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Entomological Co-infections of Arboviruses: Dengue and Chikungunya Viruses along the Coastline of Kenya

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Abstract Arthropod-borne disease outbreaks as a result of pathogen influx including arboviruses transmitted by strains of *Aedes species* occur periodically in varying spots in Kenya. However, there has been paucity of documented information on the *Aedes species* involved in transmission of different haplotypes of arboviruses. This study assessed for concomitant infection of arboviruses in different vectors. *Aedes species* were sampled by Bio gent sentinel trap. Mosquitoes were stunned at 4℃ for 5 minutes; sorted according to site, species and sex. RNA was extracted using Trizole®. cDNA was generated using one step real time PCR. Proportions of vectors and arboviruses were analyzed by R-statistics. A total of 37, 220 mosquitoes of *Aedes species* were analyzed. Vectors were: *Aedes aegypti formosus* (23,265=62.5%), *Aedes aegypti aegypti* (4,931=13.2%), *Aedes mcintoshi* (3,557=9.56%), *Aedes ochraceus* (2,156=5.79%), *Aedes pembaensis* (2,049=5.51%), *Aedes tricholabis* (487=1.31%), *Aedes albicosta* (415=1.11%), *Aedes fulgens* (200=0.54%) and *Aedes fryeri* (160=0.43%). Dengue virus (DENV) had highest entomological infections at 79% (n=1910) while Chikungunya (CHIKV) at 21%. DENV-2 had the highest frequency at 54%, while DENV-4 with the least frequency at 6%. Only ECSA genotype of CHIKV was present. Concomitant infections of arboviral haplotypes in different clades of *Aedes species* occur along the coastal region. Aedes *aegypti aegypti* is incubates all serotypes of DENV and genotype of CHIK viruses thus the major vectors of arboviruses. This information is important as it gives knowledge on areas at high risk for arboviral disease outbreaks. Consecutively, up scaled survey and implementation of control and prevention measures taken appropriately.

Keywords Co-infections; Dengue; Chikungunya; *Aedes species*

Background

Arthropod-borne viruses (arboviruses) are transmitted to humans primarily through the bites of infected hematophagous arthropods, for example mosquitoes (Lindsey et al., 2014). These viruses are maintained in the zoonotic cycle with humans as incidental dead-end host with limited role in the maintenance of this cycle (Ochieng et al., 2013). In the ecosystem, the viruses are incubated through intrinsic and extrinsic incubation. Aedes and Culex are genera of mosquitoes known to potentially get infected and transmit a range of arboviruses including Dengue, Chikungunya, Zika virus, Yellow fever, Japanese encephalitis and Venezuelan equine encephalitis among others (Lindsey et al., 2014). These viruses cause a ranging degree of clinical syndromes with different severity in humans and domestic/ wild animals (Ranjit and Kissoon, 2011). The infection can be self-limiting characterized by fevers to deadly histopathological manifestation like encephalitis and/ or hemorrhagic fevers (Ranjit and Kissoon, 2011).

Resurgence or Emergence of Epidemic arboviral diseases

In the last 20 years, there has been a significant resurgence or emergence of epidemic arboviral diseases, affecting both humans and domestic animals in various parts of the world (Gubler, 2002). Dengue fever virus was reported to be a leading cause of childhood mortality in Asia and South America (Ranjit and Kissoon, 2011). More so, it was noted as the most rapidly spreading and important arboviral disease in the world with a geographic distribution of over 100 countries (Bhatt et al., 2013); (Ranjit and Kissoon, 2011). Some arboviruses, for example, Chikungunya virus have been reported in certain geographical regions with great health and economic effects (Petersen et al., 2016). Major outbreaks of Chikungunya virus started in the Comoro Islands in January 2005 then

spread rapidly to the other islands of the region, Mayotte, Seychelles, La Reunion and Mauritius (Vazeille et al., 2010). In 2004 to 2007, an outbreak of Chikungunya virus disease was documented in the Indian Ocean islands and India (Kariuki Njenga et al., 2008). These epidemics have been caused primarily by viruses thought to be under control including Dengue, Chikungunya, yellow fever, Japanese encephalitis and Venezuelan equine encephalitis (Lindsey et al., 2014). Their epidemics are partly influenced by expansion in their geographical niche over time (Ochieng et al., 2013).

Epidemiology of *Aedes species*

Aedes species have evolved to be effective in the maintenance of viruses in the population due to their ability to get infected and transmit the viruses in three various mechanisms (da Costa et al., 2017). These mechanisms are Ⅰ) vector-host-vector interaction Ⅱ) sexual mating following emergency from pupae and Ⅲ) transovarial (Bravo et al., 2014). Chadee (2012), these vectors of arboviruses are diurnal exophagic with feeding taking place in early morning and late evening. Following feeding, *Aedes species* mosquitoes take shelter under bushes of shrubs or trees.

Aedes albopictus (also known as "Asian tiger" mosquito) is mainly found in the Asian continent and is incriminated in the transmission of arboviruses in that region (Minard et al., 2017). In Kenya, various *Aedes species* have been isolated including *Aedes aegypti, Aedes mcintoshi, Aedes ochraceous and Aedes tricholabis* (Ochieng et al., 2013). This study sought to understand the potential/ role of each *Aedes species* in the transmission of different arboviruses.

Serotypes and Biology of Dengue virus

Sutherland et al. (2011) Dengue virus (DEN) is a single stranded RNA virus comprising five distinct serotypes (DEN-1, DEN-2, DEN-3, DEN-4 and DENV-5) (Normile, 2013). These serotypes belong to the genus *Flavivirus*, family Flaviviridae. Distinct genotypes have been identified within each serotype, highlighting the extensive genetic variability of the Dengue serotypes. Among them, "Asian" genotypes of DEN-2 and DEN-3 are frequently associated with severe disease accompanying secondary Dengue infections (Kosasih et al., 2016). All Dengue virus serotypes can be transmitted by Aedes mosquitoes (Ranjit and Kissoon, 2011). For them to be transmitted, they have to get into the Aedes mosquito tissues through a bite of an infected human or animal (Sutherland et al., 2011). The virus replicates in the tissues of the mosquito and transmitted to the subsequent host through a bite as the mosquito is taking a blood meal (Harrington et al., 2014). Dengue virus can also be transmitted within the mosquito populations through mating and transovarial (da Costa et al., 2017) a phenomenon maintaining the wild arboviral population. This study explored the serotypes of Dengue virus found in Aedes mosquitoes along the Kenyan coast for a period of two years.

Genotypes and Biology of Chikungunya virus

Chikungunya virus (in Makonde meaning for "that which bends up" as a result of the stooped posture that resulted from the pain of the disease) is an arthropod-borne virus of the genus *Alphavirus*, family *Togaviridae* (Thiberville et al., 2013). Globally, three (3) genotypes of Chikungunya virus have been described: West African (WAG), East/Central/South African (ECSA) and Asian genotypes (AG) (Lanciotti and Valadere, 2014). In Kenya, only the ECSA genotype has been reported (Kariuki Njenga et al., 2008). Like other arboviruses, Chikungunya virus infection causes a fever-rash-arthralgia syndrome in humans (Petersen et al., 2016). Thiberville et al. (2013), this arthralgia can persist for months or even years depending on the immune status of the affected person. In some cases, the infection may progress to frank arthritis. Chikungunya virus infection symptoms typically occur in 72-97% of the infected individuals (Sharp et al., 2014). Only about 18% of the infected persons manifesting clinical illness associated with re-exposure do not result in clinical illness (Yoon et al., 2015). This study analyzed the genotypes of Chikungunya virus in *Aedes* mosquitoes along the Kenyan coast for a period of two years.

Concomitant infections of arboviruses in *Aedes* mosquitoes

Aedes species transmit arboviruses in the nature (da Costa et al., 2017). However, the question of vector preference by arboviruses has not been addressed. A study by Ochieng et al. (2013) isolated arboviruses in various

genera of mosquitoes with the highest number of virus reported in flood water *Aedes species*: *Aedes mcntoshi* with 18 viruses, *Aedes ochraceous* with 8 viruses and *Aedes tricholabis* with 6 viruses. Alphaviruses were isolated as follows: Ndumu virus from *Aedes mcintoshi*, *Aedes ochraceous*, and *Aedes tricholabis*. There were also 4 orthobunyaviruses from this area consisting of one Ngari virus, one Pongola and two Bunyamwera viruses isolated from *Aedes mcintoshi* only. In Rabai area of coastal Kenya, flaviviruses and alpha viruses were isolated in *Aedes aegypti* only (Odhiambo, et al., 2014). The ability of a given *Aedes species* to carry multiple viral infections was demonstrated by Vazeille et al. (2010). In their study, *Aedes albopictus* was orally infected with both Dengue and Chikungunya viruses with an ability to transmit them to their human host via its saliva. A study by Göertz et al. (2017); where *Aedes aegypti* were orally infected by Chikungunya and Zika viruses reported similar results and the vectors were highly competent, with transmission rates of up to 73% for ZIKV, 21% for CHIKV, and 12% of mosquitoes transmitting both viruses in one bite.

Study reports indicate there is a possibility that individuals may become infected by more than one arbovirus at a time (Rückert et al., 2017). This increase in human arboviruses co-infections might mean the mosquitoes could be exposed to multiple arboviruses during one feeding episode. A study by Rückert et al. (2017) exposed *Aedes aegypti* mosquitoes to Chikungunya, Dengue-2 and Zika viruses as either single, double or triple infections. Their results indicated the *Ae. Aegypti* mosquitoes could be infected with and transmit all combinations of these viruses simultaneously. In a study by Le Coupanec et al. (2017), it was demonstrated that mixed infection of Chikungunya and Dengue viruses facilitated viral replication in *Aedes aegypti* thus increasing in their numbers before exit.
It is worth noting that arboviruses can share a geographical area or overlap. Chikungunya and Zika viruses occur and co-circulate in same geographical zones (Göertz et al., 2017), while Dengue and Chikungunya overlap with Dengue endemic areas. Cases of arboviral co-infections in Aedes mosquitoes have been reported in areas with reports of arboviral co-infections in humans (Göertz et al., 2017), emphasizing the need to study co-infections in the vector mosquitoes. In this study, adult *Aedes species* mosquitoes were collected along the Kenyan coast, their species determined and screened for concomitant infections for Dengue and Chikungunya viruses over a period of two years.

1 Materials and Methods

1.1 Sentinel sites selection criteria

Study sites were identified based on their historical outbreaks of arboviral infections. In addition, the favorable climate and ecology for breeding of a variety of *Aedes species* and pathogen development within the vectors make these areas good candidates for the study.

1.2 Study area

This study was conducted in 17 sentinel sites of the four Counties along the Kenyan Coast. These Counties are Lamu, Kilfi, Mombasa and Kwale. The sentinel sites within these counties are: in Lamu (Mpeketoni); in Kilifi (Malindi, Watamu, Kilifi, Rabai, Mazeras); in Mombasa (Portreiz, Tudor, Tononoka, Nyali, Shimo-la-tewa) and in Kwale (Kwale, Tiwi, Diani, Msambweni, Lunga lunga and Vanga). In Lamu sentinel site, the collection was done in marine and periurban ecologies. In Kilifi, sampling was done in urban, marine and rural ecologies. In Mombasa, the sampling was done in urban and marine ecologies.

1.3 Study design

This was a cross sectional study which involved field and laboratory experiments as shown in Figure 1. Field work involved collection of adult *Aedes* mosquitoes in their natural habitat along the Kenyan Coast. Laboratory experiments included extraction of RNA from the *Aedes* mosquitoes and one step qRT-PCR.

1.4 Sampling technique

Adult Aedes mosquito samples were collected from the field in their natural habitat using baited Bio gent sentinel traps (BG trap) hanged at least two meters from the ground. Solid carbon dioxide (dry ice) was used as a bait as shown in Figure 2. All the caught mosquitoes were transported live in net cages to the field sorting insectary.

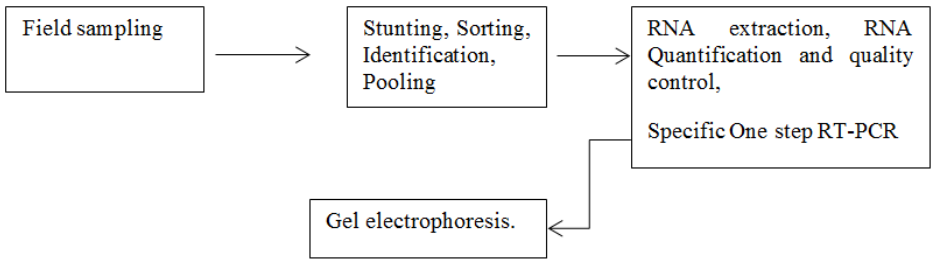

Figure 1 Flow chart of study design

Figure 2 A Bio gent sentinel trap in the field

1.5 Identification of *Aedes species*

A total of 37,220 adult *Aedes* mosquitoes were sampled along the Kenyan Coast: In Lamu County, n=7,534; in Kilifi County, n=11,646; in Mombasa County, n=7,119 and in Kwale County, n=10,921. For sorting and identification of the live mosquito samples, stunning was done at 4 ℃ for 5 minutes. Sorting was done according to sampling site, sex and *Aedes species*. Morphological identification was done using keys as described by Gillies and De Meillon (1968) and in the manual "Mosquitoes of the Ethiopian Region" (Edward, 1941; Habarch, 1988; Reinert, 2000). In addition, males and females *Aedes species* mosquito were classified as either *Aaa* or *Aaf* subspecies using scale pattern system as described earlier by McClelland GAH (1960). Briefly, mosquitoes with any white scales on the first abdominal tergite of the adult were designated *Aaa* subspecies. If the first abdominal tergite was completely lacking in white scales then the individual was designated *Aaf* subspecies. These samples were then pooled in pools of 20 (1861 pools) according to their sampling site, sex and *Aedes sub-species*. The samples were preserved in 1.5 ml cryogenic vials at -80 ℃ until molecular processing. RNA was homogenized as described by (Mackey and Chomczynski, 1995). Briefly, this was done by a mortar and pestle (cooled to temp in a liquid nitrogen bath). 1 ml of Trizol was added into the pestle with the mosquito pools and ground thoroughly. Vortex was done for one minute. An aliquot of the solution was transferred to eppendorf tubes and left in Trizol at room temperature for five minutes. 0.2 ml of chloroform was added, samples were capped and vortex for 15 seconds. Incubation of the samples was done at room temperature for 2-3 minutes. Centrifugation was done at 12,000 rpm for 15 minutes at 8 ℃. Centrifugation resulted in three phases visible within the tube. The aqueous phase at the top was transferred into a fresh tube. 0.5 ml of isopropanol was added to the new tube and incubated at room temperature for 10 minutes. This was centrifuged at 12,000 rpm for 10 minutes at 8 ℃. Following

centrifugation, the supernatant was removed and RNA pellet washed with 1 ml of 75% ethanol (vortexing). Centrifugation was done at 7,500 rpm for 5 minutes at 8 ℃. Supernatant was removed and remaining ethanol was air dried for 2-3 minutes. The pellet was eluted in elution buffer with sodium azide and vortexed for a minute. Tubes were transferred to a digital dry bath at 60 ℃ for 15 minutes before placing them on ice. Determination of the quantity and quality of extracted RNA was done using Nanodrop spectrophotometer. 1.5 µl microcuvette with OD at 260 nm and 280 nm was used determine sample concentration and purity.

1.6 Identification of Serotypes of Dengue virus

2.5 µl of the RNA samples were used in One Step real time PCR (qRT-PCR) with master mix made as per manufacturer's instructions (Applied bio systems, USA). DENV specific RNA was amplified in a total of 25 µl reaction mixture (12.5 µl of Amplitaq Gold 360 PCR master mix-Applied bio systems, USA, 50 picomoles each of forward and reverse primer, 2 µl of the cDNA and 9.5 µl of DEPC treated water to top up to 25 µl). The list of primers used (Table 1) were adapted from (Konongoi et al., 2016); (Normile, 2013) and (Ochieng et al., 2013).

Table 1 Primer sequences for serotypes of Dengue virus

Primer	Gene/ protein target	Primer sequence	Position
FU1	NS5	5′- TAC AAC ATG ATG GGA AAG AGA GAG AA-3′	9007-9032
CFD3	NS5	5′- GTG TCC CAG CCG GCG GTG TCA TCA GC-3′	9308-9283
D1	E/NS1	5′-TCAATATGCTGAAACGCGCGAGAAACCG-3′	38-65
D2	E/ NS1	5′-TTGCACCAACAGTCAATGTCTTCAGGTTC-3′	455-483
TS1		5′-CGTCTCAGTGATCCGGGGG-3′ (D1 and TS1)	
TS2		5′-CGCCACAAGGGCCATGAACAG-3′ (D1 and TS2)	
TS3		5′-TAACATCATCATGAGACAGAGC-3′ (D1 and TS3)	
TS4		5′-CTCTGTTGTCTTAAACAAGAGA-3′ (D1 and TS4)	
D5-F	NS5	5'-TCAATATGCTGAAACGCGHGAG-3'	132-153
D5-R	NS5	5'-GCGCCTTCNGNNGACATCCA-3'	764-783

The PCR plates were placed in the real time thermo cycler and were cycled at 50 ℃ for 20 minutes, 95 ℃ for 5 minutes, followed by 40 cycles of 95 ℃ for 15 seconds, 60 ℃ for 30 seconds, and 70 ℃ for 1 minute, and a final extension for 10 minute at 72 ℃. Samples were first tested using flavivirus family primers. Samples testing positive with flavivirus family primers were further tested with consensus Dengue primers D1 and D2. Samples testing positive with the Dengue consensus primers that target the E/NS1 junction of the virus genome were further tested for the 4 Dengue serotypes using the appropriate primers (Table 1). The primer sequences above were used to detect exposure to the virus using amplification conditions as described in (Kuno et al., 1998) and (Lanciotti et al., 1992). A positive control cDNA and a negative control were included during the setting up of all PCR reactions. Electrophoresis of the amplified DNA products was done on a 2% agarose gel in 1% Tris-borate EDTA buffer stained with ethidium bromide. The PCR product bands were visualized by a UV trans-illuminator and recorded using a gel photo imaging system.

1.7 Identification of Genotypes of Chikungunya virus

20.2 µl volume containing 1 µl of cDNA, 4 µl of 5X reaction buffer, 1.8 µL of 25 mM MgCl2, 1.8 µl of 10 µM dNTP, 0.5µl of 20µM of each primers, 0.3 µl of Go-Tag Flex DNA polymerase, and 10.3 µl of nuclease free water (Norgen Biotek, Canada) were used in One Step real time PCR (Applied bio systems, USA). The PCR program consisted of an initial denaturation at 95 ℃ for 2 min and 40 cycles of 30 seconds denaturation at 95 ℃, 30 seconds annealing at 55 ℃, 1 minute elongation at 72 ℃ and final extension at 72 ℃ for 5 minutes. Presence of flaviviruses was screened in all the amplified products using the pan-flavivirus primer (Ochieng et al., 2013) in Table 2. Samples testing positive with flavivirus family primers were further tested with the conventional primers for Chikungunya virus (Redd et al., 2012). A positive control cDNA and a negative control were included during the setting up of all PCR reactions. The conventional primer amplifies gene product in the Chikungunya envelope region of E1. The amplified gene products were identified by their molecular weights analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light trans-illuminator and recorded using a gel photo imaging system.

Table 2 Primer sequences for Chikungunya virus

Primer	Gene/ protein target	Primer sequence	Position
VIR 2052 F	NSP4	5′-TGG CGC TAT GAT GAA ATC TGG AAT GTT-3′	6971-6997
VIR 2052R	NSP4	5′-TAC GAT GTT GTC GTC GCC GAT GAA-3′	7086-7109
CHIKV-F	E1	5’-CGTGGTGTACAAAGGTGACG-3’	10524
CHIKV-R	E1	5’-ACG CCG GGTAGTTGACTATG-3’	11170

2 Results

2.1 *Aedes species* along the Kenyan Coast

A total of 37, 220 mosquitoes of *Aedes species* were collected from 17 sentinel sites of four Counties along the coastline of Kenya in two rain seasons as shown in Table 3.

Table 3 Mosquito collections in Sentinel sites along the Kenyan Coast

County/ Site name	GPS coordinates		No. of mosquitoes collected		
	Latitude (S)	Longitude (E)	Short rains	Long rains	Totals
Mpeketoni	02.2696 °S	40.9006 °E	2,860	4,674	7,534
Lamu			**2,860**	**4,674**	**7,534**
Malindi	03.3425 °S	40.0274 °E	2,858	2,820	5,678
Watamu	03.3425 °S	40.0274 °E	220	300	520
Kilifi	03.62940 °S	039.86605 °E	1,484	1,620	3,104
Rabai	03.92861 °S	039.57248 °E	657	1020	1677
Mazera	03.97152 °S	039.54900 °E	387	280	667
Kilifi			**5,606**	**6,040**	**11,646**
Shimo la tewa	03.95776 °S	039.74234 °E	900	1,610	2,510
Nyali	04.02749 °S	039.69432 °E	634	930	1,564
Tononoka	04.05182 °S	039.67141 °E	420	600	1,020
Tudor	04.03235 °S	039.66100 °E	187	260	447
Portreiz	04.03744 °S	039.60837 °E	638	940	1,578
Mombasa			**2,779**	**4,340**	**7,119**
Kwale	04.17489 °S	039.45586 °E	1,388	1,540	2,928
Tiwi	04.23488 °S	039.57470 °E	396	504	900
Diani	04.27956 °S	039.56773 °E	1,724	2,800	4,524
Msambweni	04.4653 °S	039.4813 °E	202	360	562
Lunga lunga	04.55573 °S	039.12374 °E	640	780	1,420
Vanga	04.66064 °S	039.2166 °E	187	400	587
Kwale			**4,537**	**6384**	**10,921**
Grand totals			**15,782**	**21,438**	**37,220**

The proportions of the *Aedes species* composition is as shown in Figure 3: *Aedes aegypti formosus* (23,265=62.5%), *Aedes aegypti aegypti* (4,931=13.2%), *Aedes mcintoshi* (3,557=9.56%), *Aedes ochraceus* (2,156=5.79%), *Aedes pembaensis* (2,049=5.51%), *Aedes tricholabis* (487=1.31%), *Aedes albicosta* (415=1.11%), *Aedes fulgens* (200=0.54%) and *Aedes fryeri* (160=0.43%). However, the proportions of these *Aedes species* along the Coastline did not differ significantly with the rain seasons (M=2,068, SD=3,520), t_8=0.03, p<0.001.

The sex of *Aedes species* collected during the two seasons was analyzed as shown in Figure 4. More males were collected compared to females for the following species: *Aedes aegypti formosus* (51%), *Aedes aegypti aegypti* (68%), *Aedes ochraceus* (51%) and *Aedes mcintoshi* (57%) were identified. However, the number of males and females were the same at 50% for *Aedes fulgens*. The number of males and female *Aedes species* did not differ significantly along the coastline with rain seasons, (M=2,068, SD=3,587), t_8=0.315, p<0.001.

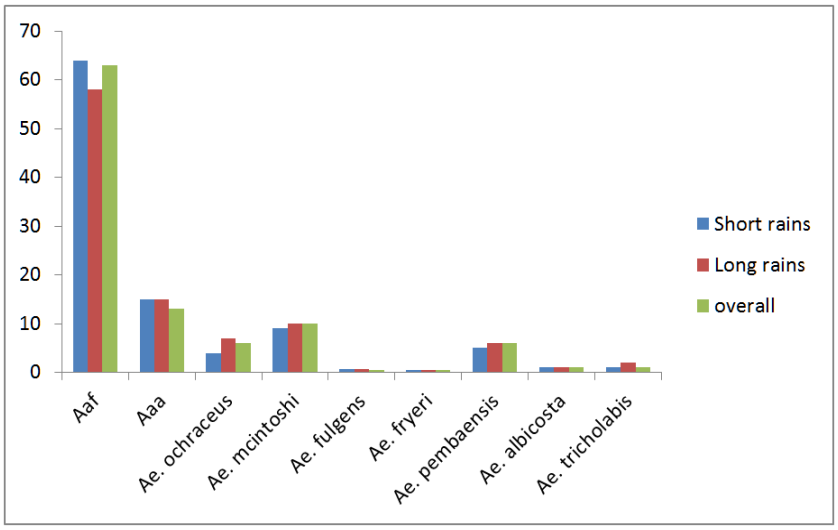

Figure 3 Composition and Proportions of *Aedes species* during short and long rain seasons

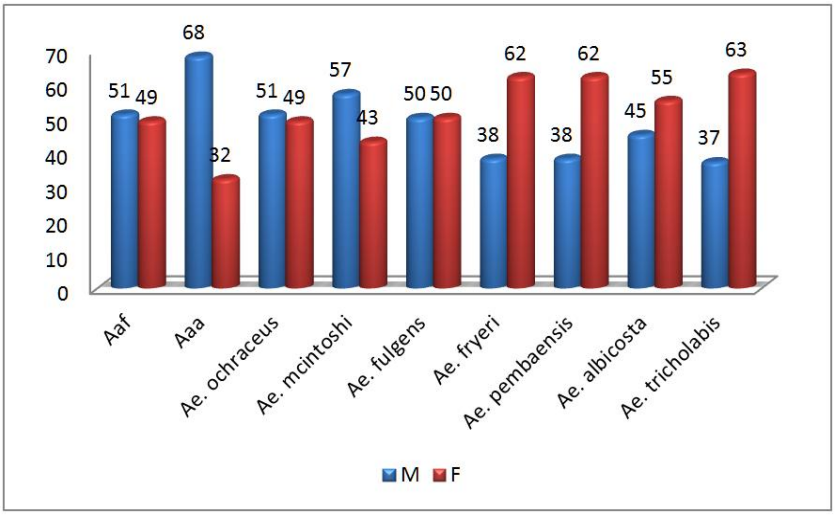

Figure 4 Comparison of sex of *Aedes species* found along the Kenyan Coast

2.2 Serotypes of Dengue virus

An assay for serotypes of Dengue virus was done in 1861 pools (n=37,220) of *Aedes species*. A total of 76/96 pools were positive for Dengue virus. The proportions of respective serotypes of dengue virus are shown in Figure 5. DENV-2 had the highest frequency among serotypes of Dengue virus at 54% (n=1,521) with DENV-4 with the least frequency at 6% (n=1,521). The infections of serotypes in *Aedes species* mosquito did not differ significantly with seasons (M=190, SD=159), t_3=0.1406, p<0.001.

2.3 Genotype of Chikungunya virus

Molecular assay for the genotypes of Chikungunya virus was done in 1861 pools (n=37,220) of *Aedes species*. A total of 20/96 pools were positive for East Central and Southern Africa (ECSA) genotype of Chikungunya virus. The abundance of the virus in the mosquito vectors was not significantly different in the short and rain seasons (M=7.25, SD=15), t_3=0.325, p<0.001.

2.4 Haplotypes of viruses in different clades of *Aedes species*

The frequency of entomological arboviral infection was highest with DENV-2 at 41%, followed by DENV-1 at 29%, CHIKV at 23%, and DENV-4 at 4% and DENV-3 at 3% as shown in Figure 6.

Aedes aegypti aegypti had the highest infection for arboviruses at 42%, followed by *Aedes aegypti formosus* at 35%, *Aedes macnitosh* at 11%, *Aedes pembaensis* at 6%, *Aedes ocharaceus* at 3%, *Aedes albicosta* at 1%, *Aedes fryeri* at 1%, *Aedes tricholabis* and *Aedes fulgens* at 0.5% each as shown in Figure 7.

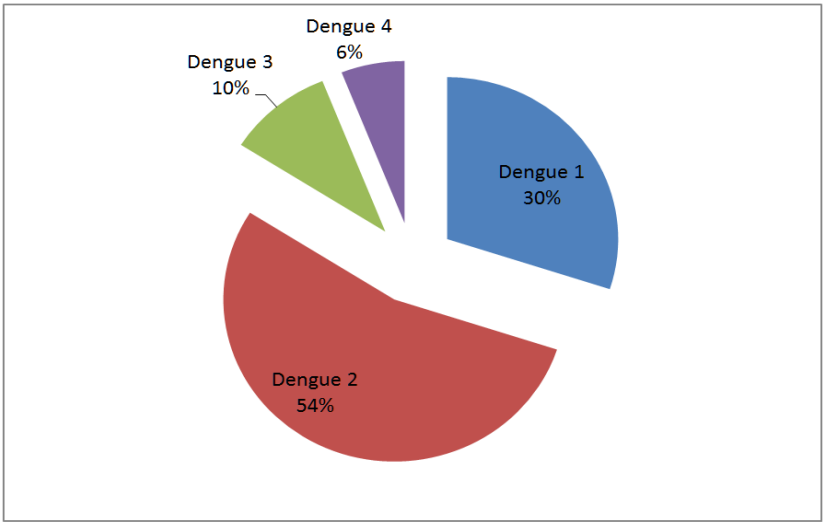

Figure 5 Serotypes of Dengue virus along the Kenyan Coast

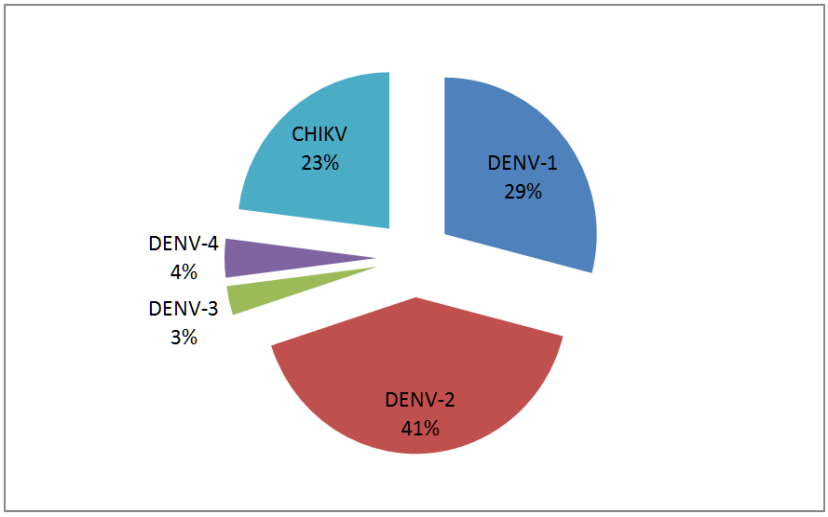

Figure 6 Proportions of Dengue and Chikungunya arboviruses in Coastal Kenya

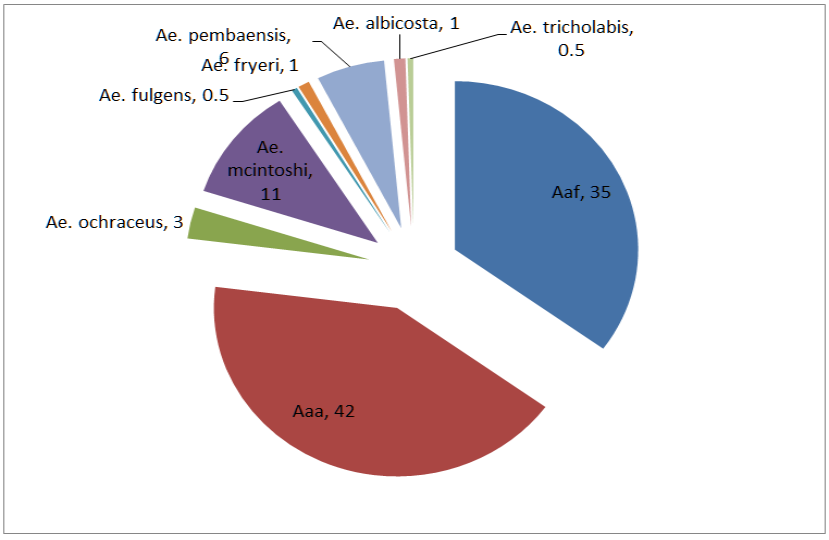

Figure 7 Proportions of *Aedes species* infected by arboviruses

Dengue and Chikungunya arboviruses were positive in males and female Aedes mosquitoes. Proportions of the arboviral infections in males and females are shown in Figure 8. More female mosquitoes were infected with viruses.

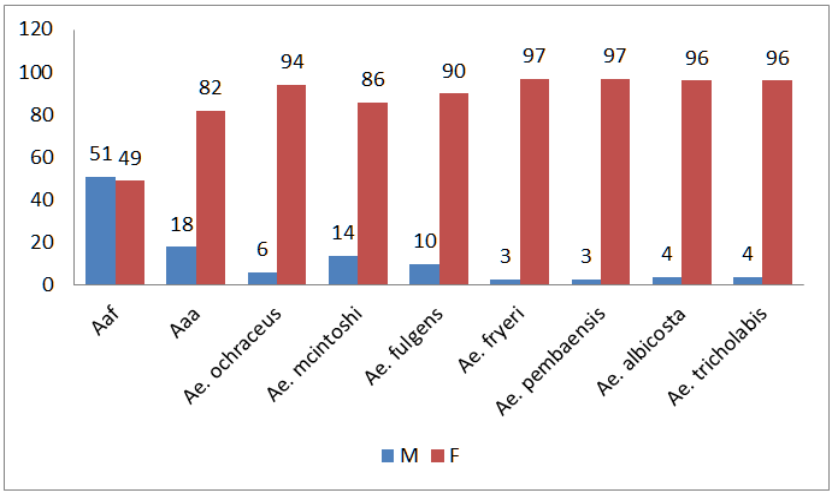

Figure 8 Arboviruses in sex of *Aedes species*

Aedes species mosquito infections by serotypes of Dengue virus and genotype of Chikungunya virus are shown in Table 4 and Figure 9. All *Aedes species* were highly infected with DENV-2 serotype. *Aedes fulgens* were the most infected with DENV-2 at 78% (n=200). DENV-1 was positively identified in all *Aedes species* except in *Aedes fryeri*. *Aedes ochraceus* were highly infected with CHIKV at 66% (n=2156). Almost all *Aedes species* except in *Aedes fulgens* and *Aedes tricholabis* were infected with CHIKV. Concomitant infections were observed in all *Aedes species* with *Aedes aegypti aegypti* and *Aedes aegypti formosus* infected by all serotypes of Dengue and Chikungunya viruses. Members of *Aedes macnitosh* were not infected by DENV-4 despite presence of this virus in the *Aedes aegypti* s.l populations within the same ecological zones in Lamu, Malindi and Kilifi. Similarly, *Aedes tricholabis* were negative for CHIKV despite this virus being in *Aedes pembaensis* within the same locality of Watamu, Tudor and Vanga. *Aedes albicosta* for DENV-3 and DENV-4 despite presence of this viruses in *Aedes aegypti* s.l populations within the same ecological zones in Malindi and Shimo la tewa. *Aedes fryeri* were not infected by DENV-1, DENV-3 and DENV-4 despite presence of the arboviruses in Portreiz. *Aedes fulgens* were not infected by DENV-3 and DENV-4 despite their presence in Nyali. *Aedes ochraceus* were not infected by DENV-4 despite presence of the arbovirus in Portreiz.

Table 4 Haplotypes of viruses in different clades of *Aedes species*

Serotypes	*Aaf*	*Aaa*	*Ae. ochraceus*	*Ae. mcintoshi*	*Ae. fulgens*	*Ae. fryeri*	*Ae. pembaensis*	*Ae. albicosta*	*Ae. tricholabis*
DENV1	28,n=148	32,n=195	30,n=6	29,n=45	22,n=2	0	47,n=42	48,n=10	72,n=5
DENV2	55,n=294	50,n=309	60,n=12	65,n=100	78,n=7	100,n=7	26,n=23	52,n=11	14,n=1
DENV3	12,n=65	13,n=78	10,n=2	6,n=10	0	0	1,n=1	0	0
DENV4	5,n=29	5,n=34	0	0	0	0	26,n=23	0	14,n=1

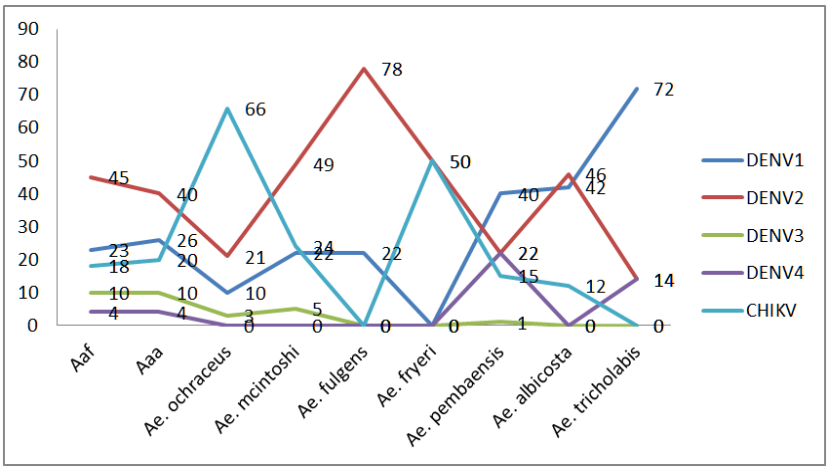

Figure 9 Haplotypes of viruses in different clades of *Aedes species*

3 Discussion

There are four serotypes of Dengue virus circulating in *Aedes species* along the coastline of Kenya. DENV-5 was not detected as it was the case by Normile (2013) in Malasyia, although they used serum for assays. This report confirms findings by Sutherland et al., (2011) but contrast reports by Konongoi et al. (2016) in which three serotypes of Dengue virus were reported (DENV-2, DENV-2 and DENV-3). This study reports higher rate of DENV-2 serotype infection in *Aedes species* along the entire coastline. This is similar to reports from serological studies by Sutherland et al. (2011) and it implies the higher human cases of DEN-2 virus are due to the highe prevalence of the viral serotype in the mosquito vectors. However, this was not the case in assays by Konongoi et al. (2016) in which DENV-1 was reported to have higher rates of infection. This indicates dynamism in population of circulating serotypes. Phylogenetic and epidemiological studies by Bravo et al. (2014) have shown Dengue serotypes with more virulence can drive out virus strains of lesser epidemiological impact. Therefore continuous survey of the pattern of these viruses is essential to asses if some of the viruses will be driven out of some areas. DENV-4 is the least circulating serotype of Dengue virus in *Aedes species* mosquitoes along the Kenya's coastline. This is in support of statistics on Dengue virus by Sutherland et al. (2011). The rates of arboviral infection in the *Aedes species* did not vary significantly along the coastline. This might mean all serotypes of Dengue virus are in circulation (Ochieng et al., 2013) and human infection can occur in similar proportions.

The genotype of Chikungunya virus circulating in *Aedes species* mosquitoes along the Kenya's coastline is the East Central and Southern Africa (ECSA) genotype. This finding is in line with reports from recent studies by Sergon et al. (2008) and by Kariuki Njenga et al. (2008). This genotype is present in *Aedes species* along the four counties under this study. The genotype is highly concentrated in the southern parts of the coastline. The population and activity of the virus increased during long rain season, although the increase was not significant. This is supported by the findings by Harrington et al. (2014) in which there was no difference in number of mosquito biting frequencies during low and high viral transmission seasons.

Aedes aegypti s.l and *Aedes pembaensis* were infected by all serotypes of Dengue and Chikungunya viruses. This confirms numerous reports that *Aedes aegypti* s.l forms the primary vector for arboviruses and can be infected by Dengue and Chikungunya viruses (Rückert et al., 2017). *Aaa* subspecies had the highest arboviral infection among all *Aedes species* and sub-species screened for the viruses. This finding supports report that members of the *Aaa* subspecies have higher vector competence compared to *Aaf* subspecies (Tabachnick et al., 1985); (Mattingly, 1967).

In this study, infection of male mosquitoes by arboviruses was found in all *Aedes species*. Similar assays done earlier by Lutomiah et al. (2016) reported infection of *Aedes aegypti* males by Dengue virus. In their study, they did not isolate other arboviruses in males and only *Aedes aegypti* were screened. Infection in males clearly demonstrates ability of male mosquitoes to play a role in transmission of arboviruses in the environment. Males acquire the virus from their parents through transovarial mode of transmission and subsequently transmit it to the females during mating as reported by da Costa et al. (2017). Males pass the virus through sperms as they mate with females few days following emergence from their pupae stage. Therefore, control measures for arboviral diseases should target both males and females.

Selective infection of *Aedes species* by arboviruses in the same ecological zone may suggest preferential association among viruses, vectors and hosts. Absence of DENV-4 in *Aedes macnitosh* in Lamu, Malindi and Kilifi may suggest this vector is not suitable for transmission of DENV-4. Similar scenarios were found in *Aedes tricholabis* for CHIKV despite the virus being detected in the *Aedes pembaensis* in the same areas of Watamu, Tudor and Vanga; *Aedes albicosta* for DENV-3 and DENV-4 despite *Aedes aegypti* s.l populations testing positive for the virus within Malindi and Shimo la tewa; *Aedes fryeri* for DENV-1, DENV-3 and DENV-4 in Portreiz; *Aedes fulgens* for DENV-3 and DENV-4 in Nyali and *Aedes ochraceus* for DENV-4 in Portreiz. This calls for more studies to establish the vector-host-pathogen relationships for effective control strategies.

Isolation of multiple arboviruses in the same pool of *Aedes species* indicates possibility of concomitant infections in the vectors. This is because mosquitoes can feed multiple times on more than one human host and require multiple bites per each gonotrophic cycle (Harrington et al., 2014). Concomitant infections were reported in studies by Göertz et al. (2017), Rückert et al. (2017), Le Coupanec et al. (2017) and Vazeille et al. (2010). However, their reports are conflicting on the aspect of vector competence. Göertz et al. (2017) reported co-infection of Zika and Chikungunya virus does not influence vector competence in *Aedes aegypti*. In contrast, report by Rückert et al. (2017) says infection, dissemination and transmission of viruses by *Aedes aegypti* is mildly affected by co-infections of Zika, Chikungunya and Dengue viruses. Report by Göertz et al. (2017) shows mixed infections of Chikungunya and Dengue virus facilitated viral replication in *Aedes aegypti*. This calls for more studies to establish the fact on effect of arbovirus co-infection on vector competence. Future studies should assay individual mosquitoes from respective *Aedes species* for multiple viral infections.

4 Conclusions

This study demonstrated the occurrence of different *Aedes species* naturally infected by arboviruses along the coastline of Kenya. Concomitant infections occur in *Aedes species* along the coastal region. *Aedes aegypti aegypti* is incubates all serotypes of Dengue and genotype of Chikungunya viruses thus the major vectors of arboviruses along the coastline. Therefore, entomological viral monitoring can contribute to the control measures and identification of new vector control methods.

Authors' contributions

N.C.J - the main reseacher of the whole study. M.R - contributed to the molecular analysis of the arboviruses. All authors read and approved the final manuscript.

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