Enzootic Circulation of Chikungunya Virus in East Africa: Serological Evidence in Non-human Kenyan Primates

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Abstract. Chikungunya virus (CHIKV) is a globally emerging pathogen causing debilitating arthralgia and fever in humans. First identified in Tanzania (1953), this mosquito-borne alphavirus received little further attention until a 2004 re-emergence in Kenya from an unknown source. This outbreak subsequently spread to the Indian Ocean, with adaptation for transmission by a new urban vector. Under the hypothesis that sylvatic progenitor cycles of CHIKV exist in Kenya (as reported in West Africa, between non-human primates (NHPs) and arboreal *Aedes* spp. mosquitoes), we pursued evidence of enzootic transmission and human spillover events. We initially screened 252 archived NHP sera from Kenya using plaque reduction neutralization tests. Given an overall CHIKV seroprevalence of 13.1% (marginally higher in western Kenya), we sought more recent NHP samples during 2014 from sites in Kakamega County, sampling wild blue monkeys, olive baboons, and red-tailed monkeys ($N = 33$). We also sampled 34 yellow baboons near Kwale, coastal Kenya. Overall, CHIKV seropositivity in 2014 was 13.4% (9/67). Antibodies reactive against closely related o'nyong-nyong virus (ONNV) occurred; however, neutralization titers were too low to conclude ONNV exposure. Seroprevalence for the flavivirus dengue was also detected (28%), mostly near Kwale, suggesting possible spillback from humans to baboons. CHIKV antibodies in some juvenile and subadult NHPs suggested recent circulation. We conclude that CHIKV is circulating in western Kenya, despite the 2004 human outbreaks only being reported coastally. Further work to understand the enzootic ecology of CHIKV in east Africa is needed to identify sites of human spillover contact where urban transmission may be initiated.

INTRODUCTION

CHIKV, a mosquito-borne alphavirus (family *Togaviridae*) that can cause debilitating arthralgia in humans, has re-emerged and rapidly spread across many parts of the world in recent years.^{1,2} This begs an understanding of the source of these urban emergence events, and the ecological drivers that lead to emergence. In 2004, CHIKV re-emerged in Kenya, after years without reported activity in east Africa, with cases detected around the coastal regions of Lamu and Mombasa; 75% of the human population was affected in Lamu.³ This re-emergence sparked a chain of outbreaks across islands in the Indian Ocean, notably Comoros and La Reunion, and the Indian subcontinent, with millions of infections.^{4,5} Viral genetic analysis linked the strains of CHIKV in both the Indian Ocean basin and India (later termed the Indian Ocean Lineage) based on independent emergences from Kenya.⁶ Adaptation for transmission by the mosquito *Aedes albopictus*, via a series of adaptive mutations in the E1 and E2 envelope glycoprotein genes, occurred as the outbreak spread from Kenya.^{7,8}

CHIKV is believed to have evolved from enzootic, arboreal transmission cycles in Africa where NHPs serve as amplification hosts.^{9,10} These cycles give rise periodically to urban CHIKV emergence into a human-peridomestic mosquito cycle.¹¹ The source of the 2004 CHIKV outbreak in coastal Kenya is unknown but potentially occurred via spillover from an enzootic source in east Africa. One hypothesis for emergence from an enzootic cycle is increased contact with pathogens maintained in previously undisturbed habitats. Kenya is experiencing extensive human population growth, with changes in land use, and many forests in the country have been

disturbed by encroachment, or are now in close proximity to humans. Increasing encroachment of human populations on wildlife reserves is known to promote new cases of zoonotic disease after exposure to infected wild or domestic animals.^{12,13} Under such circumstances, disease transmitted from humans to other animals, referred to as spillback or emergence in native wildlife, can also occur; thus understanding the origins of human disease can be informative for conservation or studies of wildlife disease.^{13,14}

In Kenya, sylvatic areas can be inhabited by diverse wildlife species, including free-ranging NHPs (monkeys and apes). The transmission of viruses between NHP and humans is favored by their broadly similar physiologic and genetic characteristics.^{13,15,16} With a wide ecological distribution covering diverse habitats, NHPs are therefore useful for surveillance of emerging infectious disease (EID) and for understanding the origins and evolution of zoonotic pathogens.

A sylvatic cycle of CHIKV, circulating between NHPs and arboreal *Aedes* spp. mosquitoes, has been studied extensively in West and South Africa.^{17,18} However, there is no direct evidence for such an enzootic cycle in east Africa. In addition, east African CHIKV isolates from human outbreaks are of a different phylogenetic lineage than those in West or South Africa.^{6,11,19} The nature of any adaptations needed to initiate efficient interhuman transmission, leading to the originating strains of the 2004 Kenya outbreak assuming they emerged from an enzootic source, is also unknown.

Antigenically and genetically, CHIKV is closely related to another alphavirus, ONNV, with disparate transmission ecology by *Anopheles* spp. vectors^{20,21}; ONNV has been sporadically reported in Kenya,^{22,23} and nearby in Uganda²⁴ and Tanzania.^{25,26} It is therefore important to distinguish neutralizing antibodies generated by infection with these viruses when considering virus exposure, particularly since human

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Understanding when and where the hypothesized transition from enzootic to urban circulation occurred during or before the 2004 CHIKV outbreak could inform future interventions to prevent pandemic emergence. We therefore conducted field studies to understand the enzootic circulation of African alphaviruses. Here we focus on NHP serosurveys to gain an indication of likely foci for enzootic CHIKV circulation in Kenya.

Collection of samples. Initially, the Institute of Primate research, Kenya, provided 252 NHP sera from their collections, which had been taken from three different species: *Papio anubis* (olive baboon), *Chlorocebus aethiops* (vervet monkey), and *Cercopithecus mitis* (blue monkey), across eight sites in three regions of Kenya between 1985 and 2000 (Figure 1, Table 1).

and two sites in the Shimba Hills (Kwale) region of coastal Kenya (December 2014). Species caught were *P. anubis*, *Cercopithecus ascanius* (red-tailed monkey) and *C. mitis* (western Kenya), and *Papio cynocephalus* (yellow baboon) (coastal Kenya); other species including *C. aethiops* and *Colobus* spp. (black and white colobus monkey) were also targeted but not successfully captured during the program. Animals were captured alive, bled once, marked, and released back to their habitat. Pregnant females, lactating mothers, and infants were not sampled. Capture was done under the auspices of wildlife study permits from the Kenya Wildlife Service and according to the Kenya Medical Research Institute (KEMRI) Ethics Review Committee guidelines.

Animals were trapped using rectangular metal cage traps (1.5 m in height and 0.9 m in width), fitted with sliding trap doors, and baited with banana or corn. Captured animals were anesthetized with a 3:7 mixture of xylazine (2%) and ketamine hydrochloride (10%) injected intramuscularly at a dose of 0.1 mg/kg estimated body weight. A physical examination was carried out by a veterinarian before 9 mL of blood was drawn by venipuncture of the femoral vein; 5 mL was placed into a sterile 5-mL BD Vacutainer Plasma Preparation Tube or BD

TABLE 1
Origin and number of biobanked NHP samples screened for chikungunya virus

Region of Kenya		
Western	Central	Coastal
Kitale (<i>N</i> = 21)	Mtito Andei (<i>N</i> = 60)	Tana (<i>N</i> = 60)
Mt.Elgon (<i>N</i> = 23)		Watumu (<i>N</i> = 31)
Kakamega (<i>N</i> = 5)		Lamu (<i>N</i> = 21)
Buyangu (<i>N</i> = 31)		

NHP = non-human primate.

serum separation tube (depending on availability; both Becton Dickinson, Franklin Lakes, NJ), and 4 mL was placed into a BD Vacutainer coated with sodium heparin (Becton Dickinson). Plasma or serum, and blood were stored at -70°C prior to antibody assays. Sampled animals were monitored for 2 hours before release back to their habitat after full recovery from anesthesia.

Analysis of samples. Plaque reduction neutralization tests (PRNTs) were used to identify antibodies against CHIKV and other viruses of interest. Following heat activation (56°C for 1 hour) and a dilution 1:10 in Dulbecco's Minimum Essential Medium supplemented with 2% fetal bovine serum, sera were mixed with an equal volume of CHIKV vaccine strain 181/clone25²⁷ at a titer of 800 plaque-forming units (PFU)/mL. After 1 hour at room temperature, the virus-serum mix (100 μL) was applied to a confluent monolayer of Vero cells in a 12-well plate, and incubated at 37°C (5% CO_2) for 1 hour, before an overlay of 0.4% agarose and 5% fetal bovine serum media was applied. After a 72-hour incubation at 37°C , plates were fixed with 10% formaldehyde and stained with 0.25% crystal violet solution after removal of the agarose. Negative and CHIKV-positive serum controls were included (positive serum from mouse antibody production was provided by the Center for Virus Research, KEMRI). Two-fold serial dilutions of the 800 PFU/mL viral stock were performed in parallel as a virus-positive control reference plate, and the number of plaques resulting from the 1:2 titration was used to determine positive samples with cutoff values for 80% and 50% reduction of PFU. A χ^2 test was used to compare the proportion of seropositive samples.

Serial 2-fold dilutions of CHIKV-positive sera were also tested against ONNV (strain SG650, Uganda),²⁸ using the above methodology, and a 4-fold or greater difference in the titer provided tentative evidence of the virus to which the antibodies had developed (otherwise reactive antibodies were considered to be against an undetermined alphavirus).

In addition, a basic screening for DENV-1 to 4 IgG was conducted on serum via ELISA using a Detect IgG ELISA kit (Inbios, Seattle, WA), according to the manufacturers' instructions. Samples with an immune status ratio score > 2.84 were considered IgG positive against DENV.

Cell culture. For virus isolation, 50 μL of NHP serum (diluted 1:10) was used to inoculate both Vero and C6/36 mosquito cell lines in 12-well plates, which were incubated at 37°C and 28°C respectively, in a 5% CO_2 environment, and monitored daily for 14 days. Samples exhibiting cytopathic effects (CPEs) were transferred to a fresh plate to confirm positive CPE, prior to harvesting viral supernatant and storing at -70°C .

Molecular screening. Extraction of viral RNA from blood or serum was performed on 2014 samples using the QIAamp viral RNA mini kit (Qiagen, Germantown, MD), according to the manufacturer's protocols. This nucleic acid was used as a template in a cDNA synthesis with 5 $\times$ first strand buffer, dNTPs (10 mM), DTT (100 mM), random hexamer, RNase inhibitor (40 U/ μL), and reverse transcriptase. The polymerase chain reaction (PCR) cycling conditions were as follows: 70°C for 10 minutes, 4°C for 5 minutes, 25°C for 15 minutes, 42°C for 50 minutes, 70°C for 15 minutes, then held at 4°C . Reverse transcription PCR (RT-PCR) amplification was performed on 2 μL of the resulting cDNA, in a 25 μL volume of AmpliTaq mastermix (ThermoFisher, Waltham, MA), primers, and water. Genus-specific primers targeting alphaviruses, flaviviruses, and bunyaviruses, along with relevant cycling conditions, are shown in Table 2.

RESULTS

Archived samples. Seventeen of 80 (21.3%) NHP sera from western Kenya were PRNT₈₀ positive to CHIKV ($\geq 1:20$ final serum dilution) and 50 (62.5%) were PRNT₅₀-positive. Of 112 sera from coastal Kenya, 11 (9.8%) were PRNT₈₀-positive, and 60 (53.6%) were PRNT₅₀ positive. Of 60 sera from Mtito Andei (central Kenya), five (8.3%) were PRNT₈₀-positive, and 29 (48.3%) were positive at PRNT₅₀. Results of virus neutralization according to sites and region are shown in Figure 1. There was a significantly different seroprevalence between regions ($\chi^2 = 6.48$, *df* = 2, *P* = 0.039). Titers were obtained for all CHIKV-PRNT₈₀-positive samples (*N* = 33) and all could be distinguished from ONNV antibodies based on a 4-fold greater titer as described earlier.

2014 surveys. A total of 67 NHPs were captured and sampled in 2014 (33 from western; 34 from coastal Kenya). Table 3 indicates the number of each species caught, age

TABLE 2
Primers and cycling conditions used to screen for arboviruses

Virus	Primer	Primer sequence	Cycling conditions			
Alphavirus	Vir2052F	5'-TGG CGC TAT GAT GAA ATC TGG AAT GTT-3'	95°C (10 minutes)	95°C (30 seconds)	72°C (10 minutes)	4°C (hold)
	Vir2052R	5'-TAC GAT GTT GTC GTC GCC GAT GAA-3'		49°C (30 seconds) 72°C (30 seconds) for 35 cycles		
Flavivirus	Fu1	5'-TAC AAC ATG ATG GGA AAG AGA GAG AA-3'	95°C (10 minutes)	95°C (30 seconds)	72°C (7 minutes)	4°C (hold)
	CDF2	5'-GTG TCC CAG CCG GCG GTG TCA TCA GC-3'		55°C (30 seconds) 68°C (45 seconds) for 35 cycles		
Bunyavirus	BCS82c	5'-ATG ACT GAG TTG GAG TTT CAT GAT GTC GC-3'				
	BCS332v	5'-TGT TCC TGT TGC CAG GAA AAT-3'				

TABLE 3
Serological summary of non-human primates (NHPs) captured in Kenya during 2014

Olive baboon (<i>Papio anubis</i>)				
ID of positive animals*	Age group	CHIKV (PRNT ₈₀ titer)	Undetermined alphavirus (+ or -)	DENV IgG (+ or -)
KAKP/10	Adult	1:160	-	+
KAKP/11	Adult	-	-	+
KAKP/15	Juvenile	1:80	+/-	-
KAKP/16	Subadult	1:320	+	-
KAKP/27	Subadult	+/-	-	+
KAKP/29	Adult	1:160	-	+
KAKP/32	Juvenile	1:320	+	-
Red-tailed monkey (<i>Cercopithecus ascanius</i>)				
ID of positive animals†	Age group	CHIKV (PRNT ₈₀ titer)	Undetermined alphavirus (+ or -)	DENV IgG (+ or -)
KAKP/25	Adult	1:160	+	-
Blue monkey (<i>Cercopithecus mitis</i>)				
ID of positive animals‡	Age group	CHIKV (PRNT ₈₀ titer)	Undetermined alphavirus (+ or -)	DENV IgG (+ or -)
KAKP/01	Adult	1:80	-	-
KAKP/03	Subadult	1:320	+	-
KAKP/04	Subadult	1:160	-	-
Yellow baboon (<i>Papio cynocephalus</i>)				
ID of positive animals§	Age group	CHIKV (PRNT ₈₀ titer)	Undetermined alphavirus (+ or -)	DENV IgG (+ or -)
KWA/01	Subadult	-	+	-
KWA/02	Subadult	-	+	+
KWA/03	Adult	-	-	+
KWA/06	Adult	-	-	+
KWA/08	Adult	-	+	+
KWA/09	Adult	-	-	+
KWA/10	Adult	-	-	+
KWA/11	Adult	-	-	+
KWA/12	Adult	+/-	+	+
KWA/13	Adult	-	+	+
KWA/15	Adult	-	-	+
KWA/19	Adult	-	-	-
KWA/20	Adult	-	+	+
KWA/21	Adult	-	+	+
KWA/23	Subadult	-	-	+
KWA/27	Subadult	-	+	+
KWA/29	Adult	-	+	-

NHP = non-human primate; PRNT = plaque reduction neutralization test. Only seropositive NHP are shown.

* From a total of 18 individuals (seven adults, five subadults, six juveniles) sampled in western Kenya. +/- indicates an equivocal PRNT titer of 1:20.

† From a total of seven individuals (five adults, one subadult, one juvenile) sampled in western Kenya.

‡ From a total of eight individuals (two adults, six subadults) sampled in western Kenya.

§ From a total of 34 individuals (27 adults, six subadults, one juvenile) sampled in coastal Kenya. +/- indicates an equivocal PRNT titer of 1:20.

strata, and their seropositive status against CHIKV, ONNV, and DENV. Evidence of co-exposure in the same individual is also indicated.

Although 21% (14/67) of NHP sera neutralized CHIKV, only nine of these (64%) had a 4-fold PRNT₈₀-positive titer greater than that for ONNV, that is, sufficient to conclude CHIKV and not ONNV seropositivity; all were from sites in western Kenya. Similarly, 21% of NHP sera neutralized only ONNV (14/67); however, all had titers (PRNT₈₀) of 1:40 or less, suggesting the possibility that they contained antibodies reactive against an unknown alphavirus. Of the nine CHIKV antibody-positive NHPs, two had PRNT₈₀ titers of 1:80, four had PRNT₈₀ titers of 1:160, and three PRNT₈₀ titers of 1:320; all were from western Kenya; five were Olive baboons (two of which were juveniles), three were blue monkeys (two were subadults), and one was a red-tailed monkey.

IgG against DENV-1 to 4 was identified in 41% (14/34) of the NHP sera from coastal Kenya, and in five NHP sera (15%) from western (all baboon species, and specifically those individuals with territory closest to human habitation). Insufficient sample volumes were available for more specific confirmatory testing.

None of the sera inoculated onto Vero or C6/36 cells produced CPE. However, viral RNA was detected in one coastal NHP sample (*P. cynocephalus*; yellow baboon) tested by RT-PCR, which was subsequently identified as a flavivirus based on the PCR specificity.

DISCUSSION

Following the 2004 Kenyan outbreaks of CHIKV, we investigated NHP as potential enzootic reservoir/amplification hosts for arbovirus maintenance and spillover, also testing for the related alphavirus ONNV. Sampling conducted in 2014 gave a more recent indication of serostatus against circulating arboviruses, based on two regions of Kenya. In contrast to sites in Kwale County, western Kenya showed increased evidence of CHIKV, which included antibody titers (1:160 and greater) in juveniles and subadults, suggesting recent circulation. Although we also tested sera for ONNV antibodies, titers were insufficient to conclude evidence of infection by this virus. Archived samples also supported higher CHIKV seroprevalence in western sites (21%), more than double the rate

at the coastal sites (10%), and higher neutralizing antibody titers. This finding was despite all detected human cases occurring on coastal Kenya in 2004 and low NHP seroprevalence in Lamu (i.e., an epicenter of the 2004 re-emergence event); none of 21 NHP captured there in 1989, prior to the major 2004 CHIKV outbreak, showed neutralizing activity against CHIKV. These samples dated 15 years earlier than the outbreak however, and the distribution of enzootic CHIKV could have changed during that period.

During the 2004 Lamu outbreak of CHIKV, there was significantly higher human seroprevalence in urban than in rural areas.³ Our study focus here was on sylvatic habitats, where little intervention by humans has occurred, to examine principally enzootic virus maintenance. Interestingly, we did detect a low presence of CHIKV antibodies in NHP species (e.g., blue monkey) fairly deep inside a forest with little human contact, including in a subadult individual, suggesting recent enzootic circulation. The tendency of *Papio* (baboon) species to leave the forest and come into contact with neighboring human developments may explain why seroprevalence was highest in that species, suggesting spillback of pathogens via a human-to-NHP direction. We did detect higher seroprevalence, particularly of DENV, in NHP (noticeably baboons) that made movements overlapping the edge of villages. Furthermore, the lack of DENV exposure in baboons captured in the interior of Shimba Hills sanctuary adds support for spillback from human to NHP populations. Additional investigation with more extensive flavivirus serologic methods is required to confirm such a hypothesis; as well as consideration of the potential role of mosquito vector species (e.g., range of peridomestic or sylvatic *Aedes* spp.). Nevertheless, reducing human encroachment on primate habitat, and vice versa, and/or effective vector control measures could limit cross-species transmission of EID and restrict future outbreaks.

NHP capture is a costly and time-consuming exercise, but we highlight the value of such collections. Although not from the same species or sites, it was beneficial to examine NHP samples collected in Kenya before and after the 2004 CHIKV outbreak. We detected CHIKV antibodies in NHP within western Kenya in 2014, and 21.3% of all archived samples from western Kenya since 1992 were positive, supporting the recent and past circulation of CHIKV in this region. Although none of the five samples previously obtained within Kakamega tested positive (PNRT₈₀) against CHIKV (specific date of collection pre-2000 unfortunately unknown), examination of additional archived samples across a variety of dates would aid interpretation. Testing via PRNT is quite specific for alphaviruses and considered a gold standard in serological testing. Although we evaluated cross-reactivity with ONNV, additional alphaviruses are known to circulate in Kenya (e.g., Semliki Forest [SFV], Ndumu [NDUV], and Sindbis viruses),^{29,30} along with possibly further unknown viruses and potential cross-reactions with these viruses should be considered in further studies. We found little or no reaction between CHIKV and either SFV or NDUV, which are in the same serocomplex (unpublished data). We are not aware of any reports of these other alphaviruses being isolated from wild NHP. Finally, mosquito surveillance collections in coastal, western, and central Kenya are needed to characterize the important sylvatic vectors involved in any enzootic arbovirus transmission; most likely being arboreal *Aedes* species.¹⁰ Establishing arbovirus infection rates and foci for vector transmission,

combined with integrating findings from studies such as ours or further vertebrate serum testing, are needed to develop a more comprehensive understanding of the role of enzootic arbovirus circulation in disease emergence.

CONCLUSION

A re-emergence of chikungunya virus (CHIKV), a mosquito-borne alphavirus, occurred on coastal Kenya in 2004. The source leading to this outbreak of human cases (severe arthralgia and fever) was unknown. Since enzootic (non-human) maintenance of the virus has been discovered elsewhere in Africa between non-human primates (NHPs) and forest mosquitoes, we examined archived samples of NHP sera from different regions of Kenya for antibodies against CHIKV, along with more current NHP samples collected during 2014 from sylvatic Kenyan habitats near Kwale (coastal) and Kakamega (western). Results from archive samples indicated historic chikungunya infection at most regions, with antibodies more frequently detected at western Kenya sites. The seropositive primates in 2014 were all from western Kenya and distinguishable from infection with the closely related o'nyong-nyong virus (ONNV). Evidence of CHIKV exposure in blue monkeys, whose habitat currently has little human contact, suggests enzootic transmission. The presence of antibodies in juvenile and subadult NHPs suggests recent viral activity. Baboons sampled closest to human habitations by Kwale showed antibodies to dengue virus (DENV), suggesting cross-species transmission from humans.

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