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Raisa Silva Raulino

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**THÈSE POUR OBTENIR LE GRADE DE DOCTEUR
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(TransVIHMI)
L'Unité Mixte Internationale (UMI 233)**

**Revisit Dogmas on Origins and Ecology of
Emerging and Re-emerging Infectious Diseases:
Arboviruses in Africa**

**Présentée par Raisa Clareana SILVA RAULINO
Le [15 Décembre 2021]**

Sous la direction de Martine PEETERS et Ahidjo AYOUBA

Devant le jury composé de

Renato S. de AGUIAR, PU, UFMG, Institut des sciences biologiques, Département de biologie
générale. [Rapporteurs]

Richard NJOUOM, PUDR, Directeur de Recherche RIIP, Service de Virologie Centre Pasteur du
Cameroun [Rapporteurs]

Vincent FOULOGNE, PU, Département de bactériologie-virologie, CHU de Montpellier
[Président]

Marcelo A. SOARES, PA-Chercheur, UFRJ, Institut National du Cancer (INCA) [Examinateur]

Martine PEETERS, DRCE, IRD [Directrice de Thèse]

Ahidjo AYOUBA, DR2, IRD [Co-Directeur de Thèse]



**UNIVERSITÉ
DE MONTPELLIER**

For the woman of my life: my mother, my sunshine
Ana Luiza.

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“The ultimate measure of a man is not where he stands in moments of comfort and convenience, but where he stands at times of challenge and controversy.” – Martin Luther King, Jr.

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Abstract

Arboviruses are responsible for a significant public health burden throughout the world with over 150 viral species having capacity to cause disease in humans. Almost all of them are zoonotic and circulate between vertebrate hosts and hematophagous vectors such as mosquitoes, ticks and midges. Almost all are maintained in sylvatic transmission cycles involving wildlife. Ever since, arboviruses have caused outbreaks around the world and new arboviruses have emerged from their sylvatic reservoirs such as West Nile, Usutu and Zika viruses as the most recent examples. Understanding the emergence of arboviral disease in humans and developing surveillance methods to predict its occurrence depends upon the understanding of the ecology of the different arboviruses and their life-cycles. During enzootic periods, arboviruses survive via sylvatic life cycles which can involve a variety of species of which many are currently not identified, especially in Africa. It is unclear which and how many animal species can contribute to the maintenance of arboviruses in nature, nevertheless the sylvatic/zoonotic cycles of many mosquito-borne viruses are presented with arboreal mosquitos feeding on non-human primates (NHPs). However, this is can be extremely oversimplified because their population numbers and number of susceptible animals at any time, are not enough to maintain sylvatic cycles and other mammals are probably also involved. Thus, the goal of this project is to provide new insights in the animal reservoir or amplifier hosts, the ecology of arboviruses in African countries and document extent of infection rates in animals. We developed a serological tool that allows simultaneous detection of IgG antibodies to multiple arboviruses in a single biological sample. With this highly sensitive and specific multiplex immunoassay, we screened more than 6000 samples from a wide diversity of primate and bat species across Central and West Africa. Primates from Cameroon and Democratic Republic of Congo showed an overall IgG prevalence is below 5%. Bat species across Guinea,

Cameroon and Democratic Republic of Congo showed an overall IgG prevalence of 8.45%. Both, primate and bat samples, reacted with at least one of the ten antigens tested, variable seroprevalences were observed according to collection sites, environmental behavior of the species and for the virus. We conclude that African NHPs are most likely not the only reservoir for the arboviruses tested and the presence of IgG antibodies in bats suggests that they could also play a role in the sylvatic cycle of arboviruses in addition to NHPs.

Key words: Arboviruses, Primates, Bats, Africa, Antibodies, Luminex

Résumé substantiel

Les maladies infectieuses émergentes et ré-émergentes (MIE) représentent une menace épidémique constante pour la politique de santé mondiale ainsi qu'une chaîne étendue de conséquences avec un impact économique et social. Environ 60% des MIEs ont une origine zoonotique et pour la plupart il existe un lien avec la faune sauvage. De ce fait, l'interconnectivité entre l'homme, l'environnement et les animaux représente un risque majeur pour l'émergence des maladies zoonotiques. Cette inter connectivité a augmenté significativement par l'accroissement des activités humaines dans les zones occupées pour la faune sauvage, comme par exemple le récent développement des industries forestières et minières dans les zones forestières reculées et l'augmentation de la chasse et de la consommation de viande de brousse. Ces zones connaissent des changements climatiques et environnementales, menant à l'exposition des humains aux vecteurs d'agents pathogènes tels que les rongeurs, les chauves-souris ou les moustiques qui migrent facilement entre la forêt et les agglomérations voisines. Une étape majeure dans la compréhension, la prédiction et le contrôle du risque des infections zoonotiques émergentes et ré-émergentes, c'est de caractériser la diversité des agents pathogènes afin de documenter l'étendue du réservoir animal à l'interface entre l'homme et l'animal basé sur l'approche « Une seule Santé ».

La grande propagation du moustique *Aedes aegypti* entre le 15^e et 19^e siècles à travers le commerce des esclaves de l'Afrique aux Amériques est responsable de la première émergence documentée des deux virus transmis par les arthropodes en Amérique ; il s'agit du virus de la Dengue et celui de la Fièvre jaune. Avec plus de 150 espèces virales ayant la capacité de causer des maladies chez l'homme, les Arbovirus constituent un problème majeur de santé publique avec un lourd tribut. La plupart d'entre eux sont zoonotiques et circulent entre l'hôte vertébré et des vecteurs hématophages comme les moustiques, tiques et moucheron. La plupart sont maintenus en cycles de transmission sylvatiques impliquant la faune sauvage. Depuis, les

arbovirus ont causé des épidémies dans le monde et des nouveaux virus ont émergé de leur réservoirs sylvatiques comme le virus du Nil Occidental, l'Usutu et le virus Zika, qui sont des exemples récents. Comprendre l'émergence de la maladie arbovirale chez l'homme et développer des méthodes de surveillance afin de prédire son apparition dépend d'une compréhension de l'écologie individuelle des chaque arbovirus et de son cycle de vie. Pendant les périodes épizootiques, les arbovirus se maintiennent via les cycles sylvatiques impliquant une grande variété d'espèces dont la plupart ne sont pas actuellement identifiés, surtout pas en Afrique. Ce n'est pas encore clairement compris quelle espèce animale contribue à la pérennisation des arbovirus dans la faune. Cependant, la plupart d'études incriminent les moustiques arboricoles se nourrissant du sang de primates non humains (PNHs) comme vecteurs majeurs des arbovirus. Mais cela reste dubitatif car le nombre de leurs populations et le nombre d'animaux sensibles à tout moment ne sont pas suffisants pour maintenir les cycles sylvatiques et d'autres mammifères sont probablement impliqués.

Ainsi, l'objectif de ce projet de thèse était de proposer de nouvelles connaissances sur le réservoir animale ou l'hôte amplificateur, l'écologie des arbovirus dans certains pays d'Afrique et documenter l'étendu des taux d'infection chez les animaux. Nous avons développé un outil sérologique qui a permis de détecter simultanément des anticorps IgG contre plusieurs arbovirus dans un échantillon biologique par la technologie Luminex capable de détecter jusqu'à 100 régions d'antigènes dans un seul puits d'une plaque 96 puits à fond plat. Des antigènes à large affinité afin de capturer des anticorps avec réaction croisée des mêmes virus ou des virus apparentés ont été combiné avec antigènes plus spécifiques pour différencier entre les différents virus et sérotypes. Nous avons testé un total de 17 protéines recombinantes (RP) pour sept différents arbovirus. Pour les *Flavivirus* nous avons utilisé les RP dérivées du domaine III de l'enveloppe (DIII) pour la Dengue, le Zika et le virus du Nil et des protéines non-structurel 1 (NS1) de la Dengue, le Zika, l'Usutu, le virus du Nil et la Fièvre jaune. Pour

les *Alphavirus* nous avons utilisé des RP pour l'enveloppe E2 du Chikungunya et O'nyong-nyong mais aussi une RP protéine non-structurale (NSP) pour le Chikungunya. Les protéines recombinantes ont été couplées à des billes magnétiques MagPlex® en différentes concentrations selon chaque antigène. Les dilutions en série des échantillons ont été exécutées à volumes finaux différents aussi bien que différents temps d'incubation et température dans le but d'obtenir « un meilleur signal par rapport au bruit de fond ». Les résultats sont donnés en intensité moyenne de fluorescence (MFI) par bille.

Pour évaluer la sensibilité, la spécificité et la précision, un panel d'échantillons positifs (n=95) et négatifs (n=66) bien documentés a été utilisé. La sensibilité, la spécificité et la précision de chacun des antigènes ont été déterminées par les courbes ROC et la formule Moyenne des contrôles négatifs + 3 fois l'écart type. Dans un panel de 161 échantillons, les antigènes NS1, CHIKV-E2 et WNV-DIII avaient > 95 % de sensibilités et spécificités, à l'exception de NS1-YFV. Pour USUV_NS1 et ONNV_E2, nous n'avons aucun contrôle positif, la spécificité était de 98,5% et 96,97 %, respectivement. Pour les autres antigènes, la sensibilité était faible, limitant l'intérêt de leur utilisation dans des études de séroprévalence. Des réactions simultanées entre les virus homologues et non homologues ont été observées avec une plage comprise entre 0% et 60%. Ensuite, nous avons utilisé les dix antigènes de notre panel hautement spécifiques et sensibles pour dépister plus de 6000 espèces de primates et de chauves-souris à travers l'Afrique centrale et occidentale.

Des milliers d'échantillons bien documentés de primates et de chauves-souris à travers l'Afrique ont été testés pour la présence d'anticorps IgG contre les arbovirus. Les échantillons de viande de brousse, de DBS et de matières fécales des primates collectés dans de nombreux sites différents au Cameroun et en RDC ont été testés. Des échantillons de DBS provenant de chauves-souris frugivores et insectivores en liberté qui ont été collectés dans des sites de repos et d'alimentation dans leur environnement naturel en Guinée, au Cameroun et en RDC ont

également été examinés. Pour l'interprétation des résultats et l'estimation de la prévalence, nous sommes appuyés sur des approches mathématiques et statistiques en l'absence d'échantillons témoins positifs et négatifs pour les différentes espèces animales.

Un total de 3,518 échantillons de 9 espèces de PNHs du Cameroun et de la République démocratique du Congo (RDC) étaient testés contre NS1 (DENV1-4/WNV/ZIKV/USUV), E2 (CHIKV/ONNV) et DIII (WNV) antigènes. Les singes (n = 2,100), la séroprévalence globale variée entre 2% et 5% pour les dix antigènes testés. Nous avons stratifié les singes par leur habitat, les espèces arboricoles ont montré réactivité élevée. Pour les singes du Cameroun, la prévalence des IgG observée contre ONNV-E2 et DENV2-NS1 était de 3.95% et 3.40% respectivement et en RDC, ONNV-E2 (6.63%) et WNV-NS1 (4.42%). Chez les grands singes (n = 1,418) la prévalence des IgG était entre 0 % pour USUV-NS1 à 2,6 % pour CHIKV-E2. Toutefois, une large différence a été observée parmi les sites des collectes et les espèces des grands singes, par exemple 18% (9/40) et 8.2% (4/49) des gorilles étaient réactifs avec CHIKV-E2 ou WNV-NS1, respectivement dans deux différents sites du Cameroun.

Nous avons testé des échantillons de 2,579 chauves-souris, représentant 1,917 frugivores et 641 insectivores, réparties en sept familles et 21 espèces. Globalement, 218/2579 (8.45%) chauves-souris, ont réagi avec au moins un des dix antigènes testés. La prévalence la plus élevée a été observée contre USUV avec 2,3% (59/2579), suivi de 1,9% (49/2579) et 1,35% (35/2579) pour les sérotypes DENV 4 et 3, respectivement. La séroprévalence globale variait selon les pays et les sites de collecte, 11% (151/1376) au Cameroun, 3,5% (20/565) en RDC et 7,3% (47/638) en Guinée. Les taux les plus élevés ont été observés chez *Hypsignathus monstrosus* (17,9%), *Rousettus aegyptiacus* (16,4%) et *Eidolon helvum* (10,7%) et chez les espèces de la famille insectivore des *Molossidés* (7,8%-8,9%). Enfin, nous avons observé des changements de séroprévalence au cours de l'année dans les colonies d'*E. helvum* et *H. monstrosus* qui pourraient être liés au comportement des populations.

En conclusion, les primates africains ne sont probablement pas les réservoirs naturels pour les arbovirus testés, mais plutôt des hôtes intermédiaires et la présence d'anticorps IgG chez les chauves-souris suggère qu'ils pourraient jouer un rôle dans les cycles sylvatiques des arbovirus en complément des primates ou d'autres animaux. En plus, tous deux contribuent au maintien des arbovirus en milieu sylvatique, rural et urbain en Afrique centrale et de l'Ouest. D'autres études de recherche séroépidémiologique et de détection moléculaire chez les animaux, mais aussi chez les humains qui habitent dans les mêmes zones sont nécessaires pour une meilleure compréhension de l'écologie des arbovirus et notamment identifier les réservoirs sylvatiques potentiels afin de prédire leur impact potentiel sur la santé humaine.

Mots clés : Arbovirus, Primates, Chauves-souris, Afrique, Anticorps, Luminex

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Abbreviations

Abs – antibodies
Ae – Aedes
BUNV – Bunyamwera virus
CCHF – Crimean-Congo Hemorrhagic Fever Virus
CF – complement fixation test
CHIKV – Chikungunya virus
CMR – Cameroon
COVID-19 – Corona virus disease 2019
Cx – Culex
DBS – dry blood spot
DENV – Dengue virus
DENV-1 – serotype 1 dengue virus
DENV-2 – serotype 2 dengue virus
DENV-3 – serotype 3 dengue virus
DENV-4 – serotype 4 dengue virus
DHF – Dengue Hemorrhagic Fever
DRC – Democratic Republic of Congo
E2 – envelope 2
ECSA – East/Central/South/African genotypes
EDIII – Envelope domain III
EIDs – Emerging infectious diseases
EEV – Equine encephalitis virus
ELISA - Enzyme-Linked Immunosorbent Assay
HI – hemagglutination inhibition test
ICTV – International Committee on Taxonomy of Viruses
IgM – immunoglobulines M
IgG – immunoglobulines G
IOD – Indian Ocean lineage
JBEV – Japanese Encephalitis
MAYV – Mayaro virus
MBV – Mosquito-borne viruses
MBFV – Mosquito-borne Flaviviruses

MFI – mean fluorescence intensity
mRNA – messenger RNA
NHPs – non-human primates
NS1 – non-structural protein 1
NSP – non-structural protein
ONNV – O’nyong-nyong virus
PAHO – Pan American Health Organization
PRNT – plaque-reduction neutralization test
RP – recombinant proteins
ROC – receiver operating characteristic curve
RT-PCR - reverse transcriptase polymerase chain reaction
RVFV – Rift Valley fever
SFV – Semliki Forest virus
SINV – Sindbus virus
SLEV – St. Louis encephalitis virus
SPONV – Spondweni virus
TBV – Tick-borne viruses
TBEV – Tick-borne encephalitis virus
USUV – Usutu virus
VNT – virus neutralization test
WA – West Africa genotypes
WEEV – Western equine encephalomyelitis virus
WNF – West Nile Fever
WNV – West Nile virus
WHO – World Health Organization
YFV – Yellow Fever virus
ZIKV – Zika virus

Section I: *General Introduction and Objectives*

Chapter I: Emerging and Re-emerging Viruses

I.A. Emerging Infectious and Zoonoses

Emerging infectious diseases (EIDs) are defined as infections that arise or have arisen from new pathogens in a population and whose incidence in humans and in geographic extent has increased during the last two decades and that seem to continue to increase. Moreover, these pathogens that are established and disseminated in the host population may reappear (or re-emerge) due to the recognition of the infectious agent that was not detected after a decline in incidence (Morse 1995; Morens, Folkers, and Fauci 2010; van Doorn H 2014). Since the beginning of the 20th century more than 300 infectious diseases have emerged or re-emerged around the world (Jones et al. 2008).

Emerging and re-emerging infectious diseases represent a constant epidemic threat to the global health policy as well as an extensive chain of consequences with economic and social impact (Binder et al. 1999; Smith et al. 2019; Fisher and Murray 2021; Jones et al. 2008). Over the years several factors have contributed and continue to contribute to emergence and re-emergence of new pathogenic agents and one of them is driven by the increased globalization through mobility, industrialization and technology. This ‘globalization big bang’ generated advances in public health policy and at the same time challenges for global health associated due to rapid environmental and social changes (Bloom and Cadarette 2019; Morens, Folkers, and Fauci 2010; Fisher and Murray 2021; Labonté, Mohindra, and Schrecker 2011; Daly 2021; Heffernan 2018).

Over the last decades, the global community has witnessed several outbreaks with viruses that lead to significant epidemics or even pandemics like the ongoing Coronavirus disease 2019 (COVID-19). The emergence and spread of these EIDs events is due to a combination of many factors such as time, increasing population densities, increased travel, encroachment on natural previously inhabited areas, global wildlife trade, ecological changes

due to agricultural (deforestation) or economic development and climate changes that represent the drivers of human behavioural activities (Morse 1995; Jones et al. 2008; Allen et al. 2017; Daly 2021; van Doorn H 2014; Fisher and Murray 2021; Scheffers et al. 2019; Heffernan 2018).

The majority of EIDs events have a zoonotic origin (60%) and the great majority originate in wildlife which shows that the interconnectivity between humans, the environment and animals represent a major risk for emergence from sylvatic reservoirs (Jones et al. 2008; van Doorn H 2014; Fisher and Murray 2021). Most zoonotic events that affect the human population are caused by viruses with an RNA genome which have high ability to adapt to changing environments and naïve hosts. A factor that facilitated the interactions among host-pathogens and the interspecies transmission of a viral zoonosis was the human encroachment into wilderness habitats for land use and mining industries in remote forest areas and increasing hunting and consumption of bushmeat (Wolfe et al. 2005; Daly 2021). This leads to exposure of humans to insect or other arthropod vectors and vertebrate reservoirs as bats and monkeys that are natural reservoirs of vector-borne diseases with arboviruses, one of the most serious and important EIDs (Morse 1995)(Karesh et al. 2012)(Morens, Folkers, and Fauci 2010).

I.B. Arbovirus

The term ‘arbovirus’ is an acronym for *Arthropod-borne virus*, referring to a diverse group of infectious agents that are transmitted by blood-sucking arthropods (mosquitoes, ticks or sandflies) from an infected individual or from an animal reservoir to susceptible hosts (Young 2018; Kuno and Chang 2005; Hanley and Weaver 2008). One of the most widely spread vectors in the world was *Aedes aegypti* and started with the slave trade in Africa to the Americas during the 15th to 19th centuries. *Ae. aegypti* and a secondary vector, *Ae. albopictus* - mosquitoes that can transmit many arboviruses, expanded throughout the 18th and 19th centuries from African countries due trade routes with Asia, Europe, Central America. The

rising globalization and increased travel, transport and migration lead to the first dengue fever epidemics in different hemispheres (Simmons, Jeremy, et al. 2012; Huang, Higgs, and Vanlandingham 2019; Daly 2021).

During the first 50 years of the 20th century many arboviruses have been identified in animals, humans and arthropods (Hubálek, Rudolf, and Nowotny 2014; Venter 2018). Over the last 30 years, there has been an increase in the emergence and / or re-emergence as well as a spread of this large group of viruses, giving rise to epidemic outbreaks in several regions of the world, with a major impact on international public health (Gould et al. 2017; Girard et al. 2020; Weaver 2018). Today, arboviruses represent a major threat for public health due to the adaptation of vectors in urban regions as well as the population density and their movements facilitated by transport but also due to climatic and environmental changes such as rainfall and temperature which apparently seems to foster mosquitoes to blood-feeding on human rather than animals (Jones et al. 2008; Daly 2021; Girard et al. 2020; Pennisi 2020).

Mosquito-borne arboviruses that have emerged throughout the tropics and caused epidemic outbreaks comprise Zika virus (ZIKV) (Grubaugh et al. 2019; Zanluca et al. 2015); Yellow fever virus (YFV) (Bryant, Holmes, and Barrett 2007; Fernandes et al. 2017); West Nile virus (WNV) (Siconelli et al. 2021; Martin and Simonin 2019); Chikungunya virus (CHIKV) (Rico-Mendoza et al. 2019; de Souza Costa et al. 2019) and Dengue virus (DENV). DENV is the most prevalent arbovirus in the world with 100-400 million infections estimated per year, (Mayer, Tesh, and Vasilakis 2017; Cabral et al. 2021).

I.C. Arbovirus Families

In the past, arboviruses were classified in different serogroups from A to D based on their ability to replicate and transmission through arthropods (De Madri and Porterfield 1974). However, they were reorganized and classified based on their manner of messenger RNA

(mRNA) synthesis (Baltimore classification) and antigenic characteristics (C. H. Calisher and Monath 1988; C. H. Calisher and Karabatsos 1988).

Arboviruses are comprising many taxonomic families with many of them being zoonotic arboviruses, with clinical importance for humans and livestock, that belong to the families of *Flaviviridae*, *Togaviridae*, *Bunyaviridae*, *Rhabdoviridae*, *Orthomyxoviridae* and *Reoviridae* (**Figure 1**) (Gubler 2002; Venter 2018; Go, Udeni B. R. Balasuriya, and Lee 2014).

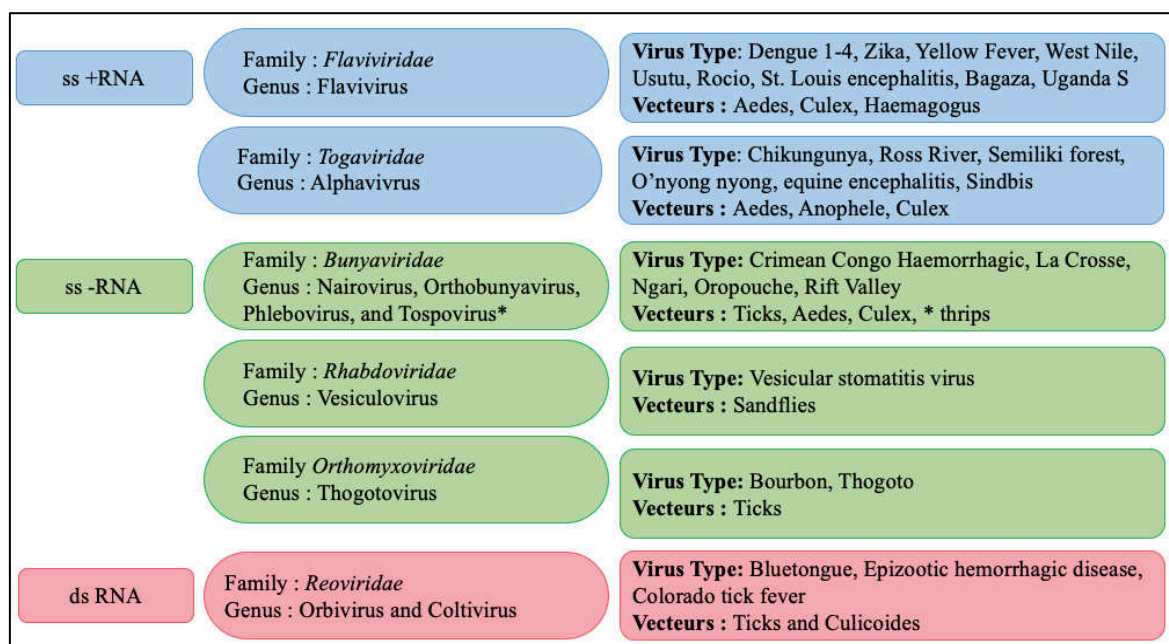


Figure 1: Classification of arboviruses. Families are grouped according to Baltimore classification: ss, single-stranded; + positive-sense; - negative-sense; ds, double-stranded. *Tospoviruses are known to infect plants but are transmitted by arthropods. **Modified from** (Go, Udeni B. R. Balasuriya, and Lee 2014).

To date, more than 500 arboviruses have been registered in the International Catalogue of Arboviruses of which about 150 are known to be pathogenic to humans and the vast majority that have an impact on public health belong to *Flavivirus* and *Alphavirus* genus, and some genera of the *Bunyaviridae* family (Gubler 2002; Young 2018).

I.C.1 Genus *Flavivirus*

The name *Flavi* is derived from the Latin word “*flavus*” that means “yellow”. This refers to the propensity of flaviviruses to cause jaundice in infected humans and from the early research done on the yellow fever vaccine in 1930s (Norrby 2007; Rathore and St. John 2020). The *Flavivirus* genus belongs to the *Flaviviridae* family which encompasses three additional genera unrelated to arboviruses; *Pestivirus*, *Hepavirus* and *Pegivirus*, and are known to cause vector-borne and hepatic disease in humans (Simmonds et al. 2017).

The *Flavivirus* genus currently comprises arboviruses linked to epidemic outbreaks of Dengue, Zika, Yellow fever, West Nile virus and tick-borne encephalitis virus. Globally distributed, well-known *flaviviruses* as well as lesser-characterized *flaviviruses* (Usutu, tick-borne encephalitis and Japanese encephalitis viruses) have been causing outbreaks in animals, humans and infecting up to 400 million people annually (Bhatt et al. 2013; Pierson and Diamond 2020).

The *Flaviviruses* comprise over 50 taxonomically recognized viruses with a very complex ecological network (Simmonds et al. 2017). They are divided in three major clusters: members of non-vector, tick-borne viruses (TBV) and mosquito-borne viruses (MBV) (**Figure 2**). *Flaviviruses* are also classified according to their antigenic similarity into serocomplexes and sub-complexes. Cross-reactions are observed and have been demonstrated by hemagglutination-inhibition (HI) and neutralization titer assay (Kuno et al. 1998; C. Calisher et al. 1989; Li, Barrett, and Beasley 2005).

I.C.1.a. Structure and Genome Organization

Flaviviruses are small (~50 nm) RNA virions, enveloped with a spherical capsid and coated with a lipid membrane derived from the host cell. Mature virions contain both

membrane and envelope coding proteins (M and E) and immature forms contain a precursor membrane protein (prM) (**Figure 3**) (Pierson and Diamond 2020; Simmonds et al. 2017).

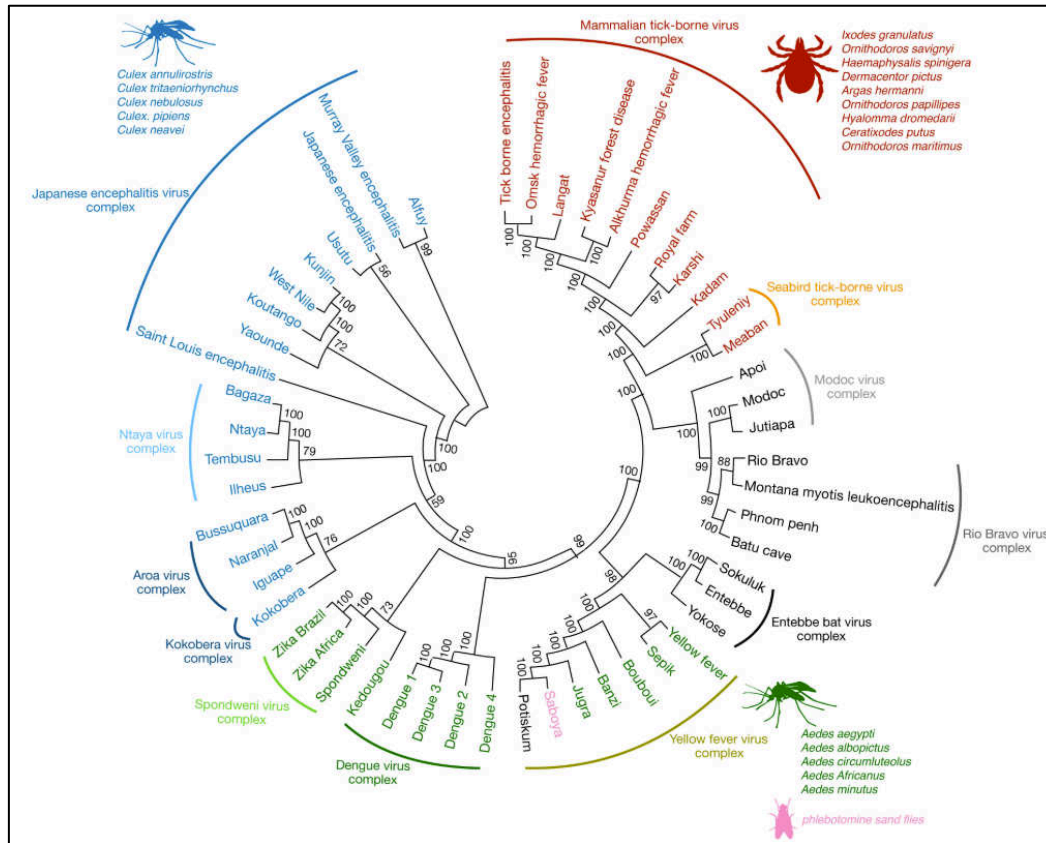


Figure 2: Phylogenetic tree illustrating the antigenic relationships among flaviviruses. Viruses from the same serocomplex are covered by arched lines and common vectors are listed also by colors. Source : *Frontiers in Immunology* (Rathore and St. John 2020).

The *Flaviviruses* genome is a positive-sense unsegmented single-stranded RNA of approximately 9.4-13 kb in length (Shi et al. 2016). The genome consists in a single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTR)s that encodes a polyprotein which results in ten functional proteins (Zmurko, Neyts, and Dallmeier 2015). These proteins are divided in three structural (capsid, C; prM/M, membrane ; envelope protein, E) as well as seven non-structural (NS) proteins that are synthesized in infected cells (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Zhang et al. 2017; Zmurko, Neyts, and

Dallmeier 2015) (Figure 4).

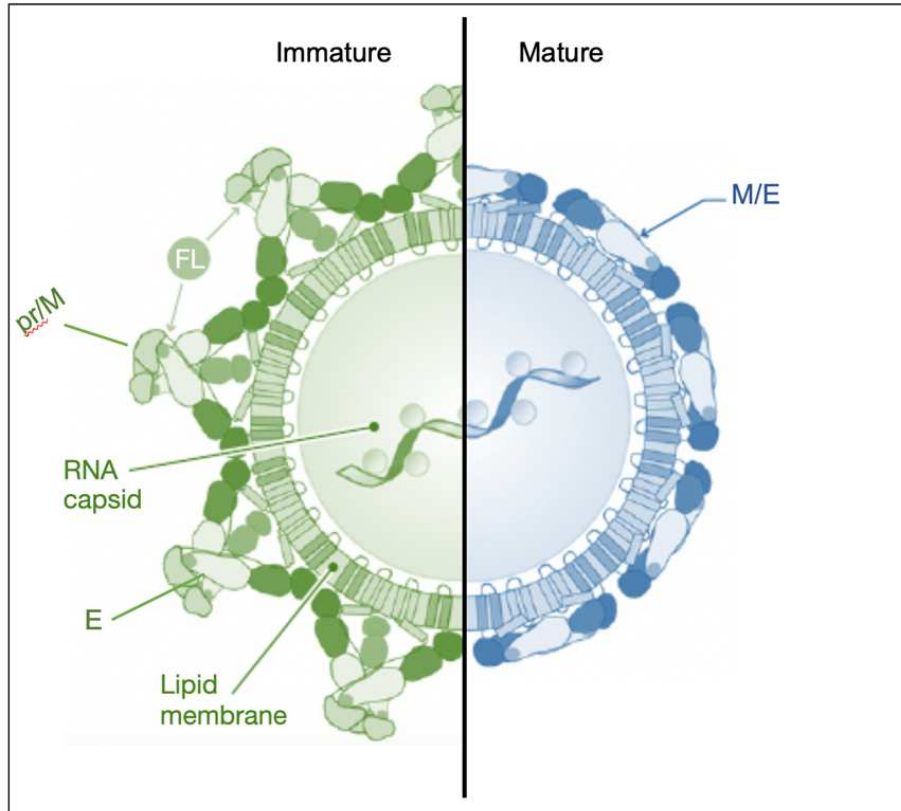


Figure 3: Typical morphology of both mature and immature virions. The structures consist in structural proteins C, E, prM and lipid membrane. Modified from : *EMBO reports* (Rey et al. 2018)

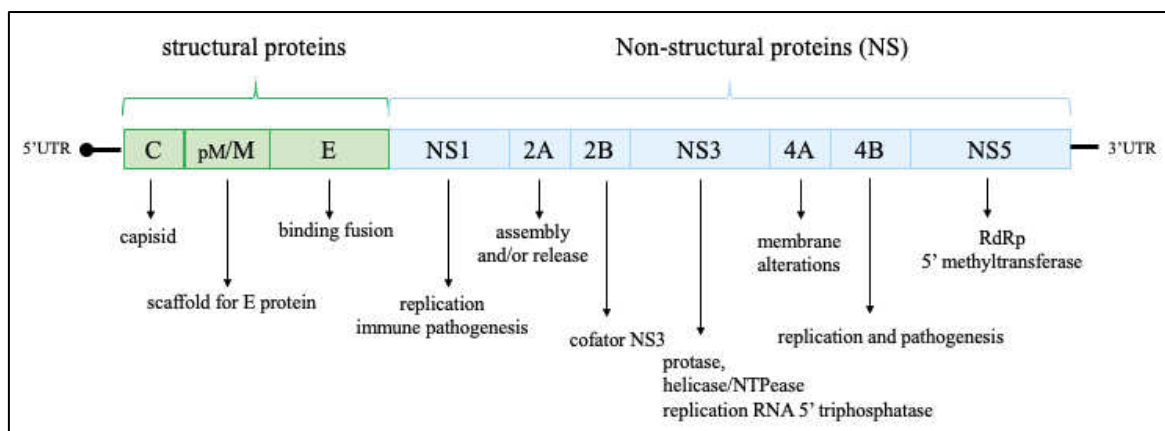


Figure 4: *Flavivirus* genome structure. Modified from: *Reviews in Medical Virology* (Zmurko, Neyts, and Dallmeier 2015).

I.C.2. Zoonotic Flaviviruses

The vast majority of zoonotic and globally important *flaviviruses* are mosquito-borne viruses (MBFV) such as Dengue, Yellow fever, West Nile, Zika but also Usutu virus that was neglected for many years and suddenly emerged in Europe far away from the area where it was initially described (H Weissenböck et al. 2010; Marion Clé et al. 2018). The first cases of these viruses were isolated at the beginning of the 20th century, i.e., Yellow fever virus that 30 years after the first isolation caused one of the most severe epidemics in Africa with 30,000 deaths in Ethiopia (Theiler and Downs 1973; H Weissenböck et al. 2010).

Mosquito-borne flaviviruses are distributed in many continents, affect millions of humans but also are pathogenic for domestic animals, and wildlife with a variety of mosquito species that feed on a wide range of vertebrate hosts (H Weissenböck et al. 2010; Gould et al. 2017).

According to The International Committee on Taxonomy of Viruses (ICTV) the MBFV are composed of 38 viruses that are divided in a “mosquito-borne” and a “probably mosquito-borne” group. They are classified based on genetic and antigenic relationships as well as geographic association, vector, host, ecological characteristics and disease association. (Simmonds et al. 2017) (**Table 1**).

Here we review and further describe only the mosquito-borne flaviviruses that were investigated in this thesis.

Table 1: List of mosquito-borne flavivirus that cause disease in domestic animals, wildlife and humans.

Group	Virus / subtypes or strains*	Abbreviation	Geographic distribution	Vertebrate host	Human disease	Full sequence available	Zoonotic reservoir	Vector
Arao virus	Arao virus	AROV	Venezuela	Sentinel hamster (boain)	Unknown	AY632536	Unknown	Unknown
	Bussaquan virus	BSOV	Brazil, Colombia, Panama	Man, Sentinel Alouatta and mouse	Fever; joint pain; headache	AF013366	Unknown	Culex spp
	Iguape virus	IGUV	Brazil	Sentinel mice, Mammals ?	Unknown	AF013375	Birds ?	Unknown
	Naranjal virus	NILV	Ecuador	Sentinel hamster	Unknown	AF013390	Unknown	Culex spp
Dengue virus	Dengue virus 1	DENV-1	Tropic and subtropics	Humans, NHP	Fever; rash; vasculopathy	U88536	Unknown	<i>Ae. aegypti, Ae. albopictus</i>
	Dengue virus 2	DENV-2				U87411		
	Dengue virus 3	DENV-3				M93130		
	Dengue virus 4	DENV-4				AF126573		
Japanese encephalitis virus	Cacipacaré virus	CPCV	Brazil	Birds, NHP, Equines, Humans	Acute febrile illness one person	KF917536	Unknown	<i>C. pipiens, Ae. aegypti, Anopheles</i>
	Japanese encephalitis virus	JEV	Asia, Australia	Pigs; Horses; Birds	Encephalitis; Meningitis; Fever	M118370	Birds, Pigs	<i>Culex spp</i>
	Koutango virus	KOVU	Senegal	Humans	Fever; rash	AF013384	Unknown	<i>Ae. aegypti</i>
	Murray Valley encephalitis virus	MVEV	Australia, Papua New Guinea	Birds, Humans	Fever; rash; encephalitis	AF161266	Birds	<i>Culex annulirostris</i>
	Ality virus*	ALFV	Australia	Unknown	Unknown	AF013360	Unknown	<i>Culex spp</i>
	St. Louis encephalitis virus	SLEV	Americas	Birds, certain mammals	Fever; encephalitis; paralysis	DQ825916	Birds	<i>Culex spp, variety species</i>
	Usutu virus	USUV	Africa, Europe	Different birds species	Encephalitis; Meningitis; Fever	AY453411	Birds	<i>Culex spp, Mansonia spp</i>
	West Nile virus	WNV	Worldwide	Reptiles, birds, mammals	Encephalitis; Meningitis; Paralysis; Fever	M12294	Birds	<i>C. pipiens, C. tarsalis</i>
	Kunjin virus*	KUNV	Australia, Indonesia, Malaysia	Birds, horses, Humans	Fever; rash; lymphadenopathy	D00246	Unknown	<i>Culex annulirostris</i>
	Yaoundé virus	YAOV	West and Central Africa	Wild Birds, 2 mammals	Unknown	AF013413	Unknown	<i>Culex spp, variety species</i>
Kokobera virus	Kokobera virus	KOKV	Australia, Papua New Guinea	Humans, horses, cattle, macropods	Acute polyarthritis	AY632541	horses ?	<i>Culex spp, Aedes spp</i>
	Stairford virus*	STRV	Australia	Humans, horses, macropods	Unknown	AF013407	macropods ?	<i>Aedes spp, Anopheles spp</i>
Mogaza virus	Bagaza virus	BAGV	Africa, Europe, India	Birds, Turkeys	Fever; acute encephalitis reported	AY632545	Unknown	<i>Culex spp, Ae. aegypti</i>
	Ilhéus virus	ILHV	Central and South America	Rodents, bats, birds, monkeys	Fever; headache; myalgia; encephalitis	AY632539	Unknown	<i>Psorophora, Ochletotatus, a variety</i>
	Rocio virus*	ROCV	Brazil	wild birds, Sentinel mice,	Fever; meningoenephalitis	AF013397	Birds ?	<i>Psorophora, Ochletotatus, a variety</i>
	Israel turkey meningoencephalitis virus	ITV	Israel, South Africa	Turkeys, wild birds	Unknown	AF013377	Unknown	<i>C. pipiens, Phlebotomus papatasi</i>
	Naya virus	NTAV	Africa	Migratory birds, domestic animals	Unknown	JX236040	Unknown	<i>Culex spp</i>
	Tembusu virus	TMUV	Malaysia, Thailand	chickens, ducks, pigeons, sparrows	Unknown	JF95923	Unknown	<i>Culex spp</i>
Yellow fever virus	Zika virus#	ZIKV	Africa, Asia, Americas	Humans, NHP, bats, rodents,...	Fever; Guillian-Barre; Microcephaly	AY632535	Unknown	<i>Aedes, Anopheles, Mansonia</i>
	Sepik virus	SEPV	New Guinea	Unknown	Fever; headache	DQ837642	Unknown	<i>Mansonia</i>
	Wesselbron virus	WESSV	Africa, Madagascar, Thailand	Sheep, goat, cattle, pigs, wild rodents	Fever; myalgia; arthralgia	EU070555	Unknown	<i>edes spp, Culex, Anopheles, Mansonia</i>
	yellow fever virus	YFV	Tropical Africa and South America	Monkeys, Humans	paupic	X03700	Monkeys (ylvatic cycle)	<i>Ae. aegypti, Haemagogus</i>
Kadugou virus	Kadugou virus	KEDV	Senegal Central African Republic	Unknown	Unknown	AY632540	Unknown	<i>Aedes spp</i>
	Banzi virus	BANV	Africa	Human, Cattle, Mastomys	Fever	DQ859056	Unknown	<i>Culex spp, Mansonia spp</i>
Edge Hill virus	Boubou virus	BOUV	Africa	Humans	Unknown	DQ859057	Unknown	<i>Anopheles paludis</i>
	Edge Hill virus	EHV	Australia	Wallabies	Myalgia, arthralgia and fatigue	DQ859060	Unknown	<i>Aedes vigilax, Culex, Anopheles</i>
	Jugra virus	JUGV	Malaysia	<i>C. brachyotis</i>	Unknown	DQ859066	Unknown	<i>Aedes spp</i>
	Soboya virus	SABV	Africa	Bats, NHP, Rodents, Reptilia	Unknown	DQ859062	Unknown	mosquito species
	Potiskum Virus*	POTV	Africa	Experimental	Unknown	DQ859067	Unknown	Unknown
Uganda S virus	UGSV	Africa	Birds, Wild monkeys	Unknown	DQ859065	Unknown	<i>Aedes spp.</i>	

This list was based on the last report of ICTV, updated in 2019 and was completed with literature-based research (Simmonds et al. 2017; Venter 2018; Pierson and Diamond 2020) (Arbovirus Catalog – CDC Division of Vector-Borne Disease (DVBD)). Dark green column represents the six groups of mosquito-borne flavivirus and light green the two groups of probably mosquito-borne flavivirus.

I.C.2.a. *Yellow Fever virus*

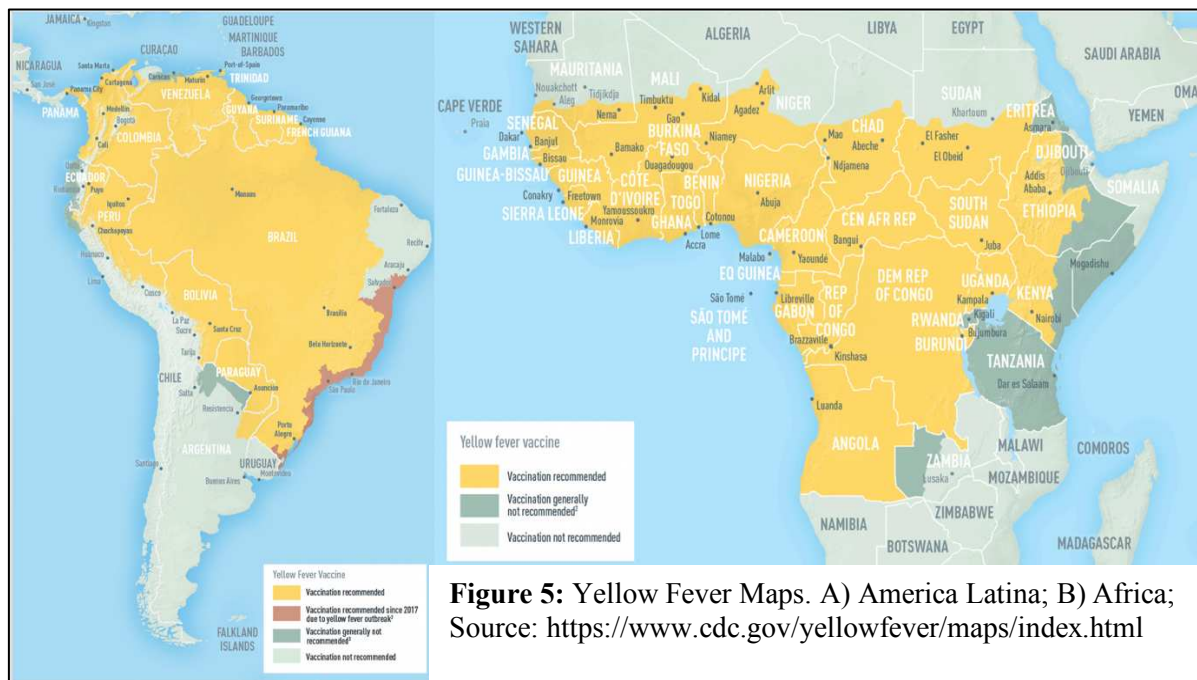
One of the first reports showing yellow fever-like disease is dated from 1495 in Hispaniola as well as the first outbreaks on the Caribbean islands in mid-1647 (Hindle 1932). Thereafter for nearly four centuries (15th to 19th) it caused numerous outbreaks in ports of North and South American cities, but also in Africa and Europe, particularly the Mediterranean coast. The disease spread then further in North America causing significant epidemic outbreaks such as in Philadelphia, where 10% of the population was wiped out by the disease (Rush 1805) and continued to expand throughout the coastal region of the Americas (Holbrook 2017; Bryant, Holmes, and Barrett 2007).

Nevertheless, the ways on how the disease spread and caused terrible epidemics remained unknown for centuries until a Yellow Fever commission was set up at the end of the 19th century. The commission members as well as the military volunteers who participated in the survey, showed that mosquitoes were the main vectors of YFV transmission, being a milestone (Finlay C. J. 1881; Norrby 2007; Holbrook 2017).

Extensive research on YFV was done in mid-1930, like viral isolation studies coordinated by Adrian Stokes and Rockefeller Foundation, showing the viral nature of the agent by inoculating the virus obtained from human samples into monkeys (Stokes, Bauer, and Hudson 1928). In the same period, efforts were made to eliminate *Aedes aegypti* in endemic areas for Yellow fever disease (Africa and Tropical America). However, in certain regions it was not successful, thus showing for the first time that the monkeys were the reservoir host of the virus and illustrating the transmission cycle between humans, monkeys and jungle-dwelling mosquitoes. Other advances were the development of a test by Max Theiler, which measured protective antibodies in mice and humans as well as the development of the attenuated 17D virus strain which gave him the Nobel prize (Norrby 2007).

During the 20th century, the scientific community declared that YFV entered in the New World and Europe through slave trade, like *Ae. aegypti*, in bilges of the ships. This theory was confirmed through evolutionary/molecular and epidemiological studies in the 21st century (Bryant, Holmes, and Barrett 2007) that estimated that YFV strains emerged in Americans about ~400 years ago, which corresponds with the timing of the slave trade. Moreover, the ancestral lineage of YFV in Africa is estimated to have emerged about 3,000 years ago (Bryant, Holmes, and Barrett 2007; Moureau et al. 2015). Genetic studies on *Ae. aegypti* showed that the American mosquitos are direct descendants of the African mosquito population, providing an additional piece on the puzzle of the origin of YFV in the Americas (Gould et al. 2017; Crawford et al. 2017; Tabachnick 1991).

Currently, yellow fever is still endemic in tropical and temperate regions, despite the creation of the vaccine (1937) and the implementation of vaccination programs, which are sometimes poorly managed. According to the World Health Organization (WHO) (WHO 2017) more than 30 countries in Africa and more than 13 in Latin America are endemic areas for YFV, and vaccination is recommended for people traveling to these countries (**Figure 5**). However, even with massive vaccination campaigns in these countries, recurrent outbreaks and re-emergences continue to occur, as for example in Angola in 2016, where no reports of urban YFV outbreaks were reported since 1971. The outbreak in Angola has further spread to the Democratic Republic of Congo, a neighboring country, but also to Kenya through international travel from individuals that were infected in Angola. Moreover, 11 cases of foreign workers infected in Angola were reported in urban centers in China, which were the first cases of travel-associated YFV introduction in Asia (Brent et al. 2018; Wilder-Smith and Monath 2017; Girard et al. 2020). Similarly, outbreaks of YFV in South American countries (Brazil and Peru) led to imported cases of YFV into Europe and the USA (ECDC 2017; Girard et al. 2020; Huang, Higgs, and Vanlandingham 2019).



Most of the endemic regions cited above have experienced the re-emergence and spillover of YFV due to factors that reflect social and surveillance problems: poverty, failure to implement vaccination programs, infected humans near naive populations in urban centers, rural areas with difficult access for vaccination, increased population density, as well as the failure of vector control in tropical and equatorial areas where mosquitoes that transmit the virus are prevalent (Bryant, Holmes, and Barrett 2007; Wilder-Smith and Monath 2017; Pierson and Diamond 2020).

Characterized as a native African virus, YFV virus is transmitted primarily by *Aedes spp* that are widely distributed across the African continent and the *Haemogogus* species that are more predominant in forests from Latin America. Although the two continents are endemic, it is estimated that 90% of the cases occur in Africa, resulting in approximately 130,000 severe cases of YFV as well as approximately 78,000 deaths per year, according to the estimation of a model (Garske et al. 2014; Hamlet et al. 2018). Progressions in disease burden change of YFV were made for 2050 and 2070 in Africa in a context of climate change. Gaythorpe et al., states

that in a less severe scenario that annual deaths will increase by about 93.0% [CI 95% (92.7, 93.2%)] in 2050 (Gaythorpe et al. 2020).

The biome is especially heterogeneous in Africa and Latin America, where recurrent outbreaks of YFV occur due the progressive combinations of factors that contributed (i.e., anthropic modifications) to the overlap of ecosystems (Possas et al. 2018). These regions harbor different species of mosquitoes capable of becoming infected with the YFV, and carry the virus between or in overlap of ecosystems and ecotone (floreys, rural and urban areas) (Possas et al. 2018). Thus, there are three main types of transmission cycles: sylvatic (or jungle), urban and intermediate:

- Sylvatic cycles: the cycle is mediated by both varieties of tree-dwelling mosquitoes and monkeys are assumed to be the primary reservoir of YFV in tropical forests. In south America, the main wild mosquitoes that bite monkeys are the *Haemogogus* and *Sabethes*, whereas transmission among African primates is vectored by arboreal mosquito species such as *Ae. formosus*, *Ae. africanus*, *Ae. simpsoni*. Occasionally, humans can become infected when working or travelling in the forest (Garske et al. 2014; WHO 2016, 2017);
- Urban cycles: It involves transmission by infected *Aedes* spp. mosquitoes that feed on humans (humans-mosquitoes-humans) and is characterized by rapid transmission due high population and mosquito density. Furthermore, the large epidemics caused by yellow fever virus in urban areas are due to the introduction of virus as a result of zoonotic spillover from the sylvatic reservoir to a susceptible population. This was reported by some studies done from outbreaks in Brazil and Uganda in 2016 (N. R. Faria et al. 2018; Kwagonza et al. 2018);

- Intermediate cycles: This type of transmission occurs in ecosystems that overlap rural and forest areas and are driven by peri-domestic mosquitoes who feed opportunistically on humans and monkeys. In Africa an example of intermediate cycle is in the moist Savannah region that is affected by a wide variety of tree-hole breeding mosquito and can transmit YFV to both humans and monkeys when density reaches high levels. These events can lead to small-scale epidemics in rural regions (Garske et al. 2014; Barrett and Higgs 2006).

Even with the existence of an effective vaccine, YFV is far from being eradicated due to the presence of a sylvatic reservoir, principally in Africa where non-human primates are hardly affected by the infection (unlike some episodes in Brazil (Garske et al. 2014; Gaythorpe et al. 2019; T. P. Monath and Vasconcelos 2015; WHO 2016). Thus, YFV infection keeps happening due/or as a result of sylvatic spillover. Other YFV burden is the climate change that can in the future interfere on the sylvatic transmission cycle and increase the cases of infection (Gaythorpe et al. 2020). Efforts in monitoring transmission and epidemiological surveillance of yellow fever are very important issues that will contribute to controlling or eliminating possible outbreaks in warmer areas or predicting outbreaks in other regions such as Asia that have never reported an outbreak of YFV but harbor species of mosquitoes that are susceptible to the West-African genotype of yellow fever virus (Lataillade et al. 2020).

I.C.2.b. *Dengue virus*

Infections with Dengue virus (DENV) increased rapidly and they represent the most prevalent mosquito-borne virus infection in the world, threatening 4 billion people in 128 countries with 70% of them residing in Asia (WHO 2021). Dengue is endemic and widespread in tropical and subtropical areas due to the efficient mosquito vector *Ae. aegypti* and to a lesser

extent *Ae. albopictus* (**Figure 6**). Currently, it is estimated that 390 million infections occur yearly, causing mainly mild illness but also dengue hemorrhagic fever disease (DHF) and deaths (Guzman and Harris 2015).



Figure 6: Global burden of dengue in the world. Modified from *The Lancet*, (Guzman and Harris 2015).

The origin of dengue remains largely debated because several studies indicate that dengue emerged from monkeys and spread to humans in both Africa and Southeast Asia. The spread of DENV through other continents, like the Americas was facilitated during the slave trade between the 15th to the 19th centuries similarly as for YFV and the corresponding competent vectors (Chen and Vasilakis 2011; Gubler 2002; Vasilakis, Tesh, and Weaver 2008; Higuera and Ramírez 2019).

Reports of the disease with similar clinical conditions was reported in a Chinese encyclopedia that dated from 265 A.D to 992 A.D. The disease was called water poison, and was reported as being associated with flying insects and water (Gubler 1998). The earliest description of febrile illness in Africa occurred in 1881 and 1883 on the coast of west Africa. Almost 100 years later, the dengue virus was isolated (DENV-1 and DENV-2) from patients with fever in Ibadan, a town located in the south west of Nigeria (Fagbami, Fabiyi, and Monath 1977; Vasilakis, Tesh, and Weaver 2008).

In the early 20th century, Athens, Greece, was hit by a large dengue epidemic resulting in one million cases. Until 1940, only mild forms of the disease, but which caused large epidemics, were documented in the medical literature. However the pattern of the disease took another turn due to the description of hemorrhagic forms of the disease after the War World II and the first cases related of epidemic DHF were described in the Philippines in the early 50s (Gubler 1998).

The picture of the dengue epidemic changed in the mid of the 20th century, and the virus expanded dramatically across continents most likely related to the spread of mosquito eggs carried in vehicle tires during shipping especially of *Ae. albopictus*. *Ae. aegypti* was the first vector to spread before the mid-20th century (Simmons et al. 2012). Even with the explosion of dengue in tropical countries, Southeast Asia managed to control the epidemic due to vector control policies implemented from 1950 to 1960 for the Malaria and Yellow Fever Programs (Gubler 2002).

Eradication programs for *Ae. aegypti* began to be implemented in the tropics and subtropics, achieving success in a short space of time. However, the mosquitoes reinvaded areas that were previously eradicated because the dengue transmission cycle could not be stopped in endemic areas (Simmons et al. 2012). Some factors directly contribute to the re-emergence of dengue and the increase in the disease burden that accelerated the dengue epidemic such as the unprecedented global population growth, urbanization (i.e., in Southeast Asia) associated to urban crowding principally in tropical urban centers and population displacement through travel were essential for the increase and spread of dengue (Gubler 1998; Huang, Higgs, and Vanlandingham 2019).

According to WHO, dengue cases increased over 8 fold over in the last two decades (2000 to 2019) with 5.9 million cases reported in 2019, with more than 3 million cases reported in the Americas (World Health Organisation 2021). Different serotypes of DENV have been

implicate with the massive outbreaks. Dengue virus comprises four serotypes (DENV-1 to DENV-4 and new DENV-5) genetically and antigenically distant, within the dengue antigenic complex, although they are epidemiologically similar (Vasilakis et al. 2011). The massive outbreaks are caused by different serotypes of dengue virus. In Guangzhou, China, serotype DENV-1 has been implicated in most outbreaks (Ma et al. 2021). The four serotypes circulate in Brazil as well as on the African continent, being DENV-1 and DENV-2 the most reported serotypes implicated in outbreaks in this sites (Cabral et al. 2021; Amarasinghe et al. 2011; Mwanyika et al. 2021).

Gubler and colleagues hypothesized that (Gubler 1998) the endemic strains of dengue that circulate today have a sylvatic ancestor involving non-human primates (NHP) and *Aedes* vectors from gallery forests. This hypothesis was tested by Whang and colleagues (Wang et al. 2000) who sequenced the complete genome of DENV-1, 2 and 4 strains, that were isolated from sentinel monkeys (*Macaca spp.* and *Presbytis spp.*) with *Ae. niveua* as the principal vector in Malaysia (Rudnick, Marchette, and Garcia 1967). In the same study Whang and colleagues sequenced some DENV-2 strains isolated from forest areas in West Africa (Rico-Hesse 1990). Thus, showed that endemic/epidemic lineages of dengue serotypes evolved independently from sylvatic dengue lineages ~1500 years ago which represents a recent event in the evolution of dengue.

In Asia all four sylvatic serotypes of dengue circulate between canopy-dwelling mosquitoes and monkeys and in West Africa until 2003 only sylvatic DENV-2 has been identified and demonstrated that sylvatic cycles are maintained between monkeys and sylvatic mosquitoes, principally *Ae. furcifer* mosquitos (Rudnick, Marchette, and Garcia 1967; M. Diallo et al. 2003). Yet, little studies are done on the sylvatic transmission cycle of dengue and the circulation of sylvatic serotypes to examine whether other mammals are involved in the maintenance of dengue virus in forests. Until 2011, three primate species (*Chlorocebus sabeus*,

Papio papio and *Erythrocebus patas*) have been described to play a role in the sylvatic transmission cycles of dengue virus as amplifiers or reservoir hosts and in a limited number of regions in Africa (Vasilakis et al. 2011). Even today, there are no studies that examined to what extent sylvatic dengue viruses still cause outbreaks in humans, even after it has been shown that sylvatic dengue is the ancestor of endemic/epidemic dengue. Sylvatic DENV-2 isolates are evolutionary distinct from the endemic/epidemic DENV-2 isolates. Thus, only a few reports have documented the pathogenicity of sylvatic dengue in humans (Rico-Hesse 1990; Wang et al. 2000; Vasilakis et al. 2008).

Within the four serotypes of dengue, distinct genotypes are described and they can co-circulate in the same area (Tchetgna et al. 2021). Infection with a particular endemic dengue serotype can cause mild illness, resulting in homologous immunity to the circulating serotype in a particular community. However, when other endemic serotypes circulate in the same community causing co-infections, the risk to develop DHF increases (Weaver and Barrett 2004; Gubler 2002). For instance, in 1964 there was the first dengue outbreak in Nigeria where the isolated virus corresponded to dengue serotypes 1 and 2, causing only classic dengue (Fagbami, Fabiyi, and Monath 1977). After some years, DENV-3 and DENV-4 were reported and DHF was identified for the first time in Nigeria. Fagbami and colleagues (2018), emphasized that presence of multiple serotypes of dengue in Nigeria enhances the occurrence of more cases of DHF and can be misdiagnosed as malaria which is common in these areas (Fagbami and Onoja 2018).

Current studies have appointed the active circulation of all dengue serotypes on the African continent. A systematic review and meta-analysis from Mwanyika and colleagues showed 17 outbreaks in East and 16 outbreaks in West Africa between 1960 and 2020, with DENV-2 and DENV-1 contributing to most outbreaks, followed by DENV-3 and DENV-4 (Figure 7). Risk factors that have been associated with outbreaks include older age, climate

change, lack of vector control, and recent travel history (Mwanyika et al. 2021).

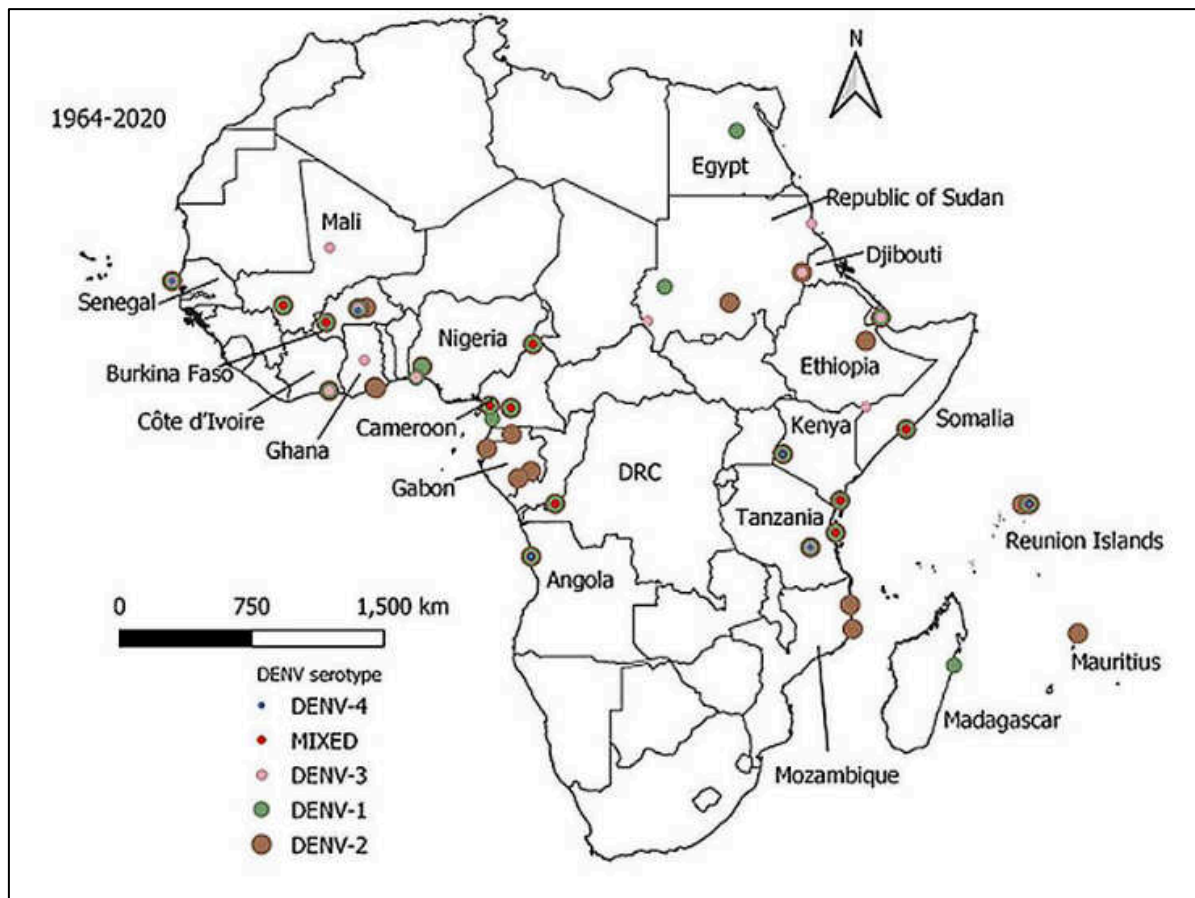


Figure 7: Distribution of dengue virus serotypes in Africa. The degree of distribution was classified by color. Source: *Viruses*, (Mwanyika et al. 2021).

In Gabon, Central Africa, DENV-2 has been implicated in outbreaks in 2007 and 2010. Surveillance studies between the 2007 and 2010 outbreaks showed the association between the circulation of DENV-1 and DENV-3 with DENV-2 in the respectively periods and clinical hemorrhagic signs, demonstrating the intensification of severe cases during the circulation of multiple serotypes (Caron et al. 2013). Recently in Douala, Cameroon, the co-circulation of DENV-1 to 3 (Tchetgna et al. 2021) was reported in acute febrile patients with 68.3% of cases infected with serotype 3 of dengue. Sylvatic DENV-2 was isolated from a patient returning to Spain after a trip to Guinea and presented a clinical condition of DHF. The authors reported then the first evidence of DHF caused by a sylvatic DENV-2 West African lineage virus (Franco

et al. 2011).

Sylvatic dengue cycles occur in many West African countries and Southeast Asia. Sylvatic DENV-2 has been detected in Senegal since 1970 in mosquitoes but also in humans and monkeys (M. Diallo et al. 2003). Moreover, there are periods in which some serotypes are silenced and re-emerge after intervals of 5-8 years, as reported by Diallo (2003), as example of DENV-2 which re-emerged in the sylvatic cycle in the Kedougou after years of silence. In the same study, 17 *Chlorocebus sabaues* were captured in a forest gallery in Kedougou. DENV-2 IgG was detected in 58% to 77% in adult and in 37% in juvenile monkeys. However, they could not conclude any relationship to the role of monkeys in the amplification of the virus, due the cross-reaction of antibodies between flaviviruses (M. Diallo et al. 2003; Saluzzo, Cornet, Castagnet, et al. 1986). In Nigeria, monkeys captured in lowland rainforest, living far away from humans, presented a high prevalence of dengue neutralizing antibody suggesting the existence of a forest cycle (Fagbami, Fabiyi, and Monath 1977).

In the Americas, the presence of heterologous DENV-1 and DENV-2 antibodies was associated with increased risk for hemorrhagic fever (Gardella-Garcia et al. 2008). In Brazil a fatal case was associated with the introduction of a new DENV-2 which was attributed to several factors like herd immunity reduction against the serotype and differences in viral replication in the host (Cunha et al. 2021). All four DENV serotypes have also been identified in wild mammals in French Guyana, in Mexico, in Colombia, Costa Rica, Equator and Brazil, thus proving the possible existence of a sylvatic transmission cycle until then not well elucidated in these regions, unlike in Africa and Asia (de Thoisy B et al. 2009; Aguilar-Setién et al. 2008; Calderón et al. 2021; Platt et al. 2000; Catenacci et al. 2018). In French Guyana 600 mammals were captured in forests surrounded by an urban area and forest sites where the disease has not yet emerged. All four DENV serotypes identified by RT-PCR, circulated in both sites and were detected in 22%, 12.6%, 12.5% of Rodentia, Marsupialia and Chiroptera,

respectively. They showed also that the DENV-2 strains found in mammals have 99% identity with the strains that circulate in humans and suggest a spillover from mammals or a possible enzootic cycle involving these animals (de Thoisy B et al. 2009). Bats in Colombia showed positivity to DENV-2 corresponding to natural infection (Calderón et al. 2021). In Brazil, primates and sloths presented antibodies against dengue virus serotypes 1, 2 and 3. These samples were collected in agroforestry environments where humans and animals share the same environment. This shows evidence for the circulation of the virus but does not confirm a sylvatic transmission cycle (Catenacci et al. 2018).

As mentioned and evidenced above the four dengue serotypes traditionally are maintained in two distinct transmission cycles. The sylvatic cycle that include non-human primates, and probably also other mammals, and a diverse range of forest-dwelling mosquitoes primarily *Aedes spp* that occurs especially in Africa and Asia. The endemic/epidemic cycle which utilizes highly efficient peridomestic mosquitos as *Ae. aegypti* and *Ae. albopictus* and human host (Fagbami and Onoja, 2018).

- In the dengue endemic/epidemic cycle, humans are the amplifier hosts in urban areas where the virus is mainly transmitted by *Aedes spp.* vectors and other mosquitos serving as secondary vectors (Wang et al. 2000; Weaver and Barrett 2004) These mosquitoes have conquered a range of territories and are very efficiently to transmit dengue but can also be co-infected and co-transmit other arboviruses (Vogels et al. 2019; Rezende et al. 2020). However, the ability of these urban *Aedes* mosquitoes to transmit sylvatic DENV is being discussed. Recently it was reported that the circulation of sylvatic DENV-4 in both *Ae. aegypti* and *Ae. albopictus* in urban areas of Peninsular Malaysia, were very close to a sylvatic strain isolated from sentinel monkeys at the same place 50

years ago. In addition, all four dengue serotypes were identified circulating in the same period of the study. This fact shows the susceptibility of mosquitoes to become infected with sylvatic dengue strains (Johari et al. 2019). Another fact that can contribute to the distribution of sylvatic strains mirrors the context of rural African villages surrounded by forests where some species of mosquitoes i.e., *Ae. furcifer*, can circulate between forest and peridomestic habitats suggesting that transfer of strains could occur regularly (Vasilakis, Tesh, and Weaver 2008).

- The sylvatic or zoonotic cycle involves especially non-human primates as vertebrate reservoir hosts in West Africa and in Southeast Asia and serological and molecular studies have suggested a secondary role of other mammals in the amplification of dengue virus. In the Americas the role of wildlife in the sylvatic dengue cycle has not yet been identified despite serological and molecular evidence (de Thoisy B et al. 2009; Calderón et al. 2021). Until now, five sylvatic serotypes have been recognized and the last has only been isolated in Asia (Mustafa et al. 2015). The sylvatic cycle involves arboreal mosquitoes that live in gallery forests, especially in tree-holes, and then maintain the cycle when they feed on NHPs (Vasilakis, Tesh, and Weaver 2008; Schatzmayr 2001; Weaver and Barrett 2004). Another fact to note is a second type of sylvatic cycle in rural areas surrounded by forest that creates an “intermediate zone” that favors primatophilic mosquitoes that live in the forest canopies and descend to ground level to feed on humans, which facilitates the transfer of sylvatic dengue strains (Vasilakis N. et al., 2011).

I.C.2.c. Zika virus

Zika virus is on the priority list of the WHO "blueprint", that means, that it is a disease that requires attention for additional surveillance and research. Zika virus is mainly transmitted by the highly anthropophilic *Ae. aegypti* mosquito that is distributed in a wide range of regions (African Region, Region of the Americas, South-East Asia Region, and Western Pacific Region) (World Health Organization 2019). Until 2007, sporadic cases of Zika virus infections were registered in the world causing only mild febrile illness in humans in Africa and Asia (Pierson and Diamond 2020). Since 2013 successive outbreaks by Zika virus have been observed and a lot of information began to be generated. Currently, a total of 86 countries and territories in subtropical and tropical climates have reported autochthonous transmission of ZIKV (World Health Organization 2019).

Zika virus was discovered in the Zika Forest of Uganda in 1947 and isolated from a sentinel rhesus monkey. The following year Zika virus was isolated from *Ae. africanus* mosquitoes in the same region, and was identified consecutively for the first time in humans in Uganda and United Republic of Tanzania in 1952 (Dick 1952). The Zika transmission cycle in Africa was initially mainly limited to enzootic circulation and sporadic spillover to humans. Zika virus expanded further to equatorial Asia from the ancestral African lineage. The Asian lineage of the virus emerged and conquered the territory of the Pacific islands and the Americas, at the beginning of the 21st century (Posen et al. 2016). A considerable number of outbreaks started to appear: in French Polynesia (2013-2014), Cape Verde Islands and Brazil (2015), Guinea-Bissau, Angola, Singapura, Vietnam and Thailand (2016). Furthermore, cases of Guillain-Barré syndrome (GBS), microcephaly, maternal-fetal transmission and also detection of Zika in semen shaped the set of factors associated with Zika virus outbreaks leading to a WHO declaration that ZIKV is a Public Health Emergency of International Concern (World Health Organization 2019; Musso, Ko, and Baud 2019) (**Figure 8**).

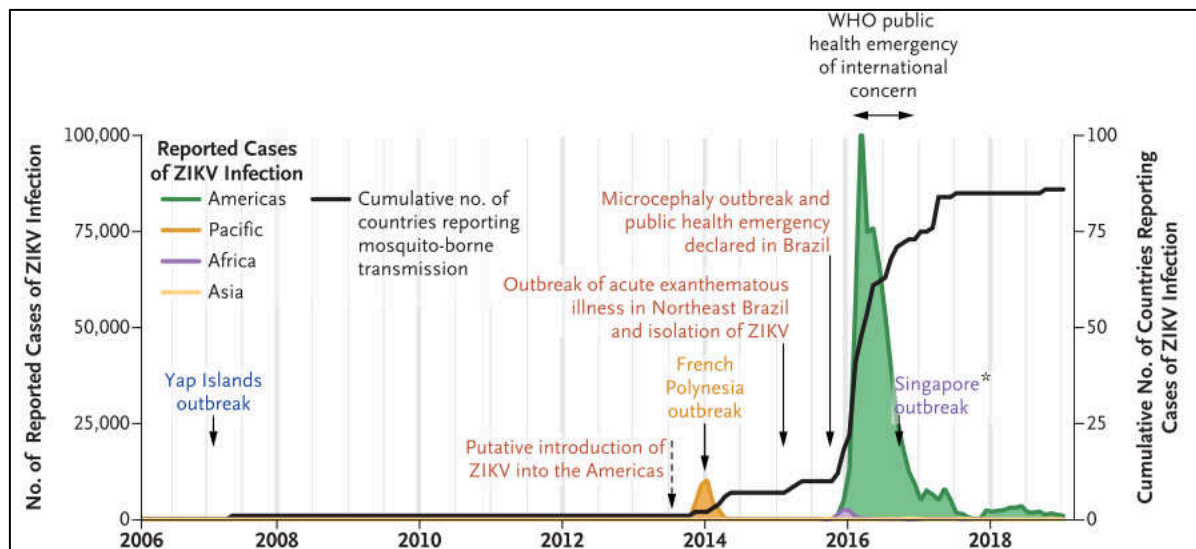


Figure 8: Number of reported and cumulative cases of Zika virus infection in countries. Source: *The New England and journal of Medicine*, Reproduced with permission from (Musso, Ko, and Baud 2019). Copyright Massachusetts Medical Society.

In a phylogenetic tree, Zika virus clusters within the Spondweni virus (SPONV) lineage that circulates only on the African continent. ZIKV strains are divided into two major lineages, the African lineage and Asian/American lineage and they differ in approximately 90% of their nucleotide sequence (Duong, Dussart, and Buchy 2017). Shen et al. (2016), revealed in their study that two countries in West Africa (Senegal and Cote d'Ivoire) played a key role in the evolution and genotypic divergence of Zika virus, since in the phylogenetic tree the Zika virus is located close to the SPONV (a virus that circulate only in Africa), suggesting this geographical place as origin of Zika (Shen et al. 2016; Marchi et al. 2020). The spread of the Zika virus across Southeast Asia gave rise to the Asian lineage which subsequently spread to French Polynesia followed by the spread to America, giving rise to American strains that emerged from the ZIKV Asian lineage (Nuno Rodrigues Faria et al. 2016; Musso, Ko, and Baud 2019). African lineages have been isolated in Central and Western Africa and contains two sublineages (East and West Africa), based on the sequences of E and NS5 genes, while Asian/American lineage are reported in Southeast Asia, Pacific region and American Islands,

the latter have formed a new American subclade (Nuno Rodrigues Faria et al. 2016; Duong, Dussart, and Buchy 2017) (**Figure 9**).

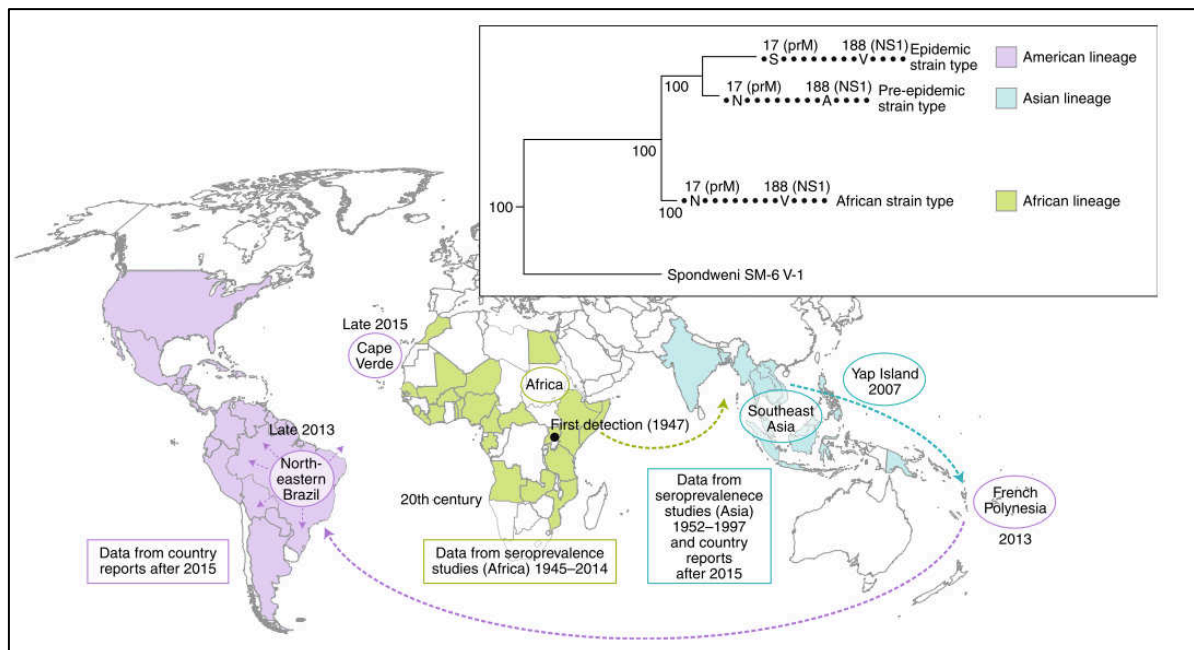


Figure 9: Distribution of Zika virus lineages after its origin. Source: *Nature ecology and evolution*, (Gutiérrez-Bugallo et al. 2019)

The lineages show differences in transmissibility and pathogenicity/virulence. While only the Asian Zika virus lineage has been implicated in all human outbreaks and fetal abnormalities, the African Zika virus lineages showed higher transmissibility in mosquitoes and lethality in mice but also higher infectivity and replication in human neural stem cell than Asian strains in experimental infections (Aubry et al. 2021; Simonin et al. 2017)

The Zika virus mosquito-borne transmission cycle involves two distinct cycles (sylvatic and urban) and the “intermediate zone” (ecotone/rural) as for other *flavivirus* cycles.

- *Sylvatic or enzootic cycle:* involve primarily NHPs as vertebrate/amplifier host and arboreal mosquitoes. Sometimes, Zika virus may spillover from the sylvatic context and initiate epidemics in humans or humans can be bitten by virus-infected mosquitoes, occasionally when they encroach the forest. Until now, no animal reservoirs were identified other than primates, despite

serological evidence of antibodies in other animal species. Therefore, other vertebrate animals can play a role in maintenance of Zika virus like birds, reptiles or bats in East Africa for example (Darwish et al. 1983; Shepherd and Williams 1964; Johnson et al. 1977; Gutiérrez-Bugallo et al. 2019; Kirya and Okia 1977).

- *Urban cycle*: the virus is transmitted from human to human via *Ae. aegypti* and *Ae. albopictus* mosquitos as vector.

Many mosquito species worldwide have been identified to be infected with Zika 31 species belonging to five genera: *Aedes*, *Culex*, *Anopheles* and *Eretmapodites* (Gutiérrez-Bugallo et al. 2019). The majority of these species have been caught in sylvatic settings (Kraemer et al. 2015). These mosquitoes are distributed in 15 countries in both enzootic and epidemic regions and they suggested that in the sylvatic Zika transmission cycle the following species *Ae. africanus*, *Ae. furcifer*, *Ae. lutocephalus*, *Ae. vittatus*, *Ae. dalzielli* and *Ae. taylori* are mostly implicated while in the urban cycles it is *Ae. aegypti* (Gutiérrez-Bugallo et al. 2019). *Ae. aegypti* remains the main vector worldwide in urban areas that transmits Zika virus. Furthermore, studies on vector competence for Zika virus transmission, as well as epidemiological and genomic surveillance in endemic and forests areas is still scarce. Recently, an experimental infection study with *Ae. aegypti* (native mosquito) and *Ae. albopictus* (invasive mosquito) in Gabon, showed that besides that they are able to replicate and transmit all Zika virus strains, they have high vector competence for the African virus lineage. The authors emphasize the strong adaptation of African Zika virus strain to both mosquito species in Gabon and the ZIKV epidemics is mainly related to related to autochthonous cases rather than non-native ZIKV (Jiolle et al. 2021). Another study in Central Africa, in Cameroon and the Republic of Congo, also analyzed the ability of native and invasive *Aedes* mosquitoes to transmit Zika virus and confirms the results obtained in the study in Gabon, thus highlighting the efficient

transmission of Zika virus by both mosquito species. However *Ae. aegypti* showed to be more transmission competent than *Ae. albopictus* (Kamgang et al. 2020). These results are important because they clarify and demonstrate the vector capacity of *Aedes* mosquito species to cause possible outbreaks in urban and rural communities. In blood donors in Cameroon the global seroprevalence was ~5% and was associated with the existence of a (peri-) sylvatic cycle of Zika virus transmission (Gake et al. 2017). In East Africa, convalescent plasma from individuals with ZIKV-like illness (fever and/or rash) were screened and very low seroprevalence of IgG antibodies against ZIKV was found during ~20-year period (Gobillot et al. 2020). In Senegal and Nigeria, samples collected from 1992 to 2016 from febrile patients, showed that 6.2% of patients had IgM antibodies to ZIKV and were infected with the African lineage of Zika virus. The study showed also the presence of 2 African ZIKV sublineages circulating throughout West Africa and demonstrate that Zika virus circulates, even if timidly, among the urban population (Herrera et al. 2017).

In Africa, enzootic cycles of Zika virus are supposed to involve NHPs and arboreal mosquitoes and circulation seems to be frequent as shown through successive studies in Senegal over the last 50 years. The frequency of sylvatic arbovirus isolations and temporal patterns of mosquito abundance, were associated with weather variables and evaluated using a uniquely rich longitudinal dataset from Senegal. ZIKV has a dominant isolation periodicity of 4.0 years (Althouse et al. 2015). However, in America and Asia, no Zika virus sylvatic cycle was identified (Gutiérrez-Bugallo et al. 2019) but some studies have reported Zika infection in NHPs in Brazil (S. R. Favoretto et al. 2019) and the death of wild marmosets infected with Zika in Rio de Janeiro (Meireles M. 2020; Silva et al. 2020). In Asia, despite of sporadic circulation and detection of Zika virus in humans, it is unknown if these cases are only driven from the human-endemic cycle or if they can occur from zoonotic spillover events to humans from not yet identified sylvatic cycles (Duong, Dussart, and Buchy 2017).

Despite evidence of previous exposure to ZIKV in various animal species as well as viral isolation reported in the literature, little is known about the effects of Zika virus infections in wild animal populations (Bueno et al. 2016; Gutiérrez-Bugallo et al. 2019). Experimental studies show that bats and monkeys are susceptible to Zika virus infections (Simpson and O’Sullivan 1968; McCracken et al. 2017; Vanchiere et al. 2018), but knowledge on the extent and the degree of viremia and possible clinical signs is still limited. Furthermore, there is also lack of data on the circulation of this virus, although it originated in Africa, on the African continent itself, as well as in Asia and America. Despite evidence of NHPs participation in enzootic cycles, other animal species may support Zika virus amplification in rural and forest areas. Many questions remain on how the cycle is sustained in wild areas, the impact of the virus on wild animal populations, the mechanism of Zika virus transmission among wild populations and on how animals and humans become affected by Zika virus spillover/spillback between urban and sylvatic cycles. Thus, entomological studies, combined with epidemiological and genomic surveillance in wild environment from field samples of wildlife are essential to know how the Zika virus emerges and his subsequent dispersion.

I.C.2.d. *West Nile virus*

More than 80 years have passed since West Nile virus (WNV) has been isolated and became a mosquito-borne flavivirus disease with widespread geographical distribution infecting a wide range of hosts and vectors. When it comes to pathogenicity, WNV is the most important veterinary pathogen among flaviviruses, however the degree of pathogenicity in humans is comparatively low when compared to other flaviviruses such as DENV, YFV and Japanese encephalitis virus (JEV) (H Weissenböck et al. 2010)

WNV was first identified in 1937 in a woman with fever in the West Nile district of Uganda (K. Smithburn et al. 1940). More than 13 years later, new isolates were identified in

the blood of febrile children from Egypt (Melnick et al. 1951) and in birds (crows and rock pigeon) in the Nile delta Region (Work and Hurlbut 1953). Sixty years after its isolation, WNV spread in the Old world and was isolated from birds (Nir et al. 1967), mosquitoes, domestic animals (Omilabu et al. 1990) and humans (Renaudet, Jan, and Ridet 1978) (Nur et al. 1999), causing only mild disease and no deaths (Chancey et al. 2015). The majority of the epidemics in this period occurred in Sub-Saharan Africa (SSA) (**Figure 10**) and during this time up to 55% of human populations seroconvert (W. Sule et al. 2015).

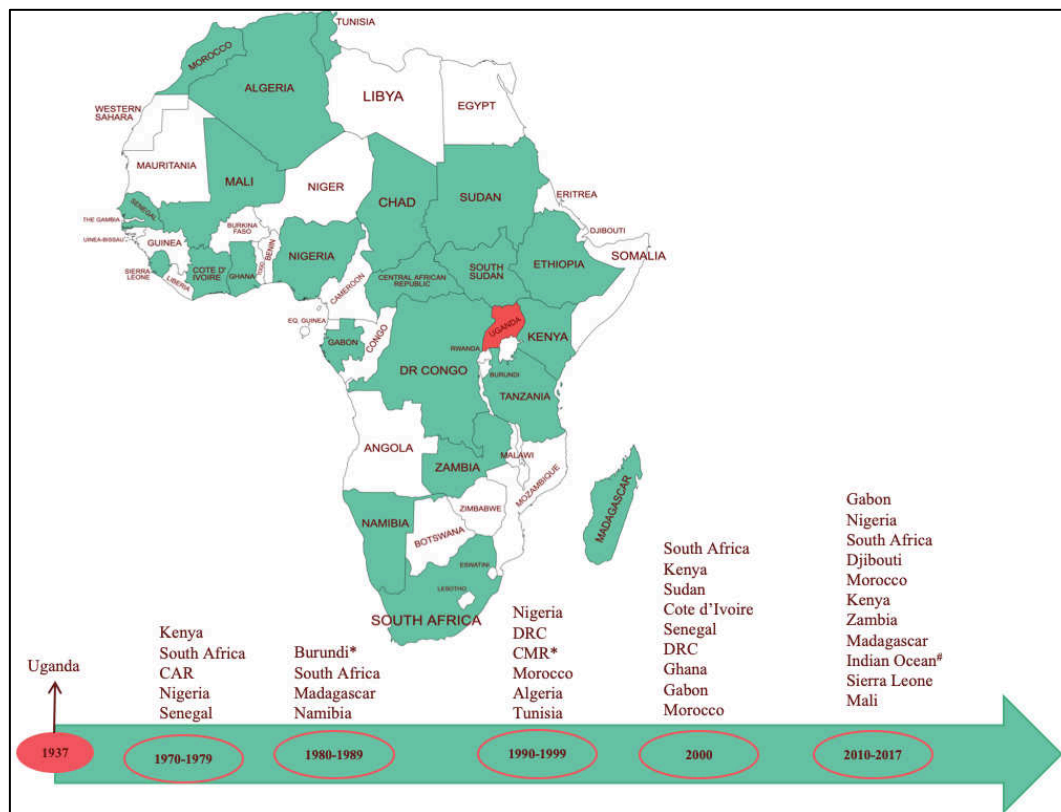


Figure 10: Distribution of cases with West Nile virus that have been isolated or detected on the African continent in the last 80 years. (This map was made with references in Sule et al. 2018).

In a serological survey in Guinea, Sudan, Uganda, Kenya and DRC, the authors found IgG antibodies against WNV in human samples in all of these countries suggesting that the virus is very active in these areas. Besides that, in the same study *bleu monkeys* from Kakamega

forest Kenya showed presence of neutralizing antibodies to West Nile and St. Louis viruses (K. C. Smithburn and Jacobs 1942). In South Africa, samples from indigenous residents in widely scattered areas, showed highest incidence of sera with neutralizing antibodies to West Nile and Sindbis viruses. Moreover, on the same study showed that 18,8% (78/413) of monkeys had neutralizing antibodies to WNV as well as domestic animals and juvenile birds (Kokernot, Smithburn, and Weinbren 1956). Interestingly, a serosurvey held in Cairo in 1951 conducted in 251 individuals, more than 50% of the children in the study, which were the vast majority, carried maternal antibodies against WNV, as well as neutralizing and complement-fixing antibodies to WNV, indicating the widespread of WNV in the population (Melnick et al. 1951). WNV continued to circulate and cause outbreaks on the African continent and Middle East during the beginning of the 20th and 21st century, causing mild fever symptoms to fatal cases of WND in humans but also equine infections (Chancey et al. 2015).

In Europe, the West Nile virus circulates since 1958 when it was first detected in the serum of an Albanian patient (Bardos et al. 1959). Then four years later an outbreak in France was recognized where cases of WNV were identified in both humans and horses (Murgue et al. 2001). West Nile virus continued to circulate, and was identified in a wide range of species of migratory birds, ticks, and mosquitoes in several countries on the European continent (Filipe 1972; Lontai and Straub 1998; Ernek E 1977). After a period of silence two major epidemics occurred in Europe, one in Romania (1996) and another one in Russia (1999) with patients presenting neurological signs and meningoencephalitis (Platonov et al. 2001; Tsai et al. 1998). Several other outbreaks of WNV have subsequently been reported in Europe between 2000 and 2009 in France, Hungary and Italie (Zeller and Schuffenecker 2004; Martin and Simonin 2019). Surveillance programs in Europe started to be implemented after the constant outbreaks with WNV, which conquered other European territories that were not yet identified, leading to the suggestion of a seasonal introduction through different routes (Esteves et al. 2006; Sambri et

al. 2013; Chancey et al. 2015).

Simultaneous outbreaks of WNV have also occurred in the Middle East in countries as Turkey, Iran, Lebanon, Jordan (Chancey et al. 2015) since the first isolation in 1951 in Israel where the first outbreak occurred on an agricultural settlement with higher morbidity in children than in adults (Bernkopf, Levine, and Nerson 1953). Following this period, another outbreak in Israel marked a new era of epidemics in the region and it was in 2000, more than 20 years without reported WNV outbreaks, that 35 fatal cases were confirmed out of 417 serologically confirmed cases of WNF (Weinberger et al. 2001). WNV in Southern and Eastern Asia as well as in Australia have been documented in humans, birds (Mackenzie and Williams 2009) and other animal species, principally in China with seropositivity ranging from 4.9% and 14.9% in dogs and cats, respectively (Lan et al. 2011). Curiously, the first case of WNV reported in China was in birds, unlike several other reports in the world where the first isolates were identified in humans. In India, cases of WNV have been reported since 1981, although sporadic cases were documented throughout 1970s - 2000s (George et al. 1984).

At the end of the 20th century, the West Nile virus has emerged in the North America and attracted attention by the rapid spread in this new territory (Gubler 2007). The epicenter of the epidemic started in the metropolitan area of New York in 1999, where cases of encephalitis and meningitis were observed (Mostashari et al. 2001). The virus had already widespread over 10 states in 2001 and had already reached 40 states in 2002, with cases of WNV and approximately 10% of fatalities. After its constant presence in 2004 the WNV was considered endemic in North America. In 2010 a study estimated that more than 3 million people were infected with WNV in the US based on antibody detection which represents a cumulative incidence since the first outbreak in 1999 (Petersen et al. 2013). WNV spread throughout continental USA and established enzootic cycles in this region. The virus is also widely established from Canada to Venezuela (Gubler 2007). The first cases in South America were

documented in Colombia, Argentina and Venezuela in horses and sylvatic birds. Serological evidence of WNV infection in Brazilian horses and birds were reported in 2008, the first human case was confirmed in 2014 and the first isolation of WNV was confirmed in 2018 from a horse that presented neurological disorders and died (de Castro-Jorge et al. 2019). However, in tropical areas such as Caribbean or in Central or South America, severe and fatal neurological cases of WNV infection have been rare in humans and horses, presenting a different epidemiologic pattern compared to the viruses from the same serogroup and which are closely related such as Japanese encephalitis virus and St. Louis encephalitis (Gubler 2007).

West Nile virus is phylogenetically related to Usutu, Murray Valley encephalitis and Kunjin viruses and is a member of the Japanese encephalitis serocomplex (Calisher et al. 1989). Multiple lineages of WNV have been identified that mostly correspond with geographic distribution and they differ by more 20-25% (May et al. 2011; Chancey et al. 2015). The two major lineages, lineage 1 and 2, emerged in the beginning of 20th century and are still predominant in the epidemics in North America and Europe (W. F. Sule et al. 2018). WNV-1 lineage crossed of the eastern migratory birds routes (Eastern or Western Mediterranean countries) which facilitated its spread northwards towards Europe, Middle East and Northern of Africa (Zehender et al. 2011; Chancey et al. 2015). Lineage 1 is the largest in world distribution and is associated with outbreaks in humans together with lineage 2 and 5. Lineage 1 is subdivided in three clades: 1a contains isolates from Africa, Europe, the Middle East, Asia and the Americas, clade 1b is found in Australia and is represented by the Kunjin virus (KUNV) and 1c (formerly lineage 5) is represented by isolates from India. A single substitution at NS3 residue 249 in lineage 1 WNV strains confers increased viremia and virulence in birds especially in American crows (Brault et al. 2007). The high levels of WNV lineage 1 seropositivity in Africa suggest an endemic mosquito-bird transmission cycle, whereas in Europa, WNV circulates seasonally causing sporadic epidemics (May et al. 2011). Lineage 2

is endemic in sub Saharan Africa and Madagascar and in South Africa and is implicated in neurological diseases in horses (P. Jupp 2001). In Europe, WNV lineage 2 strains emerged in 2005 and have been associated with bird and human outbreaks (Chancey et al. 2015) but also in Equine encephalomyelitis outbreak (Kutasi et al. 2011). Lineage 3 of WNV was isolated in mosquitos in Austria in 1997 and 1999, while lineage 4 includes isolates from ticks, mosquitoes and reptiles and has been seen circulating since 1988. Lineage 6 has been described in Spain and the lineage 7 strains were isolated from ticks and rodents in Senegal and experimentally it has been shown to be more virulent than the 1c strain 'NY99" (Fall et al. 2017).

The natural enzootic cycle of WNV is between ornithophilic/anthropophilic mosquitoes and feral birds, with mammals serving as dead-end host because of low-level or short-duration viremia. Humans and horses can also be infected with WNV, although they can develop serious illness leading to death, they are considered as dead-end or incidental host in the transmission cycle because of low viremia (P. Jupp 2001; Jeffrey Root 2013). Other vertebrate species (dogs, goats, cattle, pigs, chips and other) are also susceptible to virus infection and some reports have shown that they can develop low viremia and support arthropod-borne transmission (Meulen, Pensaert, and Nauwynck 2005). Furthermore, WNV infection have been reported in wild mammals (lemurs, rodents and bat), in livestock (sheep and goats) and sometimes with high seroprevalence rates (McLean et al. 2002; Meulen, Pensaert, and Nauwynck 2005) for example, among a group of crocodiles in Israel, 70% (14/20) showed neutralizing antibodies to WNV (Steinman et al. 2003). Other mechanisms of WNV transmission is by close contact between susceptible birds and cloacal or oral fluids from other birds but also when susceptible hosts feed on infected animals or mosquitoes (Meulen, Pensaert, and Nauwynck 2005).

Birds are the principal reservoir hosts for the WNV life cycle and mosquitos of the *Culex* genus are the principal vector, although WNV can also be transmitted by a variety of

other mosquitoes. WNV infection is very pathogenic for some species of birds principally to the crows family in Americas (Eidson et al. 2001; Brault et al. 2004). some bird mortality, although rare, has been associated with WNV in Europe, Africa, the Middle East and Asia (Kilpatrick 2011), but it can occur as was the case with young geese in Israel (McLean et al. 2002). Normally, birds develop high levels of viremia that are enough to infect feeding mosquitoes fact and thus to consider birds as host reservoirs of the virus (W. F. Sule et al. 2018). A study in South Africa showed that 92% and 70% of 30 feral bird species had hemagglutination inhibition (HI) and neutralizing antibodies to WNV, respectively. In addition these bird species were involved in the enzootic cycle of WNV without fatal cases, suggestion that these birds were the main host reservoir (P. Jupp 2001).

West Nile virus infections have been described in many mammal species with high seropositivity rates, but their role in the epidemiology of WNV is still unknown. In Louisiana, 1,692 captive rhesus monkeys, pigtail macaques and baboons had their blood tested to WNV after an epidemic of meningoencephalitis during the summer in the state. The study found a high infection rate in this captive nonhuman primate population with an overall seroprevalence of 36%, suggesting the silent circulation in nature during epidemic periods (Ratterree et al. 2003). Recently, another study conducted in mangrove forests and caves from Malaysia, showed a seropositivity of 29.6% to WNV in long-tailed macaques, and of 12% in bats (*Lesser Short-nosed Fruit* bats, *Lesser Sheath-tailed* bats, and *Thai Horseshoe* bats) (Ain-Najwa et al. 2020). Wild boars also showed antibodies to WNV by virus neutralization test (VNT) in Italy (Petruccelli et al. 2020).

I.C.2.e *Usutu virus*

Usutu virus (USUV) is a member of the Japanese encephalitis serocomplex and shares many features with WNV such as the enzootic transmission cycle that involves *Culex* mosquitos

as vector and birds as reservoir hosts, but also the pathogenicity with neurological complications (Poidinger, Hall, and Mackenzie 1996). USUV recently emerged in Europe causing epizootics among both captive and wild birds and also Usutu fever in humans (Daniel Cadar et al. 2014).

USUV, was first isolated from a pool of *Culex neavei* mosquitos in 1959 in Swaziland (Southern Africa). During the same period, it was also isolated from a bird-biting mosquito *Mansonia autites*, in Uganda (M. C. Williams et al. 1964; McIntosh 1985). Usutu virus has been reported in many countries of Sub-Saharan Africa like Senegal, Côte d'Ivoire, Uganda, Nigeria, Central African Republic (CAR) and Burkina Faso and many isolates were identified by entomological surveillance in these countries principally in Senegal (Nikolay et al. 2011). In these countries, USUV was isolated in different species of mosquitoes as *Culex neavei*, *Culex perfuscus*, *Aedes minutos*, *Aedes mansonii* and *Coquillettidia*, non-migratory birds (without fatal cases), rodents and humans (only two cases, in CAR and Burkina Faso) (Nikolay et al. 2011).

Usutu virus was introduced in Europe, similarly as for WNV by migratory bird routes from Africa through southern Mediterranean countries (**Figure 11**). USUV emerged in 2001 in Austria, where it caused significant outbreaks and mortality among many species of birds principally on Eurasian blackbirds and the virus continued to spread to neighboring countries (Herbert Weissenböck et al. 2002). However, there is serological evidence from 1996 suggesting that USUV circulated prior to 2001, and that the virus established among local mosquito species and then spread further (Herbert Weissenböck et al. 2013). After the first outbreak in Austria, a decrease in wild bird mortality was shown after a high rate of seroconversion of >50% was reached in 2005 compared to <10% between 2003 and 2004 in these birds, concluding herd immunity among the bird hosts (Meister et al. 2008). There is evidence of co-circulation of USUV and WNV in Southern European countries and some

authors showed that the seroprevalence of USUV seems to be much higher than WNV (Zannoli and Sambri 2019). In addition, these two viruses are quite similar for clinical manifestations in humans and when it comes to pathogenicity in some bird species, USUV appears to be more lethal as compared to WNV (Zannoli and Sambri 2019).

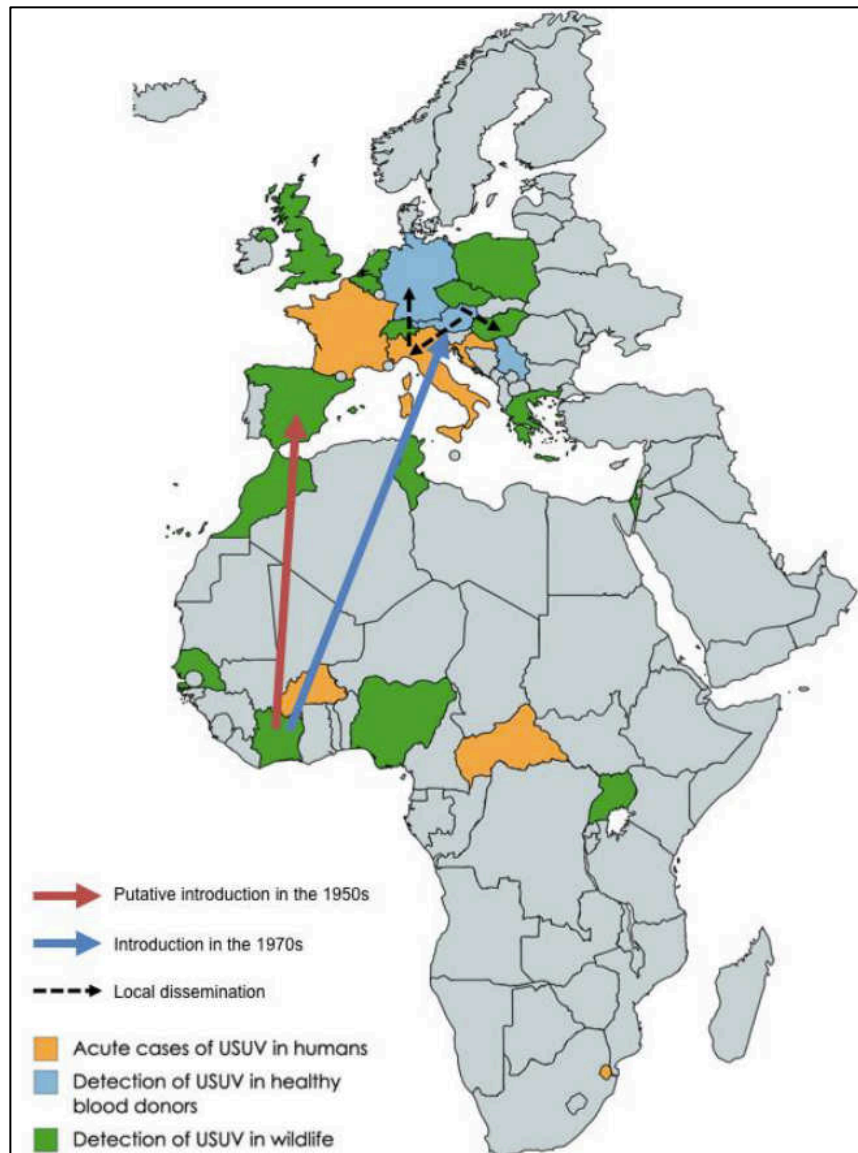


Figure 11: Identification of the Usutu virus in countries until 2019.
Source: *Viruses* (Roesch et al. 2019)

The first human cases in Europe were identified in blood donors in Italy in 2009 by serological confirmation (Gaibani et al. 2012) and the first neuroinvasive case in two immunocompromised patients in the same year by RT-PCR and sequencing (Pecorari et al. 2009; Cavrini et al. 2009). Neutralizing and IgG antibodies to USUV were found in 18.1 % of

forestry workers from Northern Italy (Percivalle et al. 2017). Until 2018, <50 human cases of USUV infection were identified with almost all the cases in Europe (Italia, Germany, Croatia, Austria and France) and two other cases in Africa (CAR and Burkina Faso) (Clé et al. 2019; Zannoli and Sambri 2019). These human USUV infections were characterized by meningo-encephalitis and neuro-invasive disease in Europe and mild clinical signs (fever and skin rash) in the two African cases. Retrospective studies in human cerebrospinal fluid and serum samples (Italy) but also in blood donors (Italy and Germany) have identified the presence of USUV RNA and past exposition to USUV through antibody detection (Grottola et al. 2017; Daniel Cadar et al. 2017). The study conducted by Grottola et al. (2017) in the Emilia-Romagna Region in northern Italy, suggested that human USUV infection is not a sporadic event (Grottola et al. 2017). These studies confirmed the existence of asymptomatic USUV infections and the necessity to better classify the clinical conditions of infections related to USUV. In reality, they observed that the human cases coincided with USUV bird epizootics which could favor the exposure of humans to this infection (Clé et al. 2019).

In Europe, in addition to evidence of infection and die-off disease in birds by USUV, other animal species were detected with evidence of USUV infection. These evidences raised question on the ability of USUV to adapt to new hosts. A study using virus-specific micro-neutralization tests and conducted in Zoo animals in France showed evidence of USUV infection in African wild dogs but also different rate of seroprevalence, 14.9% to 1.46% in birds for Usutu and West Nile viruses, respectively (Constant et al. 2020). In Italy, horses, chickens and dead wild birds were examined for the presence of USUV after episodes of high mortality of blackbirds in 2009. Samples were collected between 2008 and 2009 and horses showed higher prevalence of USUV neutralizing antibodies, 89.2% in 2008, as compared to 7.8% in 2009 in Italy. Chickens, blackbirds, rock pigeon and magpie also seroconverted to USUV and viral strains were detected in 52.9% of blackbirds (Savini et al. 2011). In southwest Germany,

USUV was identified in brain samples of 2 dead bats (*Pipistrellus pipistrellus*). The both bat USUV strains had 90.8% to 99.3% aa identity compared to detected in mosquitoes, birds and humans' strains from Europe and Africa. The authors suggest the probability of interactions between bats, mosquitoes and birds and their contribution in the epizootic cycles rather than acting as a silent reservoir to USUV (Daniel Cadar et al. 2014).

Engel et al. (2016), showed by analysis of the complete genome from different mosquitos, birds and bat species, that Usutu virus was introduced regularly from Africa into Europe during the last 50 years and this events are still ongoing (Engel et al. 2016). These events explain the broad genetic diversity of USUV strains identified in Europe (Roesch et al. 2019; Engel et al. 2016; Ndione et al. 2019; Cadar et al. 2017). So far, eight different genetic lineages have been reported on the basis of their geographic origin and the genetic variability based on partial NS5 (highly conserved) and full-genome sequences. The USUV strains are composed by African (1,2 and 3) and European groups (1,2 ,3 ,4 and 5) and they were differentiated from each other by different host species, climate and vegetation (Engel et al. 2016; Roesch et al. 2019; Cadar et al. 2017).

Similarly, as for the enzootic cycle of WNV, USUV is maintained between ornithophilic mosquito vectors, mainly of the *Culex* genus and birds as natural amplification hosts. However, information about ecology and the existence of a non-avian reservoir, that could be involved in the USUV transmission cycle, principally in Africa is still scarce (Nikolay et al. 2011). Yet, 93 bird species (wild, captive and migratory) were reported to be infected with USUV, which caused clinical manifestations as central nervous system disorders, prostration, disorientation, ataxia and weight loss and consequently led to avian mortality in Europe (Clé et al. 2019). Some species of birds such as blackbirds (*Turdus merula*), gray owls (*Strix nebulosa*), and house sparrows (*Passer domesticus*) seem to be the most affected by USUV infection. Other animal species have been reported to be infected with USUV such as horses (Savini et

al. 2011), bats (Cadar et al. 2017), wild boars (Petruccelli et al. 2020), wild ruminants (Garcia-Bocanegra et al. 2016), dogs (Montagnaro et al. 2019), rodents and shrews (Diagne et al. 2019) but they have been designated as accidental hosts.

Besides all the evidence that USUV infects a wide range of animal species, it has high adaptability to naïve vectors and vertebrate host populations in Europe and increasing virulence, limited research is conducted on USUV. Understanding its ecology, as well as other mechanisms that are involved in its transmission and maintenance, but also establishing surveillance programs in risk areas and in wildlife are necessary. This emergent virus has the potential for global spread and to become a public health burden as the example of other *flaviviruses* that originated on the same continent.

I.C.3 Genus *Alphavirus*

The *Alphavirus* genus (*Alpha* - from Greek letter α) originally got its name from its first classification within Group A arboviruses based on their antigenic cross-relationships. The *Alphavirus* genus belongs to the *Togaviridae* family and is the only genus of this family and it contains at least 32 species that can be classified antigenically and genetically into complexes (Chen R et al. 2020).

The *Alphavirus* genus comprises arboviruses that are linked to global epidemics and their members are responsible for a wide range of medically important emerging diseases in humans, but they also can infect domestic and wild terrestrial vertebrates, as well as fish (Pastorino et al. 2004; Peyrefitte et al. 2007; Pérez et al. 2019; Steyn et al. 2020; Button et al. 2020). Interestingly, *Alphaviruses* present an epidemiological pattern linked to ecological and geographical conditions, which means that some members are restricted or have been identified in just one geographic region as is the case of ONNV in Africa and Mayaro virus (MAYV) in Latin America (Powers et al. 2001; Chen et al. 2020).

Like other arboviruses of medical importance, alphaviruses are transmitted by mosquitoes, except salmon pancreatic disease virus (SPDV) and Southern elephant seal virus (SESV) i.e., SES virus found in lice collected from elephant seals (La Linn et al. 2001). Interestingly, these two marine alphaviruses are possibly linked to the evolutionary origin of the alphaviruses, because phylogenetic analysis suggest that the origin of alphaviruses occurred in the southern oceans and spread across the Old and New World (Forrester et al. 2012).

Alphaviruses are classified into Old World viruses, which are arthritogenic such as Chikungunya (CHIKV), Mayaro (MAYV), O'nyong'nyong (ONNV), Semliki Forest (SFV) and Ross River (RRV) and New World viruses, those that cause encephalitis such as Western Equine, Venezuelan Equine and Eastern Equine Encephalitis viruses. They can be also divided into eight antigenically related complexes (**Figure 12**) (Powers et al. 2000; Weaver et al. 2012; Filomatori et al. 2020).

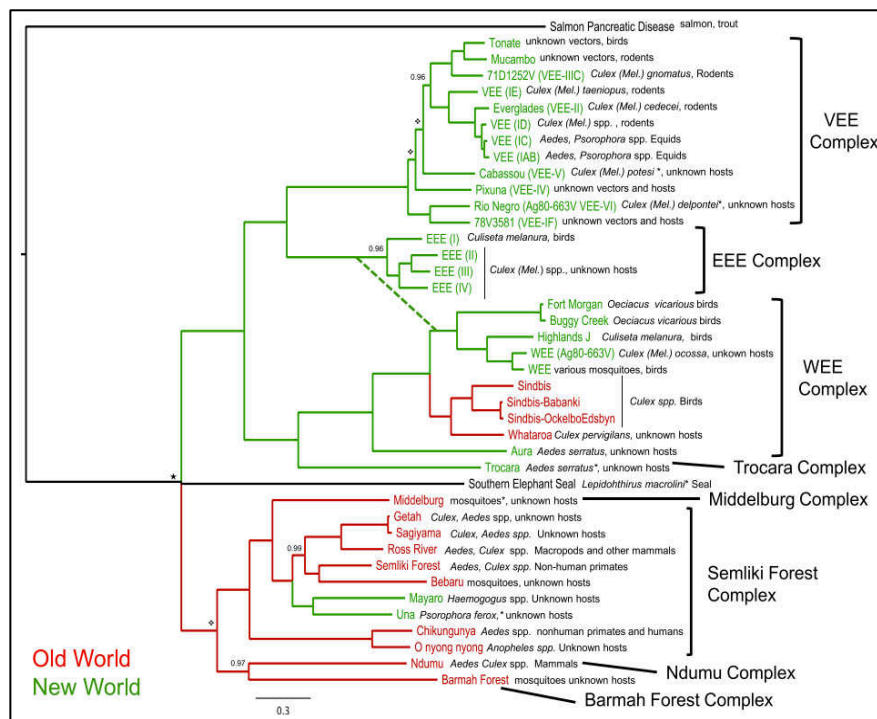


Figure 12: Phylogenetic tree of the alphaviruses divided into antigenic complexes and geographic placement. Source: *Elsevier, Antiviral Research* (Weaver et al. 2012)

1.C.3.a. Structure and Genome Organization

Alphaviruses are small RNA virions about 70 nm in diameter, spherical and include an icosahedral nucleocapsid composed of 240 capsid protein monomers, surrounded by a lipid bilayer embedded with glycoprotein spikes. The envelope contains 80 spikes, each spike is a trimer of E1 and E2 heterodimers (**Figure 13**)(Weaver and Smith 2011; Chen R et al. 2020). The cytoplasmic tails of E2 interact with capsid proteins (Button et al. 2020).

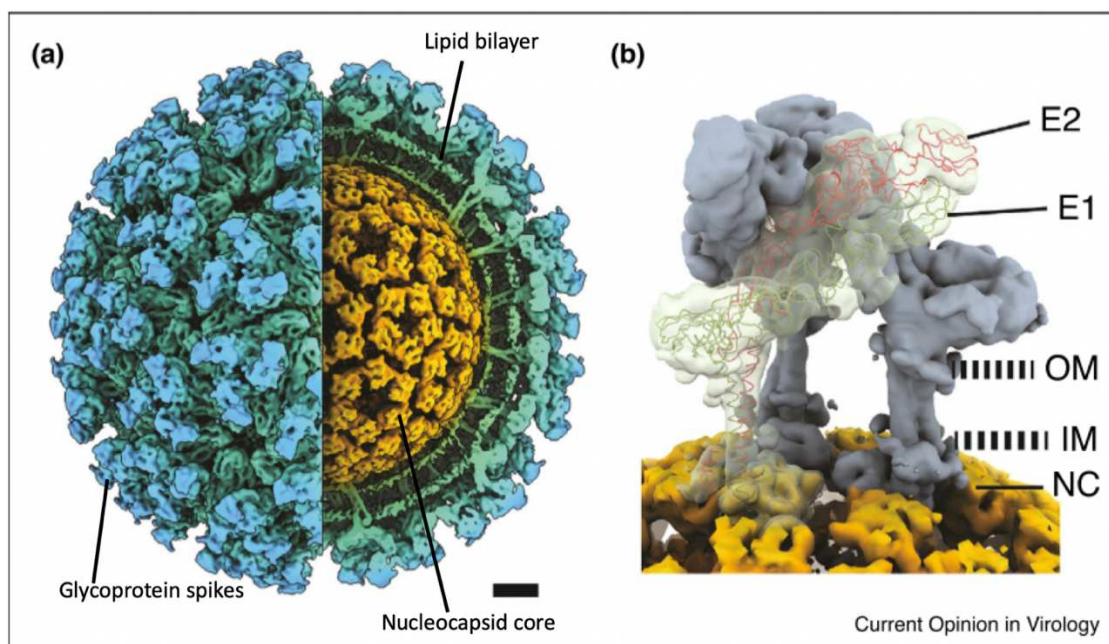


Figure 13: Alphavirus structure. (a) A representation of the structure of the Sindbis alphavirus virion; (b) Trimer of spike E1/E2. Heterodimers in gray. OM, outer membrane. IM, inner membrane. NC, nucleocapsid core. Modified from *Elsevier, Current Opinion in Virology* (Button et al. 2020).

The genome of alphaviruses is a positive sense, single-stranded RNA of approximately ~11.5 kb in length. Alphavirus RNA contains a 5' cap, and 3' poly-A tail, and encodes four nonstructural proteins and six structural proteins (**Figure 14**). The nonstructural ORF encoded nsP1-nsP4 that are important for transcription and replication. Structural ORF encodes the capsid, E3, E2, 6K/TF (transframe) and E1, in this order, that are translated as polyproteins from subgenomic mRNA. Structural proteins are involved in the assembly of an infectious virion

and E2 contains the major antigenic determinants that contribute to virulence in vertebrate hosts and infectivity in mosquitoes (Chen et al. 2020; Weaver and Smith 2011; Weaver et al. 2012; Button et al. 2020)

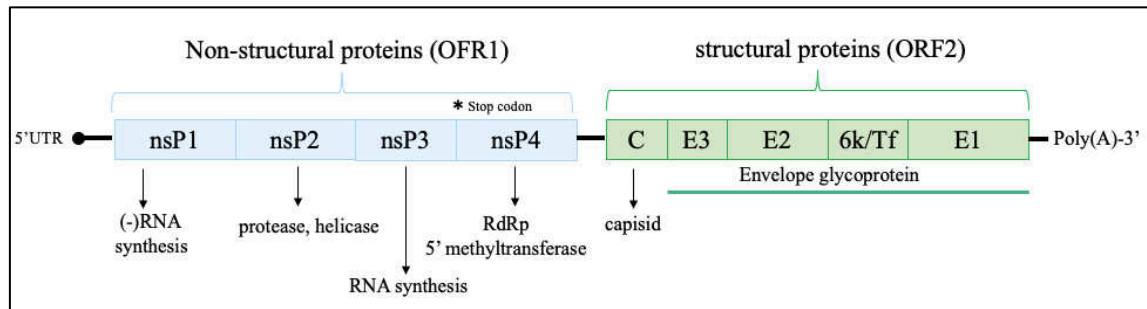


Figure 14: *Alphavirus* genome structure. Modified from, *Elsevier Antiviral Research* (Weaver et al. 2012).

I.C.4. Zoonotic *Alphaviruses*

Several zoonotic alphaviruses such as Middelburg, Sindbis, Venezuelan equine encephalitis, Mayaro, Chikungunya and O'nyong-nyong viruses have re-emerged over the past decade in Old and New World regions causing neurological disease outbreaks in wildlife, domestic animals and fever, rash, myalgia, arthralgia and neurological symptoms in humans. This group of viruses often circulates between both primatophilic and anthropophilic mosquitoes and is documented in a wide variety of mammalian, avian and other vertebrate hosts (Weaver et al. 2012; Venter 2018; Salazar-bravo and Alvis-guzma 2020). The table below lists the mosquito-borne *alphaviruses*, especially those of medical importance (**Table 2**).

Table 2: List of mosquito-borne *Alphavirus* that cause disease in domestic animals, wildlife and humans.

Antigenic Complex	Virus/subtype or strain	Abbreviation	First isolation	Geographic distribution	Vertebrate host	Human disease	Full sequence available	Vector
Barnah complex	<i>Barnah Forest virus</i>	BFV	Australia, 1974	Australia		yes	U73745	<i>Culex spp.</i> ; <i>Aedes spp.</i>
	no classified	EILV	Negev desert, 1982	Israel			JX678730	<i>Anopheles coustani</i>
Ndumu complex	<i>Ndumu virus</i>	NDUV	South Africa, 1959	Africa		yes	HM147989	<i>Aedes spp.</i>
Middelburg complex	<i>Middelburg virus</i>	MIDV	South Africa, 1957	Africa		yes	KM115530	<i>Aedes spp.</i>
Trocara complex	<i>Trocara virus</i>	TORV	Brazil, 1984	Brazil			AF252265/HM147991	<i>Culex serratus</i>
EEE complex	<i>Eastern equine encephalitis virus</i>	EEEV	Maryland, USA, 1933	North and South America	Birds, rodents	yes	EF151502/X63135	<i>Culex spp.</i> ; <i>Aedes spp.</i>
	<i>Bebaru virus</i>	BEBV	Malaysia, 1956	Malaysia			HM147985	<i>Culex spp.</i>
Semliki Forest Complex	<i>Chikungunya virus</i>	CHIKV	Tanzania, 1953	Africa, India Ocean, Asia	Humans, NHP	yes	AF369024/KP003813	<i>Ae. Aegypti</i> ; <i>Ae. albopictus</i>
	<i>Getaah virus</i>	GETV	Malaysia, 1955	Australia, Asia	Cattle and horses		A702913	<i>Culex spp.</i> ; <i>Aedes spp.</i>
	<i>Mayaro virus</i>	MAYV	Trinidad, 1954	South America, Caribbean		yes	AF237947	<i>Haemagogus spp.</i>
	<i>Onyong-nyong virus</i>	ONNV	Uganda, 1959	Africa	Humans	yes	AF079456/AF079457	<i>Anopheles spp.</i>
	<i>Ross River virus</i>	RRV	Australia, 1959	Australasia	Rodents	yes	QG433358	<i>Culex spp.</i> ; <i>Aedes spp.</i>
	<i>Semliki Forest virus</i>	SFV	Uganda, 1942	Africa		yes	X04129/JF972635	<i>Aedes spp.</i>
	<i>Una virus</i>	UNAV	Brasil, 1959	South America			U94603	<i>Psorophora</i> ; <i>Aedes spp.</i>
	<i>Cabassou virus</i>	CABV	French Guiana, 1968	French Guiana			AF075259	<i>Culex portesi</i>
	<i>Everglades virus</i>	EVEV	Florida, USA, 1963	South Florida	Birds		AF075251	<i>Culex spp.</i>
	<i>Mosso das Pedras virus</i>	MDPV	Brazil, 1978	South America			AF075257	
VEE complex	<i>Mucambo virus</i>	MUCV	Brazil, 1954	South America, Caribbean			AF075253	<i>Culex spp.</i>
	<i>Pixana virus</i>	PIXV	Brazil, 1961	South America			AF075256	<i>Anopheles spp.</i>
	<i>Tonate virus</i>	TONV	French Guiana, 1973	South and Central America			AF075254	<i>Culex portesi</i>
	<i>Rio Negro virus</i>	RNV	Argentina, 1980	South America			AF075258	<i>Culex spp.</i>
	<i>Venezuelan equine encephalitis virus</i>	VEEV	Venezuela, 1938	South and Central America	Rodents, horses, humans	yes	AF069903/L04653	<i>Aedes</i> ; <i>Culex</i> ; <i>Psorophora</i>
	<i>Aura virus</i>	AURAV	Brazil, 1959	South America			AF126284	<i>Culex spp.</i>
WEE complex	<i>Highlands J virus</i>	HJV	Florida, USA, 1960	North America	Birds		GU167952	<i>Culex melanura</i>
	<i>Madariaga virus</i>	MADV				yes	DQ241303/KJ469640	<i>Culex pedroii</i>
	<i>Sindbis virus</i>	SINV	Egypt, 1952	Global	Birds		AF429428/AF103729	<i>Culex spp.</i> ; <i>Aedes spp.</i>
	<i>Western equine encephalitis virus</i>	WEEV	California, 1930	North and South America	Rodents and birds	yes	AF214040/QG287646	<i>Culex spp.</i> ; <i>Aedes spp.</i>
	<i>Whataroa virus</i>	WHAV	New Zealand, 1962	New Zealand	Birds		HM147993	<i>Culiseta</i> ; <i>Culex spp.</i>

This list was based in the last report of ICTV, updated in 2019 (Chen R et al. 2020) and was filled also with literature-based research (Weaver et al. 2012; Powers et al. 2001; Forrester et al. 2012)(Arbovirus Catalog – CDC Division of Vector-Borne Disease (DVBD).

Despite the many reports of outbreaks caused by alphaviruses and the relative knowledge of their pathogenesis, some epidemiological features as well as the enzootic transmission cycles remain unknown or poorly understood, as well as their burden and the endemic extent of the disease in tropical countries of Africa. Here, we will focus on two *alphaviruses* that are closely related: Chikungunya and O'nyong-nyong viruses.

I.C.4. a. *Chikungunya virus*

Among the Old World alphaviruses, Chikungunya virus (CHIKV) is in fact the most important human pathogen and is involved in major outbreaks in the world causing morbidity and mortality (Weaver et al. 2012). Chikungunya, which means “to become contorted” derives from a word in the Kimakonde/Makonde language, and describes the diseases that bends up the joints (Herve Zeller, Van Bortel, and Sudre 2016; WHO 2020; Chen R et al. 2020).

Chikungunya's disease overlaps with the symptoms of other *flaviviruses* such as Dengue and Zika which can result in misdiagnosis in areas where these viruses are endemic and co-circulate. The clinical onset can include febrile illness with rash, severe pain in the palm of hands and soles of the feet. Polyarthralgia is typically a severe sequelae of the disease, that appears after recovery from the acute infection, and can persist for months or years (Herve Zeller, Bortel, and Sudre 2016; Cardona-Ospina, Diaz-Quijano, and Rodríguez-Morales 2015; Gould et al. 2017). Other severe symptoms can include neurological sequelae, myocarditis, ocular disease, acute renal disease or hepatitis. Despite the range of clinical presentations and their sequelae, fatal cases of CHIKV infection are less frequent, but in some epidemics mortality rates have been observed (Gérardin et al. 2011; Economopoulou et al. 2009), i.g. an increased mortality was reported during an epidemic peak in 2006 in Ahmedabad, India with a total of 3,056 excess deaths (Mavalankar et al. 2008) as well as on Réunion Island, in the same year of the last example, 203 death cases (244,000 cases) by CHIKV infection was reported (Renault et al.

2007).

Chikungunya virus was discovered in 1953 and it was isolated from the blood of several febrile patients and mosquito species after an outbreak reported during 1952-1953 in Tanzania, East Africa (Jupp and McIntosh 1988; Herve Zeller, Van Bortel, and Sudre 2016). After the first isolate, several outbreaks began to be reported in several African countries for about more than 30 years. Moreover, CHIKV outbreaks started to be reported in Asia concurrently with outbreaks in Africa, with the first confirmed case of CHIKV infection in late 1950. CHIKV then spread in southeast Asia where outbreaks were reported in Sri Lanka, Indonesia, India and in other eastern countries (Herve Zeller, Van Bortel, and Sudre 2016; Weaver et al. 2012). It can be said that the major first phase of the epidemics caused by CHIKV occurred between 1960-1980 and that after that first moment there was a decrease in cases and outbreaks of CHIKV in both Africa and Asia (Herve Zeller, Van Bortel, and Sudre 2016).

The second phase of CHIKV outbreaks started in 2004-2005, since then chikungunya has been identified in more than 60 countries worldwide (WHO 2020). In 2004, CHIKV emerged on Lamu Island in Kenya and started an epidemic affecting 75% of the Lamu population (Sergon et al. 2008) and also reemerged in other countries in Central and West Africa. Following these outbreaks, CHIKV widely spread in Reunion Island (Josseran et al. 2006) (Renault et al. 2007) and several other islands in the Indian Ocean (Mauritius, Seycheles, Madagascar and Mayotte) (Higgs 2006), to India (Kalantri, Joshi, and Riley 2006) and to Southeast Asia (Indonesia, Maldives, Sri Lanka, Malaysia and Thailand). In Reunion Island \approx 255,000 cases were reported between 2005-2006 with peak incidences reported during two epidemics waves (Renault et al. 2007). Following this epidemic period (2005-2006), after a gap of 32 years, CHIKV re-emerged in India, causing 1.3 million cases in 13 states (Arankalle et al. 2007). In 2006 CHIKV re-emerged in Malaysia after 7 years of silence. The authors confirmed through genomic sequences that the Asian genotype was responsible for the outbreak

supporting the idea that CHIKV is an endemic infection in Malaysia (AbuBakar et al. 2007).

In Europe, travel-related CHIKV infection cases from endemic areas started to be reported in France 2005-2006 (ECDPC 2020) and the first outbreaks started consecutively in Italy (2007 and 2017) (Rezza et al. 2007) and France (2010, 2014 and 2017) (Grandadam et al. 2011). These outbreaks highlighted the potential dissemination of CHIKV and the efficiency of transmission by *Ae. albopictus* (Vega-Rua et al. 2013).

In America and the Caribbean Islands, the first cases of autochthonous transmission of CHIKV were reported in 2013 primarily on the islands of Saint Martin and Martinique. A large epidemic of CHIKV spanned over to the other Caribbean Islands and the *Ae. aegypti* and *Ae. albopictus* mosquitoes were the main vectors with the CHIKV strains belonging to the Asian genotype, being responsible for the outbreaks (Cunha et al. 2020). In 2014 CHIKV reached continental territory in countries such as French Guiana, Venezuela, Equator, Peru, Bolivia, Argentina, Chile and Brazil. The Pan American Health Organization (PAHO) reported in 2014 more than 1 million suspected cases and 37,480 in 2015, 146, 914 in 2016 and 123,087 in 2017 autochthonous cases were confirmed in America (WHO 2020). Moreover, all these autochthonous cases on the continent were related to the Asian lineage. The first time that East/Central/South African (ECSA) genotype was notified in America was in 2015 in Bahia state, the north-eastern region of Brazil. This Brazilian ECSA lineage was closely related to an CHIKV from Angola identified in 1962 (Nunes et al. 2015). By mid-2021 more than 27.000 mil cases were confirmed on the American continent (PAHO- Pan American Health Organization 2021).

When it comes to the first major outbreaks of CHIKV, two major lineages ECSA and Asian were responsible for it. Phylogenetic analysis using partial nucleotide sequences of the E1 gene supported the hypothesis that CHIKV originated in Africa with the existence of two main genotypes i.e. the West African (WA) and ECSA (Powers et al. 2000). Currently, CHIKV

are classified into three geographically distinct genotypes: enzootic WA, enzootic ECSA and endemic/epidemic Asian (Gould et al. 2017). In addition, the Asian and ECSA genotypes caused outbreaks in worldwide. A mutation in ECSA genotypes conferred enhanced transmission by the *Ae. albopictus* which could explain the rapid spread of CHIKV during the last years. The mutation is located in the E1 glycoprotein at position 226 (E1-A226V) and increases the ability of the ECSA strain to replicate in this mosquito's species (Vazeille et al. 2007; Sam et al. 2012). Some recent sub-lineages are responsible for multiple outbreaks such as the Indian Ocean lineage (IOL) and the Asian/American lineage, that emerged within the ECSA and Asian clade, respectively (Langsjoen et al. 2018; Phadungsombat et al. 2020). These lineages and sub-lineages are distributed around the globe and with major concentration in tropical and sub-tropical zones (**Figure 15**).

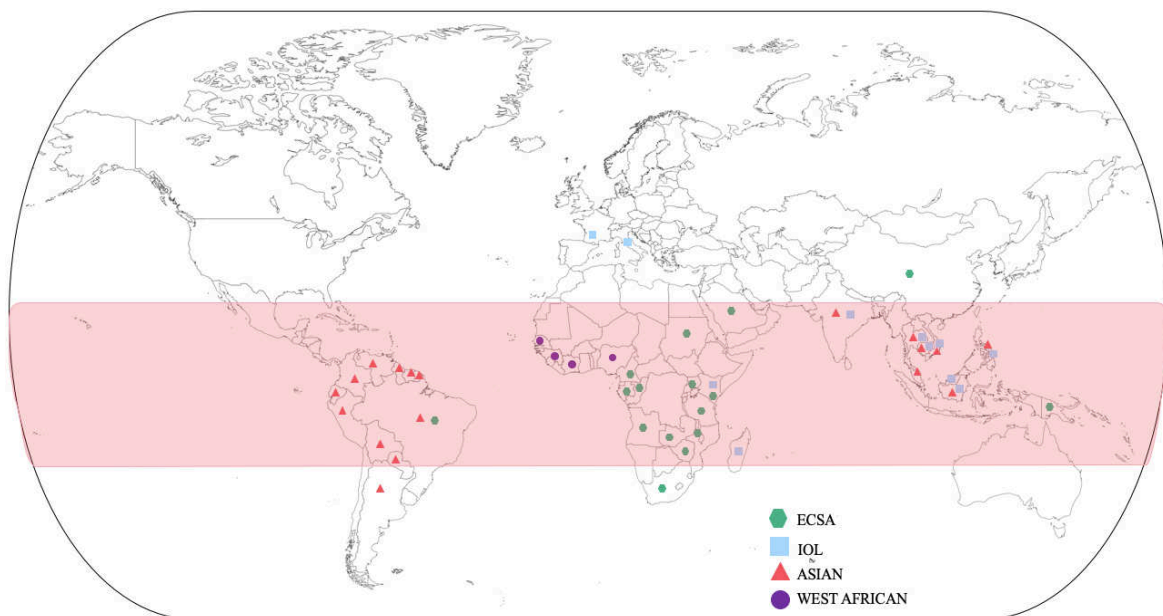


Figure 15: Chikungunya virus lineages distributed worldwide. Tropical and sub-tropical regions are highlighted in red on the map. This map was modified from *Frontiers in Microbiology* (Cunha et al. 2020).

The transmission cycle of CHIKV are divided in two mains cycles (enzootic/sylvatic; epidemic/endemic cycles) and involve vertebrate hosts and mosquito vectors (Tsetsarkin, Chen,

and Weaver 2016):

- The enzootic, sylvatic African cycles involves NHPs as principal enzootic amplification hosts and forest-dwelling mosquitos. CHIKV enzootic transmission cycles occur most in sub-Saharan Africa but are still poorly understood. (Althouse et al. 2018). In Africa, many sylvatic *Aedes* species are involved in this transmission cycles and *Ae. Africanus* appears to be the principal vector (Diallo et al. 1999). Sylvatic CHIKV have been isolated from many NHPs species such as *Chlorocebus sabaues*, *Erythrocebus patas*, *Papio papio*, *Cercopithecus aethiops*, *Galago senegalensis* and anti-CHIKV neutralizing antibodies have been documented in *Mandrillus sphinx*, *Cercopithecus ascanius schmidtii*, *Cercopithecus (Chlorocebus) aethiops sensu lato* and *Papio ursinus* in Senegal, Gabon, Uganda and South Africa (Althouse et al. 2018). An enzootic CHIKV cycle outside of Africa has not yet been confirmed, despite the evidence of CHIKV in monkeys in the Philippines and Malaysia. In the Philippines, 14.8% and 59.3% of *Macaca fascicularis* showed IgM and IgG antibodies to CHIKV, respectively (Inoue et al. 2003). CHIKV was isolated for the first time from wild long tailed macaques (*Macaca fascicularis*) in Malaysia, suggesting the existence of sylvatic cycle (Apandi et al. 2009)
- The epidemic/endemic cycles occur in urban areas and the transmissions are established by between humans and mosquitos, where *Ae. aegypti* and *Ae. albopictus* are the principal anthropophilic vectors. Moreover, *Ae. albopictus* has been identified in the last years as an excellent vector to transmit the virus among humans, which was increased by the adaptive mutation in the CHIKV

envelope glycoproteins (Tsetsarkin and Weaver 2011)(Pagès et al. 2009; Vega-Rua et al. 2013; Pereira-Dos-Santos et al. 2020).

- Otherwise, CHIKV was isolated from squirrels, bats and ticks but also rodents and birds have been reported to be seropositive to CHIKV suggesting the existence of a cycle that contribute to maintaining the virus in Senegal and Central African Republic (endemic regions), while primate are immunologically protected (Diallo et al. 1999; Althouse et al. 2018); *Artibeus jamaicensis* and *Artibeus lituratus* bat species were captured in Grenada Island (Caribbean) and were tested for neutralizing antibodies to CHIKV and 36% (15/42) were seropositive (Stone et al. 2018).

Chikungunya virus has become widely spread and became a public health threat during the last 15 years. Yet, there are still many gaps in understanding the genetics, evolution and emergence mechanism of CHIKV. It is necessary to continue sampling of wild animals and human populations in tropical and sub-tropical regions, to identifies the levels exposure and CHIKV seroconversion to better understanding the sylvatic cycle of CHIKV and to know which vertebrate host are implicate in the maintenance of the sylvatic cycle in the regions. Moreover, a global vigilance is required to predicting future CHIKV emergence and outbreaks (Tsetsarkin, Chen, and Weaver 2016; Herve Zeller, Van Bortel, and Sudre 2016).

I.C.4. b. *O’Nyong nyong virus*

O’nyong-nyong, means ‘very painful weakening of joints,’ in the language of the Acholi people of northwestern Uganda (Haddow, Davies, and Walker 1960). ONNV become endemic in sub-Saharan Africa and co-circulate in the same areas as forests and urban settlements where dengue fever and malaria are also present (LaBeaud et al. 2015).

ONNV was first isolated from human blood and anopheline mosquitos in Gulu, during an epidemic that started in northern Uganda in 1959 (Haddow, Davies, and Walker 1960; M. C. Williams and Woodall 1965). The epidemic is widespread in East Africa including Kenya, Tanzania, Malawi, Mozambique and the Democratic Republic of Congo and lasted > 4 years affecting more than 2 million people only in eastern Africa (M. C. Williams and Woodall 1965; R. Lanciotti et al. 1998; Bessaud et al. 2006). During the epidemic period, ONNV spread also westward to Senegal, Nigeria, Guinea Bissau, DRC, Cameroon and the Central African Republic (CAF) where human infections and virus isolation have been documented (Brottes et al. 1966; Taufflieb R, Cornet M, and Camicas JL 1968; Guyer 1972; Kiwanuka et al. 1999; Powers et al. 2000; Posey et al. 2005; Giovanni Rezza, Chen, and Weaver 2017). Human cases of ONNV, outbreaks and virus isolation in the African continent are shown on the map below (Figure 16) (Pezzi et al. 2019).

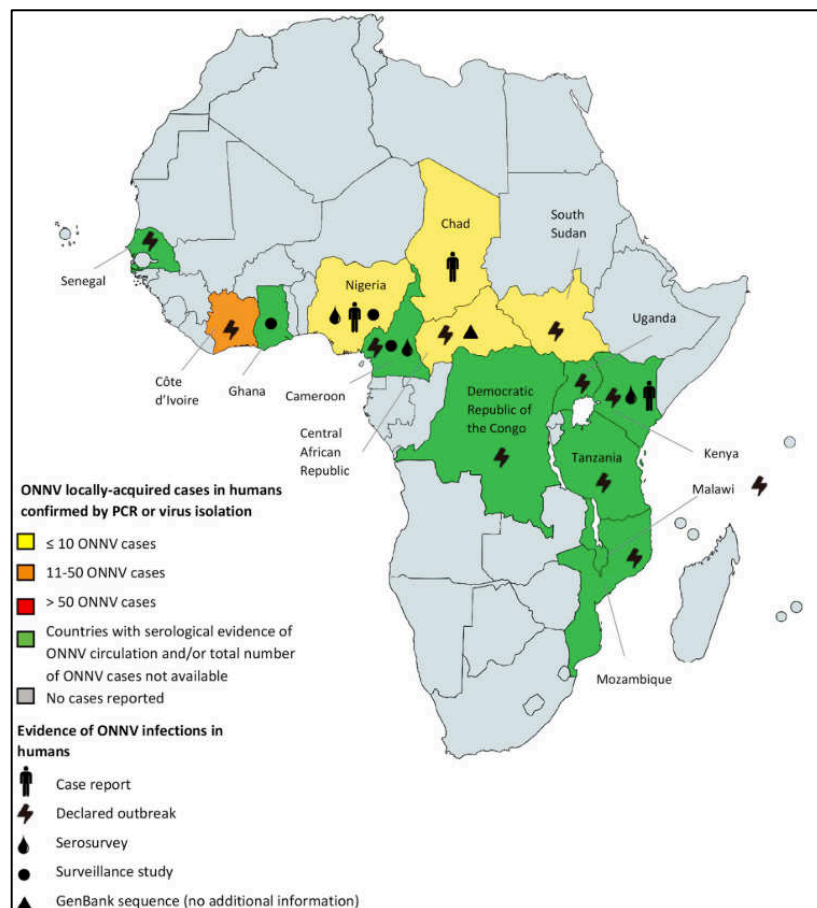


Figure 16: Cases of O’nyong-nyong virus in Africa countries. Source: *Elsevier Antiviral Research* (Pezzi et al. 2019).

A second epidemic wave started in 1996 after 35 years of absence of reports of human infections or epidemics caused by ONNV. The virus re-emerged in south-central Uganda causing sporadic outbreaks and similar symptoms to that of the first epidemic period (Rwaguma et al. 1997; R. Lanciotti et al. 1998). During this outbreak in south-central Uganda a large difference in infection and attack rates was reported. ONNV infection rate ranged from 3% to 45% and ONNV fever attack rate ranging from 3% to 29% (Sanders et al. 1999). During the same ONNV fever epidemic (1996–1997), ONNV was isolated from *Anopheles funestus* mosquitos during entomological studies in the Bbaale village, Rakai District of southcentral Uganda, assuming that this mosquito species was the main vector as it was the most abundant species during collection (80%)(Lutwama et al. 1999).

High rates of ONNV were reported in coastal Kenya in sera of non-symptomatic humans and an IgG prevalence of 56% in a total of 443 samples confirmed by PRNT as compared to 6% for CHIKV. Another fact was that younger adults were more likely to be seropositive than older adults and older age and female gender were associated with ONNV and CHIKV seropositivity (LaBeaud et al. 2015).

In Cameroon, 34.2% (29/79) of febrile patients reacted with *alphavirus* antigens by Haemagglutination-inhibition (HI) and ONNV and CHIKV being the most frequent. The samples were also positive by complement fixation and some samples showed high neutralizing antibodies titer against ONNV by PNRT (Fokam et al. 2010). Another study in a rural area of western Cameroon, no anti-ONNV antibodies were found in healthy volunteers one year after CHIKV outbreaks in 2006 (Demanou et al. 2010). Further studies of seroprevalence and distribution of arboviruses in rural Cameroonian adults were conducted and 256 samples were tested by PRNT and a high seroprevalence was observed for both ONNV (47.7%) and CHIKV (46.5%), besides that some sera had higher antibody titers to ONNV than to CHIKV suggested possible ONNV circulation in the population (Kuniholm et al. 2006). In Côte d'Ivoire

an outbreak of febrile illness started in 2003 in a refugee camp and samples were collected from patients in the acute and convalescent phase. Of those surveyed, 26% showed positivity to ONNV infection in serological and RT-PCR (Posey et al. 2005). In addition, ONNV was isolated in Chad from the blood of a convalescent patient, and anti-ONNV IgG was detected in a traveler with fever who returned to Europe from West Africa (Bessaud et al. 2006; Tappe et al. 2014).

ONN fever disease is characterized by the sudden onset of fever, severe arthralgia, that is similar to CHIK fever, but also headache, generalized maculopapular skin rash. Unlike CHIK fever, ONN fever causes more manifestations of cervical lymphadenitis and conjunctivitis than CHIK fever (Shore 1961; Kiwanuka et al. 1999; Tappe et al. 2014). The transmission of ONNV is primarily by anopheline mosquitoes, these include *Anopheles funestus* and *Anopheles gambiae*, and ONNV is thus the only virus among alphaviruses that is transmitted by this species of mosquitoes, the same vectors of malaria (M. Williams et al. 1965). The transmission cycle is limited to the African continent and during the interepidemic periods, ONNV is probably maintained through an enzootic cycle, whose actors have not been well characterized yet. Some mammal species showed neutralizing antibodies against ONNV, suggesting the existence of an enzootic cycle (Pezzi et al. 2020). These species were: duikers (*Cephalophus* and *Philantomba* spp.) in DRC, forest buffaloes (*Syncerus caffer nanus*) in DRC and Gabon and also mandrills (*Mandrillus sphinx*) in Gabon; suggesting that several species of mammals may be involved in the enzootic cycle of ONNV and serve as host/reservoir (Kading et al., 2013).

I.C.5. Typical Mechanism of Flaviviruses and Alphaviruses Transmission

The transmission cycles for several mosquito-borne virus have been established with some of them occurring in sylvatic (or jungle), urban and ecotone ecosystems in the same

region. Depending on the environment and the transmission vectors, the cycles use different species as vertebrate or reservoir hosts. Furthermore, humans and other animal species can serve as a dead-end host. Thus, for an arbovirus transmission cycle to be established is require a host in which they replicate and a vector (mosquitoes and ticks) (Weaver and Barrett 2004).

The knowledge of the natural ecology of transmission and the capacity of different vectors to transmit some arboviruses as Dengue, Yellow fever, Zika, Usutu, O'nyong-nyong and Chikungunya is essential to understanding their emergence. Some of these arboviruses have an enzootic cycle establish in Africa but for other some gaps are still open and the possibility of other animal species being involved are pertinent.

The natural YFV transmission cycle in Africa is an enzootic cycle involving *Aedes spp.* found in the forest canopy as vector and NHPs. When humans encroach the forest, they can be infected by sylvatic strains. Urban and intermediate cycles exist involving human-mosquito-human in urban cycles and human-primates-humans in the jungle border areas of intermediate zones (ecotone).

The natural Dengue virus transmission cycle in Africa is a sylvatic cycle involving non-human primates and arboreal canopy-dwelling *Aedes spp.* (i.g., *Ae. furcifer* and *Ae. aegypti formosus*). An intermediate zone (ecotone) as rural areas can facilitate the transfer of sylvatic DENV from the forest to peridomestic environments. The human cycle is mediated by *Ae. aegypti* and *Ae. albopictus*.

The natural Zika virus transmission cycle in Africa is an enzootic cycle mediated by non-human primates and arboreal mosquitoes as *Ae. vittatus*, *Ae. furcifer* and *Ae. aegypti formosus*. Zika virus also stablish an urban tansmition cycle involving human-mosquitoes-humans which *Ae. aegypti* and *Ae. albopictus* act as vectors.

The enzootic cycle of WNV is mediated by birds and ornithophilic/anthropophilic mosquitoes (i.e., *Cx. univittatus*) with mammals serving as dead-end host.

The natural cycle of transmission of the USUV is an enzootic cycle involving mainly passerine birds as amplifier host and mosquitoes ornithophilis (i.e., *Culex spp.*) as vector.

In Africa CHIKV is maintained in a sylvatic cycle involving non-human primates and mosquitoes of the genus *Aedes* (i.g., *Ae. furcifer*, *Ae. luteocephalus*, *Ae. africanus*). In this cycle's humans can be infected being an incidental host. The virus has an urban cycle well established and mediated by *Ae. aegypti* and *Ae. albopictus* mosquito species.

The sylvatic cycle of ONNV in Africa that has not been characterized yet but *Anopheles funestus* and *Anopheles gambiae* are involved as natural vectors. Humans serving as amplification hosts during epidemics.

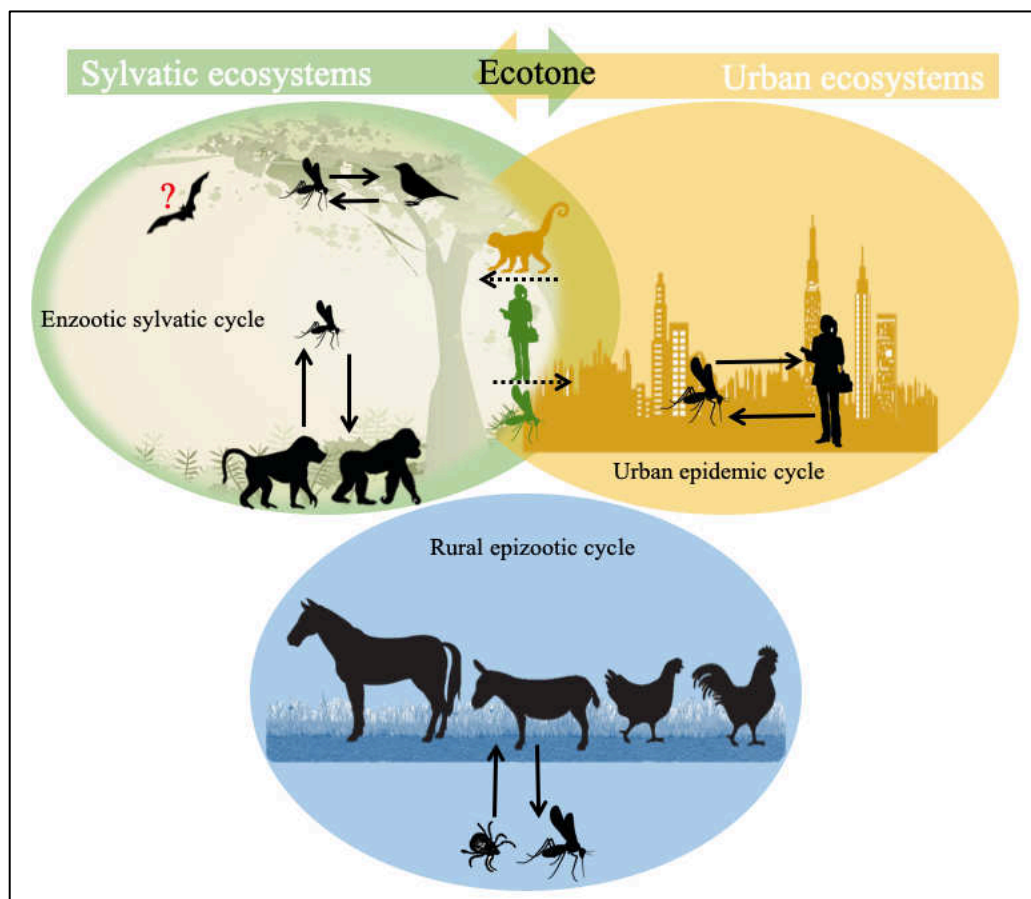


Figure 17: Typical Flavivirus and Alphavirus transmission cycles. The enzootic transmission cycles of some zoonotic arboviruses are maintained involving birds, rodents, NHPs and perhaps bats, as yet unconfirmed and bridge vectors. Some ecosystems can overlap and facilitate the movement of vectors, animals and humans between sylvatic, rural and urban areas, facilitating the movement, for example, of DENV strains of sylvatic or endemic origin. Some arboviruses use domestic animals as amplifying hosts within a rural epizootic cycle.

I.D. Arbovirus Distribution

I.D.1. The Global Epidemic

During the last two decades, the emergence and resurgence of epidemic arboviral diseases have affected humans and animals around the world. They have become a concern and a burden for public health worldwide. Arbovirus epidemics were mainly caused by Yellow fever, Dengue, Zika, Chikungunya viruses and more recently by the emerging viruses like West Nile in the Americas and Europe and Usutu in Europe. As an example, Dengue virus is one of the most common infections globally and is distributed in at least 128 countries with active transmission putting billions of people at risk (Kraemer et al. 2015; Stanaway et al. 2016; Fritzell et al. 2018).

I.D.2. The epidemic in Africa

More than 600 arboviruses are cataloged around the world and comprise several families. A large number of these arboviruses, which are pathogenic to humans and cause various clinical manifestations are suggested to originate in Africa (Burt, Goedhals, and Mathengtheng 2014; Young 2018). The principal arboviruses that cause diseases in humans and different clinical signs are transmitted by mosquitoes. Mosquito-borne diseases have caused more than 700,000 deaths yearly across the globe (Burt, Goedhals, and Mathengtheng 2014; Nyaruaba et al. 2019). Some and the most common clinical syndromes are acute benign febrile illness, rash, headache, conjunctivitis, neurological disease, polyarthritis, hemorrhagic fevers (Young 2018). In South Africa for example, mosquito-borne viruses as Sindbis, Rift Valley, West Nile cause large outbreaks in periods of heavy rainfall. In addition, other less known *flaviviruses* and *alphaviruses* such as Usutu and Middelburg viruses have been shown to have a high potential for emergence and to cause outbreaks, however knowledge on their

medical importance and diagnostic capacity is still limited (Burt, Goedhals, and Mathengtheng 2014). Mosquito-borne viruses such as CHIKV, ZIKV and WNV were isolated for the first time in East Africa, and are now causing outbreaks worldwide (Nyaruaba et al. 2019).

In sub-Saharan Africa, acute febrile illnesses is one of the most widely distributed disease. However, distinguishing which pathogen is implicated in the acute febrile illnesses becomes challenging because familiar diseases as malaria and typhoid fever can be over-diagnosed and the real extend of arboviruses can be underestimated (Sow et al. 2016). Serological surveys have been an important tool in the identification and distribution of arboviruses in the general population, as well as in domestic animals and wildlife mainly in the identification of vertebrate/reservoirs host, since many of these arboviruses still do not have an identified reservoir source (Gilbert et al. 2013).

I.D.2.a. In Guinea

Several arboviruses have been identified in various regions of Guinea. Serological surveys were conducted between the 70s and 80s in febrile patients and a prevalence of 40% for YFV is found (Butenko 1996). YFV has caused outbreaks throughout the country and the largest outbreak in the population was in 2000 and 2001 in Conakry (Nathan et al. 2001; Jentes et al. 2010). Several arboviruses were also isolated from arthropods, bats, birds, non-human primates (Butenko 1996). A considerable proportion of febrile illnesses remains unidentified in Guinea. Thus, serum of 47 suspected cases of arboviruses were tested by Immunoglobulin M antibody capture enzyme-linked immunosorbent assays and plaque-reduction neutralization assays. The study found a seropositivity of 63% (30/47) in the patients and the arboviruses included WNV, YFV, DENV, CHIKV and Thayna virus (Jentes et al. 2010). Seroepidemiological studies of arboviruses in human populations are still scarce and even despite evidence of circulation throughout the African continent, laboratory diagnoses do not

seem to be part of the routine, even in Guinea. Furthermore, it leaves an open gap in the history of arboviruses.

D.2.b. In Cameroon

Cameroon is a country located in central Africa with a vast tropical rain forest being favorable for dissemination of many infectious diseases as malaria (endemic in the region) and the arboviruses due to presence of a variety of hematophagous arthropods. Many mosquito species have been identified in the field like *Aedes spp*, *Anophele spp*, and *Culex spp* supporting the circulation of arboviruses (Demanou et al. 2010; Braack et al. 2018).

Serosurvey showed the circulation of many arboviruses in the country, even silently (Fokam et al. 2010). Febrile patients (with negative laboratory findings for malaria and typhoid fever) and anthropophilic mosquitoes (4,764) were tested for arboviruses by hemagglutination inhibition (HI), complement fixation test (CF) and virus isolation. The patients reacted with both *alphavirus* (36.7%) and *flavivirus* (58.2%) by HI. The most *alphavirus* positive by HI were CHIKV (34.2%) and ONNV (34.2%). All serotypes of dengue were detected being DENV-2 (39.2%) the most seroprevalent followed by ZIKV, YFV and WNV by HI. No virus was isolated (Fokam et al. 2010). Another serosurvey in samples from 234 acutely febrile patients have been screened for arboviruses and antibodies to CHIKV, DENV 1–4, YFV, WNV were detected by HAI and cross-reactivity among the *flavivirus* was also observed. 44% of serum samples showed antibodies to CHIKV (Ndip et al. 2004).

A serosurvey for arboviruses was conducted in healthy rural Cameroonian adults. A total of 256 serum samples were tested by PRNT and different positive rates were found; CHIKV (46.5%), ONNV (47.7%), DENV-2 (12.5%), 26.9% to YFV and 6.6% to WNV. The samples were also seropositive for Sindbis and Thayna viruses. Age was significantly related to increased rates of YFV and CHIKV (Kuniholm et al. 2006).

Different serotypes of dengue have been identified among acute febrile patients and in children of Cameroon. A cross-sectional study using a Dengue NS1-IgG/IgM Rapid Test was conducted in 961 children of pediatric clinics and observed a prevalence of 6.14% acute dengue virus in children with febrile illness. A significant difference was seen between populations who practiced vector protection measures among those who did not (Tedjou et al. 2019). In Douala, Cameroon, where *Ae. aegypti* is predominant in downtown and *Ae. albopictus* more common in suburban areas the presence of dengue was investigated in acute febrile patients. In 2017 a total of 114 plasma were tested by PCR, showing that serotype 1 of dengue was predominant and responsible of 10% of the acute febrile diseases of which 63.2% were females (Yousseu et al. 2018). More recently, 320 acute febrile patients were screened for DENV using RT-PCR and 12.8% (41/320) were positive with DENV-3 (68.3%) the most common followed by DENV-2 (19.5%) and DENV-1 (4.9%) (Tchetgna et al. 2021).

Chikungunya was first isolated in Cameroon in 2006 where the virus was identified in Douala and Yaoundé during an outbreak of a dengue-like syndrome in the same year. Samples were collected from patients (1 to 54 years) and tested for CHIKV, DENV and WNV using ELISA/IgM/IgG. Specific real time RT-PCR showed that one sample was positive for CHIKV and phylogenetic analysis showed that it fell in cluster of viruses from the Democratic Republic of the Congo. The authors suggest the existence of urban cycles since the patients were infected in urban or peri-urban centers (Peyrefitte et al. 2007). A serological and entomological survey was conducted in 2006 during an outbreak of CHIKV in a rural area in Western Cameroon, one year after the identification of the initial outbreak. ELISA IgM and IgG was used to discriminate CHIKV, ONNV and DENV. A total of 105 sera were screened and 51.4% had detectable IgM anti-CHIKV antibodies and 39% had IgG. All sera were negative to DENV and ONNV. The mosquitoes captured were *Ae. africanus* and *Culex spp* (Demanou et al. 2010).

I.D.2.c. In the Democratic Republic of Congo

The Democratic Republic of Congo (DRC) located in central Africa, is the second largest country in Africa. DRC comprising an area of more than 230 thousand hectares with primary ecosystems such as tropical and subtropical grassland, savanna, shrubland, tropical and subtropical moist broadleaf forest. The country has an incredible biodiversity with many species of apes, elephants, white rhino etc (Simons 2013; Mbanzulu et al. 2020). Their biodiversity contributed to the emergence of numerous viral diseases. Arboviruses circulate since 1912 when the first outbreak of YFV was reported (Deprez 1920). A number of mosquito-borne viruses have been documented in humans but also in livestock and domestic animals and multiple outbreaks of CHIKV and YFV have been reported in the country (Nur et al. 1999; Pastorino et al. 2004; Willcox et al. 2018; Makiala-Mandanda et al. 2018; Selhorst et al. 2020; Mbanzulu et al. 2020)

Chikungunya virus has caused repeated outbreaks in DRC. The virus was first isolated in 1958 and two years later, Pastorino and colleagues (2004) reported the resurgence of CHIKV after 39 years during an urban epidemic in Kinshasa in 1999-2000 (Pastorino et al. 2004). The virus was isolated from nine patients with clinical symptoms and all sequences clustered in the central African lineage (Pastorino et al. 2004). In 2019 another outbreak of CHIKV was reported in DRC and 49.7% (87/175) of the samples from patients with chikungunya-like symptoms collected in Kinshasa (capital) and Kongo Central province were positive for CHIKV by real-time RT-PCR. In addition, CHIKV was found in 41.7% (14/34) pools of mosquitoes more predominantly in *Ae. albopictus* than *Ae. Aegypti* from the same area. ECSA2 lineage was identified by whole-genome sequencing and the new viruses did not cluster with sequences from the previously reported outbreak. The authors suggested that CHIKV is adapting in function of *Ae. albopictus* (Selhorst et al. 2020).

A serosurvey was conducted using dried blood spots (DBS) from 978 children and

screened using ELISA and neutralization assays. A global seroprevalence of 18.7% (183/978) was shown and the tests showed evidence of past infection to DENV and ZIKV. Antibodies to serotypes 1, 2 and 3 of dengue were identified. Furthermore, the authors were surprised by the positive result for YFV, showing the failure of the vaccination program with only 18.4% were positive by ELISA (Willcox et al. 2018). YFV in DRC is responsible for more than 95% of acute febrile jaundice cases, according to the program of Yellow fever surveillance in DRC. Real-time PCR was used to screen 453 patients that were IgM negative to YFV for presence of DENV, WNV, CHIKV, ONNV, ZIKV, RFV and YFV. Patients were positive for DENV and CHIKV, and some of them showed co-infection with both viruses (Makiala-Mandanda et al. 2018). West Nile virus caused sporadic outbreaks in some regions of DRC and in military patients with neurologic signs, abdominal pain, arthralgia and headache, IgM antibodies to the following arboviruses were detected; WNV (66%), CHIKV (34%) and DENV (3%). The results confirmed the outbreaks of WNV in Kapalata military camp in Kisangani, DRC (Nur et al. 1999).

A review on mosquito-borne viral disease in DRC showed that arboviral infections increased over time and space in the country and YFV, DENV, ZIKV, WNV, CHIKV and ONNV were more frequently recorded across the DRC. Serological investigations and molecular methods were used on the majority of human samples. Nevertheless, African buffalo, chimpanzees and elephants from Haut-Uelé Province have been identified as potential hosts or reservoirs to CHIKV based on serological evidence only. Neutralizing antibodies against ONNV were found in buffaloes and duikers in the Garamba National Park and Ituri Rain Forest (Kading et al. 2013) respectively, but infections in humans have never been identified in DRC. They also highlight factors that are drivers for the occurrence of mosquito-borne viruses as heavy rains, human movements, forest encroachment and deforestation (Mbanzulu et al. 2020).

I.E. Animal Reservoir and the Ecology of Arboviruses

A reservoir of an infectious agent is life beings (humans, animals) or environments in which the agent normally lives, grows and multiplies and can persist for long periods (CDC 2012). In the case of wildlife populations, these may be an important reservoir of infectious agents and provide a “zoonotic pool”, from which pathogens, hitherto unknown in humans, can emerge (Morse 1995). Regarding reservoirs of arboviruses, there are still vast discussions and controversial issues concerning vertebrate reservoirs (Kuno et al. 2017). Arboviruses have a unique mode of transmission using arthropods as vectors of transmission to vertebrate hosts and these vertebrates may play a role as reservoirs, as defined by the WHO (WHO 1985). However, arboviruses represent a heterogeneous group of viruses that involve multiple vectors and/or animal hosts, which is intriguing and a puzzle to identify vertebrate reservoirs.

Another element is the variable time of the viremic period in infected vertebrate hosts despite some studies have shown persistence viral RNA in different periods of time. As examples, WNV was detected in urine of convalescent patients for 2452 days (Murray et al. 2010) and for up to 5.5 months in the brain of monkeys after inoculation (Pogodina et al. 1983). Therefore, to identify the vertebrate reservoirs of arboviruses, three commonly empirically-learned conditions have been stated by Kuno and colleagues (2017) “(i) either continued or intermittent isolation of an arbovirus in question from the suspected vertebrates during the period of complete cessation of vector’s feeding activity (a minimum of 2 but preferably ≥ 3 months) (hereafter called the “interrupted period”), such as during the dry season in the tropics or the cold season in temperate regions; (ii) the total absence of the introduction of infected vertebrates and/or vectors during the interrupted period; and (iii) the detection of the infectious virus from blood after the interrupted period. Ideally, a consensus among multiple groups of independent researchers for a given virus-host relationship is desirable”(Kuno et al. 2017).

The maintenance mechanism and amplification cycle of arboviruses is still incompletely elucidated and sometimes becomes complex because it can involve several vectors and infect numerous host species depending on the environment. Despite the knowledge of certain actors in the role of arbovirus maintenance, infected humans may be considered as a “transient reservoir” involved in the urban transmission cycles of Dengue, Chikungunya and Zika viruses (Kuno et al. 2017; Weaver and Barrett 2004). However, the natural maintenance mechanism of certain arboviruses in the context of sylvatic/enzootic transmission cycles remains unknown as well as the animal/vertebrate reservoir. Because of the recent observations of a sylvatic cycle of Zika virus in Brazil, it is suggested that neotropical primates could be reservoirs for Zika virus in Brazil. Hence, sera and oral swabs from twenty four neotropical primates were tested by real-time PCR and seven (29%) were positive (Favoretto et al. 2016). In another study in Brazil, 82 carcasses of free-living NHP that were found dead in urban and peri-urban areas were investigated for YFV and ZIKV by molecular analysis and thirty nine percent (32/82) were ZIKV-positive in at least one tissue sample and all samples were negative for YFV (Terzian et al. 2018). The obtained nucleotide sequences from these samples were phylogenetically related to the American lineage of ZIKV.

Bats have been suspected to play a role as reservoir for DENV, because both RNA and antibodies to NS1 proteins have been detected in samples from Neotropical bat species in French Guiana, Mexico and Costa Rica (de Thoisy, Dussart, and Kazanji 2004; de Thoisy B et al. 2009; Moreira-Soto, Soto-Garita, and Corrales-Aquilar 2017; Thompson et al. 2015)

The maintenance mechanism of WNV and the reservoir hosts in certain regions, such as the USA, are well described. In epizootic and enzootic cycles of WNV transmissions, several species of birds are involved and act as reservoirs/amplifying hosts (Taieb et al. 2020). However, the possibility of other animal species participating in the WNV transmission cycle or even acting as amplifier hosts cannot be ruled out. Although the determination and/or the

identification of animal species serving as reservoirs and/or amplifying hosts in a given environment is a complex process, elucidating this is essential for the understanding of the origin of arboviruses and to predict potential outbreaks.

II.A. Non-human Primates

The risk of pathogen transmission between humans and non-human primates (NHPs) increased in the last years due the increasing proximity between them. The natural barriers between them were reduced by several means such as road development, encroachment of humans into ecosystems for agriculture, hunting, travel which made contacts between wildlife and humans more frequent, allowing thus the transmission of infectious diseases (Devaux et al. 2019; Scheffers et al. 2019; Jones et al. 2008). Africa is estimated to host 70 species of NHP including apes and monkeys (Perelman et al. 2011). Some are terrestrial, like apes, others, like some guenons, are arboreal. These NHPs live in contrasted areas in lowland forests, secondary forests and in the forest/savannah mosaics. For example, Central Africa is composed for 90% of tropical forest, concentrating thus a vast diversity of NHPs. In this part of Africa, hunting and eating of NHPs as bushmeat is common and wildlife remains the primary source of meat for most Central African villagers (Wolfe et al. 2005). This practice is associated with an increased risk for zoonotic transmissions because humans can be exposed to infected blood, secretions or tissues of bushmeat during hunting and butchering (Steve et al. 2017; Peeters et al. 2002; Filippone et al. 2015).

Zoonotic transmission of pathogens from NHPs to humans can also occur by other routes such as droplets, fecal-oral contamination, through bites or by an arthropod vector. The encroachment of humans in forest habitats increased also the contact with many species of mosquito vectors that feed on NHPs that can be potentially infected by arboviruses, and infect humans who can bring the disease to more populated urban areas (Possas et al. 2018). It is

assumed that many emerging arboviruses originated in NHPs and that they are maintained in natural forests by sylvatic transmission cycles involving arboreal mosquitoes as vectors (Weaver and Barrett 2004; Vasilakis et al. 2011).

NHPs typically develop viremia without clinical signs (Jones et al. 2008), but this is based on limited data. However, some NHP species such as *Alouatta spp.* from South America, are susceptible to YFV and developed clinical signs and even deaths (Possas et al. 2018). Therefore, the circulation of humans between forests, urban and rural areas can contribute to the spread of arboviruses in both sylvatic or urban cycles, leading to events of “spill back” and “spill over (Vasilakis et al. 2011; Weaver and Barrett 2004; Hanley et al. 2013).

NHPs are suspected to play a role in the sylvatic lifecycle because they have been shown to be infected by many arboviruses (DENVs, CHIKV, YFV and ZIKV) primarily across the African continent. Arboviruses have been detected in several primate species either by virus isolation, RNA detection, and serological testing. Moreover, NHPs can also be experimentally infected (Valentine, Murdock, and Kelly 2019). Different species of NHPs cohabit with forest-dwelling mosquitoes that feed on them contributing to the maintenance of the sylvatic cycles (Althouse et al. 2018; Eastwood et al. 2017; Kading et al. 2013; D. Diallo et al. 2012; Cornet et al. 1984).

Natural infection of NHPs with arboviruses have been reported in Africa for more than 50 years (Valentine, Murdock, and Kelly 2019). A serosurvey in Nigeria found neutralizing antibodies to DENV-2 in 45% of Nigerians, 25% (3/12) of *Galagos* species, an arboreal species, showed neutralizing antibodies to DENV-2 and also highest percentage of DENV antibodies was observed in rainforest zone and savannah zone (Fagbami, Fabiyi, and Monath 1977). In Senegal a serosurvey showed positivity to DENV-2 in *Erythrocebus patas*, *Cercopithecus aethiops*, *Papio papio* (PP) by complement-fixation test (Saluzzo, Cornet, Adam, et al. 1986) and 58.8% (10/17) of *African Green Monkeys* showed antibodies by ELISA

(Diallo et al. 2003). DENV was isolated from *E. patas* (Cornet et al. 1984). Eastwood et al. (2017) showed that 57.1% (4/7) of *Papio anubis* and 82.3% (14/17) of *Papio cynocephalus* in East Africa showed IgG antibodies to all serotypes of DENV. The authors suggested possible spillback from humans to *Papio species* due to close contact of these primates with humans in forest areas (Eastwood et al. 2017). Evidence of exposure to DENV-2, WNV, YFV and CHIKV was also demonstrated in *Mandrillus sphinx* in Gabon (Kading et al. 2013).

CHIKV also has been isolated in several NHP species and sylvatic mosquitoes since 1964 (McIntosh et al. 1964; McIntosh 1970). Evidence of epizootic cycles was observed in the forest canopies in Uganda, where sylvatic mosquitoes and *Cercopithecus ascanius schmidtii* were positive to CHIKV by hemagglutination-inhibition with *C. ascanius schmidtii* showing high seropositivity (83%;25/30) (McCrae et al. 1971). *Papio papio*, *Erythrocebus patas*, *Galago senegalensis*, *Chlorocebus sabaesus* have been confirmed to be naturally infected with CHIKV in Senegal (Diallo et al. 1999; Sow et al. 2018; Althouse et al. 2018). These monkey species showed high levels of neutralizing antibody to CHIKV by PRNT, where 87% (66/76), 75% (25/33) and 71% (5/7) of *Papio papio*, *C.sabaesus*, and *E. patas* were positive, respectively (Sow et al. 2018). In addition to the evidence of natural CHIKV infection in mandrills in Gabon, this species also presented neutralizing antibodies to ONNV by PRNT (16%; 4/25) (Kading et al. 2013). In Kenya, an overall CHIKV seroprevalence of 13.4% (9/67) was found in sera from blue monkeys, olive baboons, red-tailed monkeys and yellow baboons by PRNT (Eastwood et al. 2017). It is evident that CHIKV has established a sylvatic transmission cycle involving NHPs in Africa, but it is not yet defined whether other vertebrates have a role in maintaining the virus, removing the status of NHPs as the only host vertebrates.

In Uganda (McCrae and Kirya 1982; Dick 1952), Nigeria (T. Monath and Kemp 1973), Senegal (Althouse et al. 2015), Tanzania and Gambia (Buechler et al. 2017). ZIKV has been detected in multiple species of NHPs. In Uganda, where the virus was first identified in a

sentinel macaque monkey, high seropositivity to ZIKV was observed in *Cercopithecus ascanius schmidti* (38.1%; 54/142), *Colobus abyssinicus uellensis* (45.4%; 4/11) and *Cercocebus albigena johnstoni* (50%; 2/4) were by HAI but ZIKV was also isolated in *Aedes africanus* (McCrae and Kirya 1982). High seropositivity to ZIKV was observed in Nigeria in multiple monkey species of the *Cercopithecus* genus (*Cercopithecus mona*, *C.nictitans martini*), *Cercocebus torquatus*, *Chlorocebus aethiops*, *Papio anubis* and *Erythrocebus patas* with rates that varied between 11% to 100% by HAI and 5.9% to 100% by serum neutralization (SN) (Monath and Kemp 1973) *Papio sp* and *Chlorocebus sp* showed evidence of exposure to ZIKV by ELISA, in Tanzania and Gambia, respectively (Buechler et al. 2017).

II.B. Bats

Bats are traditionally grouped in the *Chiroptera* order (meaning “hand-wings” in Greek) and represent 20% of all mammalian species, the second largest order of mammals in terms of number of species (Woo and Lau 2019; Moratelli and Calisher 2015; Melaun et al. 2014). More than 1300 species of bats are found worldwide and are divided into suborders, i.e. Megachiroptera (Old World fruit bats) and Microchiroptera (echolocating bats). Most microbats are insectivorous (Jones et al. 2002) (Woo and Lau 2019). Being the only mammals with the capacity to fly, they help dissemination of seeds and pollen, playing a role in insect control, and they have thus an important role in ecosystems. Their gregarious roosting behavior and population densities increase the dissemination and transmission of viruses within and between species (Calisher et al. 2006; Moratelli and Calisher 2015; Melaun et al. 2014). Bats have high diversity and are known to harbor a variety of zoonotic viruses. These viruses can cross species barriers and infect humans as well as domestic and wild animals (Olival et al. 2017; Moratelli and Calisher 2015).

Africa is home for a diversity of bat species where they live in complex ecosystems,

exploit a variety of roosts and share ecological niches with other wild animals but also with many hematophagous arthropods like ticks, mosquitoes, flies, etc (Kunz et al. 2011; Calisher et al. 2006). This close ecological relationships of bats with hematophagous arthropods makes that bats are exposed to a diversity of arthropod-borne viruses of medical and veterinary importance. Furthermore, the interaction of bats with other species extends to contact with humans. This human-bat interaction depends on the behavior and life traits of bats, where different species roost on urban buildings, feed on fruit trees or insects in urban and rural areas (Moratelli and Calisher 2015). Another form of interaction takes place because of human cultural characteristics, which often use bats as a food source through hunting or medicine mainly in African countries (Kamins et al. 2011; Anti et al. 2015; Rocha et al. 2021). All this ecological configuration sets up a favorable scenario for the transmission of zoonotic viruses favoring both spillover and spillback (Guthid et al. 2020).

Serological and viral isolation studies reported the exposure of bats to arboviruses worldwide (Shepherd and Williams 1964; Simpson et al. 1968; M. C. Williams, Simpson, and Shepherd 1964; Kading et al. 2018; Reagan et al. 1956; Price 1978; Sotomayor-Bonilla et al. 2014; Stone et al. 2018; Vicente-Santos et al. 2017; Gard, Marshall, and Woodroffe 1973) and speculations arose regarding their roles as reservoir hosts for arboviruses (Calisher et al. 2006; Fagre and Kading 2019) Although the evidence of susceptibility to infection by arboviruses exists, the role of bats in the sylvatic or urban transmission cycles remains understood.

Bats exposure to arboviruses have been evidenced by serological and molecular tools for a wide diversity of viruses; i.e. *Nairoviridae* such as Crimean-Congo Hemorrhagic Fever Virus (CCHF), *Peribunyaviridae* like Bunyamwera virus (BUNV), *Phenuviridae* (Rift Valley fever (RVFV), *Flaviviridae* (Banza virus (BANV); JBEV; SLEV; TBEV; DENV; USUV; WNV; YFV; ZIKV), *Reoviridae* (Bukakata (BUKV), *Togaviridae* (Babanki virus (BBKV); CHIKV; EEV; ONNV; Sindbus virus (SINV); Semlink Forest virus (SFV)) (Fagre and Kading

2019). However, studies on emerging and medically important arboviruses in bats are still scarce in African countries.

Yellow fever virus circulates in very low titre in *Eidolon helvum* that also developed neutralizing antibodies (Shepherd and Williams 1964). ZIKV and CHIKV were inoculated to *Rousettus angolensis*, but only ZIKV replication was observed (Shepherd and Williams 1964). In East and West Africa, strains of Group B virus (*flaviviruses*) have been isolated from salivary glands of the *Chaerephon* genus and from brain of *Eidolon helvum* (M. C. Williams, Simpson, and Shepherd 1964). Chikungunya virus was also detected in *Chaerephon* bats by HAI. Shepherd and Williams also tested 172 bats captured in Uganda and found presence of antibodies to Group A (Alphavirus), Group B (flavivirus) and Bunyamwera virus. Simpson and O'Sullivan (1968) reported studies conducted in East Africa with bats and viral inoculations and they state that bats play a role in the natural history of arboviruses and that cycles of direct transmission among bats through urine, feces and saliva may be the most likely that only the involvement of mosquitoes (Simpson and O'Sullivan 1968). This study supported another study performed by the same authors where they also reported isolations of Group B viruses from *Chaerephon* bats and demonstrated that antibodies circulate in a large variety of bats collected in Kenya and Uganda (Simpson et al. 1968). Studies of natural arbovirus infection in bats were carried out in Senegal and a dozen different arboviruses have been isolated since 1961, among them CHIKV and different Group B viruses (Brès 1970). Studies on yellow fever in Ethiopia showed that bats were seropositive to YFV, ZIKV, WNV and other arboviruses (Sérié et al. 1968).

More recent studies identified also the circulation of arboviruses in Old World bats. CHIKV was detected in *Scotophilus sp.* bats in Kédougou in Senegal where it seems that sylvatic transmission cycles are periodic with silent intervals lasting approximately three years (Diallo et al. 1999). Neutralizing antibodies against *flaviviruses* were common in 626 Ugandan

fruit and insectivorous bats. *Little free-tailed* bat and *Angolan free-tailed* bat showed neutralizing antibodies to DENV-2. African straw-colored fruit bat showed neutralizing antibodies to WNV. *Little epauletted* fruit bat was seropositive to WNV, DENV-2 and for BBKV and RVFV. *Egyptian rousette* was positive for YFV and many *flaviviruses* and *alphaviruses* (Kading et al. 2018).

II.C. Other Animals

Arboviruses have also been detected in other animal species beside bats and primates, such as rodents, shrews, livestock, domestic animals and in domestic and wild birds, the latter considered as reservoirs for WNV (Kuno et al. 2017; Nikolay et al. 2011; Jori et al. 2021; Hubálek, Rudolf, and Nowotny 2014; Diagne et al. 2019; Escribano-Romero et al. 2015; Bażanów et al. 2018). In the WNV transmission cycle, equids do not participate in the cycle but are dead-end host and develop febrile illness and encephalomyelitis with ataxia, paralysis and mortality. Moreover, camels, dogs, geese, feral pigeons and free-living birds can be clinically affected by the disease (Tee, Horadagoda, and Mogg 2012; Dunkel et al. 2004; Cannon et al. 2006; Bakonyi et al. 2004).

It is common within the African context that infectious diseases in animals circulate at the wildlife-livestock interface, like the African Swine Fever (ASF) virus that threatens the global swine industry and the wildlife reservoir species are warthogs and ticks in East and Southern Africa (Jori et al. 2021; Dixon et al. 2020). For example, disease due to arboviruses in Nigeria, had an impact on livestock resulting in economic losses and on public health for decades (Oluwayelu, Adebisi, and Tomori 2018). Anti-WNV antibodies were detected in 90% (131/145) of horses in Nigeria by ELISA suggesting an enzootic cycle and a strong exposure of horses to WNV (W. Sule et al. 2015).

Rodents have been identified as possible reservoir hosts for some arboviruses. CHIKV

and WNV have been found in rodents in Guinea. In the same study rodents presented antibodies to 18 different arboviruses with 13 of them pathogenic for human (Konstantinov et al. 2006). Antibodies specific to CHIKV have been detected also in rodents in Senegal, and the authors suggested that rodents could be an alternate reservoir to CHIKV, contributing to maintain the cycle in the region (Diallo et al. 1999; Althouse et al. 2018). Another virus suspected of using rodents as reservoir/vertebrate hosts in addition to birds is the USUV (Clé et al. 2019). USUV was detected in three different species of rodents and shrews in Senegal by qRT-PCR and at the time of capture no signs of the disease were observed in the animals and the authors concluded to the possibility of involvement of rodents in the USUV replication cycle as an amplifying or reservoir host (Diagne et al. 2019).

Neutralizing antibodies against ONNV, CHIKV, WNV and DENV-2 have been reported in African forest buffalo in DRC and Gabon. Four species of duikers in the DRC had also neutralizing antibodies specific to ONNV. All the Elephants tested showed neutralizing antibody against WNV but also 67% (2/3) to CHIKV in Zambia and 10% (2/21) in DRC (Kading et al. 2013). The sample size of these studies is however low to make the results not representative. ONNV was also isolated in Senegal from sentinel mice (Lhuillier et al. 1988). Anti-CHIKV antibodies have been detected in several other wild animal species including palm squirrels and birds in Senegal (Diallo et al. 1999) or birds and reptiles in Zimbabwe (McIntosh et al. 1964). Domestic animals in Nigeria have also been detected with antibodies against CHIKV by HAI (Dickinson et al. 1965) and horses reacted positively to complement fixation (FC) antigens for several other arboviruses (Olaleye et al. 1989). WNV was detected in cattle and sheep animals by neutralizing test in South Africa (Dickinson et al. 1965). A study in Kenya on rodents, birds, cattle, goat, dog and reptile, carried out viral isolation and serological tests in all mentioned animal species. Birds were actively positive to WNV and cattle and sacred ibis exhibited antibodies against many *flaviviruses*. Rodents in general showed low prevalence of

antibodies against all antigens tested, being more positive to ONNV and ZIKV. The white toothed shrew showed a antibodies rate of 71.4% against WNV (Johnson et al. 1977).

Chapter III: Diagnosis in Arboviruses

Molecular and serological tools to diagnose arboviral infections have significantly improved and today high-quality assays to detect antigens and antibodies are available (**Figure 18**). Although viral RNA detection has higher specificity than antibodies (Abs) detection due to the short acute phase, the majority of arbovirus assays are focused on antibody detection such as enzyme-linked immunosorbent assays (ELISAs), immunofluorescence assay (IFA), and plaque-reduction neutralization test (PRNT) (Fritzell et al. 2018; Kerkhof et al. 2020).

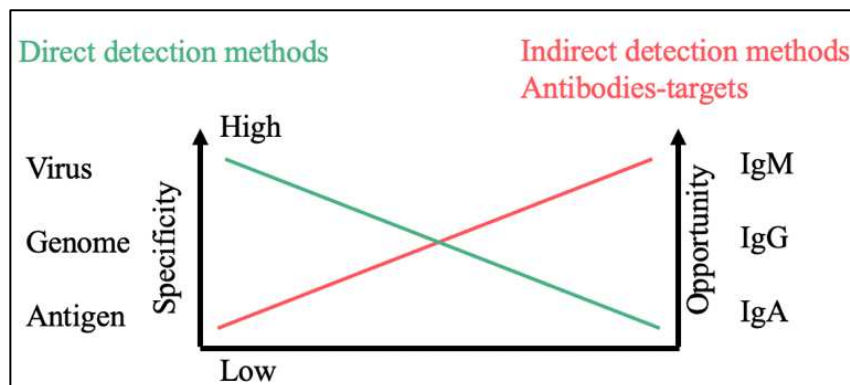


Figure 18: Diagnostic methods for the detection of arboviruses. Modified from, *Trends in Microbiology* (Kerkhof et al. 2020).

Arbovirus infection starts with an asymptomatic incubation period depending on the virus followed by a relatively short viremic phase. Antibodies appear a few days after infection and IgG can persist for months or years. In dengue infections for example, the virus can be isolated during the incubation period between 0 to 5 days of the primary infection. The onset of symptoms starts from the 5th day where IgM can be detected reaching a peak two weeks after disease onset. IgM can persist for up to 6 months. Several days after IgM development,

IgG Abs are developed during primary infection. During a secondary infection it appear can reaching a peak six weeks after onset of symptoms persisting even lifelong (Dussart, Cesaire, and Sall 2012; Kerkhof et al. 2020).

ELISAs are effective to identify seroconversions and have been applied in many studies. A review retrieved public papers from 2000 to 2018 addressing the seroprevalence of some arboviruses and found that the majority of the studies used IgG ELISAs (77%). IgG ELISAs was performed in the majority of studies as compared to IgM ELISA using NS1 antigens and/or RT-PCR that were often combined (Fritzell et al. 2018). IgG ELISAs measure past exposure to arboviruses and are widely used as commercial diagnostic kits. These test tend to have a high sensitivity but often a low specificity due the cross-reactivity with other arboviruses and can lead to false-positive results (Fritzell et al. 2018; Sergon et al. 2008; Blaylock et al. 2011; Andayi et al. 2014).

Plaque reduction neutralization test (PRNT) are considered as the gold standard and are often used as confirmatory tests for antibody/antigen assays. This test measures in *vitro* the capacity of antibodies to neutralize the infecting virus. In contrast, this test is time-consuming, labor-intensive and expensive, and only a limited number of samples can be tested at the same time (Fritzell et al. 2018; Kerkhof et al. 2020). PRNT have been performed to detection of neutralizing antibodies in different serum specimens. Sera of neotropical bats were analyzed by PRNT to detect dengue virus and 21.2% (51/241) of bats showed neutralizing anti-DENV antibodies (Vicente-Santos et al. 2017). Another study conducted in bats from Uganda used PRNT to detect antibodies against different *flaviviruses* and *alphaviruses*. Neutralizing antibodies against WNV, YFV, DENV, BBKV, RVFV were identified in bats but also showed a significant neutralizing antibody titers against an undetermined *flavivirus* (Kading et al. 2018). In Kenya, serum samples from humans were tested for *alphavirus* exposure using standardized CHIKV IgG ELISA. After 26% (486/1,848) of participants tested positive for IgG

ELISA, PRNT confirmed positives to CHIKV (6%) and ONNV (56%), illustrating thus the difficulty to develop specific Elisa's (LaBeaud et al. 2015).

Many molecular technologies to detect arboviruses are available and are in general PCR-based (end-point; real time), but isothermal techniques (LAMP: Loop-mediated isothermal amplification; TMA: transcription mediated amplification) as well as next generation sequence (NGS) are also used. PCR-based and especially the isothermal amplification (IA) methods have the advantage of being very specific, sensitive and robust and many commercial diagnostic molecular assays are available. PCR-assays targeting single or multiple viruses of the same serocomplex have been showed to be highly sensitive (Lanciotti et al. 1992; Scaramozzino et al. 2001).

Multiplex Immunoassays have been developed for simultaneous detection of multiple virus but also to distinguish between past or recent infections on closely related viruses (Boyd et al. 2015; Merbah et al. 2020; Tyson et al. 2019; Beck et al. 2015). This technology can capture specific antibodies against i.g., *flavivirus/alphavirus* antigens using recombinants antigens covalently coupled to microspheres. Some advantages compared to virus neutralization assays and real time RT-PCR are that they are less labor intensive, allow high throughput screening, and require only a small sample volume and can be adapted to different biological samples like, whole blood, plasma, serum, dried blood spots, fecal samples etc (Basile et al. 2013; Glushakova et al. 2019; Venkateswaran et al. 2020).

The EDIII envelope protein (a structural protein of Flavivirus) is used as an antigen for serological diagnosis because is the major target of neutralizing antibodies. It was observed that most DENV-neutralizing antibodies (Abs) targeted EDIII in a DENV study (Dowd and Pierson 2011; Zhang et al. 2017). Other proteins secreted by Flaviviruses and used in serological diagnosis and commercial kits is the non-structural protein NS1 (involved in the virus lifecycle) and it was proposed to be the most specific antigen for detecting specific immune responses

(Mora-Cárdenas et al. 2020). Detection of anti-NS1 IgM Abs has shown higher sensitivity in serum samples of infected patients compared with real-time RT-PCR (Huhtamo et al. 2010). Purified NS1 antigens have been used for detection of IgG Abs and showed limited cross reactivity to related viruses (Cleton et al. 2015). Furthermore, Mora-Cárdenas and colleagues (2020) analyzed the sensitivity and specificity of NS1 as an antigen to detect antibodies against *flaviviruses* using sera from exposed individuals. They found a high specificity and sensitivity of NS1-based assays confirming that recombinant NS1 proteins are an excellent option for a variety of diagnostic formats (Mora-Cárdenas et al. 2020). For the detection of specific antibodies against *alphaviruses* such as CHIKV, MAYV and ONNV, E2 glycoprotein that is involved in binding and entry of the virus in host cells as well as induces neutralizing antibodies for host protection have been widely used in diagnostic serologic tests (Fumagalli et al. 2018; Weger-Lucarelli et al. 2016; Fox et al. 2015). A recent study compared ELISAs using recombinant E2 protein and a CHIKV-specific neutralization assay on serum samples from humans with suspected CHIKV infection. They found different percentage of sensitivity and specificity. While in samples that were positive for IgG (26/59) the sensitivity and specificity was 89,66% and 100% respectively, in IgM positive samples (24/59) the sensitivity and specificity was 92.48% and 79.04%, respectively (Fumagalli et al. 2018).

Serological diagnosis of arbovirus infections is a challenging due their antigenic cross-reactivity but especially in regions or countries where important different flavivirus or alphavirus infections co-circulate and are endemic. For Zika, Chikungunya, Mayaro or dengue viruses the clinical diagnosis is difficult because they have similar clinical features, and this is even more complicated when these viruses co-circulate (Daly 2021; Fritzell et al. 2018). Seroprevalence studies can provide a picture of the immunity landscape for infectious diseases (Metcalf et al. 2016). Thus, multiplex immunoassays are effective to rapidly screen for multiple infections in humans and wildlife, in many biological specimens and both endemic or non-endemic areas

where arboviruses co-circulating.

Objective of the thesis work

In almost any review or research article, the zoonotic cycles for four well known arboviruses (Zika, dengue (DENV), yellow fever (YFV), and chikungunya (CHIKV)) are presented with arboreal mosquitos feeding on non-human primates (NHP). This is extremely oversimplified because today, there are only extremely limited or no data at all available on the extent of these arboviral infections in NHP. However, the population size and the number of susceptible NHP at any time, are most likely not high enough to maintain sylvatic arbovirus cycles during extended epizootic periods and we hypothesize that other mammal species with large numbers of offspring to ensure sufficient naïve animals at any time, play also a role in sylvatic cycles (ex. bats, rodents or others).

THE GENERAL OBJECTIVE of the work presented in this thesis is to study the extent of exposure of primates and bats to arboviruses by studying the prevalence of antibodies against arboviruses of public health importance such as Chikungunya (CHIKV), O’Nyongnyong (ONNV), Dengue (DENV 1-4), ZIKA (ZIKV), West Nile (WNV) and Usutu (USUV), Yellow Fever virus and to provide new insights in the ecology and animal reservoir of arboviruses in African countries with high likelihood for (re)emergence of these zoonotic infections.

Specific objectives:

- Develop a sensitive and specific high throughput assay to simultaneously
- Evaluate extent of exposure in non-human primates (NHP) and bats by studying the prevalence of antibodies
- Evaluate whether NHPs and bats play a role in sylvatic cycles and contribute to maintenance of arboviruses in Africa

Section II: *Experimental Memory*

II: MATERIALS AND METHODS

II.A. *Study design*

The project used samples from non-human primates and bats that have been collected in Guinea, Democratic Republic of Congo (DRC) and Cameroon. These countries have favorable ecosystems to maintain sylvatic cycles of arboviral infections. The project was built up further on long standing multidisciplinary collaborations involving TransVIHMI (INSERM-University of Montpellier/IRD); CREMER, in Yaoundé, Cameroon; INRB and University of Kinshasa, in DRC; national veterinary services, CERFIG and INSP in Conakry, Guinea. Moreover, from our previous studies on other pathogens (HIV, malaria, Ebola...) at the animal/human interface, several thousands of samples were already available from a wide diversity of African mammals (NHP and bats). This allowed us to do a retrospective screening for arboviruses in existing samples.

II.B. *Luminex xMAP Technology as high-throughput immunoassay tool*

The Multiple Analyte Profiling technology (xMAP; Luminex) is a flow cytometry-based system that enables simultaneous detection of up to 100 analytes in a single well of a 96-flat bottomed plate (Vignali 2000; Reslova et al. 2017). This technology is generally more sensitive than standard ELISA and Western blot to detect antibodies, has a broad dynamic range, requires only small volumes of scarce biological samples and is readily adapted to high throughput (Beck et al. 2015; Ahuka-Mundeke et al. 2011; Ayouba et al. 2017; Satterly et al. 2017). We extended the xMAP technology, that we developed successfully for the detection of antibodies to Ebolaviruses and for a wide diversity of HIV/SIV lineages (Ahuka-Mundeke et al. 2011; Ayouba et al. 2017), with antigens from the following arboviruses: Dengue (DENV), Zika (ZIKV), Usutu (USUV), West Nile (WNV), Yellow fever (YFV), Chikungunya (CHIKV)

and O'nyong-nyong (ONNV) viruses. The test was evaluated for sensitivity, specificity and accuracy for each virus on a reference panel of well documented positive and negative samples from humans. Our test was then adapted to screen a wide diversity of wild species with specific anti-IgG conjugates and for use on plasma, whole blood, dried blood spots (DBS) and fecal samples stored in RNA-later.

II.B.1. Human Reference Panel

In order to assess the sensitivity, specificity and accuracy of our multiplex immunoassay based on Luminex xMAP we used arbovirus positive and negative human serum samples as controls for the immunoassay. We constructed an assay panel composed of 161 samples of which 95 were serum samples with antibodies to the following viruses: Chikungunya (n=27), Zika (n=16), Dengue (n=23), West Nile (n=11), Yellow Fever (n=12) and 66 samples known to be negative for arboviruses serving as negative controls. The reference samples were provided by the Virology Department of the University Hospital from Montpellier and from the Institute of Tropical Medicine, Antwerp, Belgium. More information in **Article 1 (page 125)**.

II.B.2. Antigen coupling to magnetic beads and dilution of samples

Broadly reactive antigens to capture cross-reactive antibodies of the same or related viruses were combined with more specific antigens to differentiate between the different viruses and genotypes. We tested a total of 17 recombinant proteins (RP) for seven different arboviruses. For flaviviruses we used RP derived of the envelope domain III (DIII) from Dengue, Zika and West Nile and nonstructural protein 1 (NS1) from Dengue, Zika, Usutu, West Nile and Yellow fever. For the Alphaviruses we used RP derived of the envelope E2 region from Chikungunya and O'nyong-nyong and a non-structural protein (NSP) from Chikungunya.

Recombinant proteins were coupled to MagPlex® microspheres in different concentrations according to each antigen. Serial dilutions of samples were made to different final volumes as well as different incubation times and temperatures in order to give the best signal to noise ratio. The results are given as mean fluorescence intensity (MFI) per bead. The list of recombinant proteins and details of RP-coupled magnetic beads can be consulted in **Article 1 (page 128)**.

II.C. Non-Human primate and bat samples used in the study

Thousands of well documented samples (fecal, whole blood and DBS) from NHP and bats across Africa were available from previous studies on the origin of HIV and/or Ebolaviruses allowing thus rapid retrospective screening for the presence of antibodies to arboviruses in order to study the ecology of arboviruses in wildlife. For each animal sample, date and details on the collection site (GPS coordinates), visual species identification and information on the ecological environment are available. Molecular species confirmation was also done on a large subset of samples.

II.C.1. *Non-human Primates* (NHPs)

With a history of samples collected for over 17 years, the laboratory has currently >12,000 fecal samples from apes (chimpanzees, gorillas, bonobos) and > 5,000 whole blood samples from > 25 monkey species collected from pets or bushmeat. We screened samples that were collected in many different sites in Cameroon and DRC (**Article 1, page 108**). Fecal samples were collected around night nets and feeding sites and also opportunistically on track. All samples were in stored in optimal conditions (-20°C, -80°C) and in sufficient quantity to perform the tests.

II.C.2. *Bats*

The African continent contains about 321 extant species of bats according to the African Bats NPC and this comprises 25% of the global diversity of bats

(<https://africanbats.org/>). For the study in this thesis, our laboratory already had more than 8,000 samples (DBS, oral and anal swabs) from bats available from at least 70 species. Free-ranging frugivorous and insectivorous bats were captured at night using ground mist nets or harp traps in roosting and foraging sites in their natural environment in Guinea, Cameroon and the Democratic Republic of Congo (DRC) **Article 2 (page 133)**. They were released after sampling. We screened DBS samples that were air-dried and preserved separately in plastic bags containing desiccant silica desiccant and well-stocked in -20°C. More details can be found in **Article 2 (page 137)**.

II.D. Interpretation of Results: Calculation of Cut-Off

Human Panel: In order to interpret and evaluate the effectiveness of the arbovirus multiplex microsphere immunoassay, we used two statistical methods to assess sensitivity, specificity and accuracy: ROC (XLSTAT) curve and the formula "Mean + 3 X standard deviation". To calculate the *cut-off* we used the results that are expressed as median fluorescence intensity (MFI). Each bead-coupled RP in the set is identified by two dyes and with a third dye used to read out binding of the analyte via both biotin and streptavidin-conjugated. Then, when a sample is captured an MFI for each bead is recorded. The *cut-off* was defined for each RP by calculating the threshold value corresponding to the mean of MFI for the negative samples + 3 times the standard deviation (+3SD). Thus, the *cut-off* value that defined a sample as positive or negative, corresponds to the consensus MFI for all the RPs used. ROC curve analysis was also used to determine the *cut-off* values for each antigen (except for ONNV and USUV). their sensitivity, specificity and accuracy which corresponds to the area under the curve (AUC). The ROC curve was performed in (XLSTAT).

Wildlife samples: In absence of positive and negative samples for the different animal species, we will rely on mathematical and statistical approaches (i.g., change point analysis and binomial-exponential distributions) for prevalence estimates.

Section III : Results

Chapter II : Article 1 – Multiplex detection of antibodies to Chikungunya, O’nyong-nyong, Zika, Dengue, West Nile and Usutu viruses in diverse nonhuman primate species from Cameroon and the Democratic Republic of Congo

II.A. Goal of the study

During recent centuries many human epidemic mosquito-borne arboviruses have emerged and re-emerged in different parts of the world. These mosquito-borne arboviruses such as Zika virus, Dengue virus, West Nile Virus, YFV, Chikungunya virus, Usutu virus and others have affected not only the health of humans but also a wide range of domestic animals, birds. Many of these arboviruses as ZIKV, DENV, YFV and CHIKV for example, are present in sylvatic (forest) transmission cycles involving NHPs and forest-dwelling mosquitoes. The presence of humans in natural forest and the contact with forest animals has increased significantly. Human encroachment in forest habitats of NHPs creates conditions that they become infected after being bitten by mosquitoes carrying an arbovirus. Limited data are available on the extent of these infections in NHP as well as on their precise role especially in African countries where many arboviruses cocirculate in the same geographic area. Identifying the extent of arbovirus infection in sylvatic transmission cycles is a challenge for serological diagnostic and epidemiological surveillance mainly because these viruses are antigenically related and can cross-react. Our aim was to develop and validate a high-throughput serological screening tool to study the circulation of certain arboviruses representing a significant potential impact on human health in NHPs of Central Africa in a large sample size representing numerous species.

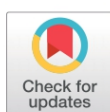
RESEARCH ARTICLE

Multiplex detection of antibodies to Chikungunya, O'nyong-nyong, Zika, Dengue, West Nile and Usutu viruses in diverse non-human primate species from Cameroon and the Democratic Republic of Congo

Raisa Raulino¹, Guillaume Thaurignac¹, Christelle Butel¹, Christian Julian Villabona-Arenas¹, Thomas Foe¹, Severin Louf², Simon-Pierre Ndimbo-Kumugo³, Placide Mbala-Kingebeni³, Sheila Makiala-Mandanda³, Steve Ahuka-Mundeke³, Karen Kerkhof⁴, Eric Delaporte¹, Kevin K. Ariën^{4,5}, Vincent Foulongne⁶, Eitel Mpoudi Ngole², Martine Peeters¹, Ahidjo Ayouba^{1*}

1 Recherches Translacionnelles sur le VIH et Maladies Infectieuses/INSERM U1175, Institut de Recherche pour le Développement et Université de Montpellier, France, **2** Centre de Recherches sur les Maladies Émergentes, Ré-émergentes et la Médecine Nucléaire, Institut de Recherches Médicales et D'études des Plantes Médicinales, Yaoundé, Cameroun, **3** Institut National de Recherche Biomédicales, Kinshasa, République Démocratique du Congo, **4** Department of Biomedical Sciences, Virology Unit, Institute of Tropical Medicine, Antwerp, Belgium, **5** Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium, **6** Département de bactériologie-virologie, CHU de Montpellier, Montpellier, France

* ahidjo.ayouba@ird.fr



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Abstract

Background

Epidemic arbovirus transmission occurs among humans by mosquito bites and the sylvatic transmission cycles involving non-human primates (NHPs) still exists. However, limited data are available on the extent in NHPs infections and their role. In this study, we have developed and validated a high-throughput serological screening tool to study the circulation of multiple arboviruses that represent a significant threat to human health, in NHPs in Central Africa.

Methodology/Principal findings

Recombinant proteins NS1, envelope domain-3 (DIII) for the dengue (DENV), yellow fever (YFV), usutu (USUV), west nile (WNV) and zika (ZIKV) and envelope 2 for the chikungunya (CHIKV) and o'nyong-nyong (ONNV) were coupled to Luminex beads to detect IgG directed against these viruses. Evaluation of test performance was made using 161 human sera of known arboviral status (66 negative and 95 positive). The sensitivity and specificity of each antigen were determined by statistical methods and ROC curves (except for ONNV and USUV). All NS1 antigens (except NS1-YFV), CHIKV-E2 and WNV-DIII had sensitivities and specificities > 95%. For the other DIII antigens, the sensitivity was low, limiting the interest of their use for seroprevalence studies. Few simultaneous reactions were observed between

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the CHIKV+ samples and the NS1 antigens to the non-CHIKV arboviruses. On the other hand, the DENV+ samples crossed-reacted with NS1 of all the DENV serotypes (1 to 4), as well as with ZIKV, USUV and to a lesser extent with YFV. A total of 3,518 samples of 29 species of NHPs from Cameroon and the Democratic Republic of Congo (DRC) were tested against NS1 (except YFV), E2 (CHIKV/ONNV) and DIII (WNV) antigens. In monkeys ($n = 2,100$), the global prevalence varied between 2 and 5% for the ten antigens tested. When we stratified by monkey's biotope, the arboreal species showed the highest reactivity. In monkeys from Cameroon, the highest IgG prevalence were observed against ONNV-E2 and DENV2-NS1 with 3.95% and 3.40% respectively and in DRC, ONNV-E2 (6.63%) and WNV-NS1 (4.42%). Overall prevalence was low in apes ($n = 1,418$): ranging from 0% for USUV-NS1 to 2.6% for CHIKV-E2. However, a very large disparity was observed among collection site and ape species, e.g. 18% (9/40) and 8.2% (4/49) of gorillas were reactive with CHIKV-E2 or WNV-NS1, respectively in two different sites in Cameroon.

Conclusions/Significance

We have developed a serological assay based on Luminex technology, with high specificity and sensitivity for simultaneous detection of antibodies to 10 antigens from 6 different arboviruses. This is the first study that evaluated on a large scale the presence of antibodies to arboviruses in NHPs to evaluate their role in sylvatic cycles. The overall low prevalence (<5%) in more than 3,500 NHPs samples from Cameroon and the DRC does not allow us to affirm that NHP are reservoirs, but rather, intermediate hosts of these viruses.

Author summary

In the last decades, chikungunya, zika, yellow fever, usutu and dengue viruses have (re-)emerged in different parts of the world and many of these outbreaks occur in resource-limited countries with limited or under-equipped health facilities and where endemic malaria with very similar clinical symptoms confounds surveillance. Most arboviruses that circulate today likely originated in Africa where sporadic human outbreaks occur. In this work, we developed a serological tool that allows simultaneous detection of IgG antibodies to multiple arbovirus in a biological sample. With this highly sensitive and specific multiplex assay, we screened more than 3,500 samples collected from 29 species of monkeys and apes in Africa. We found a global IgG antibody prevalence of less than 5%. However, this seroprevalence varied by collection site, NHPs species and virus type. Given these findings, we concluded that African non-human primates are most likely not the reservoirs, but rather are intermediate hosts.

Introduction

Ecology and human behavior play a major role in the increasing emergence and re-emergence of infectious diseases and several factors are required for this to occur [1]. These include prevalence of pathogens in the natural host, transmission mode of the pathogens, frequent contact between humans and wildlife, capacity to adapt to a new host and conditions for subsequent epidemic spread into the human population [2,3]. In particular, the increased contact between humans and wildlife lead to increased risk for disease emergence in humans [4,5].

In the last decades, chikungunya (CHIKV), zika (ZIKV), yellow fever (YFV), usutu (USUV) and dengue (DENV) viruses have (re)-emerged in different parts of the world [6–8], many of these outbreaks occur in resource-limited countries with limited or under-equipped health facilities and where endemic malaria with very similar clinical symptoms confounds surveillance [9]. Most arboviruses that circulate today likely originated in Africa where sporadic human outbreaks occur: YFV is known to circulate endemically in Sub-Saharan Africa for centuries [10]; west Nile virus (WNV) and ZIKV were first identified in 1937 and 1947, respectively, in Uganda [11]; CHIKV in 1952 in Tanzania [12] USUV in 1959 in South Africa. The precise origins of DENV remain unknown but it is widely prevalent in East, Central and South Africa.

While large outbreaks of arbovirus diseases in human populations are well documented [13–15], many questions remain unanswered on their sylvatic-cycles, in particular, which wildlife species could be involved. In almost any review or research article, the zoonotic cycles for ZIKV, DENV, YFV CHIKV are presented with arboreal mosquitos feeding on non-human primates (NHPs) but this is based on very limited data. There is only very limited evidence of arbovirus infection or exposure in NHPs but also in wildlife in general. Studying the animal reservoir of arboviruses is a challenging question, because (i) there are difficulties inherent in wildlife sampling, (ii) there are limited high throughput screening technologies, (iii) the antigenic proximity among arboviruses hinders specificity and (iv) arboviruses cause acute infections which limits the detection of the virus to a very narrow window of time, rarely exceeding 3 weeks [16]. The detection of antibodies against arboviruses antigens represent an alternative to virus detection but information on seroprevalence in wildlife are very limited. A few studies reported prevalence of antibodies (mostly IgG) in different African non-human primates (NHPs) species (reviewed in Valentine and colleagues in 2019) [17], which varied from 0% to 100%, depending on the species, the targeted arbovirus, the detection method used, the country of sample's origin and the number of samples tested.

In the present work, we have addressed some of these challenges. We first developed a high throughput serological screening tool based on the Luminex technology. Next, we screened more than 3,500 samples of a wide diversity of NHPs species from Cameroon and the Democratic Republic of Congo (DRC) for presence of antibodies to multiple arboviruses in order to evaluate their potential role in sylvatic cycles. Our data show an overall low seroprevalence of IgG antibodies to arboviruses unevenly distributed according to NHPs species and to sample collection site.

Materials and methods

Human panel samples and ethics statement

We used a panel of 161 samples of known arbovirus serostatus to validate our Luminex based serological test (S1 Table). All the human samples used in this study were anonymized, and there is no way to link back these leftovers to the 161 patients. The panel consisted of 66 arbovirus negative leftover plasma samples from the Virology department of the University Hospital, Montpellier, France and the Institute of Tropical Medicine, Antwerp, Belgium. The patients were referred to these laboratories for various illnesses and were tested for the presence of an arboviral infection by IgM and IgG Immunofluorescence assay or ELISA assays, PCR and sero-neutralization for some of them. All 66 negative control samples used in the present study were negative with all these assays. Positive control samples for CHIKV, DENV, WNV, YFV and ZIKV originated from patients in France, Belgium, Colombia and the DRC. The samples were obtained as follows: during outbreaks (CHIKV and YFV in the DRC, DENV in Colombia and WNV in France); returning travelers in Europe from countries with

outbreaks (ZIKV, DENV) or vaccinees (YFV in Colombia). DENV positive sera consisted of six DENV-1, seven DENV-2, four DENV-3, three DENV-4 and three DENV-1,2,3,4 reactive samples. Outbreak and returning travelers' samples were confirmed positive by PCR and follow-up serum samples, confirmed by commercial serological assays, were used in the present study. For USUV, we had only three samples from experimentally infected mice that were serially bled. We were not able to get positive controls for o'nyong nyong virus (ONNV).

Samples from monkeys and apes

We tested samples from bushmeat and pet monkeys from studies that were conducted between 1999 and 2016 on simian retroviruses and the origin of HIV [18–20] in 14 different sites in southern Cameroon and the DRC (Fig 1 and S2A Table). Whole blood was collected from monkey bushmeat, either by intracardiac puncture and subsequent storage at -20°C , or as a dried blood spot (DBS) on Whatman 903 filter paper (GE Healthcare) at the points of hunting injury and spotting, as described previously [21]. Blood was drawn on EDTA tubes from pet monkeys by venipuncture after tranquilization with ketamine [20]. Species were visually identified in the field and confirmed on a subset of samples by 12S sequence analysis, as previously described [22]. Fecal samples were collected between 2005 and 2017 from wild common chimpanzees (*Pan troglodytes troglodytes*), western lowland gorillas (*Gorilla gorilla gorilla*), eastern lowland gorillas (*Gorilla beringei graueri*) and bonobos (*Pan paniscus*) at 18 different sites in Cameroon and DRC as part of studies on the origin of HIV [23] (Fig 1 and S2B Table). Feces were stored in RNA-later (Sigma-Aldrich, Saint-Quentin Fallavier, France), kept at ambient temperature in the field for a maximum of three weeks, and then stored at -20°C or -80°C in central repository laboratory.

Screening for IgG antibodies to arboviruses

Recombinant proteins. We used different commercially available recombinant proteins derived from the envelope or non-structural proteins of CHIKV, ONNV, DENV, ZIKV, USUV, YFV and WNV viruses (S3 Table). The proteins were purchased already purified (all

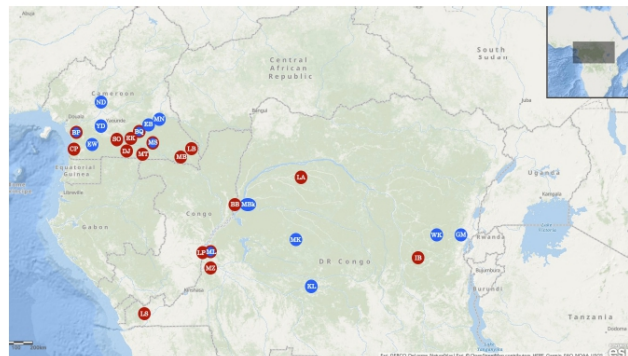


Fig 1. Sample collection sites. Sites where samples from non-human primates (NHPs) were collected are highlighted with circles on the maps, as follows: blue indicates sites where bushmeat samples from monkeys were collected; red, sites where fecal samples from apes were collected; blue and red, sites where bushmeat samples from monkeys and fecal samples from apes were collected. Abbreviations of sites are as follows: BP, Bipindi, north of Dja; EB, Eboumetoum; EW, Ebolowa; GM, Goma; KL, Kole; MBk, Mbandaka; MK, Monkoto; ML, Malebo; MN, Mindourou; MS, Messok; ND, Nditam; WK, Walikale; YD, Yaoundé.

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above 95% of purity, except DENV4_NS1 and CHIKV_NSP at >90%) as lyophilized powders, and resuspended in a buffer at concentration as per manufacturer's instructions, aliquoted and stored frozen at -20°C until use.

Protein coupling to Luminex beads. We used our previously described protocol for coupling primary amines bearing moieties (peptides and proteins) to Luminex beads [21,24]. Briefly, recombinant proteins (1–4µg/1.25 x10⁶ beads) were covalently coupled on carboxyl functionalized fluorescent magnetic beads (Luminex Corp., Austin, TX) with the BioPlex amine coupling kit (Bio-Rad Laboratories, Marnes-la-Coquette, France) according to the manufacturer's instructions. We blocked unreacted sites with blocking buffer from the amine coupling kit. Protein-coupled microsphere preparations were washed with PBS, and stored in storage buffer (Bio-Rad) at 4°C in the dark until use.

Multiplex screening for IgG antibodies to arbovirus in plasma and fecal dialysates.

Before use, recombinant protein-coupled beads were vortexed for 30s and diluted to 2,000 beads/µl of assay buffer (Phosphate Buffered Saline (PBS) containing 0.75 mol/L NaCl, 1% (wt/vol) bovine serum albumin (Sigma Aldrich, Saint-Quentin Fallavier, France), 5% (vol/vol) heat-inactivated fetal bovine serum (Gibco-Invitrogen, Cergy Pontoise, France), and 0.2% (vol/vol) Tween-20 (Sigma-Aldrich). Tests were performed in 96-well flat-bottom chimney plates (Greiner bio one, Frickenhausen, Germany). Fifty microliters of bead mixture were added to each well. Preliminary experiments on different plasma dilutions (1/50–1/1,000) and different incubation times and temperatures showed that the dilution 1/200 and +4°C overnight incubation gave the best signal to noise ratio. Liquid was aspirated with an automatic plate washer (BioTek 405TS Microplate washer) and wells were then incubated with 100 µl of plasma (diluted 1/200 in assay buffer) for 16h at 4°C in the dark on a plate shaker at 300 rpm/min. After 3 washings with 100 µl of assay buffer, 50 µl of biotin-labeled anti-human IgG was added (BD-Pharmingen, Le Pont De Claix, France) at a concentration of 4 µg/ml in each well and incubated for 30 min in the dark while shaking at 300 rpm. Plates were washed 3 times as above, and 50 µl of streptavidin-R-phycoerythrin (Fisher Scientific/Life Technologies, Illkirch, France) at 1 µg/ml were added per well and incubated for ten min with shaking at 300 rpm. As in previous studies from our group and others, anti-human IgG was also used for NHPs [21,25]. Antigen-antibody reactions were then read on BioPlex-200 equipment (Bio-Rad, Marnes-la-Coquette, France). At least 100 events were read for each bead set, and the results were expressed as median fluorescence intensity (MFI) per 100 beads. To detect IgG antibodies to arboviruses in fecal samples, RNA-later-precipitated immunoglobulins were first resolubilized by diluting the fecal/RNA-later mixture (2 mL) with PBS–0.05% Tween 20 (7 mL), followed by incubation for 1 hour at 60°C, centrifugation (3900g for 10 minutes) to clarify the solution, and dialysis against PBS overnight at 4°C under a continuous stirring. The reconstituted extracts were then tested in the Luminex (diluted three volumes of dialysate for one volume of buffer) as previously described [24].

Calculation of cut-off, sensitivity, specificity and accuracy. For the samples of the panel, we used receiver operating characteristics (ROC) curve analysis to determine the cut-off values for each antigen, its sensitivity, specificity and accuracy which corresponds to the area under the curve (AUC). The ROC curve analysis was performed with the Life module of XLSTAT (Addinsoft, Paris, France) implemented in Microsoft Excel. We also determined the sensitivity, specificity and accuracy by calculating the mean MFI of negative controls for each antigen, the standard deviation to the mean (SD) and using as cut-off the mean plus three times the SD because for ONNV and USUV, no or only limited positive control samples were available. We used the Wilson method [26] to calculate online the 95% confidence intervals (CI) around the proportions (http://ww3.ac-poitiers.fr/math/prof/resso/cali/ic_phrek.html).

In the absence of positive controls for NHPs samples, we analyzed the data obtained from plasma and DBS samples with different statistical methods to determine MFI cut-off values for each antigen as reported in our previous studies on ebolavirus in NHPs [27]. We used a change-point analysis with the R package “changepoint” and calculated one single shift in the arithmetic mean with the AMOC (at most one change) method. We also fitted univariate distributions to our data and defined the cut-off based on a 0.05 risk of error [28]. The set of candidate distributions was reduced with a bootstrapped skewness-kurtosis analysis [29]. Maximum likelihood estimation was performed to select the best-fit distribution based on AIC (Akaike information criterion) using the R library “fitdistrplus” [30]. The best-fit distributions were negative binomial and negative exponential distributions and both were considered in data analyses. Data were bootstrapped 10,000 times and averaged for each antigen. Analyses were done with R software version 3.3.6. We then compared the cut-off values identified by the 3 different methods and calculated their mean as a consensus cut-off that we used in this study (S4 Table). We calculated separately cut-off values for samples collected as DBS (samples from the DRC) and those collected as blood in EDTA tubes (samples from Cameroon) because of the wide disparity of blood quantity collected with DBS. We considered a sample antigen reactive if MFI was above the cut-off value. Likewise, for samples collected as feces, we calculate MFI cut-off values separately on the data generate from the dialysates (S5 Table). We considered samples positive for a given antigen if they presented MFI above the cut-off value for this antigen.

Results

Performance of the arbovirus Luminex assay on a reference panel of human samples

Before proceeding to the screening of NHPs samples, we first evaluated the performance of the novel Luminex-based serological assay on the panel of human samples with known status (S1 Table). To determine the assay performances, we first calculated cut-off values by two methods for 15 of the 17 antigens included. For USUV and ONNV, it was not possible to use ROC analysis because of the absence of positive controls and cut-off values were determined as 3XSD of negative samples. Results of cut-off determinations are summarized in Table 1. Regardless of the cut-off method used to determine the sensitivity, specificity and accuracy of each antigen, the specificity is in general high, > 95%, for the majority of the antigens. The sensitivity, on the contrary, depended on the virus and on the recombinant protein used. The sensitivity of NS1 recombinant proteins as determined by the ROC analysis method was 100% for WNV, DENV-2, DENV-3, ZIKV and >95% for DENV-1; 87% for DENV-4 and only 44% for YFV proteins. We also observed a 100% sensitivity for WNV-DIII protein. Sensitivity of CHIKV-E2 envelope protein was also high, >95% by ROC analysis. Of note, DIII recombinant proteins from the envelope, except WNV-DIII, presented weaker sensitivity, compared to NS1 recombinant proteins.

One hallmark of sero-detection of antibodies to arboviruses is cross-reaction due to antigenic homology between the different viruses. For the evaluation of the level of cross-reaction on our panel of 95 positive samples, we used cutoff values obtained with the ROC analysis method (except for ONNV and USUV). S6 Table shows the proportion of CHIKV, ZIKV, DENV, WNV and YFV positive samples that also react present with heterologous antigens. By doing so, 14/27 (52%) CHIKV+ positive samples cross-reacted with closely related ONNV-E2, and also with others flavivirus, varying from 2/27 (7.4%) to 16/27 (60%). On the other hand, flavivirus positive samples presented no or weak cross-reactions with antigens from the alphaviruses, CHIKV and ONNV, and variable rates of cross-reactions were observed among the

Table 1. Cut-off, Sensitivity, Specificity and Accuracy of antibody detection to the 17 different arbovirus recombinant proteins on the reference panel of 161 human samples.

Antigens	Cut-off calculation method	Cut-off value (MFI ^a)	N tested/ N negative	Specificity (%)	95% CI ^b	N tested/ N negative	Sensitivity (%)	95% CI	Accuracy (%)	95% CI
CHIKV_E2	Mean+3SD	324	66/65	98.48	91.0–99.0	27/24	88.89	71.0–96.0	96	92.0–97.0
	ROC	229	66/63	95.45	87.0–98.0	27/26	96.3	40.0–75.0	96	92.0–97.0
CHIKV_NSP	Mean+3SD	395	66/65	98.48	0.91–99.0	27/12	44.44	27.0–62.0	83	75.0–84.0
	ROC	246	66/65	98.48	0.91–99.0	27/20	74.07	55.0–86.0	91	86.0–93.0
ONNV_E2	Mean+3SD	362	66/64	96.97	0.91–99.0	NA/NA	NA	NA	NA	NA
ZIKV_DIII	Mean+3SD	485	66/65	98.48	0.91–99.0	16/0	0	0.0–19.0	79	70.0–80.0
	ROC	145	66/59	89.39	79.0–94.0	16/02	12.5	03.0–36.0	74	65.0–75.0
ZIKV_NS1	Mean+3SD	74	66/65	98.48	91.0–99.0	16/16	100	80.0–100	0.99	96.0–100
	ROC	345	66/66	100	94.0–100	16/16	100	80.0–100	100	100–100
YFV_NS1	Mean+3SD	421	66/65	98.48	91.0–99.0	18/5	27.78	12.0–50.0	83	75.0–85.0
	ROC	176	66/62	93.94	85.0–97.0	18/8	44.44	24.0–66.0	83	75.0–85.0
DENV1_DIII	Mean+3SD	134	66/66	100	94.0–100	23/17	73.91	53.0–87.0	93	88.0–95.0
	ROC	132	66/66	100	94.0–100	23/18	78.26	58.0–91.0	94	90.0–96.0
DENV2_DIII	Mean+3SD	842	66/65	98.48	91.0–99.0	23/4	17.39	06.0–37.0	78	69.0–78.0
	ROC	631	66/63	95.45	87.0–98.0	23/8	34.78	18.0–55.0	80	71.0–81.0
DENV3_DIII	Mean+3SD	197	66/63	95.45	87.0–98.0	23/17	73.91	53.0–87.0	90	84.0–91.0
	ROC	79	66/57	86.36	76.0–92.0	23/21	91.3	76.0–97.0	88	81.0–89.0
DENV4_DIII	Mean+3SD	544	66/64	96.97	91.0–99.0	23/6	26.09	12.0–46.0	79	70.0–80.0
	ROC	112	66/53	80.3	69.0–88.0	23/18	78.26	58.0–91.0	80	71.0–81.0
DENV1_NS1	Mean+3SD	673	66/65	98.48	91.0–99.0	23/21	91.3	73.0–97.0	97	93.0–98.0
	ROC	550	66/64	96.97	91.0–99.0	23/22	95.65	79.0–99.0	97	93.0–98.0
DENV2_NS1	Mean+3SD	812	66/64	96.97	91.0–99.0	23/23	100	85.0–100	98	95.0–99.0
	ROC	996	66/66	100	94.0–100	23/23	100	85.0–100	100	100–100
DENV3_NS1	Mean+3SD	54	66/64	96.97	91.0–99.0	23/23	100	85.0–100	98	95.0–99.0
	ROC	200	66/66	100	94.0–100	23/23	100	85.0–100	100	100–100

(Continued)

Table 1. (Continued)

Antigens	Cut-off calculation method	Cut-off value (MFI ^a)	N tested/ N negative	Specificity (%)	95% CI ^b	N tested/ N negative	Sensitivity (%)	95% CI	Accuracy (%)	95% CI
DENV4_NS1	Mean+3SD	471	66/65	98.48	91.0–99.0	23/19	82.61	62.0–93.0	94	90.0–96.0
	ROC	462	66/65	98.48	91.0–99.0	23/20	86.96	67.0–95.0	96	91.0–97.0
USUV_NS1	Mean+3SD	89	66/65	98.48	91.0–99.0	NA/NA	NA	NA	NA	NA
WNV_NS1	Mean+3SD	100	66/64	96.97	91.0–99.0	11/11	100	74.0–100	97	94.0–99.0
	ROC	1297	66/66	100	94.0–100	11/11	100	74.0–100	100	100–100
WNV_DIII	Mean+3SD	309	66/65	98.48	91.0–99.0	11/11	100	74.0–100	99	96.0–100
	ROC	817	66/66	100	94.0–100	11/11	100	74.0–100	100	100–100

(CHIKV: Chikungunya virus); (ZIKV: Zika virus); (DENV: Dengue virus); (USUV: Usutu virus); (WNV: West Nile virus); (YFV: Yellow Fever virus); (ONNV: O’Nyong Nyong virus).

^a MFI: median fluorescence intensity

^b CI: confidence interval.

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different flaviviruses. Unsurprisingly, DENV+ positive samples highly cross-reacted with all DENV-NS1 proteins, regardless the dengue virus serotype not allowing to differentiate among dengue serotypes. Cross reactivity was also seen between the DIII proteins of the dengue serotypes; for example, of the six DENV-1+ samples, five reacted with DENV-1, DENV-3 and DENV-4 DIII and only DENV-2 DIII antigen presented no reaction with the six DENV-1 positive samples. DENV+ samples also reacted at high proportion with NS1 antigens from ZIKV (14/23, 60%), YFV (19/23, 82.6%) and WNV (9/23, 39%). All WNV+ samples cross-reacted with NS1 antigens from USUV and at different levels (0–36%) with other arboviral antigens. Finally, YFV+ samples cross-reacted with other NS1 antigens at levels equal or superior to that of cognate YFV NS1 antigen. To summarize, cross-reactions in our control panel with the different arbovirus antigens is variable and ranged between 0% and 60%.

Overall, accuracy of the different antigens evaluated was excellent (>95%) for all NS1, except YFV-NS1. CHIKV-E2 and WNV-DIII recombinant proteins also presented high accuracy. We thus decided to use only antigens with high sensitivity and specificity (i.e. CHIKV-E2, ONNV, ZIKV-NS1, DENV1-4-NS1, WNV-NS1 and DIII, USUV-NS1) for the screening of wildlife samples.

Seroprevalence of IgG antibodies to arboviruses in multiple monkey species from Cameroon and the DRC

Overall, 2,100 samples collected from 26 different monkey species were screened (S2A Table and S1 Data). Total IgG antibodies to the ten selected recombinant proteins varied between 2% to 5% (Table 2). We analyzed more in detail the seroprevalence of IgG antibodies to the different arbovirus antigens, stratifying by monkey species (Table 2). Seven of 26 species screened were IgG negative towards all the antigens tested, i.e. *red-capped mangabey* (n = 7), *Hamlyn’s monkey* (n = 6), *L’Hoest’s monkey* (n = 38), *Preuss’s monkey* (n = 1), *drill* (n = 1), *mandrills* (n = 24) and *olive baboon* (n = 16). It should be noted that for four of these seven species, less than ten samples were tested. For the remaining 19 monkey species presenting

Table 2. Seroprevalence of IgG antibodies to Chikungunya (CHIKV), O'nyong nyong (ONNV), Zika (ZIKV), Dengue (DENV), Usutu (USUV) and West Nile (WNV) viruses stratified by monkey species.

Species	Total	CHIKV E2	ONNV E2'	ZIKV NS1	DENV1 NS1	DENV2 NS1	DENV3 NS1	DENV4 NS1	At least 1 DENV NS1	USUV_NSI*	WNV NSI*	WNV DIII*	Range (%)
Allan swamp monkey	41	- ^a	-	1/41 (2.4) ^b	-	-	-	-	-	1/41 (2.4)	2/41 (4.8)	3/41 (7.3)	(0.0–7.3)
Agile mangabey	128	3/128 (2.3)	4/61 (6.5)	2/128 (1.5)	2/128 (1.5)	-	1/128 (0.8)	2/128 (1.5)	3/128(2.3)	1/112 (0.8)	1/112 (0.8)	2/112 (1.7)	(0.0–6.5)
Red capped mangabey	7	-	-	-	-	-	-	-	-	-	-	-	(0.0–0.0)
Angolan colobus	25	-	-	1/25 (4.0)	2/25 (8.0)	1/25 (4.0)	2/25 (8.0)	2/25 (8.0)	2/25 (8.0)	2/21 (9.5)	2/21 (9.5)	-	(0.0–9.5)
Mantled guereza	34	1/34 (2.9)	1/27 (3.7)	1/34 (2.9)	1/34 (2.9)	1/34 (2.9)	1/34 (2.9)	1/34 (2.9)	1/34 (2.9)	1/34 (2.9)	1/34 (2.9)	1/34 (2.9)	(0.0–3.7)
Black colobus	7	2/7 (28.0)	nt ^c	1/7(14)	1/7 (14)	2/7 (28.0)	1/7 (14)	1/7 (14)	2/7 (28.0)	-	-	-	(0.0–28.0)
Tsuapa red colobus	86	2/86 (2.3)	3/85 (3.5)	1/86 (1.1)	1/86 (1.1)	1/86 (1.1)	-	1/86 (1.1)	3/86 (3.4)	3/85 (3.4)	1/85 (1.1)	5/85 (5.8)	(0.0–5.8)
Red tailed monkey	234	8/234 (3.4)	23/181 (12)	11/234 (4.7)	6/234 (2.6)	6/234 (2.6)	5/234 (2.1)	7/234 (3.0)	12/234 (5.1)	8/181 (4.5)	8/181 (4.5)	13/181 (7.1)	(2.6–12.0)
Mustached monkey	504	21/504 (4.1)	7/148 (4.7)	12/504 (2.3)	7/504 (1.3)	15/504 (2.9)	6/504 (1.2)	9/504 (1.7)	20/504 (3.9)	7/369 (1.8)	5/369 (1.3)	10/369 (2.7)	(1.2–4.7)
Hamlyn's monkey	6	-	nt	-	-	-	-	-	-	nt	nt	nt	(0.0–0.0)
l'Hoest's monkey	38	-	nt	-	-	-	-	-	-	nt	nt	nt	(0.0–0.0)
Blue monkey	51	16/51 (31.0)	nt	1/51 (1.9)	-	-	1/51 (1.9)	-	1/51 (1.9)	nt	nt	nt	(0.0–31.0)
Mona monkey	9	-	1/9 (11)	-	-	-	-	-	-	-	-	-	(0.0–11.0)
De Brazza monkey	59	-	2/43 (4.6)	2/59 (3.3)	-	-	-	1/59 (1.7)	1/59 (1.7)	2/53 (3.7)	3/53 (5.6)	4/53 (7.5)	(0.0–7.5)
Greater spot-nosed	385	6/385 (1.5)	9/210 (4.2)	17/385 (4.4)	7/385 (1.8)	18/385 (4.7)	9/385 (2.3)	10/385 (2.6)	29/385 (7.5)	12/315 (3.8)	6/315 (1.9)	7/315 (2.2)	(1.5–7.5)
Crested mona monkey	182	2/182 (1.0)	3/77 (3.8)	6/182 (3.3)	7/182 (3.8)	8/182 (4.4)	5/182 (2.7)	5/182 (2.7)	9/182 (4.9)	3/137 (2.1)	4/137 (2.9)	2/137 (1.4)	(1.0–4.9)
Preuss monkey	1	-	-	-	-	-	-	-	-	-	-	-	(0.0–0.0)
Wolf's monkey	71	1/71 (1.4)	2/55 (3.6)	1/71 (1.4)	2/71 (2.8)	2/71 (2.8)	3/71 (4.2)	1/71 (1.4)	5/71 (7.0)	1/55 (1.8)	1/55 (1.8)	-	(0.0–7.0)
Tantalus monkey	14	-	-	-	-	-	-	-	-	-	-	1/14 (7.1)	(0.0–7.1)
Patas monkey	16	-	-	-	-	-	-	-	-	-	-	1/16 (6.2)	(0.0–6.2)
Grey checked mangabey	110	5/110 (4.5)	-	7/110 (6.3)	5/110 (4.5)	5/110 (4.5)	5/110 (4.5)	8/110 (7.2)	9/110 (8.1)	2/74 (2.7)	1/74 (1.3)	3/74 (4.0)	(0.9–8.1)
Black mangabey	33	-	1/29 (3.4)	-	-	-	-	1/33 (3.0)	1/33 (3.0)	-	-	-	(0.0–3.0)
Drill	1	-	-	-	-	-	-	-	-	-	-	-	(0.0–0.0)
Mandrill	24	-	-	-	-	-	-	-	-	-	-	-	(0.0–0.0)
Northern talapoin	18	-	-	1/18 (5.5)	2/18 (11.0)	1/18 (5.5)	1/18 (5.5)	1/18 (5.5)	2/18 (11)	1/18 (5.5)	-	2/18 (11.1)	(0.0–11.0)
Olive baboon	16	-	-	-	-	-	-	-	-	-	-	-	(0.0–0.0)

(Continued)

Table 2. (Continued)

Species	Total	CHIKV E2	ONNV E2 ^a	ZIKV NS1	DENV1 NS1	DENV2 NS1	DENV3 NS1	DENV4 NS1	At least 1 DENV NS1	USUV-NS1 ^b	WNV NS1 ^c	WNV DIII ^c	Range (%)
Total	2100	67/2100 (3.2)	56/1109 (5.0)	65/2100 (3.0)	43/2100 (2.0)	60/2100 (2.8)	40/2100 (1.9)	50/2100 (2.3)	100/2100 (4.6)	44/1613 (2.7)	37/1613 (2.3)	54/1613 (3.3)	(1.9–4.9)

^a -: no positive samples detected

^b n/N tested (percentages)

^c: nt, not tested.

*The total of samples tested on ONNV, Usutu and WNV antigens are different to those tested on the other antigens for certain species.

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IgG reaction against at least one antigen, seroprevalence varied greatly by monkey species and virus antigen. Hence, the *blue monkey* presented the highest proportion of positive samples, 31% (16/51), against CHIKV and were only marginally positive (1/51) against ZIKV and DENV-3 NS1 recombinant proteins. The next monkey species presenting high proportions of reactive samples are black colobus with 28% (2/7) reactive against CHIKV-E2 and DENV-NS1 recombinant proteins, angolans colobus and northern talapoin with 8% (2/25) and 11% (2/18) reactive samples against DENV-NS1 and WNV-DIII antigens, respectively. Red-tailed and mona monkeys presented 12% (23/181) and 11% (1/9) reactive samples against ONNV_E2 antigen. The remaining 13 monkey species presented less than 8% of reactive samples against any of the tested antigens.

Thereafter, IgG antibodies were stratified by collection sites and prevalences showed variation between 1.46% (WNV-NS1 in Cameroon) and 6.63% (ONNV_E2 in the DRC) (Table 3). The proportions of ZIKV and DENV positive samples were in general higher in samples collected in Cameroon than in DRC, while IgG antibodies against CHIKV, ONNV, USUV and WNV antigens were higher in samples collected in DRC than in Cameroon (Table 3). For DENV-2 and WNV, the difference was statistically significant ($p = 0.0321$ for DENV-2, 0.0007 and 0.0039 for WNV-NS1 and WNV-DIII, respectively). The proportion of positive samples is also unevenly distributed among the sampling sites within countries. A clear difference was observed between samples collected from pets and those obtained from feral monkeys. Hence 8/175 (4.5%) samples from pets, all from infant or juvenile monkeys living in urban areas, reacted with at least one of the antigens, while 5.2–46.6% of samples reacted with at least one antigen in samples collected from feral monkeys (>90% adults). IgG antibodies to CHIKV-E2 protein were detected at high proportion (35%) in WK, eastern DRC and in lesser extent, in GM also in eastern DRC and in EB, EW and BP in Cameroon.

We then stratified the proportion of positive samples by NHP biotopes and split NHPs into three groups: arboreal ($n = 1,749$), terrestrial ($n = 264$) and semi-terrestrial ($n = 87$) species (Fig 2 and S7 Table). Following this grouping, in the arboreal group positive samples were detected against all the ten antigens tested. Another remarkable observation is the quasi-absence of DENV positive samples in semi-terrestrial monkeys, with the exception of one sample (1/87) with antibodies against DENV-4 NS1 antigen. Finally, in this group of semi-terrestrial NHPs, despite the relative low number of samples tested ($n = 87$), high proportions of reactive samples were observed against WNV and USUV virus antigens.

We also checked for simultaneous reactions to multiple viruses (Table 4). For example, in Cameroon, 10% of CHIKV-E2 positive samples ($n = 40$), were also reactive with ONNV-E2, ZIKV-NS1 and DENV-2 NS1 antigens; 40% of USUV NS1 reactive samples ($n = 22$) reacted also with WNV NS1 antigen. In DRC, 18% of CHIKV-E2 reactive samples ($n = 27$) were reactive with ONNV-E2 and 11% with ZIKV-NS1 antigens. In both countries, DENV-NS1 reactive samples reacted with other NS1 antigens in proportions ranging between 27 and 63%. Among the USUV-NS1 reactive samples ($n = 18$), 72% reacted with WNV-NS1 antigen.

Table 3. Seroprevalence of IgG antibodies to Chikungunya (CHIKV), O'nyong nyong (ONNV), Zika (ZIKV), Dengue (DENV), Usutu (USUV) and West Nile (WNV) viruses in monkeys stratified by collection site shown in Fig 1.

	CHIKV_E2	ONNV_E2 ^a	ZIKV_NSI	DENV1_NSI	DENV2_NSI	DENV3_NSI	DENV4_NSI	> 1 DENV_NSI	USUV_NSI	WNV_NSI [†]	WNV_DIII [†]	Total ^a (%)
Cameroon (n = 1470)												
Pets (n = 175)	- ^b	2 (1.1%) ^c	-	2 (1.1)	-	1 (0.5)	1 (0.5)	2 (1.1)	-	1 (0.5)	5 (2.8)	8/175 (4.5)
BP (n = 19)	1 (5.2)	1 (5.2%)	1 (5.2)	1 (5.2)	1 (5.2)	-	1 (5.2)	1 (5.2)	1 (5.2)	-	-	3/19 (15.7)
ND (n = 65)	-	1 (1.5%)	1 (1.5)	1 (1.5)	1 (1.5)	1 (1.5)	2 (3.0)	1 (6.2)	1 (1.5)	1 (1.5)	1 (1.5)	5/65 (7.69)
YD (n = 96)	-	-	2 (2.0)	1 (1.0)	1 (1.0)	-	3 (3.1)	2 (2.0)	1 (1.0)	1 (1.0)	-	5/96 (5.20)
BQ (n = 51)	1 (1.9)	4 (7.8%)	2 (3.9)	3 (5.9)	5 (9.8)	3 (5.9)	5 (9.8)	4 (4.2)	2 (3.9)	4 (7.8)	-	13/51 (25.4)
EW (n = 228)	12 (5.2)	11 (4.66) ^d	10 (4.4)	5 (2.1)	14 (6.1)	6 (2.6)	5 (2.1)	8 (3.5)	4 (2.9) ^e	3 (1.72) ^e	10 (5.7) ^e	44/228 (19.2)
EB (n = 236)	11 (4.66)	nt ^f	7 (3.0)	3 (1.2)	5 (2.1)	3 (1.2)	4 (1.7)	4 (4.5)	2 (1.6) ^g	1 (0.6) ^g	3 (1.8) ^g	25/236 (10.5)
MS (n = 94)	3 (3.2)	6 (6.4%)	7 (7.4)	4 (4.2)	6 (6.4)	4 (4.2)	4 (4.2)	4 (4.5)	6 (6.4)	5 (5.4)	1 (1.0)	17/94 (18.0)
MN (n = 506)	12 (2.3)	12 (7.6%) ^h	17 (3.3)	12 (2.3)	17 (3.3)	10 (1.9)	12 (2.3)	14 (2.8)	9 (2.7) ⁱ	1 (0.3) ⁱ	9 (2.7) ⁱ	68/506 (13.4)
Total	40 (2.72)	26 (3.95)	47 (3.19)	32 (2.17)	50 (3.40)	28 (1.90)	37 (2.51)	40 (2.72)	26 (2.23)	17 (1.46)	29 (2.49)	
DRC (n = 630)												
MBk (n = 125)	2 (1.6)	6 (4.8%)	3 (2.4)	-	2 (1.6)	1 (0.8)	-	-	5 (4.0)	8 (6.4)	6 (4.8)	20/125 (16.0)
ML (n = 45)	-	12 (2.7%)	5 (11)	2 (4.4)	1 (2.2)	2 (4.4)	1 (2.2)	2 (4.4)	3 (6.6)	4 (8.8)	10 (22)	21/45 (46.6)
MK (n = 29)	-	1 (3.4%)	1 (3.4)	1 (3.4)	2 (6.8)	1 (3.4)	4 (13)	1 (3.4)	2 (6.8)	2 (6.8)	2 (6.8)	8/29 (27.5)
KL (n = 280)	5 (1.8)	11 (4.3%) ^h	7 (2.5)	7 (2.5)	5 (1.8)	6 (2.1)	8 (2.8)	6 (2.1)	8 (3.1) ⁱ	6 (2.3) ⁱ	7 (2.7) ⁱ	32/280 (11.4)
WK (n = 39)	14 (35)	nt	2 (5.1)	1 (2.5)	-	1 (2.5)	-	-	nt	nt	nt	15/39 (38.4)
GM (n = 112)	6 (5.3)	nt	-	-	-	1 (0.9)	-	-	nt	nt	nt	7/112 (6.2)
Total	27 (4.28)	30 (6.63)	18 (2.85)	11 (1.74)	10 (1.58)	12 (1.89)	13 (2.06)	9 (1.42)	18 (3.96)	20 (4.42)	25 (5.53)	

^a Total number of samples reactive with one or more antigens.

^b -: no positive samples were identified.

^c number of positives (percentages).

^d Total numbers tested are different: Cameroon: n = 1161 (EW = 174; EB = 158; MN = 329); DRC: n = 452 (KL = 253).

^e Total numbers tested are different: Cameroon: n = 657 (MN = 157); DRC: n = 452 (KL = 253).

^f -: nt = not tested.

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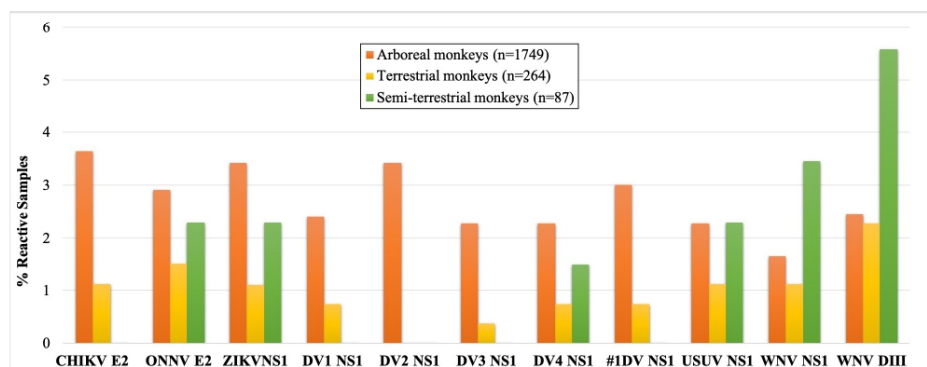


Fig 2. Proportions of positive samples to several arboviruses and stratified by monkey biotope/habitat. The figure shows the proportion (vertical axis) of samples positive to the different antigens reported in the horizontal axis, in the different monkey species biotopes as detailed in supplementary S7 Table.

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Low seroprevalence of IgG antibodies to arboviruses antigens in apes from Cameroon and the DRC

African apes, including gorillas, chimpanzees and bonobos are endangered species and are on the red list of IUCN. Thus, only non-invasive sampling methods are allowed as alternative

Table 4. Percentages of monkey samples reacting simultaneously with different arboviral recombinant proteins.

Cameroon	Number of reactive samples	ONNV_E2	ZIKV_NS1	DENV1_NS1	DENV2_NS1	DENV3_NS1	DENV4_NS1	USUV_NS1	WNV_NS1	WNV_DIII
CHIKV_E2	40	10.0	10.0	2.5	10.0	2.5	2.5	2.5	2.5	2.5
ONNV_E2	26		7.7	3.8	3.8	3.8	3.8	7.7	7.7	7.7
ZIKV_NS1	47			27.7	36.2	31.9	27.7	29.8	12.8	2.1
DENV1_NS1	26				69.2	50.0	80.8	23.1	19.2	3.8
DENV2_NS1	39					41.0	41.0	17.9	10.3	2.6
DENV3_NS1	22						50.0	22.7	22.7	0.0
DENV4_NS1	37							21.6	16.2	2.7
USUV_NS1	22								40.9	0.0
WNV_NS1	10									0.0
WNV_DIII	15									
DRC	Number of reactive samples	ONNV_E2	ZIKV_NS1	DENV1_NS1	DENV2_NS1	DENV3_NS1	DENV4_NS1	USUV_NS1	WNV_NS1	WNV_DIII
CHIKV_E2	27	18.5	11.1	3.7	3.7	3.7	3.7	3.7	3.7	0.0
ONNV_E2	30		13.3	6.7	10.0	3.3	10.0	13.3	16.7	23.3
ZIKV_NS1	18			22.2	16.7	27.8	27.8	33.3	33.3	11.1
DENV1_NS1	11				54.5	63.6	63.6	36.4	27.3	9.1
DENV2_NS1	10					50.0	50.0	40.0	40.0	20.0
DENV3_NS1	12						50.0	41.7	41.7	8.3
DENV4_NS1	13							30.8	30.8	7.7
USUV_NS1	18								72.2	16.7
WNV_NS1	20									10.0
WNV_DIII	25									

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Table 5. Seroprevalence of IgG antibodies to Chikungunya (CHIKV), Zika (ZIKV), Dengue (DENV), Usutu (USUV), O’Nyong nyong (ONNV) and West Nile (WNV) viruses stratified by ape species and collection site as shown in Fig 1.

Country	Sampling site	CHIKV	ONNV	ZIKV	DV1	DV2	DV3	DV4	USUV	WNV	WNV
		E2	E2	NS1	NS1	NS1	NS1	NS1	NS1	NS1	DIII
Bonobo											
DRC ^a	LP (n = 14)	- ^b	-	-	-	1 (7.14) ^c	-	-	-	2 (14.2)	-
DRC	ML (n = 18)	-	-	1 (5.55)	-	-	-	-	-	-	-
DRC	MZ (n = 183)	1 (0.54)	-	-	-	-	-	-	-	-	-
DRC	LA (n = 137)	-	-	-	-	3 (2.1)	-	2 (1.4)	-	12 (8.75)	-
Subtotal	n = 352	1 (0.28)	-	1 (0.28)	-	4 (1.13)	-	2 (0.56)	-	14 (3.97)	-
Chimpanzee											
CMR	EK (n = 35)	-	2 (5.71)	1 (2.85)	-	-	-	-	-	-	-
CMR	BQ (n = 29)	-	2 (6.89)	2 (6.9)	-	-	-	-	-	2 (6.89)	-
CMR	CP (n = 5)	-	-	-	-	-	-	-	-	-	-
CMR	MB (n = 113)	1 (0.88)	-	-	-	-	-	-	-	1 (0.88)	-
DRC	BB (n = 10)	-	-	-	-	-	-	-	-	2 (20.0)	-
DRC	LS (n = 71)	-	-	-	-	-	-	-	-	-	-
Subtotal	n = 263	1 (0.38)	4 (1.52)	3 (1.14)	-	-	-	-	-	5 (1.90)	-
Gorilla											
DRC	IB (n = 47)	-	-	-	-	-	-	-	-	2 (4.25)	-
CMR	EK (n = 65)	-	-	-	-	-	-	-	-	-	-
CMR	BQ (n = 127)	9 (7.08)	7 (5.51)	10 (7.8)	-	-	-	1 (0.78)	-	-	3 (2.36)
CMR	SO (n = 49)	3 (6.12)	2 (4.08)	2 (4.08)	2 (4.08)	2 (4.08)	2 (4.08)	2 (4.08)	1 (2.04)	4 (8.16)	2 (4.08)
CMR	BP (n = 85)	-	-	-	-	-	-	-	-	-	-
CMR	CP (n = 195)	6 (3.07)	3 (1.53)	2 (1.02)	1 (0.51)	1 (0.51)	-	-	-	-	1 (0.51)
CMR	MS (n = 48)	-	1 (2.08)	2 (4.16)	-	-	-	-	-	-	-
CMR	DJ (n = 104)	6 (5.76)	2 (1.92)	-	-	-	-	-	-	-	-
CMR	MT (n = 40)	9 (18.3)	7 (17.5)	2 (5.00)	-	-	-	-	-	-	3 (7.5)
CMR	LB (n = 20)	-	-	-	-	-	-	-	-	-	-
CMR	MB (n = 23)	2 (8.69)	1 (4.34)	1 (4.34)	-	-	-	-	-	-	-
Subtotal	n = 803	35 (4.35)	23 (2.82)	19 (2.36)	3 (0.37)	3 (0.37)	2 (0.25)	3 (0.37)	1 (0.12)	6 (0.74)	9 (1.12)
Total	n = 1418	37 (2.60)	27 (1.90)	23 (1.62)	3 (0.21)	7 (0.49)	2 (0.14)	5 (0.35)	1 (0.07)	25 (1.76)	9 (0.63)

^a CMR, Cameroon; DRC, Democratic Republic of Congo

^b no positive samples were identified

^c number of positives (percentages)

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methods to study pathogens in these animals. Here we tested dialysates of 1,418 fecal samples from bonobos, chimpanzees and gorillas on the same antigens used for samples from monkeys (S1 Data). Overall, the proportions of IgG positive samples against any given arbovirus antigen was low (<5%) (Table 5). Gorillas, with 4.35% (35/803) reactive samples against CHIKV-E2 antigen, presented the highest proportion of IgG positive samples. There was no DENV positive sample in the 263 chimpanzees’ fecal dialysates tested. Many samples collected in the remaining 12 collection sites presented IgG antibodies against multiples arboviral antigens. For example, samples collected from gorillas in SO (Cameroon) reacted against all the ten antigens. As for monkeys, simultaneous reactions to multiple virus antigens were also observed with samples from apes. For example, 59%, 19% and 24% of CHIKV-E2 positive samples reacted also with ONNV-E2, ZIKV-NS1 and WNV-DIII antigens, respectively (Table 6).

Table 6. Percentages of ape samples reacting simultaneously with different arboviral recombinant proteins.

	Number of reactive samples	ONNV_E2	ZIKV_NS1	DENV1_NS1	DENV2_NS1	DENV3_NS1	DENV4_NS1	USUV_NS1	WNV_NS1	WNV_DIII
CHIKV_E2	37	59.5	18.9	2.7	2.7	2.7	2.7	2.7	8.1	24.3
ONNV_E2	27		23.1	3.8	3.8	3.8	3.8	3.8	11.5	34.6
ZIKV_NS1	23			13.0	13.0	8.7	8.7	4.3	8.7	17.4
DENV1_NS1	3				100	66.7	66.7	33.3	66.7	33.3
DENV2_NS1	7					28.6	57.1	14.3	85.7	14.3
DENV3_NS1	2						100	50.0	100	50.0
DENV4_NS1	5							20.0	80.0	20.0
USUV_NS1	1								100	4.5
WNV_NS1	25									8.0
WNV_DIII	9									

<https://doi.org/10.1371/journal.pntd.0009028.t006>

Likewise, DENV-1 NS1 reactive samples reacted with 33%-100% of other DENV, WNV and USUV NS1 antigens.

Discussion

In this study, we used a novel high throughput multiplex immunoassay that included ten antigens derived from six different arboviruses, including the four Dengue serotypes, to evaluate prevalence of IgG antibodies to these viruses in a large number of samples from NHPs species from Cameroon and the DRC. Overall, we showed that IgG antibodies to arboviral antigens are low and unevenly distributed in a wide diversity of the different NHPs species and in the different sites of the two countries where the samples were collected.

Validation of a serological screening tool

First, we developed a screening tool to detect IgG antibodies to CHIKV, ONNV, DENV, ZIKV, USUV, YFV and WNV viruses using 17 purified recombinant viral proteins. After performance evaluation, we kept only ten of these antigens for the screening of wildlife samples that presented the highest sensitivities and/or with high specificities (Table 3). In our assay, we found that the commonly used NS1 (DENV1-4, ZIKV, WNV) and E2 (CHIKV) antigens together with WNV-DIII recombinant proteins are the most sensitive antigens to detect IgG antibodies in our panel, with sensitivity and specificity $\geq 95\%$ (Table 1). DIII antigens from ZIKV and DENV1-4 viruses and NSP for CHIKV were excluded because of low sensitivities. This could be explained by several parameters, including antibody kinetics, specificity restricted to one or two epitopes or recombinant protein structure impairment due to *in vitro* production issues. YFV-NS1 was not further used because of its low sensitivity, however it cannot be excluded that positive controls from vaccinated people (15/18) are not the most appropriate controls for assay development.

Sensitivity and cross-reactions are the recurrent concerns in the detection of antibodies to arbovirus infection. ELISAs, Immunofluorescence tests (IFT), multiplex microspheres assays (MIA) or Rapid Diagnostic Tests (RDT) have been used for the detection of IgG or IgM to various arbovirus [16,31]. Reported sensitivity and specificity varied greatly (39–100%) by the assay, the antibody isotype detected, the geographical environment, the time since clinical symptom onset of the infection [16]. For example, in a study performed in French Guiana in 2019 on a panel of 199 samples, including 90 ZIKV positives [32], the authors evaluated two commercially available ELISA assays and observed sensitivities of 71% and 79% and

specificities of 70% and 62% for IgG detection. In our current work, the level of cross-reaction varied between 0% and 60%, depending on the antigen (Table 3) and correspond to what was observed in previous studies [33,34].

Variable prevalence of IgG antibodies to arboviral antigens in monkeys from Cameroon and DRC

The overall IgG prevalence is <5% for all the six arboviruses tested in both Cameroon and the DRC. Combining all the DENV serotype NS1 antigens as one target increased IgG prevalence for some monkey species (Table 2). This strategy could thus be a valuable alternative of screening, especially if ELISA, not MIA, is to be used. This sero-prevalence to arboviruses in general is lower than in other published papers. For example, a work by Diallo and colleagues [35] reported 58.8% (10/17) prevalence of DENV in African Green Monkeys (AGMs) in Senegal using an ELISA assay for screening. In Zambia 34.4% (33/96) of samples from NHPs were positive for ZIKV using Plaque Reduction Neutralization Test (PRNT) [36]. Another study investigated the enzootic cycle of CHIKV in monkeys (*C. sabaues*, *E. patas*, and *P. papio*) collected over 3 years in Senegal. They used PRNT and found 72% (479/667) seropositivity for CHIKV, and 40% of 42 randomly selected samples were also positive for ONNV [37]. Buechler et al investigated the prevalence of ZIKV exposure in wild baboons and AGMs from South Africa by ELISA assay and found that 4.9% (2/41) and 16% (4/25) had antibodies against ZIKV, respectively [38]. Another work, performed on samples collected in diverse African mammals including mandrills from Gabon, Kading [39] and colleagues used PRNT to evaluate the presence of antibodies to flaviviruses (DENV-2, WNV, YFV) and alphaviruses (CHIKV, ONNV). In the set of 25 mandrills tested, the authors found that 80% and 76% presented neutralizing antibodies to flaviviruses and alphaviruses, respectively. Proportion of neutralizing antibodies as determined PRNT, at the level of different virus was also high, ranging from 16% for ONNV to 48% for YFV. However, sample dilutions to reach 80% reduction of plaque formation are often low (1/10 to 1/100) for most of the antigens tested. In our current work, we used stringent cut-off calculation methods to determine positivity criteria and the observed prevalence could thus underestimate the actual situation in wildlife. The difference in the proportion of positive samples in mandrills from our study and the one reported by Kading and colleagues [39] in Gabon, could also be due to the location or the detection assay used (Luminex versus PRNT). While we only detect IgG antibodies, PRNT rely on the reaction of all immunoglobulin isotypes, including IgM. In other monkey species, we observed high prevalence of IgG antibodies to some antigens. For example, 31% (16/51) (Table 2) samples from blue monkeys, presented antibodies to CHIKV-E2 recombinant protein, 7.3% (3/41) samples from Allan swamp monkey presented IgG to WNV-DIII proteins. Variability by monkey species could be due to the ecology of the monkey species. For example, the blue monkey is an arboreal species that dwells high in the canopy of contiguous and fragmented lowland and montane tropical moist forests, riverine and gallery forests and could thus be exposed to different mosquito vectors than baboons. At higher level of classification (by biotope, S7 Table and Fig 2), lower prevalence was observed in terrestrial and semi-terrestrial NHPs species, probably reflecting the ecology of vectors of studied viruses. However, this can only be ascertained in conjunction with future entomological investigations. The variability we observed in the proportion of positive samples by monkey species could also be due to geography, *i.e.*, collection site, and different ecological environment ranging from forest savannah to tropical rain forest. Age can also play a role, as can be the case for pets whose low prevalence could be rather explained by their younger age. Indeed, monkeys kept as pets are generally juveniles whose mothers have been killed by hunters in the wild. In addition, these pets were mainly living in

urban areas. On the other hand, the large majority (> 90%) of bushmeat samples are from adult animals. While one would be expecting significant differences between USUV and WNV viruses and the other arboviruses because birds instead of NHP, are thought to be the reservoirs of USUV and WNV viruses, no differences were observed in the present work.

Low prevalence of IgG antibodies to arboviruses in apes from Cameroon and the DRC

In apes, the overall IgG prevalence to the different arbovirus antigens we tested in the present work was generally lower than what was observed for monkeys. A primary possibility to explain this difference is the biological source of the sample. Hence, samples from monkeys were plasma or dried blood spots, while ape samples were feces dialysates and IgG concentration in this latter medium is lower than in plasma. Nevertheless, we showed in our earlier works on HIV/SIV and Ebola that IgG antibodies to these viruses can be detected in fecal dialysates of apes for SIV [19,23] and of survivors to Ebola virus disease, although at lower sensitivities [25]. The lower prevalence in apes can also be due to their terrestrial and semi-terrestrial behavior as observed in monkeys. In apes, we observed two distinct patterns of IgG reactivity towards arboviral antigens. In one side, in bonobos and chimpanzees, antibodies were mainly directed against WNV-NS1 antigen, and in gorillas against CHIKV-E2 antigen (Table 5). Significant difference for CHIKV IgG prevalence was also observed between chimpanzees and gorillas' samples collected on the same site (MB site in Cameroon, 0.88% vs 8.69%, $p = 0.039$). This dichotomy could be explained by differential behaviors between the *Pan* genus and gorillas. Indeed, while gorillas spend their nights in nests on the ground, common chimpanzees and bonobos have their nests in the trees. They are thus probably exposed to different mosquito vectors. Like for monkeys, seroprevalence of IgG positive samples is also unevenly distributed in sampling sites and can thus also explain differences in species. For example, most of WNV-NS1 positive bonobos samples were from LA site. This variability by sampling site warrants further investigation, especially at the entomological level.

Limitations of the present study

A first limitation of our study is the absence of control panel with a set of well documented samples for each of the viruses we studied here, e.g. USUV and ONNV. This could have improved the evaluation of the sensitivity of our assay. A second limitation of this novel assay is the absence of sylvatic virus antigens. Including these antigens in our Multiplex assay could have increased the chances to detect antibodies against viruses circulating in wildlife. However, DENV serotypes for which sylvatic counterparts have been identified (DENV-1,-2, and 4), amino acids identity in NS1 ranged from 91% to 97% and for the envelope, between 93% and 98% (S8 Table). Thus, based on these sequence identities, it is likely that sylvatic infections have been detected with our assay. Finally, for some species (e.g. drills and Preuss's monkey) the sample size was very limited, making it impossible to estimate prevalence of arboviruses in these monkey species.

In summary, this is the first study that evaluated on a large scale the presence of antibodies to arboviruses in NHPs to evaluate their role in sylvatic cycles. We used in this work archived samples from a diverse set of NHPs from Cameroon and the DRC to investigate the circulation of different arboviruses in these wild animals. Archived samples have helped to address multiple questions relevant to the field of infectious diseases [37]. Because of the overall low IgG prevalence observed in our study, we cannot conclude that monkeys are an important wildlife reservoir for arboviruses to maintain the sylvatic viral cycle, especially given the relative low numbers of NHPs living in close proximity to humans as compared to birds, bats or rodents

which are also suspected to play a role in the sylvatic life cycle for some of these viruses. Rather, NHPs might be intermediate hosts of these pathogens. Additional research is still needed in the improvement of detection tools and to elucidate the sylvatic reservoirs of arboviruses and their potential impact on human health.

Supporting information

S1 Table. Characteristics of the panel of human plasma samples used to validate the arbovirus Luminex assay.

(DOCX)

S2 Table. A: Number of monkey samples collected from different sites in Cameroon and the Democratic Republic of Congo (DRC). Location of sites are shown in Fig 1 with the same abbreviations. **B:** Number of bonobo, chimpanzee and gorilla fecal samples collected from different sites in Cameroon and the Democratic Republic of Congo (DRC). Location of sites are shown in Fig 1 with the same abbreviations.

(DOCX)

S3 Table. Recombinant proteins used in the study.

(DOCX)

S4 Table. Cut-off values obtained with the different methods for each antigen for monkey samples collected in Cameroon (CMR) as whole blood and as dried blood spots (DBS) in the Democratic Republic of Congo (DRC).

(DOCX)

S5 Table. Cut-off values obtained with the different methods for each antigen on ape fecal samples.

(DOCX)

S6 Table. Level of cross-reactivity with recombinant antigens from other arboviruses in the reference panel of human plasma samples.

(DOCX)

S7 Table. Number and proportions of positive monkey samples to arboviruses and stratified by biotope/habitat.

(DOCX)

S8 Table. Amino acid identity matrix between diverse human and sylvatic Dengue viruses envelope and NS1 proteins.

(PDF)

S1 Data. Raw data used for the manuscript.

(XLSX)

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Author Contributions

Conceptualization: Martine Peeters, Ahidjo Ayouba.

Data curation: Raisa Raulino, Guillaume Thaurignac, Martine Peeters, Ahidjo Ayouba.

Formal analysis: Raisa Raulino, Martine Peeters, Ahidjo Ayouba.

Funding acquisition: Eric Delaporte, Kevin K. Ariën, Eitel Mpoudi Ngole, Martine Peeters, Ahidjo Ayouba.

Investigation: Raisa Raulino, Guillaume Thaurignac, Christelle Butel, Thomas Foe, Karen Kerkhof.

Methodology: Christian Julian Villabona-Arenas, Kevin K. Ariën, Martine Peeters, Ahidjo Ayouba.

Project administration: Kevin K. Ariën, Martine Peeters.

Resources: Severin Loul, Simon-Pierre Ndimbo-Kumugo, Placide Mbala-Kingebeni, Sheila Makiala-Mandanda, Steve Ahuka-Mundeke, Kevin K. Ariën, Vincent Foulongne.

Supervision: Martine Peeters, Ahidjo Ayouba.

Validation: Martine Peeters, Ahidjo Ayouba.

Visualization: Raisa Raulino, Martine Peeters, Ahidjo Ayouba.

Writing – original draft: Raisa Raulino, Christian Julian Villabona-Arenas, Martine Peeters, Ahidjo Ayouba.

Writing – review & editing: Raisa Raulino, Guillaume Thaurignac, Christelle Butel, Christian Julian Villabona-Arenas, Thomas Foe, Severin Loul, Simon-Pierre Ndimbo-Kumugo, Placide Mbala-Kingebeni, Sheila Makiala-Mandanda, Steve Ahuka-Mundeke, Karen Kerkhof, Eric Delaporte, Kevin K. Ariën, Vincent Foulongne, Eitel Mpoudi Ngole, Martine Peeters, Ahidjo Ayouba.

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II.A.1. Supporting Information

S1 Table. Characteristics of the panel of human plasma samples used to validate the arbovirus Luminex assay.

Sample serostatus	France	Belgium	Colombia	DRC
Negative (n=66)	61	5	0	0
CHIKV+ (n=27)	1	5	0	21
DENV+ (n=23) *	5	6	12	0
WNV+ (n=11)	11	0	0	0
YFV+ (n=18)	0	10	5	3
ZIKV+ (n=16)	2	14	0	0

CHIKV, Chikungunya virus ; ONNV, O'nyong nyong virus, ZIKV, Zika virus ; DENV, Dengue virus; USUV, Usutu virus; WNV, West Nile virus

*among the 23 DENV, 6 were DENV-1, 7 DENV-2, 4 DENV-3, 3 DENV-4 and 3 DENV1-4 serotypes.

S2A Table: Number of monkey samples collected from different sites in Cameroon and the Democratic Republic of Congo (DRC). Location of sites are shown in Figure 1 with the same abbreviations

Genus	Species	Common name	Cameroon									Democratic Republic of Congo						Total	
			Pets	BP [§]	ND	YD	BQ	EW	EB	MS	MN	MBk	ML	MK	KL	WK	GM		
<i>Allenopithecus</i>	<i>nigroviridis</i>	Allan swamp monkey	-	-	-	-	-	-	-	-	-	-	38	-	2	1	-	-	41
<i>Cercocebus</i>	<i>agilis</i>	Agile mangabey	11	-	-	1	9	-	31	13	60	3	-	-	-	-	-	-	128
	<i>torquatus</i>	Red capped mangabey	2	-	-	-	-	5	-	-	-	-	-	-	-	-	-	-	7
<i>Colobus</i>	<i>angolensis</i>	Angolan colobus	-	-	-	-	-	-	-	-	-	-	-	-	4	21	-	-	25
	<i>guereza</i>	Mantled guereza	1	-	15	8	-	-	-	-	8	1	-	-	1	-	-	-	34
	<i>satanus</i>	Black colobus	-	-	-	-	-	7	-	-	-	-	-	-	-	-	-	-	7
<i>Ptilocolobus</i>	<i>tholloni</i>	Tsuapa red colobus	-	-	-	-	-	-	-	-	-	-	-	-	2	84	-	-	86
<i>Cercopithecus</i>	<i>ascanius</i>	Red tailed monkey	-	-	-	-	-	-	-	-	-	-	35	42	12	106	6	33	234
	<i>cephus</i>	Mustached monkey	27	6	9	16	24	108	158	14	142	-	-	-	-	-	-	-	504
	<i>hamlyni</i>	Hamlyn's monkey	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	6
	<i>lhoesti</i>	l'Hoest monkey	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	32	38
	<i>mitis</i>	Blue monkey	-	-	-	-	-	-	-	-	-	-	-	-	-	-	21	30	51
	<i>mona</i>	Mona monkey	8	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	9
	<i>neglectus</i>	De Brazza monkey	4	-	-	4	8	2	11	-	3	18	2	1	6	-	-	-	59
	<i>nictitans</i>	Greater spot-nosed	41	8	21	51	-	69	7	35	144	9	-	-	-	-	-	-	385
	<i>pogonias</i>	Crested mona monkey	5	1	10	14	10	21	27	17	76	-	-	-	-	-	-	1	182
	<i>preussi</i>	Preuss monkey	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
	<i>wolfi</i>	Wolf's monkey	-	-	-	-	-	-	-	-	-	21	1	5	30	6	8	-	71
<i>Chlorocebus</i>	<i>tantalus</i>	Tantalus monkey	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14
<i>Erythrocebus</i>	<i>patas</i>	Patas monkey	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16
<i>Lophocebus</i>	<i>albigena</i>	Grey checked mangabey	6	-	10	-	-	1	2	15	73	-	-	1	-	-	2	-	110
	<i>aterrimus</i>	Black mangabey	-	-	-	-	-	-	-	-	-	-	-	2	31	-	-	-	33
<i>Mandrillus</i>	<i>leucophaeus</i>	Drill	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
	<i>sphinx</i>	Mandrill	15	1	-	-	-	8	-	-	-	-	-	-	-	-	-	-	24
<i>Miopithecus</i>	<i>talapoin</i>	Northern talapoin	7	3	-	1	-	7	-	-	-	-	-	-	-	-	-	-	18
<i>Papio</i>	<i>anubis</i>	Olive baboon	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16
Total			175	19	65	96	51	228	236	94	506	125	45	29	280	39	112	2100	

§ abbreviations of sites are as follows: BP, Bipindi; BQ, north of Dja; EB, Eboumetoum; EW, Ebolowa; GM, Goma; KL, Kole; MBk, Mbandaka; MK, Monkoto; ML, Malebo; MN, Mindourou; MS, Messok; ND, Nditam; WK, Walikale; YD, Yaoundé

S2B Table: Number of bonobo, chimpanzee and gorilla fecal samples collected from different sites in Cameroon and the Democratic Republic of Congo (DRC). Location of sites are shown in Figure 1 with the same abbreviations.

Country	Collection site#	Bonobo <i>Pan paniscus</i>	Chimpanzee <i>Pan troglodytes troglodytes</i>	Gorilla <i>Gorilla gorilla gorilla*</i>	Total
Cameroon	SO	-	-	49	49
Cameroon	BP	-	-	85	85
Cameroon	CP	-	5	195	200
Cameroon	MS	-	-	48	48
Cameroon	DJ	-	-	104	104
Cameroon	MT	-	-	40	40
Cameroon	LB	-	-	20	20
Cameroon	EK	-	35	65	100
Cameroon	BQ	-	29	127	156
Cameroon	MB	-	113	23	136
DRC	LP	14	-	-	14
DRC	ML	18	-	-	18
DRC	MZ	183	-	-	183
DRC	LA	137	-	-	137
DRC	BB	-	10	-	10
DRC	LS	-	71	-	71
DRC	IB*	-	-	47	47
Total		352	263	803	1,418

abbreviations of sites are as follows: BB, Bobangi; BP, Bipindi; BQ, north of Dja; CP, Campo; DJ, Djoum; EK, Ekom; IB, Ibanga; LA, Lomako-Yokokala; LB, Lobéké; LS, Lusanga; MB, Mambelé ; ML, Malebo; MS, Messok; MT, Mintom; MZ, Manzana; LP, Lempu; SO, Somalomo.* Gorilla samples collected at IB in eastern DRC are from *Gorilla berengei graueri* species.

S3 Table. Recombinant proteins used in the study.

Virus	Protein	Antigen	Provider	Reference	Purity by SDP-PAGE	
Chikungunya	Envelope	CHIKV_E2	Interchim	A2YI70	> 95%	
	Non Structural Protein 1234	CHIKV_NSP	Cusabio	RPC23292	> 90%	
O'nyong-nyong	Envelope	ONNV_E2	Centaur	IT-022-005Ep	> 95%	
Zika	Envelope Domain 3	ZIKV_DIII	Interchim	A2YHT0	> 95%	
	Non Structural protein 1	ZIKV_NS1	Interchim	40544-V07H	> 95%	
Yellow Fever	Non Structural protein 1	YFV_NS1	Interchim	80-1547	> 95%	
Dengue	Envelope Domain 3	DENV1_DIII	Interchim	BWZ880	> 95%	
	Envelope Domain 3	DENV2_DIII	Interchim	40471-V08Y3	> 95%	
	Envelope Domain 3	DENV3_DIII	Interchim	OPPA02169	> 95%	
	Envelope Domain 3	DENV4_DIII	Interchim	OPPA02021	> 95%	
	Non Structural protein 1	DENV1_NS1	Biorad	PIP047A	> 95%	
	Non Structural protein 1	DENV2_NS1	Biorad	PIP048A	> 95%	
	Non Structural protein 1	DENV3_NS1	Biorad	PIP049A	> 95%	
	Non Structural protein 1	DENV4_NS1	Biorad	PIP050A	> 90%	
	Usutu	Non Structural protein 1	USUV_NS1	Abcam	Ab218552	> 95%
	West Nile	Non Structural protein 1	WNV_NS1	Interchim	40346-V07H	> 95%
Envelope Domain 3		WNV_DIII	Interchim	40345-V08Y	> 95%	

S5 Table. Cut-off values obtained with the different methods for each antigen on ape fecal samples.

Antigen	Binomial	Exponential	ChangePoint	Mean of the 3 methods
CHIKV_E2	49	47	78	48
CHIKV_NSP	23	22	24	22
ONNV_E2	26	35	43	30
ZIKV_DIII	14	17	3	15
ZIKV_NS1	25	25	38	25
YFV_NS1	14	19	10	16
DENV1_DIII	13	14	2	13
DENV2_DIII	14	22	13	18
DENV3_DIII	14	15	10	14
DENV4_DIII	17	19	21	18
DENV1_NS1	13	15	2	14
DENV2_NS1	15	15	10	15
DENV3_NS1	13	14	2	13
DENV4_NS1	13	15	2	14
USUV_NS1	13	16	2	14
WNV_NS1	25	19	50	22
WNV_DIII	14	28	24	21

S6 Table. Level of cross-reactivity with recombinant antigens from other arboviruses in the reference panel of human plasma samples

Plasma sample status	CHIK V_E2	CHIKV _NSP	ONNV E2 *	ZIKV _DIII	ZIKV _NS1	YFV _NS1	DENV 1_DIII	DENV 2_DIII	DENV 3_DIII	DENV 4_DIII	DENV 1_NS1	DENV 2_NS1	DENV 3_NS1	DENV 4_NS1	USUV _NS1 *	WNV _NS1	WNV _DIII
CHIKV+ (n=27)	26/27	19/27	14/27	0/27	2/27	14/27	12/27	8/27	10/27	16/27	11/27	5/27	4/27	6/27	14/27	5/27	1/27
ZIKV+ (n=16)	0/16	0/16	0/16	2/16	16/16	7/16	2/16	0/16	8/16	1/16	5/16	4/16	5/16	5/16	0/16	2/16	0/16
DENV+ (n=23)	1/23	0/23	1/23	2/23	14/23	19/23	18/23	8/23	21/23	18/23	22/23	23/23	23/23	20/23	15/23	9/23	1/23
WNV+ (n=11)	0/11	0/11	0/11	0/11	1/11	0/11	2/11	2/11	1/11	3/11	3/11	3/11	2/11	4/11	11/11	11/11	11/11
YFV+ (n=18)	2/18	0/18	0/18	1/18	4/18	8/18	7/18	2/18	7/18	4/18	7/18	5/18	7/18	7/18	6/18	2/18	0/18
NEG (n=66)	3/66	1/66	2/66	7/66	0/66	5/66	0/66	3/66	9/66	13/66	2/66	0/66	0/66	1/66	1/66	0/66	0/66

*Results based on cut-off determined with Mean+3xSD method instead of ROC analysis due to absence of positive control samples. (CHIKV: Chikungunya virus); (ZIKV: Zika virus); (DENV: Dengue virus); (USUV: Usutu virus); (WNV: West Nile virus); (YFV: Yellow Fever virus); (NEG: Negative).

S7 Table. Number and proportions of positive monkey samples to arboviruses and stratified by biotope/habitat.

Common species name		Number of Sample reactive to indicates antigens (%)											Total ^a	
	N	Country	CHIKV E2	ONNV E2	ZIKVNS1	DV1 NS1	DV2 NS1	DV3 NS1	DV4 NS1	> 1 NS1 DENV*	USUV NS1	WNV NS1	WNV DIII	N+ (% , range)
Arboreal (n=1749)														
<i>Angolan colobus</i>	25	DRC	- ^b	-	1/25 (4.0) ^c	2/25 (8.0)	1/25 (4.0)	2/25 (8.0)	2/25 (8.0)	2 (8.0)	2/21 (9.5)	2/21(9.5)	-	3 (12.0; 4.1-29.9)
<i>Mantled guereza</i>	34	DRC§ / CMR	1/34 (2.9)	1/27 (3.7)	1/34 (2.9)	1/34 (2.9)	1/34 (2.9)	1/34 (2.9)	1/34 (2.9)	1 (2.9)	1/34 (2.9)	1/34 (2.9)	1/34 (2.9)	3 (8.82; 12.4-40.0)
<i>Black colobus</i>	7	CMR	2/7 (28.0)	nt ^d	1/7 (14)	1/7 (14)	2/7 (28.0)	1/7 (14)	1/7 (14)	1 (14)	-	-	-	3 (42.0; 15.8-74.9)
<i>Tsuapa red colobus</i>	86	DRC	2/86 (2.3)	3/85 (3.5)	1/86 (1.1)	1/86 (1.1)	1/86 (1.1)	-	1/86 (1.1)	-	3/85 (3.4)	1/85 (1.1)	5/85 (5.8)	11 (12.7; 7.2-21.4)
<i>Red tailed monkey</i>	234	DRC	8/234 (3.4)	23/181 (12)	11/234 (4.7)	6/234 (2.6)	6/234 (2.6)	5/234 (2.1)	7/234 (3.0)	6 (2.6)	8/181 (4.5)	8/181 (4.5)	13/181 (7.1)	49 (20.9; 16.2-26.6)
<i>Mustached monkey</i>	504	CMR	21/504 (4.1)	7/148 (4.7)	12/504 (2.3)	7/504 (1.3)	15/504 (2.9)	6/504 (1.2)	9/504 (1.7)	11 (2.1)	7/369 (1.8)	5/369 (1.3)	10/369 (2.7)	69 (13.6; 10.9-16.9)
<i>Blue monkey</i>	51	DRC	16/51 (31.0)	nt	1/51 (1.9)	-	-	1/51 (1.9)	-	-	nt	nt	nt	17 (33.3; 21.9-47.0)
<i>Mona monkey</i>	9	CMR	-	1/9 (11)	-	-	-	-	-	-	-	-	-	1 (11.1; 1.9-43.5)
<i>Greater spot-nosed</i>	385	DRC§ / CMR	6/385 (1.5)	9/210 (4.2)	17/385 (4.4)	7/385 (1.8)	18/385 (4.7)	9/385 (2.3)	10/385 (2.6)	11 (2.8)	12/315 (3.8)	6/315 (1.9)	7/315 (2.2)	58 (15; 11.8-18.9)
<i>Crested mona monkey</i>	182	DRC§ / CMR	2/182 (1.0)	3/77 (3.8)	6/182 (3.3)	7/182 (3.8)	8/182 (4.4)	5/182 (2.7)	5/182 (2.7)	7 (3.8)	3/137 (2.1)	4/137 (2.9)	2/137 (1.4)	17 (9.3; 5.9-14.4)
<i>Wolf's monkey</i>	71	DRC	1/71 (1.4)	2/55 (3.6)	1/71 (1.4)	2/71 (2.8)	2/71 (2.8)	3/71 (4.2)	1/71 (1.4)	1 (1.4)	1/55 (1.8)	1/55 (1.8)	-	8 (11.6; 8.8-25.6)
<i>Grey cheecked mangabey</i>	110	DRC§/ CMR	5/110 (4.5)	-	7/110 (6.3)	5/110 (4.5)	5/110 (4.5)	5/110 (4.5)	8/110 (7.2)	5 (4.5)	2/74 (2.7)	1/74 (1.3)	3/74 (4.0)	17 (15.4; 9.8-23.3)
<i>Black mangabey</i>	33	DRC	-	1/29 (3.4)	-	-	-	-	1/33 (3.0)	-	-	-	-	2 (6.0; 1.6-19.6)
<i>Northern talapoin</i>	18	CMR	-	-	1/18 (5.5)	2/18 (11.0)	1/18 (5.5)	1/18 (5.5)	1/18 (5.5)	2 (11)	1/18 (5.5)	-	2/18 (11.1)	4 (22.2; 9.0-45.2)
Total arboreal	1749		64 (3.6)	50 (2.8)	61 (3.5)	41 (2.3)	60 (3.4)	39 (2.2)	47 (2.7)	47(2.7)	40 (2.7)	29 (1.6)	43 (2.5)	
Terrestrial (n=264)														
<i>Allan swamp monkey</i>	41	DRC	-	-	1/41 (2.4)	-	-	-	-	-	1/41 (2.4)	2/41 (4.8)	3/41 (7.3)	7 (17.0; 8.5-31.2)
<i>Agile mangabey</i>	128	DRC§/ CMR	3/128 (2.3)	4/61 (6.5)	2/128 (1.5)	2/128 (1.5)	-	1/128 (0.8)	2/128 (1.5)	2 (1.5)	1/112 (0.8)	1/112 (0.8)	2/112 (1.7)	11 (8.5; 4.8-14.7)
<i>l'Hoest monkey</i>	38	DRC	-	nt	-	-	-	-	-	-	nt	nt	nt	0
<i>Preuss monkey</i>	1	CMR	-	-	-	-	-	-	-	-	-	-	-	0
<i>Patas monkey</i>	16	CMR	-	-	-	-	-	-	-	-	-	-	1/16 (6.2)	1 (6.25; 1.1-28.3)
<i>Mandrill</i>	24	CMR	-	-	-	-	-	-	-	-	-	-	-	0
<i>Olive baboon</i>	16	CMR	-	-	-	-	-	-	-	-	-	-	-	0
Total terrestrial	264		3 (1.1)	4 (1.5)	3 (1.1)	2 (0.8)	0	1 (0.4)	2 (0.8)	2 (0.8)	2 (0.8)	3 (1.1)	6 (2.2)	
Semi-terrestrial (n=87)														
<i>Red capped mangabey</i>	7	CMR	-	-	-	-	-	-	-	-	-	-	-	0
<i>Hamlyn's monkey</i>	6	DRC	-	nt	-	-	-	-	-	-	nt	nt	nt	0
<i>De Brazza monkey</i>	59	DRC/CMR	-	2/43 (4.6)	2/59 (3.3)	-	-	-	1/59 (1.7)	-	2/53 (3.7)	3/53 (5.6)	4/53 (7.5)	9 (15.2; 8.2-26.5)
<i>Tantalus monkey</i>	14	CMR	-	-	-	-	-	-	-	-	-	-	1/14 (7.1)	1 (7.1; 1.2-31.4)
<i>Drill</i>	1	CMR	-	-	-	-	-	-	-	-	-	-	-	0
Total semi-terrestrial	87		0	2 (2.3)	2 (2.3)	0	0	0	1 (1.1)	0	2 (2.3)	3 (3.4)	5 (5.7)	

^a Total number of samples reactive with more Denv NS1antigens ; ^b no positive samples were identified; ^c number of positives (percentages); ^d nt, not tested

*Reactive to more than one DENV antigen; § less than 10 sample

S8 Table. Amino acid identity matrix between diverse human and sylvatic Dengue virus envelope and NS1 proteins.

	Envelope (% amino acid identity)		
	Sylvatic DV1	Sylvatic DV2	Sylvatic DV4
hDV1*	97,6	69,5	64,2
hDV2	68,5	93,7	64,0
hDV4	63,7	64,2	95,7

*: human dengue virus

	NS1 (% amino acid identity)		
	Sylvatic DV1	Sylvatic DV2	Sylvatic DV4
hDV1	97,0	73,0	68,7
hDV2	73,0	91,4	72,0
hDV4	68,5	71,1	93,0

Chapter III : Article 2 - Seroprevalence of IgG antibodies against multiple arboviruses in bats from Cameroon, Guinea, and the Democratic Republic of Congo.

III.A. Goal of the study

Bats harbor a wide diversity of zoonotic viruses and are natural reservoir hosts for various pathogens, such as Marburg virus, Nipah virus, and Hendra virus. Bats have a high potential for viral dispersion, and transmit viruses to humans and other domestic or wild animals. Furthermore, bats have been reported to be exposed to many arboviruses in both urban and forest environments, but limited data on the extend of these infections are available from African countries which harbor a vast diversity of bat species. In this context, we hypothesize that bat species could also play a role in the sylvatic arbovirus cycles during extended epizootic periods because they have a large number of offspring to ensure sufficient naïve animals at any time. Our aim was to apply our multiplex immunoassay to screen retrospectively a large number from different bat species to evaluate the circulation of certain arboviruses in bats from Cameroon, DRC and Guinea. This allows us to evaluate the extent and degree of exposure of bats to arboviruses.

Seroprevalence of IgG antibodies against multiple arboviruses in bats from Cameroon, Guinea, and the Democratic Republic of Congo (Vector-Borne and Zoonotic Diseases)

Raisa Raulino¹, Guillaume Thaurignac¹, Alpha K. Keita^{1,2}, Amandine Esteban¹, Souana Goumou², Ramadan Diallo³, Simon-Pierre Ndimbo-Kumugo^{4,5}, Innocent Ndong Bass⁶, Placide Mbala Kingebeni^{4,5}, Abdoulaye Toure^{2,7,8}, Eric Delaporte¹, Steve Ahuka-Mundeke^{4,5}, Jean-Jacques Muyembe Tamfum^{4,5}, Eitel Mpoudi-Ngole⁶, Martine Peeters^{1*}, Ahidjo Ayouba^{1*}

¹ TransVIHMI ; University of Montpellier, Institut de Recherche pour le Développement, INSERM, Montpellier, France

² Centre de Recherche et de Formation en Infectiologie de Guinée (CERFIG), Conakry, Guinea.

³ Laboratoire de Virologie, Ministère de l'Élevage et des Productions Animales, Republic of Guinea

⁴ National Institute of Biomedical Research (INRB), Kinshasa, Democratic Republic of the Congo

⁵ Service de Microbiologie, Cliniques Universitaires de Kinshasa, Kinshasa, Democratic Republic of Congo.

⁶ Laboratoire de Virologie, Institut de Recherches Médicales et d'Études des Plantes Médicinales (IMPM), Yaoundé, Cameroon-IRD, Yaounde, Cameroon.

⁷ Université de Conakry, Conakry, Guinée

⁸ Institut National de Santé Publique (INSP), Conakry, Guinée

* : Co-supervised the work

Running Title: Antibodies to arboviruses in African bats.

ABSTRACT

Background: Emergence of mosquito-borne arboviruses have caused significant public health burden. The life cycle of arboviruses comprises sylvatic and urban cycles, including a wildlife reservoir, a human host and an arthropod vector. However, many questions remain on the sylvatic cycles of arboviruses. Here, we investigate the prevalence of IgG antibodies to arboviruses of public health importance in African bats. **Material and methods:** We collected dried blood spots from bats in Cameroon, Guinea and the Democratic Republic of the Congo (DRC). To detect IgG antibodies to ten antigens of six arboviruses (Dengue, Zika, West Nile, Usutu, Chikungunya and O'nyong nyong viruses) we adapted a previously validated multiplex detection assay based on the Luminex technology. **Results:** We tested samples from 2,579 bats, representing 1,917 frugivorous and 641 insectivorous bats distributed in seven families and 21 species. Overall, 218/2579 (8.45%) bat samples reacted with at least one of the ten antigens tested. The highest prevalence was observed against Usutu virus with 2.3% (59/2579), followed by 1.9% (49/2579) and 1.35% (35/2579) for the Dengue virus serotypes 4 and 3, respectively. The global seroprevalence varied by country and collection site, 11% (151/1376) in Cameroon, 3.5% (20/565) in Democratic Republic of the Congo and 7.3% (47/638) in Guinea. The highest rates were observed in *Hypsignathus monstrosus* (17.9%), *Rousettus aegyptiacus* (16.4%) and *Eidolon helvum* (10.7%) and in species from the insectivorous Molossidae family (7.8%-8.9%). Finally, we observed changes in seroprevalence over the year in *E. helvum* and *H. monstrosus* colonies which could be related to population structure. **Conclusion:** On more than 2,500 bat samples tested, we showed variable IgG seroprevalences against multiple arboviruses. Overall, the prevalence of IgG antibodies of 8.45% against arboviruses found in bats suggest that they could play a role in arboviruses cycles in the wild, in addition to other animal species.

Keywords: arboviruses, bats, Africa, antibodies, Luminex.

INTRODUCTION

The number and impact of emerging infectious diseases (EIDs) has significantly increased over time. The large majority of EIDs have a zoonotic origin, and more than 70% originate from wildlife (Jones, et al. 2008). Among them, mosquito-borne arboviruses constitute a significant threat to public health in worldwide. Arboviruses are predominantly RNA viruses and approximately one third have pathogenic potential for humans, such as Dengue, Zika, West Nile, Usutu, Chikungunya, O'nyong-nyong and many other viruses (Guzmán, et al. 2020). The life cycle of arboviruses comprises in general a sylvatic or enzootic cycle and a urban cycle, that include a wildlife reservoir, a human host and an arthropod vector in a complex interplay. Many arboviruses causing epidemics today have their origin in Africa or were first identified in Africa; i.e. Yellow Fever, West Nile, Zika, Chikungunya and Usutu viruses (Shope 1994, Dick 1953, Mason and Haddow 1957). For many of them, like Zika, Dengue, or Chikungunya, non-human primates (NHPs) were considered to play a major role in sylvatic cycles, but this was based on limited data. We recently showed that the observed seroprevalence for arboviruses on a large number of NHPs in Africa was not sufficient for them to be considered as the main reservoir species. Thus, other species most likely also play a role to maintain these viruses in the sylvatic cycles in Africa (Valentine, et al. 2019, Raulino, et al. 2021).

Bats are known to harbor many viruses and arboviruses have also been detected in bats (Luis, et al. 2013, Melaun, et al. 2014, Fagre and Kading 2019, Ayhan, et al. 2021, Calderon A 2016). Bats represent 20% of all mammalian species, with more than 1400 species worldwide, have high population densities and roosting behavior that favor the likelihood of transmission of pathogens (Omatsu, et al. 2007). The majority of studies reporting on arboviruses in bats are from southern America (Hernández-Aguilar, et al. 2021, Thompson, et al. 2015, Ubico and

McLean 1995). Very few studies have been conducted in Africa, the majority were done before 1970 and reported antibodies to Zika virus or Yellow Fever in several bat species in east Africa (Williams, et al. 1964, Simpson, et al. 1968).

The limited data available on this important question is probably due to lack of assays for bat's immune typing, the difficulty of arbovirus serology because of antigenic proximity among different arboviral lineages and limited sample volumes that can be drawn from bats. Recently, our group has developed a high throughput serological tool to detect IgG antibodies to different arboviruses of global interest that circumvent some of the above mentioned difficulties (Raulino, et al. 2021). In the present report, we adapted this serological tool for the detection of IgG antibodies to six arboviruses (Chikungunya, O'nyong nyong, Dengue, Zika, West Nile and Usutu viruses) and tested bat samples collected in Cameroon, Guinea and DRC. Our data showed that seroprevalence varied by country, by collection site, by bat families and species, and ranged from less to 1% to up to 20%. These findings suggest that bats could play a role in sylvatic life cycles of some arboviruses.

MATERIAL AND METHODS

Bat sampling and sites

Samples from free-ranging frugivorous and insectivorous bats were collected between 2016 and 2019 from in Cameroon, DRC and Guinea in the framework of a study on Ebola and other viruses in bats. Bats were captured using mist nets or harp traps in roosting and foraging sites and immediately released after sampling as previously described (De Nys, et al. 2018). Dried blood spots (DBS) were prepared on Whatman 903 filter paper (GE Healthcare, Feasterville-Treose, PA, USA) from whole blood samples collected by venipuncture of the propatagial or brachial vein. DBS samples were preserved as described in De Nys, et al. 2018, stored in the

filed at ambient temperature and then at -20°C. For each sample, information on capture site (GPS coordinates (Supplementary Table S5), ecological environment), capture method, morphology (body measurements, weight, color), sex, age class (adult, juvenile), reproduction cycle and visual species identification were recorded in the field. Permissions to perform this research were obtained from administrative authorities of Cameroun, Guinea and the DRC.

Detection of antibodies to arboviruses

Samples were tested with our previously developed assay based on the Luminex technology with sensitivity and specificity and accuracy > 95% on a reference panel of well documented human samples (Raulino, et al. 2021). We used 10 commercially available recombinant proteins from six arboviruses: CHIKV-E2 (Chikungunya virus Envelope protein 2), ONNV-E2 (O'nyong nyong virus envelope protein 2), DENV-NS1 (Dengue virus-Nonstructural protein 1) (1 to 4 serotypes), USUV-NS1 (Usutu virus-Non Structural protein 1), WNV-NS1 (West Nile virus Non Structural protein 1) , WNV-DIII (West Nile virus Domain-3 protein) and ZIKV-NS1 (Zika virus Non-Structural protein 1)(Raulino et al. 2021). The assay was performed as previously described and optimized for bats using goat anti-bat biotin-labeled IgG (Euromedex, France) and Streptavidin-R- phycoerythrin (Fisher Scientific/Life Technologies, Illkirch, France) (Raulino, et al. 2021). Whole blood was reconstituted from DBS as previously described and a final plasma dilution 1/200 was used (Ayoub, et al. 2019). The results were expressed as median fluorescence intensity (MFI) per 50 beads. Each plate included a blank well, positive samples for Chikungunya, Dengue, Zika and West Nile viruses and also 2 negative samples to validate inter-assay repeatability.

Calculation of cut-off values for the different antigens

In the absence of positive and negative control samples from bats, we analyzed our data with different statistical methods to determine MFI *cut-off* values for each antigen as previously described (Raulino, et al. 2021, De Nys, et al. 2018, Ayoub, et al. 2019, Peel, et al. 2013, Gilbert, et al. 2013). We used a change-point analysis with the R package “changept” and calculated one single shift in the arithmetic mean with the AMOC (at most one change) method (Lardeux, et al. 2016, Killick and Eckley 2014). We also used fitted univariate distributions (binomial and negative exponential distributions) to our data and defined the cut-off based on a 0.001 risk of error. Data were bootstrapped 10,000 times and averaged for each antigen. Analyses were done with R software version 3.3.6. We then compared the *cut-off* values identified by the 3 different methods and calculated their mean as a consensus *cut-off* that we used in this study (**Supplementary Table S1**). A sample was considered reactive if MFI was above the *cut-off* value.

Molecular confirmation of bat species

Species identification recorded in the field was molecularly confirmed on a subset of samples by sequence analysis of an 800 bp fragment of the mitochondrial cytochrome b (CytB) like in our previous studies (De Nys, et al. 2018, Lacroix, et al. 2020). Sequences were submitted to NCBI for BLAST analysis to identify the most similar bat species. For samples with none or low similarity (<97%) hits with species in Genbank, phylogenetic tree analysis was done using maximum likelihood methods implemented in PhyML (Guindon and Gascuel 2003) with reference sequences in order to approach genus identification. To discriminate between *Epomophorus gambiensis* and *Micropteropus pusillus*, we used also morphologic details on forearm and weight measurements because species identification was not possible based on

CytB sequences (Nesi, et al. 2011). Species identification was extrapolated by per capture method, GPS coordinate and morphological descriptions for the other samples.

RESULTS

Distribution and diversity of bat species

We tested a total of 2,579 bats captured in 21 different sites in Guinea (n=8), Cameroon (n=4) and DRC (n=9) (**Figure 1**). The samples were from bats living in urban areas (52%), villages (26%), forest (10.5%) and caves (10.9%). For 2,313 (89.6%) samples, the species identification in the field was confirmed by sequence analysis, however it was not possible to clearly discriminate between certain *Chaerophon* and *Mops* species from the *Molossidae* family based on CytB sequences and morphological data. We tested at least 12 different species of frugivorous bats, and six different families of insectivorous bats. Details on the different bat species that were collected in each country are shown in **Table 1**.

Overall, 1193 bats were male, 1381 female and for five data were not available. Among females, 77% were adults, 8.0% were reproductively immature and 14% were juveniles. For males, 70%, 12% and 17% were adults, reproductively immature and juveniles respectively. For the remaining samples information on age and sex were not available.

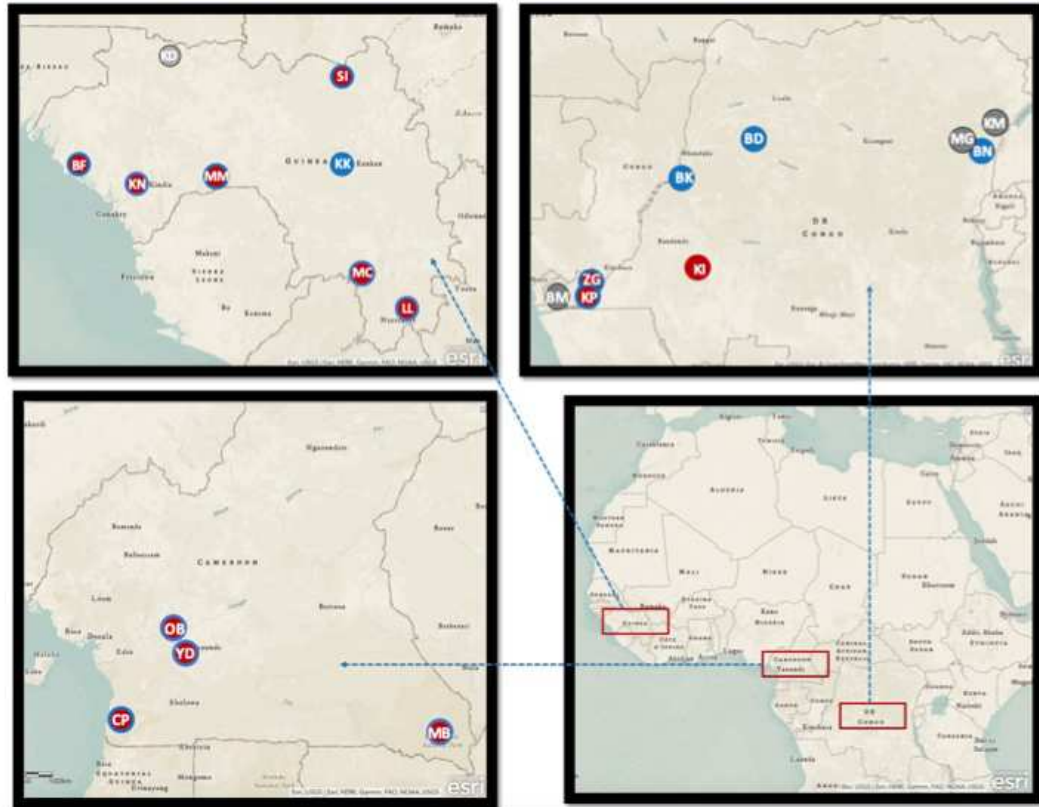


Figure 1: Bat collection sites and presence of arboviruses in Cameroon, Guinea and DRC.

The maps represent Africa and the 3 countries (Guinea, top; Cameroon, center; and the DRC, bottom) where studied samples have been collected. Grey dots indicate sampling sites where no antibodies to arboviruses were detected; red dots, sites where antibodies to alphaviruses were detected; blue dots, sites where antibodies to flaviviruses were detected; red dots with blue circles, sites where antibodies to flavi- and alpha viruses were detected. Site abbreviations: **Cameroon** – Village: CP/Campo; MB/Mambele, Urban area: OB/Obala ;YD/Yaoundé. **DRC** – Caves: BM/Boma; KP/Kimpse; ZG/Zongo, Forest: ZG/Zongo; BD/Boende, Urban area: BN/Beni; BK/Bikoro; BM/Boma; KP/Kimpse; KM/Komanda; MG/Mangina, Village: BD/Boende; KI/Kikiwit. **Guinea** – Caves: KK/Kankan; KN/Kindia; MC/Macenta; MM/Mamou; SI/Siguiiri, Forest: MC/Macenta, Urban area: KN/Kindia; MM/Mamou; Villages: BF/Boffa; KK/Kankan; KN/Kindia; KD/Koundara; LL/Lola; MC/Macenta; MM/Mamou; SI/Siguiiri.

Table 1: Number of different bat families and species collected in each country

Family/ genus/species	Cameroon n (%)	DRC n (%)	Guinea n (%)	Total n (%)
Pteropodidae				
<i>Casinonycteris arginnis</i>	1 (0.07)	4 (0.7)	-	5 (0.19)
<i>Eidolon helvum</i>	815 (59)	153 (27)	20 (3.1)	988 (38)
<i>Epomophorus gambianus</i>	-	41 (7.2)	44 (6.8)	85 (3.2)
<i>Epomops sp*</i>	121 (8.8)	56 (9.9)	21 (3.2)	198 (7.6)
<i>Hypsignathus monstrosus</i>	249 (18)	5 (0.8)	2 (0.3)	256 (9.9)
<i>Lissonycteris angolensis</i>	41 (2.9)	6 (1.0)	3 (0.4)	50 (1.9)
<i>Megaloglossus woermanni</i>	6 (0.43)	5 (0.8)	-	11 (0.4)
<i>Micropteropus pusillus</i>	5 (0.36)	95 (16)	19 (2.9)	119 (4.6)
<i>Myonycteris torquata</i>	26 (1.8)	31 (5.4)	-	57 (2.2)
<i>Nanonycteris veldkampii</i>	-	-	1 (0.1)	1 (0.03)
<i>Rousettus aegyptiacus</i>	1 (0.07)	-	145 (22)	146 (5.6)
<i>Scotonycteris zenkeri</i>	1 (0.07)	-	-	1 (0.03)
Subtotal frugivorous bats	1266 (92)	396 (70)	255 (33)	1917 (74)
Hipposideridae				
<i>Hipposideros sp</i>	1 (0.07)	102 (18)	78 (12)	181 (7.0)
Miniopteridae				
<i>Miniopterus sp</i>	-	49 (8.6)	5 (0.7)	54 (2.0)
Molossidae				
<i>Chaerephon sp</i>	-	2 (0.3)	154 (24)	156 (6.0)
<i>Chaerephon or Mops</i>	94 (6.8)	-	35 (5.4)	129 (5.0)
<i>Mops sp</i>	-	-	56 (8.7)	56 (2.1)
Nycteridae				
<i>Nycteris sp</i>	-	1 (0.1)	2 (0.3)	3 (0.11)
Rhinolophidae				
<i>Rhinolophus sp</i>	-	7 (1.2)	35 (5.4)	42 (1.6)
Vespertilionidae				
<i>Neoromicia sp</i>	-	4 (0.7)	-	4 (0.15)
<i>Scotophilus sp</i>	-	3 (0.5)	13 (2.0)	16 (0.6)
Subtotal insectivorous bats	95 (6.9)	168 (29)	378 (59)	641 (24)
Indeterminate species	15 (1.0)	1 (0.1)	5 (0.7)	21 (0.8)
Total	1376 (53.3)	565 (21.9)	638 (24.7)	2579 (100)

Evidence of antibodies to Alphaviruses and Flaviviruses in bats

Antibodies to alphavirus (Chikungunya and O'nyong-nyong) and flaviviruses (Dengue 1 to 4, Usutu, West Nile and Zika) tested in this study were observed in *Eidolon helvum*, *Epomophorus gambianus*, *Epomops* sp, *Hypsignathus monstrosus*, *Lissonycteris angolensis*, *Micropteropus pusillus*, *Myonycteris torquata*, *Rousettus aegyptiacus*, *Hipposideros* sp, *Miniopterus* sp, *Chaerephon* sp, *Mops* sp and *Rhinolophus* sp bats from the three countries (Figure 1). Overall, 8.45% (218/2,579) of the samples reacted with at least one arbovirus antigen (Table 2). Globally, 2.3% of the bats had antibodies to Usutu virus, 0.85% to 1.35% to serotypes 1 to 4 of Dengue virus and the lowest proportions were observed for Zika virus (0.54%). Global rates and stratified per virus varied among countries and within countries. In Cameroon, 11% (151/1,376) of bat samples had antibodies to arboviruses, ranging between 0.58% for Zika virus to 4.0% for Usutu virus. In Guinea, we also observed antibodies to all viruses with a total seroprevalence of 7.3% (47/638) and a predominance to O'nyong-nyong (2.35%). In DRC, 3.5% (20/565) of the bat samples tested had antibodies with a predominance to antigen NS1 of West Nile virus, and in contrast to the two other countries, no antibodies to Chikungunya virus were observed.

Antibodies to at least one arbovirus were observed in 14 (70%) of the 21 species tested, i.e. in all species with sufficient samples (>20) (**Table 3**). The highest rates were observed among the following frugivorous bat species, *Hypsignathus monstrosus* (17.9%), *Rousettus aegyptiacus* (16.4%) and *Eidolon helvum* (10.7%) and in species from the insectivorous *Molossidae* family, *Mops* sp (8.9%) and *Mops/Chaerophon* sp (7.8%). *Eidolon helvum* is a common species, which showed IgG antibodies against all viruses tested, but with different positivity rates for each virus in different sites tested, which is probably related to differences in the numbers of samples tested. *Hypsignathus monstrosus* is also a common species, but large

numbers were only tested in Cameroon, and antibodies were observed against all viruses tested with 10% of samples reactive to Usutu virus. In *Rousettus aegyptiacus*, that were highly represented in the sample set from Guinea, antibodies against almost all viruses were detected reaching 8.9% for O'nyong-nyong virus. Also, in bats from the Molossidae family, antibodies against almost all alphaviruses and flaviviruses were observed with 7.4% of samples reactive against to serotype 4 of Dengue in Cameroon. In other species, for example *Micropteropus pusillus*, IgG antibodies were only observed against flavivirus, and in *Myonycteris torquata* only IgG antibodies to alphavirus were detected (**Table 3**). Overall, antibodies were observed in 10% (192/1917) of frugivorous bats, versus 3.9% (25/641) in insectivorous bats. Frugivorous bats had antibodies to all viruses tested, and in insectivorous bats no antibodies were observed against the Usutu and to antigen NS1 of West Nile virus (**Table 3**). Only for the serotype 2 of Dengue virus, the difference between the positivity in frugivorous and insectivorous was statistically significant ($p=0.028$; OR:4.5626; CI 95%: 1.41-39.7).

Ten percent of frugivorous and insectivorous bats captured in urban areas had antibodies to arboviruses versus 6.6 % in villages. *Eidolon helvum* bats were captured in cities, villages and forests with the following positivity rates, 10.7%, 18% and 9.8% respectively. Similar rates were observed for *Hypsignathus monstrosus* captured in urban areas (18.5%) and villages (16.6%). *Rousettus aegyptiacus* bats were also positive in all settings with rates between 11.5% to 30%. Most insectivorous bats were captured in more remote areas and showed lower rates, except for species of the Molossidae family, with 7.7% of *Chaerephon/Mops*, that were captured in villages having antibodies to arboviruses (**Table 4**). The difference in positivity rates of antibodies to arboviruses between frugivorous and insectivorous might reflect the different biotopes. **Supplementary Table S2** shows in detail the reactivity with the different viruses according to the ecological settings independently from the species. Overall, antibodies

to almost all viruses studied are observed in all settings, although at different rates with a trend of lower rates in caves as compared to the three other settings.

Table 2: Prevalence of antibodies to Alphaviruses (CHIKV and ONNV) and Flaviruses (ZIKV, DENV1-4, USUV and WNV) in bats in the different collection sites shown in Figure 1.

		CHIKV_E2	ONNV_E2	ZIKV_NS1	DENV1_NS1	DENV2_NS1	DENV3_NS1	DENV4_NS1	USUV_NS1	WNV_NS1	WNV_D3	Total *positive (%)
Cameroon	n	N Positive (% / CI)										
CP	81	1 (1.23)	1 (1.23)	-	1 (1.23)	2 (2.46)	-	1 (1.23)	-	-	-	4 (4.9)
MB	143	1 (0.69)	2 (1.39)	-	2 (1.39)	-	1 (0.69)	7 (4.89)	-	-	1 (0.69)	11 (7.6)
OB	295	2 (0.67)	1 (0.33)	2 (0.67)	1 (0.33)	1 (0.33)	1 (0.33)	2 (0.67)	4 (1.35)	2 (0.67)	4 (1.35)	15 (5.0)
YD	857	11 (1.3)	9 (1.0)	6 (0.7)	16 (1.9)	19 (2.2)	18 (2.1)	26 (3.0)	52 (6.0)	9 (1.0)	5 (0.6)	121 (14.1)
SUB-TOTAL	1376	15 (1.0 / 0.66 - 1.80)	13 (0.94 / 0.55 - 1.61)	8 (0.58 / 0.29 - 1.15)	20 (1.4 / 0.94 - 2.24)	22 (1.6 / 1.05 - 2.41)	20 (1.4 / 0.94 - 2.24)	36 (2.6 / 1.89 - 3.61)	56 (4.0 / 3.14 - 5.25)	11 (0.8 / 0.44 - 1.43)	10 (0.72 / 0.39 - 1.34)	151 (11)
DRC												
BN	77	-	-	-	-	-	1 (1.29)	-	-	-	-	1 (1.2)
BK	43	-	-	-	-	-	-	1 (2.32)	-	-	1 (2.32)	2 (4.6)
BD	136	-	-	-	-	-	1 (0.73)	3 (2.20)	1 (0.73)	-	2 (1.51)	6 (4.4)
BM	41	-	-	-	-	-	-	-	-	-	-	0 (0.0)
KI	42	-	1 (2.38)	-	-	-	-	-	-	-	-	1 (2.3)
KP	43	-	1 (2.32)	1 (2.32)	-	-	-	-	-	-	-	2 (4.7)
KM	20	-	-	-	-	-	-	-	-	-	-	0 (0.0)
MG	21	-	-	-	-	-	-	-	-	-	-	0 (0.0)
ZG	142	-	1 (0.70)	1 (0.70)	1 (0.70)	1 (0.70)	2 (1.40)	1 (0.70)	1 (0.70)	6 (4.22)	-	8 (5.6)
SUB-TOTAL	565	0 (0.0 / 0.00 - 0.68)	3 (0.53 / 0.18 - 1.55)	2 (0.35 / 0.09 - 1.29)	1 (0.17 / 0.03 - 1.00)	1 (0.17 / 0.03 - 1.00)	4 (0.70 / 0.27 - 1.81)	5 (0.88 / 0.37 - 2.06)	2 (0.35 / 0.09 - 1.29)	6 (1.06 / 0.48 - 2.30)	3 (0.53 / 0.18 - 1.55)	20 (3.5)
Guinea												
BF	93	-	1 (1.07)	1 (1.07)	-	1 (1.07)	5 (5.37)	1 (1.07)	-	-	1 (1.07)	9 (9.6)
KK	91	-	-	-	-	1 (1.09)	-	-	-	-	2 (2.19)	3 (3.2)
KN	114	2 (1.75)	3 (2.63)	-	1 (0.87)	1 (0.87)	1 (0.87)	1 (0.87)	-	-	-	6 (5.2)
KD	7	-	-	-	-	-	-	-	-	-	-	0 (0.0)
LL	36	-	1 (2.77)	1 (2.77)	-	-	1 (2.77)	-	1 (2.77)	2 (5.55)	1 (2.77)	5 (13.8)
MC	98	-	7 (7.14)	-	-	1 (1.02)	3 (3.06)	4 (4.08)	-	-	3 (3.06)	12 (12)
MM	99	3 (3.00)	2 (2.00)	1 (1.00)	-	1 (1.00)	-	1 (1.00)	-	-	-	8 (8.0)
SG	100	-	1 (1.00)	1 (1.00)	-	1 (1.00)	1 (1.00)	1 (1.00)	-	-	1 (1.00)	4 (4.0)
SUB-TOTAL	638	5 (0.78 / 0.33 - 1.83)	15 (2.35 / 1.42 - 3.85)	4 (0.62 / 0.24 - 1.61)	1 (0.15 / 0.02 - 0.89)	6 (0.94 / 0.43 - 2.04)	11 (1.75 / 0.96 - 3.07)	8 (1.25 / 0.63 - 2.46)	1 (0.15 / 0.02 - 0.89)	2 (0.31 / 0.08 - 1.14)	8 (1.25 / 0.63 - 2.46)	47 (7.3)
Total	2579	20 (0.77 / 0.50 - 1.20)	31 (1.2 / 0.84 - 1.71)	14 (0.54 / 0.32 - 0.91)	22 (0.85 / 0.56 - 1.29)	29 (1.12 / 0.78 - 1.62)	35 (1.35 / 0.97 - 1.89)	49 (1.9 / 1.44 - 2.51)	59 (2.3 / 1.77 - 2.94)	19 (0.73 / 0.47 - 1.15)	21 (0.81 / 0.53 - 1.25)	218 (8.45)

CHIKV, Chikungunya virus; ONNV, O'nyong nyong virus; ZIKV, Zika virus; DENV, Dengue virus; USUV, Usutu virus; WNV, West Nile virus; CI = 95% confidence interval; *total means number of samples reactive with at least one antigen

Table 3: Seroprevalence of IgG antibodies to arboviruses in bat species.

Species / genus	N tested	CHIK_E2	ONNV_E2	ZIKV_NS1	DENV1_NS1	DENV2_NS1	DENV3_NS1	DENV4_NS1	USUV_NS1	WNV_NS1	WNV_D3	N Pos£	% ; CI95%
		N positive (%)											
<i>Casinonycteris sp</i>	5	-	-	-	-	-	-	-	-	-	-	0	0.0 ; 0-43
<i>Eidolon helvum</i>	988	9 (0.91)	9 (0.91)	5 (0.50)	16 (1.61)	21 (2.12)	16 (1.61)	26 (2.63)	32 (3.23)	15 (1.51)	9 (0.91)	106	10.7 ; 8.9-12
<i>Epomophorus gambianus</i>	85	-	1 (1.17)	-	-	-	1 (1.17)	-	-	-	-	2	2.35 ; 0.6-8.1
<i>Epomops sp*</i>	198	1 (0.52)	-	3 (1.51)	-	-	-	1 (0.52)	-	-	1 (0.52)	6	3.0 ; 1.3-6.4
<i>Hypsignathus monstrosus</i>	256	3 (1.17)	2 (0.78)	3 (1.17)	3 (1.17)	3 (1.17)	7 (2.76)	6 (2.34)	26 (10.1)	4 (1.56)	2 (0.78)	46	17.9 ; 13-23
<i>Lissonycteris angolensis</i>	50	-	-	-	-	-	-	1 (2.0)	-	-	-	1	2.0 ; 0.3-10
<i>Megaloglossus woermanni</i>	11	-	-	-	-	-	-	-	-	-	-	0	0.0 ; 0-25
<i>Micropteropus pusillus</i>	119	-	-	1 (0.84)	-	-	1 (0.84)	1 (0.84)	1 (0.84)	-	1 (0.84)	5	4.2 ; 1.8-9.4
<i>Myonycteris torquata</i>	57	1 (1.75)	2 (3.5)	-	-	-	-	-	-	-	-	3	5.2 ; 1.8-14
<i>Nanonycteris veldkampii</i>	1	-	-	-	-	-	-	-	-	-	-	0	0.0 ; §
<i>Rousettus aegyptiacus</i>	146	4 (1.37)	13 (8.96)	1 (0.68)	1 (0.68)	3 (2.06)	4 (2.73)	5 (3.44)	-	-	4 (1.37)	24	16.4 ; 11-23
<i>Scotonycteris zenkeri</i>	1	-	-	-	-	-	-	-	-	-	-	0	0.0 ; §
Frugivorous bats	1917	17 (0.88)	27 (1.4)	13 (0.67)	20 (1.04)	27 (1.4) §	28 (1.46)	40 (2.0)	59 (3.0)	19 (0.99)	17 (0.88)	192	10 ; 8.7-11
<i>Hipposideros sp</i>	181	-	1 (0.55)	-	-	1 (0.55)	-	-	-	-	1 (0.55)	3	1.65 ; 0.5-4.7
<i>Miniopterus sp</i>	54	-	-	1 (1.85)	-	-	-	-	-	-	-	1	1.85 ; 0.3-9.7
<i>Chaerephon sp</i>	156	1 (0.64)	1 (0.64)	-	-	-	1 (0.64)	2 (1.28)	-	-	-	5	3.2 ; 1.3-7.2
<i>Chaerephon or Mops</i>	129	1 (0.77)	2 (1.55)	-	2 (1.55)	-	1 (0.77)	7 (5.42)	-	-	-	10	7.75 ; 4.2-13
<i>Mops sp</i>	56	-	-	-	-	1 (1.92)	4 (7.69)	-	-	-	1 (1.92)	5	8.9 ; 3.8-19
<i>Nycteris sp</i>	3	-	-	-	-	-	-	-	-	-	-	0	0.0 ; 0-56
<i>Rhinolophus sp</i>	42	-	-	-	-	-	-	-	-	-	1 (2.38)	1	2.38 ; 0.4-12
<i>Neoromicia sp</i>	4	-	-	-	-	-	-	-	-	-	-	0	0.0 ; 0-49
<i>Scotophilus sp</i>	16	-	-	-	-	-	-	-	-	-	-	0	0.0 ; 0-19
Insectivorous bats	641	2 (0.31)	4 (0.62)	1 (0.15)	2 (0.31)	2 (0.31) §	6 (0.93)	9 (1.4)	0 (0.0)	0 (0.0)	3 (0.46)	25	3.9 ; 2.6-5.7
Indeterminate species	21	-	-	-	-	-	-	-	-	-	1 (4.76)	1	4.8 ; 0.8-22

*In Cameroon and DRC, *Epomops franqueti*, in Guinea *Epomops buetikoferi*; § = p -value=0.02825 ; CI= confidence interval; §= confidence interval was not calculated; CHIKV, Chikungunya virus; ONNV, O'nyong nyong virus; ZIKV, Zika virus ; DENV, Dengue virus ; USUV, Usutu virus ; WNV, West Nile virus , £ number positive with a t least one antigen.

Table 4: Proportion of samples with antibodies to arbovirus for each species according ecological site where they were captured.

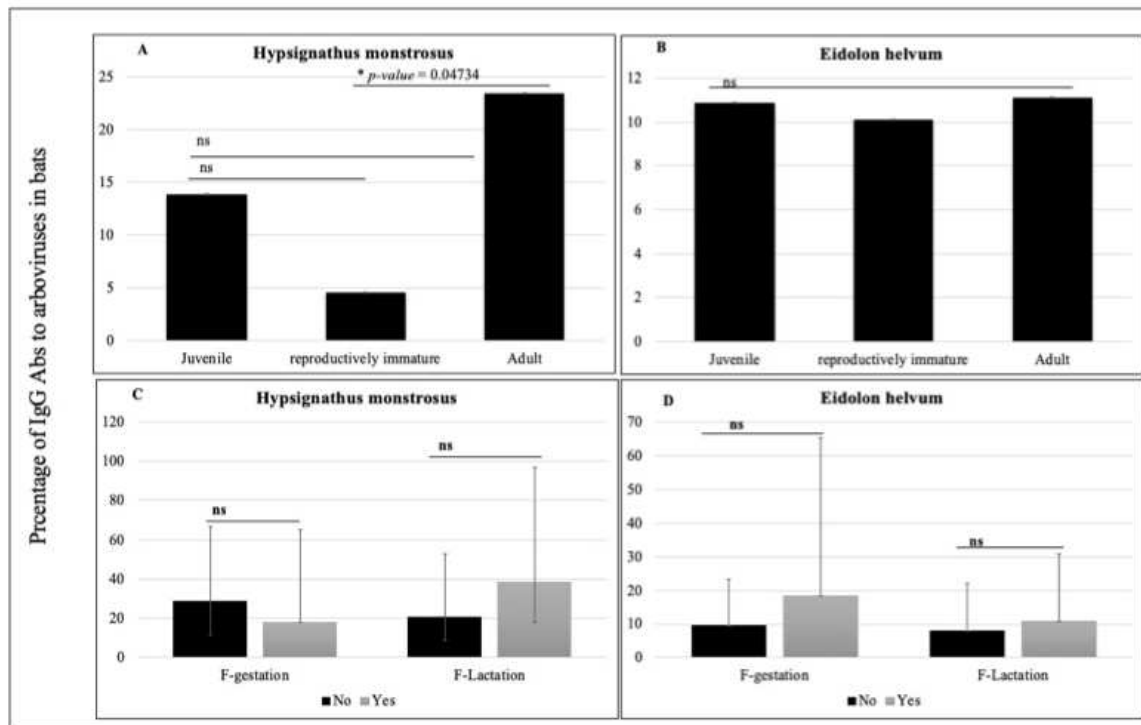
	Caves	Forest	Urban area	Village
	N pos / N tested (%)			
Pteropodidae				
<i>Casinonycteris sp</i>	-	0/4 (0.0)	-	-
<i>Eidolon helvum</i>	0/2 (0.0)	14/143 (9.8)	88/821 (10.7)	4/22 (18)
<i>Epomophorus gambianus</i>	0/1 (0.0)	-	1/42 (2.4)	1/42 (2.4)
<i>Epomops sp*</i>	-	0/11 (0.0)	4/89 (4.5)	2/98 (2.0)
<i>Hypsignathus monstrosus</i>	-	0/5 (0.0)	42/227 (18.5)	4/24 (16.6)
<i>Lissonycteris angolensis</i>	-	1/3 (33)	0/43 (0.0)	0/4 (0.0)
<i>Megaloglossus woermanni</i>	-	-	0/1 (0.0)	0/10 (0.0)
<i>Micropteropus pusillus</i>	0/3 (0.0)	0/39 (0.0)	3/61 (4.9)	2/16 (12.5)
<i>Myonycteris torquata</i>	-	0/12 (0.0)	1/27 (3.7)	1/18 (5.5)
<i>Nanonycteris veldkampii</i>	-	-	-	0/1 (0.0)
<i>Rousettus aegyptiacus</i>	7/23 (30)	5/41 (12)	3/26 (11.5)	9/56 (16)
<i>Scotonycteris zenkeri</i>	-	-	-	0/1 (0.0)
Subtotal Frugivorous bats	7/29 (24)	20/258 (7.7)	142/1337 (10.6)	23/293 (7.9)
Hipposideridae				
<i>Hipposideros sp</i>	3/154 (1.9)	0/6 (0.0)	0/19 (0.0)	0/2 (0.0)
Miniopteridae				
<i>Miniopterus sp</i>	1/51 (1.9)	0/3 (0.0)	-	-
Molossidae				
<i>Chaerephon sp</i>	0/2 (0.0)	-	0/1 (0.0)	5/153 (3.3)
<i>Chaerephon or Mops</i>	-	-	-	10/129 (7.7)
<i>Mops sp</i>	-	-	-	5/56 (8.9)
Nycteridae				
<i>Nycteris sp</i>	0/2 (0.0)	0/1 (0.0)	-	-
Rhinolophidae				
<i>Rhinolophus sp</i>	1/38 (2.6)	0/4 (0.0)	-	-
Vespertilionidae				
<i>Neoromicia sp</i>	-	-	-	0/4 (0.0)
<i>Scotophilus sp</i>	-	-	0/2 (0.0)	0/14 (0.0)
Subtotal Insectivorous bats	5/245 (2.0)	0/14 (0.0)	0/22 (0.0)	20/376 (5.3)
Indeterminate species	0/5 (0.0)	-	0/1 (0.0)	1/15 (6.6)
Total/ (% [95%CI])	12/281 (4.2 [2.4-7.3])	20/272 (7.35 [4.8-11])	142/1360 (10.4 [8.9-12])	44/666 (6.6 [4.9-8.7])

*In Cameroon and DRC, *Epomops franqueti*, in Guinea *Epomops buetikoferi*

Antibodies in E. helvum and H. monstrosus according to seasons, age and reproductive cycle

The 815 *Eidolon helvum* and 227 of the 249 *Hypsignatus monstrosus* samples in Cameroon, were part of colonies roosting in Yaoundé and foraging in neighboring areas. Samples were collected on a monthly basis between December 2018 and November 2019. For *E. helvum*, we tested 221 juvenile, 218 reproductively immature and 378 adult bats, and comparable seroprevalence rates were observed in all age categories ranging from 10.1% to 11.1%. For *H. monstrosus*, differences according to age category were observed, with highest proportion of positives among adults (31/132, 23.5%), 10/72 (13.9%) of juveniles and lowest proportions in reproductively immature (1/22; 4.6%) (**Figure 2**). Seropositivity rates were also significantly higher in adult *H. monstrosus* as compared to adult *E. helvum* bats, 23.5% versus 11.1% respectively (p-value <0.0001) (**Supplementary Table S3**). We also examined whether antibody status can vary in females during gestation and lactation. Among the female *H. monstrosus* bats, 2/11 (18.1%) of the gestating bats were positive versus 28.5% (18/63) of the other adult female bats, and for *E. helvum* the opposite trend was observed with 18.1% (2/11) gestating bats positive versus 9.3% (15/160) for the other adult female bats. The differences observed were not significant statistically. Similarly, there were also differences between female adults during lactation period, as shown in **Figure 2**, but they were also not statistically significant. Taken together, the data from the *E. helvum* and *H. monstrosus* bat colonies showed fluctuations over time with a minimum rate in June (4.26%) and maximum in February (34.78%) (**Supplementary Table S4**), however, the number of samples are low for certain months and the population structure at a given period could influence positivity rates as shown above.

Figure 2: Percentage of positive samples in colonie of bats, according to age and reproductive cycle



The figure represents the proportions of positive sample in two species (*H.monstrosus* and *E.helvum*) of fruit bats followed-up over a year in Yaoundé, Cameroon. Panels A and B (top) represent the proportions of positive samples at three categories of age. Panels C and D (bottom) compare the proportion of positive samples in the same two bat species, stratified by female reproduction/lactation status. (ns) stands for non-significant statistically (p-value above 0.05).

Simultaneous reaction to multiple alphavirus and flavivirus in bats

We observed simultaneous reactivity to antigenically-related viruses, but also to genetically diverse and less antigenically related viruses (**Table 5**). The highest simultaneous reactivity was observed for NS1 antigens of the four Dengue virus serotypes, reaching 68% between the serotypes 1 and 4 of Dengue virus. The results showed also that 30% of the E2 of Chikungunya virus positive samples reacted with E2 of O'nyong-nyong virus, and 5% with NS1 of Usutu virus and to the serotype 3 of Dengue virus. Among the 31 positive samples of

O'nyong-nyong virus, 6 (19%) had simultaneous reactions with the E2 of Chikungunya virus.

For the other antigens, simultaneous reactions with other antigens were in general below 15%.

Table 5: Proportion of simultaneous reactivity to antigenically-related virus and less antigenically related ones.

	N tested	CHIK_E2	ONNV_E2	ZIKV_NS1	DV1_NS1	DV2_NS1	DV3_NS1	DV4_NS1	USUV_NS1	WNV_NS1	WNV_D3
		N pos (%)									
CHIK_E2	n=20		6 (30)	0 (0)	0 (0)	0 (0)	1 (5)	0 (0)	1 (5)	0 (0)	0 (0)
ONNV_E2	n=31	6 (19)		0 (0)	0 (0)	0 (0)	2 (6.4)	3 (9.6)	0 (0)	0 (0)	4 (12)
ZIKV_NS1	n=14	0 (0)	0 (0)		1 (7)	1 (7)	0 (0)	1 (7)	1 (7)	1 (7)	2 (14)
DV1_NS1	n=22	0 (0)	0 (0)	1 (4.5)		12 (54)	3 (13)	15 (68)	1 (4.5)	0 (0)	0 (0)
DV2_NS1	n=29	0 (0)	0 (0)	1 (3.4)	12 (41)		4 (13)	12 (41)	1 (3.4)	0 (0)	0 (0)
DV3_NS1	n=35	1 (2.8)	2 (5.7)	0 (0)	3 (8.5)	4 (11)		5 (14)	11 (31)	7 (20)	2 (5.7)
DV4_NS1	n=49	0 (0)	3 (6.1)	1 (2)	15 (30)	12 (24)	5 (10)		3 (6.1)	7 (20)	1 (6.1)
USUV_NS1	n=59	1 (1.6)	0 (0)	1 (1.6)	1 (1.6)	1 (1.6)	11 (18)	3 (5.0)		7 (11)	1 (1.6)
WNV_NS1	n=19	0 (0)	0 (0)	1 (5.2)	0 (0)	0 (0)	7 (36)	1 (5.2)	7 (36)		1 (5.2)
WNV_D3	n=21	0 (0)	3 (14)	2 (9.5)	0 (0)	0 (0)	2 (9.5)	3 (14)	1 (4.76)	1 (4.76)	

DISCUSSION

The objective of this study was to estimate the prevalence of antibodies to arboviruses of public health importance in a large number of bats in Central and West Africa using the same serological assay. We tested 2,579 samples from at least 21 bat species, representing to our knowledge one of the most comprehensive report on arboviruses in bats in Africa. Our data showed spatial and temporal variation of IgG antibodies to diverse arboviruses. We observed an overall seroprevalence to arboviruses of 8.45%. This is lower than what was reported from (the few) previous seroprevalence studies in African bats, but different serological methods were used (i.e. agglutination versus Luminex EIA), the geographical location and the study period can also have an impact (Shepherd and Williams 1964).

Bats are flying mammals that nightly fly up to 20 km for foraging and up to hundreds or thousands of kilometers during seasonal migrations for some species (Schloesing, et al. 2020, Roberts, et al. 2012). Comparing prevalence in different collection sites is thus only meaningful if they are sufficiently distant. Here, almost all our study sites are 20-300 km apart (see **Figure 1**). IgG antibodies to at least one arbovirus antigen were detected in 17 of the 21 sites investigated and, in the four sites with no positive samples, the sample size was low (i.e. < 50). Furthermore, the positivity rate profile for antibodies to arboviruses was statistically different according to the ecological settings (see **Supplementary Table S2**). These differences could be related to bat species, environment, human factors or a combination of those. For example, in Yaoundé and Obala where most bat samples studied were collected are urbanized areas, that could favored a vector population to interact between bats and humans in the same environment, thus contributing to sustain the transmission cycle of arboviruses.

Similar to other studies, we found variable prevalence by bat species, most likely corresponding to different ecology and exposure to arthropod vectors (Hernández-Aguilar, et al. 2021, Shepherd and Williams 1964, Kading, et al. 2018). We observed the highest prevalences in three frugivorous bat species; *Eidolon helvum*, *Hypsignatus monstrosus* and *Rousettus aegyptiacus*. *E. helvum* is widespread in equatorial Africa (https://animaldiversity.org/accounts/Eidolon_helvum/; accessed July, 23rd, 2021), and is roosting in both urban and rural areas representing thus a good intermediate between sylvatic and urban cycles of arboviruses. Combined entomological and bat ecology studies are therefore urgently needed for a better understanding of the role of Chiroptera in the lifecycle of arboviruses in Africa.

Contrasting with the recurrent outbreaks of Chikungunya virus in DRC (Selhorst, et al. 2020), we did not observe IgG antibodies to Chikungunya virus in bat samples from this

country, which might be due to the overall limited number of bat samples tested in this large country. Nevertheless, antibodies to Chikungunya virus have been readily detected in free-ranging NHPs in DRC, but in different areas as where we tested bats in this study (Raulino, et al. 2021). It is also possible that bats are not equally exposed to the same array of mosquito vectors than NHPs that are involved in the sylvatic cycles of arboviruses as suggested by others (Guzmán, et al. 2020, Selhorst, et al. 2020, Simo, et al. 2019, Pastorino, et al. 2004).

Usutu virus has been isolated from mosquitoes, birds and some mammals in Europe and in some countries in Africa such as Senegal, Central African Republic, South Africa and Uganda but data on Usutu virus in Africa are still very limited (Diagne, et al. 2019, Clé, et al. 2019, Nikolay, et al. 2011). Here, we observed antibodies against to Usutu virus, indicative of the circulation of this virus in at least three African fruit bat species (**see Table 3**). To the best of our knowledge, this is the first evidence of Usutu virus circulation in bats from Africa, suggesting that they might contribute to the enzootic cycle of Usutu virus in Africa but additional investigations to clarify the roles of bats in the viral cycle in Africa is needed.

The 12-month longitudinal follow-up of bat colonies in the same geographical location, allowed to study impact of age, reproduction cycle and eventual seasonality. We observed higher prevalence in adults than in immature or juvenile adults for *H. monstrosus* species, but not for *E. helvum*, suggesting a possible difference in behavior and ecology and subsequent exposure to vectors. During the female reproductive cycles, i.e. gestation or lactation, no statistically significant difference was observed for both species.

Nevertheless, our work has some limitations, first of all the lack of confirmation of some of the seroreactivity by affinity-based virus neutralization assay or affinity measurement assays which was hampered by the limited volumes of blood samples, i.e. only a few drops of blood on filter paper. Another limitation is the absence of sequence confirmation of circulation of

arboviruses that induced IgG antibodies detected during this work. Arbovirus RNA can only be detected during a limited period during acute infection, therefore prospective studies are needed that should include blood samples in adequate storage conditions for molecular analysis.

Conclusion

Overall, the presence of antibodies suggests that bats could play a role in the sylvatic cycle of arboviruses in addition to non-human primates and other mammals or animal species. Population size and the number of susceptible NHP at any time, are most likely not high enough to maintain sylvatic arbovirus cycles and therefore other species like bats with large numbers of offspring, i.e. naïve animals at any time, could thus also play also a role in sylvatic cycles. Moreover, several bat species live close to human settlements and create thus opportunities for contacts via an arthropod vector. More studies are still needed to document diversity of animal species susceptible to arboviruses and their role in sylvatic cycles and emergence of new viruses in humans.

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Authorship Confirmation Statement

All authors participated to the work presented here and all approved the final version of the manuscript.

- Conceived the study: AA, MP.
- Collected samples: SPNK, INB, RD, SG, AKK.
- Performed experiments: RR, GT, AE.
- Analyzed the data: RR, GT, AA MP.
- Drafted the manuscript: RR, AA, MP.
- Get funding: AA, MP.
- Reviewed preliminary and final versions of the manuscript: All the authors

Author Disclosure Statement

- All the authors declared no conflicting financial interests.

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III.A.1. Supporting Information

Supplementary Table S1: Cut-off values in MFI (Medium Fluorescence Intensity) used to discriminate between antibody positive and negative bat samples.

	Change Point	Binomial	Exponential	Mean
CHIK_E2	609	138	89	279
ONNV_E2	747	253	162	387
ZIKV_NS1	223	46	30	100
DENV1_NS1	391	168	106	222
DENV2_NS1	473	248	153	291
DENV3_NS1	29	17	14	20
DENV4_NS1	268	223	146	212
USUV_NS1	548	237	102	296
WNV_NS1	926	168	93	396
WNV_D3	356	67	58	160

Supplementary Table S2 : Percentage of samples with antibodies to the different arboviruses according to ecological setting where they were captured.

	N pos/N tested	%	<i>P</i>-value	CHIK_E2	ONNV_E2	ZIKV_NS1	DENV1_NS1	DENV2_NS1	DENV3_NS1	DENV4_NS1	USUV_NS1	WNV_NS1	WNV_D3
Caves	12/281	4,7	>0,001	2 (0.71)	6 (2.1)	1 (0.35)	0 (0.0)	2 (0.71)	1 (0.35)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.71)
Forest	20/272	7,3		0 (0.0)	5 (1.8)	1 (0.36)	1 (0.36)	1 (0.36)	3 (1.1)	5 (1.8)	2 (0.73)	6 (2.2)	3 (1.1)
Urban area	142/1360	10,4		15 (1.1)	10 (0.73)	8 (0.58)	17 (1.25)	20 (1.5)	21 (1.5)	29 (2.1)	56 (4.1)	11 (0.8)	10 (0.73)
Village	44/666	6,6		3 (0.45)	10 (1.5)	4 (0.73)	4 (0.73)	7 (1.0)	10 (1.5)	15 (2.2)	1 (0.15)	2 (0.3)	6 (0.9)

(%) the percentage of positive samples

Supplementary Table S3: Number and percentage of samples with antibodies to arboviruses in colonies of *Eidolon helvum* and *Hypsignatus monstrosus* bats according to age category and reproductive status for adult females.

	<i>Hypsignatus monstrosus</i>		<i>Eidolon helvum</i>		Inter-species
	N tested	N pos (% ; 95%CI)	N tested	N pos (% ;95%CI)	<i>p-value</i>
Juvenile	72	10 (13.9 ; 7.7-23.7)	221	24 (10.9 ; 7.4-15.0)	0.52593
Immature adult *	22	1 (4.6 ; 0.8-21)	218	22 (10.1 ; 6.7-14.8)	0.70359
Adult *	132	31 (23.5 ; 17-31)	376	42 (11.1 ; 8.3-14.7)	0.000413
<hr/>					
F-gestation	11	2 (18.1 ; 5.1-47)	11	2 (18.1 ; 5.1-47)	1
F-no gestation	63	18 (28.5 ; 17-38)	160	15 (9.3 ; 5.7-14)	0.00061
<hr/>					
F-lactation	21	8 (38.0 ; 20-59)	65	7 (10.7 ; 5.3-20)	0.00776
F-no lactation	58	12 (20.6 ; 12-32)	125	10 (8.0 ; 4.4-14)	0.02549

95% CI= confidential interval; * indicated a *p-value* less than 0.05 among Immature adult and Adult on *Hypsignatus monstrosus*

Supplementary Table S4: Seroprevalence of antibodies to arbovirus in colonies of *Eidolon helvum* and *Hypsignatus monstrosus* bats from Yaoundé and Obala over the months.

Months	<i>E. helvum</i>		<i>H. monstrosus</i>		Total			Rains mm
	N Tested	N pos (%)	N Tested	N pos (%)	N Tested	N pos (%)	CI 95%	
Dec-18	20	3 (15.0)	34	6 (17.7)	54	9 (16.7)	9.0 ; 28	13
Jan-19	95	10 (10.5)	40	10 (25.0)	135	20 (14.8)	9.7 ; 21	18
Feb-19	8	0 (0.0)	15	8 (53.3)	23	8 (34.8)	18 ; 55	26
Mar-19	52	6 (11.5)	25	3 (12.0)	77	9 (11.7)	6.2 ; 20	56
Apr-19	47	6 (12.8)	16	1 (6.3)	63	7 (11.1)	5.4 ; 21	104
May-19	149	22 (14.8)	21	5 (23.8)	170	27 (15.9)	11 ; 20	95
Jun-19	81	3 (3.7)	13	1 (7.7)	94	4 (4.3)	1.6 ; 10	83
Jul-19	130	21 (16.2)	5	0 (0.0)	135	21 (15.6)	10 ; 22	41
Sep-19	50	5 (10.0)	19	4 (21.1)	69	9 (13.0)	7.0; 22	144
Oct-19	69	7 (10.1)	17	3 (17.7)	86	10 (11.6)	6.4 ; 20	106
Nov-19	114	5 (4.4)	22	1 (4.6)	136	6 (4.4)	2.0 ; 9.3	56
Total	815	88 (10.8)	227	42 (18.5)	1042	130 (12.5)	10-14	

Supplementary Table S5 : GPS coordinates of the study sites in Cameroon, Guinea and the DRC

Sites	Latitude (N or S)	GPS	Longitude (E or W)	GPS
CMR				
Campo	N	2,37278	E	10.10434
Mambele	N	2,44172	E	15.43393
Obala	N	4,0306	E	11.53149
Yaoundé	N	3,86401	E	11.48763
DRC				
Beni	N	0.4898	E	29.45981
Bikoro	S	0.73664	E	18.1378
Boende	S	00.38007	E	20.85815
Boma	S	5,57029	E	13.045
Kikwit	S	5.03754	E	18.81946
Kimpese	S	5,4821	E	14,45868
Komanda	N	1.34869	E	29.75669
Mangina	N	0.62339	E	29.61268
Zongo	S	4,72927	E	14.88844
Guinea				
Boffa	N	10.32175	W	14.26312
Kankan	N	10.37856	W	9.37333
Kindia	N	9.52728	W	13.68561
Koundara	N	12.32034	W	12.78523
Lola	N	7.91639	W	8.49855
Macenta	N	8.53663	W	9.50049
Mamou	N	10.2849	W	12.23256
Siguiriri	N	11.5565	W	8.80697

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Section IV: *General Discussions and Perspectives*

In the last decades epidemics caused by arboviruses, and especially those transmitted by mosquitoes, affected millions of humans as well as animals, highlighting the importance of public health policies for these arboviruses, which are often poorly characterized. Many of these mosquitoes-borne arboviruses that (re)emerged in different parts of the world are known to have their origin in Africa such as WNV and USUV, isolated from wild birds and sylvatic mosquitoes, as well as ZIKV isolated from a sentinel rhesus monkey in Uganda. Despite the evidence that these mosquito-borne arboviruses are maintained in a sylvatic transmission cycle involving a range of sylvatic mosquitoes and a group of vertebrate hosts, data are still limited on the extent of the animal reservoir and on the different animal that are involved in addition to NHPs to maintain the sylvatic transmission cycle. Thus, studying the ecology of arboviruses in wildlife is crucial especially in African countries where recurrent outbreaks occur and where the resources and equipment are limited to distinguish similar febrile illnesses caused by malaria and arboviruses.

This whole scenario was the major objective of our work on epidemiological investigations of arboviruses in wildlife in Central and West African countries. In order to better understand and document the circulation of arboviruses in wildlife in Africa we have developed a multiplex serological assay, with high specificity and sensitivity for simultaneous detection of IgG antibodies against 10 antigens from 6 different arboviruses. This allowed us to consciously track the degree of exposure to arboviruses in more than 6000 thousand samples representing more than 50 animal species with a focus on NHPs and bats.

Three topics will be debated: multiplex immunoassay as a tool for detecting antibodies to arboviruses and to estimate exposure to arboviruses in wild NHPs and bats.

- **Multiplex immunoassay as a tool for detecting antibodies to arboviruses**

Serological tests are used as a tool for epidemiological surveillance of exposure to

infectious diseases in wildlife populations (Boyd et al. 2015; Beck et al. 2015; Nys et al. 2018; Ayouba et al. 2019). It is an important tool for inferring the infection history to specific viruses, monitoring and predicting possible pathogens to emerge. Despite the ubiquitous use of serology in the investigation of infections in wildlife, methodological limitations and interpretation of results must be considered (Gilbert et al. 2013; Peel et al. 2013). In this work we developed a highly specific and sensitive test for simultaneous detection of antibodies to many arboviruses that can be applied in serosurveys in humans and wildlife populations using a variable type of biological samples and with only scarce sample volumes that are needed.

Firstly, the recombinant proteins chosen to screen for antibodies were structural (EIII; *Flavivirus* and E2; *Alphavirus*) and non-structural (NS1; *flavivirus* and NSP; *alphavirus*) proteins whose target role is to induce neutralizing antibodies as well as to serve as a specific antigen, respectively. Even more, these recombinant proteins are often used in commercial serological kits for the serological diagnosis of ZIKV, CHIKV and DENV. With this approach we were able to capture broad and diverse antibody responses against *flavivirus* and *alphavirus* infections. To validate the test performance, we used a panel of human serum samples known to be positive for the specific arboviruses from this study (except for ONNV and USUV) as well as negative samples for these arboviruses. It is known that positive and negative control samples are important in determining the sensitivity and specificity of a test as well as using an appropriate method to determine the *cut-off* point and define a sample as being “positive” or “negative” (Peel et al. 2013).

In our assay we were able to obtain a specificity above 95% for most of the recombinant proteins tested within the *flavivirus* and *alphavirus* family regardless of the method used (ROC curve or Meas+3SD) on the reference panel of human samples. This is important because these values are comparable to tests considered as the gold standard of specificity like PRNT and molecular tests. Besides that, the sensitivity was above 95% for NS1 proteins from *Flaviviruses*

and for E2 from CHIKV, except for YFV (<44%) and serotype 4 (>86%) of Dengue. However, the sensitivity was highly variable and weak for most recombinant proteins from flaviviruses in the envelope domain III except for WNV. The sensitivity for WNV is in agreement with data found by Beck (2015) where the recombinant EDIII used to capture specific antibodies against WNV reached a sensitivity of 98% versus 100% in our study (Beck et al. 2015). This variable and/or low sensitivities are in contrast with the characteristics of many immunological assays that are often more sensitive than specific (Fritzell et al. 2018). On the other hand a study showed an epitope on ZIKV envelope domain III protein with high sensitivity (92%) that could discriminate between past and current infections by DENV and ZIKV (Denis et al. 2019). All these counterparts can be explained by several parameters, including antibody kinetics, specificity restricted to one or two epitopes or compromised recombinant protein structure due to *in vitro* production issues. Unfortunately there was a lack of positive controls for USUV and ONNV to evaluate the sensitivity of our test for these viruses.

One of the strengths and limitations for the interpretation of serological tests and which impacts on the results is the *cut-off* point for cross-reactions mainly in human sera positive for arboviruses that belong to the same family, which sometimes may require additional tests (De Alwis et al. 2014; Mantke et al. 2004; Fritzell et al. 2018). Denis et al. (2019) emphasized that the use of recombinant proteins can partially improve the specificity of IgG-based diagnostic assays which theoretically can improve test reliability in relation to cross-reactions (Denis et al. 2019). In our test, although highly specific, we observed simultaneous reactivity between heterologous and homologous viruses. Chikungunya for example reacted simultaneously and expressively with ONNV (51%) and USUV (51%). This may be due to the fact that most of these CHIKV samples were collected in regions with co-circulating arboviruses and after outbreaks or that the proteins used are not specific enough. It can be hypothesized that there was exposure to multiple

arboviruses in this samples. Among the *flaviviruses*, WNV-NS1, ZIKV-NS1, DENV-NS1 and YFV-NS1 reacted simultaneously with homologous antigens in varying proportions. This can be explained because the *flaviviruses* can induce cross-reactive IgG in samples/humans from endemic area for DENV or ZIKV for example because the viruses are closely related (Denis et al. 2019). Flavivirus antigens practically did not cross-react with the *alphavirus* antigens which proves the specificity of the test. Another flaw that could be reformulated was the use of positive samples for YFV from vaccinated patients as positive controls which probably interfered with the test sensitivity. Unfortunately, we were not able to apply antibody neutralization tests to discriminated the cross-reactivity.

It would be interesting for future studies to acquire positive samples for ONNV and USUV to assess the sensitivity of the test for the respective recombinant proteins and YFV antibody positive samples from patients who developed the disease. Moreover, the possibility of including other viruses within the group of mosquito-borne *thealphaviruses* such as Middelburg virus (MIDV) and Semliki Forest virus (SFV) that have ruminants (sheep, goat).and wild birds, rodents, insectivores bats as vertebrate host (Hubálek, Rudolf, and Nowotny 2014), respectively, and which circulate mainly in Africa, thus, with the potential to cause outbreaks. More important would be to acquire also well documented positive samples from wildlife to see if there is a difference in cross-reactivity rate, sensitivity and specificity. The test needs also to be improved for detection of IgM antibodies so that both IgG and IgM can be identified in the same assay.

Even despite some unresolved issues, our assay has high specificity, sensitivity and accuracy for several recombinant proteins. This allows us to efficiently investigate the exposure of primates and bats to arboviruses in their natural habitat to better understand the ecology and role of different species in the sylvatic transmission cycle of arboviruses.

- **Prevalence of IgG antibodies against arbovirus in NHPs**

Primates are known to be animals reservoirs for several arboviruses such as YFV, ZIKV and CHIKV, and despite having viremia, they usually do not show clinical signs of infection (Valentine, Murdock, and Kelly 2019). However, the data are still very simplified about the sylvatic transmission cycle of several other arboviruses and on the role of NHPs in the sylvatic transmission cycle as well as the degree of exposure of NHPs to arboviruses.

Studying viruses/arboviruses in wild populations and to identify animals reservoirs of pathogens in general is a challenge and arduous task for several reasons:

- i) Collecting samples from wild animal samples is difficult, requiring many logistics and needs to adhere to ethical issues related to conservation of the animal populations, especially when highly endangered species are studied like NHPs;
- ii) Specificity of the assay due to the antigenic proximity of many arboviruses;
- iii) Regions where several arboviruses co-circulate and/or are endemic may hinder the interpretation of the assay and differentiate between cross-reactivity or exposure to multiple viruses;
- iv) Virus detection is only possible during a due to the short window period for the majority of arboviruses, because viruses are cleared after infection;
- v) A high throughput screening that is highly efficient in detecting antibodies for is needed because sample volumes can be significantly reduced;
- vi) furthermore, in the absence of a well-characterized positive and negative controls, determining an efficient *cut-off* point is challenging.

With this work we assessed and solved some of these challenges through our high throughput multiplex screening assay for detection of antibodies.

In general, the overall prevalence of antibodies was low in NHPs and bats and this may

have been influenced by several factors, like the stringent *cut-off* calculation methods such as changepoint, exponential-binomial-fitted distribution. These methods were applied in order to identify samples as being true positive and negative at MFI values in the absence of standard controls and are thus most likely minimal estimates of prevalences. While changepoint analysis acts in the identification and analysis of time series, allowing the identification of abrupt points of change, which defines the *cut-off* value, the fitted distribution evaluates the probability distribution of a series of data related to the measurement repetition of a variable phenomenon. Distributional models have been applied to serological studies in wildlife when there are no standard controls to use "traditional" methods such as the ROC curve but which can cause biased underestimates of seroprevalence, as reported by Peel (Peel et al. 2013) and Gilbert (Gilbert et al. 2013).

Another parenthesis are the types of samples tested (plasma, DBS, feces dialysates) in different primate species, this may have an influence on the sensitivity of antibody detection rate and consequently on the global prevalence, it has already been shown that sensitivity of IgG detection is lower in stool dialysates (Ayouba et al. 2019). It is important to develop statistical methods that allow more accurate *cut-off* calculation to allow more reliable prevalence estimates, because here we report most likely minimal estimates and underestimate thus real exposure in NHP and bats to arboviruses.

When we look at the prevalence of IgG antibodies in the different species, we observed a high variability in the distribution of seropositivity among the different species, but also among collection sites, which could reflect different habitat and behavior. However, it is important to the dynamics of antibodies within species, which could allow estimate time since last exposure and the total number of times a species has been exposed over a lifetime and to how many different arboviruses because many of them are antigenically related (Fischer et al. 2021; Peel et al. 2013). It would be interesting to follow longitudinally different species of infantile monkeys and in

different seasonal periods to observe the dynamics of exposure to arboviruses in order to measure antibody titers as well as the collection of sylvatic mosquitoes that cohabit with the NHPs.

The results obtained from our serosurvey in the different monkey species provided us with more information about the enzootic cycle of arboviruses. Firstly, the interesting result of seropositivity for ONNV which predominated in DRC and Cameroon. ONNV is known to circulate in humans in Africa, including in Cameroon and DRC, causing several outbreaks in the continent (Pezzi et al. 2019). However, ONNV has a sylvatic transmission cycle that is poorly understood with many gaps. Although antibodies against ONNV have been identified in duikers and buffaloes in DRC and Gabon, so far there is only one report of seropositivity of ONNV in Mandrills in Gabon (Kading et al. 2013). Mandrills in our study were not positive for ONNV, but numbers of samples were limited (n=25). Althouse et al. investigated the seropositivity for ONNV in 12 *C. sabaesus*, 25 *P. papio*, and 5 *E. patas* by PNRT, but the results were ambiguous, according to the author, suggesting that the positivity could also reflect reactivity to CHIKV rather than ONNV (Althouse et al. 2018). Our results provide thus evidence of a potential enzootic cycle of ONNV in DRC and Cameroon involving different monkey species and includes for the first-time information about the circulation of the ONNV in Guinea. A second interesting fact is that we describe for the first time the detection of anti-USUV antibodies in monkeys. USUV is known to circulate between birds and *Culex spp* and human infections have been reported in few countries of Africa and Europe (Roesch et al. 2019). Despite that other mammals have demonstrated antibodies against USUV they are not considered as hosts that participate in the transmission cycles of USUV. At least 50% (13/26) of the monkey species had IgG antibodies against USUV. This demonstrates that the virus circulates in many monkeys species in both arboreal, terrestrial and semi-terrestrial and in different environments. *Red tailed monkeys* for example, showed a positive rate of 4.5% (8/181). However, we can not exclude cross-reaction between other flavivirus

antigens not tested in the study, despite the high specificity in our test. Nevertheless, when we observe the behavior and habitat of these monkeys, we cannot rule out the possibility of exposure to USUV. This monkey species is found in East and Central Africa and is mainly arboreal but may also come to the ground, thus circulating in many different environments and having contact with various types of arthropods, and thus also to mosquitoes that feed on birds in threetops. Their diet is fruit-based but they are considered omnivorous because they eat leaves, flowers or insects in times of fruit shortage (additional evidence of contact with diverse arthropods). They also are very social and have been observed interacting with *blue monkeys* (a species that showed 31% of IgG antibodies against CHIKV). Both species were sampled in DRC where there are recurrent outbreaks of CHIKV. Thus, in this ecological context we cannot rule out the possibility that these species are exposed, infected and serve also as a host for viruses such as USUV as well as for other arboviruses already confirmed. To date, no case of infection or detection of the Usutu virus has been reported in Cameroon, Guinea and DRC. Our results provide evidence of exposure of monkeys to USUV and demonstrates that Usutu is a virus that may be neglected on the African continent.

Other observations can be taken into account and discussed towards the results. We certify that among all species tested, most are positive for at least one antigen tested, as well as in all collection sites, which confirms the wide distribution of arboviruses. In Cameroon, according to literature and serological studies, serotype 2 of dengue is involved in outbreaks of dengue in the country. We observed the circulation of serotype 2 of dengue in monkeys. Ebolowa is an urban community surrounded by a tropical forest with cocoa production as a source of income and Mindourou is a rural community located in the middle of a forest with an equatorial climate and around 90% of the population lives from agriculture. Both sites shared common species of monkeys as greater spot-nosed (*Cercopithecus nictitans*) and moustached monkeys (*Cercopithecus cephus*) and were among the species that showed considerable positivity of IgG

antibody for serotype 2 of dengue. The circulation of DENV-2 in rural and urban areas in Cameroon has been reported, but unfortunately we do not know if the strains circulating in urban and rural areas are endemic or sylvatic dengue strains since spillback and spillover can happen. Molecular investigations are required. Another interesting observation is that if we combine reactivity to the WNV antigens NS1 and DIII, the global seroprevalence reaches 5.6% (91/1613), being the most prevalent virus in the study. Furthermore, if we focus on DRC where high seroprevalence rates of WNV have already been reported in humans (Nur et al. 1999), as well as the presence of neutralizing antibodies in animal species such as elephants, buffaloes, horses and mountain gorilla (Kading et al. 2013), WNV stands out in monkey samples from DRC and with an seroprevalence of 7.1% (45/630) when reactivity to both antigens are combined.

In apes, the investigation strategy for detecting antibodies against arboviruses was in feces dialysates, because only non-invasive sampling is allowed for these highly endangered species, even though the concentration of antibodies and the sensitivity is reduced in this type of sample. Thus, we obtained a low overall prevalence of IgG antibodies in apes, which may have been influenced by the type of samples, in addition to behavior, and exposure to different species of mosquitoes. The first interesting fact is the non-detection of antibodies against DENV in practically all species. Is detection of DENV in faeces less effective? Or the behavioral characteristic of the apes, which, like most other terrestrial monkeys, did not show antibodies against dengue, unlike arboreal species? Apes don't develop enough viremia to infect mosquitoes during the meal? These are questions that remain open and that perhaps an entomological analysis can help to elucidate and fill in some gaps. We also found IgG antibodies against WNV in gorillas in DRC like Kading et al.,(Kading et al. 2013). IgG antibodies to WNV were found also in gorillas in Cameroon, in chimpanzees in Cameroon and DRC, and in bonobos in DRC. This emphasizes the wide spread and distribution of the West Nile virus in apes in Central Africa. Bonobos from

the Lomako-Yokokala natural reserve, which is located in northern DRC, had a seropositivity rate of 8.75% (12/137) to WNV. This species lives in moist tropical forest, dry forest, savannah woodland and marshy grassland and has the characteristic of building nests in trees, which can contribute to the exposure to ornithophilic mosquitoes and explain the serostatus. *Gorillas* and chimpanzees showed IgG antibodies against *flavivirus* and *alphavirus*. In chimpanzees we detected IgG antibodies to CHIKV, ONNV, ZIKV and WNV in Cameroon and only against WNV in DRC. Curiously, these species were the only that did not show antibodies against DENV. Information in the literature about arboviruses and chimpanzees is scarce, with only so far the identification of YFV detection, which makes the discussion difficult. *Gorillas* were the ones that showed IgG antibodies against all antigens arboviruses tested. In general, high seroprevalence rates against CHIKV (18.3%) and ONNV (17.5%) in Minton (CMR) in gorillas, providing evidence of enzootic circulation of *alphavirus* in this region and gorillas are used as a reservoir host. In Somalomo in CMR, gorillas reacted against all antigens tested. This town is agricultural and it is localized in a dense area of equatorial forest and being close to the Dja forest reserve, with a high biodiversity. Perhaps the biodiversity of the region can favor the exposure of these gorillas to different species of peridomestic and/or sylvatic mosquitoes that transit between the ville and the forest environment.

Several *alphaviruses* and *flaviviruses* have already been identified to use primates as both reservoir or amplifiers host and for their contribution to the sylvatic transmission cycle. The evidence of exposure to multiple *alphaviruses* and *flaviviruses* in different regions observed in our study reinforces this idea of the range of existing enzootic cycles, although some NHPs do not show evidence of previous exposure to some arboviruses. Our results also add up because they generated data on other viruses that are still poorly characterized and poorly studied, such as ONNV and USUV, which have a high power to cause outbreaks. Given the lowprevalence in

NHPs they are probably not the only natural reservoirs of arboviruses but in fact contribute as reservoirs hosts of the enzootic cycles.

- **Prevalence of IgG antibodies against arbovirus in bats**

Serosurvey of arboviruses in bat populations increased during the last decade, but only in neotropical regions (Hernández-Aguilar et al. 2021) even knowing that arboviruses that circulate around the globe and that impact human and animal public health arose from tropical areas of Africa (20). Data on the role of bats in the ecology arboviruses like transmission, maintenance and prevalence of arboviruses in sylvatic cycles in African countries are scarce, since most studies are of entomological surveys (JF et al. 2017; M. Diallo et al. 2003) Our study was among the first to investigate a large number of *frugivorous* and *insectivorous* bats of different species and demonstrated that these bats were exposed to different flaviviruses and alphaviruses in almost all sites where samples were collected.

From the serological investigation of more than 6 arboviruses in more than 20 species of bats, we were able to demonstrate the possibility of these mammals of play a role in the sylvatic transmission cycle of arboviruses in addition to primates and birds. Our high throughput multiplex immunoassay revealed a variable seroprevalence by country (3.5-11%), among insectivorous and frugivorous bats (3.4-10%), by ecological sites (4.4-10.4%) and in colonies of *E. helvum* and *H. monstrosus* (10.7-18.5%) reaching an overall seroprevalence of 8.5%.

The overall arbovirus seroprevalence (8.45%) in bats in our study is distinct from the rates found in previous studies in Uganda and Ethiopia (M. C. Williams, Simpson, and Shepherd 1964; Shepherd and Williams 1964; Simpson et al. 1968; Andral et al. 1968; Kading et al. 2018) and the comparison between studies is thus limited due to the lack of studies of arboviruses in bats.

The circulation profile of arboviruses in the three countries may reflect the behavior of

the species as well as the type of habitat. An interesting result was the 4.0% IgG antibody rate (54/1376) to USUV in Yaoundé, Cameroon. Bats also showed antibodies IgG against USUV in Guinea and DRC, which is perhaps the first report of identification of specific antibodies in bats in Africa or at least in Cameroon, Guinea and DRC (there are reports of USUV in wildlife in Senegal, Uganda, Nigeria and Côte d'Ivoire (Nikolay et al. 2011). Little is known about the pathogenicity of USUV in bats, even once identified in the brains of two dead bats in Germany. Usutu virus is suggested to be neurotropic and has been detected in wild birds and several other species of wildlife in Africa and Europe. The point is that here we have serological evidence that bats are exposed to this virus and respond to an infection by producing long-lasting antibodies. In addition, they could contribute to the enzootic cycle of USUV in Africa beyond wild birds. In Guinea, a coastal country in West Africa that is divided roughly into four distinct geographic regions between lowlands, mountains, savannah and rainforest (Jentes et al. 2010), the majority of bats were captured in villages, caves and forest, where most of them had IgG antibodies to both alphaviruses and flaviviruses. An interesting result was the seropositivity to ONNV being the most predominant. The majority of bats that were positive to ONNV (7.14%) were captured around the village of Macenta (forest region). Data on seroprevalence of arboviruses in this country is very scarce. Recurrent YFV outbreaks occurred in Guinea including in Macenta (2003) (Nathan et al. 2001) and IgG antibodies to WNV, DENV, CHIKV were reported in humans (Jentes et al. 2010). Data on the exposure of mammals in Guinea to arboviruses and their role as a reservoir were cited in two articles (Konstantinov et al. 2006) (Butenko 1996). However, it was only available to read the abstract of available articles, not having access to more specific information. In the abstract of paper by Butenko, 127 arbovirus strains were isolated from mosquitoes, Ixodidae ticks, wild birds, bats, monkeys and other mammals and blood samples collected from febrile patients were examined in 1978-1989. However, no information was obtained regarding the 2,700 bat samples

he tested (Butenko 1996). To date, no serological evidence of ONNV circulation or an outbreak has been reported in Guinea so our results add information to the enzootic cycle of arboviruses in the Guinea region, especially the likely role of bats in the maintenance of ONNV in the nature.

We observed highest rates among some frugivorous bat species as *Eidolon helvum*, *Hypsignathus monstrosus* and *Rousettus aegyptiacus*. *Eidolon helvum* and *Hypsignathus monstrosus*, predominant species in Yaounde, Cameroon showed positivity for all six antigens with Usutu virus being the most prevalent, which explains that these species could act as enzootic hosts for this virus in the region. The same can be said of *Rousettus aegyptiacus* and the seropositivity for ONNV, since this species was collected predominantly in Guinea which mirrors the role of this species as potential enzootic host in the region. In some insectivorous bats low rates of IgG were observed and according to Kading et al. this is suggestive of possible accidental exposure (Kading et al. 2018). *Insectivorous* bats has been reported to be exposed to dengue virus in Uganda which is in agreement with our results (Kading et al. 2018). However, this study in Uganda only detected neutralizing antibodies against dengue serotype 2, which, in our study was low. Natural DENV-2 infection has been reported in neotropical *insectivorous* bats (Mexico, Colombia, Caribbean) however some studies propose that bats are rather dead-end-hosts for dengue and they are not competent reservoirs for dengue.

We were able to study longitudinally a colony of bats in Yaoundé and Obala, which allowed us to evaluate and observe the dynamics arboviruses in this bat colony as well as the impact of age, reproduction cycle and eventual seasonality. Bat species showed antibodies against arboviruses during all monthly collection periods between Dec-2018 and Nov-2019, except *Eidolon Helvum* in February and *Hypsignathus monstrosus* in July, which is referring to the months of low precipitation. Perhaps this simply reflects the small number of samples tested in these months. Longitudinal entomological investigations would be needed. As already discussed,

Hypsignathus monstrosus adults were significantly more seropositive than all other age groups and *Eidolon Helvum* which can reflect the behavior and ecology of this bats.

This study provides a broad insight into the dynamics of exposure of various bat species to arboviruses and how complex wild arbovirus transmission cycles can be in wildlife. Bats were sampled in three countries and geographically regions favorable for epidemic outbreaks. We used different antigens from six viruses that make up two large families of arboviruses that cause epidemics around the world. This allowed us to assess on a large scale the presence of antibodies against arboviruses in bats and also to revisit some arboviruses that appeared in the Old World and until then had not been documented in bats. More studies are still needed, where samples of bats, humans and mosquito vectors that inhabit the same niche can be screened in order to assess the degree of exposure and infection in humans and bats. In conclusion, the presence of antibodies suggests that bats could play a role in the sylvatic transmission cycle of arboviruses in addition to non-human primates and other mammals or animal species.

General conclusion

The goal of this work was to document and understand the enzootic cycle of arboviruses in African countries, with the aim to provide new insights into the ecology and the possibility of other animal reservoirs being involved in the life cycle of arboviruses. We first developed a large-scale multiplex antibody detection assay. We obtained a specificity and sensitivity above 95% for 10 antigens from 6 different arboviruses. Second, we applied this technique on more than 6000 samples of NHPs and bats from the Old World which allowed us to observe the degree of exposure of NHPs and bats to arboviruses. Our observations indicate that arboreal monkeys are more exposed to arboviruses and may contribute more to the sylvatic transmission cycle of arboviruses than apes. Assuming that the population size and the number of susceptible NHPs are not high enough to maintain the enzootic cycle during epizootic periods, we investigated also bats as an alternative in the enzootic arbovirus transmission cycle. Our study suggest that bats can play a role in sylvatic transmission cycles for some arboviruses. Moreover, some species may be more susceptible to exposure to a certain virus type such as Usutu, in which it has been shown to circulate extensively among *H. monstrosus* and *E. helvum*. ONNV and USUV were first documented in some bat and primate species. This demonstrates how widespread arboviruses circulate in African countries and that research on these arboviruses with high potential to cause outbreaks is scarce or neglected. Our data once again show the complexity of the ecology of arboviruses, especially on the sylvatic transmission cycles. More studies are important to combine samples of human populations, mosquitoes and animals that cohabit in the same region in order to further improve the knowledge of the extent of infection of arboviruses in human and animal species, and fill more gaps about possible animal reservoirs and enzootic cycles.

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