Although malaria is a common cause of fever in many developing countries, bacterial, fungal and viral pathogens are also implicated. A recent systematic review on the etiology of AFI in developing countries over the last three and half decades showed that 28.5% of AFI cases were due to malaria, 14% were due to bacteria or fungi and 17.4% were viral (Prasad et al., 2015). Due to the effective global efforts to control malaria, the burden of non-malaria pathogens in AFI etiology is increasing (Murray et al., 2014, Mueller et al., 2014). Some of these pathogens are emerging and re-emerging and are unlikely to have detection tests readily available.

5.5 Diagnosis of acute febrile illnesses

AFIs present with similar clinical manifestations but a diverse array of causative pathogens. The common diagnostic approach by clinicians is to test for agents that are known to occur in a particular geographical area such as malaria or diseases associated with a particular outbreak such as dengue and chikungunya. In surveillance studies, most studies report an array of specific infections using serological assays and polymerase chain reaction (Schoepp et al., 2014, Mueller et al., 2014, Liu et al., 2015).

This approach is chosen for its pragmatism in the backdrop of limitations in resources, but it does not detect causes of AFI that are not part of the array being tested. There are numerous multiplex tests for common causes of AFI but in the developing world, these are mainly found in research settings rather than at the point of care where they are most needed (Piantadosi and Kanjilal, 2020, Rhee et al., 2019).

Diagnosis of arbovirus infection usually requires knowledge of arboviral epidemiology in the area. Laboratory diagnosis is usually done through serology (ELISA or Immunofluorescence assay) or by PCR. Serological assays are prone to cross-reactivity among the various species hence the need to confirm positive results by plaque neutralization assays (Hilgenfeld and Vasudevan, 2018, Piantadosi and Kanjilal, 2020, Rhee et al., 2019).

High-throughput sequencing (HTS) is increasingly being used to detect pathogens, mainly in research labs. A major advantage of HTS is that it can be done without a priori knowledge of the pathogen's genome which makes the technique ideal for detection of any pathogen causing AFI, both known or novel. The main challenges of HTS are low sensitivity (compared to PCR), high cost, and complexity requiring highly trained staff (Feng et al., 2015, Zhang et al., 2015, Saelens et al., 2015, Stremlau et al., 2015, U.S. Food and Drug Administration, 2015, Piantadosi and Kanjilal, 2020)

5.6 Mitigation against emergence and reemergence of arboviral infections

The WHO has made recommendations on how to mitigate against EIDs. These are (1) national epidemic preparedness, laboratory capacity, early warning alert and response systems (2) training of healthcare workers in epidemic preparedness and response, (3) standardization of preparedness and response measures (4) strengthening biosafety, biosecurity and readiness for dangerous emerging pathogens such as viral hemorrhagic fevers and (5) coordination and support of member states for pandemic preparedness and response (World Health Organization, 2015).

This requires a one health approach - a multidisciplinary approach encompassing human, animal and environmental spheres; coupled with national, regional and international collaboration and coordination.

6.0 MATERIALS AND METHODS

6.1 Study Design

This was a descriptive cross-sectional study on patients presenting with fever in Kibera Informal Settlement (Nairobi) and Taita-Taveta County. It was part of a larger study on zoonotic and arboviral infections which was investigating transmission dynamics of emerging and reemerging infections in Kenya by studying humans, arthropod vectors, rodents, bats and the environment

6.2 Study Setting

We conducted this study in Kibera slums in Nairobi city and rural and semi-rural Taita–Taveta County. In Kibera, we collected samples from Ushirika Medical Clinic in Kianda village, Kibera South Health Centre in Lindi and Amref-Kibera Community Health Centre in Laini Saba village. In Taita-Taveta County, we collected samples from Moi County Referral Hospital in Voi town, Mwatate Sub-County Hospital in Mwatate and Wundanyi Sub-County Hospital in Wundanyi.

Kibera Sub-County in Nairobi has a population of at least 185,000 people in an area of 12 km^2; a population density of 15,311 people/km^2 (KNBS, 2019). Kibera informal settlement borders more affluent neighborhoods (which provide employment), the Nairobi dam and Ngong forest. The slum has twelve villages, the eastern part (Laini-Saba, Soweto East and Silanga) is mainly inhabited by single persons living alone or sharing a shack. Many of these are unmarried or have left their families in the countryside to seek employment in Nairobi. They often visit their families in various parts of the country. The east of Kibera is nearest to Nairobi's industrial area where many of the people work. Towards the west of Kibera, the proportion of singles living together or living alone decreases. In the most westerly villages (Lindi, Soweto West and Raila), more than three-quarters of the households are inhabited by households with children (0 -17 years) who form 50% of the population as compared to 36% in the whole of Kibera (Desgroppes and Taupin, 2011). Kibera is congested with insufficient drainage and sanitation, stagnant pools of water, and poorly lit shanties (Scott et al., 2017).

There are several dispensaries, clinics, nursing homes and health centers within the slum which are mainly run by charity organizations, faith-based organizations, private

owners and some by the government. Three of these were purposively selected for patient recruitment. Kibera Community Health Centre (KSHC) in Laini-saba, eastern Kibera, which is run jointly by Amref and Nairobi County Health Department; Ushirika Medical Clinic (UMC) in Kianda, in the western part of Kibera, which is run by a community-based organization and Kibera South Health Centre in Lindi, in the central part of Kibera, which was being run by Médecins Sans Frontières during the time of the study. These facilities were selected to represent all parts of Kibera (Figure 5).

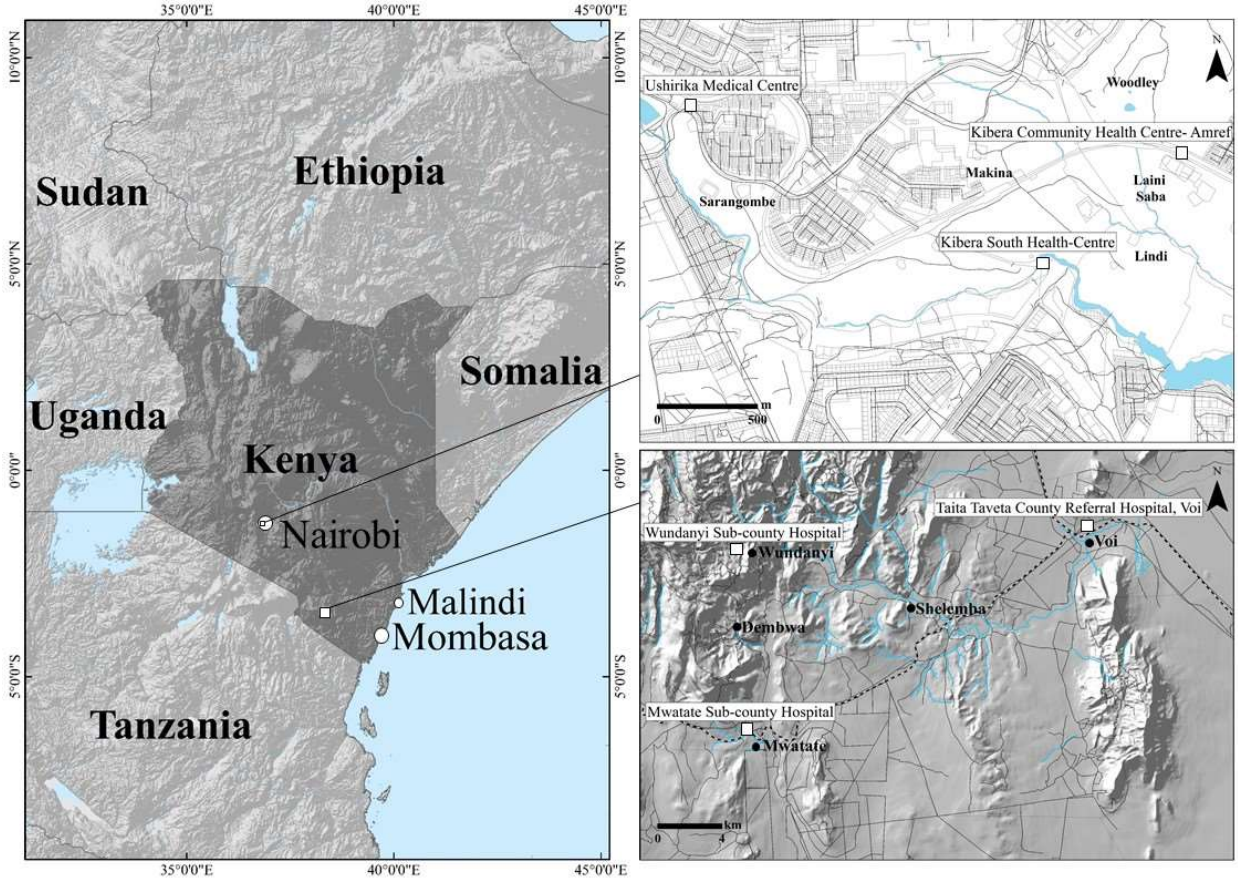

Figure 5. Map of showing the location of study sites in Kibera and Taita-Taveta County. Map courtesy of Ruut Uusitalo.

Taita-Taveta County is located in southern Kenya near the border with Tanzania. The county covers an area of 17,000 km^2; stretching over lowland savannah and highlands. Tsavo East and West National Parks also form a significant part of the County. It has a population of 340,000 people (20 people/km^2) (KNBS, 2019). It has about 100 health facilities which include one county referral hospital and four sub-county hospitals

(Taveta, Wesu, Mwatate and Wundanyi). The rest are Health centers, dispensaries and clinics; some of these facilities are run by faith-based organizations and private entities (Ministry of Health, 2015). This study was conducted in Voi, Mwatate and Wundanyi hospitals in order to cover both lowlands and highlands as well as rural and urban population in the county. Moi County Referral Hospital (Voi) is the largest in the county and is located in the lowland town of Voi (altitude of 600 m), next to the Mombasa – Nairobi highway and Tsavo East National Park. It is a level 4, 112-bed facility which serves as the referral hospital for the county. Wundanyi Sub-County Hospital is a 16-bed level-3 facility in the highland town of Wundanyi (altitude = 1400 m). Mwatate Sub-County Hospital is a 17-bed, level 3 facility in between Voi and Wundanyi in a small eponymous town at the foot of Taita Hills (Figure 5).

Taita–Taveta has is ecologically diverse with highly variable topography, vegetation and climate. The county includes lowland savannah, highlands with an Afromontane forest, Tsavo national parks and several conservancies (Erdogan et al., 2011). Taita-Taveta is traversed by a railway and several roads and is home to Tsavo National Parks as well as Lakes Jipe and Chala which attract both Kenyan and international tourists. It is also proximal to the Kenyan coastline, where arboviral infections such as DENV and CHIKV are known to be endemic (Ochieng et al., 2015, LaBeaud et al., 2015).

6.3 Study Population

We studied patients with acute febrile illness presenting in three hospitals in Taita–Taveta from Apr to Aug 2016 and another three health facilities in Kibera slums, Nairobi, from Feb to Jun 2017. Consenting patients with a fever (defines temperature of $\geq$37.5 °C) were enrolled into the study.

6.4 Sample Size Determination

We used the prevalence of DENV, the most common arbovirus, to estimate the required sample size. The prevalence reported in previous studies in Kenya was highly variable, ranging between 0 – 70% depending on the location and population tested (Obonyo et al., 2018, Konongoi et al., 2016, Mease et al., 2011, Sutherland et al., 2011). We therefore assumed that seroprevalence of arboviruses in Taita-Taveta would be about 30% and half of that (15%) for Nairobi. This was informed by the fact that Taita-Taveta being nearer the coast where arbovirus infections are most common, it was likely to

have a higher prevalence than Nairobi. We set a confidence interval of 95%, power of 80% and a margin of error of 5%. The sample size was estimated using the OpenEpi online sample size calculator for a single proportion (Sullivan, 2003):

$$n = (Z_{\alpha/2})^2 * (p(1-p) / e^2$$

Where Zα/2 is the critical z-score value when using a confidence interval of 95% and is equal to 1.96, and p is the expected sample proportions of the parameter of interest.

$$\text{Taita-Taveta: } 1.96^2 * 0.3 * 0.7 / 0.05^2 = 323$$

$$\text{Nairobi: } 1.96^2 * 0.85 * 0.15 / 0.05^2 = 196$$

Therefore, the minimum sample size for Taita –Taveta and Nairobi was 323 and 196 respectively. Sample collection was done over both dry and rainy months to capture seasonal variations.

6.5 Eligibility Criteria

6.5.1 Inclusion Criteria

Any patient presenting with a febrile illness that had lasted up to 14 days before the hospital visit and gave consent to participate in the study. Febrile children whose parents or guardians gave informed consent were also included.

6.5.2 Exclusion Criteria

Patients who needed emergency life-saving care at admission were not enrolled to avoid delay management.

6.6 Study Procedures:

Clinicians identified patients who fit the inclusion criteria and briefed them about the study. Once informed consent was obtained, sociodemographic and clinical data was collected using a clinician-administered questionnaire. Blood samples (5 ml) were collected from study participants using EDTA or serum-separator vacutainers. Samples were kept at -20°C at the facility for a maximum to three weeks then transported on ice to KAVI-ICR lab for storage at -80°C. At KAVI-ICR samples were centrifuged at 1200 g for 10 minutes to extract serum (from Taita-Taveta samples) and plasma from Kibera samples. Two aliquots (500 – 1000 µL) were then prepared from each sample, bar-

coded and stored at -80˚C before shipment to the Department of Virology, University of Helsinki (UH) on dry ice for laboratory analysis.

6.7 Laboratory Procedures

6.7.1 Antibody and Antigen Testing

6.7.1.1 Flavivirus Serology

Testing for flavivirus IgM and IgG antibodies was done using an in-house immunofluorescence assay (IFA) with DENV-3 antigen as described previously (Vene et al., 1995). Briefly, dengue type 3 virus was added to Vero E6 cells in a 25 mm^3 tissue culture flask with Eagle's minimal essential medium supplemented with penicillin-streptomycin/ml and 5% fetal bovine serum. The cells were incubated at 37°C for five days then mixed with uninfected Vero E6 cells at a ratio of 1:3 in a cell culture media. Slides were prepared by seeding cleaned 12-well slides followed by incubation for sixteen hours at 37°C and 5% carbon dioxide. The slides were then fixed using acetone and stored at -70°C till use (Masika et al., 2020, Vene et al., 1995).

Serum/plasma samples were diluted 1:20 in phosphate-buffered saline (PBS), added to pre-spotted slides, and incubated at 37 °C for half an hour. The slides were washed 4 times (the first three with PBS and the fourth with distilled water) and incubated at 37 °C for 30 minutes with FITC-anti-human IgG conjugate (Jackson Immuno Research Laboratories, West Grove, PA, USA) diluted 1:100 in PBS then washed again as described above, and studied using a fluorescence microscope (Figure 6). Positive samples were diluted two-fold serially from 1:40 to 1:2560 (1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560) until the last positive dilution was attained. The reciprocal of the weakest dilution that showed fluorescence was recorded as the titer (Masika et al., 2020).

GullSORB IgG-inactivation reagent (Meridian Bioscience, OH, USA) was added to dengue IgG antibody-positive samples before testing for DENV IgM by IFA. For samples positive by IgM antibodies on IFA, dengue IgM ELISA (Capture DXSelect, Focus Diagnostics, Diasorin Molecular LLC, CA, USA) was performed as per manufacturer’s instructions. Samples that tested positive for DENV IgM antibodies on ELISA, IgG antibodies on IFA or DENV RNA by PCR were additionally tested for DENV

NS1 antigen using Platelia Dengue NS1 Ag ELISA test (Bio-Rad Laboratores, CA, USA). Samples positive for DENV IgG antibodies by IFA were tested for Zika virus IgG using Anti-Zika Virus ELISA (Euroimmun, Germany); DENV IgM-antibody positive samples were also tested for ZIKV IgM antibodies if there was enough sample. The presence of any DENV antibodies was interpreted as flavivirus antibodies due to cross-reactivity between flaviviruses (Masika et al., 2020).

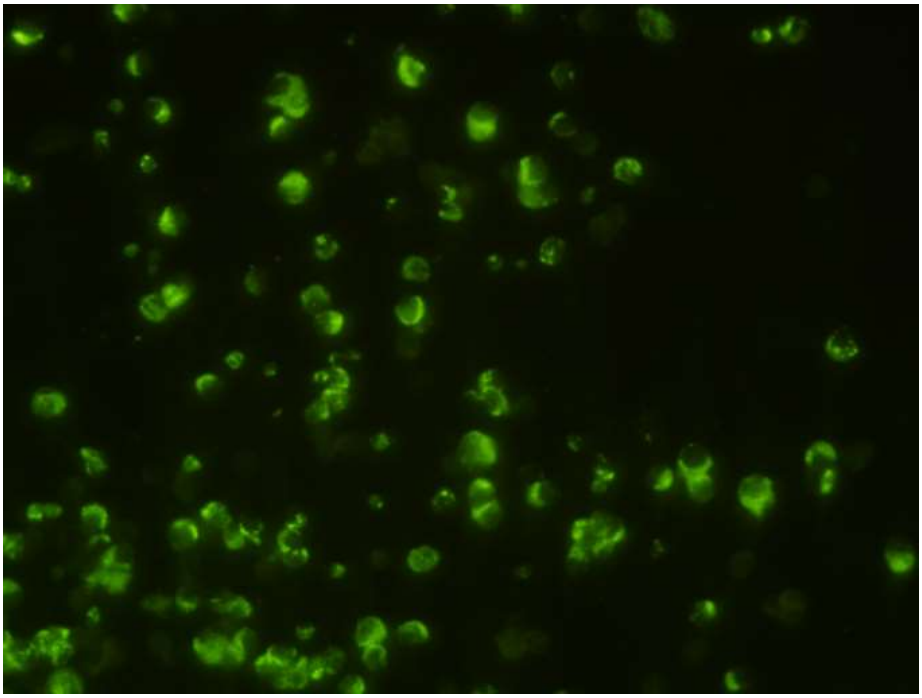

Figure 6. A sample positive for Dengue IgG antibodies on Immunofluorescence assay

6.7.1.2 Alphavirus Serology

Samples were tested using an in-house IFA for Chikungunya IgG antibodies as described previously (Kallio-Kokko et al., 2006, Masika et al., 2022). In summary, Vero E6 cells were infected with CHIKV (Ross strain) and mixed with uninfected Vero E6 cells as control, seeded on slide spots then fixed with acetone and stored at −70°C till use.

Patient serum/plasma samples were diluted 1:20 in PBS, added to pre-spotted slides, and incubated at 37 °C for 30 minutes. The slides were washed as described above and incubated at 37 °C for 30 minutes with FITC-antihuman IgG conjugate (Jackson

Immuno Research Laboratories, PA, USA) diluted 1:100 in PBS then washed again and examined for fluorescence using an ultraviolet light microscope. To obtain CHIKV titers by IFA, positive samples were diluted serially (2-fold) from 1:40 to 1:2560 (1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560) and tested for immunofluorescence. The reciprocal of the weakest dilution that showed fluorescence was recorded as the titer.

The samples were also tested for CHIKV IgM antibodies using CHIKV IgM ELISA (Euroimmun, Germany) as instructed by the manufacturer.

6.7.1.3 Plaque Reduction Neutralization Assay for Alphaviruses

Depending on the availability of sample volume, a subset of CHIKV-IgG and/or IgM antibody-positive samples were analyzed at Umeå University, Sweden using PRNT for CHIKV, ONNV and SINV. The PRNT was performed using a method described previously (LaBeaud et al., 2015, Masika et al., 2022). Briefly, 50 µL of patient samples (diluted two-fold from 1:40, 1:80, 1:160 to 1:320) was added to 50 µL of the respective virus, incubated at room temperature for one hour, then transferred to 12-well plates with a layer of Vero-B4 cells. After additional incubation for one hour at 37°C in 5% CO_2, the cells were covered with 100 µL of Dulbecco's Modified Eagle Medium and 2% carboxymethylcellulose (CMC; Sigma Life Science, MO, USA) for three days. The CMC overlay was removed, cells fixed with 4% paraformaldehyde, stained with crystal violet and plaques enumerated. The reciprocal of the weakest serum dilution that showed at least 80% plaque reduction was recorded as the titer (Masika et al., 2022, LaBeaud et al., 2015). A PRNT titer >40 was considered positive. Samples were considered positive for ONNV if ONNV titers were at least 2 times higher than CHIKV titers, and CHIKV positive if the CHIKV titer was >40 and 4 times higher than ONNV titer. If titers for both ONNV and CHIKV were >40 and the difference was less than two-fold, the sample was considered positive for both ONNV and CHIKV. This is because CHIKV and ONNV exhibit a characteristic cross-reactivity with CHIKV antibodies are more likely to cross-react with ONNV antigens than ONNV antibodies with CHIKV antigens (Hozé et al., 2021). The virus strains used to infect Vero B4 cells for PRNT were CHIKV LR2006-OPY1 (Accession: EU224268), ONNV IbH12628 and SINV Lovanger strain (Accession: KF737350).

6.7.2 Arbovirus RNA Detection

6.7.2.1 Flaviviral RNA Detection

Nucleic acid extraction was done from serum or plasma samples (200 µl) using an automated MagNa Pure LC instrument (Roche, Switzerland), eluted to a volume of 50 µl and stored at -70 ˚C before RT-PCR (Masika et al., 2020).

A pan-flavivirus RNA screening was performed using a SYBR green RT-PCR assay using a Stratagene Mx3005p rt-PCR machine (Agilent Technologies, CA, USA). The products were then amplified using a second semi-nested conventional PCR step (Bio-Rad T100 Cycler, Bio-Rad Laboratories, CA, USA) and visualized on an agarose gel as described before (Moureau et al., 2007, Moureau et al., 2010, Masika et al., 2020). TBEV strain Kumlinge-A52, which has not been reported in Kenya, was used as a positive control. We used degenerate primers as described by Moureau et. al. First-round primers were PF1s 5' TGYRTBTAYAACATGATGGG-3' (forward) and PF2R-bis 5' GTGTCCCAXCCN GCNGTRTC-3 (reverse), while second-round, semi-nested primers were PF3S 5' ATH TGGTWYATGTGGYTDGG-3' (forward) and PF2R-bis 5' GTGTCCCAXCCNGCNGTR TC-3 (reverse) (Moureau et al., 2010, Moureau et al., 2007). These primers target part of the RNA polymerase domain within the flavivirus NS5 gene which is highly conserved (Kuno, 1998).

Additionally, we performed a nested RT-PCR (targeting the E-gene) for selected PCR-positive samples. Reverse transcription was done using Maxima RT, random hexamers and RiboLock RNase inhibitor polymerase. Amplification was performed using DreamTaq DNA polymerase (Thermo Fisher Scientific, MA, USA) as per the manufacturer's instructions. We used primers targeting the DENV-2 E-gene: First-step: 5'-CAT GGA TGT CAT CAG AAG GG-3' and 5'- CCT TTR ATG TCT CCT GTC AT-3', nested-step: 5'- GGR TYY TGA GAC ATC CAG G-3' and 5' –TCT GRT GTT ATY TGT TTC CAC-3'.

The final products of the pan-flavivirus RT-PCR and the DENV-2 E-gene RT-PCR (~ 150 bp and ~ 1500 bp respectively) were submitted to the Institute of Biotechnology (UH) for Sanger sequencing. Sequences obtained were analyzed using the NCBI Basic Local Alignment and Research Tool (BLAST) to identify the specific virus (Masika et al., 2020).

6.7.2.2 Alphavirus RNA Detection

Nucleic acids extraction was done from serum or plasma samples (200 µl) using a MagNa Pure LC instrument (Roche, Switzerland), and eluted to a volume of 50 µl. For cDNA synthesis, 5µL of RNA template was added to a 9.5 µL mix of random hexamers (1 µL), dNTPs (1µL) and nuclease-free water (7.5µL) and heated at 65˚C for 5 minutes. A 5.5 µL mix of RiboLock RNase inhibitor (0.5 µL), Maxima RT (1µL) and 5X buffer (4 µL) was then added. The total volume of 20 µL was cycled through 25 ˚C (10 minutes), 50 ˚C (30 minutes) and 85 ˚C (5 minutes) then kept at 4˚C pending PCR amplification.

We then performed a nested PCR using degenerate primers targeting the nsP4 gene of the alphavirus genome as described previously (Sánchez-Seco et al., 2001). In the first step of the PCR, 2µL of cDNA was added to a 48 µL mix of 10x DreamTaq buffer (5 µL), DreamTaq polymerase (0.25 µL), dNTPs (2 µL), first-round primers (5 µL each) and nuclease-free water (30.75 µL). The 50 µL reaction mix was taken through 40 cycles of 94 ˚C (3 min), 94 ˚C (30 sec), 52˚C (1 min), 68˚C (30 sec) and a final extension step at 68 ˚C for five minutes. For the nested-PCR step, 2 µL of the first-round product was added to a 45 µL mix of 10x DreamTaq buffer (5 µL), DreamTaq polymerase (0.25 µL), dNTPs (2 µL), second-round forward primer (5 µL), reverse primer (2.5µL) and nuclease-free water (30.25 µL). The reaction mix was then taken through 40 cycles of 94 ˚C (3 min), 94 ˚C (30 sec), 52 ˚C (1 min), 72˚C (30 sec) and a final extension step at 72 ˚C for five minutes. We used chikungunya virus (Ross Strain, European Virus Archive) as a positive control for the PCR.

First-round primers were 5'-GAYGCITAYYTIGAYATGGTIGAIGG-3' and 5'-KYTCYTCIGTRTGYTTIGTICCIGG-3' and the nested-step primers were 5'-GIAAYTGYAAYGTIACICARATG-3' and 5-GCRAAIARIGCIGCIGCYTYIGGICC-3' respectively (Sánchez-Seco et al., 2001).

After amplification, 10µL of the final amplicon was analyzed through gel electrophoresis using a 2% agarose gel with 0.5 µg/ml of ethidium bromide in Tris/Borate/EDTA (TBE) buffer which was imaged using the AlphaImager™ Gel Imaging System (Alpha Innotech, UK). The final product (from the second amplification) was approximately 200 bp. One PCR-positive sample was submitted for Sanger sequencing at the Finnish

Institute for Molecular Medicine (FIMM), University of Helsinki. Output sequences were analyzed using the NCBI BLAST.

6.7.3 Next-generation Sequencing

We analyzed a subset of samples using high throughput sequencing using Illumina MiSeq™ (Illumina). A total of 119 samples were analyzed including 110 randomly selected samples that were run in 22 pools of 5 samples each and 9 samples that were run individually including 6 that tested positive for DENV on PCR, 1 that had tested positive for CHIKV on PCR and 2 that were negative for either.

Sample preparation for sequencing was done using a modified protocol that has been reported previously (Conceição-Neto et al., 2015, Conceição-Neto et al., 2018). In summary, plasma/serum samples were ultrafiltered using 0.4µM polycarbonate membrane filters to eliminate any cellular debris. This was followed by treatment with benzonase endonuclease (Millipore, MA, USA) and micrococcal exonuclease (New England Biolabs, United Kingdom) enzymes to degrade free nucleic acids within the sample (spares viral nucleic acids which are protected within viral capsids) and precipitation with polyethylene glycol (Abcam, PEG virus precipitation kit, United Kingdom) to concentrate virions. Nucleic acid extraction was done using QIAamp® Viral RNA Mini Kit (Qiagen, Germany), then reverse transcription and amplification performed using Whole Transcriptome Amplification Kit (Sigma Aldrich, MO, USA). The amplicons were purified using GeneJet PCR Purification Kit (Life Technologies, CA, USA) followed by library preparation using Nextera XT kit (Illumina, CA, USA) as per the manufacturer's instructions. Library fragment size was assessed through electrophoresis and the DNA was quantified using a Qubit Broad-Range dsDNA Assay Kit (Life Technologies, MA, USA) and NEBNext Library Quantification kit (New England BioLabs, UK). Prepared libraries were sequenced using an Illumina MiSeq™ V2 reagent kit (Illumina, CA, USA) as per the manufacturer's instructions (Masika et al., 2020).

6.7.4 Sequence Analysis

Sanger sequences obtained from RT-PCR products were analyzed using NCBI BLAST to identify the virus species and type.

We used an in-house bioinformatics analysis pipeline (Lazypipe) to analyze Illumina MiSeq™ sequence data (Plyusnin et al., 2020). Briefly, de-novo assembly was done using MIRA assembler version 4.9.5 (Chevreux et al., 1999) followed by re-assembly against the de-novo assembled sequences using BWA-MEM algorithm executed in SAMTools version 1 (Li, 2011). Virus sequences were categorized into different taxonomic units (families, genera and species). Additional DENV-2 complete coding sequences (CDS) were retrieved from the NCBI GenBank (accessed in 31.08.2021) for phylogenetic analysis.

6.7.5 Virus Isolation by culture

Virus isolation through culture was attempted from three DENV-2 PCR-positive and one CHIKV PCR-positive sample. This was performed as described previously (Huhtamo et al., 2008). In brief, virus cultures were conducted concurrently in two cell lines - Vero E6 monkey kidney cells grown in Minimal Essential Medium at 37 °C and 5% carbon dioxide, and in C6/36 cells grown in Leibovitz L-15 medium at room temperature. Cells in 25cm^2 flasks were incubated with 50µL of sample for one hour and observed for 24 days for cytopathic effects (CPE). When CPE was evident, cells were harvested for testing on IFA and RT-PCR. If positive on PCR, the culture supernatant was analyzed using next-generation sequencing (Masika et al., 2020).

6.8 Data Management

6.8.1 Data collection, analysis and dissemination

Data was captured using questionnaires and laboratory result sheets and entered into IBM SPSS Statistics 22 software for analysis. Questionnaires were administered by trained clinicians to capture patient demographics, signs and symptoms and pertinent medical history such as recent travel. Univariate analysis to summarize data was done using measures of central tendency (means and standard deviation or median and interquartile range) for numerical variables, and frequencies/proportions for categorical data. Bivariate analysis for a preliminary assessment of association between dependent variables and independent variables was done using Pearson’s Chi-squared test or Fisher’s Exact Test (FET) for categorical variables such as infection status and age category (Children vs adult) and facility location (Nairobi vs Taita-Taveta). We student t-test to assess any association between the outcome variable and numerical

independent variables such as age. Factors with a p value of <0.2 in the bivariate analysis (Alyousefi et al., 2016) were further assessed using simultaneous binary logistic regression.

Data was stored in a password-protected laptop and backed up on a DropBox™ folder accessible to the investigators only. Deidentified data may be available to other researchers on request. The findings were also disseminated through two peer-reviewed publications, conference and journal club presentations and seminars with health workers in the study sites.

6.8.2 Laboratory Data Analysis

All samples were analyzed for flavivirus and alphavirus IgG (using IFA) and IgM (ELISA) antibodies and categorized as IgG/IgM antibody positive or negative. Where the sample volume was sufficient, alphavirus IgG and IgM positive samples and randomly selected negative controls were analyzed using PRNT and categorized as CHIKV positive, ONNV positive, CHIKV+ONNV positive or negative for all. All samples were also analyzed for flavivirus and alphavirus RNA by group-consensus PCR followed by specific virus identification by Sanger sequencing and categorized as either PCR-positive or negative. Randomly selected samples and some individual samples of interest (PCR-positive) were analyzed used next-generation sequencing and the results analyzed using a bioinformatics pipeline.

6.9 Ethical considerations

This study was approved by the Kenyatta National Hospital-University of Nairobi Ethics & Research Committee (KNH-UoN ERC P707/11/2015 and P618/08/2016). We obtained authorization to conduct research from the health services departments of Taita-Taveta County and Nairobi City County. We obtained informed consent from all participants or guardians (for children) as well as assent for children aged 10 -17 years. No identifiable personal information was analyzed or published. All clinicians and lab technologists who participated in data collection were working at the study sites and were trained on the study procedures as well as research ethics. Blood samples for any tests required for clinical management of the patients were prioritized before research samples could be collected. Shipping permits were obtained from KNH-UoN ERC and the Ministry of Health.

7.0 FINDINGS

7.1 Characteristics of participants

We analyzed 557 samples from individual patients; 326 serum samples from Taita-Taveta and 231 plasma samples from Kibera. From Taita Taveta, we enrolled participants from Wundanyi Sub-County Hospital (155), Mwatate Sub-County Hospital (125) and Moi Country Referral Hospital, Voi (46). From Kibera in Nairobi, we enrolled at Ushirika Medical Clinic (111), Kibera Community Health Centre (81) and Kibera South Health Centre (39). Half (52%) of the participants were female. The mean age was 22.4 years (SD = 20.2), ranging from 2 months to 85 years (Figure 7). The mean body temperature at enrolment was 38.6 ˚C (SD =0.63). The median duration since the onset of fever was 2 days (range = 1 – 14 days). Participant characteristics are summarized in Table 2.

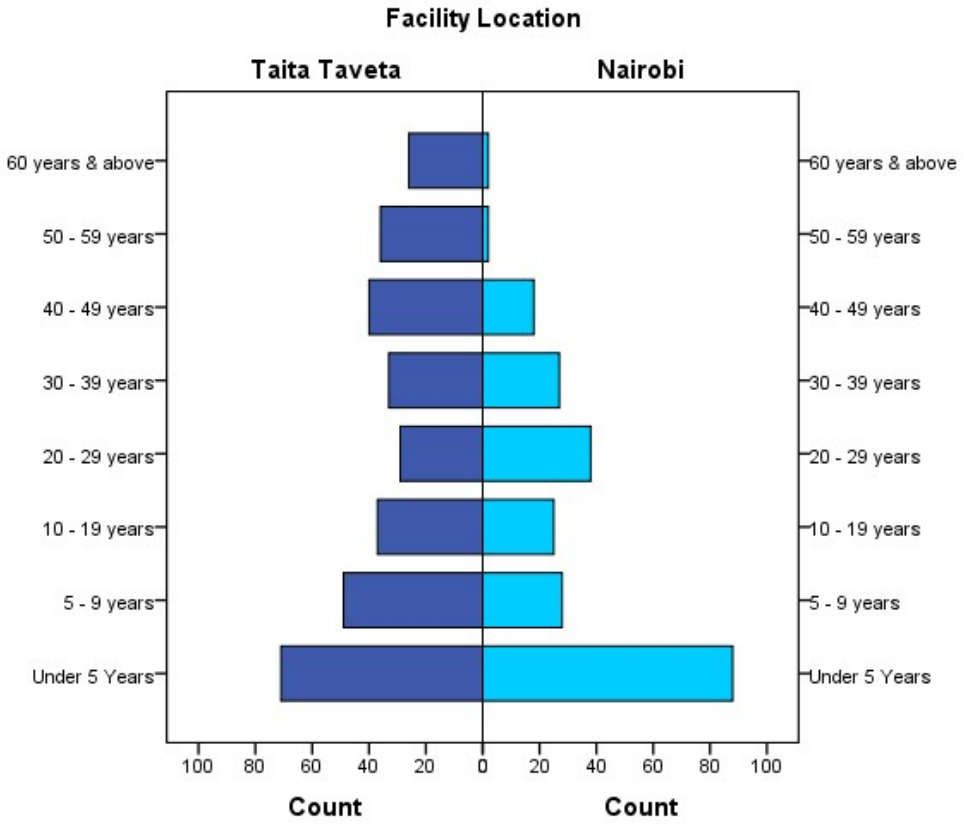

Figure 7. Age distribution among patients with febrile illness by facility location

Table 2. Characteristics of Patients with Acute Febrile Illness in Health Facilities in Taita-Taveta and Nairobi

Parameter	Taita Taveta	Nairobi	Total (%)
n	326 (59%)	231 (41%)	557
Gender (n=547)			
Male	146 (46%)	115 (50%)	261 (48%)
Female	172 (54%)	114 (50%)	286 (52%)
Age (n=546)			
Mean (Standard Deviation)	26.8 (22.1)	16.2 (15.3)	22.4 (20.2)
Median (Interquartile Range)	20.6 (39.5)	10.2 (24.4)	16.4 (33.6)
Range (Youngest – Oldest)	6 months – 85 yrs	2 months – 77 yrs	2 months – 85 yrs
Age Groups (n=545)			
0 - 5 years	69 (22%)	87 (39%)	156 (29%)
5 - 17 years	81 (25%)	45 (20%)	126 (23%)
18 years & above	169 (53%)	94 (41%)	263 (48%)
Education Level in adults (n=245)			
None	15 (10%)	0 (0%)	15 (6%)
Primary school	77 (50%)	27 (30%)	104 (42%)
Secondary school	29 (19%)	36 (40%)	65 (27%)
Tertiary level	34 (22%)	27 (30%)	61 (25%)
History of travel (n=552)	54 (17%)	88 (38%)	142 (26%)
Contact with animals (n=557)			
Contact with goats	134 (41%)	25 (11%)	159 (29%)
Contact with cattle	96 (29%)	26 (11%)	122 (22%)
Contact with sheep	26 (8%)	11 (5%)	37 (7%)
Contact with chicken	190 (58%)	43 (19%)	233 (42%)
Contact with dogs	53 (16%)	41 (18%)	94 (17%)
Contact with cats	61 (19%)	87 (38%)	148 (27%)
Contact with rodents	217 (67%)	127 (55%)	344 (62%)
Contact with bats	94 (29%)	6 (3%)	100 (18%)
Contact with any animal	268/326 (82%)	166/231 (72%)	434/557 (80%)
Signs & symptoms (n=557)			
Temperature in ℃ (Mean/SD)	38.8 (0.56)	38.4 (0.67)	38.6 (0.63)
Range (Lowest – Highest ℃)	37.5 – 40.4	37.5 – 40.9	37.5 – 40.9
Duration of fever - Median (range)	2 (1-7) days	2 (1-14) days	2 (1-14) days
Joint pain	139 (43%)	70 (30%)	209 (38%)
Myalgia	123 (38%)	65 (28%)	188 (34%)
Headache	53 (16%)	108 (47%)	161 (29%)
Cough	44 (13%)	95 (41%)	139 (25%)
Vomiting	53 (16%)	68 (29%)	121 (22%)
Diarrhoea	29 (9%)	47 (20%)	76 (14%)
Rash	18 (6%)	16 (7%)	34 (6%)

7.2 Serology results

7.2.1 Flavivirus serology

Dengue IgG

Screening for dengue IgG antibodies was done using immunofluorescence assay (IFA). Fifty-one samples (9%) were positive for dengue IgG antibodies (n=557). The IFA titers for CHIKV Igg-positive samples ranged from 10 to 2560 (Figure 8). Dengue IgG prevalence was higher in Taita Taveta at 13.5% than in Kibera at 3.0% (χ^2, $p < 0.001$).

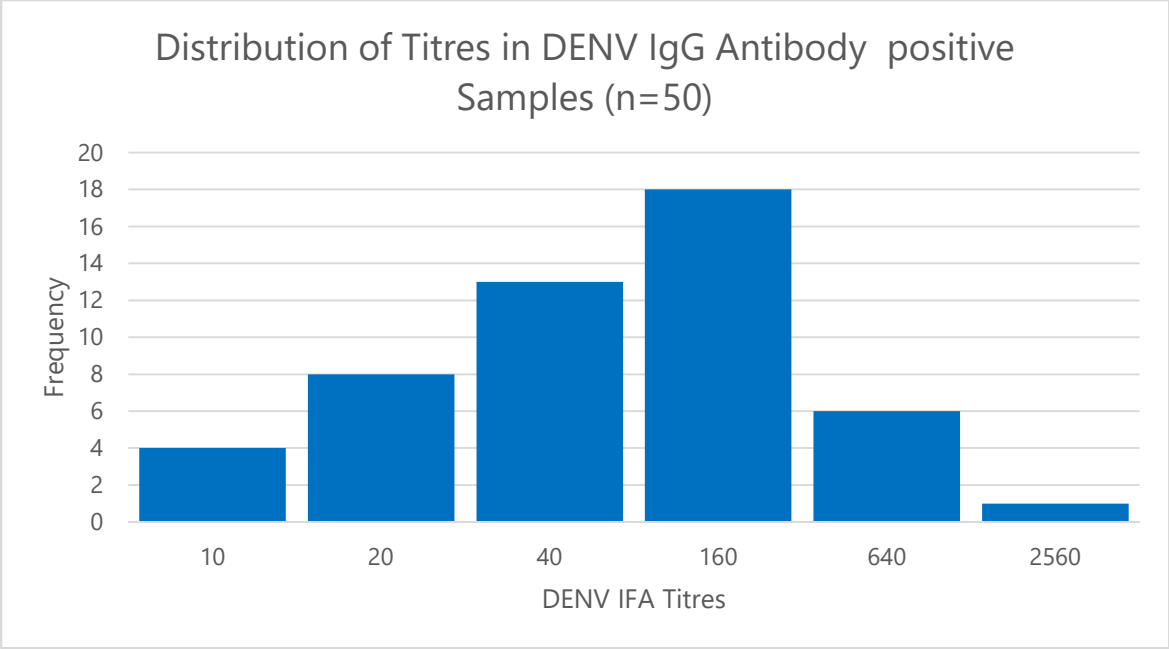

Figure 8. Distribution of Dengue IgG IFA antibody titres

Dengue IgM antibodies

Screening for DENV IgM antibodies was done using IFA and 57/557 samples that were DENV IgM IFA-positive were re-tested using DENV IgM ELISA. Three (0.5% of 557) of these 57 samples were positive on Dengue IgM ELISA; two (0.6% of 326) in Taita-Taveta and one (0.4% of 231) in Kibera.

Dengue NS1 Antigen ELISA

A total of 57 samples that were positive for either Dengue IgG IFA, IgM ELISA, or Dengue RNA PCR were tested for NS1 Antigen using ELISA. Five samples tested positive for NS1 Antigen. All five were from Taita-Taveta.

Overall, 8 samples (1.4%) tested positive for acute dengue infection (DENV IgM ELISA, NS1 antigen or PCR). Seven were from Taita-Taveta and 1 from Kibera. Table 3.

Table 3. Dengue Serology Results

Parameter	Taita-Taveta		Nairobi		Overall	
	n	% (95%CI)	n	% (95%CI)	n	% (95%CI)
Dengue IgG IFA Positive	44/326	14(10.0-17.7)	7/23	3(1.2-6.1)	51/557	9(6.9-11.9)
Dengue IgM IFA Positive	34/325	11(7.4-14.3)	42/229	18.3(13.5-24.0)	76/554	14(11.0-16.9)
Dengue IgM ELISA Positive	2/34	6(0.7-19.7)	1/42	2.4(0.1-12.6)	3/76	4(0.8-11.1)
Dengue NS1 Ag ELISA Positive	5/45	11(3.7-24.1)	0/12	0(0.0-26.5)	5/57	9(2.9-19.3)
Dengue PCR Positive	5/326	1.5(0.9-4.4)	0/231	0(0.0-1.6)	5/557	1(0.3-2)
Acute Dengue (IgM ELISA, NS1 Ag, RNA PCR) Positive	7/326	2.1(0.9-4.4)	1/231	0.4(0.0-2.4)	8/557	1.5(0.6-2.8)
Dengue Antibody Titers (IFA)						
10	0/44	0 (0.0-8.0)	4/6	67(22.3-95.7)	4/50(8%)	8(2.2-19.2)
20	8/44	18(8.2-32.7)	0/6	0(0.0-45.9)	8/50(16%)	16(7.2-29.1)
40	12/44	27(15.0-42.8)	1/6	17(0.4-64.1)	13/50(26%)	26(14.6-20.3)
160	17/44	39(24.4-54.5)	1/6	17(0.4-64.1)	18/50(36%)	36(22.9-50.8)
640	6/44	14(5.2-27.4)	0/6	0(0.0-45.9)	6/50(12%)	12(4.5-24.3)
2560	1/44	2(0.1-12.0)	0/6	0(0.0-45.9)	1/50(2%)	2(0.1-10.6)

7.2.2 Alphavirus Serology

Chikungunya IgG Antibodies by IFA

We screened 557 serum or plasma samples for chikungunya IgG antibodies on immunofluorescence assay. Overall, 49 (9%) were positive. CHIKV IgG seroprevalence was 7% in patients from Taita-Taveta and (12%) in Kibera (χ^2, p= 0.04).

Chikungunya IgM antibodies by ELISA

We tested 555 serum/plasma samples for Chikungunya IgM antibodies on ELISA; 33 of these (6%) were positive with 6% and 7% seropositivity for Taita-Taveta and Kibera respectively. The difference was not significant (χ^2, p= 0.59).

Table 4. Prevalence of Chikungunya Virus IgG (IFA) and (IgM) antibodies by location

Parameter	Taita-Taveta		Nairobi		Total	
	n	% (95%CI)	n	% (95%CI)	n	% (95%CI)
CHIKV IgG IFA	22/326	7(4.3-10.0)	27/231	12(7.8-16.5)	49/557	9(6.6-11.5)
CHIKV IgM ELISA	18/326	6(3.3-8.6)	15/228	7(3.7-10.7)	33/554	6(4.1-8.3)
CHIK IgG or IgM	40/326	12(8.9-16.3)	36/227	17(12.0-22.1)	76/553	14(11.3-17.2)
CHIKV IgG Titers by IFA						
<100	8/22	36.4(17.2-59.3)	21/25	84(63.9-95.5)	29/47	62(46.4-75.5)
100 - 300	6/22	27(10.7-50.2)	4/25	16(4.5-36.1)	10/47	21(10.7-35.7)
>300	8/22	36(17.2-59.3)	0/25	0(0.0-13.7)	8/47	17(7.6-30.8)

Alphavirus Plaque Reduction Neutralization Assay Results

Sixty-nine samples were tested on PRNT for CHIKV, ONNV and SINV antibodies. This included 31 CHIKV IgG IFA positive samples, 23 CHIKV IgM ELISA positive samples, 4 samples positive for both CHIKV IgG (IFA) and IgM (ELISA) and 11 samples that were negative for both CHIKV IgG and IgM (Table 13).

No samples were positive for SINV. Fourteen samples were positive for ONNV only, one was positive for CHIKV only, 14 were positive for both ONNV and CHIKV and 39 samples were negative for both. Therefore, a total of 30 samples were positive for any alphavirus antibodies. Four samples tested positive for alphavirus IgM antibodies on PRNT which indicates ongoing infection at the time of sample collection.

Thirteen of 15 samples that were positive for ONNV on PRNT had tested positive for CHIKV IgG on immunofluorescence and none had tested positive for CHIKV IgM on ELISA suggesting previous exposure to ONNV (

Table 5).

Four patients (0.2%) had alphavirus IgM antibodies on both ELISA and PRNT.

Table 5. Alphavirus Plaque Reduction Neutralization Assay Results

		PRNT Results					
Sample characteristics	**n**	**CHIKV & ONNV Positive**	**CHIKV Positive**	**ONNV Positive**	**SINV Positive**	**Positive for any alphavirus**	**Negative**
CHIKV IgG positive (IFA)	31	12	0	13	0	24	6
CHIKV IgM positive (ELISA)	23	0	1	0	0	1	22
Both CHIKV IgG & IgM positive	4	2	0	1	0	3	1
Negative	11	0	0	1	0	1	10
TOTAL	**69**	**14**	**1**	**15**	**0**	**30**	**39**

Co-infection

None of the samples were detected to have acute infection for both flaviviruses and alphaviruses while two samples had IgG antibodies for both. Overall, 85 (15%) samples tested positive for at least one arbovirus.

7.2.3 Bivariate and Multivariable Analysis:
Factors associated with Flavivirus infection

On bivariate analysis, eleven factors showed potential for association with Dengue IgG IFA seropositivity (p value <0.2), as shown on Table 6. A summary of bivariate analysis of potential predictors of exposure to flaviviruses and alphaviruses among febrile patients in Nairobi & Taita-Taveta. On simultaneous binary logistic regression, only three of these had a significant association with Dengue IgG seropositivity – age, facility location and recent history of travel to the coast. See Table 9.

The prevalence of flavivirus (DENV) IgG antibodies was higher in adults (16.9%) than in children under 18 years (2.2%), OR (95%CI) 7.34 (2.687 – 20.266), p <0.001. In children under 5 years, including 57 children who were under two years old, DENV IgG prevalence was zero, rising to 5% in children aged 15 to 17 years, 16% in adults aged below 60 years and 23% in persons above 60 years of age. Table 9.

DENV IgG prevalence was higher in Taita-Taveta (13.8%) than in Kibera (3.1%) OR (95%CI) 3.43 (1.198 – 9.808), p < 0.022). In addition, recent travel to coastal Kenya was significantly associated with exposure to flavivirus infection, OR (95%CI) 5.22 (2.103 – 12.979), p<0.001).

Dengue IgG seroprevalence was higher in females (12.5%) than in males (5.9%) but this was not statistically significant, OR (95%CI) 1.86 (0.913 – 3.788), p=0.088). There was no association between dengue IgG seropositivity and contact with any animals. Table 9.

Factors associated with Alphavirus Infection

On bivariate analysis, nine factors showed potential for association with alphavirus IgG (PRNT) seropositivity (p value <0.2), as shown in Table 6. On simultaneous binary logistic regression analysis, only three of these factors had a significant association with alphavirus IgG seropositivity– age and facility location. See Table 10.

Alphavirus IgG antibodies (PRNT) were more frequent in adults (9.6%) than in children (1.1%), OR (95%CI) 10.86 (2.808 – 41.973), p <0.001. (Table 10). The mean age for participants with alphavirus IgG antibodies was 34.9 years (SD=14.5) versus 21.5 years (SD=20.0) in seronegative participants; p<0.001 (Figure 9).

Alphavirus IgG seroprevalence was also higher in Kibera (9.5%) than in Taita-Taveta (2.0%), OR (95%CI) 4.99 (1.334 – 18.675), p = 0.04).

Travel to western Kenya (former Nyanza and Western provinces) was associated with higher seroprevalence of alphavirus IgG antibodies, 10.0% and 4.6% respectively, but this was not statistically significant, OR (95%CI) 1.02 (0.340 – 3.035) p<0.997. (Table 10). There was no association between alphavirus IgG seroprevalence and gender. (Table 6).

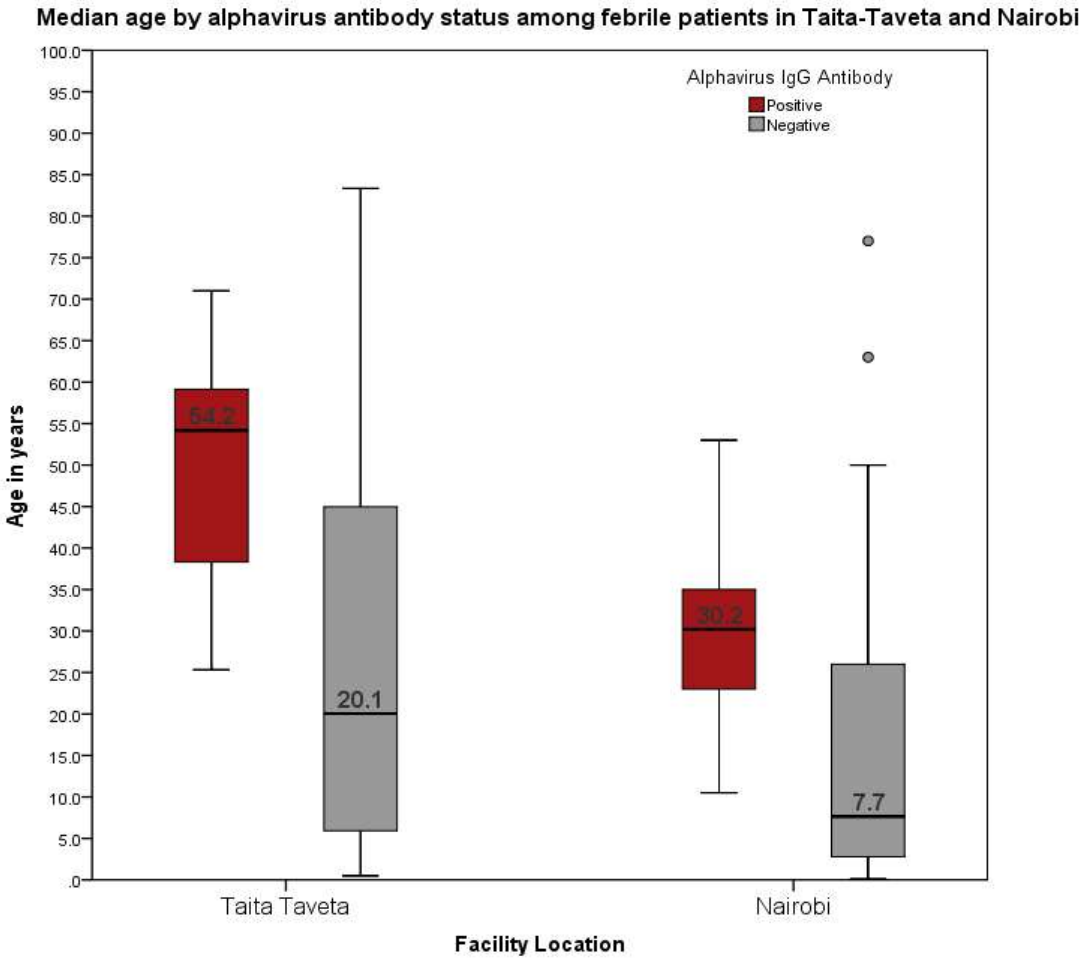

Figure 9. Median age by Alphavirus IgG antibody status and Location

Table 6. A summary of bivariate analysis of potential predictors of exposure to flaviviruses and alphaviruses among febrile patients in Nairobi & Taita-Taveta

Characteristic	Flavivirus IgG Positive (IFA [α])	P value (chi-square test)	Alphavirus IgG Positive (PRNT [β])	P Value (chi-square test)
Age Group				
Children (<18 years)	6/283 (2.1%)	**<0.001**	3/275 (1.1%)	**<0.001**
Adults	44/263 (16.7%)		24/253 (9.5%)	
Gender				
Female	35/286 (12.2%)	**0.01**	15/274 (5.5%)	0.86
Male	16/261 (6.1%)		13/253 (5.1%)	
Facility Location				
Nairobi	7/231 (3.0%)	**<0.001**	21/228 (9.2%)	**<0.001**
Taita-Taveta	44/326 (13.5%)		7/309 (2.3%)	
History of Travel [δ]				
Yes	16/142 (11.3%)	0.29	9/137 (6.6%)	0.35
No	34/410 (8.3%)		18/395 (4.6%)	
Joint Pain				
Yes	33/209 (15.8%)	**<0.001**	17/201 (8.5%)	**0.009**
No	18/348 (5.2%)		11/336 (3.3%)	
Myalgia				
Yes	27/188 (14.4%)	**0.002**	12/180 (6.7%)	0.28
No	24/369 (6.5%0		16/357 (4.5%)	
Headache				
Yes	14/161 (8.7%)	0.81	16/157 (10.2%)	**0.002**
No	37/396 (9.3%)		12/380 (3.2%)	
Cough				
Yes	7/139 (5.0%)	**0.052**	5/136 (3.7%)	0.35
No	44/418 (10.5%)		23/401 (5.7%)	
Contact with goats				
Yes	23/159 (14.5%)	**0.006**	4/148 (2.7%)	**0.11**
No	28/398 (7.0%)		24/389 (6.2%)	
Contact with cattle				
Yes	11/122 (9.0%)	0.95	4/117 (3.4%)	0.32
No	40/435 (9.2%)		24/420 (5.7%)	
Contact with chicken				
Yes	31/233 (13.3%)	**0.004**	6/223 (2.7%)	**0.02**
No	20/324 (6.2%)		22/314 (7.0%)	
Contact with cats				
Yes	14/148 (9.5%)	0.88	12/142 (8.5%)	**0.04**
No	37/409 (9.0%)		16/395 (4.1%)	
Contact with rodents				
Yes	33/344 (9.6%)	0.65	15/331 (4.5%)	0.37
No	18/213 (8.5%)		13/206 (6.3%)	
Contact with bats				
Yes	17/100 (17.0%)	**0.003**	3/91 (3.3%)	0.37
No	34/457 (7.4%)		25/446 (5.6%)	
Any animal contact				
Yes	47/434 (10.8%)	**0.01**	19/419 (4.5%)	**0.18**
No	4/123 (7.4%)		9/118 (7.6%)	

[α] IFA = Immunofluorescence assays; [β] PRNT = Plaque Reduction Neutralization Test
[δ] History of travel out of home county within the last 30 days
The p values <0.2 for variables that were further assessed using logistic regression are in bold

Table 7. Association between travel and risk of flavivirus infection among patients with febrile illness in Kenya

Parameter		Flavivirus IgG IFA	Odds ratio (CI)	P value (FET)
Any history of travel				
	Yes	16/142 (11.3%)	1.4 (0.75 – 2.63)	0.31
	No	34/410 (8.3%)		
Travel to western Kenya				
	Yes	3/65 (4.6%)	0.5 (0.14 – 1.48)	0.25
	No	48/492 (9.8%)		
Travel to coastal Kenya				
	Yes	12/39 (30.8%)	5.4 (2.57 – 11.61)	<0.001
	No	39/518 (7.5%)		
Travel to the coast or western				
	Yes	15/104 (14.4%)	1.9 (1.03 – 3.72)	0.057
	No	36/453 (7.9%)		

Table 8. Association between travel and risk of alphavirus infection among patients with febrile illness in health facilities in Taita-Taveta and Kibera

Parameter		Alphavirus IgG PRNT	Odds ratio (CI)	P value (FET)
Any history of travel				
	Yes	9/137 (6.6%)	1.5 (0.65 – 3.36)	0.37
	No	18/395 (4.6%)		
Travel to western Kenya				
	Yes	7/62 (11.3%)	2.8 (1.12 – 6.77)	0.03
	No	21/475 (4.4%)		
Travel to coastal Kenya				
	Yes	1/38 (2.6%)	0.5 (0.06 – 3.58)	0.71
	No	27/499 (5.4%)		
Travel to the coast or western				
	Yes	8/100 (8%)	1.8 (0.775 – 4.24)	0.21
	No	20/437 (4.6%)		

In Taita-Taveta, over two-thirds of patients who reported a history of recent travel (37/54, 69%) had traveled to the coast (mainly Mombasa County). In Kibera, 56/88 (64%) with a history of travel had traveled to western Kenya (mainly Kisumu, Kakamega and Siaya Counties). Kibera residents were more likely to travel to western Kenya than Taita-Taveta residents, who were more likely to travel to coastal Kenya

Overall, a recent history of travel to any part of Kenya was not significantly associated with arbovirus infection but travel specifically to western or coastal Kenya was associated with risk of infection. Travel to coastal Kenya was associated with flavivirus IgG seropositivity; odds ratio (OR) = 5.5 (Confidence interval (CI) = 2.57 – 11.61); χ^2,

p=0.001 (FET) (Table 7). Travel to western Kenya was associated with alphavirus IgG seropositivity; OR = 2.8 (CI 1.12 – 6.77); χ^2, p = 0.03 (FET) (Table 8).

Table 9. Multivariable Analysis of Factors Associated with Dengue IgG Seropositivity using Simultaneous Binary Logistic Regression.

Variable		DENV IgG Positive, n (%)	Odds Ratio (95% CI)	*p* value
Gender				
	Female	35/281 (12.5%)	1.86 (0.913 - 3.788)	0.088
	Male	15/255 (5.9%)	Reference	
Age Group				
	Adult	44/260 (16.9%)	7.34 (2.687 - 20.266)	**<0.001**
	Child	6/276 (2.2%)	Reference	
Facility Location				
	Taita-Taveta	43/312 (13.8%)	3.43 (1.198 - 9.808)	**0.022**
	Nairobi	7/224 (3.1%)	Reference	
Travel to Coast				
	Yes	12/38 (31.6%)	5.22 (2.103 - 12.979)	**<0.001**
	No	38/498 (7.6%)	Reference	
Joint Pain				
	Yes	32/203 (15.8%)	1.66 (0.768 - 3.566)	0.198
	No	18/333 (5.4%)	Reference	
Myalgia				
	Yes	26/182 (14.3%)	0.82 (0.370 - 1.804)	0.616
	No	24/354 (6.8%)	Reference	
Cough				
	Yes	7/133 (5.3%)	1.50 (0.540 – 4.148)	0.438
	No	43/403 (10.75)	Reference	
Contact with goats				
	Yes	22/153 (14.4%)	1.03 (0.498 - 2.121)	0.942
	No	28/383 (7.3%)	Reference	
Contact with chicken				
	Yes	30/227 (13.2%)	0.99 (0.453 - 2.200)	0.996
	No	20/309 (6.5%)	Reference	
Encountered bats				
	Yes	17/97 (17.5%)	1.14 (0.509 - 2.561)	0.749
	No	33/439 (7.5%)	Reference	
Contact with Any Animal				
	Yes	46/422 (10.9%)	1.32 (0.375 - 4.622)	0.667
	No	4/114 (3.5%)	Reference	

Table 10. Multivariable Analysis of Factors Associated with Alphavirus IgG (PRNT) Seropositivity using Simultaneous Binary Logistic Regression.

Variable	Alphavirus IgG Positive (PRNT), n (%)	Odds Ratio (95% CI)	*p* value
Facility Location			
Nairobi	21/221 (9.5%)	4.99 (1.334 – 18.675)	**0.017**
Taita-Taveta	6/297 (2.0%)	Reference	
Age Group			
Adult	24/250 (9.6%)	10.86 (2.808 – 41.973)	**0.001**
Child	3/268 (1.1%)	Reference	
Travel to western/Nyanza			
Yes	6/60 (10.0%)	1.02 (0.340 – 3.035)	0.977
No	21/458 (4.6%)	Reference	
Joint Pain			
Yes	16/196 (8.2%)	1.45 (0.584 – 3.572)	0.425
No	11/322 (3.4%)	Reference	
Headache			
Yes	16/153 (10.5%)	0.84 (0.299 – 2.369)	0.744
No	11/365 (3.0%)	Reference	
Contact with goats			
Yes	4/143 (2.8%)	0.86 (0.213 – 3.455)	0.829
No	23/375 (6.1%)	Reference	
Contact with Cats			
Yes	12/139 (8.6%)	1.69 (0.576 – 4.953)	0.340
No	15/379 (4.0%)	Reference	
Contact with chicken			
Yes	6/218 (2.8%)	0.64 (0.182 – 2.270)	0.493
No	21/300 (7.0%)	Reference	
Contact with Any Animal			
Yes	19/408 (4.7%)	0.64 (0.190 – 2.178)	0.479
No	8/110 (7.3%)	Reference	

Table 11. Intra-county variation in prevalence of flavivirus and alphavirus IgG antibodies.

Location	Health Facility	Dengue IgG Positive	p value (Chi-square)	Alphavirus IgG Positive	p value (Chi-square)
Taita-Taveta	Wundanyi	14/155 (9%)		0/148 (0%)	
	Mwatate	24/125 (19.2%)		5/116 (4.3%)	
	Voi	6/46 (13%)	0.046	2/45 (4.4%)	0.037
Nairobi	Ushirika	5/111 (4.5%)		19/109 (17.4%)	
	Kibera South	1/39 (2.6%)		2/38 (5.3%)	
	Kibera Community	1/81 (1.2%)	0.419	0/81 (0%)	<0.001

As shown in Table 11, there was significant intra-county variation in both Nairobi and Taita-Taveta for alphavirus IgG seroprevalence. Dengue IgG seroprevalence showed significant intra-county variation in Taita-Taveta but not in Nairobi.

7.3 PCR Results

Flavivirus RNA detection

All 557 samples were tested for RNA using pan-flavivirus PCR. Five samples (1%) were positive. Sanger sequencing of the positive amplicons identified all five as dengue type 2 virus. All five DENV RT-PCR-positive samples were collected in Taita Taveta in May (four) and June (one) 2016 (*Table 12*).

Table 12. Characteristics Dengue virus PCR-positive samples

Sample ID	DENV PCR	DENV IgG IFA	DENV IgG Titer	DENV IgM IFA	DENV IgM ELISA	DENV NS1 ag ELISA	Collection Date (Duration of symptoms)	Sex	Age (Years)	Travel History
85A	+ve	+ve	640	+ve	Neg	+ve	10.05.2016 (2 days)	Female	16	Yes
222	+ve	+ve	40	-ve	NT	+ve	04.05.2016 (2 days)	Male	17	NR
509	+ve	-ve	NT	-ve	NT	NT	24.06.2016 (3 days)	Male	46	No
76	+ve	-ve	NT	-ve	NT	NT	16.05.2016 (2 days)	Female	3	Yes
96	+ve	-ve	NT	-ve	NT	+ve	04.05.2016 (2 days)	Female	19	Yes

Key:
DENV = Dengue virus; IgG = Immunoglobulin G; IgM= Immunoglobulin M; Ag= antigen;
+ve = Positive; -ve = Negative; NT = Not tested; NR = Not recorded

Dengue virus isolation

Three dengue PCR-positive samples were cultured on Vero E6 cells and C6/36 *Aedes albopictus* cell line. No growth was observed on Vero E6 cells; two of the samples grew on C6/36 cells and showed cytopathic effect (rounding and detachment) on days 20 and 24 respectively.

Alphavirus PCR results

All 557 samples were tested for pan-alphavirus RNA using nested conventional PCR and any positive samples were confirmed through Sanger sequencing. Only one sample (0.2%) tested positive on PCR and was confirmed to be Chikungunya virus (Figure 10). The sample had high CHIKV IgG antibody titers (640) on IFA and no CHIKV IgM antibodies. We did not have enough sample volume to run PRNT assay.

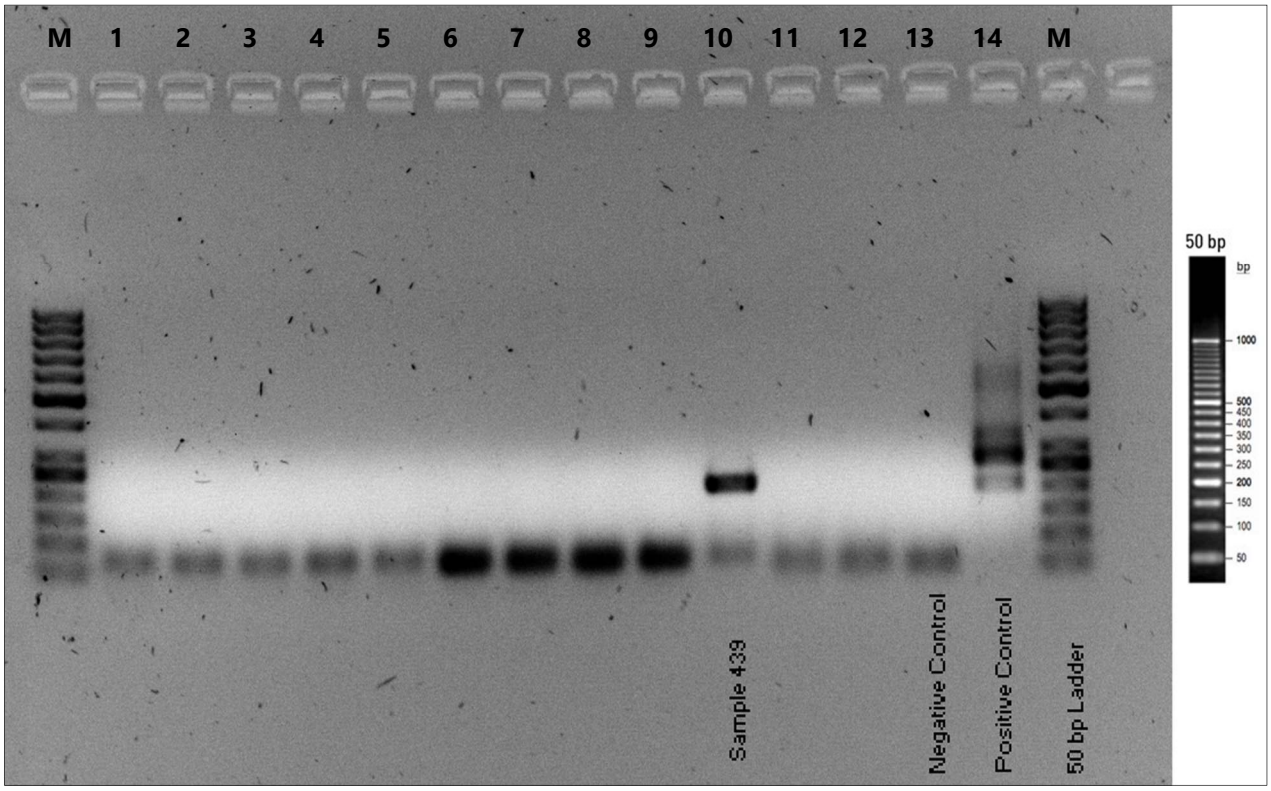

Figure 10. An image of an electrophoresis gel showing a positive sample (lane 10) on pan-alphavirus PCR

Chikungunya Virus isolation

Pan-alphavirus PCR-positive sample was cultured in Vero E6 and C6/36 cell lines but no growth was observed over a period of three weeks.

Table 13. Characteristics of samples that tested positive for Alphavirus antibodies on PRNT

Sample ID	CHIKV IgG IFA	CHIKV IgG IFA Titers	CHIKV IgM ELISA	CHIKV PRNT	CHIKV PRNT Titers	ONNV PRNT	ONNV PRNT titers	SINV PRNT Titers	SINV PRNT	CHIKV RNA PCR	Sex	Age (years)	Travel History	Fever duration (days)	Location
1061	Pos	Not Done	Not Done	Pos	80	Pos	80	40	Neg	Neg	Female	34	No	1	Kibera
785	Pos	40	Pos	Pos	200	Pos	320	40	Neg	Neg	Female	16	No	4	Kibera
795	Pos	160	Pos	Pos	240	Pos	160	40	Neg	Neg	Male	11	Yes	3	Kibera
408	Pos	2560	Neg	Pos	240	Pos	320	40	Neg	Neg	Female	50	No	1	Taita-Taveta
477	Pos	160	Neg	Pos	60	Pos	80	40	Neg	Neg	Male	58	No	4	Taita-Taveta
485	Pos	640	Neg	Pos	100	Pos	160	40	Neg	Neg	Female	25	No	3	Taita-Taveta
846	Pos	40	Neg	Pos	60	Pos	80	40	Neg	Neg	Male	25	Yes	3	Kibera
1062	Pos	Not Done	Neg	Pos	320	Pos	320	40	Neg	Neg	Female	23	No	2	Kibera
1078	Pos	160	Neg	Pos	240	Pos	320	40	Neg	Neg	Male	32	No		Kibera
1089	Pos		Neg	Pos	240	Pos	320	40	Neg	Neg	Female	29	No	2	Kibera
732	Pos	40	Neg	Pos	240	Pos	320	40	Neg	Neg	Female	24	No	4	Kibera
780	Pos	160	Neg	Pos	160	Pos	240	40	Neg	Neg	Female	40	Yes	2	Kibera
897	Pos	160	Neg	Pos	200	Pos	320	40	Neg	Neg	Male	22		7	Kibera
770	Pos	40	Neg	Pos	240	Neg	80	40	Neg	Neg	Male	35	No	1	Kibera
277	Neg	Not Done	**Pos**	Pos	320	Neg	40	40	Neg	Neg	Female	19	Yes	1	Taita-Taveta
767	Pos	40	**Pos**	Neg	60	Pos	320	40	Neg	Neg	Male	53	Yes	2	Kibera
243	Pos	640	Neg	Neg	40	Pos	320	40	Neg	Neg	Female		Yes	2	Taita-Taveta
252	Pos	640	Neg	Neg	60	Pos	320	40	Neg	Neg	Male	38	Yes	5	Taita-Taveta
416B	Pos	160	Neg	Neg	60	Pos	240	40	Neg	Neg	Male	59	No	1	Taita-Taveta
506	Pos	160	Neg	Neg	60	Pos	120	40	Neg	Neg	Female	71	No	2	Taita-Taveta
749	Pos	40	Neg	Neg	100	Pos	240	40	Neg	Neg	Female	30	No	3	Kibera
703	Pos	40	Neg	Neg	40	Pos	160	40	Neg	Neg	Female	22	No	2	Kibera
748	Pos	40	Neg	Neg	80	Pos	320	40	Neg	Neg	Male	43	Yes	2	Kibera
757	Pos	40	Neg	Neg	120	Pos	320	40	Neg	Neg	Female	30	No	2	Kibera
763	Pos	40	Neg	Neg	80	Pos	320	40	Neg	Neg	Male	17	No	2	Kibera
768	Pos	10	Neg	Neg	40	Pos	80	40	Neg	Neg	Female	48	No	1	Kibera
774	Pos	10	Neg	Neg	40	Pos	240	40	Neg	Neg	Male	40	Yes	4	Kibera
777	Pos	40	Neg	Neg	160	Pos	320	40	Neg	Neg	Female	32	No	2	Kibera
786	Pos	10	Neg	Neg	40	Pos	240	40	Neg	Neg	Male	34	Yes	1	Kibera
703	Neg	Not Done	Neg	Neg	40	Pos	160	40	Neg	Neg	Female	26	Yes	2	Kibera
Summary	Pos=28, Neg=2		Pos=4, Neg=25	Pos=15, Neg=15		Pos=15 Neg=2			Yes=0 No=30	Pos=0 Neg=30	Female=17 Male=13	Mean=34 years	Yes=11 No=18	Mean= 2.4 days	Taita Taveta = 8, Kibera = 22

KEY:
PRNT: Plaque reduction neutralization test; CHIKV: Chikungunya virus, ONNV: Onyong-nyong virus; SINV: Sindbis virus; IFA: Immunofluorescence assay; Pos: Positive, Neg: Negative

7.4 High-throughput Sequencing

We used Illumina MiSeq™ next-generation sequencing to analyze five DENV RT-PCR-positive samples, and one CHIKV RT-PCR-positive sample. Out of the five samples that had tested positive for DENV on PCR, we recovered 3 complete coding sequences via NGS which had a length of ~10,200 bp. One of these samples was retrieved from a viral culture on C6/36 *Ae. albopictus* cell line (Accession number MK473384.1) while the other two were retrieved directly from serum samples (MK473386.1, MK473385.1). We also recovered one envelope gene sequence which was 1646 bp (MK473383.1).

We did not retrieve any CHIKV sequence from the one sample that had tested CHIKV-positive on PCR or the 110 pooled samples.

We undertook unbiased, target-independent sequencing of twenty-two pools comprising 110 samples (five samples for each pool) that had tested negative for CHIKV and DENV on PCR. Out of these, we recovered coding sequences from at least 16 unique families of viruses at a varying frequency (Table 14). Three of these families were of clinical significance – *Flaviviridae Picornaviridae* and *Retroviridae*. These were pegivirus A and pegivirus C (*Flaviviridae*), HIV-1 (*Retroviridae*) and enterovirus A (*Picornaviridae*) (Table 15). Other viruses that we detected were in the families *Anelloviridae, Circoviridae, Mimiviridae, Phycodnaviridae, Reoviridae, Cruciviridae, Parvoviridae* and other unclassified viruses. The unclassified viruses were alphasatellites, archaeal viruses, *Autographiviridae*, cyclovirus, DNA satellites, kryptosalinivirus, lavidavirus, and podovirus among others. (Table *16*)

7.5 Bioinformatic analysis for Dengue virus

All sequences were dengue virus type 2. For comparison, we retrieved complete coding sequences from NCBI Genbank from Kenya. The majority (35/41 or 85%) of these were DENV type 2 virus coding sequences, four were DENV type 1 and one each for DENV-3 and DENV-4. We included 33 DENV-2 complete coding sequences from Genbank in our phylogenetic analysis. The DENV sequences retrieved in this study formed a monophyletic cluster of cosmopolitan dengue type 2 viruses and were closely related to other DENV-2 viruses responsible for infections in Malindi, Kilifi and Mombasa between 2014 and 2017 (Figure 11).

All DENV-2 complete-coding sequences from Kenya that are available in the NCBI GenBank form a single cluster with the sequences obtained in this study. Another strain, T90-S83 MH822949 from New Delhi, India, 2014 formed an outgroup for the Kenyan dengue sequences (Masika et al., 2020). The Kenyan DENV-2 strains also clustered with another strain (RGCB880) reported in India in 2010. Analysis of *env* gene showed that strain c96 (MK473384.1) which was isolated from Wundanyi (in Taita-Taveta county) in 2016 was closely related to a strain isolated from someone who traveled to Taiwan from Kenya in March 2016. Additionally, the strain 509 (MK473383.1) isolated from Mwatate, 85A (MK473385.1) from Wundanyi and 222 (MK473386.1) from Voi in 2016 were similar to strains that were isolated in Malindi, in the Kenyan coast, in 2017. Through molecular clock analysis, we dated the most recent common ancestor (tMRCA) of the Kenyan dengue virus strains isolated in this study, in the year 2012; a year before a dengue outbreak that took place in Mombasa in 2013 (Masika et al., 2020) (Figure 12).

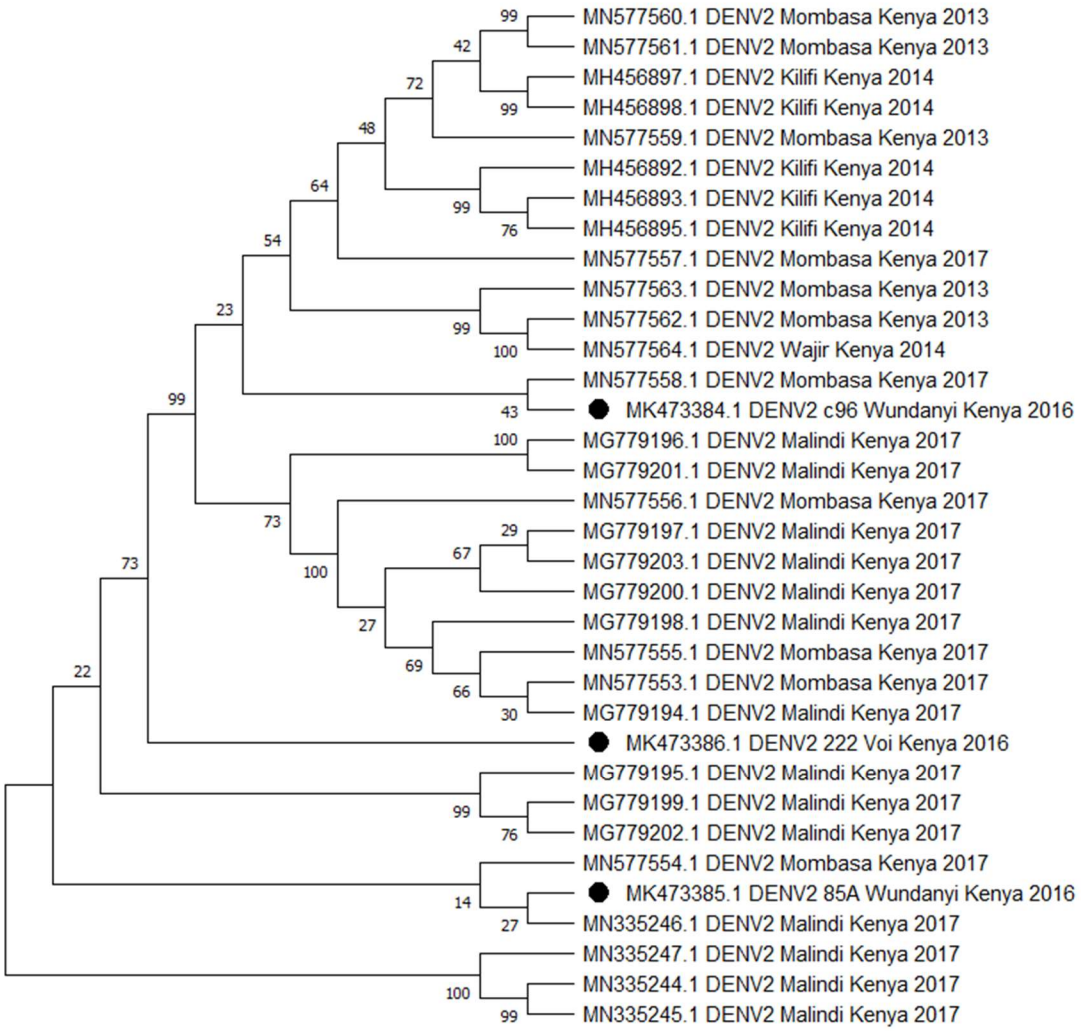

Figure 11. Phylogenetic tree of complete coding sequences of Dengue virus type 2 from Kenya

Evolutionary analysis of 34 Kenyan DENV-2 complete coding sequences using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993) in MEGA X software (Kumar et al., 2018).

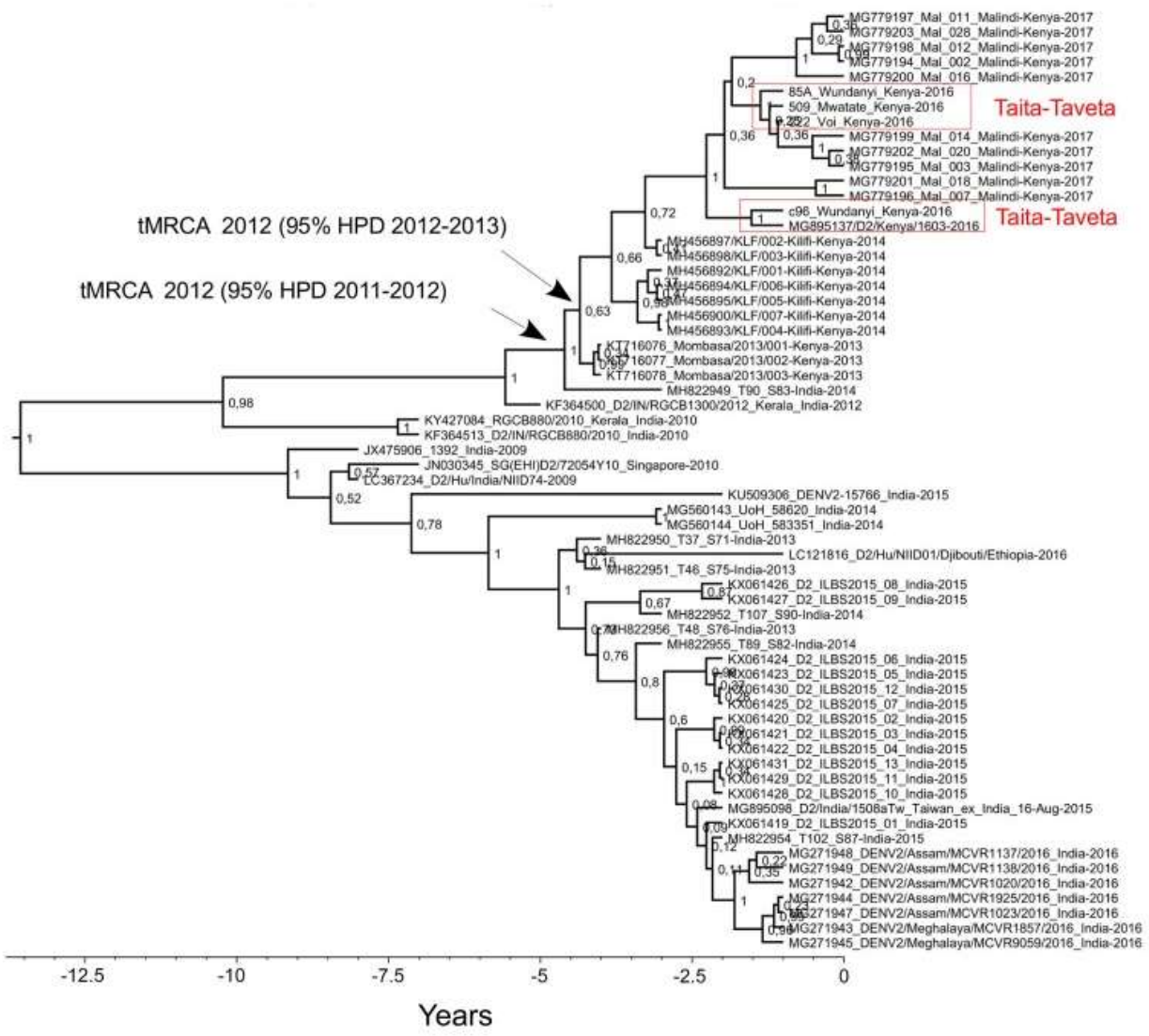

Figure 12. Phylogenetic trees of Dengue virus 2 envelope gene sequences

Phylogenetic tree of dengue virus 2 envelope gene sequences. The nodes of the Most Recent Common Ancestors (tMRCA) of the Kenyan sequences obtained in this study are shown with arrows.

7.6 Bioinformatic Analysis of Sequences from Pooled Samples

Using an in-house bioinformatics pipeline for assembly and taxonomic profiling of virus sequences, we identified 15 virus families in 22 pools of samples each. The most commonly detected families were *Anelloviridae* (in 20 pools), *Circoviridae* (19/22), *Mimiviridae* (13/22), *Phycodnaviridae* (13/22) and *Reoviridae* (10/22) (*Table 14*).

We also detected partial coding sequences of human pegiviruses (*Flaviviridae*) in five pools of samples. Pegivirus coding sequences were short, most being <1000bp and the longest five ranging from ~1000 to ~3000 bp. We selected two representative coding sequences (2954 and 1396 bp respectively) for phylogenetic analysis. Both sequences had a similarity of 85 – 93% to pegivirus sequences from various parts of Africa, Far East and Americas. (Figure 13)

We retrieved one complete coding sequence of Enterovirus A from a pool of five samples collected in Taita-Taveta in 2016. The sequence was significantly dissimilar from other enterovirus sequences in the NCBI Genbank. The closest match (93.7%) was a Coxsackie A16 virus sequence isolated in Kilifi, Kenya in 2015 (Phan et al., 2022). The Taita-Kenya Enterovirus A also had a similarity of 85 – 90% with a set of 38 complete enterovirus sequences isolated in China, Taiwan, France and Australia between 2008 and 2018, these were included in the phylogenetic analysis (Figure 14). Compared with the other 39 sequences, the Taita-Kenya strain has multiple unique insertions and deletions with the largest insertion being 78 bp long.

A short fragment of HIV-1 sequence (333 bp) was recovered from one pool of samples. This was a fragment of the HIV-1 clade A *vif* gene and its closest match was a complete coding sequence (AY322191.1) isolated from a Kenyan patient in 1997 (97% similarity). All the top ten matches were isolated from Kenyan patients except one from Tanzania in 2001 (AF361872.1).

Table 14. Virus families detected in pooled samples from Kenyan febrile patients using high-throughput sequencing

	Pool ID																					
Virus Family	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**	**P10**	**P11**	**P12**	**P13**	**P14**	**P15**	**P16**	**P17**	**P18**	**P19**	**P20**	**P21**	**P22**	**Frequency**
1. *Anelloviridae*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	20 (91%)
2. *Circoviridae*	Yes	Yes	Yes	Yes	No	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	19 (86%)
3. *Mimiviridae*	Yes	No	Yes	Yes	No	No	Yes	No	No	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	No	Yes	13 (59%)
4. *Phycodnaviridae*	Yes	Yes	Yes	Yes	No	No	No	No	No	Yes	No	Yes	Yes	Yes	No	Yes	Yes	No	Yes	No	Yes	Yes	13 (59%)
5. *Reoviridae*	Yes	No	Yes	Yes	No	No	No	No	No	Yes	No	Yes	Yes	Yes	No	No	No	Yes	No	Yes	Yes	No	10 (45%)
6. *Cruciviridae*	Yes	No	No	No	No	No	No	No	No	No	No	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes	Yes	No	8 (36%)
7. *Parvoviridae*	Yes	No	Yes	No	No	No	No	No	No	No	No	No	No	Yes	No	Yes	No	Yes	No	Yes	Yes	No	7 (32%)
8. *Flaviviridae*	Yes	No	No	Yes	No	No	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No	Yes	5 (23%)
9. *Pithoviridae*	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	Yes	No	No	2 (9%)
10. *Retroviridae*	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No	1 (5%)
11. *Baculoviridae*	No	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No	No	1 (5%)
12. *Iridoviridae*	No	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	1 (5%)
13. *Nanoviridae*	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No	1 (5%)
14. *Nudiviridae*	No	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	1 (5%)
15. *Picornaviridae*	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No	1 (5%)
16. Unclassified	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	22 (100%)

Table 15. List and frequency of potentially clinically-relevant viruses in sample pools of patients with acute febrile illness in Kenya

Family	Genus	Species	Frequency (n = 22 pools)
Anelloviridae	*Alphatorquevirus*	Torque teno virus	15 (68%)
	Betatorquevirus	Torque teno mini virus	9 (41%)
		TTV-like mini virus	12 (55%)
	Gammatorquevirus	Torque teno midi virus	9 (41%)
	Anellovirus	Chimpanzee anellovirus	3 (14%)
		Gorilla anellovirus	5 (23%)
		Opossum torque teno virus	3 (14%)
		Small anellovirus	1 (5%)
		Torque teno virus	17 (77%)
Flaviviridae	*Pegivirus*	Pegivirus A	2 (9%)
		Pegivirus C	5 (23%)
Parvoviridae	*Dependoparvovirus*	Adeno-associated virus	1 (5%)
	Parvovirus	Parvovirus NIH-CQV	7 (32%)
Picornaviridae	*Enterovirus*	Enterovirus A	1 (5%)
Reoviridae	*Seadornavirus*	Kadipiro virus	10 (45%)
Retroviridae	*Lentivirus*	Human immunodeficiency virus 1	2 (9%)

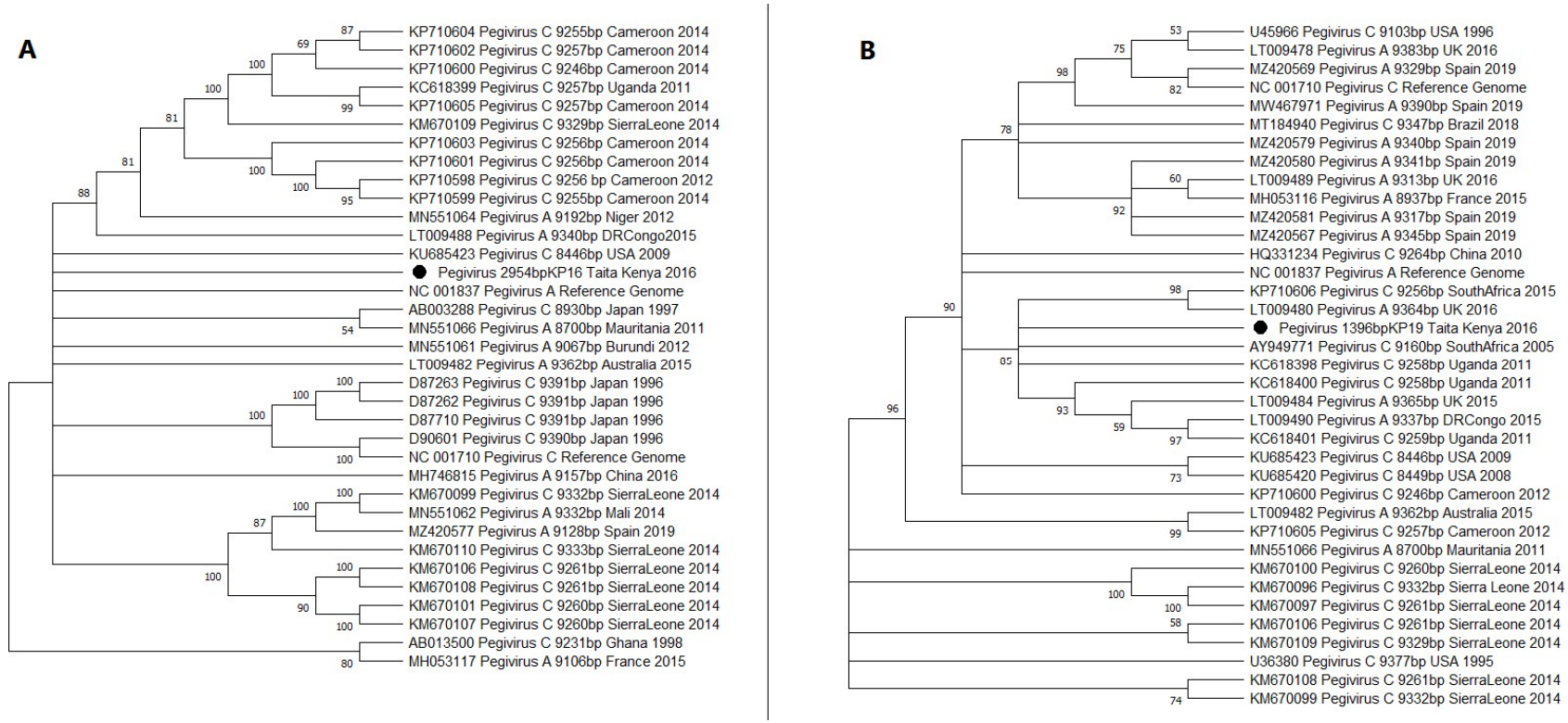

Figure 13. Phylogenetic analysis of Pegivirus sequences isolated from febrile patients in Kenya

We analyzed two fragments of pegivirus sequences A and B. A (2954 bp) had 90-93% similarity with 34 pegivirus sequences while B (1396 bp) had 85 – 90 % similarity with 36 other pegivirus sequences. The closest matches for both fragments were mainly from Africa and Asia with a few matches from Europe, Australia and the United States of America. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) *on MEGA-X Software (Kumar et al., 2018)*.

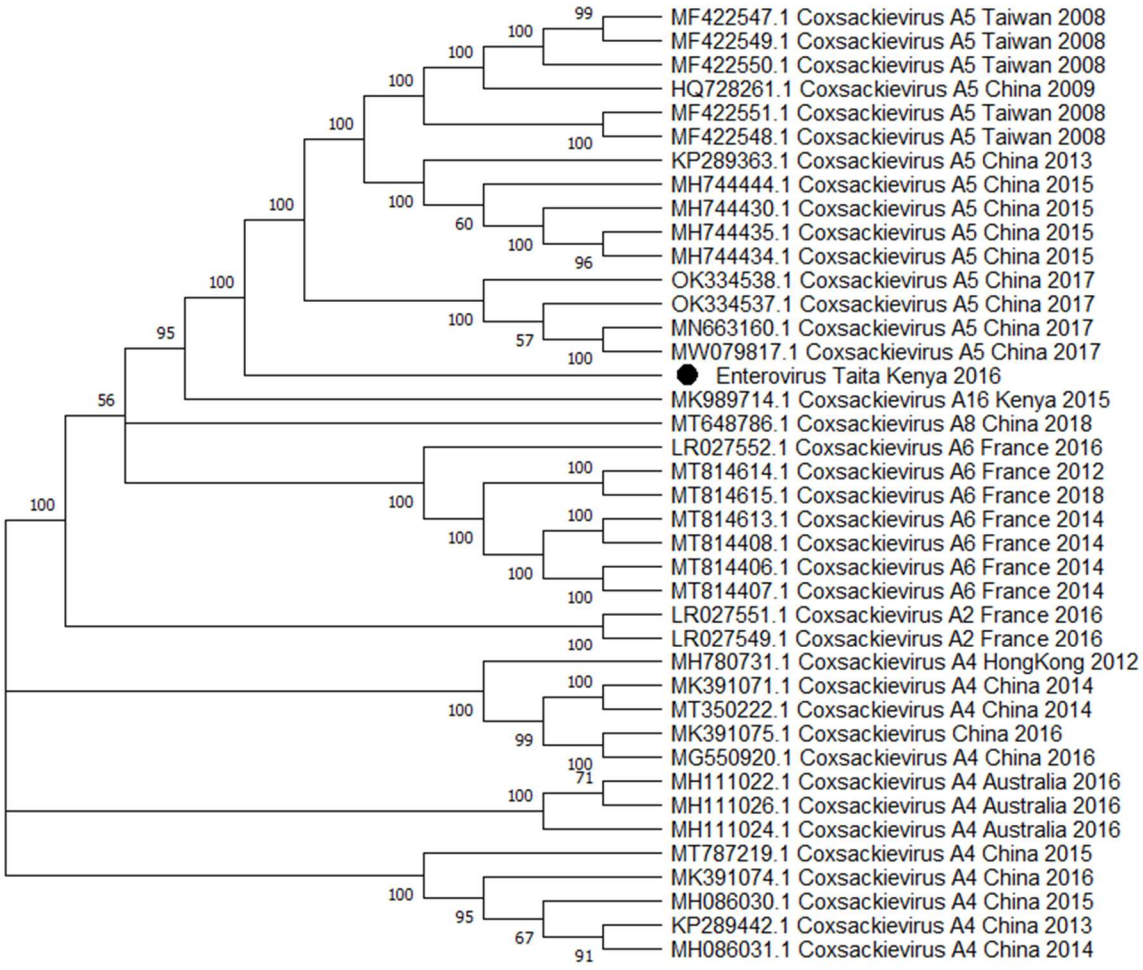

Figure 14: Phylogenetic analysis of Enterovirus complete coding sequences

Evolutionary analysis of 40 Enterovirus complete coding sequences using the Neighbor-Joining method (Saitou and Nei, 1987) on MEGA-X Software (Kumar et al., 2018).

Table 16. List and frequency of other viruses identified through next-generation sequencing in sample pools of patients with acute febrile illness in Kenya

Family	Genus	Species	Frequency (n=22 pools)
Autographiviridae	*Sednavirus*	Cyanophage KBS-P-1A	1 (5%)
		Cyanophage KBS-S-2A	1 (5%)
Baculoviridae	*Alphabaculovirus*	Choristoneura fumiferana DEF multiple nucleopolyhedrovirus	1 (5%)
Circoviridae	*Circovirus*	Anguilla circovirus	3 (14%)
		Avon-Heathcote Estuary-associated circular virus	7 (32%)
		Bat circovirus	8 (36%)
		Calanoida copepod-associated circular virus	5 (23%)
		Circoviridae-10 LDMD-2013	1 (5%)
		Circoviridae-13 LDMD-2013	1 (5%)
		Circoviridae LDMD-2013b	2 (9%)
		Circovirus-like genome DHCV	14 (64%)
		Circovirus-like genome RW-E	3 (14%)
		Circovirus-like NI/2007	2 (9%)
		Dromedary stool-associated circular ssDNA virus	1 (5%)
		Gammarus amphipod-associated circular virus	14 (64%)
		Hermit crab-associated circular virus	7 (32%)
		Mytilus clam-associated circular virus	1 (5%)
		Mississippi grass shrimp-associated circular virus	1 (5%)
		Primnoa pacifica coral-associated circular virus	1 (5%)
	Cyclovirus	Dragonfly associated cyclovirus 4	4 (18%)
		Dragonfly larvae-associated circular virus	4 (18%)
		Dragonfly-associated microphage 1	2 (9%)
Cruciviridae	*Crucivirus*	Cruciviridae sp.	8 (36%)
Iridoviridae	*Ranavirus*	Frog virus 3	1 (5%)
Lavidaviridae	*Lavidavirus*	Qinghai Lake virophage	2 (9%)
Mimiviridae	*Catovirus*	Catovirus CTV1	1 (5%)
	Hokovirus	Hokovirus HKV1	9 (41%)
	Klosneuvirus	Bodo saltans virus	1 (5%)
		Klosneuvirus KNV1	5 (23%)
	Mimivirus	Acanthamoeba polyphaga mimivirus	1 (5%)
		Hirudovirus strain Sangsue	1 (5%)
		Megaviridae environmental sample	1 (5%)
		Megavirus chiliensis	2 (9%)
		Mimivirus AB-566-O17	1 (5%)
Myoviridae	*Senquatrovirus*	SEN virus	5 (23%)
Nudiviridae	*Deltanudivirus*	Tipula oleracea nudivirus	1 (5%)
Parvoviridae	*Ambidensovirus*	Blattodean ambidensovirus 2	1 (5%)
		Densovirus SC116	1 (5%)
	Dependoparvovirus	Corn snake parvovirus	1 (5%)
Pithoviridae	*Pithovirus*	Pithovirus sibericum	2 (9%)
Phycodnaviridae	*Chlorovirus*	Acanthocystis turfacea Chlorella virus	7 (32%)
		Paramecium bursaria Chlorella virus	4 (18%)
	Phycodnavirus	Aureococcus anophagefferens virus	1 (5%)

		Chrysochromulina ericina virus	1 (5%)
		Organic Lake phycodnavirus 1	1 (5%)
	Prasinovirus	Ostreococcus lucimarinus virus 7	8 (36%)
		Ostreococcus tauri virus	2 (9%)
		Yellowstone lake phycodnavirus 1	2 (9%)
	Prymnesiovirus	Prymnesium parvum DNA virus BW1	1 (5%)
	Raphidovirus	Heterosigma akashiwo virus 01	4 (18%)
Siphoviridae	*Kryptosalinivirus*	Salinibacter virus M8CRM-1	1 (5%)
Satellites	*Alphasatellites*	Papaya leaf curl alphasatellite	2 (9%)
	Clostunsatellite	Sophora yellow stunt virus	1 (5%)
	DNA satellites	Sewage-associated circular DNA molecule	13 (59%)
		Sewage-associated circular DNA virus	20 (91%)
Unclassified	Unclassified Virus	Beihai picorna-like virus 70	2 (9%)
		Carascovirus SF1	1 (5%)
		Changjiang tombus-like virus 4	1 (5%)
		Changjiang tombus-like virus 6	1 (5%)
		Diporeia sp. associated circular virus	2 (9%)
		Diporeia-associated CRESS-DNA virus LH481	1 (5%)
		Hubei tombus-like virus 7	1 (5%)
		Hudisavirus sp.	2 (9%)
		Idotea virus IWaV278	1 (5%)
		Lake Sarah-associated circular virus	100%
		Mollivirus sibericum	5%
		Odonata-associated circular virus-13	2 (9%)
		Pacific flying fox associated multicomponent virus	1 (5%)
		Rodent stool-associated circular genome virus	1 (5%)
		Sanxia picorna-like virus 7	1 (5%)
		Shahe tombus-like virus 1	1 (5%)
		Skeletonema virus LDF-2015a	1 (5%)
		Unidentified circular ssDNA virus	14%
		Unknown phycodnavirus	1 (5%)
		Virus PK2111	2 (9%)
		Virus Rctr	45%
		Wenzhou tombus-like virus 1	2 (9%)
		Nepavirus	1 (5%)
		Uncultured marine virus	16 (73%)
		Uncultured virus	21 (95%)
		ANMV-1 virus	2 (9%)

8.0 DISCUSSION

Emergence and re-emergence of arboviral infections is a significant threat to public health. Arboviruses present with a non-specific acute febrile illness which makes identification of specific etiological agents difficult especially in resource-limited settings. In this study, we assessed the prevalence of arbovirus infection and exposure in febrile patients in both rural and urban settings in Kenya. We found the prevalence of acute flavivirus infection and previous exposure to be 1.4% and 8% respectively. Acute alphavirus infection and previous exposure was 0.2% and 9% respectively. None of the patients had acute co-infection with both virus groups. We also recovered three DENV complete coding sequences which formed a monophyletic cluster of cosmopolitan dengue type 2 viruses. The sequences were closely related to other DENV-2 viruses responsible for infections in coastal Kenya between 2014 and 2017 as well as sequences isolated in India between 2010 and 2016. Through unbiased high-throughput sequencing, we also detected human pegiviruses, HIV and enterovirus A in pooled blood samples which are of clinical importance.

We utilized serological, PCR and sequencing techniques which allowed us to screen for a wide range of emerging viral infections. Serological assays such as ELISA and IFA for arboviruses tend to have cross-reactivity within specific virus families. Although this reduces the specificity of the assays within the genus, we leveraged the cross-reactivity to detect a broader range of pathogens, then used neutralization assays for speciation where the sample was sufficient. Additionally, rather than using virus-specific primers, we used degenerate primers targeting entire arbovirus groups (alphavirus and flavivirus) which made it possible to screen for more viruses simultaneously. To screen for other viral pathogens, including novel viruses, we used target-independent, high-throughput Illumina MiSeq™ sequencing. This enabled detection of some viruses which could not have been identified using specific immunoassays or group-consensus PCR.

In this study, we found a low prevalence of acute flavivirus infection in febrile patients (1.4%) with the prevalence being four times higher in rural Taita (1.5%) than the urban Kibera in Nairobi (0.4%). The prevalence of CHIKV IgM was moderately high at 6% with

both Taita and Nairobi having similar numbers at 6% and 7% respectively, but only 0.2% of this was confirmed by PRNT making further analysis impossible.

This prevalence of acute arbovirus infection is lower in the study areas than what has been reported in similar studies conducted in coastal and western Kenya which are endemic for arboviruses and other vector-borne infections such as malaria. A study on adults presenting with fever at various hospitals in coastal Kenya in 2014-2015 reported a prevalence of acute dengue (on PCR) of 8.8% (Ngoi et al., 2016a). Another study on 868 samples collected in Nairobi, northern (Mandera and Wajir) and coastal (Mombasa, Malindi and Lamu) Kenya from 2011 to 2014 during an outbreak reported an acute dengue infection of 46.5%. All cases reported by the study in Nairobi were associated with travel to the two other areas (Konongoi et al., 2016).

Although the two study areas have not had any reported arbovirus outbreaks in the past, exposure to dengue in a rural population (Taita –Taveta) was four times higher than in an urban population (Nairobi). This could be because Taita-Taveta is closer to coastal areas where flaviviruses are endemic and regularly cause outbreaks (LaBeaud et al., 2015, Vu et al., 2017a). On the other hand, previous exposure to alphaviruses was nearly double in Nairobi (12%) as compared to Taita-Taveta (7%). This suggests that the transmission pattern for alphaviruses in Kenya is different from that of flaviviruses, even though both tend to be co-endemic in parts of the country such as the coastal strip. Previous studies reported a higher seroprevalence of Onyong-nyong in western Kenya than at the coast (Inziani et al., 2020, Waggoner et al., 2017). A 2019 study on the seroprevalence of arbovirus antibodies in children in western Kenya (Teso) reported a seroprevalence of 5.6% and 1.4% for Chikungunya and dengue respectively (Inziani et al., 2020). A household-based study in western Kenya conducted from 2010 to 2012 reported a seroprevalence of 66.9% for alphaviruses and 1.6 for flaviviruses (Grossi-Soyster et al., 2017). Population studies conducted at about the same time (2009) in coastal Kenya reported a seroprevalence of 48% for flaviviruses and 22% for alphaviruses (LaBeaud et al., 2015, Vu et al., 2017a). Another study on pools of Aedes mosquitoes collected in western and coastal Kenya in 2014-2016 reported a prevalence of 5.9% and 0.4% for chikungunya and dengue viruses respectively. All the dengue-positive pools were detected in samples from the coast and none from western Kenya

while the rate of infection with chikungunya was similar in both places (Heath et al., 2020). These studies corroborate our findings that the risk of infection with dengue is lower in western than in coastal Kenya while the risk of alphavirus infection is higher in western than in the coast region. Kibera in Nairobi has more social and travel links with western than coastal Kenya, which may explain the higher prevalence of alphavirus prevalence in Kibera.

We report that arboviruses are an important cause of febrile illness even during inter-epidemic periods, in both rural and urban areas in Kenya as reported in previous studies (Grossi-Soyster et al., 2017) and that exposure to arboviral infections increases with age (LaBeaud et al., 2015, Inziani et al., 2020). The fact that children under five had arbovirus antibodies suggests recent outbreaks or endemicity within the study population. Such arboviruses that are circulating in the population portend a risk of causing outbreaks from time to time as has happened in the recent past. This also puts other countries at risk due to global connectivity and travel. For instance, during a yellow fever outbreak in Angola in 2016, workers returning to China from Angola were diagnosed with yellow fever placing billions of people in Asia at risk of infection (Wasserman et al., 2016).

All the dengue viruses we detected in the study population were DENV-2 which corresponds to previous studies that show DENV-2 to be dominant in Kenya over the last decade (Langat et al., 2020, Gathii et al., 2018, Konongoi et al., 2016). In contrast, in a study on children in western Kenya in 2014 – 2015, the most common type was DENV-1 (70%) followed by type 3 (15.9%) with DENV-2 and DENV-3 at 2.4% (Vu et al., 2017b). It is likely that all serotypes are circulating at different levels in the country and flare up sporadically causing outbreaks (Konongoi et al., 2016). The close relation to sequences previously detected in India underlines the well-documented role that international travel plays in the spread of arboviruses (Masika et al., 2020).

Using high throughput sequencing we detected human pegiviruses (HPGV) in a fifth of the pools analyzed. Human pegiviruses are in the *Pegivirus* genus, *Flaviviridae* family (ICTV, 2022). Previously HPGV was known as Hepatitis G or GB virus C. Pegiviruses cause persistent infection in humans and are common globally but no causal link with

any clinical disease has been identified (Simmonds et al., 2017, Yang et al., 2020). A similar study on febrile patients in coastal Kenya also reported pegiviruses in 67% of pools analyzed (Ngoi et al., 2016b). In HIV patients, pegivirus infection has been associated with better outcomes such as higher CD4 count, lower viral load and slower clinical progression (Singh and Blackard, 2017, Xiang et al., 2001). The presence of HPGV has also been associated with lower mortality in Ebola patients, suggesting that HPGV may have a general antiviral effect not specific to HIV alone; a hypothesis that requires further investigation (Singh and Blackard, 2017). We also detected enterovirus A (also known as Coxsackie A5 virus) in one of the pools. It is in the *Picornaviridae* family, *Enterovirus* genus which contains enterovirus A – L and rhinovirus A – C (ICTV, 2022). Enteroviruses are known to cause large outbreaks in children, especially in Asia. Their clinical presentation is diverse, ranging from respiratory tract infections, rash, meningitis, encephalitis, myelitis and acute flaccid paralysis to hand-foot and mouth disease (Gonzalez et al., 2019). The role of enteroviruses in undifferentiated fever is not well elucidated and requires further investigation.

In addition to pegiviruses and enteroviruses, we also identified HIV in one sample pool which contained some samples from HIV patients on treatment. It is possible that the HIV sequences detected were from patients on treatment with breakthrough viremia or from undiagnosed HIV patients. We were not able to unpool the samples for further analysis due to insufficient sample volume.

Anelloviridae were the most common viruses detected in plasma through HTS in this study. Anelloviruses are small (30 – 50 nm), naked, single-stranded DNA (~2-4kb) viruses (Hino, 2002). It is a highly diverse family with 155 species in 31 genera; the largest three of these genera are *Alphatorquevirus*, *Betatorquevirus* and *Gammatorquevirus* (ICTV, 2022). They are ubiquitous in human plasma, forming the largest component of the human plasma virome and are not linked to any pathology. They cause chronic infections in multiple organ systems and the prevalence is not linked to health status, age, or socioeconomic status (Focosi et al., 2016). Anellovirus viral load is directly linked to the level of immunosuppression in the host (De Vlaminck et al., 2013, Fernández-Ruiz et al., 2019).

Circoviridae, Mimiviridae, Phycodnaviridae, Reoviridae, Cruciviridae, Parvoviridae and other unclassified viruses which we detected in a majority of the pools are likely due to contamination or “normal flora” found in human blood. Contamination is a well-documented challenge in high throughput sequencing of viruses. The contaminants are derived from some of the reagent kits used in HTS (the so called *kitome*) especially silica-based RNA extraction kits as well as from environmental contaminants. A good example is Kadipiro virus, a reovirus whose presence in human blood samples was initially thought to be due to infection but was later attributed to contamination (Fitzpatrick et al., 2021, Salter et al., 2014, Ngoi et al., 2017, Ngoi et al., 2016b). These viruses have not been linked with any particular disease and their clinical importance is a question for further research.

Strengths and limitations of this study

In this study, we leverage a rich set of assays – ELISA, IFA, group-consensus PCR and unbiased high throughput sequencing to screen for a broad range of viral pathogens. Despite the broad-spectrum approach, we took measures to preserve the specificity of the assays by use of Sanger sequencing of PCR positive samples and where sufficient sample was available, through the use of neutralization assays.

Since we conducted the study during an inter-epidemic period when no known outbreak was in progress, the data we report here provides evidence of arbovirus circulation outside of reported epidemics and in areas where outbreaks have not been reported before.

One of the limitations of this study was the inability to undertake neutralization assays for flaviviruses due to insufficient blood samples. We partially mitigated this by screening Dengue IgM positive samples with NS1 antigen ELISA to improve specificity. We also detected DENV by PCR and isolated it in viral cultures in some of the samples. Another limitation was that we collected samples at only one-time point which meant we could have missed some arboviral markers which may take several days to appear in blood. We could have also missed seasonal viral causes of fever since we only collected samples for a limited period of the year. To limit the impact of cross-sectional sampling we enrolled patients during both dry and wet seasons in both study areas. We may have missed other pathogens responsible for febrile illness but not usually

detectable in blood; this could have been detected in other samples such as stool, respiratory swabs or cerebrospinal fluid.

In addition, target-independent high-throughput sequencing has lower sensitivity than PCR and may not detect all viruses present in a sample especially if the viral load is low (Lau et al., 2017, Perlejewski et al., 2020). To improve the sensitivity, we enriched viral RNA in samples through, ultrafiltration, precipitation with polyethyleneglycol and nuclease digestion of free nucleic acids. That notwithstanding, HTS only detected dengue virus in 2 out of 5 PCR-positive plasma samples. This indicates that HTS may have missed other viral pathogens as well. On the other hand, HTS provided additional sequence data as well as a broader number of pathogens than PCR making it a suitable complementary assay. Also, detection of pathogens in febrile patients by NGS does necessarily prove causation. The significance and clinical relevance of such pathogens would need to be investigated further using appropriately designed studies.

Selection of Kibera and Taita-Taveta was done through convenience sampling which limits the generalizability of the data to the rest of the country. Nonetheless, the two areas present an opportunity to compare prevalence of arboviruses in patients in a rural and an urban area.

Conclusion

Arboviruses are an important cause of acute febrile illness even during inter-epidemic periods. Exposure to both alphaviruses and flaviviruses is moderate in both Taita-Taveta and Nairobi which are geographically disparate areas in the country. It is likely that humans play the role of a reservoir from which arbovirus outbreaks may arise without the need for a sylvatic transmission which used to be the source of most arbovirus outbreaks in the past.

Diagnosis of acute febrile illness remains a challenging task, especially in resource-limited settings. High-throughput sequencing is a viable tool for the identification of both known and novel pathogens and as it becomes cheaper and more accessible, it will be useful to have it as part of the surveillance tools. However, HTS is still very expensive and slow (takes several days to generate results) and has relatively lower sensitivity than PCR limiting its use for clinical diagnosis (Titcomb et al., 2019, Li et al., 2021).

Recommendations

We need to enhance surveillance for emerging and re-emerging viral pathogens by screening febrile patients for arboviruses and other pathogens. We should also undertake targeted sampling by identifying locations that are most likely to experience EIDs or patients with specific symptoms and collecting the appropriate samples for testing. We should explore emerging tools such as high-throughput sequencing which is increasingly becoming easier to implement and more affordable. HTS may make it easier to screen human, vector and environmental samples such as sewage samples in such areas for emerging pathogens. This (one health) approach would increase our capacity and optimize sharing of resources at the global, national or local level, to detect emerging infections promptly enabling timely interventions to control outbreaks of zoonotic pathogens, both known and unknown (Forbes et al., 2021, Kareinen et al., 2020, Forbes et al., 2019).

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APPENDICES

Appendix 1: Information & Consent Form - Questionnaire

KAVI-ICR

University of Nairobi

STUDY TITLE: Zoonotic and Arboviral Infections in Kenya

INFORMATION AND CONSENT FORM

Introduction & Purpose of Study:

This is a study being conducted by KAVI Institute of Clinical Research. The aim is to assess the disease burden of zoonotic and arboviral infections in Kenya. These are infections that are transmitted from animals to man, usually by arthropods such as mosquitoes and ticks, animal bites or close contact with infected animals or humans.

Inclusion Criteria:

You have been approached because you have presented to a health facility with an acute febrile illness. The study shall recruit 323 patients in your county.

Procedures:

- You will be informed about the study and asked to give written consent if you choose to participate. For children, consent will be sought from their guardian but they will be asked to give assent before participating in the study.
- You will be asked questions relating to your health and undergo a physical examination
- A blood sample (10ml – about 1 table spoon) will be drawn from you for laboratory testing. You will be asked to provide urine and saliva samples as well.
- The samples will be tested for several infections; Dengue virus, West Nile virus, Crimean-Congo Hemorrhagic Fever virus, Chikungunya virus, O'nyong'nyong virus, Marburg virus, malaria, typhoid, rickettsia and brucellosis. The results will be communicated to you through a clinician in this health facility.

Sample analysis and storage:

Screening tests shall be done at University of Nairobi. Samples that test positive on the screening tests shall be stored and shipped to the University of Helsinki for further tests.

Samples will be stored for a period of 20 years at KAVI-ICR, University of Nairobi.

Risk

There may be a risk involved in drawing blood for the tests in this study; you may have pain from the needle at the site of drawing blood, or you may feel dizzy and/or faint after the procedure.

There is no serious physical harm anticipated in this study but should any arise you will be offered treatment by free of charge.

Benefits:

You will know if you are currently infected or ever exposed to any of the ten diseases being tested. You will also assist in generating knowledge about zoonotic and arboviral infections.

Voluntary Participation and Right to Withdraw from the Study:

Participation in this study is voluntary, you may refuse to participate or withdraw at any point in time. There will be no consequences if you refuse to participate or pull out of the study.

Cost:

You will not be expected to pay to take part in this study. It will take about 30 minutes of your time for history taking, examination and obtaining samples.

Confidentiality:

You will be identified by a unique number for purposes of this study. Your personal information and test results shall not be divulged to anyone else outside the research team or research regulators.

Ethical Approval:

This study been reviewed and approved by the Kenyatta National Hospital-University of Nairobi Ethical Review Committee. If you have any complains about the study please contact the committee chairperson, *Prof. Anastacia Guantai* on 020 2726300 or make an appointment to see her at the University of Nairobi School of Pharmacy.

Contacts:

If you have any questions or need to contact the investigator on any matter relating to the study please call 0737 770306 or email *mosmasika@uonbi.ac.ke*

Thank you.

APPENDIX 1b: Information & Consent Form – Questionnaire (SWAHILI)

KAVI-ICR

University of Nairobi

Utafiti: Magonjwa yanayotokana na virusi vinavyoenezwa kwa binadamu na wanyama na wadudu

MAELEZO KUHUSU UTAFITI

Madhumuni ya utafiti huu:

Utafiti huu unafanywa na taasisi ya utafiti ya KAVI Institute of Clinical Research, Chuo Kikuu cha Nairobi.

Madhumuni ya utafiti huu ni kutathmini athari za Magonjwa yanayotokana na virusi vinavyoenezwa kwa binadamu na wanyama na wadudu.

Wanaoshiriki kwa utafiti:

Unaombwa kushiriki kwa utafiti huu kwa sababu umekuja kwa hospitali kwa shida ya homa. Utafiti huu utahitaji washiriki 323 kwa kaunti yako.

Utaratibu:

- Kwanza, utaelezwa madhumuni na matarajio ya utafiti huu na athari zinazoweza kutokana nao, kasha utaombwa kupeana idhini yako kwa kuweka sahihi kwa fomu ya kupeana idhini. Kwa watoto, mzazi au mlezi wao ndiye atakayeombwa kupeana idhini.
- Utaulizwa maswali kuhusu afya yako halafu utapimwa mwili na daktari
- Utatolewa sampuli ya damu, kiasi cha 10ml au kijiko kimoja; pia utaombwa kutoa sampuli ya mkojo na mate
- Sampuli hizi zitapimwa magomjwa kumi yanoyoenezwa na wadudu au wanyama: Virusi vya *Dengue, West Nile, Crimean-Congo, Chikungunya, O'nyong'nyong,* na *Marburg;* pamoja na magonjwa ya *malaria, homa ya matumbo, Riketsia and Brusela.* Utapashwa matokeo ya vipimo hivi kupitia daktari wako

Vipimo na uwekaji wa sampuli

Vipimo vya kwanza vitafanyiwa Chuo Kikuu cha Nairobi, sampuli zitakapatikana na ugonjwa wowote unaofanyiwa utafiti zitapelekwa Chuo Kikuu cha Helsinki kwa vipimo zaidi.

Sampuli zitawekwa kwa muda wa miaka 20 years kwa friji za taasisi ya KAVI-ICR, Chuo kikuu cha Nairobi.

Hatari au usumbufu

Kuna uwezekano kwamba kutoa damu yangu kunaweza kukuathiri; unaweza kuhisi uchungu wakati shindano inakudunga mkononi au pia kuzirai unapoona damu.

Hatutarajii kwamba utapata athari kubwa kimwili ila jambo lolote likitokea utatibiwa na madakatri wetu bila malipo.

Manufaa ya kushiriki:

Ukishiriki kwa utafiti huu, utanufaika kwa kujua matokeo ya vipimo vyetu bila malipo yoyote. Pia utachangia kwa ujuzi juu ya magonjwa haya.

Kushiriki kwa hiari na kuondoka kwenye utafiti huu:

Unashiriki kwa utafiti huu kwa hiari yako na unaweza kujiondoa wakati wowote na bila kushurutishwa kueleza sababu zako wala kupoteza haki zako.

Gharama ya utafiti huu kwako:

Hautakiwi kulipa chochote ili kushiriki kwa utafiiti huu.

Itakuchukua muda wa dakika 30 kujibu kupimwa na dakatri na kutoa sampuli.

Usiri:

Jina lako halitatumika kwa utafiti huu ila utatambulika kwa nambari yako ya utafiti. Habari zozote kukuhusu na uchunguzi wote tutakaofanya zitahifadhiwa kwa usiri na hazitafichuliwa kwa watu wengine ila watafiti na idara za udhabiti na uendeshaji wa utafiti nchini, ikiwa watahitaji kukagua utafiti wetu.

Ukaguzi na kibali:

Taratibu za utafiti huu zimekaguliwa na kupewa kibali za kamati ya maadili ya utafiti ya Hospitali Kitaifa ya Kenyatta na Chuo Kikuu cha Nairobi.

Ukipata swali au shida yoyote kutokana na utafiti huu unaweza kumpigia simu mwenyekiti wa kamati hii *Prof. Anastacia Guantai* kwa simu nambari 020 2726300 au kupanga kumuona kwa ofisi ya kamati hii kwa Chuo kikuu cha Nairobi.

Jinsi ya kuwafikia watafiti:

Ukiwa na swali lolote au ukiitaji kwafikia wanaofanya utafiti huu kwa jambo lolotekuhusu utafiti huu, piga simu nambari 0737770306 ama utume barua pepe kwa *mosmasika@uonbi.ac.ke*

Appendix 2: Statement of Consent

Zoonotic and Arboviral Infections in Kenya

STATEMENT OF CONSENT:

Participant:

I, (Name of participant) _____

Of (address) _____

Agree to participate in this study titled: Zoonotic and Arboviral Infections in Kenya

I have been given enough information on the study and any questions I had have been answered.

I understand what is required of me and give my consent voluntarily; I know I can withdraw from the study at any point without losing any of my rights.

Name of Study Participant: _____

Signature: _____ Date: _____

Person Obtaining Informed Consent:

I _____

have explained the nature and purpose of this study and the foreseeable risks to the participant and answered all his/her questions

Name: _____

Signature: _____ Date: _____

Appendix 2b: Statement of Consent

WARAKA WA IDHINI (WATOTO):

Mshiriki

Mimi, (Jina la anayeshiriki): _____

Kutoka (anwani) _____

Ninakubali kushiriki kwa utafiti huu unaoitwa:

Magonjwa yanayotokana na virusi vinavyoenezwa kwa binadamu na wanyama na wadudu

Nimeelezwa kwa undani taratibu za utafiti huu na nikajibiwa maswali yote. Nimeelewa matarajio ya utafiti huu kwangu na kwamba najiunga na utafiti huu kwa hiari yangu na ninaweza kujitoa wakati wowote bila kuathiri haki zangu nilizonazo kwa sasa.

Jina la Mshiriki: _____

Sahihi/Kidole: _____ Tarehe: _____

Anayechukua idhini:

Mimi (Jina) _____,

Nimemwelezea mzazi/mlezi huyu madhumuni na matarajio ya utafiti huu pamoja na athari zinazoweza kutokana nao; pia nimemjibu maswali yake.

Jina: _____

Sahihi: _____ Tarehe: _____

Appendix 3: Statement of Consent (Minors)

STATEMENT OF CONSENT (MINORS):

Parent/Guardian:

I, (Name of parent/guardian) _____

Of (address) _____

Agree for my child to participate in this study titled: Zoonotic and Arboviral Infections in Kenya

I have been given enough information on the study and any questions I had have been answered.

I understand what is required of my child and give my consent voluntarily; I know I can withdraw the child from the study at any point without losing any legal rights.

Name of parent/guardian: _____

Signature: _____ Date: _____

Person Obtaining Informed Consent:

I _____,

have explained the nature and purpose of this study and the foreseeable risks to the participant and answered all his/her questions

Name: _____

Signature: _____ Date: _____

Appendix 3b: Statement of Consent (Minors) - Swahili

WARAKA WA IDHINI (WATOTO):

Mzazi/mlezi wa mtoto anayeshiriki:

Mimi, (Jina la Mzazi au mlezi wa mtoto): _____

Kutoka (anwani) _____

Ninakubali mtoto watu ashiriki kwa utafiti huu unaoitwa:

Magonjwa yanayotokana na virusi vinavyoenezwa kwa binadamu na wanyama na wadudu

Nimeelezwa kwa undani taratibu za utafiti huu na nikajibiwa maswali yote. Nimeelewa matarajio ya utafiti huu kwa mtoto wangu na kwamba anajiunga na utafiti huu kwa hiari yake na anaweza kujitoa wakati wowote bila kuathiri haki zake alizonazo kwa sasa.

Jina la mzazi/mlezi: _____

Sahihi/Kidole: _____ Tarehe: _____

Anayechukua idhini:

Mimi (Jina) _____,

Nimemwelezea mzazi/mlezi huyu madhumuni na matarajio ya utafiti huu pamoja na athari zinazoweza kutokana nao; pia nimemjibu maswali yake.

Jina: _____

Sahihi: _____ Tarehe: _____

Study ID: _____

Appendix 4: Questionnaire (To be filled by study clinician)

QUESTIONNAIRE

STUDY TITLE: Zoonotic and Arboviral Infections in Kenya

Date: _ _ | _ _ | _ _ _ _

Facility Name: …….

Patient Hospital Number: _ _ _ _ _

1. Gender: ☐ Male ☐ Female
2. Date of Birth: (Day/Mon/Year): _____
3. Residence: Village: _____
   Sub-County: _____
4. Marital status:

   ☐ Single ☐ Married ☐ Divorced ☐ Other (Specify):_____

5. Number of years completed in school:

   _____ Years completed in primary school

   _____ Years completed in secondary school

   _____ Years completed in tertiary education

6. History of recent travel (within the last one month):

   Destination: _____

   County/Country: _____

History and Medical Examination

1. Indicate presence and duration of any of the following:

   ☐ Fever (Temp: ____^{0}) Duration: _____

   ☐ Rash Duration: _____

   ☐ Joint pains Duration: _____

☐ Myalgia Duration: _____

☐ Vomiting Duration: _____

☐ Diarrhea Duration: _____

☐ Bleeding tendency Duration: _____

☐ Other (specify):_____ Duration: _____

2. Family history of similar illness

_____

3. Any history of contact (outside family) of similar illness

_____

4. Comments:

_____

_____

_____

5. Which of the following animals do you have at home? (Tick if present)

☐ Goats ☐ Cattle ☐ Sheep ☐ Swine ☐ Chicken

☐ Other:_____

6. Have you encountered any of the following in your dwelling or work?

☐ Rodents (rat/mice)

☐ Bats

☐ Other Wild animal: _____

Clinicians Name:_____ Signature: _____

Date: _____