Molecular detection of arboviruses in *Aedes* mosquitoes collected from Kyela district, Tanzania

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ABSTRACT

Arboviruses belong to a group of viruses that are transmitted by arthropods, mainly mosquitoes and ticks causing clinical disease symptoms in humans and animals ranging from febrile illnesses to hemorrhagic fevers. The present study aimed at examining the circulation of Chikungunya, Dengue, Yellow fever and Rift valley fever viral genomes in *Aedes* mosquitoes from Kyela district in Tanzania. A systematic vector surveillance spanning two months and covering 5 sites in Kyela district was carried out in order to evaluate the potential role of *Aedes spp* in arbovirus transmission in the study area. Mosquitoes were collected, identified to species level by using morphological keys, pooled in respect species and collection sites and screened for arboviruses by RT-PCR. Adult mosquitoes were collected from April to May, 2015 using CO$_2$ -baited CDC light traps, magnet traps as well as human landing collection (HLC). The study sites included Kyela town, Kajunjumele, Ipida, Matema and Njisi villages. A total of 480 blood-feed *Aedes ssp* were collected, identified and grouped in to 24 pools (1-20 mosquitoes per pool) according to species level and location. Out of the 480 *Aedes* spp collected, *Aedes aegypti* represented the most abundant species totaling 338 (70.4%), followed by *Aedes africanus* 102 (21.2%) and *Aedes natalensis* being the minority 40 (8.3%). Arboviruses were detected in 9 pools (37.5%) including *Alphaviruses* (8 pools) and *Flaviviruses* (1 pool). No sample was positive for *Bunyaviruses*. Chikungunya virus (CHIKV) was detected in 6 (75%) *alphavirus* positive pools that were collected mostly in the areas where rice cultivation was common. The findings of this study suggest that people from this region are highly likely to be exposed to arbovirus infections which may represent significant public health concerns.

Keywords: arboviruses, *Aedes*, molecular diagnosis, Kyela district, abundance

RESUME

Detection moléculaire des arboviruses dans les moustiques de genre *Aedes* collectées dans la zone de Kyela en Tanzanie.

Les arbovirus appartiennent à un groupe de virus qui sont transmis par des arthropodes, principalement des moustiques et des tiques, responsables de maladies chez l'homme et animaux. Cette étude visait à examiner la circulation des génomes viraux de CHIKV, de DENV, de YFV et de RVFV chez des moustiques du genre *Aedes* collectés dans la zone de Kyela en Tanzanie. Une surveillance systématique de deux mois et couvrant 5 différents sites dans la zone de Kyela a été effectuée afin d'évaluer le rôle potentiel des espèces d'*Aedes* dans la transmission d'arbovirus dans la zone d'étude. Les moustiques ont été collectés, identifiés en utilisant des clefs morphologiques et rassemblés selon les espèces et les sites de collection. La détection des arbovirus a été effectuée par la technique de RT-PCR. Les moustiques adultes ont été capturés d'Avril à Mai 2015 dans la ville de Kyela, les villages de Kajunjumele, d'Ipida, de Matema et de Njisi. Quatre cents quatre-vingts moustiques du genre *Aedes* ont été capturés, identifiés et groupés dans 24 lots (1-20 moustiques par lot) selon les espèces et l'endroit de collecte. Sur les 480 moustiques attrapés, *Aedes aegypti* a représenté l'espèce la plus abondante (338 spécimen, 70.4%), suivi de *Aedes africanus* (102 spécimen, 21.2%) et *Aedes natalensis* (40 specimen, 8.3%). Des arbovirus ont été détectés dans 9 lots (37.5%) comprenant alphavirus (8 lots) et flavivirus (1 lot). Aucun échantillon n'était positif pour bunyavirus. Le virus de Chikungunya (CHIKV) a été détecté dans 6 lots qui étaient positifs pour l'alphavirus (soit 75%) qui ont été collectes pour la plupart dans les sites où la culture de riz était commune. Les résultats de cette étude suggèrent que les animaux et habitants de cette région seraient fortement exposés aux infections par les arbovirus qui peuvent représenter des problèmes significatifs de santé publique.

Mots-clés : arbovirus, moustiques, *Aedes*, diagnostic moléculaire, Tanzanie

Introduction

Arboviruses (Arthropod-borne viruses) compose a large group of viruses that are commonly transmitted to humans and animals mainly by the bite of mosquitoes and less frequently by ticks, which results in diseases [3]. They are classified into *Togaviridae, Flaviviridae, Bunyaviridae* and *Reoviridae* families having mostly single-strand ribonucleic acid (RNA) genome with spherical morphology and a diameter that ranges from 45-120 nm [3]. Once confined to limited geographic areas, several of these viruses have currently spread well beyond their historically endemic regions to become pathogens of global importance [7].

The Chikungunya virus (CHIKV), Dengue virus (DENV), Yellow fever virus (YFV) and Rift Valley fever virus (RVFV) are included in the group of these viruses. They are considered to be the most common emerging pathogens transmitted to humans by *Aedes* mosquitoes and cause major disease burdens in tropical and subtropical countries worldwide [2]. Most of these viruses are maintained in zoonotic cycles and humans are usually incidental dead-end hosts with an insignificant role in maintaining the cycle of the virus. They constitute a growing international public health problem for which a licensed vaccine, therapeutic drugs, and effective vector control programs are lacking [7].

Tanzania has had multiple arbovirus outbreaks resulting in economic and public health distress including: RVFV reported for the first time in 1930 followed by periodic epidemics of 10-20 years in 1947, 1957, 1977, 1997 and 2007 [17]. By the end of 2007, the disease had claimed thousands of

cases in ruminants and several hundred human cases [5]; CHIKV was reported in 1953 where patients were described to have acute onset of fever associated with rigor headache, joint pain and rash [5].

Febrile diseases caused by arboviruses cause high rates of morbidity and mortality in human because they are often mis-diagnosed and treated as malaria. Much of this confusion stems from the difficulty of clinical discrimination between different arboviral infections and those caused by often hyperendemic *Plasmodium falciparum* [19]. The lack of efficient prophylactic and therapeutic measure make infection with these pathogens a serious public health concern not only in endemic developing countries, but also in many non-endemic industrial countries.

No studies have been conducted in Kyela district to investigate the presence of the selected arboviruses in their potential vectors. However, their diagnosis is still a challenge due to clinical manifestation similarity to other diseases; in most cases the diseases are underreported. Thus, the present study is aimed to examine the presence of CHIKV, DENV, YFV and RVFV viral genome in *Aedes* mosquito from Kyela district. Detection of the presence of virus in their arthropod hosts are important for monitoring of viral activity and provide quantitative information that could be useful for modeling the transmission dynamics. Indeed, findings of this could provide important information for understanding arbovirus disease status and their transmission patterns in regions with no history of disease outbreaks in Tanzania.

Material and methods

STUDY AREA

This study was conducted in Kyela district of Mbeya region, located in the South Western corner of the Southern Highlands of Tanzania. The district lies between longitudes 33°41' and 33°30' East of Greenwich and between latitudes 9°25' and 9°40' South of Equator (Figure 1). Kyela's rainy season is between November and June, with the heaviest rains falling in April and May. The district lies in the flood plains of Lake Nyasa and thus receives heavy rains of about 3000 mm per year. Kyela has a hot and humid climate with a mean daily temperature of 23°C. The natural vegetation is of tropical savanna forest and grasslands with lagoon vegetation on the swamps and river mouths to the lakes. The study site was chosen to include the coastal cities: Kyela (town), Kajunjumele, Njisi, Ipinda, and Matema because of their location to low altitude (500m) with availability of having mosquito favourable habitats. Furthermore, their locations on the proximity to the Kyela flood plains and Lake Malawi as important factors which can contribute to arbovirus emergences. .

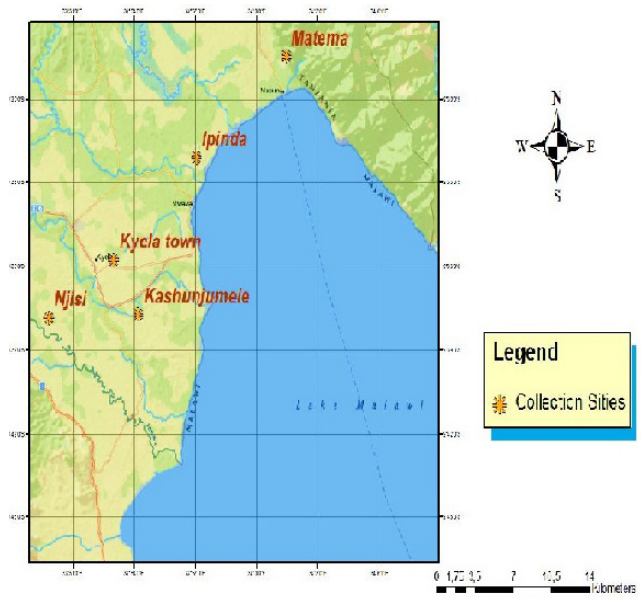

FIGURE 1: Map of Kyela district showing sampling sites. Source: GIS coordinates

STUDY DESIGN AND SAMPLE SIZE

The present study utilized a cross-sectional panel design and was conducted from April to May 2015, which is rainfall months suitable for mosquitoes breeding; approximately, 1830 mosquitoes were collected from which 480 were the *Aedes spp*. They were selected and analysed for virus detection after morphological identification. For the outdoor mosquito collection, the Mosquito Magnet (MM) trap was used, which is battery operated trap and runs on propane gas that is catalytically converted to produce carbon dioxide which attracts the mosquitoes. The trap was run from 17hours to 06 hours outdors. The Center for Disease Control (CDC) light traps and human landing collection (HLC) aspirators were also used for collection of indoors mosquitoes.

MOSQUITO COLLECTIONS AND IDENTIFICATION

The adult mosquitoes trapped were killed using alcohol 100%, sorted by genus level, packed in labeled 1.5 ml eppendorf tubes and transported in dry ice to the Sokoine University of Agriculture (SUA) molecular biology laboratory where they were kept in freezer at -20°C before identification. Then after, identification by using a standard morphological identification keys [13] under a stereo light microscope. Mosquitoes were pooled (up to 20 mosquitoes per pool) by species and collection sites.

MOLECULAR ANALYSIS

Viral RNA extraction and cDNA synthesis

RNA was extracted from pooled mosquito samples using the QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany) according to manufacturer's recommendations. Briefly, 140 µL of serum was added to 560 µL AVL buffer containing

carrier RNA into a 1.5 mL micro-centrifuge tube and mixed by pulse-vortexing for 15 sec followed by incubation at room temperature for 10 min. Protein precipitation was followed by adding 560µL of absolute alcohol mixed by pulse-vortexing for 15 sec. The lysate was then passed through the silica-gel column, and the column was washed twice with 500 µL of washing buffers AW1 and AW2, respectively. Finally, RNAs were carefully eluted by 60 µL of buffer AVE equilibrated temperature and the extracted mosquito RNA was stored in -20˚C at Southern African Centre for Infectious Diseases Surveillance (SACIDS) molecular biology laboratory located at SUA Morogoro before amplification. To convert extracted RNA into cDNA, 4 µL of RNA template was combined into 16 µL of a master mix containing 4µL of 5XVILO Reaction Mix, 2µL of 10xSuperscript Enzyme Mix and 10µL nuclease free water; the whole volume was brought up to 20µL. The tube contents were mixed by vortexing for 15 seconds and the incubated in the thermocycler at the following conditions: 25°C for 10 min, 42°C for 60 min; the reaction was terminated at 85°C for 5 min and 4°C as hold temperature.

Arbovirus detection by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The cDNA amplicons were used for RT-PCR amplification (AgPath-ID™ One-Step RT-PCR Kit, Applied Biosystems, USA) using primers targeting virus genera or specific arboviruses. A total of 20µL of master mix was prepared containing 10µL of 2xDream Taq green PCR Master Mix (Thermo Scientific, Calsband, USA), 1µL of both forward and reverse primer, 1µL of cDNA and 7 µL of nuclease-free water up to 20µL. Primers targeting *Bunyavirus*, *Alphavirus* and *Flavivirus* (Table I) were used to detect the presence of arbovirus specific genera. For a samples which tested positive with genus primers, were tested further with primers that target conserved genes in the specific viruses belonging to the genus in question (Table II). The PCR cycling performed was the following: an initial denaturation step at 94°C for 15 min, following by 35 cycles of denaturation at 94°C for 30 sec, annealing temperatures at 57°C for 60 sec each, extension at 72°C for 30sec. The reaction mixture in each PCR tube was then subjected to a final extension step at 72°C for 10 min. The PCR amplification of targeted gene for specific virus in the cDNA was performed in a 25 µL reaction containing: 12.5 µl of 2xDream Taq Green PCR master mix (Thermo Scientific), 0.5 µL each of forward and reverse primer, 2 µl of the cDNA and 9.5 µl of water to top up to 25 µl. Thermal profiles were performed on a GeneAmp PCR system 9700 (Applied Biosystems, USA). PCR products were separated by electrophoresis and visualized under UV light.

STATISTICAL ANALYSIS

Data were listed, selected, sorted and manipulated using Microsoft Excel sheet. A statistical tool, Epi Info7 software (Centers for Disease Control and Prevention) was used to calculate the proportion of infected mosquito pools and the difference of infection according to each study village at $P < 0.05$. Chi-square test and Fisher exact tests were used to evaluate heterogeneity of infection rates among the different villages.

Virus	Target gene or protein	Primer	Sequence (5'→3')	Position	PCR product size (bp)	Reference
Alphavirus	NSP4	VIR2052F	TGG CGC TAT GAT GAA ATC TGG AAT GTT	6971-6997	150	Esho *et al.*, 2005
		VIR2052R	TAC GAT GTT GTC GTC GCC GAT GAA	7086-7109		
Bunyavirus	N Protein	BCS82C	ATC ACT GAG TTG GAG TTT CAT GAT GTC	86-114	251	Bryant *et al.*, 2007
		BCS332V	GCTGT TCC TGT TGC CAG GAA AAT	309-329		
Flavivirus	NS5	FU1	TAC AAC ATG ATG GGA AAG AGA GAG AA	9007-9032	220	Kuno *et al.*, 1996
		CFD2	GTG TCC CAG CCG GCG GTG TCA TCA GC	9308-9283		

Table I: DNA sequences of the primers used for detection of arbovirus genera

Virus	Primers	Sequence (5'-3')	Region, position	Reference
CHIKV	CHIK3F	CAC ACG TAG CCT ACC AGT TTC	5'NTR, 14-112	Smith *et al.*, 2009
	CHIK3R	GCT GTC AGC GTC TAT GTC CAC		
DENV	D1 38-65	TCA ATA TGC TGA AAC GCG CGA GAA ACC G	3'UTR, 10520-10541	Lanciotti *et al.*,1992
	D2 455-483	TTG CAC CAA CAG TCA ATG TCT TCA GGT TC	3'UTR, 10674-10694	
RVFV	RVF3	CAG ATG ACA GGT GCT AGC	Gn, 876	Testuro, 2012
	RVF4	CTA CCA TGT CCT CAA T	GlyM, 2817-2840	
YFV	CAG	CGA GTT GCT AGC AAT AAA CAC ATT TGG A	Polypro. 43 - 71	Weidmann *et al.*,2010
	YF7	AAT GCT CCC TTT CCC AAA TA	Polyprot. 1293-1312	

Table II: DNA sequence of the primers used for screening of specific viruses

Results

MOSQUITO ABUNDANCE AND DISTRIBUTION

During this investigation, a total of 1830 mosquito belonging to different species were collected. In order to screen arboviruses, *Aedes ssp* were considered. Such, 480 blood-feed *Aedes ssp* were collected, identified and pooled in to 24 pools (up to 20 mosquitoes in each pool) according to species level and location. Using morphological keys, in overall, 3 species of *Aedes* genera were identified including *Aedes natalensis, Aedes aegypti* and *Aedes africanus*. Out of 480 mosquito *Aedes* collected, *Aedes aegypti* represented the most abundant specie 338 (70.4%), followed by *Aedes africanus* 102 (21.2%) and *Aedes natalensis* being the minority 40 (8.3%) (Table III).

In general, there is significantly different for mosquito abundance between sampling sites ($P < 0.05$; IC 9.54 – 30.57). The most abundant mosquito collection by site was recorded in Kyela (n= 601) followed by Kajunjumele (n= 391); Njisi (n=365); Ipinda (n= 293) and Matema village giving the least collections (n=180) (Fig.2).

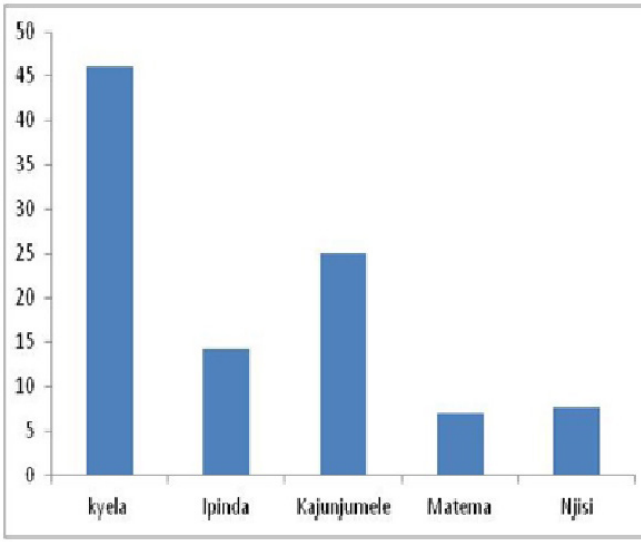

Figure 2: Abundance and distribution of the adult *Aedes* mosquito collected according to the sampling sites

DETECTION OF ARBOVIRUSES IN MOSQUITOES BY RT-PCR

Specific arbovirus (*Bunyavirus*, *Alphavirus* and *Flavivirus* were screened from the 24 *Aedes* pools; arboviruses were detected in 9 pools (37.5%) including *Alphavirus* (8 pools) and *Flavivirus* (1 pool). No sample has been positive for *Bunyavirus*. The positive mosquito samples were then further tested using primers targeting conserved genes in the specific virus belonging to the concerned genus (Table IV).

From a total of 8 *Aedes* mosquito pools tested positive for *Alphavirus* genus, Chikungunya virus was identified into 6 pools by RT-PCR (Fig. 3), giving an infection rate of 75% (n=6). The highest number of Chikungunya infection was detected in mosquitoes sampled in Kyela town 66.6% (n= 4) followed by Kajunjumele 16.6% (n=1) and Njisi 16.6% (n=1). No infection was found in Matema and Ipinda. The statistic test showed that positivity rates were not significantly different among the different villages ($P > 0.05$). However, the higher rates of infection were found in the more geographically central study sites, areas surrounding rice plantations.

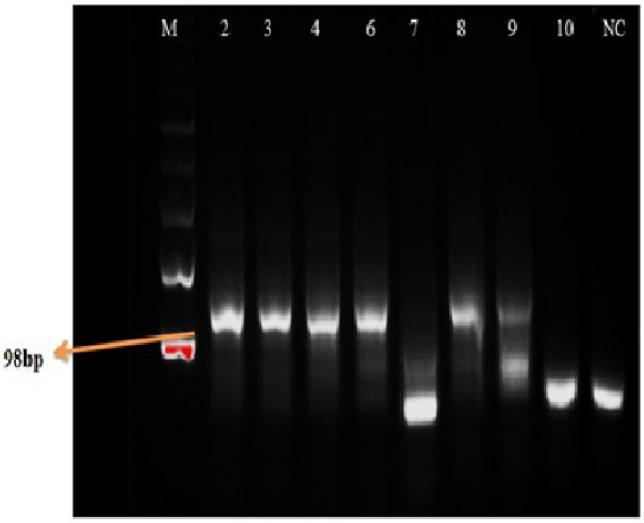

Figure 3: Visualization of RT-PCR products 5'NTR for Chikungunya virus. The expected PCR product size was 98 bp. M: DNA marker. Lines 2-10: Mosquito pools, NC: Negative control. Pools 2, 3, 4, 6, 8 and 9 were positive, while pool 7 and 10 were negative.

Discussions

A geographical assessment of arbovirus vectors and their role in virus transmission is a fundamental aspect for the determination of high risk areas where emergence and circulation of arthropod viral diseases might occur. The present study aimed to determine mosquito competence in arbovirus transmission in Kyela district. To our knowledge, this is the first study to be conducted in the above mentioned district on this topic. The sampling in all sites targeted the

Species	Kyela town	Ipinda	Kajunj.	Matema	Njisi	Total
Ae. aegypti	120	59	98	26	35	**338**
Ae. africanus	66	10	18	6	2	**102**
Ae. natalensis	34	0	4	2	0	**40**
Total	220	69	120	34	37	**480**

Table III: Different *Aedes* species identified and their abundance at each sampling site.

rainy season when vector abundance is usually expected to be highest resulting in increased host/reservoir and vector contact hence increased virus activity. This research showed that mosquito vectors of arboviruses endemic or epidemic in East Africa such *Aedes spp* are distributed throughout the Kyela district, although in varying densities. In some cases, distribution of these vectors was restricted to certain areas probably due to the ecological and environmental adaptation.

From April to May 2015, *Aedes* mosquitoes were collected. *Aedes aegypti* represented the most abundant specie followed by *Aedes africanus* and *Aedes natalensis* being the minority. The abundance of these primary vectors and floodwater *Aedes* in the semi-arid district may be attributed to the nature of the terrain, soil types and vegetation cover, and rainfall which may influence availability of favorable vector breeding and resting grounds [9, 16].

Aedes aegypti the principal vector of the mentioned viruses was predominated in the Kyela town where there is high range of temperature and uncontrolled level of population growth. This is due mostly by the anthropophilic behavior of *Ae. Aegypti* [8] feeding where there is high level of human activity, mismanagement of container after use, presence of old tyres attributing to lack of environmental hygiene. This is confirmed by the report from Pakistan showing that Tyres trade had contributed to the reinvading of *Ae. Aegypti* major vector of emerging diseases such as dengue in the area where it was already eradicated [13]. Evidence that East African *Ae. aegypti* are among the most yellow fever competent species in the world [20] also means that the Kyela Tanzanian district is consistently at higher risk of YF transmission.

The importance of mosquitoes in arbovirus disease transmission and maintenance in Tanzania cannot be overemphasized. Distinct arboviruses were detected in *Aedes* mosquito pools collected in Kyela by RT-PCR. From 24 pools of *Aedes* species, arboviruses were detected in 9 including *Alphavirus* (8) and *Flavivirus* (1) genus; no sample was tested positive for *Bunyavirus*.

These results correlate with the recent studies carried out in the same district reporting high seroprevalence of *Alphaviruses* and *Flaviviruses* in human serum collected particularly in Kyela and Kajunjumele [21]. This suggests that *Aedes* mosquitoes may have played an important role in the transmission of arbovirus in Kyela district.

The infectivity rates differed according to the different sampling sites. More infected pools of arboviruses were

Location	Mosq. genus	**Pool #**	**AlphaV**	**BunyV**	**FlaviV**	**CHIKV**	**DENV**	**RVFV**	**YFV**
Kyela	*Aedes aegypti*	1	-	-	-	-	-	-	-
	Aedes aegypti	2	+	-	+	+	-	-	-
	Aedes aegypti	3	+	-	-	-	-	-	-
	Aedes aegypti	4	+	-	-	+	-	-	-
	Aedes aegypti	5	-	-	-	-	-	-	-
	Aedes aegypti	6	+	-	-	-	-	-	-
	Aedes africanus	7	+	-	-	-	-	-	-
	Aedes africanus	8	+	-	-	+	-	-	-
	Aedes africanus	9	-	-	-	-	-	-	-
	Aedes africanus	10	-	-	-	-	-	-	-
	Aedes natalensis	11	-	-	-	-	-	-	-
Kajunjumele	*Aedes aegypti*	12	-	-	-	-	-	-	-
	Aedes aegypti	13	+	-	-	+	-	-	-
	Aedes aegypti	14	-	-	-	-	-	-	-
	Aedes aegypti	15	-	-	-	-	-	-	-
	Aedes aegypti	16	-	-	-	-	-	-	-
	Aedes africanus	17	-	-	-	-	-	-	-
Ipinda	*Aedes aegypti*	18	-	-	-	-	-	-	-
	Aedes aegypti	19	-	-	-	-	-	-	-
	Aedes africanus	20	-	-	-	-	-	-	-
Matema	*Aedes aegypti*	21	-	-	-	-	-	-	-
	Aedes aegypti	22	-	-	-	-	-	-	-
Njisi	*Aedes aegypti*	23	-	-	-	-	-	-	-
	Aedes aegypti	24	+	-	-	+	-	-	-

Table IV: List of arboviruses detected in *Aedes* mosquitoes using RT-PCR

reported from Kyela town, Njisi and Kajunjumele; these three regions are classified as semi-arid, surrounding flood plains of Lake Malawi and are inhabited by communities whose economy mainly depend on rice or paddy cultivation. These conditions provide an ideal habitat for different *Aedes* species considered as major vectors for most arboviruses. These finding concur with previous studies suggesting that most of arboviruses primarily affect inhabitants of the dry lands of the Rift Valley regions and the outlying semi-arid and arid grazing lands[1]. In addition, despite the differences in geographic locations, arbovirus infectivity rate may be explained by the anthrophilic behavior of *Aedes* mosquitoes. Furthermore according to the latest research [8], showing that, Chikungunya IgG was more evenly distributed in the two Kyela sub-districts and was also common in other sites nearby watercourse; it is estimated that apart from the trans-ovarial transmission which is common for most vectors, *Aedes* mosquitoes collected in these areas may feed on infected people and then become infected.

Amplification with specific primers of distinct viruses from suspected positive arbovirus genera showed the presence of Chikungunya virus. However, no sample was found to be positive for Dengue fever, Rift valley fever virus as well as yellow fever virus. This could be attributed to the effect of distribution of mosquito nets by some campaigns in both pregnant mothers and infants which may have reduced mosquito to feed on infected people. Studies carried out in New California [13] and in Argentina [4] confirm the implication of *Aedes aegypti* in Chikungunya and Dengue transmission during epidemics. *Aedes ssp* were showed also to be responsible for the 2004-2006 Chikungunya outbreaks in the Indian Ocean Islands [16], where 75% of the population was affected [15]. While *Ae. aegypti* is usually responsible for urban yellow fever outbreaks, the first reported outbreak in 1992-1993 in the Rift Valley Province of Kenya was sylvatic in nature and was associated with *Ae. (Stegomyia) africanus*. In addition, five cases of human CHIKV infection were confirmed in Uganda during a 3-month period in 1968 when multiple CHIKV isolations were made from *Aedes africanus* [10]. Further study conducted in Kinshasa, showed that Ae. Aegypti is present in high densities and has been incriminated in CHIKV transmission [9].

Indeed, detection of infection of arboviruses in this area suggests that *Ae. ssp* have the potential to transmit Chikungunya and Dengue virus that they may play a major role during epizootics/epidemics. The low positivity obtained for genera-specific could be due either to the mosquito preservation methods used as well as the time between sample collection and laboratory analysis. *Aedes* mosquito pools used in this investigation was frozen at – 20 ˚C and RNA extraction was done 4 months after collection. These situations can increase the chance for RNA degradation as cold storage availability allows samples to be stored not longer than a week after field collection [18]. The most recommended preservation method for RNA extraction is RNAlater as well as Liquid nitrogen which is a nontoxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA. No pool was positive for Dengue fever, Rift valley fever and Yellow fever virus and this could be ascribed to absence of outbreaks reported for these diseases in the region suggesting that mosquitoes could be feeding on uninfected people.

In conclusion, this study has shown the presence of different arboviruses vectors present in Kyela district in Tanzania. Molecular analysis confirmed an evidence of active circulation of Chikungunya virus in the region. This study suggests that people from Kyela, especially in certain geographic locations, are highly likely to be exposed to arboviruses over the course of their lifetime.

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