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DIFFERENTIAL INFECTIVITIES OF O'NYONG-NYONG AND CHIKUNGUNYA VIRUS ISOLATES IN *ANOPHELES GAMBIAE* AND *AEDES AEGYPTI* MOSQUITOES

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Abstract. O'nyong-nyong virus (ONNV) and chikungunya virus (CHIKV) are closely related alphaviruses that cause human disease in Africa and Asia. Like most alphaviruses, CHIKV is vectored by culicine mosquitoes. ONNV is considered unusual as it primarily infects anopheline mosquitoes; however, there are relatively few experimental data to support this. In this study, three strains of ONNV and one strain of CHIKV were evaluated in *Anopheles gambiae* and *Aedes aegypti* mosquitoes and in four cell lines. As predicted, CHIKV was not infectious to *An. gambiae*, and we observed strain-variability for ONNV with respect to the ability of the virus to infect *An. gambiae* and *Ae. aegypti*. The species specificity *in vivo* was reflected by *in vitro* experiments using culicine and anopheline-derived cell lines.

INTRODUCTION

O'nyong-nyong virus (ONNV) and chikungunya virus (CHIKV) are closely related viruses in the Semliki Forest antigenic complex (family Togaviridae, genus *Alphavirus*).[1] ONNV is unique among mosquito-borne alphaviruses in being primarily transmitted by mosquitoes of the genus *Anopheles*, whereas the primary vectors for CHIKV are from the genus *Aedes*.[2,3] The basis for the unusual vector-specificity of the former is unknown.

Both CHIKV and ONNV can cause febrile illness in humans. Clinically, the symptoms of chikungunya are difficult to distinguish from those of dengue fever and so may be misdiagnosed.[4–6] CHIKV infections are characterized by fever, headache, nausea, vomiting, myalgia, rash, and arthralgia.[7] The clinical features of ONNV infections include a low-grade fever, symmetrical polyarthralgia, lymphadenopathy, generalized papular or maculopapular exanthema, and joint pain.[5,8]

CHIKV was first isolated in 1953 in Tanzania from the serum of a febrile human.[9] There have been numerous isolates made from both humans and mosquitoes in central, western, and southern Africa as well as in Asia.[10] CHIKV is believed to have originated in Africa where it is maintained in a sylvatic cycle involving wild primates and forest-dwelling mosquitoes such as *Aedes furcifer.* It was subsequently introduced into Asia where, as in the African urban cycle, it is transmitted by *Ae. aegypti* mosquitoes.[3] CHIKV has been the cause of substantial epidemics in both Southeast Asia and in Africa.[11,12,13]

ONNV, which is the most closely related virus to CHIKV, was initially isolated in northern Uganda from anopheline mosquitoes and human serum during a 1959 epidemic.[4,7] This virus has been associated with relatively few but large-scale epidemics.[14] A major epidemic involving more than 2 million cases, with no recorded fatalities, was caused by ONNV in 1959 in East Africa.[15,16] After an absence of 35 years another epidemic, in 1996, occurred in southern Uganda.[17] A more recent epidemic (2002) was reported near the shores of Lake Wamala in the Mubende District, central Uganda (Julius Lutwama, personal communication). ONNV is transmitted by anopheline mosquitoes such as *Anopheles gambiae*, the most important vector for human malaria, and *An. funestus*.[15,18] Humans may be the only natural host of ONNV, as other vertebrate reservoirs have not been identified. Igbo Ora virus was initially thought to be a separate but closely related virus based on cross-complement fixation tests.[19] However, sequence analysis of this virus indicates that it is a strain of ONNV.[17]

Johnson suggested that mutations in CHIKV led to the emergence of ONNV and its ability to be transmitted by anopheline mosquitoes.[16] More recent sequence analysis by Lanciotti and others and Powers and others indicate that CHIKV and ONNV are "genetically distinct."[7,16,17] Lanciotti and others examined the complete nucleotide sequence of five alphaviruses: 3 ONNV (SG650, Gulu, and Igbo Ora), Semliki Forest (SFV), and Ross River virus (RRV). A comparison of the coding structural region of the viruses was also made, capsid through E1 genes, of six alphavirus isolates: 3 ONNV (SG650, Gulu, and Igbo Ora), CHIK, SFV, and RRV.[17] Powers and others examined the areas of the E1 envelope glycoprotein and 3′ noncoding region from 24 alphavirus isolates: 18 CHIKV, 3 ONNV, 2 Sindbis, and 1 SFV.[7] Both of these studies confirmed that CHIKV and ONNV are separate but related viruses in the Semliki Forest antigenic complex. Powers and others revealed two CHIKV lineages[7]; one lineage contained all isolates from western Africa, and the second contained all southern and eastern African strains and the Asian isolates. Data were interpreted as indicating an African origin for CHIKV with divergence of ONNV and CHIKV from a common ancestor thousands of years ago.[7]

Here we report results of a comparison of these viruses and strains *in vitro* and *in vivo*. The three strains of ONNV and one strain of CHIKV were grown on anopheline (Mos 55) and *Aedes* (C6/36) derived cell culture and two vertebrate cell lines (BHK-21 and Vero) to analyze their ability to grow *in vitro*. The viruses were also compared for their ability to infect and disseminate in *An. gambiae* and *Ae. aegypti* mosquitoes. This new information will provide the foundation for studies on the basis/mechanism of viral-vector species specificity.

MATERIALS AND METHODS

Viruses. The SG650, Gulu, and Igbo Ora strains of ONNV and CHIKV strain 37997 were obtained from the World Ref-

erence Center for Arboviruses at the University of Texas Medical Branch (Galveston, TX). Strain SG650 was isolated from human serum in Uganda in 1996[17] and has been passed once in Vero cells (GenBank accession no. AF079456). ONNV Gulu strain (30839), was isolated in northern Uganda in 1959 from human serum[20]; this virus was passed 14 times in suckling mouse brain and once in Vero cells. The Igbo Ora isolate (IBH12628) was obtained from a febrile patient in Nigeria in 1966[21,22] and has been passed six times in suckling mouse brain and once in Vero cells; this isolate has been sequenced and is similar genetically to IBH10964 (GenBank accession no. AF079457). CHIKV strain 37997 was originally isolated from the mosquito *Ae. furcifer* in Kadougou, Senegal, in 1983 (GenBank accession no. AY726732). This isolate has been passed one time in AP-61 (*Ae. pseudoscutellaris*) cells and two times in Vero cells.

Stock viruses were produced after a single passage in Vero cells maintained at 37°C in Leibovitz L-15 medium with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell supernatants were harvested when 75% of the cells showed cytopathic effect (3 + CPE), aliquoted, and stored at –80°C for use in all experiments.

***In vitro* growth kinetics of viruses.** Two vertebrate-derived, BHK-21 (hamster kidney) and Vero (green monkey kidney), and two mosquito-derived, C6/36 (*Ae. albopictus*) and MOS-55 (*An. gambiae*), cell lines were used for these studies. All cells were maintained in Leibovitz L-15 medium with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Vertebrate and mosquito cells were maintained at 37°C and 28°C, respectively. For each virus, confluent cell monolayers in 25 cm^2 flasks were infected with a standard 1 mL inoculum by rocking at room temperature for 1 hour. The inoculum was then removed and after three washes with 5 mL L-15, 5.5 mL of medium was added per flask. A sample of 0.5 mL was removed immediately. Additional 0.5 mL samples were collected at 24-hour intervals and replaced with 0.5 mL of fresh medium. Samples were stored at –80°C until titrated. Experiments were repeated three times. Data represents virus production for a standardized monolayer area (25 cm^2). Due to a difference in the size of individual cells, the multiplicity of infection varied for the different cell lines, for example, 0.25 for C6/36 cells and 0.63 for BHK-21 cells.

Mosquitoes. The white-eyed Higgs variant of the Rexville D strain of *Ae. aegypti* and the G3 strain of *An. gambiae* were reared at 27°C and 80% relative humidity under a 16-hour light:8-hour dark photoperiod, as previously described.[23–25] Adults were supplied with a cotton wool pad soaked in a 10% sucrose solution *ad libitum* and fed on anesthetized hamsters once per week for egg production.

Virus infections of mosquitoes. Four-day-old adult female *Ae. aegypti* or *An. gambiae* mosquitoes were fed a blood meal containing one of the three strains of ONNV or the CHIKV to be analyzed. Fresh virus was grown from stock and harvested from Vero cells when 75% of the cells showed CPE. The viral supernatant was mixed with an equal volume of defibrinated sheep blood (Colorado Serum Company, Denver, CO). As a phagostimulant, adenosine triphosphate at a final concentration of 2 mM was added to the blood meal. Mosquitoes were fed using an isolation glove box located in a Biosafety Level 3 insectary. Infectious blood was heated to 37°C and placed in a Hemotek feeding apparatus (Discovery Workshops, Accrington, Lancashire, UK), and mosquitoes were allowed to feed for 1 hour.[26] Fully engorged females were separated from unfed females and were placed into new cartons. Three to eight mosquitoes were removed for titration on Days 0, 1, 2, 3, 7, and 14 postinfection (p.i.) and were stored at –80°C. Day 0 samples, collected immediately after feeding, were used to determine the titer of virus imbibed and to evaluate continuity between experiments.

Titrations. Viral samples harvested from cell culture and in mosquitoes were quantified as tissue culture infectious dose 50 end-point titers ($\log_{10}$ $TCID_{50}$/mL) using a standardized procedure.[27] Briefly, 100 μL samples of cell culture supernatant/mosquito triturate were pipetted into wells of the first column of a 96-well plate, serially diluted in a 10-fold series, seeded with Vero cells, and incubated at 37°C for 7 days.[27] Prior to titration, each mosquito was triturated in 1 mL of L-15 medium and filtered through a 0.22-μM syringe filter (Millipore, Carrigwohill, Cork, Ireland).[27] Differences in viral infection rates between *Ae. aegypti* and *An. gambiae* were tested for significance by Fisher's exact test using SPSS version 11.5 (SPSS Inc. Chicago, IL).

Immunofluorescence assay. Salivary glands were dissected from 7- and 14-day p.i. mosquitoes for analysis to determine dissemination rates. The mosquitoes were dissected on glass microscope slides in phosphate-buffered saline. Salivary glands were air dried, fixed in cold acetone for 10 minutes, and stained using a cross-reactive mouse hyperimmune ascitic fluid raised against CHIKV as the primary antibody and amplifying the signal using indirect immunofluorescence assay (IFA) protocols previously described.[27–29]

RESULTS

Analysis of viruses *in vitro*. All three strains of ONNV were infectious to all four cell lines (Figures 1A–1C). In contrast, CHIKV strain 37997 failed to infect the *An. gambiae* cell line, MOS-55, but was able to infect the two vertebrate cell lines, Vero and BHK, and *Ae. albopictus* cells, C6/36 (Figure 1D). Cytopathic effect developed rapidly within 48 hours p.i. in both vertebrate cell lines, with subsequent total monolayer destruction, but was not observed in either of the mosquito cell lines.

Infectivity and replication *in vivo*. The abilities of the viruses to infect *Ae. aegypti* and *An. gambiae* were found to vary among the ONNV strains and between ONNV and CHIKV. ONNV strains SG650 and Gulu were fed in four separate experiments to both species of mosquitoes; Igbo Ora was fed in two separate experiments; CHIKV was fed in one experiment. Two types of comparisons were made of the viral infection rates in mosquitoes using Fisher's exact test. The first analyzed differences in infection rates between *Ae. aegypti* and *An. gambiae* mosquitoes for each individual strain of ONNV and one strain of CHIKV (Table 1). The second analyzed the differences in infection rates for either *Ae. aegypti* or *An. gambiae* mosquitoes between the three strains of ONNV (Table 1). No significant difference ($P > 0.05$) was found in the infection rates for both species of mosquitoes on Days 0 and 1 p.i. for all three ONNV strains and CHIKV (Table 1). Therefore, differences in infection rates observed at later time points p.i. cannot be attributed to the initial titer of virus imbibed.

ONNV SG650 infected both species of mosquitoes at a high rate. After the initial exposure of *Ae. aegypti* and *An. gambiae*

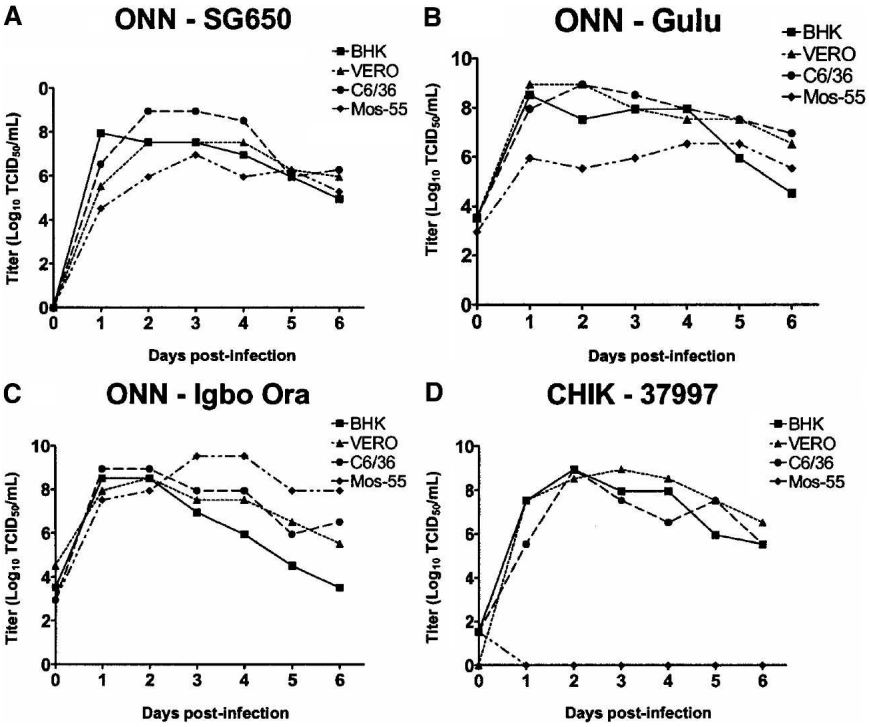

FIGURE 1. Representative growth curves based on triplicate experiments for three strains of ONNV and one strain of CHIKV virus in two vertebrate (BHK-21 and Vero) and two mosquito (C6/36 and Mos-55) cell lines.

to an infected blood meal, fully engorged females were found to have imbibed an average of 5.0 $\log_{10}$ $TCID_{50}$/mosquito (Table 1). There was decline in viral titer on Days 2 and 3 p.i., which was then followed by an increase in the titer and the number of mosquitoes infected; by Day 14 p.i., the mean titer was 4.3 and 3.8 $\log_{10}$ $TCID_{50}$/mosquito for *Ae. aegypti* and *An. gambiae*, respectively. One hundred percent of the mosquitoes were infected on 14 days p.i. (Table 1).

TABLE 1
Average mosquito body titers and infection rates using three strains of ONNV and CHIKV in *Ae. aegypti* and *An. gambiae*

		Ae. aegypti		*An. gambiae*	
Virus strain	Day postinfection	Titer* (± SD)	Infected/total (%)	Titer* (± SD)	Infected/total (%)
ONN	0†	5.0 (± 0.5)	25/25 (100)	5.0 (± 0.7)	25/25 (100)
SG650	1†	4.3 (± 0.4)	12/13 (92)	4.3 (± 0.3)	16/16 (100)
	2	1.0 (± 1.4)	2/15 (13)	3.5 (± 0.5)	18/18 (100)
	3	1.7 (± 2.4)	3/18 (17)	3.0 (± 0.7)	12/16 (75)
	7	3.3 (± 0.3)	21/37 (57)	3.5 (± 0.6)	38/40 (95)
	14†	4.3 (± 0.4)	42/42 (100)	3.8 (± 0.6)	34/34 (100)
ONN	0†	6.1 (± 0.5)	22/22 (100)	6.1 (± 0.1)	21/21 (100)
Gulu	1†	3.3 (± 1.0)	17/22 (77)	4.4 (± 0.5)	11/16 (69)
	2†	2.4 (± 3.4)	5/13 (38)	2.6 (± 1.0)	7/14 (50)
	3†	1.4 (± 2.0)	6/16 (38)	2.4 (± 1.2)	9/16 (56)
	7	1.3 (± 2.0)	4/28 (14)	2.7 (± 0.5)	22/32 (69)
	14	1.9 (± 2.6)	3/30 (10)‡	1.7 (± 1.2)	14/32 (44)‡
ONN	0†	5.3 (± 0.1)	11/11 (100)	5.0 (± 0.2)	11/11 (100)
Igbo Ora	1†	4.0 (± 0.8)	8/8 (100)	2.8 (± 0.3)	8/8 (100)
	2†	2.4 (± 0.3)	5/8 (63)	2.0 (± 0.3)	6/8 (75)
	3†	0	0/8 (0)	0	0/8 (0)
	7†	1.8 (± 1.2)	5/16 (31)	2.9 (± 0.2)	10/16 (63)
	14	3.4 (± 0.9)	4/16 (25)‡	3.7 (± 0.1)	12/16 (75)‡
CHIK	0†	6.7 (± 0.3)	3/3 (100)	6.5 (± 0.0)	3/3 (100)
37997	1†	5.9 (± 0.8)	8/8 (100)	4.9 (± 0.6)	8/8 (100)
	2	4.5 (± 0.0)	5/5 (100)	0	0.7 (0)
	3	4.8 (± 0.2)	5/5 (100)	0	0/8 (0)
	7	6.8 (± 0.6)	8/8 (100)	1.4 (± 2.0)	3/7 (43)
	14	5.0 (± 0.4)	7/7 (100)	0	0/8 (0)

* Average blood meal titers were ONN SG650, 7.3 (± 0.6) $\log_{10}$ $TCID_{50}$/mL; ONN Gulu, 7.7 (± 0.4) $\log_{10}$ $TCID_{50}$/mL; ONN Igbo Ora, 7.7 (± 0.3) $\log_{10}$ $TCID_{50}$/mL; CHIK, 8.0 $\log_{10}TCID_{50}$/mL.
† No significant difference in infection rates between species (for a virus strain) on indicated days postinfection using Fishers's exact test ($P > 0.05$).
‡ No significant difference ($P > 0.05$) in Day 14 p.i. infection rates of the three strains of ONNV in *Ae. aegypti* or *An. gambiae* for strains indicated.

Although Gulu replicated in both mosquito species, the number of infected mosquitoes and the titer of the individual mosquitoes decreased steadily from days 0 to 14 p.i. A comparison of infection rates between Gulu and SG650 Day-14 p.i. revealed a significantly lower rate in the Gulu infection rates in both *Ae. aegypti* and *An. gambiae* mosquitoes (Table 1). A significant difference was also found for this virus between the two mosquito species on Days 7 and 14 p.i. (Table 1). On Day 14 p.i., 10% of the *Ae. aegypti* and 44% of the *An. gambiae* mosquitoes were infected.

Comparison of infection rates of Igbo Ora using Fisher's exact test indicated that there was not a significant difference ($P > 0.05$) in the replication of this strain in the two mosquitoes tested until Day 14 p.i. (Table 1). At 14 days p.i., 25% of *Ae. aegypti* mosquitoes and 75% of *An. gambiae* mosquitoes were infected (Table 1). Igbo Ora infection rates on Day 14 p.i. did not show a significant difference from the Gulu infection rates in *Ae. aegypti* or *An. gambiae.* However, both Gulu and Igbo Ora produced significantly lower infection rates than SG650 on Day 14 p.i. (Table 1).

The most significant difference in infection rates was found between *Ae. aegypti* and *An. gambiae* infected with CHIKV strain 37997. The blood meal titers were identical, 7.95 $\log_{10}$ $TCID_{50}$/mL of CHIKV, and the initial amount of virus imbibed were similar for both *Ae. aegypti* and *An. gambiae* (Table 1). However, the infection rates on Days 2, 3, 7, and 14 p.i. for *Ae. aegypti* and *An. gambiae* with CHIKV varied significantly (Table 1). On Day 14 p.i. 100% of *Ae. aegypti* were infected, whereas none of the *An. gambiae* mosquitoes were infected (Table 1).

***In vivo* infection and dissemination.** Salivary glands of individual mosquitoes were analyzed by IFA on Days 7 and 14 p.i. to determine dissemination rates for the two species of mosquitoes with the three strains of ONNV (Table 2). ONNV strains SG650, Gulu, and Igbo Ora disseminated in both species of mosquitoes by Day 14 p.i. (Table 2). For each strain of ONNV, there was not a significant difference in dissemination rates between *Ae. aegypti* and *An. gambiae* ($P < 0.05$), and no difference was found in *Ae. aegypti* between strains. However, there was a significant difference when virus strains were compared with each other in *An. gambiae*. SG650 and Igbo Ora disseminated in significantly more mosquitoes than the Gulu strain in *An. gambiae* (Table 2).

TABLE 2

Dissemination rates based on antigen detection by immunofluorescence assay for three strains of ONNV and one strain of CHIKV in *Ae. aegypti* and *An. gambiae* mosquitoes

Virus strain	Day postinfection	*Ae. aegypti** positive/total (%)	*An. gambiae* positive/total (%)
ONN	7	0/10 (0)	0/10 (0)
SG650	14	2/10 (20)	4/7 (57)†
ONN	7	0/10 (0)	0/10 (0)
Gulu	14	2/15 (14)	1/15 (7)
ONN	7	0/10 (0)	0/10 (0)
Igbo Ora	14	2/5 (40)	3/5 (60)†
CHIKV	7‡	3/8 (38)	0/8 (0)
37997	14‡	5/8 (63)	0/8 (0)

* No significant difference ($P > 0.05$) in Day 14 postfeed dissemination rates among ONNV strains in *Ae. aegypti.*

† No significant difference ($P > 0.05$) in Day 14 postfeed dissemination rates between indicated ONNV strains in *An. gambiae.*

‡ Significant difference in dissemination rates between *Ae. aegypti* and *An. gambiae* using Fishers's exact test ($P < 0.05$).

Analysis of CHIKV salivary glands by IFA indicated a significant difference in dissemination between the two species of mosquitoes. CHIKV disseminated in 38% and 63% of *Ae. aegypti* mosquitoes on Days 7 and 14 p.i., respectively. CHIKV did not disseminate in any of the *An. gambiae* mosquitoes analyzed on Days 7 or 14 p.i. CHIKV was the only virus studied that disseminated by Day 7 p.i. in either mosquito tested (Table 2).

DISCUSSION

Although relatively closely related in nature, ONNV and CHIKV are transmitted by mosquitoes in different genera.[3,7,16,17] Although the alphaviruses and flaviviruses are typically transmitted by culicine mosquitoes (e.g., *Aedes* and *Culex* species), ONNV is relatively unusual in that it is primarily transmitted by anopheline mosquitoes and has even been isolated from *Mansonia uniformis*.[17] In contrast, viruses in several other genera, including 22 bunyaviruses (e.g., Cache Valley), Orungo virus (orbiviridae), bovine ephemeral fever virus, and barur virus (rhabdoviridae), have been isolated from *Anopheles* spp.[1] This study aimed to determine the vector specificity of three strains of ONNV and one strain of CHIKV under laboratory conditions and to determine if this vector specificity is consistent for different viral strains.

Previous experimental growth curves with ONNV and CHIKV have used various cell lines. Vertebrate lines that have been infected include BHK,[30–32] HeLa cells,[33] Vero cells,[34,35] LLC-MK_2,[34] and XTC-2 cells.[36] Numerous arthropod-derived lines have also been tested, and it seems that those derived from *Aedes* are more sensitive than those from *Culex*.[37] Buckley used *Ae. aegypti* and *Ae. albopictus* cell lines and concluded that although both viruses replicated in *Ae. albopictus* cells, CHIKV replicated in *Ae. aegypti* cells whereas ONNV did not.[31] Unpublished data cited by Karabatsos reported infection of several cells lines including *An. stephensi*.[1] Igbo Ora virus behaved similarly to ONNV, as might be predicted based on the recent phylogenetic analysis of this isolate. Igarashi described the sensitivity of the C6/36 clone of Singh's *Aedes albopictus* cells (SAAL) to CHIK infection.[38] A peak titer of approximately 8.5 logs occurred at 2 days p.i., with a gradual decline to 7 logs by 6.5 days p.i. Some CPE was reported in these cloned cells but not in the uncloned (SAAR) line.[38]

Differential growth of ONNV and CHIKV in mosquito cells has been documented.[39] Both viruses grew in the SAAL, AM-60 (*Aedes malayensis*) and AP-61 cell lines (*Aedes pseudoscutellaris*), whereas only CHIKV grew in AA-20A (*Ae. aegypti*) and Singh's *Ae. aegypti* (S.AA) lines. By raising the incubation temperature from 28°C to 32°C, these workers observed plaque formation by ONNV in AP-61 cells. Ironically, the *An. gambiae* cell line AG-55 (= MOS-55) was included in these studies but not tested for susceptibility to ONNV or CHIKV. The original publication describing the generation of this cell line commented that "The cells were infected with O'nyong-nyong virus (which *An. gambiae* transmits in nature) without any cytopathic effect" but provides no supporting data.[40] Both viruses replicate in the RA243 cell line from the tick *Rhipicephalus appendiculatus*.[41]

Our data are the first to compare the growth of ONNV and CHIKV in the MOS-55 cell line, which previous analysis has

confirmed to be genotypically authentic with *An. gambiae*.[42] All three strains of the ONNV replicated in the four cell lines, including MOS-55; however, CHIKV strain 37997 failed to replicate in MOS-55. The more rapid decline in viral titers from the vertebrate lines probably reflects less sustained virion production due to cell death. Our observations demonstrate that the vector specificity of CHIKV, which has been found in nature and confirmed in our *in vivo* experiments, are apparently retained in *in vitro* systems. CHIKV failed to infect the *An. gambiae* derived cell line, in contrast with ONNV, which can infect and replicate in both of the *Ae. albopictus* and *An. gambiae* derived cell lines.

Our experiments demonstrated that all three of the ONNV strains used were able to infect and replicate in both *An. gambiae* and *Ae. aegypti*, but strain variations were apparent. Although both the individual mosquito viral titers and the proportion of infected mosquitoes declined over time for the Gulu and Igbo Ora strains, dissemination data indicates that the viruses replicated in both species of mosquito. Based on titration, significantly higher rates of infection were observed with ONNV SG650 in *Ae. aegypti* and *An. gambiae* when compared with the Gulu and Igbo Ora strains. The dissemination rates between these three strains were not found to be significantly different in *Ae. aegypti*; however, the dissemination rate of Gulu in *An. gambiae* was significantly lower than for the other two ONNV strains. In *An. gambiae*, SG650 titers remained relatively high even early during infection, without the obvious eclipse phase that was observed in *Ae. aegypti* on Days 2 and 3 p.i. A possible explanation for the observed ONNV strain variation is that since they were first isolated, both Gulu and Igbo Ora have been passaged repeatedly through suckling mice. Selection for neurovirulence may therefore have compromised infectivity for mosquitoes. Because strain SG650 had only been passed once on Vero cells, one would speculate that it is more likely to have retained its natural phenotype. Of the three ONNV strains, it replicated most efficiently. The Gulu strain has been passed more frequently than the Igbo Ora strain (14 mouse-brain passes versus 6 passes), replicated relatively poorly in mosquitoes, and was associated with significantly lower infection rates in both species of mosquito when compared with SG650 and a significantly lower dissemination rate in *An. gambiae* when compared with SG650 and Igbo Ora.

Our data for CHIKV clearly demonstrated that this virus is infectious to *Ae. aegypti* but not *An. gambiae*. In *Ae. aegypti*, we observed that viral titers remained high throughout the 14-day experimental period with 100% infection rates and 38% dissemination rates by Day 7. Early laboratory experiments concluded that *An. gambiae* and *An. stephensi* are barely susceptible to CHIKV and although *An. albimanus* can be infected, it cannot transmit the virus.[43–46] Our data for *An. gambiae* are supportive of these observations, and although we found some mosquitoes to be still infected at 7 days p.i., titers were extremely low. By Day 14 p.i., none of the *An. gambiae* mosquitoes remained infected, and there was no dissemination into the salivary glands.

In conclusion, both our *in vitro* and *in vivo* data support and extend previous work that has demonstrated the unusual capability of ONNV to infect both anopheline and culicine mosquitoes. Infection and dissemination rates were found to vary between the three strains of ONNV analyzed and between the two species of mosquitoes analyzed. Data from *in vitro* and *in vivo* infection with CHIKV strain 37997 determined that this strain will only infect and disseminate in *Ae. aegypti* mosquitoes and not in *An. gambiae* mosquitoes.

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