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KOUTANGO:
UNDER REPORTED ARBOVIRAL DISEASE IN WEST AFRICA

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University
and Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Veterinary Medicine
through the Department of Pathobiological Sciences

by
Jaime Matias de Araujo Lobo
D.V.M. Faculty of Veterinary Medicine, Brno, Czech Republic 1992
August 2012

Dedication

This dissertation is dedicated to the almighty God, my grandparents Matias Félix Feliciano and Joaquina Matias Félix and, my parents Jaime de Araújo Lobo and Ana Matias Félix.

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First of all, I want to give thanks to Almighty God without whom none of this research would have been possible. His guidance and blessings surrounded me and my environment. At all times when problems seemed not solvable, he was there. I am grateful to my wife Biruk, for emotional support whenever I needed during my PhD; to my son Prince, for making me to be a good listener. To all my relatives for their love, and support during the tough times spent here when I lost my parents and close relatives far from home while at school and taking the exams.

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Appendix: Commonly Used Abbreviations

Ae. spp. Aedes species

C57 BL/6 C57 black 6

CBP CREB binding protein

CCHF Crimean Congo hemorrhagic fever

CHK Chikungunya

CHKV Chikungunya virus

CSF Cerebrospinal fluid

DEN Dengue

DENV Dengue virus

DF Dengue fever

DHF Dengue hemorrhagic fever

DSS Dengue Shock Syndrome

EEE Eastern equine encephalitis

IFA Immunofluorescence Assay

IFNα Interferon alpha

IFNβ Interferon beta

IgA Immunoglobulin A

IgG Immunoglobulin G

IgM Immunoglobulin M

IRF Interferon Regulatory Factor

ISGF3 Interferon-stimulated gene factor 3

ISRE Interferon-sensitive response element

KOUT	Koutango
KOUTV	Koutango virus
MAB	Monoclonal antibody
NF-κB	Nuclear Factor-KappaB
PRNT	Plaque Reduction Neutralization Test
TLR	Toll like receptor
USU	Usutu
USUV	Usutu virus
VEE	Venezuelan equine encephalitis
WEE	Western equine encephalitis
WN	West Nile
WNV	West Nile virus
YF	Yellow fever
YFV	Yellow fever virus
ZF	Zika fever
ZIKV	Zika virus

Abstract

Arthropod-borne viruses (arboviruses) are among the most common agents of human febrile illnesses worldwide. As crucially important emerging pathogens, they have caused multiple, notable epidemics of human disease and unnoticed epizootics over recent decades. Despite the public health relevance, very little is known about the geographic distribution of the agents and vectors, relative impact, and risk factors associated to the arboviral infection in many regions of the world and in the tropics in particular. Presented in this dissertation is an experimental study that explores the serology screening of serum samples from 151 patients whom were diagnosed with undifferentiated febrile illness in Sierra Leone, after ruling out endemic malaria and Lassa fever. Related to the laboratory results of the testing, three exploratory experiments on Koutango virus were developed. The experiment directed special interest into the vector mosquito *Aedes aegypti* and its ability to uptake, disseminate, and transmit the virus. The study of the early events occurring during the interaction between the virus and cells performed in the laboratory was another area of interest with the objective to predict the disease outcome. In order to explore the vertebrate viremia profile, we attempted to develop a suitable animal model for the Koutango virus study in the laboratory. The overall hypothesis of this research is that arboviruses circulating in West Africa are the cause of undiagnosed febrile illnesses. To investigate the hypothesis, this research explores the poorly understood epidemiological features and geographic range of certain endemic arboviruses, particularly the Koutango virus, and whether or not they circulate in the region with the *Aedes aegypti* mosquito being the competent vector that transmits the virus.

The research in this dissertation contributes to the understanding of the epidemiological features and the actual expanding geographical range of many arboviruses. It describes the gold standard laboratory technique for the serology diagnostic of diverse arboviral diseases. In addition, it explores novel laboratory research techniques that may serve as an important tool for the implementation of effective surveillance programs necessary to explore and control the circulation of diverse arboviruses, particularly those associated with human illnesses in West Africa.

Chapter 1: Overview and Literature Review

Introduction

Arthropod-borne viruses (arboviruses) are enveloped RNA viruses that are transmitted by blood-sucking arthropods (mosquitoes, sand flies, fleas, ticks, and lice) to vertebrates. The most common mode of transmission is biological involving factors that enhance encounters between virus, competent vector, and susceptible vertebrate host. Over the past few decades there has been a worldwide emergence of arthropod-borne viral pathogens (arboviruses) [1, 2], particularly those transmitted by mosquitoes. Despite the public health importance, the geographic range of the pathogens, their relative impact, and the epidemiologic characteristics linked to arbovirus infection are poorly defined in many regions of the world [1]. Arboviruses are a heterogeneous group, but the medically relevant ones belong to a few virus genera, including Flavivirus and Alphavirus. Examples of emerging arboviruses include West Nile virus (WNV; Flavivirus) in North America, Japanese encephalitis virus (JEV; Flavivirus) in Asia, chikungunya virus (CHIKV; Alphavirus) in the Indian Ocean region and dengue viruses (DENV; Flavivirus) globally. One shared characteristic of many emergent arboviruses is the ability to expand host and geographical range, due in part to the plasticity of the RNA genome [3]. Some arboviruses have developed the ability to colonize humans as their primary reservoir, while others depend on birds or peridomestic animals, with human infection resultant from spill-over from zoonotic replication cycles. The increase in activities on forested areas worldwide is likely to increase human exposure to sylvatic or forest arbovirus cycles. Tropical areas in particular, with the year round hot and humid conditions, are well suited for maintenance of arboviruses that have the potential to emerge as significant human pathogens [4]. In the neotropics alone, greater than 145 distinct arbovirus species have been recognized [4].

Arboviruses are known to circulate in the human population in Africa. In the 1980s, for example, there was an increase in urban DENV activity in Nigeria, Senegal, and Republic of Guinea [5-7]; in the 1990s, DENV 1, 2, and 4 were isolated from mosquitoes and humans [6, 8-12] in Guinea and other neighboring West African countries [13]. In addition, there has been historical and recent evidence of a sylvatic cycle of DENV in Senegal and the Republic of Guinea. Further there was evidence that dengue had reached the human populations, as neutralizing antibody for DENV was detected in both humans and monkeys [11, 14-16]. Koutango virus (KOUTV), like Yellow Fever (YF) and DENV, belongs to the family Flaviviridae and was first isolated from gerbils caught in Senegal in 1968 [13]. The virus is ecologically associated with rodents, and *Aedes aegypti* mosquitoes were experimentally shown to be competent vectors of this virus, though field reports also implicate other *Aedes spp.* in the transmission cycle [17].

YF is transmitted in its urban cycle by *Ae. aegypti* mosquitoes and is the cause of the almost 200,000 cases and 30,000 deaths annually with nearly 90% of cases occurring in sub-Saharan Africa [18]. Since 2000, YF has been a recurring problem for neighboring Guinea with reported outbreaks in 2001 and 2005. In September 2003 and December 2008, there were laboratory confirmed cases of YF reported from Sierra Leone, and a mass vaccination campaign was put in place [19].

Yellow Fever

Yellow fever is a viral hemorrhagic fever which infects 200,000 people and causes 30,000 deaths globally per year [20]. There are three different epidemiological patterns of Yellow Fever virus (YFV) transmission: the sylvatic pattern, the urban cycle, and an intermediate

cycle that bridges these two patterns [21]. The main vector of the urban cycle YF is the female *Aedes aegypti* [22-26]. In the sylvatic cycle of YFV monkeys and humans are the primary and accidental hosts, respectively; humans become infected with sylvatic YFV when bitten by the primary mosquito vector, *Ae. africanus, Ae. bromeliae* or one of several other mosquito species. Most of these mosquitoes breed and live in holes and cracks in the upper part of the trees in the forest [22, 24-26]. In South America, YF is an occupational disease found in the forestry industry workers [26]. Intermediate epidemics are a mixture of human-to-human and monkey-to-human transmission characterized by focal outbreaks separated by areas without human cases [27]. Patients that recover from an infection by YF develop a solid, long-lasting immunity against reinfection [28].

In humans, the incubation period for the disease is generally three to six days after the bite from an infected mosquito. The patient is infectious to mosquitoes for the first three to four days after the onset of symptoms [24] characterized by a sudden onset of fever, headache, backache, general muscle pain, nausea, vomiting, and [29] bradycardia in relation to the increase in temperature (Faget's sign) [30, 31]. About 15% of those infected develop a serious illness with acute, remission, and toxic phases. The acute phase lasts about three days with the sudden onset of fever, headache, myalgia, nausea, and vomiting; then, remission for up to 24 hours (characteristic "saddle-back" fever) [31] followed by a toxic phase of jaundice and vomiting (black vomitus) in which hemorrhaging of the gums and nose, hematuria, albuminuria, and oliguria (reduction of urine production) may occur. At least half of the individuals who reach the toxic phase do not survive and die between the seventh and tenth day after onset [24, 26, 27, 32].

Malaria and YF may coexist in a region [33], and malaria usually shows clinical symptoms nearly identical with those of the early stages of YF. In the beginning during the acute

febrile phase of an infection, there is little to distinguish the illness from a number of other febrile conditions such as typhoid fever, rickettsial infections, influenza, leptospirosis, viral hepatitis, infectious mononucleosis, and other arboviral fevers like DEN, Lassa fever and chikungunya [21, 24, 33]. The definitive diagnosis of YF is made by serology or virus isolation, which requires trained health care personnel, proper laboratory equipment, and special reagents for the interpretation of the test results [27, 33].

In the 34 countries of Africa with a combined population of 468 million, YF is endemic. Luckily the YF vaccine has proven safe and efficacious, [23] but must be transported and stored frozen [24]. However, once a vial is opened, the vial must be kept cold and used within one immunization session and it must be discarded after that (in this case, one immunization session is considered to be six hours) [34]. In 95% of persons vaccinated, one dose of YF vaccine provides immune protection for at least 10 years and possibly life-long [35, 36].

The strategies to better monitor YF in Africa are epidemic control, mass immunization, routine childhood immunization, and surveillance [24, 37]. Emergency vaccination takes place as soon as an outbreak has been confirmed, in an attempt to limit the spread of infection by immunizing the population in the focus, regardless of their immune status. Active surveillance is essential in at-risk countries for the early detection of cases allowing for fast action to control an outbreak. Often it has proved difficult to identify early, isolated cases before they trigger an epidemic because of the difficulties of distinguishing YF from diseases with similar symptoms (e.g. malaria) [36]. Due to a small risk of adverse reactions, YF vaccine should not be administered to children less than six months of age; therefore it is usually administered at the time of the measles vaccination at nine months of age. Older children should also be vaccinated routinely in areas at high risk for YF epidemics [35, 36, 38].

Since the late 1980s, there has been a dramatic resurgence of YF. Vaccination activities in many of the countries at risk, which include the poorest in the world, are generally weak. Outbreaks were reported in several countries in West Africa in 1994 and in 1995. Only five of 34 African countries at risk reported YF vaccine coverage data in 1996.

History

The first account of a sickness presented itself as a fever aboard a vessel off the coast of Senegal in 1768, commonly accepted as the first report of YF in Africa. Although there was not a clinical description of the fever, evidence suggests its occurrence first in men who had been ashore, followed with propagation aboard the ship. The tropical and subtropical Americas were devastated with epidemics of YF for more than 200 years and serious outbreaks occurred as far north as Spain, France, England, and Italy. In the United States, 20, 15, 8, and 7 epidemics were reported in Philadelphia, New York City, Boston, and Baltimore, respectively, decimating populations and paralyzing the economy [28].

In 1848, American Josiah Clark Nott was the first to suggest that YF was spread by mosquitoes [39], but it was the Cuban physician C. J. Finlay who, in 1881, circulated the first really serious theory of mosquito transmission of YF. During the Spanish-American war, the YF Commission with Walter Reed, concluded that the mosquito was the vector of YF. Furthermore, it was determined that YF could be produced experimentally by the subcutaneous injection of blood taken from the general circulation of a YF patient during the first and second days of his illness and that YF was not conveyed by fomites [28, 40]. The Commission also demonstrated for the first time that a virus was the causative agent of the disease [28].

In former French Africa, cases of YF were apparently rare between 1906 and 1922 increasing during 1922 and 1927 with numerous small, disconnected outbreaks reported in West

Africa [41]. From 1927 to 1931, disease incidence decreased markedly seemingly disappearing from one colony after another; however, in 1931 YF reappeared. The almost simultaneous reappearance in cases of YF, without any connection between them, in many places throughout West Africa and in countries where the disease had not been reported at all for several years, explained the persistence of latent YF foci in these countries [42].

In laboratory diagnostics, the number of serological studies increased considerably after Theiler discovered that mice could be used instead of monkeys *Macacus rhesus*, for protection tests [41]. The results of these tests were positive in West African countries such as Sierra Leone, Nigeria, Senegal, and along the former Upper Volta Territory (Burkina Faso) [41]. Positive results were also obtained in East African Sudan, Uganda, Kenya, Tanganyika, and Northern Rhodesia [41]. By 1928, certain eminent epidemiological features of YF had evolved indicating that the disease followed the trade routes such as rivers, roads, and railways; was pre-eminently urban; however, outbreaks often occurred in isolated spots in the jungle; almost inevitably, outbreaks followed the arrival of large numbers of non-immunes; newcomers to endemic foci suffered disease almost exclusively, with high attack rates in non-immunes, while the locals presented a high level of immunity, and attack rates were higher when infected localities were visited at night [43].

Vaccination Program

Typical epidemics of YF occurred in Nigeria, Ghana, and Gambia in 1925, 1926, and 1934, respectively, before mass immunization campaigns started in French Africa south of the Sahara. The first large-scale vaccination involved live-attenuated YF vaccines, the neurotropic vaccine from human virus passaged in mouse brain and the 17D vaccine from human virus passaged in embryonated chicken eggs, developed in the 1930s. Over 38 million doses were administered during the period from 1939 to 1952 in West African countries, and the incidence

of YF declined dramatically; however, in 1961, the neurotropic vaccine was no longer administered to children under 10 years due to a high incidence of encephalitic reactions. Production of this vaccine ceased in 1980. Today, 17D is the only type of YF vaccine produced because of its mild side effects 5 to 8 days post vaccination. Currently in Africa, 34 countries are at risk for the disease, but only 17 have set up an immunization policy. In the years 2003 and 2008, laboratory confirmation of YF cases where reported in Sierra Leone prompting mass immunization campaigns to begin [26].

Epidemiology

In tropical areas of Africa and South America, YF is prevalent; however areas of the Americas and Europe infested by the mosquito *Ae. aegypti* are still at risk of introduction and spread of the disease [22]. The reservoir of YFV is the susceptible vector mosquito species that remains infected throughout life and transmits the virus transovarially [27]. With nonhuman primates responsible for maintaining the infection, YF can persist as a zoonosis in tropical areas of Africa and America [27]. Man and monkey play the role of amplifiers of the amount of virus available for the infection of mosquitoes [27].

The causative agent of YF is an arbovirus from the *Flavivirus* genus of the family Flaviviridae. It is a single-stranded virus with a positive polarity RNA genome. Viral particles are 43 nm in size made up of a ribonucleoprotein core and a lipoprotein envelope [27]. Although, there is very little evidence for differences in virulence between wild strains of YF virus, considerable heterogeneity between isolates from Africa and South America has been observed [44].

The fundamental understanding is that there are only two genotypes of YF in Africa, one represented by West African viruses and the other by Central and East African strains [45], and

one or possibly two in South America, found by sequencing wild-type YFV strains of different geographic origin. The YFV database includes the entire genome sequences of the Asibi and French viscerotropic viruses (Ghana and Senegal, 1927), partial sequences of the E gene, the 5' and 3' termini, and of the NS4a-NS4b region of multiple isolates from South America and Africa isolated over a 60-year period. South American viruses fall into one major phylogenetic group with respect to the E gene sequence. In contrast to the situation in Africa, the two South American genotypes do not segregate into discrete geographic distributions [45].

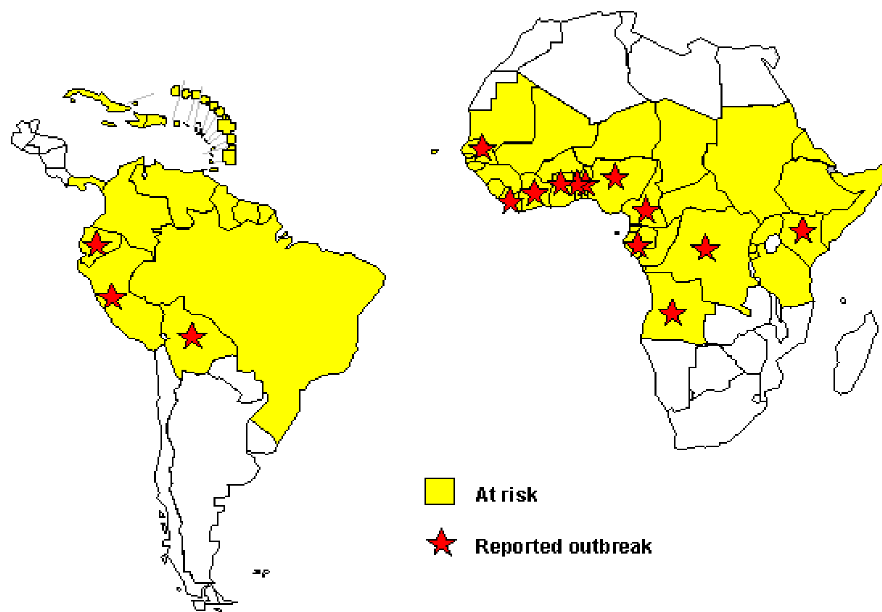


Figure 1.1 Countries at risk for Yellow Fever and having reported at least one outbreak from 1985-1998. Yellow Fever is endemic in 34 countries of Africa with a combined population of 468 million. (WHO/EPI/GEN/98.11 Yellow Fever)

Transmission Cycles

Vertical Transmission in Mosquito Vector

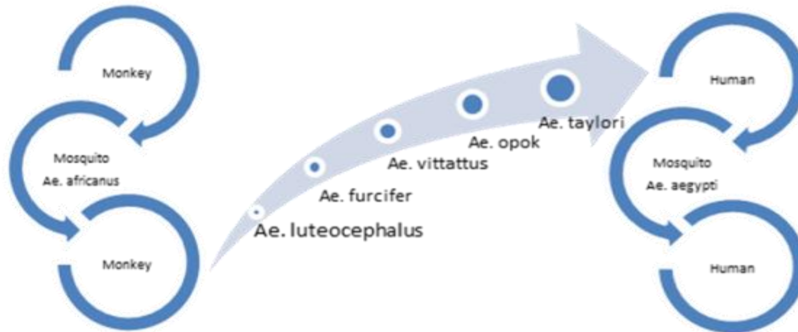
In the 1980s, vertical transmission of the YFV in *Haemagogus equinus* and *Ae. aegypti* was demonstrated [46], as well as the recovery of YFV from male *Aedes furcifer* in Senegal [47]. These evidences of vertical transmission might explain YFV survival in nature without the need for alternate vectors, prolonged survival, retarded transmission by long-lived, drought-resistant, adult female mosquitos, persistent infections of vertebrates, or reintroduction of virus from distant enzootic foci [26]. Recently, natural YFV vertical transmission has been demonstrated in *Ae. aegypti* in Senegal. It was thought that vertical transmission played a major role in the spread of the epidemic [48]. Its efficiency is increased by the possibility of venereal infection of females by males [49]. In this way, the vector maintains the virus for very long periods suggestive of a true reservoir [50]. Epidemiological implications of vertical transmission are evidenced by the virus being transmitted early after the emergence of *Ae. aegypti* females persisting inside infected eggs laid in peridomestic breeding sites until the next rainy season [48].

Horizontal Transmission by Mosquito Vector

Horizontal transmission may occur in maintenance or amplification cycles depending on ecological factors that affect the level of contact with susceptible hosts (Figure 1. 2) [49]. The maintenance cycle is characterized by a relatively stable prevalence of infection in which the vector-vertebrate contact is loose; whereas in the amplification cycle, an increase in the amount of circulating virus is noted, due in part to close vector-vertebrate contact. In the former cycle, YF appears in enzootic or endemic form, while appearing epizootic or epidemic in the latter. The ecological factors that may affect horizontal transmission are dependent on the degree of contact

between vectors and susceptible vertebrate hosts; therefore the mode of transmission hinges on the amount of virus, the abundance of vectors and vertebrates [49].

(A) Sylvatic , Urban, and Intermediate Yellow Fever in Africa



(B) Sylvatic and Urban Yellow Fever in South America

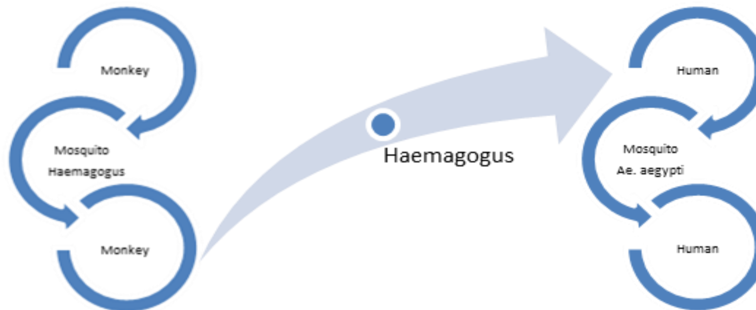


Figure 1.2 Transmission Cycles of Yellow Fever (WHO/EPI/GEN/98.11 Yellow Fever)

Distribution in Africa

The distribution of YF in Africa is best understood in terms of vegetation zones which reflect rainfall patterns and determine the abundance and distribution of mosquito vectors and vertebrate hosts. The vegetation zones are the equatorial rain forest, the humid/semi-humid savannah, and the dry savannah. Typically, year-round enzootic YF transmission between monkeys and *Ae. africanus* occurs in the Equatorial rain forest (mainly sylvatic) zone. The virus

activity is generally at a low level and sporadic cases or focal outbreaks have been observed, in a manner similar to sylvatic YF in South America. Transmission is predominantly monkey-to-monkey, and human infection is sporadic. The humid/semi-humid savannah with either monkey-to-monkey or monkey-to-human transmission is a major area of risk especially during the rainy seasons where it is prone to repeated emergence of YF activity, which may occur at a high rate of transmission due to the presence of vector and host populations. Sylvatic *Aedes* (e.g., *Ae. furcifer, Ae. luteocephalus, Ae. vittatus*) reach very high densities during the rainy season, and are responsible for cyclic epizootics in monkey populations and epidemics with inter-human transmission [51]. This zone is also known as the intermediate zone of transmission. Vertical transmission in these mosquitoes assures virus survival and continuation of epizootic waves. It is in this vegetation zone that most epidemics of YF have occurred. In the dry savannah zones the sylvatic vector populations are too low or active for too short of a period to sustain an epizootic mainly human-to-human transmission occurs with the potential for epidemics. The virus may nonetheless be introduced into a cycle of inter-human transmission by *Ae. aegypti*, either if an epizootic extends from the humid savannah, or if infected individuals move to villages with the domestic vector in the dry savannah. If the virus is introduced into urban or very dry savannah regions where the human population stores water and lives in association with domestic *Ae. aegypti*, explosive outbreaks of *Ae. aegypti*-borne YF (urban-type transmission) may result [26].

The Mosquito Vector in Africa

The main vectors of YF in Africa are mosquitos of the genus *Aedes*, subgenera *Stegomyia* and *Diceromyia*. Seven species, divided into 3 groups according to the contact with humans, are believed to play an important role in nature: the domestic vector *Aedes (Stegomyia) aegypti* co-habiting around the household, the semi-domestic wild vectors that can acquire domestic habits

Ae. (Stegomyia) africanus, Ae. (Stegomyia) luteocephalus, and *Ae. (Diceromyia) furcifer,* and the wild vector *Ae. (Stegomyia) opok, Ae. (Stegomyia) simpsoni group,* and *Ae. (Diceromyia) taylori* [49]. Vectors lay eggs resistant to desiccation that remain dormant during the dry season and hatch only when rain fills the breeding places. In savannah areas there are no adults during the dry season and transmission is discontinuous. While primates are implicated in the natural transmission cycle of YF, monkeys remain the main vertebrate hosts involved in the circulation of YF virus in Africa, but galagoes (bush babies) may also play an important role. The viraemia in the monkeys is a period of two to five days, with a maximum of nine days [52], developing into life-long immunity after infection, thus preventing becoming virus reservoirs [49]. Monkeys that inhabit the top of the forest trees are the main vertebrate hosts in the wild cycle (e.g. *Cercopithecus mitis*), while those which come to ground level (*Cercocebus*) or leave the forest to enter plantations (*Cercopithecus aethiops*) are the link between the wild cycle and humans. In savannah areas, monkeys usually live at ground level, but sleep in trees where they are exposed to mosquito bites. There, monkeys such as patas or baboons easily disseminate the virus because their territory is very large [49].

Dengue Fever/Dengue Hemorrhagic Fever

Dengue fever/dengue hemorrhagic fever/dengue shock syndrome (DF/DHF/DSS) is caused by an infection with one of four dengue virus serotypes, DEN-1, DEN-2, DEN-3 and DEN-4, that are antigenically closely related to each other [53]. However, these serotypes are serologically distinct so that infection with one serotype provides life-long protective immunity against the homolog serotype, but does not provide cross-protective immunity against the others;

thus, persons living in an endemic area can be infected with each of the four dengue serotypes during their lifetime [54]. This leads to extensive cross-reactivity in serological tests.

Epidemic DF has been known clinically for over 200 years [55], but the etiology of the disease was not discovered until 1944. It was characterized during most of its history by periodic, often infrequent, epidemics. In the past 17 years, however, there has been a dramatic re-emergence of epidemic DF activity in the tropics worldwide. This amplified epidemic activity caused by all four virus serotypes has been linked with the geographical expansion of the mosquito vector and the virus, the expansion of hyperendemicity (the co-circulation of multiple virus serotypes in an area), and the emergence of DHF. Hyperendemicity is the most persistent factor associated with the evolution of epidemic DHF in a geographical area [54].

Transmission Cycle

DENV uses the sylvatic cycle pattern characterized by the interaction between the lower primates and forest *Aedes* species, a transition pattern connecting humans and peridomestic *Aedes* species, and an urban cycle involving humans and domesticated *Aedes* species [16, 56, 57]. There may be some overlap between each of these cycles, in accordance to where they occur and the mosquito species involved.

Humans become infected with DEN viruses by the bite of an infected mosquito [58]. *Ae. aegypti*, the principal DENV vector, is a small, black-and-white, extremely domesticated tropical mosquito which prefers to lay the eggs in man-made containers usually found in and around households, such as the flower vases, old automobile tires, buckets that collect rainwater and coconut shells. These containers used for water storage, are crucial in generating large numbers of adult mosquitoes in close vicinity to human residences. The adult mosquitoes rest preferably indoors and choose to feed on humans at the daylight hours with intense biting activities early in

the morning for 2 to 3 hours and in the afternoon for several hours before dark. However, these mosquitoes will feed throughout the day if indoors. Female mosquitoes are very anxious blood feeders, with a discontinuous feeding process in reaction to the slightest movement, returning to the same or to a different person to continue the feeding process later. This behavior enables *Ae. aegypti* females to feed on several persons during a single blood meal transmitting DENV to multiple persons in a short time [59-62]. This feeding behavior enables several members of the same household to become ill with DF in a short 24-to-36 hour time period and proves *Ae. aegypti* to be an efficient epidemic vector [63].

Studies in Nigeria in the 1970s, involving humans who were living in areas where *Ae. aegypti* was not established and monkeys from a lowland rain forest and a gallery forest that had DEN neutralizing antibody provided the first evidence of a forest enzootic cycle of DEN in Africa [64, 65]. Recently in the 1980s, [66, 67] the forest enzootic cycle was confirmed by isolation of over 300 DENV-2 viruses from five species of wild-caught mosquitoes in West Africa: *Ae. africanus, Ae. leuteocephalus, Ae. opok, Ae. taylori, and Ae. furcifer.* Moreover, two isolates collected from pools of male *Ae. furcifer-taylori* mosquitoes in the forests of the Ivory Coast [67] and Senegal, proposed transovarial transmission in the natural maintenance of DENV. The major mosquito vectors of DEN, *Ae. aegypti* and *Ae. albopictus,* have been comprehensively studied in the laboratory incriminating *Ae. albopictus* as a highly susceptible and more efficient host for DENV than *Ae. aegypti [68-71].* However, there is a little doubt that *Ae. aegypti* is the most important epidemic vector of DEN and DHF globally, largely because of its domesticated habits and close association with humans. *Ae. aegypti* must feed on persons with high viraemia to become infected and only those viruses associated with high viraemia can be transmitted by this species, while those viruses producing low viraemia are not able to be

transmitted [68, 72]. In contrast, viruses in semi-rural, rural, and forested areas can be maintained by more efficient vector species of *Aedes* in a cycle combining transovarial transmission with periodic amplification in humans and monkeys. Field evidence from Africa and Malaysia involves lower primates in forest maintenance cycles of DENVs. Likewise, chimpanzees, gibbons, and macaque monkeys are laboratory predisposed to infection with DENVs developing detectable viraemia in absence of clinical illness [66, 73-76].

Clinical Presentation

DENV infection in humans causes a spectrum of illness ranging from unapparent or mild febrile illness to severe and fatal hemorrhagic disease. Clinical presentation in both children and adults may vary in severity, depending on the strain and serotypes of the infecting virus, and the immune status, age, and the genetic background of the patient. In dengue endemic areas, acute DEN infections are often clinically nonspecific, especially in children, with signs and symptoms of a viral syndrome. Classical DF is primarily a disease of older children and adults, characterized by a sudden onset of fever and one or more of non-specific signs and symptoms such as frontal headache, retro-orbital pain, myalgias, arthralgias, nausea and vomiting, weakness, and rash. DF is generally self-limiting and rarely fatal, the acute illness lasting 3 to 7 days. Convalescence, however, may be prolonged for weeks with weakness and depression. No permanent sequelae are known, and immunity for the infecting virus serotype is lifelong [58, 77, 78].

DHF is primarily a disease of children under 15 years of age, although it may occur in older children and adults as well. Similar to DF, it is characterized by a rapid onset of fever and non-specific signs and symptoms, and is difficult to discriminate from DF and other illnesses during the acute phase. The critical stage in DHF occurs at the time of defervescence when the

patient develops a capillary-leak syndrome, with signs of circulatory failure and hemorrhagic manifestations, primarily skin hemorrhages. DHF can be a very dramatic disease with the patient's condition worsening very rapidly with the onset of shock and resulting in death if the plasma leakage is not detected and corrected with fluid replacement treatment. Risk factors for developing severe hemorrhagic disease are not entirely understood but include the DENV strain and serotype, the patient immune status, age, and genetic background [79].

Methods for Assay

DENV belong to the most difficult arboviruses to isolate and propagate. They do not grow well in any of the laboratory animals or mammalian cell cultures normally used in virology laboratories requiring adaptation of the virus to the cell culture [80]. The development of the mosquito inoculation technique provided a highly sensitive and relatively rapid method for isolation and assay of DENVs [81]. The direct fluorescent antibody test provides a simple technique to determine DENV infection in the mosquito [82]. Mosquito cell lines are highly susceptible to DENV infection and immunofluorescence has been used to detect virus infection in these cells because cytopathic effect (CPE) is not reliable to detect all DENVs.

The studies on the levels of virus-neutralizing antibodies on DENV were confirmed with non-human primates [83-86]. To measure virus-neutralizing and protective antibodies, the plaque reduction neutralization test (PRNT) is the most commonly accepted method. Mediated by antibody, virus neutralization is a result of the inactivation of the virus in such a way that is no longer able to infect and replicate in cell cultures or animals. The flaviviral E glycoprotein is a fusion protein of a Class II [87] responsible for viral attachment to host-cell receptors and virus-mediated cell membrane fusion. It is the most important viral protein for viral infectivity, eliciting all virus-specific neutralizing antibodies. In the current PRNT, it is the anti-E

glycoprotein antibody that is investigated. The non-E glycoprotein specific antibodies (e.g., anti-NS1 antibody) can produce virus protective effects in vivo in small animal models; however these effects are not facilitated by virus-antibody interactions [88-90].

Great progress in understanding the structure and function of the flaviviral E glycoprotein has recently been made [91-95]. The E glycoprotein is 90 “head-to-tail” homodimers on the virion surface. The E glycoprotein monomer is divided into three structural domains: DI, DII, and DIII. The DII (the dimerization domain) is a long finger-like structure containing the hydrophobic membrane-fusion sequence at its tip. The fusion tip is protected during replication by a combination of DIII of the associated monomer, E protein glycosylation, and the prM protein [96]. The DIII has been shown to be involved in virus attachment to Vero cells in culture [97]. The DI contains the E glycoprotein molecular hinge. The E glycoprotein undergoes an acid-catalyzed oligomeric reorganization to a fusogenic homotrimer [98-101]. This event occurs in the endosome, permitting the viral nucleocapsid to escape into the cytoplasm and initiate the viral RNA and protein synthesis. Similarly, the prM protein participates as a chaperone protein for the E glycoprotein during viral maturation, helping to maintain the E glycoprotein structure until viral morphogenesis is complete and the virion escapes the acidic exocytic vesicles [87]. The virus is neutralized by the antibodies to both DII and DIII however; the anti-DIII antibodies tend to be powerful neutralizing antibodies, and more virus-type specific. Anti-DII antibodies are more virus cross-reactive, and although they can neutralize virus infectivity, they are less potent than anti-DIII antibodies [102]. The known mechanisms of flaviviral neutralization, the blocking attachment of virus to cells, and the blocking of the virus fusion process were identified [97, 103] however; it is not known how many antibody molecules are needed to neutralize the infectivity of a single virion. Studies suggest that the prM protein may also cause virus-neutralizing

antibodies. The ability of anti-prM antibodies to attach to the virion is directly related to the extent of "prM $\rightarrow$M" processing, that is cell-type specific and is mediated by furin-like cell-associated enzymes. The prM protein does not function as an attachment protein [104].

The adaptive immune response to DENV infection is determined by the presence or absence of neutralizing antibodies to the virus and T-cell responses participating by helping antibody synthesis. After infection, DENV stimulates for the IgM, IgG, and IgA antibody responses being Immunoglobulin M (IgM) response early, often before onset of symptoms. IgM can be detectable in serologic assays seven to eight days after the onset of symptoms [105]. IgA antibodies can also be detectable and have half-lives similar to IgM [106]. IgG antibodies are detectable soon after infection and maintain at the high neutralizing level for years [107]. Infection with any specified DENV serotype develops immunity to that particular serotype; however there is no long-term protection against infection with the other three DEN serotype viruses [108]. Although, re-infection of individuals with a distinct second or third serotype of DENV may result in DF or a more severe infection, DHF and/or dengue shock syndrome (DSS).

A number of hypotheses explaining the severe manifestation of disease following secondary DENV infections have been presented. It is thought that pre-existing DENV-reactive antibody might be one factor involved in mediating DHF/DSS [109-113]. The antibodies that are most likely involved are those that cross-react with, but do not neutralize diverse serotypes of DENV. Non-neutralized DENV-antibody complexes can be escorted into DENV-susceptible cells via surface expressed Fc-receptors [114-116]. This occurrence known as antibody-dependent enhancement (ADE) of DENV replication has been studied for a number of years. It is known that antibodies produced by DIII of the E glycoprotein are more virus-type specific and neutralizing. However, antibodies produced by either DI or DII are more cross-reactive among

viruses, and exhibit lower or no virus-neutralizing ability. Recently, it was proven that the West Nile virus (WNV) primarily infected humans, whose lymphocytes were used to make human monoclonal antibodies, showing that the early antibody response may be engaged towards DII [117]. By applying this hypothesis to the early antibody response in DENV, then the primary humoral response will probably consist of cross-reactive non-neutralizing antibodies. It is also possible that after secondary infection with a diverse DENV serotype, there will be a fast memory response that consists of DENV-cross reactive antibodies as explained by the shared epitopes between DENV serotypes and the abundance of memory B-cells specific for DI/DII cross-reactive epitopes [83, 84, 118, 119].

There are a diverse number of serological tests for the measurement of anti-flaviviral antibody. These tests measuring different antibody activities are as follows: hemagglutination-inhibition test, complement fixation test, fluorescent antibody test, enzyme-linked immunosorbent assay (ELISA), and PRNT. The only test that measures the biological parameter of in vitro virus neutralization is PRNT, the most serologically virus-specific test between flaviviruses, and serotype-specific test between dengue viruses. PRNT correlates well to serum levels of protection from virus infection [120] and remains the laboratory standard against which other newer tests will need to be validated.

PRNT allows for virus-antibody contact to happen in a test tube or plate, and then measures the antibody effects on viral infection by plating the mixture on virus-susceptible cells. These cells are overlaid with a semi-solid media that acts on restriction of the spread of progeny virus. Each virus that initiates a productive infection makes a distinguished area of infection, a plaque, which can be detected in many ways. During the direct staining of cells, the plaques are visualized in such a way that the cells are colored with vital dyes that permits observation of the

progress of viral plaques as uncolored holes in the cell monolayer. Neutral red is cytotoxic at high concentrations and light sensitive; therefore the dye concentration in the overlay is necessarily limited, and plates stained with neutral red should be kept in light-tight containers or incubators. Plaques are frequently counted manually and for the direct staining of cells, the plaques are counted 24 hours after the second overlay. The number of plaques formed is compared to the initial virus concentration to determine the percent reduction in total virus infectivity. In addition, many diverse computer analysis packages exist (e.g., SPSS or GraphPad Prism), and the reliability in the analysis technique is as vital as the selected method for analysis. To validate a particular test, the sample must meet the criteria: the integrity of the uninfected cell monolayer control (negative control), the appropriate plaque counts per well as determined by back-titration of input virus, little or no decrease in plaque counts with negative serum control, the appropriate PRNT titer of positive control sera, and no serum toxicity noticed in low serum dilutions [120, 121].

PRNT is calculated in a way that end-point titers are considered reciprocal of the last serum dilution, showing the desired percent reduction in plaque counts. The $PRNT_{50}$ titer should be calculated based on a 50% or greater reduction in plaque counts and $PRNT_{80}$ should be calculated on 80% or greater reduction in plaque counts. The $PRNT_{50}$ titer is preferred over the ones with higher cut-offs ($PRNT_{90}$) however, the $PRNT_{50}$ titers are more variable. The $PRNT_{80}$ titers use higher cut-offs though provide more precise results from the linear portion of the titration curve. The more rigorous $PRNT_{80}$ titers are more useful in DENV endemic areas for epidemiological studies or diagnostic purposes, by decreasing the background serum cross-reactivities among flaviviruses. A drawback of the PRNT is that it is labor intensive, making it difficult to use for large-scale investigation and vaccine trials [120].

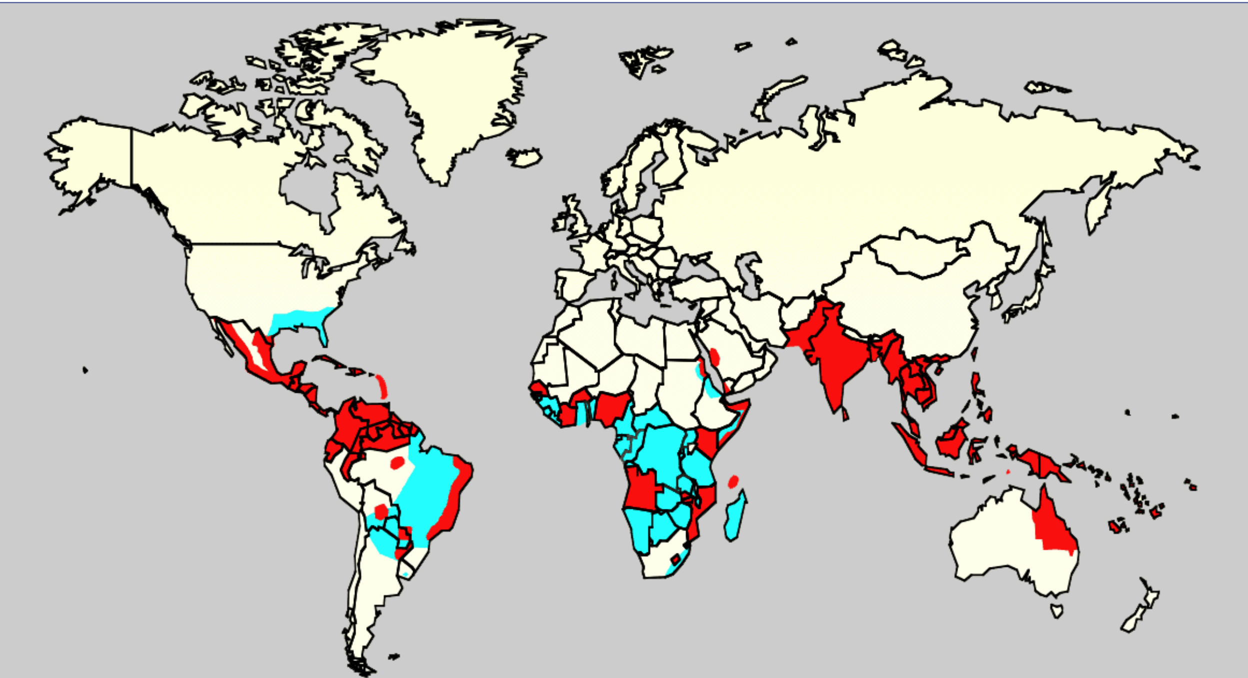


Figure 1.3 World distribution map of dengue and *Ae. aegypti* in 2006. In blue are the areas infested with the mosquito vector *Ae. aegypti* and in red are areas with *Ae. aegypti* and dengue epidemic activity [122].

Geographical Distribution and Incidence

Dengue fever and *Ae. aegypti* mosquitoes have a worldwide distribution in the tropical areas of the world, with over 2.5 billion people living in DEN endemic areas [123-125]. With an estimated 50 to 100 million cases of DF and several hundred thousand cases of DHF occurring each year, depending on epidemic activity, DF/DHF is recognized as the most important arboviral disease of humans [123, 126, 127]. Currently, DHF is a leading cause of hospitalization and death among children in many southeast Asian countries where epidemics first occurred in the 1950s [128].

DEN was reported in Africa in the late 19th and early 20th centuries. The epidemic in South Africa was confirmed by retrospective neutralizing antibody testing in the mid-1950s, but the other reported epidemics were not laboratory confirmed and therefore may not have been

DEN. During the 50 years from 1960 to 2010, twenty laboratories confirmed DEN outbreaks in 15 countries in Africa, the majority in eastern Africa. DENV was first isolated in Nigeria in the 1960s [129]. Subsequently, all 4 DENV serotypes have been isolated in Africa [130, 131]. DENV-2 has been reported to cause most epidemics, followed by DENV-1 [131]. Available data strongly suggest that DENV transmission is endemic to 34 countries in all regions of Africa. Of these countries, 22 have reported local disease transmission, 20 have reported laboratory confirmed cases, and 2 have reported only clinical cases that were not laboratory confirmed. In the remaining 12 countries, DEN was diagnosed only for travelers returning to countries to which DEN was not endemic; DEN was never reported as occurring locally in these 12 countries [130].

Reports of epidemic DF have increased dramatically since 1980 and surveillance for DEN in Africa has poorly improved during this century. Limited outbreaks have occurred in West Africa (Angola, 1986 and Senegal, 1990), but the most recent epidemic activity occurred in East Africa and the Middle East, including Seychelles (1977), Kenya (1982), Mozambique (1985), Sudan (1985), Djibouti (1991), Somalia (1982, 1993), and Saudi Arabia (1994) [130]. All four DEN serotypes have been involved. Sporadic cases of the disease clinically compatible with DHF have been reported from Mozambique, Djibouti, and Saudi Arabia [54, 130]. However, very recently a report described an imported DHF case caused by a DENV-2 West African sylvatic strain in a healthy man returning to Madrid from Guinea Bissau through Senegal. The patient was classified as experiencing grade II DHF with a risk of developing into grade III [132]. As indicated through the IgG avidity test, the patient responded with high avidity antibodies suggesting a secondary flavivirus infection.

Factors responsible for global resurgence

The dramatic re-emergence of epidemic DF/DHF in the 20th century is very complex and not completely understood [123, 133]. The scientific community associates the re-emergence with the demographic and societal changes that have occurred over the last 50 years [54, 134]. The first factor identified is the major global demographic changes coupled with the exceptional population growth primarily in tropical developing countries. Likewise, unrestrained and unplanned urbanization, which has resulted in large and congested human agglomerations near urban centers in deficient housing with inadequate water, canalization, and disposal organization systems, favors the increased transmission of mosquito-, rodent- and water-borne infectious diseases. The second factor is caused by the plastic or cellophane materials that are non-biodegradable; discarded into the environment, they collect rain-water and serve as ideal larval habitats for the vector mosquito. Moreover, in the past 20 years, used automobile tires and coconut shell refuse have increased dramatically making ideal larval habitats. All of these factors have participated in the expansion of the geographical distribution and of the increased population densities of the main mosquito vector *Ae. aegypti*. Effective *Ae. aegypti* mosquito control is practically nonexistent in the majority of the dengue-endemic countries. Over the past 25 years, ultra-low-volume space insecticide sprays for adult mosquito control [54, 63] have been emphasized, but shown to be ineffective [54, 63, 135]. Thus, hundreds of millions of people in urban centers of the tropics are living in intimate association with large populations of an efficient epidemic mosquito vector of DENVs.

A) The distribution of dengue serotypes in the year 1970.

B) The distribution of dengue serotypes in the year 2004.

Figure 1.4 The change in distribution in dengue serotypes [136].

One factor with a huge impact on the emergence of DF/DHF is the increase in air travel. The frequent epidemics and increased transmission of DEN that successively occurred in the American tropics, Asia, and the Pacific provided increased opportunity for the viruses to move

between countries and regions. Improvements in airplane design, incubating the virus, offer an ideal way of transporting DENVs between population agglomerates of the tropics resulting in a permanent exchange of DENVs and other arboviruses.

As a final point, the public health infrastructure that is needed to deal with epidemic vector-borne infectious diseases has in the past 30 years in many countries been destroyed. Added limited financial and human resources have resulted in a 'crisis of mentality' between the public health workers where there is emphasis on the implementation of emergency control measures in response to epidemics instead of developing programs to prevent epidemic transmission [54]. These have had detrimental effects on DEN prevention and control measures already lacking due to the poor surveillance system of the countries. This passive surveillance system relies upon reports by local physicians, who often have a low guide of suspicion and do not consider DEN in their differential diagnosis of DEN-like illness. As a result, the epidemic has often spread before detection and emergency control events are executed, with no impact on the course of the epidemic [54].

Prospects for the future

There is currently no vaccine for DF/DHF. Although live, attenuated vaccine candidates for all four virus serotypes have been developed [137], prediction for reversing the trend of increased epidemic DF/DHF must rely on mosquito control. New DENV strains and serotypes will likely continue to move between areas where *Ae. aegypti* occurs in infected air travelers, resulting in continued hyperendemicity, increased frequency of epidemic activity and increased incidence of DHF, if effective prevention programs are not implemented.

Effective, sustainable prevention programs for DF/DHF must have several components [138] such as an active, laboratory-based surveillance system, a rapid-response contingency plan

for prevention, education of the medical community and community-based, integrated *Ae. aegypti* control.

Other Arboviruses of Potential Interest in Africa

Zika Virus

Zika virus (ZIKV) is an enveloped, icosahedral virus with a non-segmented, positive sense, and single-stranded RNA genome. It is one of the two viruses of the Spondweni virus clade very closely related to the Spondweni virus within the mosquito-borne cluster of flavivirus. ZIKVvirus is a member of Flaviviridae family which includes DENV, YFV, West Nile virus (WNV), and Japanese encephalitis virus (JEV).

The virus, first isolated in 1947, was found in the serum of the rhesus monkey in the Zika Forest of Uganda [139], and in 1948 was isolated from *Ae. africanus* mosquitoes in the same forest [140]. While the first well-documented case of ZIKV was in 1964, the virus was isolated for the first time from humans in 1968 and subsequently in 1971-1975 in Nigeria; 40% of persons tested had neutralizing antibody to ZIKV [141]. During the period from 1951 to 1981 reports point to evidence of human infection by the virus in the African countries of Central African Republic, Uganda, Egypt, Tanzania, Sierra Leone, and Gabon. In Asia, there is evidence of human infection by ZIKV in Malaysia, India, the Philippines, Thailand, Indonesia, and Vietnam [142-146]. The virus was additionally isolated from *Ae. aegypti* mosquitoes in Malaysia and the Ivory Coast and from a human in Senegal [147].

ZIKV was isolated in nature from the *Aedes* mosquitoes from the genus as follows: *Ae. aegypti* an urban cycle vector, *Ae. africanus* a sylvatic cycle vector and from intermediate cycle vectors *Ae. furcifer, Ae. vittatus, Ae. apicoargenteus,* and *Ae. luteocephalus* [147-150]. To date

there is no solid evidence of a non-primate reservoir of ZIKV; however in one study, antibody to ZIKV was found in rodents [151]. The vertebrate hosts of the virus include monkeys in the sylvatic and humans in the urban cycles. It was proven in 2009 that ZIKV can be sexually transmitted among humans. Professor Brian Foy, a biologist from the Colorado State University, contracted Zika fever (ZF) through mosquito bites during a mosquito research trip to Senegal. A few days after his return to the USA and after having sexual intercourse with his wife, he and his wife fell ill with ZIKV, causing him and his wife a ZF, along with extreme sensitivity to light. Foy became the first person known to have transmitted an insect-borne virus to another human by sexual contact [152].

The virus first infects dendritic cells near the site of inoculation, spreading to lymph nodes, and the bloodstream [153]. Even though, flaviviruses replicate in the cytoplasm, one study suggested that ZIKV antigens might be found within the infected cell nuclei [154]. ZIKV antigens have been found in human blood the day of the onset of illness and were isolated from monkey serum nine days after the onset.

The common symptoms of ZIKV infection registered from the patients begin with mild headache, followed by maculopapular rash, fever, malaise, conjunctivitis, and arthralgia. Two days after the beginning of the first symptoms, the rash starts disappearing, and within 3 days the fever disappears and only the rash remains [155]. ZF is a relatively mild disease with 49 confirmed cases and 59 unconfirmed cases. There is no death and no hospitalizations reported [156]. So far, it presents as a mild febrile disease but with true potential as a virus and as an agent of unknown disease. Currently, there is no vaccine or any drug for ZIKV, and the only treatment is symptomatic.

In April 2007, outside of the African and Asian continent, the first outbreak of the disease was documented in the island of Yap in the Federated States of Micronesia. Originally it was assumed that the disease was DEN, CHIK or Ross River. However, serum samples from patients in the acute phase of illness contained RNA of ZIKV. ZIKV illness has been, to date, minor and self-limiting but WNV was before considered a relatively innocuous pathogen until large outbreaks with neuroinvasive disease were reported from Romania and North America. ZIKV might be considered an emerging pathogen because of the first confirmed infection outside its traditional geographic range of Africa and Asia in 2007 [156, 157]. The spread of ZIKV in the pacific could be problematic to detect because of cross-reactivity in the serological diagnostic assays. ZIKV can be easily confused with DEN and might contribute to illness during DEN outbreaks. The recognition of the virus requires collaboration between public health professionals, clinicians, and high-quality reference laboratories [155].

Usutu Virus

Usutu virus (USUV) is an African mosquito-borne virus of the family *Flaviviridae*, genus *Flavivirus* and belongs to the Japanese encephalitis serocomplex [158]. Flaviviral species, USUV and West Nile virus (WNV) in Africa, Asia and Europe; Japanese encephalitis virus (JEV) in Asia; Murray Valley encephalitis virus in Australia and Saint Louis encephalitis virus in the American continent developed from an ancestral *flavivirus* with a bird/mosquito natural cycle. Originally, in 1959 in South Africa, USUV was isolated from a mosquito vector *Culex neavei*. Later in subsequent years, USUV strains were identified from different bird and mosquito species in Africa, but only once has the disease presenting with rash and fever, been reported in humans in the Central African Republic [159, 160]. In the past, USUV was not seen as a potential risk for humans; the virus had never been related to severe or fatal diseases in

animals or humans, and it had never before been detected outside tropical and subtropical Africa [159].

In the summer 2001, several species of resident birds, especially of the order *Passeriformes,* were major victims of the emergence of USUV in Austria [161-163]. In the years that followed, the virus was isolated from dead birds and/or from mosquitoes in several European countries, including Hungary in 2005 [164], Switzerland in 2006 [165], Italy in 2009 [166], and Spain in 2006 and 2009 [167, 168]. In wild birds, USUV infection has been serologically isolated in England (2001–2002) [169], Spain (2003–2006) [170], Czech Republic (2005) [171], Poland (2006) [172], Switzerland (2006) [165], Germany (2007) [173], and Italy (2007) [174]. The recurrence of the virus over several years in Austria (2001–2006), Hungary (2003–2006), Italy (2006–2008), and Spain (2006, 2009) suggests frequent reintroduction of the virus or, more probable, persistence of transmission in the affected areas, most possibly by the overwintering mosquitoes. Findings supported by partial nucleotide sequence analysis found >99% identity between the viruses that emerged in Vienna in 2001, in Budapest in 2005, and in Zurich and Milan in 2006. The one-time invasion of USUV from Africa to Europe (Vienna) is highly agreeable, and this particular strain has since then been spreading in Central Europe [165]. However, in Italy a serological and virological study monitoring the USUV circulation proposes a different setting. This study carried out with sentinel horses and chickens, wild birds, and mosquitoes in 2008 and 2009 [175] proved that the virus had circulated in Italy in these two years. In addition, the data confirmed USUV infection in horses for the first time in Europe. The sequence assessment of USUV detected from different species in different counties indicated that there were two different strains of USUV circulating in Italy in 2008 and 2009, and these strains adapted to new hosts and vectors to become established in new areas [175].

In the summer of 2009, the virus was associated with neurological disorders of two immunocompromised patients that received blood transfusions in Italy [176, 177] and was isolated from the serum obtained from one of the patients during the acute phase of the disease. The two infections could occur with local transmission, either directly by a mosquito bite or indirectly by an infected donor. The two patients were the first reported human cases worldwide of USUV with neuroinvasive illness. The common clinical symptoms were persistent fever, headache, and impaired neurological functions. One patient developed hepatitis, a rare organ damage case described previously in WNV infection [178, 179]. The clinical picture showed a clear involvement of the central nervous system, similar to the related WNV neuroinvasive disease. Whether this new tropism was associated with a new inoculation route by transfusion, and/or to the fundamental diseases of the patients, remains unclear, but these findings support further investigations. In recent sequencing studies of USUV strains obtained in Italy in 2009 from mosquitoes, birds and humans, the sequences obtained from humans matched with the sequences obtained from birds, indicating an endemic distribution of USUV in Europe [175].

USUV infection requires that the laboratory confirmation distinguish between direct methods detecting the virus by cell culture or genomic amplification and indirect methods that detect the antibody response to the infection. Serological diagnosis of USUV infections in humans requires a similar approach to the one used for WNV. Because of the lack of experience with USUV infection in humans, it is estimated that the incubation period spans from two to 14 days, but the exact time frame is unknown; USUV can be in the initial stage of the disease noticeable in CSF and serum. The IgM antibody appears five days after onset of fever and persists in serum for many months after infection [180]. Diagnosis of USUV will be complicated, particularly due to flavivirus cross-reactions, especially in the case of WNV and

tick-borne encephalitis virus (TBEV) in several European countries [181]. It is also believed that cross-reactivity will be higher for IgG than for IgM detection; hence, improvement of tests for USUV-specific IgM is needed. As an available alternative, the seroconversion test of IgG antibodies for the acute and convalescent sera using in-house or commercial ELISA tests based on WNV antigens should be used. Serum is tested by the low specific hemagglutination inhibition [182] or ELISA tests [174]. However, PRNT must be performed to confirm positive sera, and is complex, costly, time-consuming and not accessible for laboratories lacking high bio-containment facilities.

In Europe there is a risk that potential emerging infectious diseases caused by WNV or USUV will not be recognized in time by existing surveillance infrastructures of the various European countries [183]. The circulation of USUV in consecutive years in Austria, Hungary, Italy, and Spain, the seroconversion in sentinel animals, and the virus detection in wild birds makes these regions appropriate to sustain USUV circulation between vectors and vertebrate hosts, , establishing the endemic cycles. This illustrates a need for active surveillance measures and early warning systems to detect WNV and USUV activity, and to assess the risk for public health. A multidisciplinary methodology for the assessment of risk of USUV and WNV transmission, and the impact of the diverse components (mosquitoes, birds, horses, and humans) must always be carefully considered.

Koutango Virus

Koutango Virus (KOUTV) is an arbovirus from the family Flaviviridae, genus *Flavivirus*. It is a single-stranded virus with a positive sense RNA genome. Viral particles are around 40-70 nm in diameter comprised of a ribonucleoprotein core, with icosahedral nucleocapsid, and lipoprotein envelope. The virus belongs to group B at the WNV group [132]. While searching for

an evolutionary relationship of the WNV viral strains between Africa, the Middle East, and Europe, Charrel et al. (2003) considered the whole genomes of three WNV strains from France (horse-2000), Tunisia (human-1997), and Kenya (mosquito-1998), and the envelope, NS3 and NS5 genes of KOUTV. The phylogenetic analyses, including all accessible full-length sequences, discovered that KOUTV is a distant variant of WNV, the three characterized strains belong to lineage 1, clade 1a, and the Tunisian strain is the origin of the lineage of viruses introduced in North America [132].

The most comprehensive phylogenetic analyses of flaviviruses, based on NS5 sequences [158, 184], demonstrated that the West Nile/Kunjin group belongs to the evolutionary lineage of Japanese encephalitis viruses, together with KOUTV and other viruses, such as the Murray Valley encephalitis and St. Louis encephalitis viruses. This is also supported by a recent analysis of envelope and NS5 sequences [158, 184]. Distances between KOUTV and lineage 1 and 2 viruses are <25%, while distances between Japanese encephalitis virus and other viruses are >30%. This shows that KOUTV is more closely related to the West Nile/Kunjin virus than to Japanese encephalitis virus, and may be considered as a distant variant of WNV. It is of note that no human case of KOUTV infection in nature has been reported to date. Although its capacity for infecting humans is not known, a single infection was confirmed in humans from a laboratory accident of a worker [17] who presented with a mild febrile illness. The disease caused by KOUTV results in flu-like symptoms such as two-day fever accompanied by achiness and retro-bulbar headache, to erythematous eruption on the flanks [17].

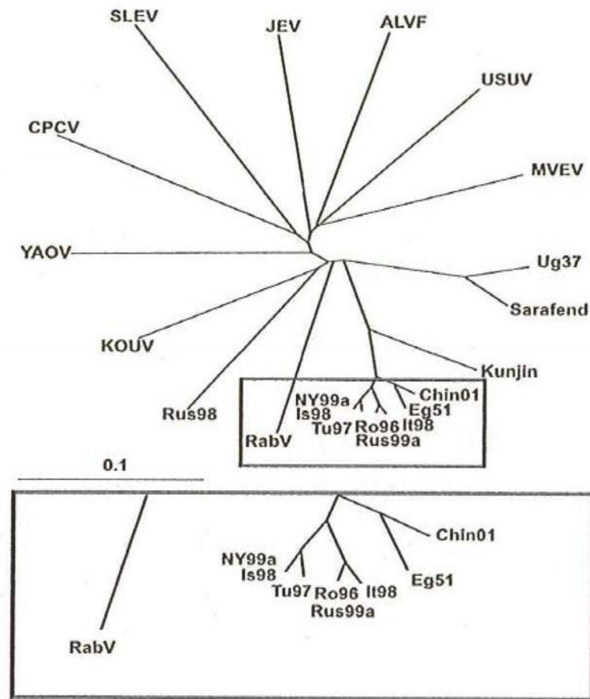


Figure1.5 Phylogenetic tree illustrating the genetic relationship between representatives of Japanese encephalitis virus and selected WNV strains based on partial genome sequences of the NS5 protein gene. Bar on the left demonstrates the genetic distance [164].

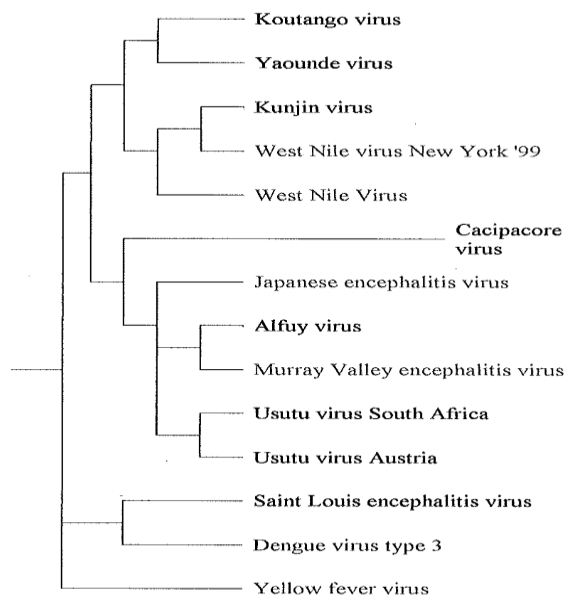


Figure 1.6 Phylogenetic tree illustrating the common ancestor of various arboviruses demonstrating that KOUTV is a distant ancestor of WNV [163].

KOUTV was first isolated in 1968 from the blood of the wild rodent reservoir *Tatera kempi* in Senegal in the Koutango region and from the rodent *Mastomys* in Central African Republic; in 1974 the virus was also isolated from gerbils in Somalia [5]. The virus is transmitted in nature by the mosquito vector *Aedes spp.* Intra-cerebral inoculation of the virus to suckling mice causes death on days 3 to 4 post infection [17]. It was confirmed from the infected field isolates that *Aedes spp.* mosquitoes are implicated in the KOUTV transmission cycle [17, 185] and the *Aedes aegypti* mosquito is a competent vector during the KOUTV transmission cycle in the laboratory [17].

The particular emphasis to arboviruses in general and KOUTV in particular is based on the worldwide re-emergence of arthropod-borne viral pathogens and the ability of the emergent arboviruses to expand host and geographical range, partly due to the plasticity of the RNA genome [3]. We are inclined to pay special attention to the increase in activities in forested areas worldwide with consequential human exposure to sylvatic arbovirus cycles. Likewise, year round hot and humid conditions in the tropics suits well for arboviral maintenance with the potential to emerge as significant human pathogens [4], and the recognition of hundreds of distinct arbovirus species with many of them being associated with humans. In West Africa, there have been reports of increased arboviral activities coupled with the isolation of diverse arboviruses since the 1980s and registered outbreaks followed by the mass vaccination campaigns [8, 13]. The recent and important evidence of a sylvatic cycle of DEN in human populations in Senegal and the Republic of Guinea added to the vertical transmission of *Aedes aegypti* and the ability to persistently infect vertebrates was reinforced by inadequate surveillance and substandard reporting on arboviral illnesses in Africa.

Objective and Rationale

Even though arboviruses are known to exist in the region, lack of efficient surveillance systems prohibit definite diagnosis of arboviruses that may have circulated for many years. It is therefore possible that malaria and typhoid fever are over-diagnosed, because of this lack of more sophisticated equipment, reliable testing machinery, and well trained personnel. In 2006, malaria and Lassa fever (LF) were first ruled out as causative agents of patients who presented to the hospital in Sierra Leone, with acute febrile illnesses. Patients were subsequently diagnosed with a fever of unknown origin. We propose that arboviruses may contribute considerably to the numbers of undiagnosed febrile illnesses in Sierra Leone.

From a Tulane University Health Care team, 151 serum samples of patients with acute febrile illness, ruled out to have malaria or LF (endemic diseases of the West Africa), were collected from Kenema hospital in Sierra Leone and sent to our laboratory at Louisiana State University, USA. To determine the exact cause of the febrile disease of unknown etiology circulating in Sierra Leone, our laboratory first attempted a virus cultivation assay from serum samples whose RT-PCR was negative. Later, the sera were tested for IgM/IgG antibody levels against DENV, a presumable flavivirus potentially circulating in West Africa and may be the cause of febrile illnesses to humans.

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Chapter 2: Serological Evidence of Co-Circulating Dengue Virus Serotypes and Koutango Virus, Under-Reported Arboviral Diseases in Sierra Leone

Introduction

Over the last few decades, there has been a worldwide re-emergence of arthropod-borne viral pathogens (arboviruses) [1, 2], particularly those transmitted by mosquitoes. Despite the public health importance, the geographic range of the pathogens and their relative impact, the epidemiologic characteristics linked to the arbovirus infection, are poorly defined in many regions of the world. Arboviruses are a heterogeneous group, but the medical relevant ones belong to a few virus genera, including Flavivirus, Alphavirus, and Orthobunyavirus. Examples of emerging arboviruses include West Nile virus (WNV; Flavivirus) in North America, Japanese encephalitis virus (JEV; Flavivirus) in Asia, chikungunya virus (CHIKV; Alphavirus) in the Indian Ocean region and dengue viruses (DENV; Flavivirus) globally.

One shared characteristic of many emergent arboviruses is the ability to expand host and geographical range, due in part to the plasticity of the RNA genome [3]. Some arboviruses have developed the ability to colonize humans as their primary reservoir, while others depend on birds or peridomestic animals, with human infection resultant from spillover from zoonotic replication cycles. The increase in activities on forested areas worldwide is likely to increase human exposure to sylvatic arbovirus cycles; at particular risk are year round hot and humid regions found mainly in tropical areas, which provide suitable grounds for arboviral maintenance and potential for emergence as significant human pathogens [4]. In the neotropics alone, greater than 145 distinct arbovirus species have been recognized [4], many of them have already been associated with human illnesses.

The limitation of implementing surveillance for arboviral diseases is the generic nature of disease presentation. Whereas severe disease can result, such as hemorrhagic manifestations (DENV and YFV) or neurological disease (WNV, JEV, and VEEV), arbovirus infection usually

manifests itself with mild to moderate febrile illness [2, 5, 6]. In early stages of disease development, patients very often present with unrecognized febrile illness [5, 7] making the clinical diagnosis erratic [8]. For example, in DENV-endemic areas, diseases caused by co-circulating arboviral pathogens have often been misdiagnosed [8, 9]. With similar clinical presentation and a variety of potential etiologic agents, laboratory support has become an integral part of effective surveillance programs. The impact on human health in endemic regions and the potential for the spread of disease emphasize the importance of improving understanding of arbovirus transmission.

Currently, the epidemiological features and geographic range for many endemic arboviruses in West Africa are poorly understood. Malaria, YF, LF, DEN and many yet undiagnosed arboviral illnesses may coexist in a region [10]. The definitive diagnosis made by serology or virus isolation requires trained health care personnel, proper laboratory equipment, and special reagents for the interpretation of results. To begin to address this gap, we conducted a laboratory-supported clinical-based study to identify certain etiologic agents associated with undifferentiated febrile illness in Sierra Leone. Herein, we describe the serological testing of blood samples on arboviruses and their relative contribution to human febrile illness.

Materials and Methods

Blood Samples

The enrollment of patients in the study and collection of blood was conducted under the approved study by the Tulane University Internal Review Board and the Ethics Committee of Sierra Leone Ministry of Health. Blood samples were collected at the Kenema District hospital in Sierra Leone from patients in an acute stage of the disease and on subsequent days 7 (late

acute) and 28 (convalescence), if available. At every visit, 5 ml of blood was drawn by venipuncture and collected in red-top Vacutainer® tubes. Because of Lassa virus endemnicity to the area, precautions were taken to make potential LF infected serum non-infectious. Thus, all samples were inactivated by heating (56 ºC for 30 min) before manipulation [11]. The serum was then separated from the clot by centrifugation and stored at approximately -11°C in labeled cryovials in a solar-powered freezer until testing. Serum samples were then transported on cold-packs by road to Côte d'Ivoire, where they were shipped on dry ice to Tulane University in New Orleans, LA. Finally, they were transferred to the School of Veterinary Medicine at Louisiana State University (SVM-LSU). These patients were determined not to have malaria or LF infections by the methods outlined in Moody, et al. and Bausch et al., respectively [11, 12].

Cells and Viruses

African monkey kidney-derived Vero cells were propagated in 1X M199 Earle's supplemented with fetal bovine serum (FBS) and Penicillin, Streptomycin, and Fungisome (P/S/F). Vero cells were grown at 37°C in a 5.1% CO_2 environment. We first attempted to isolate the virus by inoculating Vero cell culture with 100 µl of serum. However, detection of the live virus by either visualization of cytopathic effects or RT-PCR and qRT-PCR of the cell culture supernatant was unsuccessful.

We then used the sera to determine immune reactivity to DENV, which would indicate the presence of antibody against these viruses in the human population of Sierra Leone. Serological IgM/IgG testing using a $PRNT_{50}$ protocol was utilized [13]. We tested serum for neutralization using stock representatives of DENV (Table 2.1). Further, we utilized a more stringent, cross-neutralization assay to differentiate from flaviviruses: WNV, YFV, KOUTV,

ZIKV, and USUV. Viruses were tested for viral concentration by plaque assay to determine what dilution of stock virus would yield 100 plaque forming units per 100 µl.

Table 2.1 Viral strains used to conduct the PRNT

Virus type	**Strain**
DENV - 1	West Pacific 74
DENV - 2	16803 Thailand
DENV - 3	CH 55
DENV - 4	LN 634441 human Malaysia, 1988
KOUTANGO	DAK Ar D 5443 Suckling mouse 8 (30525), May 21, 1989
WNV	WN02
YELLOW FEVER	17D Vaccine TVP-9447 Vero1, Sept.21, 2004
USUTU	TVP-10675 DAK PM173269 Vero 1 Sep 14 2007
ZIKA	TVP-1565 - C6/36, 1 Dec. 1987

Plaque Reduction Neutralization Test (PRNT)

Using the PRNT, samples were screened for reaction to DENV by utilizing Vero cell culture grown on 6-well plates for a period of 2-4 days until the confluence reached at least 80%. After the media was removed from the 6-well plates leaving the attached cell sheet, 100 µl of a virus-serum mixture, previously incubated for an hour at 37°C, was gently pipetted into each well. The plates were incubated for an hour at 37°C in a 5.1% CO_2 environment, and gently rocked every 15 min. The first overlay was prepared using 2X M199 Earle's, FBS, P/S/F, and agar powder dissolved in distilled water. The agar mixture was poured into the media mixture or vice-versa and swirled for approximately 2 min; then 3 ml was overlaid onto each well of a 6-well plate with care taken not to burn the cell sheet.

After the first overlay, the second overlay was added on day 4 for WNV, ZIKV, and KOUTV, day 5 for YF and USU, and day 6 for all DENVs. The second overlay was prepared with the same media and agar preparations as in the first overlay, with the addition of neutral red

stain and 0.5% NaCl. After mixing, 1.5 ml was overlaid onto each well of a 6-well plate, which was then stored overnight at 37°C in a 5.1% CO_2 environment. Plaque formation was determined 24 hours after the second overlay.

Owing to the great deal of cross reactivity of flaviviruses, we increased the specificity of the PRNT testing to determine which DENV serotype reaction was occurring in these patients [14-16]. Thus, an endpoint titration and cross-neutralization reaction study of the $PRNT_{50}$ DENV positive samples was performed. Samples were serially diluted to 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320 with serum:BA1 diluent (Hank's M-199 salts, 1% bovine serum albumin, 350 mg/l sodium bicarbonate, 100 units/ml penicillin, 100 mg/l streptomycin, 1mg/l amphotericin B in 0.05 M, Tris, pH 7.6). Samples were then serologically tested against the uniform viral concentration of DENV 1, 2, 3, and 4, as well as ZIK, KOUT, USU, YF, and WN viruses.

The plaque reduction neutralization was interpreted as follows: Three positive control wells were made per viruses. The cell sheets were pipetted with virus only and were checked and counted for the average number of plaques formed by the virus. Wells that had been treated for testing were pipetted with serum/virus mixtures and were read and plaques counted. Plaque reduction by patient antibodies to virus was expressed as the proportion of plaques formed in the serum/virus samples divided by the average number of plaques in the positive controls. This proportion was then expressed as a percent reduction. Reduction percentage values equal to or greater than 50% ($PRNT_{50}$) were considered to have a positive result according to standard methods; those with values of 80% ($PRNT_{80}$) were considered highly neutralizing [17].

Results

Dengue virus screening with $PRNT_{50}$

We tested 151 human serum samples from Sierra Leone for reaction to DENV. Of the 151 samples tested, 118 (78.15%) had at least a 50% reduction in plaque formation. Forty-seven samples (39.83%) showed neutralization to a single DENV at the $PRNT_{50}$ level: 13 (8.61%) each to DENV-1 and DENV-4; 20 (13.25%) to DENV-2 only, and 1 to DENV-3 only (0.66%). Thirty samples (25.42%) neutralized a combination of two DENV serotypes, 33 samples (27.97%) neutralized a combination of three DENV serotypes, and 8 samples (6.78%) neutralized all four DENV serotypes. 33 serum samples did not neutralized any of the DENV serotype.

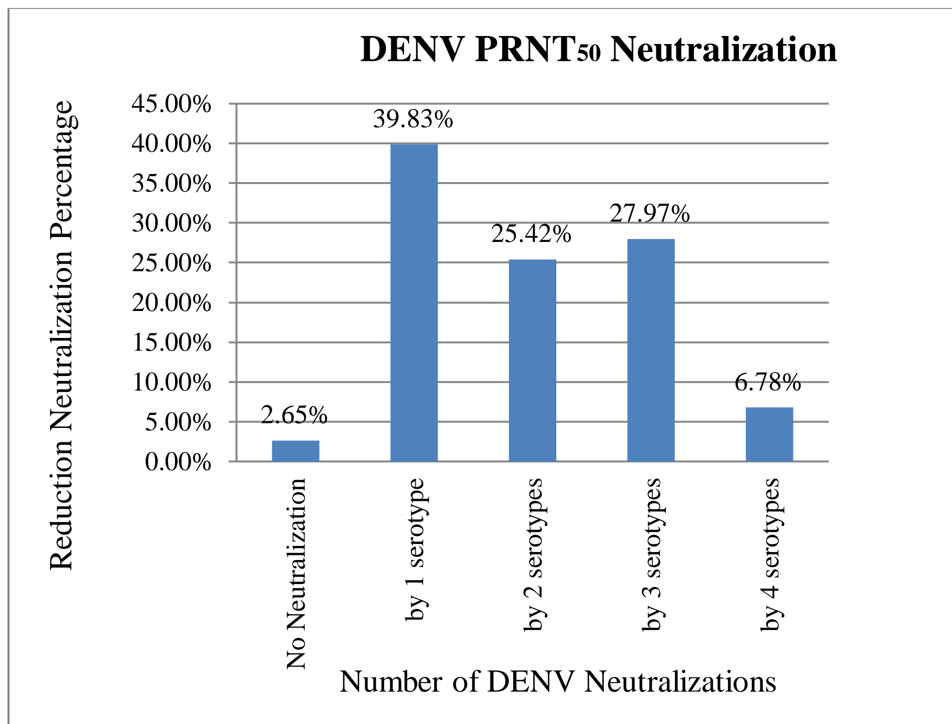

Figure 2.1 Overall $PRNT_{50}$ results based on the combination of positivity of serum samples to dengue virus serotypes. The highest percentage (39.83%) of samples neutralized DENV by one serotype of DENV-1, DENV-2, DENV-3, and DENV-4 followed by 27.97% of samples neutralizing three out of four serotypes of DENV; More than a quarter (25.42%) of samples neutralized two combinations and 6.78% neutralized all four DENV serotypes.

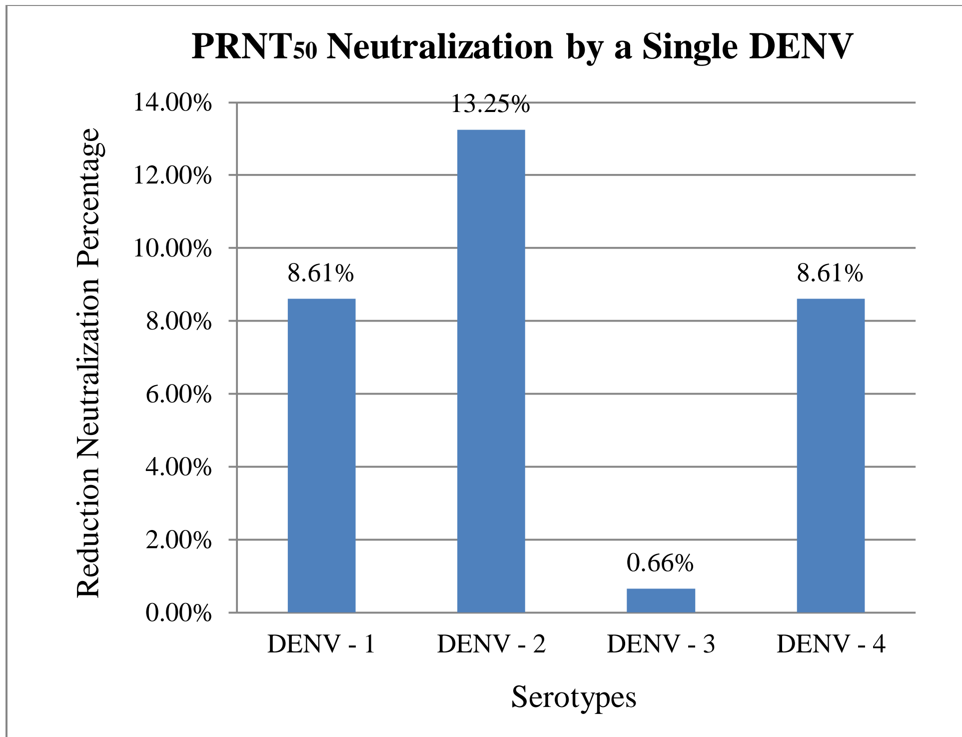

Figure 2.2 $PRNT_{50}$ neutralization profile of 47 samples that reduced the plaque formation of single dengue virus serotypes.

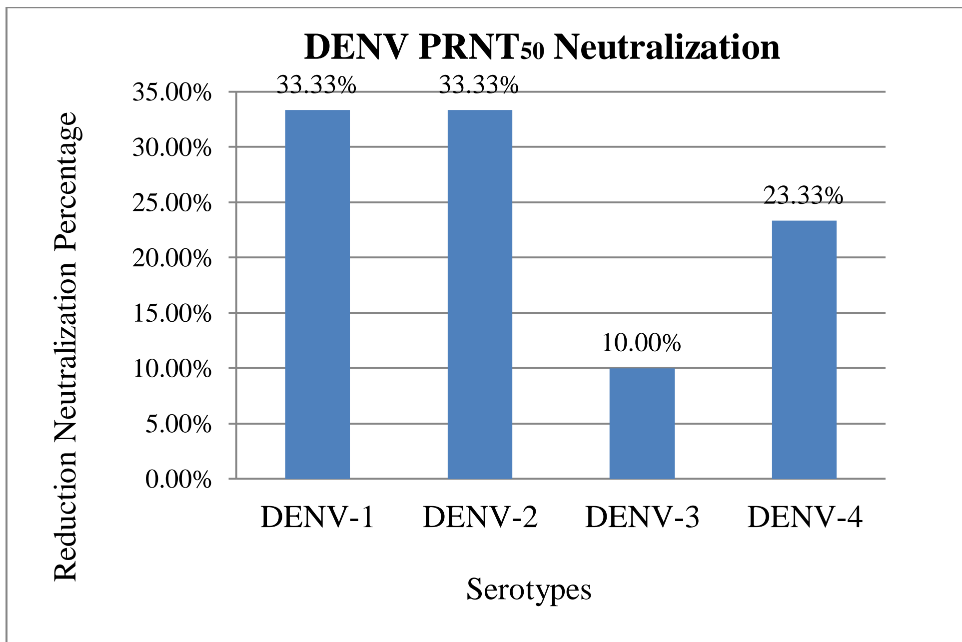

Figure 2.3 $PRNT_{50}$ results of diverse DENV serotypes neutralized by the 118 serum samples.

The PRNT assay is considered a standard serological test for detecting antibody [18, 19]. However, the cross-reactivity of antibodies to antigenic epitopes common to all flaviviruses is an important constraint in serological diagnoses [20-22]. In order to increase the specificity of the PRNT against DENV serotypes and possibly other flaviviruses, endpoint titration reactions were performed on a total of 52 of the 118 $PRNT_{50}$ positive samples.

Endpoint titration

Patients with serum available for further testing after the DENV screening were then tested for levels of antibody to all four DENV, YFV, ZIKV, WNV, USUV, and KOUTV. Serum was considered to be moderately neutralizing if it reduced plaques by 50-79% and highly neutralizing if it reduced plaques by greater than or equal to 80%. In figure 2.4, we report the qualifying neutralization at the highest level of dilution.

Nine patients had moderate or strong neutralizing antibody to DENV-1, and seven of those had highly neutralizing antibody in diluted serum. Twelve patients had neutralizing antibody to DENV-2 in diluted serum, seven with high levels of neutralization. Four patients neutralized DENV-3, two with high levels of neutralization; seven neutralized DENV-4 with five at the $PRNT_{80}$ level.

Twelve patients had neutralizing antibody to YF from which three highly neutralizing. Interestingly, nine patients had neutralizing antibody to KOUTV, an arbovirus previously undetected in the human population; six of these patients had strong neutralizing antibody to KOUTV, and one patient had a moderate level of neutralizing antibody to ZIKV, which has also been unreported in this region. There was no neutralization to either WNV or USUV.

Fourteen patients had endpoint titrations that indicated moderate ($PRNT_{50}$) and/or high ($PRNT_{80}$) neutralization to more than one flavivirus.

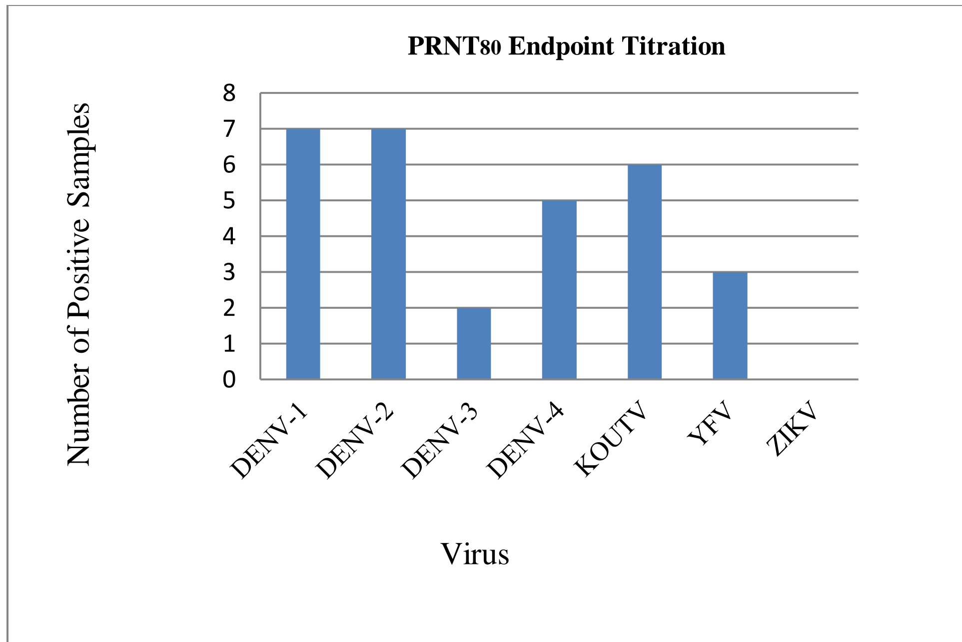

Figure 2.4 $PRNT_{80}$ endpoint titration of serum samples to diverse arboviral serotypes circulating in West Africa.

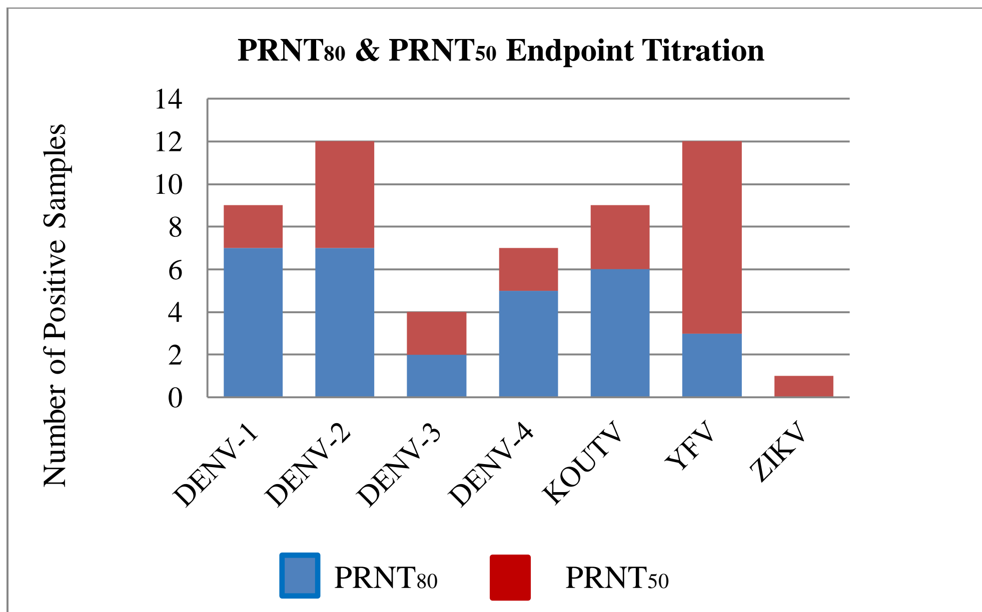

Figure 2.5 $PRNT_{80}$ and $PRNT_{50}$ endpoint titration of serum samples to diverse arboviral serotypes circulating in West Africa. Blue denotes ($PRNT_{80}$) high level of neutralization and red represents ($PRNT_{50}$) moderate plaque formation reduction.

Table 2.2 $PRNT_{80}$ neutralization titers to arboviruses.

Sample #	DENV-1	DENV-2	DENV-3	DENV-4	KOUTV	YFV	WNV	ZIKV
G – 105^{-1}	≥ 1/320							
G – 151^{-1}	≥ 1/320							
G – 216^{-1}	≥ 1/320							
G – 224^{-1}	≥ 1/320							
G – 116^{-1}		≥ 1/320						
G – 137^{-1}		≥ 1/320						
G – 140^{-1}		≥ 1/320						
G – 179^{-2}		≥ 1/320						
G – 190^{-1}		≥ 1/320						
G – 056^{-1}			≥ 1/320					
G – 204^{-1}			≥ 1/320					
G – 084^{-12}				≥ 1/320				
G – 143^{-1}				≥ 1/320				
G – 186^{-1}				≥ 1/320				
G – 201^{-1}				≥ 1/320				
G – 077^{-1}					≥ 1/320			

Table 2.3 $PRNT_{50}$ neutralization titers to arboviruses.

Sample #	DENV-1	DENV-2	DENV-3	DENV-4	KOUT	YF	WNV	ZIK
G – 152^{-1}	≥ 1/320							
G – 232^{-1}	≥ 1/320							
G – 142^{-1}			≥ 1/320					
G – 110^{-1}					≥ 1/320			
G – 055^{-1}					≥ 1/320			
G – 049^{-1}					≥ 1/320			
G – 061^{-1}							≥ 1/320	
G – 108^{-1}							≥ 1/320	
G – 119^{-1}							≥ 1/320	
G - 154^{-1}								≥ 1/320

Table 2.4 $PRNT_{80}$ (*) and $PRNT_{50}$ (**) neutralization titers to arboviruses

Sample #	DENV-1	DENV-2	DENV-3	DENV-4	KOUT	YF	WNV	ZIK
G – 163^{-1}	≥ 1/320*					≥ 1/320**		
G – 205^{-1}	≥ 1/320*					≥ 1/320**		
G – 213^{-1}	≥ 1/320*					≥ 1/320**		
G – 129^{-2}		≥ 1/320*		≥ 1/320**				
G – 155^{-1}		≥ 1/320*				≥ 1/320**		
G – 173^{-2}				≥ 1/320*		≥ 1/320**		
G – 037^{-1}		≥ 1/320**			≥ 1/320*			
G – 062^{-1}		≥ 1/320**			≥ 1/320*			
G – 130^{-1}		≥ 1/320**			≥ 1/320*			
G – 144^{-1}			≥ 1/320**		≥ 1/320*			
G – 121^{-1}					≥ 1/320*	≥ 1/320**		
G – 047^{-1}		≥ 1/320**				≥ 1/320*		
G – 181^{-1}				≥ 1/320**		≥ 1/320*		
G – 174^{-2}		≥ 1/320**				≥ 1/320*		

Table 2.5 Summary of PRNT endpoint high neutralization titers to arboviruses.

	DENV-1	DENV-2	DENV-3	DENV-4	KOUT	YF	WNV	ZIK
$PRNT_{80}$	7	7	2	5	6	3	-	-
$PRNT_{50}$	2	5	2	2	3	9	-	1
TOTAL	**9**	**12**	**4**	**7**	**9**	**12**	**-**	**1**

Discussion

Arboviruses have been isolated from arthropod vectors and vertebrates such as bats, birds, and non-human primates in West Africa where outbreaks of arboviral diseases have been reported [23-28]. Often, patients present with late stages of febrile illness and, since isolation of virus is difficult at this stage, malaria and typhoid fever may be over-diagnosed in the absence of other etiologic agents [29]. Patients presenting to the Mano River Union-Lassa Fever Network

(MRU-LFN) health care facility were tested for malaria and Lassa fever and, when those agents were ruled out, were given a diagnosis of fever of unknown origin.

Our initial screening of patient serum at the $PRNT_{50}$ level indicated that any of the four serotypes of DENV was likely underdiagnosed and could be an etiologic agent of these fevers of unknown origin. Due to the cross reaction of antibodies, we performed the endpoint titration test to get a more specific description of the immune status of these patients. Interestingly, we found evidence that not only are the four serotypes of DENV co-circulating in this area, but KOUT and YF viruses are likely circulating as well. While KOUT has been reported in other vertebrates, this is the first report of transmission to the human population. The number of patients with strong neutralizing antibody ($\geq$80%) further indicates that transmission of KOUT is occurring in this region and that PRNT results are not due to cross-neutralization.

One patient was found to have moderate neutralizing antibody to ZIKV. Although the level was only moderate ($\geq$50%, but $\leq$ 80%), there was no cross reaction in any of the other patients' serum samples, leading us to conclude that this is actual neutralization and not cross-reactivity of antibodies. However, the number of patients with neutralizing antibody to YFV may overemphasize the relative importance of YF in the area, as vaccination to YF is widely used, and antibody titers are known to persist.

Our study is not without limitations. Power outages in Africa posed a challenge to maintaining frozen sera and proper storage of specimens and reagents. This potential obstacle could have resulted in the deterioration of antibodies, which raises the possibility that even our report underestimates the frequency of infection in patients [29]. While the PRNT does not differentiate IgM from IgG antibody, there is speculation that the infections by these flaviviruses caused the febrile illness, demonstrating the importance of arbovirus surveillance in the areas.

Reports suggest that arboviruses circulate frequently in Sub-Saharan Africa [29]. Through PRNT assay confirmation, we have provided evidence that neutralizing antibodies to DENV, YFV, ZIKV, and KOUTV exist in patients in Sierra Leone. Endpoint titrations confirmed high levels of antibodies neutralizing DENV 1, 2, and 4, YFV and KOUTV, and moderate levels to DENV-3 and ZIKV (Figure 2.4).

The $PRNT_{50}$ DENVs results from the laboratory added to the report of DENV circulation in West Africa. By taking into consideration the cross-reactivity of serum antibodies to multiple flaviviruses [14], we determined that a $PRNT_{80}$ endpoint titration gave more specific results, especially important in the context of multi-serotype viruses such as DENV and the general cross-reactivity of flaviviruses. Our endpoint $PRNT_{80}$ results of the patients from Kenema Hospital in Sierra Leone suggests that the flaviviruses DEN, YF, and KOUT viruses circulate in the region and are likely the etiological agent of at least some of these fevers of unknown origin. Additionally, we are the first to confirm the circulation of a human serum neutralization of KOUT virus, a previously understudied and undiagnosed arbovirus of medical importance. More detailed study of the arbovirus presence in this region is needed to detect the possibility of transmission of these and other arboviruses into the human populations.

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Chapter 3: Flow Cytometry-based Assay for Titrating Koutango virus and Immuno-Fluorescence Assay

Introduction

The serological testing of blood samples on arboviruses determines the PRNT. As the gold standard for measuring the biological parameter of in vitro DENV neutralization, the PRNT data can be correlated to determine serum levels of protection from virus infection [1]. However, this process is very laborious, poorly automatable, and requires several hours to several days to complete. Additionally, some virus isolates do not form a solid cytopathic effect on cell monolayers presenting difficulties for cells growing in suspension [2].

The early events that occur during the interaction between the virus and the cell can have reflective influence on the disease outcome. Defining the factors that effect this interaction could lead to improved understanding of disease pathogenesis and influence strategies for vaccine or therapeutic development. Although different methods have been developed to calculate the amount of diverse infectious viruses in a variety of samples, specific advancements for this particular interaction would be valuable. Recent marked improvements in fluorophores development [3-5] and imaging technology [6] have helped visualize and basically understand the KOUTV-target cell interaction, leading to a reduction in millions of infections of unknown etiology that occur annually.

Many of the techniques implemented in Flow Cytometry-based Assay (FACS) are centered on the identification of infected cells at the single cell level using immunodetection of structural viral proteins (glycoproteins) [7-10]. The KOUTV external scaffold consists of envelope glycoprotein (E) dimers protecting the nucleocapsid shell containing a positive sense single-strand RNA genome [11]. These protein subunits on the virus surface can thus be labeled with an amine reactive dye and detected by the FACS machine or pictured through immuno-fluorescent microscopy by a simple method of labeling of KOUTV with Alexa Fluor 488. Flow

cytometry can be used to estimate the degree of labeling for fluorophores that can be excited and detected by the FACS machine. Alexa Fluor dyes have high photostability and are less pH-sensitive than the common dyes, the fluorescein and rhodamine [4], making them perfect for studies on cellular uptake and endosomal transport of the virus. The conjugation of Alexa Fluor dye did not affect the recognition of labeled KOUTV by virus-specific antibody and its putative receptors in the host cells [12]. Such titration procedures have already been developed for many other viruses such as influenza viruses, adenoviruses, HIV-1, SV40, human coronaviruses, hepatitis A virus, as well as for recombinant and/or virus-like particles [7, 9, 10, 13-21].

Basically, the FACS technique calculates the virus titer from the proportion of infected cells (i.e. positively labeled) after exposure of a given number of indicator cells to a given virus suspension volume. The hypothesis is that a single virus particle infects one single cell. However, at an extremely high multiplicity of infection (MOI) such an hypothesis becomes inaccurate, leading to an underestimation of virus titers. Certainly, the higher the MOI, the higher the probability of multiple viruses infecting a single cell becomes. Similarly, during the assay secondary infections of cells by newly formed viruses lead to overestimation of the virus titer.

We have monitored the conditions for the use of flow cytometry for virus quantification with a specific focus on DENV-2 and KOUTV, while taking into consideration the subsequent parameters: (i) cells infected by a particular virus must be easily distinguished from the uninfected cells by the emitted fluorescent signal above the auto-fluorescence background; (ii) in order to avoid secondary infection, the measurement should be best conducted at, or just before to, the conclusion of the first viral replication cycle. The assay could be optimized to end near the duration of a single replication cycle [16]. This method was validated by the correlation found in the DENV-2 and KOUTV FACS results.

In a favorable comparison, this assay was concluded in one day or less compared with the nearly one week period for PRNT methods in use for DENV and KOUTV serological assays. In order to validate this rapid assay for DEN and KOUT virus titrations, visualization confirmation of viral particles by Indirect Immuno-fluorescence Assay (IFA) to the corresponding viruses was accomplished. An increasing amount of virus samples quantified in correlation with the cumulative time-point technique showed a strong connection, indicating the accuracy of the novel method. This technique was applicable using MOI 0.1 for both viruses.

Although plaque assays for titrating KOUTV are suitable for analyzing the DAK ArD5443 Suckling mouse 8 (30525) strain three days post inoculation (dpi), flow cytometry is an innovative improvement that follows the infection at the early stage and determines the viral titer [9, 22-25]. The flow cytometry-based assay can also be used to detect KOUTV in clinical samples and to measure the ability of the virus to infect a diverse number of cell types [26-31]. The PRNT and the viral neutralization assay are being replaced by FACS for many arboviruses and could be introduced to KOUTV infection testing as well. Here, we report on an in vitro technique with FACS for titrating KOUTVs and on IFA confirming the early interaction event between the virus and cells.

Materials and Methods

Cells and Viruses

African Green monkey kidney cells were maintained in 1X M199 Earle's supplemented with 10% FBS and 2% P/S/F at 37°C in a 5% CO_2 environment.

The stock viruses KOUTV DAK Ar D 5443 Suckling mouse 8 (30525), May 21, 1989, and DENV-2 16803 Thailand were used for the FACS experiment. The KOUTV was obtained

from Robert B.Tesh, M.D. from the Center for Biodefense and Emerging Infectious Diseases, University of Texas Medical Branch (CBEID-UTMB).

Antisera and Fc Blocking Reagent

The mouse anti-flavivirus group antigen monoclonal antibody 4G2 (IgG2a) (Millipore, Temecula, CA) was used as the primary antibody. Alexa Fluor 488 goat anti-mouse IgG (Millipore, Temecula, CA) was used as the secondary antibody. The IgG antibody is labeled with a fluorescent Alexa Fluor 488 dye which reacts with IgG heavy chains in all classes of immunoglobulin light chain from the mouse. Human Fc Blocking reagent supplied in the buffer containing stabilizer and 0.05% sodium azide was purchased from Miltrnyi Biotec GmbH.

FACS Titration Assay

Infection of Vero Cells

Vero cell culture was seeded onto a 75 ml flask and incubated at 37°C in a 5% CO_2 environment until cells were approximately 90 to 95% confluent. Cells were harvested by aspirating the medium after the addition of 10 ml of PBS (1%; pH7.2). Cells were gently swirled to rinse the cell monolayer and the fluid was aspirated. This procedure was repeated two to three more times. The 0.1 MOI KOUTV concentration diluted in BA-1 diluent (Hank's M-199 salts, 1% bovine serum albumin, 350 mg/l sodium bicarbonate, 100 units/ml penicillin, 100 mg/l streptomycin, 1mg/l amphotericin B in 0.05 M, Tris, pH 7.6) to a final volume of 100 µl was added to the cells. The virus-cell complex was incubated at 37 ° C for 1 h, and plates were rocked every 15 min. The medium was removed, and cells were washed in phosphate-buffered saline (PBS). With 10% FBS and 2% P/S/F, 10 ml of 1X M199 was added to the flask, and the virus-cell complex was incubated for further labeling and visualization after 6, 12, 18, and 24 h at 37°C in a 5% CO_2 environment.

FACS Procedures on Vero Cells

After 6, 12, 18, and 24 h, the medium was aspirated and the cells were washed and trypsinized (detached from the bottom of flask) and re-suspended into PBS. An aliquot of cells was counted in Trypan blue using a hemocytometer. The remaining cells were centrifuged at 400 rcf for 5 min and re-suspended, fixed and permeabilized in 250 µl of 1:10 cytofix/cytoperm solution (BD Biosciences, San Jose, CA) diluted in dH_2O and set on ice for 20 min. The cells were centrifuged at 400 rcf for 5 min and washed twice before re-suspending in 100 µl of wash buffer that was added to 5 µl of mouse anti-flavivirus group antigen monoclonal antibody 4G2 *primary antibody* (1:20) and set for 20 min on ice. The cells were again centrifuged at 400 rcf for 5 min, washed twice, and later re-suspended in the wash buffer solution before 100µl of the 1:500 diluted goat anti-mouse Alexa Fluor 488 *secondary antibody* (BD Co.) was added. Then, the cells were set for 20 min on ice in darkness. The cells were later centrifuged at 400 rcf for 5 min, washed twice and a final 450µl of PBS was added to the tubes, vortexed, and transferred into 48-well plates for testing in the FACScan flow cytometry analysis. For each sample, at least 10,000 events were recorded.

Infection of U937Monocytic Cells

Cell cultures were counted in Trypan blue and suspended into a RPMI 1640 medium with 10% FBS and 2% P/S to a concentration of 2 x 10^6 cells per well. A volume of 0.1 MOI KOUTV was inoculated onto the suspended cells and incubated at 37 $^{\circ}$C for 2 h in a 5% CO_2 environment. After centrifugation for 5 min at 400 rcf, the supernatant was discarded and the pellet was re-suspended in wash buffer. The cells were washed twice. Then, 10 ml of RPMI 1640 medium with 10% FBS and 2% P/S were added to the tube, and the cells were incubated for 24, 48, 72, 96, and 144 h at 37 $^{\circ}$C in a 5% CO_2 environment.

FACS Procedures on U937 Monocytic Cells

This procedure is similar to the vero cells titration assay except with longer incubation times (24, 48, 72, 96, and 144 h) and the addition of 10 µl of human FcR Blocking reagent after the fixation and permeabilization procedure. Incubation with FcR Blocking reagent increases the specificity of antibody labeling, thus improving the purity of target cells by blocking the binding of antibodies to the Fc receptor of human Fc receptor-expressing cells.

Indirect Immuno-Fluorescence assay (IFA)

Infection of vero and monocytic cells with KOUTV and DENV-2 followed the same procedures as the FACS titration assay.

IFA Procedures on Vero Cells

A volume of 5 µl of vero cells infected with KOUTV and DENV were washed, trypsinized, and dispensed onto Teflon-coated, 12-well slides. The slides were air dried under the hood for 30 min, then fixed in chilled acetone for 15 min. After removing the slides from the acetone bath and air drying for approximately 30 s, the wells were overlaid with 10 µl of mouse anti-flavivirus group antigen monoclonal antibody 4G2 *primary antibody* (1:50 dilution). The slides were incubated in a moist thermomixer chamber at 37 ° C for 30 min followed with three washes in PBS. The bound antibody was detected in the dark with 10 µl of goat anti-mouse Alexa Fluor 488 *secondary antibody* (1:100 dilution), incubated in a moist thermomixer chamber at 37°C for 20 min, and washed three times in PBS. To stain the nuclear material, mounting medium containing DAPI fluorescent was added to each well and covered with a 22x50 mm cover slip (BioMerieux). After overnight solidification of the mounting medium, the slide was examined under a confocal microscope.

IFA Procedures on U937 Monocytic Cells

This procedure is similar to the vero cells IFA except that 10 µl of Human FcR Blocking reagent are added before the mouse anti-flavivirus group antigen monoclonal antibody 4G2 *primary antibody*.

Results

A prompt, improved, precise, and consistent technique for titration of KOUTV was developed based on the detection of virus infected cells by flow cytometry. The kinetics of the method delineate the number of infected cells in defined time points allowing for the estimation of the duration of the replication cycle, and therefore, the ideal infection time. The assay was developed to quantify DENV and KOUTV using antibody labeling of viral glycoprotein.

FACS

In order to correlate fluorescence reactions with biological events during virus replication, KOUTV-infected vero cells and U937 cells were harvested as described and reacted with mouse anti-flavivirus group antigen monoclonal antibody (4G2) and visualized with goat anti-mouse Alexa Fluor 488.

As seen in Figure 3.1, the vero cell line monoclonal antibody detected KOUTV antigens by 6, 12, 18, and 24 hours post infection (hpi) with 1%, 11.5%, 21.6%, and 80% infection rates, respectively. DENV antigens in the vero cell line were detected also by 6, 12, 18, and 24 hpi with infection rates of 1%, 9.6%, 21.6%, and 80%, respectively (Figure 3.2). The U937 monocytic cells detected KOUTV antigens by 96, 120, and 144 hpi with infection rates of 1%, 94% and 96% after respectively (Figure 3.3).

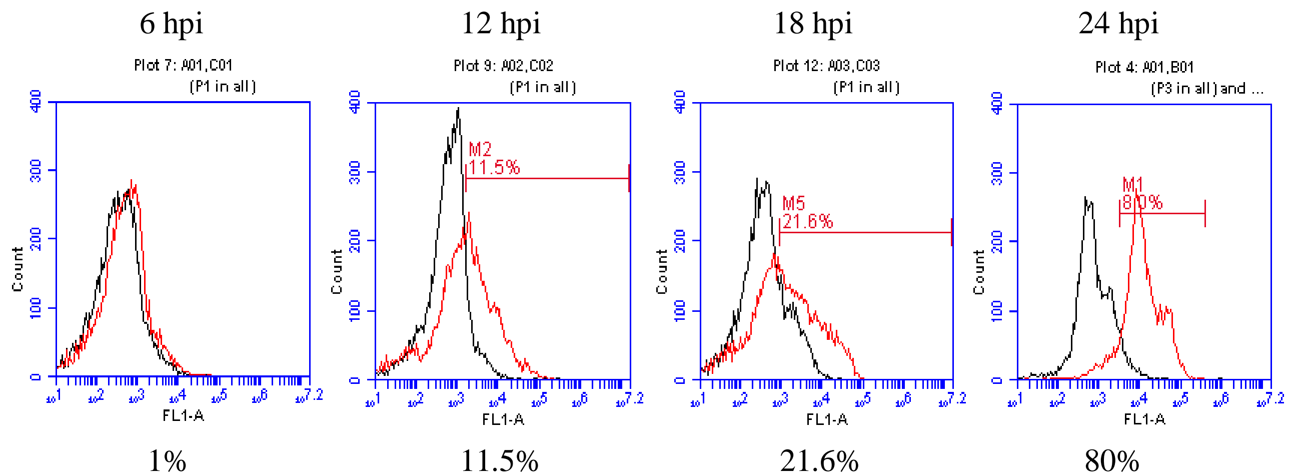

Figure 3.1 Koutango virus FACS on vero cells. The graphs illustrate the replication cycle kinetics of KOUTV on vero cells showing kinetic similarities to DENV replication cycle.

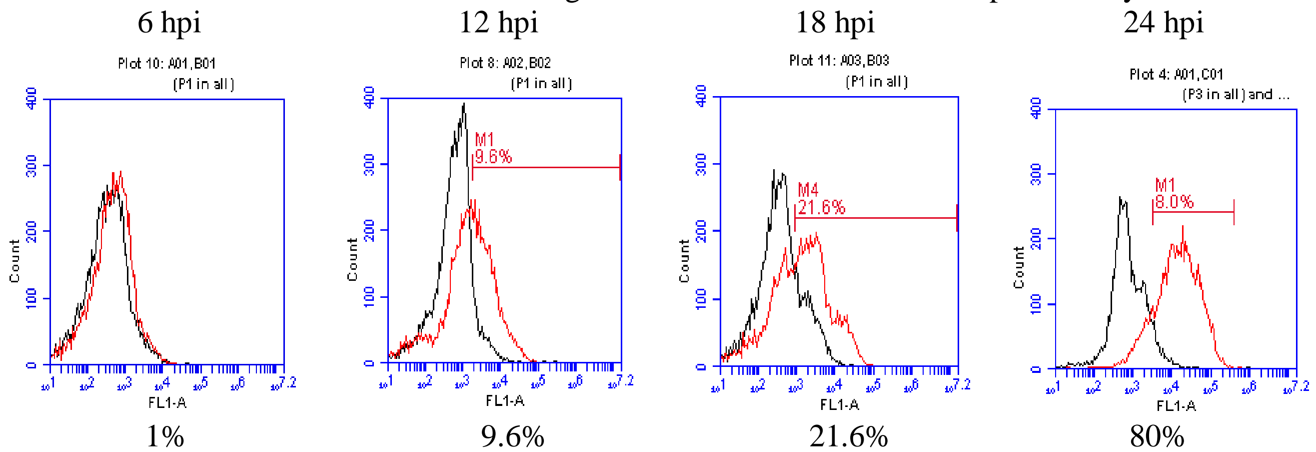

Figure 3.2 Dengue virus FACS on vero cells. The graphs illustrate the replication cycle kinetics of KOUTV on vero cells.

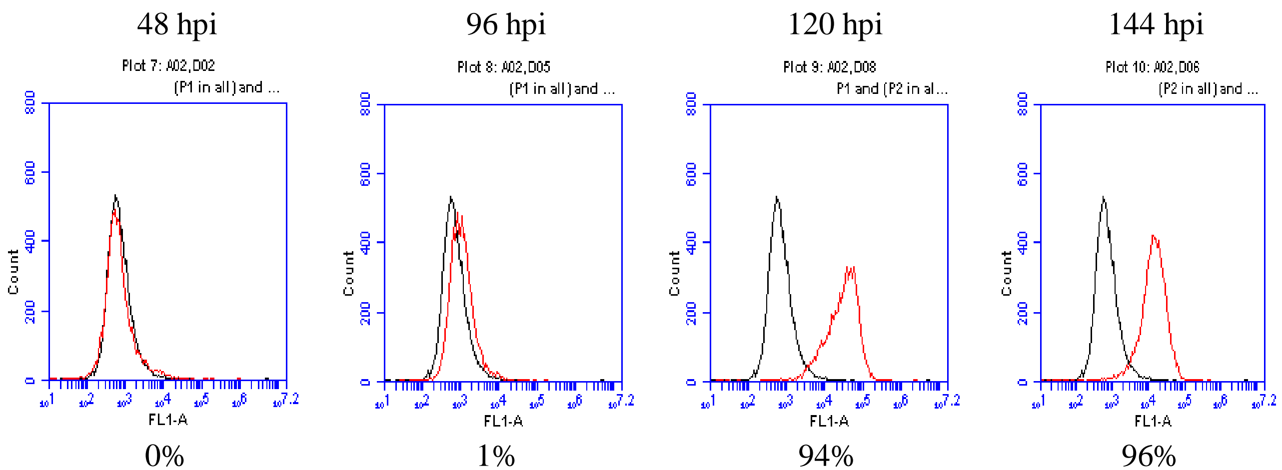

Figure 3.3 Koutango virus FACS replication cycle kinetics on U937 cells.

IFA

The KOUTV and DENV infection was confirmed by inoculation in vero cells and in U937 cells. The cytopathic effect (CPE) of KOUTV in vero cells appeared between 1 to 3 dpi. The cells became round and enlarged, then small aggregates appeared; after several hours to days, multinucleated giant cells, syncytia, and many degenerated cells and cell debris were identified. Some cells showed necrosis and became detached from the cell culture at later stages of infection. The KOUTV in U937 was characterized by the ability of these cells to enlarge and twist, increasing the number of viral particles within and outside the cells after 3 to 5 dpi.

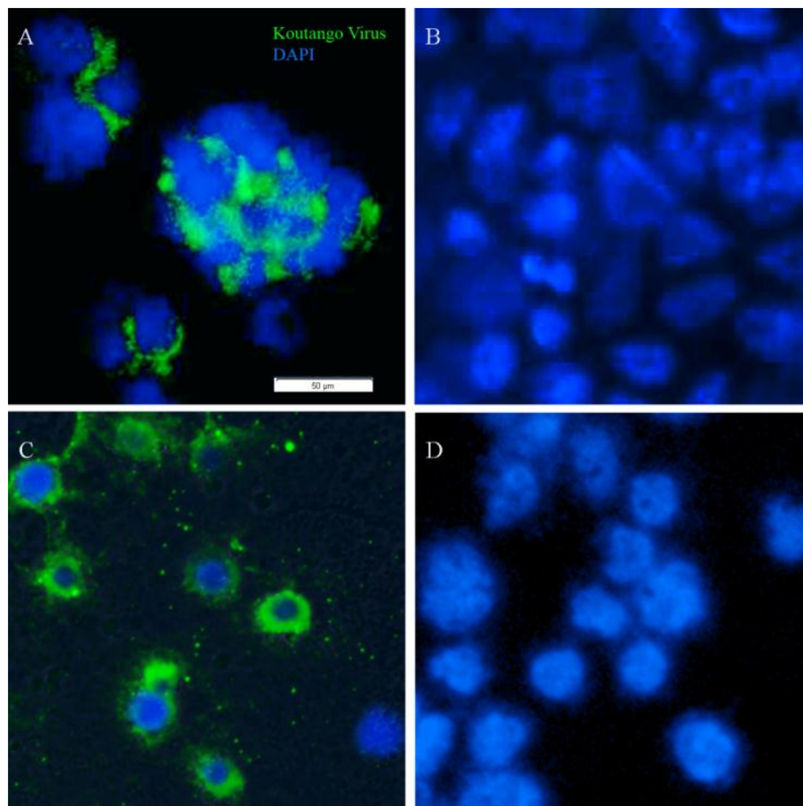

Figure 3.4 IFA of KOUTV on vero and U937 cells after incubation for 24 h and 144 h, respectively. Stained blue is the cell nucleus after DAPI fluorescent of mounting medium. Stained green is the cytoplasmic viral fluorescence. The bar scale applies to all assays.
A = KOUTV after 24 h incubation in vero cells, B = Vero cell negative control,
C = KOUTV after 144 h incubation in U937 cells, and D = U937 cell negative control.

Infected cells were harvested and tested by IFA using the mouse anti-flavivirus group antigen monoclonal antibody 4G2 *primary antibody* (1:50 dilution), goat anti-mouse Alexa Fluor 488 *secondary antibody* (1:100 dilution), and covered by DAPI stain for nuclear visualization. Positive cultures exhibit clear positive viral cytoplasmic fluorescence.

With IFA stain in vero cells, KOUTV and DENV were detected at MOI 0.1 by goat anti-mouse Alexa Fluor 488 after 6, 12, 18, and 24 hpi. In U937 cells, KOUTV was detected after 96, 120, and 144 h. It was found that with the increasing in the number of days that the number of infected cells was greater. These results indicate that both the primary and the secondary antibodies can be used in the early detection of KOUT and DEN virus-infected vero and KOUTV-infected U937 cells.

Discussion

As presented, the assay takes only 24 hours to complete for the DENV and KOUTV strains. The step-by-step FACS procedure used can be applicable to accurately quantify any other virus of similar importance, including the viral genetic product being confirmed by IFA. In order to compare and validate the KOUTV FACS and IFA testing, we simultaneously tested DENV and KOUTV.

Early laboratory confirmation of the interaction between the suspected KOUTV and the cells facilitates identification and control of KOUTV circulation in West Africa as well as other arboviruses that still go unnoticed, limiting the spread of other related febrile illnesses and reducing the incidence of outbreaks. Virus isolation is crucial, especially in early viraemia [32, 33], in not only providing information concerning the virus demarcation but also preserving the virus isolates derived from different clinical manifestations for future virological and molecular

epidemiological studies [29]. Detection of virus antigens is generally faster and 2-to-10 fold more sensitive than the quantification of infectious virions by using a plaque assay [34].

We have demonstrated in this study that the FACS testing methodology can successfully be used in the early detection of virus-infected vero cells from virus stock preparations and from patient serum samples using both the IFA staining and flow cytometry. Flow cytometry and IFA, which detects viral antigens either on the surface or within infected cells, has been successfully used in the rapid detection of herpes simplex virus and rotavirus in clinical samples after virus amplification in tissue culture [24, 25]. Two major factors, the permeabilization method using cytoperm and cytofix and the selection of directly or indirectly stained monoclonal antibodies (MABs), are involved in the detection of intracellular virus using flow cytometry. We developed the IFA and flow cytometry methods to detect the KOUTV antigen at the higher percentage of KOUT virus antigen-positive vero cells within the less time course. This was consistent with the findings of the flow cytometry on vero and U937 cells using panflavivirus 4G2 as primary antibody and the Alexa Fluor 488 as secondary antibody.

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Chapter 4: Koutango Virus Vector Competence by *Aedes aegypti* Mosquitoes

Introduction

Aedes aegypti mosquitoes, the main DENV vector, originated from Africa. In the 17th and 18th centuries, the virus spread to many countries in Africa and other tropical areas worldwide [1, 2]. Several other *Aedes* mosquito species, including *Ae. albopictus*, *Ae. africanus*, and *Ae. luteocephalus*, found in Africa, are potential vectors of diverse arboviral infections. Urbanization is a major element enabling the increase of *Aedes* spp. mosquito populations [1]. Since the 1950s, a three-fold increase in urban human population density has occurred in Africa, larger increases have occurred in Asia and the Americas [3]. With these demographic changes and subsequent increases in *Aedes* spp. populations, biting rates seem sufficient to result in outbreaks in Africa [4] and for the reemergence of diverse arboviral infections as well as the spread of other arboviral diseases throughout the tropics and sub-tropics globally [4].

In 1968, KOUTV was first isolated in Senegal in an unnamed *Aedes* species whereby implicating mosquitoes in the KOUT virus transmission cycle. Utilizing this previous research, we hypothesize the pan-tropically distributed *Ae. aegypti* mosquito (the major vector involved in the transmission of other flaviviruses) as the competent vector for KOUTV as well. In order to better characterize the potential for this emerging virus to be transmitted in the same sylvatic, urban or/and intermediate cycle as DEN and YF, we tested the *Ae. aegypti* mosquito vector competence for KOUTV.

Materials and Methods

Mosquitoes

The mosquito strain used in the experiment was *Ae. aegypti* (Linnaeus) Rockefeller from SVM-LSU. In the water environment, mosquito nymphs were gathered into cartons at the

density of 100 mosquitoes per carton and kept in an incubator at 75-80% humidity, 28°C, and a 16:8 light:dark regime. After the adult mosquitoes emerged, they were fed sugar water until blood-feeding time. During the remaining period of the experiment after the blood feeding, mosquitoes were supplied with the same sugar-water solution as stated above.

After separating mosquitoes by sex, 187 female *Ae. aegypti* mosquitoes were fed a blood meal of 10^9 plaque forming units per ml (pfu/ml) of KOUTV while 128 female *Ae. aegypti* mosquitoes were fed a blood meal of 10^6 pfu/ml. Both were tested for infection and dissemination rates.

Blood Meal and Mosquito Processing

After 3 to 5 days post emergence (dpe), mosquitoes were fed an infectious blood meal titer of 10^6 and 10^9 pfu/ml. The blood meal consisted of bovine blood in Alsevier's anticoagulant (Hemostat, Dixon, CA) mixed in a 2:1 blood-virus proportion in a total volume of approximately 3 ml per carton at 37°C. It was kept warm via the Hemotek device (Discovery Workshops, Arrington, Lanceshire, UK) during the 45 min of blood feeding.

Mosquito Infection

Mosquitoes were then sorted and only fully engorged females, identified by the presence of red blood in the abdomen visible to the naked eye, were kept for further experiments. All other mosquitoes were discarded. Mosquitoes were sampled at 3, 5, 7, 9, and 11 dpe for infection and dissemination status.

The infection rate was the percentage of all mosquitoes tested having infected bodies. The dissemination rate was the percentage of mosquitoes with infected bodies that also had infected legs. These rates have been used to assess the vector competence of DENV and many other arboviruses in mosquito vectors [5-8]. During the dissemination rate study, mosquito legs

were separated from the body and put into separate vials containing 900 µl of BA-1 diluent [9]. After homogenization at 20 Hz for 2 min using the tissuelyzer (Qiagen), the RNA was extracted using the MagMax-96 kit (Ambion) on a King Fisher nucleic acid extraction, according to the manufacturer's instructions (Thermo Scientific). The samples were then tested for the presence of KOUT viral RNA via qRT-PCR using the following protocol: RT step (1 cycle) 48°C for 2 min, 95°C for 2 min, amplification and data recording step (40 cycles) 95°C for 15 sec, 60°C for 30 sec. Primers were designed and obtained via Integrated DNA technologies with 5' FAM fluorophore and 3' Black-Hole quencher for KOUTV.

Virus Assays

The strain utilized in this experiment was the KOUTV DAK Ar D 5443 Suckling mouse 8 (30525) May 21, 1989 received from Robert B. Tesh, M.D. from the CBEID-UTMB. The low-passage viral strain was propagated by inoculating 100 µl of viral stock in the T-75 flask of confluent Vero cells. After the 45 min incubation, 10 ml of M199-1X medium with 10% FBS and 2% P/S/F was added. For the harvest of virus at the peak level when more than 90% of cells were infected, the T-75 flasks were incubated for four days at 37°C in a 5% CO_2 environment. A plaque assay was developed for the viral standard curves and concentrations; titers were verified throughout the experiment, including the qRT-PCR blood-meal testing as previously described [10]. The viral stocks were stored at -70°C. The SuperScript III One-step qRT-PCR kit (Invitrogen, Carlsbad, CA) was the option of choice as per manufacturer.

Table 4.1 Primer and probe sequences for Koutango virus DAK Ar D 5443. The sequences are on 5'→3' direction.

Strain	Forward Primer Sequence	Reverse Primer Sequence	Probe Sequence
DAK Ar D 5443	accaggaggcaagatttacg	cgctttggttatccgtgtg	accaggaggcaagatttacgcagaccgctggct gggacacacggataaccaaagcg

Results

Midgut Infection Dynamics and Legs Disseminated Infection

The midgut is one of the most important barriers for viral infection, replication, and transmission [11]. In order to assess the infection and disseminated rate, the abdomen was dissected from the legs. Mosquitoes fed a KOUT viral concentration of 10^9 pfu/ml of blood developed midgut infections of 4.45%, 31.91%, 24.14%, 46.67%, and 83.33% on 3, 5, 7, 9, and 11 dpe, respectively; while the disseminated infection was assessed at 2.22%, 8.51%, 17.24%, 10.00%, and 55.56% on 3, 5, 7, 9, and 11 dpe, respectively. Mosquitoes fed a KOUT viral concentration of 10^6 PFU/ml of blood did not develop any infection.

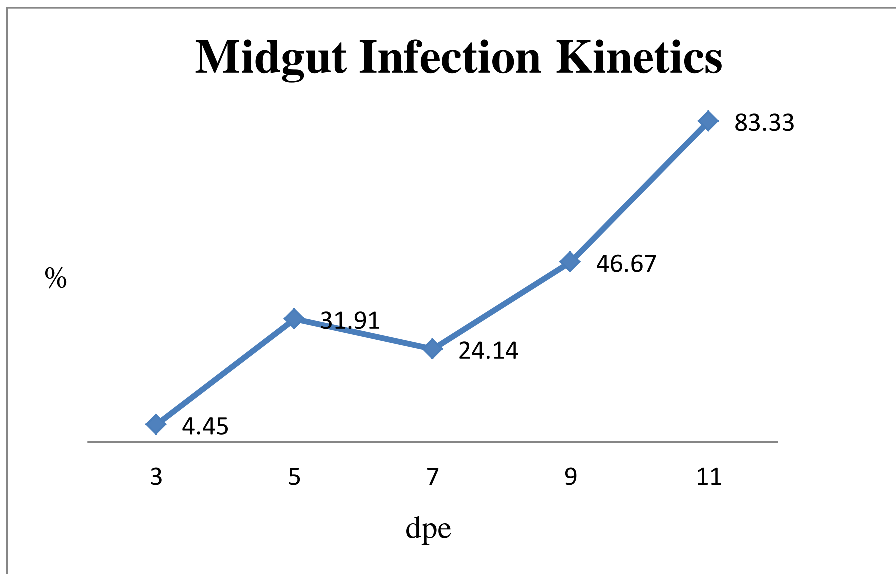

Figure 4.1 Graph of midgut infection rates after 3, 5, 7, 9, and 11 dpe.

Susceptibility to Infection

After 11 dpe at 10^9 pfu, 55.56% of mosquitoes were susceptible to infection; however we did not detect virus in any bodies or legs of the *Ae. aegypti* infected with a viral concentration of 10^6 PFU/ml of blood. The patterns of viral dissemination were similar to those for infection

rates, with *Ae. aegypti* mosquitoes having the highest dissemination rates at 11 dpe. This variation indicates a moderate midgut escape barrier for KOUTV in this species of mosquito.

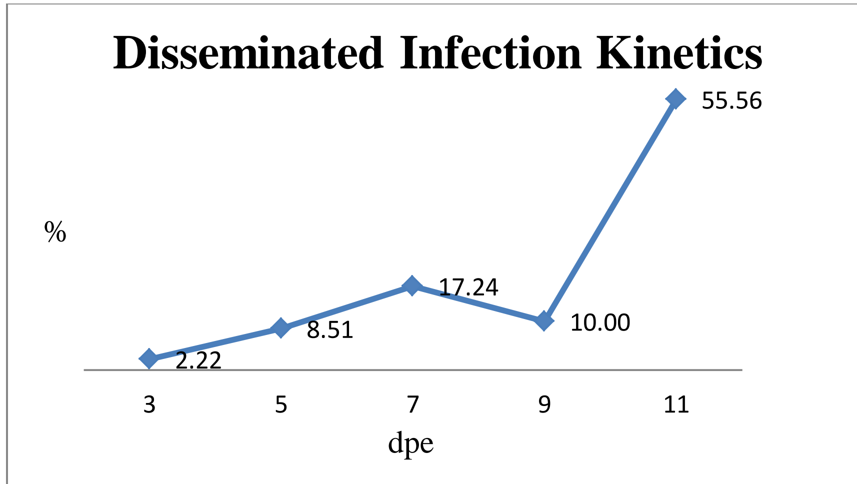

Figure 4.2 Graph of dissemination rates after 3, 5, 7, 9, and 11 dpe.

Table 4.2 Dissemination rates and sample sizes for Koutango virus at 3,5,7,9, and 11 dpe.

Strain	**Origin**	**Dissemination rates (sample size)**				
		3 dpe	**5 dpe**	**7 dpe**	**9 dpe**	**11 dpe**
DAK Ar D 5443	Senegal, human	2.22 (45)	8.51 (47)	17.24 (29)	10 (30)	55.56 (36)

Table 4.3 Table of Koutango virus infected and uninfected *Aedes aegypti* mosquitoes in the abdomen and legs after 3, 5, 7, 9, and 11 dpe.

dpe	Abdomen		Legs		Total
	uninfected	infected	uninfected	infected	
3	43	2	44	1	45
5	32	15	43	4	47
7	22	7	24	5	29
9	16	14	27	3	30
11	6	30	15	21	36
Total	119	68	153	34	187

Statistical Analysis

The data were coded as a binary response using the SAS version 9.1.3. The code "1" was used for the mosquito pool that was positive while the code "0" for the mosquito pool that was negative. All tests were applied the confidence level of 95%; a stepwise selection process was invoked to cull out non-significant effects from the model.

Discussion

The important role of *Ae. aegypti,* the vector of flaviviruses: DENV, YFV, and presumably KOUTV in West Africa can be explained by the competence of this mosquito species in the uptake, development, and transmission of those flaviviruses. This vectorial competence is determined by the intrinsic (genetically determined) and extrinsic (vary spatially) factors such as the insects physiology, immune defense system, biting and resting habits, and the microclimate of its habitat [12]. Mosquito strains of *Ae. aegypti* in Africa have homogenously revealed low susceptibility to all 4 DENV serotypes in laboratory settings [13-15]. In addition, it has been documented that there are different predispositions of the vector to different DENV genotypes; *Ae. aegypti* mosquitoes tend to be more predisposed to infection with DENV-2 of the Southeast Asian genotype than to the American genotype [16]. Similarly, it has been defined for YFV suggesting as an explanation for the absence of YF in Asia because of the reduced vector competence of *Ae. aegypti* mosquitoes strains [17, 18]. The reduced DENV vector competence in Africa may explain some of the presumable low prevalence of DENV infection in Africa, even though this explanation must be confirmed in appropriate studies. [15, 19, 20]. Experimental studies with *Ae. albopictus* mosquitoes have demonstrated that geographic variations in susceptibility to DENV infection occur among different species [14, 15]. Differences in host

preferences and reduced vector competence, which decreases the probability of sustained disease transmission, make *Ae. albopictus* mosquitoes appear to be less efficient as epidemic vectors [21].

Related to the vector competence research on WNV Turrel (2006) concluded that the ability of the mosquito species to transmit the virus by bite is dependent on transmission rate and that the detention of the virus from a mosquito does not confirm the vector competence of WNV. Although, the mosquito transmits the virus in the laboratory, the species does not have to necessarily play a significant role in nature. The transmission of a virus in nature by arthropod vectors depend on factors such as population density of the mosquitoes, susceptibility of amplifying hosts, environmental temperature, and the feeding preferences and habits [22]. In Sierra Leone, the viral fitness of *Ae. aegypti* mosquitoes to YF, DEN, and KOUT viruses is an important factor in determining if the vector can transmit the virus between hosts. The species, *Ae. Aegypti,* may be an important vector for KOUTV in one particular geographical area but may not be so important in other areas.

In nature, KOUTV circulates in a rodent-mosquito cycle involving an *Aedes spp.* vector. However, *Aedes spp.* and perhaps the cosmo-tropical mosquito *Ae. aegypti,* may serve as a bridge vector by becoming infected while feeding on non-human primates, toward furthering the anthropophilic KOUTV cycle. Indeed, the *Ae. aegypti* mosquito serves as the most important domestic vector of the YF and DEN virus urban cycle [1, 23]. Hence, it is essential to account for the vector feeding preference change to humans, depending on season and host availability. Reports by Trpis mention coconut holes, rock holes, snail shells, tins, and tires as the more common breeding sites for *Ae. aegypti* mosquitoes in the East African region, particularly in Tanzania [24]. This conclusion may apply for Sierra Leone in West Africa, as well.

However, to determine the potential of the *Ae. aegypti* mosquito to become involved in transmitting KOUTV, it would be necessary to ponder not only laboratory transmission testing, but also its abundance of *Ae. aegypti* mosquito in Sierra Leone, the reservoir/definitive host-feeding preference, the association with other viruses of similar transmission cycles, such as YF and DEN virus, and whether KOUTV has been isolated from *Ae. aegypti* species under natural conditions [22].

In the laboratory, the development of midgut and disseminated infection confirms that the midgut and escape barriers [25] are the principal factors for vector competence. The mosquito species, *Ae. aegypti,* were infected by ingestion of a virally loaded blood meal and tested on 3, 5, 7, 9, and 11 dpe for infection and dissemination rates. Maintaining them for a longer period of time at 28°C, increases dissemination. Although, we did not test *Ae. aegypti* transmission rate for KOUTV by testing the salivary gland barrier, it is assumed that due to the lack of evidence on salivary gland infection barrier, mosquitoes are capable of transmission once the virus has disseminated into the hemolymph out of the midgut [5, 8, 25].

Blood meals of 10^6 pfu/ml seem not to infect the mosquito *Ae. aegypti* or perhaps the mosquito immune system successfully clears the infection. Additional studies are needed to determine the impact of environmental temperature and reservoir/definitive host availability for the *Ae. aegypti* mosquito ability to transmit KOUTV in the same way as DEN and YF viruses. By having an urban cycle vector habit and circulating in a highly unpopulated area of Sierra Leone, KOUTV still does not seem to have a massive impact to infect humans and to be detected.

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Chapter 5: Koutango Virus Infection of Interferon Regulatory Factor, IRF 3/7 Double Knockout Mice

Introduction

In 1968, KOUTV, an arbovirus, was first isolated from the blood of wild rodent reservoirs *Tatera kempi* and *Mastomys* from the family Muridae in West, Central, and East Africa [1]. The virus is transmitted in nature by the mosquito vector *Aedes spp.* Although no human case of KOUTV infection in nature has been reported to date, a single infection from a laboratory accident of a worker was confirmed in humans [2]. Its capacity for infecting humans still needs to be investigated. From the accidental infection, it was confirmed that KOUTV causes a mild febrile illness in humans presenting as a two-day fever accompanied by achiness, retro-bulbar headache, and erythematous eruption on the flanks [2]. Investigating transmission and factors that could contribute to the spread of this virus are vital to regulating and understanding virus expansion. Modeling of KOUTV infection *in vivo* may be complicated by the lack of clinical manifestation of naturally-infected humans, perhaps adding to the difficulty of the virus in successfully establishing infections in immunocompetent mice. Studies involving KOUTV and knockout (KO) mice do not exist. AG129 mice models have most often been used to model DEN disease and pathogenesis, especially the severe manifestations [3-5]. Mouse model constraints encountered are transient viremia levels or not reaching adequate levels that represent DENV infections in humans; the laboratory adapted virus strains that are necessary to establish infection and/or that routes of exposure do not resemble natural routes (i.e. intravenous or intracranial inoculations) [6].

Toll Like Receptors (TLR) trigger the induction of type I IFN (IFN-alpha/beta) by providing a crucial mechanism of anti-viral defense [7]. The mechanism of anti-viral defense activates two transcription factors belonging to the interferon regulatory factor (IRF) family, IRF-3, and IRF-7. TLR-3 and TLR-4 induce IFN-beta by activating IRF-3; TLR-9 induces IFN-alpha and IFN-beta through IRF-7, at least when engaged by type A CpG oligonucleotides

(CpG-A) in plasmacytoid DC (pDC) [8, 9]. It is demonstrated that TLR-9 induces IFN-beta when engaged by type B CpG oligonucleotides (CpG-B) in myeloid DC and macrophages. This response is independent of IRF-3/7 and requires another IRF family member, IRF-1. IRF-1 is recruited by TLR-9 through the adaptor MyD88. Deficiency of the TLR-9→IRF-1→IFN-beta pathway results in compromised anti-viral responses *in vitro* and *in vivo* [10]. It demonstrates that TLR induces IFN-alpha or IFN-beta responses by activating distinct IRF, depending on the TLR ligand and the cell type. These distinct TLR-IRF pathways allow the immune system to modify its responses to viral pathogens (Figure 5.1) [11, 12].

Although there is only one IFN-β gene, there are many members of the IFN-α gene family, including murine and human pseudogenes, which are all located on the same chromosome (9p in humans and 4q in mice) [13, 14]. During viral infection, transcriptional induction of IFN-α/β genes is accomplished by the activation of two transcription factors of the IRF family, IRF-3 and IRF-7 [15-18]. Viral infections lead to the phosphorylation of the constitutively expressed IRF-3 at its carboxyl-terminal region, converting then to its active form [37, 38]. The phosphorylated IRF-3 then undergoes nuclear translocation interacting with co-activators CREB binding protein (CBP) and histone acetyltransferase p300, and primarily activates the IFN-β promoter [19-23]. Once IFN-β is produced, it leads the cell to activate interferon-stimulated gene factor 3 (ISGF3), which in turn induces IRF-7 gene expression by binding to an interferon-sensitive response element (ISRE) in the first intron [53]. Subsequently the newly produced IRF-7 undergoes virus-induced phosphorylation, similar to IRF-3, and activates IFN-α/β promoters [24-27] massively producing IFN-α/β by this positive-feedback loop. In the absence of IRF-7, IRF-3 acts on the IFN-β gene, while IRF-7 acts on both IFN-α and IFN-β genes by forming homodimers, and both IRF-3 and IRF-7 are necessary, working as

heterodimer, for amplifying the induction of IFN-β and certain IFN-α family genes (Figure 5.1) [27, 28].

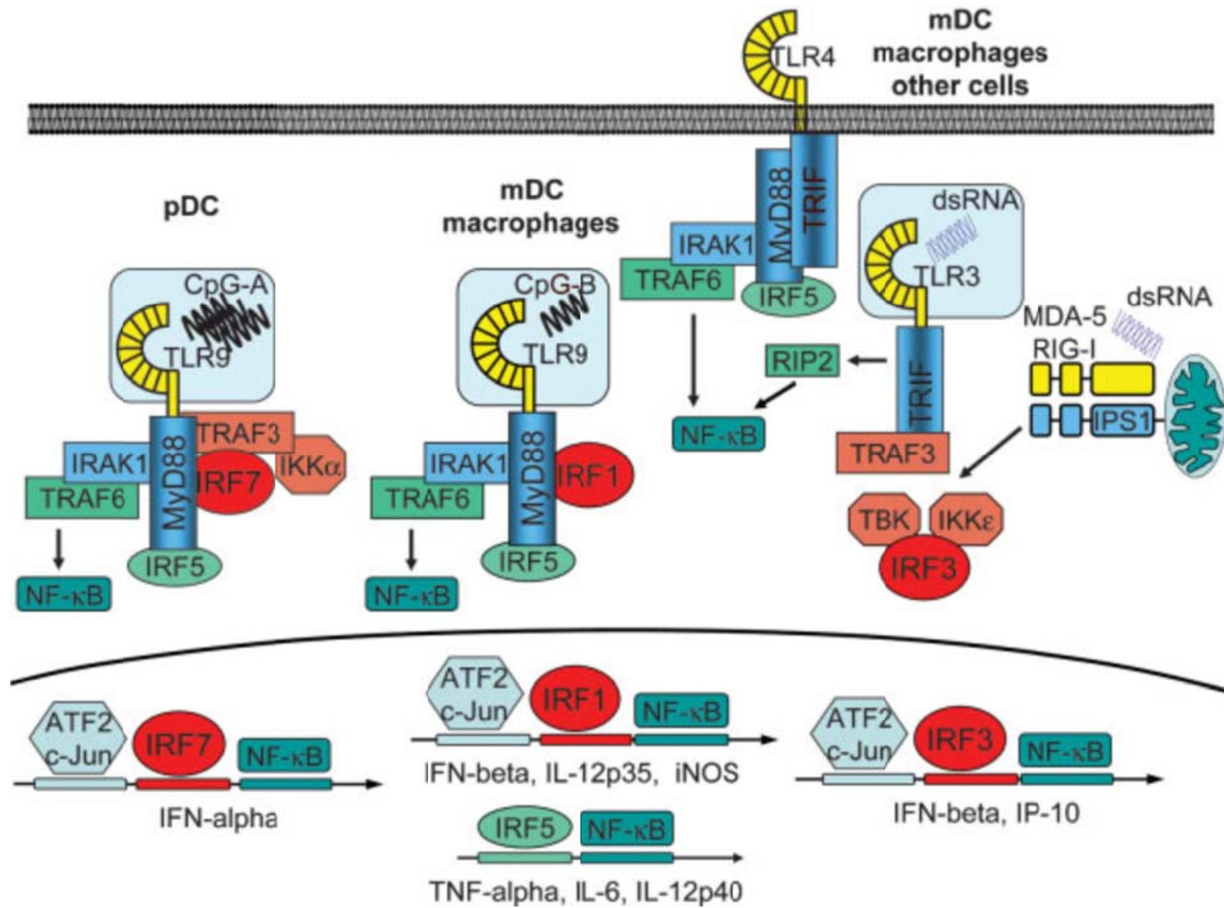

Figure 5.1 Toll Like Receptor (TLR) pathways for type I (IFNα and IFNβ) IFN induction in response to TLR ligand and the cell type. [29]

Mice that are IRF deficient are also deficient in IRF 3 and 7. We hypothesize that type I interferon only, not type II, is critical for the inhibition of KOUTV infection establishment. We tested this hypothesis using an IRF3/7 double knockout strain C57 BL/6 mice for the study of KOUTV infection establishment.

Materials and Methods

Mice

The Mice were a gift from Drs. T. Taniguchi and M. Diamond. Deficient in IRF 3 and 7, IRF3/7$^{-/--/-}$ double knockout (DKO) mice have a significantly abrogated type I interferon; type II interferon and all other immune responses are intact. Cultivated on vero cells, the KOUT virus stock was used to infect the IRF 3/7 DKO mice. All experiments met the approval and conditions of the LSU Institutional Animal Care and Use Committee (approved protocol # 09-077).

Cells

African monkey kidney-derived Vero cells were propagated in M 199-1X supplemented with FBS and P/S/F. Vero cells were grown at 37°C in a 5.1% CO_2 environment. The KOUTV stock DAK Ar D 5443 Suckling mouse 8 (30525) May 21, 1989 received from Robert B. Tesh, M.D. from Center for Biodefense and Emerging Infectious Diseases, University of Texas Medical Branch was the strain utilized in this experiment. The low-passage viral strain was propagated by inoculating 100 µl of viral stock in the T-75 flask of confluent Vero cells. After the 60 min incubation, 10 ml of M 199-1X medium with 10% FBS and 2% P/S/F was added. A protocol consisting of four days of incubation the T-75 flasks at 37°C in the 5% CO_2 environment was applied for the harvest of virus at the peak level when more than 90% of cells were infected. A plaque assay was developed for the viral standard curves and concentrations to determine the dilution of stock virus that yield 100 pfu per 100 µl; titers were verified throughout the experiment, including the one-step qRT-PCR kit (Invitrogen, Carlsbad, CA) as per manufacturer's protocol.

Virus Inoculation and Serum Collection

Four 36-week-old IRF 3/7 deficient mice were anesthetized with isofluorane and then intraperitoneally (i.p.) infected with 100 µl of supernatant (100 pfu/mouse) DAK Ar D 5443 Suckling mouse 8 (30525) from the virus-inoculated cell culture. During the observation period, mice were normally active and did not show any sign of indifference to the virus infection. Mice were bled 3 dpi; blood from two mice was captured via intrathoracic technique (direct from the heart) while blood from the remaining two mice was collected from blood clotting within the thoracic cavity. Blood was allowed to clot for 30 min on bench top, and then centrifuged at 6 rcf for 5 min. Clarified serum was collected and placed into clean tubes for viral quantification analysis.

Virus Detection

Viral RNA was extracted using the QIamp Viral RNA kit (Quiagen) as per manufacturer's instructions. The detection of KOUT viral RNA was performed using one-step Taqman qRT-PCR (Superscript III) with the same protocol as for the KOUTV Vector Competence testing. Primers were designed and obtained via Integrated DNA technologies with 5' FAM fluorophore and 3' Black-Hole quencher for KOUTV. Primers and probes did not cross react among them.

Results

There were significant differences in viremia intensity detected among the four mice infected with KOUTV and the different ways in which the mice were bled. The highest viremia level was recorded from the IRF-deficient mice bled intrathoracically, where two mice infected with KOUTV 100 pfu/ml developed 3.48×10^9 and 1.46×10^9 viral particles/ml against the virus

stock of 2.8×10^8. The mice with intraperitoneally-extracted blood presented lower viremia with KOUTV developing 2.76×10^5 and 4.26×10^5 viral load /ml. All four mice did not exhibit noticeable morbidity or mortality. Thus, the mice appeared to adapt well to the viral infection and no behavioral changes were noted. Mice were bled post i.p. inoculation to assess the antigen response to the KOUTV. Mice inoculated with 100 pfu KOUTV produced measurable amounts of KOUTV antigens detectable by lightcycler Roche 480 as per manufacturer's instructions.

Statistical Analysis

Statistical analysis for this experiment requires a much higher sample size, ranging from 25 to 40 mice. The sample size we worked with reflects the availability of IRF DKO mice in our laboratory. More work to reach the desired conclusions will be done in future.

Discussion

The development of a suitable animal model for the KOUTV study was made difficult by the lack of any change in behavior in response to the viral infection. Although the C57 BL/6 mice support some level of KOUTV replication, this tested model, after KOUTV infection by needle, did not develop any noticeable clinical signs. KOUTV infection in these mice may induce limited KOUTV specific pathogenesis with signs of liver damage seen as increased liver enzymes and increased white blood cell counts [30-32].

We have shown that IRF-deficient mice with IFNα and β responses may be susceptible to a peripheral infection with KOUTV and can serve as a model for KOUT virus isolation and amplification studies in the laboratory. IRF-deficient mice demonstrated 100% survival even though the virus amplification *in vivo* was confirmed. By utilizing this small-animal model, we

have shown that the inoculation with KOUTV Ar D 5443 suckling mouse elicited high levels of virus particles and did not appear to cause harm to the laboratory animals, allowing for the evaluation of KOUTV to replicate in IRF-deficient mice.

We concluded that blood collected from the intrathoracic KOUTV Ar D 5443 suckling mice elicited higher KOUTV antigen titers when compared to the virus stock produced in vero cells. These results indicate that in IRF-deficient mice, the KOUTV antigens were able to significantly increase the viral load, without causing any noticeable change. Whereas IRF-deficient mice have deficit-functioning immune systems, it is evident that they would be unable to clear the viral infections. The viral amplification response of these mice to KOUTV is of interest and, although it is beyond the scope of this study, it should be considered for future investigations. This is the first known report of DKO mice for a KOUTV-infection establishment study.

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Chapter 6: Discussion and Conclusions

Introduction

The most common mode of transmission of arboviruses is by blood-sucking arthropods [1, 2]. Arboviruses have the ability to expand host and geographical range, due in part to the plasticity of the RNA genome [3]. Some arboviruses have developed to colonize humans; others depend on birds or peridomestic animals with human infection resultant from spillover from zoonotic replication cycles. Arboviral maintenance in the tropics is well-suited to year round, hot and humid conditions allowing for the potential emergence as significant human pathogens [4]. In the neotropics alone, greater than 145 distinct arbovirus species have been recognized [4].

Recently, arboviruses are being confirmed to circulate in the human population in Africa. Currently, there is an increase in urban DENV activity, including evidence of a sylvatic cycle of DEN in Senegal and the Republic of Guinea, and YF outbreaks in West Africa [5-8]. KOUTV, like YF and DENV, might be ecologically associated with *Ae. aegypti* mosquitoes as vectors of human disease. It has been experimentally shown that the *Ae. aegypti* mosquito is a competent vector of KOUTV. Field reports also implicate other *Aedes spp.* in the transmission cycle of KOUTV. Viral perpetuation in nature, without the need for alternate vectors, is evidenced by the isolation of YFV from male mosquitoes *Ae. furcifer taylori* in Senegal; vector competence laboratory work proves vertical transmission in *Ae. aegypti [9].* In addition, the perpetuation of the KOUTV might be facilitated by the reintroduction of the virus from distant enzootic foci [10] playing a major role in the spread of the epidemics in West Africa, but being undiagnosed and misdiagnosed with other endemic diseases such as malaria, typhoid fever, and Lassa fever [11]. There have been cyclic resurgences of epidemic activities in the tropics worldwide linked with the geographical expansion of the mosquito vectors and the viruses, leading to the expansion of hyperendemicity, and ending in the emergence of more severe diseases. The emergence of

arboviral epidemics is linked to demographic changes, with exceptional population growth, being the most important factor. Unrestrained and unplanned urbanization causes congested human agglomerations in urban centers. Furthermore, man-made larval habitat, particularly non-biodegradable material, contributes to the development and expansion of the mosquito vector coupled with the ineffective *Ae. aegypti* mosquito control, expanding the geographical distribution and increasing population densities of the *Aedes* mosquito. Increased travel by jet plane and the destruction of the public health infrastructure in many countries supports extension and increased, undetected viral transmission.

Table 6.1 Table of the mosquito vector on selected arbovirus diseases.

Viral Disease	Mosquito Vector		
	Urban	Intermediate	Sylvatic
Yellow Fever	Ae. aegypti	Ae. furcifer, Ae. taylori, Ae. luteocephalus, Ae. vittatus, Ae. opok, Ae. simpsoni	Ae. africanus, Ae. bromeliae
Dengue	Ae. aegypti & Ae. albopictus	Ae. opok,	Ae. furcifer, Ae. taylori, Ae. luteocephalus, Ae. vittatus, Ae. africanus
Zika	Ae. aegypti	Ae. furcifer, Ae. luteocephalus, Ae. vittatus, Ae. apicoargenteus	Ae. africanus,
Koutango	? Ae. aegypti (laboratory)	? Aedes spp.	?Aedes spp.
Usutu	Culex neavei		

Summary of the Results

Implementing surveillance on arboviral diseases is greatly limited by the generic nature of disease presentation. Severe disease can result in hemorrhagic manifestations or neurological diseases, but arbovirus infection practically presents as mild to moderate unrecognized febrile illness [2, 12, 13]. With the lack of undifferentiated clinical presentation and the variety of the

etiologic agents such as malaria, YF, LF, DEN, and many other arboviruses, laboratory support has become a key part of effective surveillance programs. In the laboratory, we first tested 151 human serum samples against all four DENV serotypes at the $PRNT_{50}$ level. In order to increase the specificity of the PRNT against DENV serotypes and possibly other flaviviruses, endpoint titration reactions to all four DENV, YF, ZIK, WNV, USU, and KOUT viruses were performed on a total of 52 positive samples. The highest level of neutralization reduction registered was 7, 7, 2, and 5 samples for DEN-1, DEN-2, DEN-3, and DEN-4, respectively. In addition, 3 samples neutralized plaque formation of KOUTV and 6 samples neutralized YFV plaque formation. By a moderate level of neutralization ($PRTN_{50}$), 2, 5, 2, and 2 samples neutralized DEN-1, DEN-2, DEN-3, and DEN-4, respectively. In addition, 9 samples moderately neutralized the plaque formation of KOUTV, 3 samples neutralized the YFV plaque formation and 1 sample moderately neutralized ZIKV plaque formation.

The direct analysis of KOUTV included FACS and IFA on cell cultures, the vector competence testing of *Aedes aegypti* mosquitoes, and *in vivo* testing of IRF DKO mice. The early events that occur during the interaction between the virus and the cell can have reflective influence on the disease outcome. As the need for understanding disease pathogenesis increases, the development of methods to probe this interaction lead to marked improvements in fluorophores progress [14-16] and imaging technology [17] by visualizing and comprehending basic virus-target cell interaction, decreasing infections of unknown etiology that occur annually. In the FACS and IFA testing of vero cell culture, the virus-cell complex was incubated for further labeling and visualization after 6, 12, 18, and 24 h whereas, in the infection of U937 monocytic cell cultures, the virus-cells complex was incubated for 48, 72, 96, and 144 h at $37^{\circ}C$ in a 5% CO_2 environment with registration of the events occurring within the cells.

Based on previous research from the first isolation of KOUTV in Senegal in 1968 implicating unnamed *Aedes* species mosquitoes in the transmission cycle, we characterized the potential for this emerging virus to be transmitted in the same cycles as DEN and YF by testing *Ae. aegypti* mosquito vector competence for KOUTV. Mosquitoes infected with a KOUT viral concentration of 10^9 pfu/ml of blood developed midgut infections at rates of 4.55%, 31.91%, 24.14%, 46.67%, and 83.33% at 3, 5, 7, 9, and 11 dpe, respectively, while the disseminated infection was assessed as 2.22%, 8.51%, 17.24%, 10.00%, and 55.56% at 3, 5, 7, 9, and 11 dpe, respectively. This variation indicates a moderate midgut escape barrier of this species for KOUTV.

In the testing of IRF DKO mice, there were significant differences in the viremia intensity detected among the four mice infected with KOUTV. The highest viremia level was registered from the IRF-deficient mice bled intrathoracically, whereas the mice whose blood was extracted from a blood clot within the thoracic cavity presented with a lower viremia level compared to the stock virus as shown in the qRT-PCR testing. All four mice did not exhibit noticeable morbidity or mortality by KOUTV antigens.

Conclusions

Our initial screening of patient serum at the $PRNT_{50}$ level indicated that any of the four serotypes of DENV was likely underdiagnosed and could be an etiologic agent of these fevers of unknown origin in West Africa. We found evidence that not only are the four serotypes of DENV co-circulating in this area, but KOUT and YF viruses are likely circulating as well. This is the first report of KOUTV transmission to the human population. The number of patients with neutralizing antibody to YF may overemphasize the relative importance of YF in the area, as

vaccination to YF is widely used, and antibody titers are known to persist. Our endpoint $PRNT_{80}$ results of the patients from Kenema Hospital in Sierra Leone suggest that the flaviviruses DEN, YF, and KOUT circulate in the region and are likely the etiological agent of at least some of these fevers of unknown origin.

Originating from Africa, the *Ae. aegypti* mosquito is the main vector of DENV and YF. In addition, several other *Aedes* mosquito species, including *Ae. albopictus*, *Ae. africanus*, and *Ae. luteocephalus*, are found in Africa and are potential vectors of diverse arboviral infections. With the demographic changes and subsequent increases in *Aedes* spp. populations, biting rates seem sufficient to result in outbreaks [18] of diverse arboviral infections as well as the global spread of other arboviral diseases over Africa, the tropics, and sub-tropics [18]. Although, the mosquito transmits the virus in the laboratory, the species does not have to necessarily play a significant role in nature. The transmission of a virus in nature by the arthropod vector depends on factors such as population density of the mosquitoes, the susceptibility of amplifying hosts, the environmental temperature, and the feeding preferences and habits [19]. In Sierra Leone, the viral fitness of the *Ae. aegypti* mosquito to YF, DEN, and KOUT viruses is one of the factors important for vector transmittance of the virus between hosts. The *Aedes spp.* and perhaps the cosmo-tropical mosquito *Ae. aegypti* may serve as a bridge vector, by becoming infected while feeding on non-human primates, for the supposed further anthropophilic cycle of the KOUTV. Coconut holes, rock holes, snail shells, tins, and tires are the most common breeding sites for *Ae. aegypti* mosquito in Africa.

Investigating transmission and factors that could participate in the transmission of KOUTV are vital to regulating and understanding the virus expansion. Modeling of KOUTV infection *in vivo* may be complicated by the lack of clinical manifestation of naturally-infected

humans and perhaps adding to the difficulty of the virus to successfully establish infections in immunocompetent mice. Serving as a model for KOUTV isolation and amplification studies in the laboratory, IRF-deficient mice in IFN α and β responses may be susceptible to a peripheral infection with KOUTV. Even though virus amplification *in vivo* was confirmed, IRF-deficient mice demonstrated 100% survival. It is evident that IRF-deficient mice, with deficient functioning immune systems, are able to clear viral infections. This is the first report of utilizing DKO mice for a KOUTV-infection establishment study.

In summary this dissertation proposes for an active surveillance approach of arbovirus diseases in tropics and sub-tropics, with a special focus in the West African region through a better understanding of the complexity of the current events in nature. We offered the gold standard technique for serology testing and the traditional virus isolation methodology, as well as a novel laboratory technique for KOUTV identification added to the use of IRF DKO mouse model.

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Vita

Jaime Matias de Araújo Lobo, was born in Calulo-Libolo, Kwanza Sul province, Republic of Angola in Africa. In the 1980s, Jaime went to the former Socialist Republic of Czechoslovakia, where after a total of 11 years of study, he graduated from High School and Faculty of Veterinary Medicine in Brno in the already named Czech Republic. There he not only had remarkable academic moments, but in 1993 left good memories for Czech people through the best movie in Czech cinematography " Kurva hosi and Guten Tak", where with very well-known and all-time best artist Bolek Polivka made a history in the screens.

In the mid-1990s Jaime went back home and started his 5-year career as a veterinarian. His passion for hard work and people in need gave Jaime the opportunity to experience working at the United Nations World Food Programme in Angola. There Jaime saved many lives by working all over the country and providing them with basic necessities for life. In 2005, Jaime reached the top of his professional career in the UN while working for the United Nations Office for the Coordination of Humanitarian Affairs "Technical Coordination Unit," in Angola. He was coordinating the Social Mobilization Group for the dangerous hemorrhagic viral disease "Marburg Outbreak" in Uige province. After the end of an outbreak in his country he felt encouraged to further his education. Today Jaime is more than grateful for the opportunity the American Society and the PBS gave him. Certainly the PhD degree he is seeking gave him more knowledge and made him ready to provide better services towards humanity.