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Entomologic Investigations of a Chikungunya Virus Epidemic in the Union of the Comoros, 2005

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Abstract. From January to April 2005, an epidemic of chikungunya virus (CHIKV) illness occurred in the Union of Comoros. Entomological studies were undertaken during the peak of the outbreak, from March 11 to March 31, aimed at identifying the primary vector(s) involved in transmission so that appropriate public health measures could be implemented. Adult mosquitoes were collected by backpack aspiration and human landing collection in homes and neighborhoods of clinically ill patients. Water-holding containers were inspected for presence of mosquito larvae. Adult mosquitoes were analyzed by RT-PCR and cultivation in cells for the presence of CHIK virus and/or nucleic acid. A total of 2,326 mosquitoes were collected and processed in 199 pools. The collection consisted of 62.8% *Aedes aegypti,* 25.5% *Culex* species, and 10.7% *Aedes simpsoni* complex, *Eretmapodite*s spp and *Anopheles* spp. Seven mosquito pools were found to be positive for CHIKV RNA and 1 isolate was obtained. The single CHIKV mosquito isolate was from a pool of *Aedes aegypti* and the minimum infection rate (MIR) for this species was 4.0, suggesting that *Ae. aegypti* was the principal vector responsible for the outbreak. This was supported by high container (31.1%), household (68%), and Breteau (126) indices, with discarded tires (58.8%) and small cooking and water storage vessels (31.1%) registering the highest container indices.

INTRODUCTION

Chikungunya virus (CHIKV) is a member of the genus *Alphavirus* in the family *Togaviridae* and is most closely related to o'nyong nyong virus (ONNV) of the Semliki forest antigenic complex. CHIKV was first isolated by Ross in 1953 during the epidemic of a dengue-like illness that occurred in Tanzania.[1] The name 'chikungunya,' Makonde meaning "that which bends up" was given to the virus to describe the symptoms of the illness, which causes severe and persistent pain in the joints. Since that initial outbreak, CHIKV has continued to cause periodic and widespread epidemics in Africa and Southeast Asia.[2–8] Within the past 2 years, CHIKV outbreaks in East Africa and the Indian Ocean have become more frequent and show a distinct line of movement; they have affected the Kenyan islands of Lamu (April to August 2004)[9] and Mombasa (November to December 2004), and subsequently appeared in the Indian Ocean island of Comoros and Mauritius (January to May 2005).[10]

In January 2005, public health officials noted increased reports of dengue-like febrile illness with severe debilitating joint pain in Grande Comore (Ngazidja) of the Union of the Comoros. Initially, screening for dengue virus infection was performed on a subset of the samples but the percentage of dengue-confirmed patients was too low to account for the increase in febrile illness. In February 2005, the World Health Organization (WHO) African Regional Office (AFRO) was informed of the outbreak and 25 human serum samples were delivered to the Kenya Medical Research Institute (KEMRI) in Nairobi where preliminary results indicated that an alphavirus was responsible for the illnesses. IgM antibody testing was performed on the samples against both CHIKV and ONNV. CHIKV-specific IgM antibodies were detected in 9 samples and antibodies against ONNV were present in 1 specimen. Additionally, sequencing analysis on RT-PCR amplicon products indicated that the infecting virus was indeed CHIKV.

In early March 2005, a team including members from CDC, KEMRI, WHO, and public health officials from Comoros initiated an investigation of the outbreak in which over 1,100 cases had already been reported.[10] This report describes the results of field work focusing on entomological investigations conducted from March 11 through March 31; during this period, approximately 2,500 additional cases were documented. Fortuitously, this time frame corresponded to the peak of the outbreak as determined by the number of clinical cases reported suggesting that timing was optimal for carrying out our objectives of identifying the primary vector(s) involved in transmission of the virus and noting entomological parameters that contributed to the epidemic.

MATERIALS AND METHODS

Description of the study sites. The Comoros Islands are an archipelago of 4 tropical volcanic islands in the Mozambique Channel of the Indian Ocean with a population of over 650,000. They are composed of Grande Comore, Anjouan, Mohéli, and Mayotte (French) and together form the Union of the Comoros. All the entomologic investigations were carried out on Grande Comore, the island where virtually all cases were identified. The main city on Grande Comore, Moroni was the most affected locale; therefore, the majority of the entomologic activities were focused here although sampling was carried out in 2 other communities as well.

Grande Comore is covered with lava exhibiting a porous surface that is unable to maintain ground water. The island has no rivers or wells that can provide reliable water sources. Additionally, there are no piped water or sanitation systems in place; therefore, the inhabitants of this island harvest rain-

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water that is stored in large concrete cisterns. Most of the cisterns are either only partially covered or are completely uncovered. Predatory fish to control mosquito larvae were found in only some cisterns. Apart from large cisterns for more extended storage, water is also commonly stored in smaller containers including plastic and metal reservoirs at individual households for cooking and drinking purposes. Additionally, similar small containers that serve as ornamental plant holders and decorative vessels are a common feature around homes and business premises in the city. In contrast, the other 3 islands of the Comoros have rivers and piped water systems resulting in a reduced need for cisterns and small water-storage vessels. However, the effect of these differences on virus transmission has not been confirmed.

Garbage collection facilities do not widely exist in the cities of the island. For this reason, discarded plastic and metal water-holding containers, used tires, aluminum soda cans, and empty food cans were found widespread throughout the city. This was particularly pronounced near the urban center and dock areas of Moroni.

Adult mosquito collection. An adult mosquito survey was carried out using two main methods. Host-seeking mosquitoes were collected using human landing collections while resting mosquitoes were collected using vacuum aspiration; both methods have been found to be highly productive for collecting adult *Ae. aegypti* mosquitoes.[11,12] In the landing collection method, mosquitoes were captured in glass vials when they arrived to feed on exposed skin of public health and/or mosquito control team members. All volunteers were only involved in collections if they had previously recovered from CHIKV-like symptoms and collections were performed with approval by WHO and Comorian Health Office personnel. All investigations reported here were approved by the Comoros Ministry of Health as part of the emergency public health response to the outbreak and determined by this body to not represent research requiring review by an ethical review group. Collections were performed during peak daytime biting hours from 12:00 to 18:00 each day for 3 weeks starting March 11, 2005 at the peak of the outbreak. A 3-week time period allows the investigation of nearly 2 life cycles of *Ae. aegypti*. This amount of time is more than sufficient to obtain information regarding density and infection rates particularly when the timing of the collections is found to correspond to the peak of the epidemiologic curve. Collections were done near the homes of clinically ill patients primarily within the transmission areas of Moroni where the majority of the clinical cases were identified; some collections were also made in Mitsamiouli and Foumbouni on Grande Comore. For the vacuum aspiration method, a battery-operated backpack aspirator was used to aspirate mosquitoes resting in bedrooms and living rooms of houses in the neighborhoods of affected individuals. Landing collections and aspiration collections were performed in the same localities to collect the complete range of mosquitoes present in outbreak areas. The use of alternate collection techniques was attempted but unsuccessful for several reasons. For example, using CDC light traps was frustrated by lack of access to dry ice as bait and the overwhelming abundance of containers serving as oviposition sites precluded the use of gravid traps.

All collected mosquitoes were sorted by sex, species, and collection method into cryovials at a temporary facility set up in Moroni. Samples were initially frozen at –20°C then later transferred to liquid nitrogen charged dry shipping tanks and transported to the CDC at Fort Collins, Colorado, where they were stored at –70°C until processing.

Larval surveys. All indoor and outdoor water-containing receptacles at randomly selected domestic and business centers within 5 distinct zones in the city of Moroni were inspected for mosquito larvae from March 27 through March 31. The 5 zones were selected to ensure no bias existed in the collections due to differences in habitat. The zones included the old/central part of Moroni with dense housing, coastal Moroni adjacent to the ocean and ports with little vegetation, "suburban" housing areas with more ornamental vegetation, northern Moroni where houses are more dispersed, and eastern Moroni where the city transitions to a mountain habitat. A total of 100 houses were sampled in the city with 20 sampled per zone. Live larvae observed in positive receptacles were sampled and examined for identification. Containers positive for *Aedes aegypti* larvae were recorded. The larval indices calculated from the collected data include container (percentage of water-holding containers examined that contained *Ae. aegypti* larvae), household (percentage of houses examined that have *Ae. aegypti* larvae in at least one container), and Breteau (total number of containers with *Ae. aegypti* larvae per 100 houses) indices.

Mosquito processing. Mosquitoes were retrieved from storage at –70° and held on a chill table while being sorted and identified to species using appropriate taxonomic keys and references.[13–17] Specimens were assigned to pool by species, sex, location, trap method, and date of collection. Each pool (not exceeding 40 mosquitoes) was homogenized in 2 mL DMEM diluent.[18] The homogenate was clarified by centrifugation and used for virus isolation and nucleic acid detection. Minimum infection rate (MIR), or the [number of positive pools / total specimens tested] × 1,000, was calculated for all species cohorts that were found to be positive for virus or viral nucleic acid.

Virus isolation. For virus isolation, 1 mL of undiluted homogenized mosquito suspension was inoculated onto 25-cm^2 flasks of Vero cells. Cells were incubated for 1 hour at 37°C to allow attachment of virus; then, 4 mL of DMEM diluent was added to inoculated cells. Cells were incubated at 37°C in a 5% carbon dioxide incubator and observed daily for 10 days. Supernatant was harvested when cytopathic effects (cpe) were observed. All harvested supernatants were titrated using a standard plaque assay to confirm the presence of virus and to determine titer.[19]

Virus detection by RT-PCR. Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect and identify CHIKV-specific nucleic acid. RNA was isolated from mosquito homogenates by using QIAamp viral RNA minikit (QIAGEN, Valencia, CA), following the manufacturer's protocol. RT-PCR assays were performed using Titan One tube RT-PCR kit (Roche Molecular Biochemicals, Indianapolis, IN). Each reaction contained 10 μL of RNA and 20 pmol of each CHIKV-specific primer, 7028 forward (5′TGCGCGGCCTTCATCGGCGACTAC 3′) and 8288 reverse (5′CCAGGTCACCACCGAGAGGG 3′), in a 50 μL total reaction. Amplified product was analyzed by gel electrophoresis and all positive samples were repeated for confirmation using the above primers or a CHIKV-specific real-time RT-PCR assay. CHIKV specificity was confirmed by sequencing the amplicons.

RESULTS

Adult mosquito collections. A total of 2,326 mosquitoes were collected, mainly by backpack vacuum aspirators (23% of the total mosquitoes collected) and landing collections (77% of all mosquitoes collected), in neighborhoods where clinically ill patients resided in the capital city of Moroni. The majority of mosquitoes collected were *Aedes aegypti* (62.8%), *Ae. simpsoni* complex (4.9%), and *Culex* species (26.5%). *Eretmapodites* (3.2%) and *Anopheles* (0.2%) species were also collected. In total, at least 9 distinct species were identified from these collections. The two prominent mosquito groups, *Ae. aegypti* and *Cx.* species, were collected predominantly by landing collections and backpack aspirator, respectively. Because of the collection technique, virtually all of the *Culex* spp. mosquitoes were missing too many identifying characters to allow identification to species. A complete list of the species identified along with the numbers collected is presented in Table 1.

Nucleic acid detection and virus isolation. From the 199 pools, CHIKV amplicons of the correct size were obtained from 7 pools and CHIKV nucleic acid was confirmed by sequence analysis. Four of these were from *Ae. aegypti* females, 2 from *Aedes* species females, and 1 from *Culex* species females. From these data, the minimum infection rate (MIR) for the *Ae. aegypti* overall was calculated to be 4.0 when including both landing collection and vacuum aspiration methods. This number is 5.7 if the two positive pools that were identified to *Aedes spp*. were also *Ae. aegypti*; this is a likely scenario given the percentage of *Ae. aegypti* identified relative to other aedine species collected. The 3 positives from pools identified only to genus were all collected by backpack aspirator and may have contained engorged specimens still replete with blood. Ideally, the mosquitoes containing blood would have been processed separately from the non-engorged mosquitoes or maintained as adults until the blood was digested. Unfortunately, limited facilities and supplies did not allow adult maintenance for this purpose and although these positive samples were a definite indication of active CHIKV transmission, the possible inaccuracies of MIR's in the *Culex* spp. pool must be noted.

Pools were further tested for the presence of CHIKV by attempting virus isolation in Vero cells. All cultures that showed any cytopathology were then harvested and re-examined for CHIKV in a plaque assay. One culture, from a pool of 4 *Ae. aegypti* females, contained viable virus and an isolate was obtained. This pool corresponded to one of the positive pools from the RT-PCR analysis. The inability to isolate virus from other pools that were RT-PCR positive may have been due to the different sensitivities of the assays or possibly the loss of virus viability due to suboptimal temperature maintenance conditions of the mosquitoes during collection and shipment. Sequence analysis (data not shown) confirmed CHIKV that was found to be most closely related to strains from the Central/East African genotype.[8,20]

Larval survey. Because a large proportion of the mosquitoes were suspected to be *Ae. aegypti* and it was unknown if *Ae. albopictus* were present, a larval index study was conducted to provide a measure of risk to the population due to exposure to mosquitoes in household areas in Moroni by randomly sampling in and around homes in 5 distinct zones exhibiting different ecological patterns. The 5 zones examined ranged from urban settings in central Moroni, neighborhoods adjacent to the coast and port areas, sparsely populated regions approaching the mountains, and rural or suburban habitats with more dense vegetation. Selected homes were examined for the presence of containers with standing water and mosquito larvae in those collection vessels. There were 9 general groups of water-containing vessels identified including natural sites (e.g., puddles, split coconuts) as well as artificial containers (e.g., cisterns, tires, cans, jars, cooking pots). All of these, with the exception of waste pits/septic tanks, had larvae that were collected from them in at least one home.

Several indices were calculated to estimate the *Ae. aegypti* population density including the house index, container index, and Breteau index.[12] The overall percentage of homes

TABLE 1
Mosquito species collected by vacuum aspiration (Asp) and human landing collection (HLC)

Species	Collection method	Male/Female	Number collected	% of total mosquitoes	No pools	CHIKV-positive pools	MIR*
Aedes aegypti	Asp	Male	60	2.6	8	0	—
Aedes aegypti	Asp	Female	65	2.8	11	2	30.8
Aedes aegypti	HLC	Male	392	16.9	34	0	—
Aedes aegypti	HLC	Female	944	40.6	81	2	2.1
Aedes simpsoni complex	HLC/Asp	Female	41	1.8	3	0	—
Aedes simpsoni	HLC	Female	65	2.8	6	0	—
Aedes bromeliae	HLC	Female	10	0.4	1	0	—
Aedes vittatus	Asp	Female	1	< 0.1	1	0	—
Aedes spp.	Asp	Female	42	1.8	4	2	47.6
Aedes spp.	Asp	Male	9	0.4	1	0	—
Anopheles gambiae complex	HLC/Asp	Female	5	0.2	5	0	—
Eretmapodites spp.	HLC	Female	33	1.4	4	0	—
Eretmapodites quinquevittatus	HLC	Female	26	1.1	4	0	—
Eretmapodites quinquevittatus	HLC/Asp	Male	7	0.3	2	0	—
Eretmapodites inornatus group	HLC	Female	6	0.3	2	0	—
Eretmapodites chrysogaster group	HLC	Female	3	0.1	2	0	—
Culex spp.	HLC	Female	74	3.2	5	0	—
Culex spp.	Asp	Female	354	15.2	16	1	2.8
Culex spp.	Asp/HLC	Male	189	8.1	9	0	—
TOTALS:	—	—	2326	—	199	7	—

* MIR = minimum infection rate. MIR for each species is calculated as the [number of positive pools/total specimens tested] × 1000.

examined that had *Ae. aegypti* larvae in containers (house index) was 68% with a container index of 31%. Additionally, the Breteau index was 126; a Breteau index above 50 historically has been used to indicate a high risk of urban yellow fever virus transmission while more recent estimates indicate YFV epidemic risk with Breteau indices of 5–50[12,21] and dengue virus outbreak risk has been noted with a Breteau index of ≥ 1.[22]

In addition to the overall high density figures, several types of containers were found to have extremely high infestation rates. The most significant of these were all artificial containers present at virtually every home examined. Discarded tires were the containers that had the highest prevalence of *Ae. aegypti* larvae with almost 60% of those containing water positive for larvae. Only slightly less important were temporary water storage jars with 52% of these positive (Table 2). Natural water collection receptacles (e.g., split coconuts) were found to have *Ae. aegypti* larvae far less frequently than the extremely abundant artificial containers. A complete listing of containers and infestation rates is presented in Table 2.

DISCUSSION

Chikungunya virus has been identified in almost all of Africa and in numerous Southeast Asian countries. The transmission of this virus has been reported to occur by a variety of species with most of these belonging to the genus *Aedes*.[23,24] Transmission cycles of the virus are distinctly different in rural and urban settings that reflect the diverse mosquito fauna present in each ecological setting. In Africa, CHIKV appears to be largely maintained in sylvatic cycles involving wild primates and forest dwelling *Aedes* species mosquitoes. A number of sylvatic vector species have been implicated including *Ae. africanus* in East Africa,[3] *Ae. furcifer*, *Ae. taylori*, *Ae. dalzieli,* and *Ae. luteocephalus* in West Africa,[2,24] and *Ae. cordellieri* in South Africa.[25] In contrast, transmission of CHIKV in Asia has been documented to occur mainly in urban areas where *Ae. aegypti* and *Ae. albopictus* are the identified vectors.[5,26,27]

By performing entomological collections throughout this outbreak, we had an opportunity to evaluate which mosquitoes may be vectors of recent epidemic CHIKV in East Africa. During the first documented CHIKV outbreak in 1952–1953, *Ae. aegypti* was considered the principal vector,[28] particularly in urban settings. In Comoros, the outbreak occurred largely in urban areas suggesting the possible involvement of *Ae. aegypti*. Our investigations therefore focused upon optimizing collections of these and other domestic or peridomestic mosquitoes. Both immature and adult collections contained significant numbers of *Ae. aegypti* with high larval indices and a high percentage of total adults collected; abundance of a particular species is certainly one criteria for determining vector status during an outbreak. Recent literature has questioned the predictive value of these larval indices for risk of *Ae. aegypti*-borne infections[22,29] and numerous attempts to identify or develop more appropriate pupal and other indices has been undertaken.[30–33] However, to date, there is no solid consensus as to which immature indices best provide this risk estimate and the container and Breteau indices in particular are still frequently used as population den-

TABLE 2
Mosquito larval population density indices and their main breeding sites

Sector/Zone	I		II		III		IV		V		Overall	
Breeding sites	No. with water	No. with *Ae. aegypti* larvae	No. with water	No. with *Ae. aegypti* larvae	No. with water	No. with *Ae. aegypti* larvae	No. with water	No. with *Ae. aegypti* larvae	No. with water	No. with *Ae. aegypti* larvae	No. with water	No. with *Ae. aegypti* larvae
Cisterns, tanks	21	8	20	6	16	3	8	2	16	4	**81**	**23**
Jars, bowls, basins, drums	21	10	6	5	25	9	16	12	11	5	**79**	**41**
Waste pits, septic tanks	21	0	7	0	5	0	4	0	6	0	**43**	**0**
Buckets, saucepans, cooking pots	30	7	25	8	23	7	17	4	16	6	**111**	**32**
Tires	7	3	0	na	3	2	4	2	3	3	**17**	**10**
Cans, tins	7	1	13	6	8	3	9	2	7	3	**44**	**15**
Bottles	11	0	2	0	6	1	2	0	3	0	**24**	**1**
Puddles, potholes	0	na	0	na	1	1	1	0	1	0	**3**	**1**
Coconut,* vehicle hood,** discarded car battery***	0	na	1*	1	0	na	1**	1	1***	1	**3**	**3**
Total	**118**	**29**	**74**	**26**	**87**	**26**	**62**	**23**	**64**	**22**	**405**	**126**
Households (HH) with breeding sites	20	17	20	19	20	20	20	19	20	19	**100**	**94**
HH with positive breeding sites		14		12		17		13		12		**68**
Container Index (RI)		**24.6**		**35.1**		**29.9**		**37.1**		**34.4**		**31.1**
Household Index (HI)		**70**		**60**		**85**		**65**		**60**		**68**
Breteau Index (BI)		**145**		**130**		**130**		**115**		**110**		**126**

Container Index: Percentage of water holding containers that contain larvae; *Household Index*: Percentage of houses that have larvae in water holding containers; *Breteau Index*: number of containers with larvae per 100 households; na = not applicable.

sity indicators. Furthermore, because *Ae. aegypti* are daybiters, anthropophilic, and display interrupted feeding patterns, even a moderate mosquito population (assuming there are infected mosquitoes present) could contribute significantly to the spread of disease. Finally, the fact that infectious virus and nucleic acid were obtained virtually exclusively from *Ae. aegypti* further supports the hypothesis that this species was the primary vector during the outbreak in 2005.

Because limited options for mosquito collections precluded the examination of mosquitoes not closely associated with human habitation, the involvement of other species in the transmission of CHIKV is still a possibility. Previous reports have implicated members of other genera including *Anopheles rufipes*, *An. coustani,* and *Culex ethiopicus* in West Africa[2] as well as *Coquillettidia fuscopennata*[3] and *Mansonia africana*.[34] Additionally, although virus has not been isolated from wild collections, *Eretmapodites chrysogaster* has been shown to have a higher vector potential than *Ae. aegypti* through experimental infection studies in the laboratory.[35,36] Our collections did contain some *Eretmapodites* spp. and *Ae. simpsoni* complex mosquitoes but unfortunately, the number of specimens was too small to provide meaningful information on infection potential. Further, our collections did not extensively consider species that preferentially feed on nonhuman vertebrates. Previous seroprevalence studies have frequently detected CHIKV-specific antibodies in monkeys and on occasion in rodents and birds suggesting that enzootic transmission cycles may also be present.[2] This possibility could have implications for long-term or repeated outbreaks of CHIKV in the human populations. However, Grande Comore is not known to have any monkeys on the island so further studies to characterize any enzootic maintenance of the virus are warranted.

One finding of these entomological investigations was the identification of prime *Ae. aegypti* breeding sites. Considering the extent to which large water cisterns were used to provide water for the residents of Moroni, it was expected that these were the key premises for *Aedes aegypti* larvae. Because collections from the cisterns were not practical, the role of these storage vessels in mosquito production is still unknown. However, the smaller, common household containers and discarded tires yielded significant numbers of larvae. Although further characterization of the large vessels is still warranted, the information concerning abundance of larvae in "disposable" breeding sites provided some concrete information on sources of vectors that allowed the development of both long-term and short-term control strategies for public health officials in Comoros.

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