

DETERMINANTS OF VECTOR SPECIFICITY OF O'NYONG NYONG AND CHIKUNGUNYA VIRUSES IN *ANOPHELES* AND *AEDES* MOSQUITOES

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Abstract. The alphaviruses o'nyong nyong virus (ONNV) and chikungunya virus (CHIKV) provide a unique system to study the viral genes involved in vector specificity. ONNV infects both anopheline and culicine mosquitoes, whereas CHIKV infects only culicine mosquitoes. In this study, chimeric viruses were constructed that contained genes from both ONNV and CHIKV. These chimeras and previously described full-length infectious clones of ONNV and CHIKV were evaluated in *Anopheles gambiae* and *Aedes aegypti* mosquitoes. Virus derived from the infectious clones of ONNV and CHIKV retained the vector specificity of the parental viruses. All six of the chimeras were found to infect *Ae. aegypti* mosquitoes at high rates but only the chimera containing viral genes encoding all of the structural proteins of ONNV was able to infect *An. gambiae* mosquitoes. These data indicate that all of the viral structural proteins are necessary for ONNV to infect *An. gambiae* mosquitoes.

INTRODUCTION

O'nyong nyong virus (ONNV) and chikungunya virus (CHIKV) are mosquito-borne alphaviruses in the Semliki Forest antigenic complex, family Togaviridae.¹ Alphaviruses contain a linear, positive sense, single-stranded RNA genome that is ~11.8 kb in length.² The RNA genome consists of a capped 5' non-coding region (NCR) and a 3' polyadenylated NCR. There are four nonstructural proteins (nsP1–nsP4) that are encoded at the 5' end. The structural proteins are encoded at the 3' end of the virus from a subgenomic 26S RNA and consist of the viral capsid (C), two envelope glycoproteins (E1 and E2), and two peptides (E3 and 6K).² A total of 89% of the 11,822-nucleotide genome of ONNV (SG650) is conserved when compared with the 11,881-nucleotide genome of CHIKV (37997). Within these conserved areas, the sequence identity is between 77% and 85%.

ONNV, which is transmitted by anopheline mosquitoes such as *Anopheles gambiae* and *An. funestus*,^{3–5} has been involved in large scale epidemics in Africa.^{6–8} Although no fatalities occurred, the 1959–1962 epidemic involved more than two million cases.^{3,4} In 1996, ONNV was the cause of another epidemic in Uganda after an absence of 35 years.⁹ ONNV produces symptoms such as fever, althralgia, and rash that generally last for less than one week.¹⁰

CHIKV is transmitted by *Aedes furcifer* mosquitoes in Africa¹¹ and *Culex quinquefasciatus*, *Ae. aegypti*, and *Ae. albopictus* mosquitoes in Asia.^{12,13} This virus has caused substantial epidemics in both Africa and southeast Asia.^{14,15} Epidemic infection with CHIKV decreased on both continents in the 1970s and 1980s, but has recently re-emerged. A 1999 outbreak in Indonesia was the first epidemic transmission of CHIKV in this location since 1982.¹⁶ A second outbreak of infection with CHIKV occurred in Indonesia from 2001 to 2003.¹³ After a 39-year absence, CHIKV was found to be the cause of an urban epidemic in the Democratic Republic of the Congo.¹⁷ Infection with CHIKV is characterized by fever, nausea, althralgia, and rash.

Chimeric viruses based on ONNV and CHIKV provide useful tools to study the viral genetic determinants of vector specificity. Three strains of ONNV (SG650, Gulu, and Igbo Ora), have been shown to be infectious to varying degrees to both *Ae. aegypti* and *An. gambiae* mosquitoes and to two invertebrate cell culture lines derived from *Anopheles* (Mos 55) and *Aedes* (C6/36) mosquitoes.¹⁸ ONNV strain SG650 was found to have the highest infection rate in both species of mosquitoes compared with the two other strains. CHIKV (37997) is able to infect *Ae. aegypti* mosquitoes and C6/36 cells; however, *An. gambiae* mosquitoes and Mos 55 cells have been found to be refractory to infection with this virus.¹⁸

Six chimeric viruses based on ONNV and CHIKV were developed and characterized in *Ae. aegypti* and *An. gambiae* mosquitoes. These chimeric viruses possessed nonstructural genes from CHIKV and partial or full structural genes derived from ONNV. Complimentary chimeras were also constructed in which the nonstructural genes were obtained from ONNV and the structural genes were derived from CHIKV. The ability of these viruses to infect two species of mosquitoes was compared with virus rescued from the full-length infectious clones of ONNV and CHIKV.

MATERIALS AND METHODS

Viruses. ONNV (SG650) and CHIKV (37997) were obtained from the Centers for Disease Control and Prevention arbovirus reference center in Fort Collins, Colorado and the World Reference Center for Arboviruses at the University of Texas Medical Branch in Galveston, Texas. ONNV (SG650) was isolated from human serum in Uganda in 1996 and has been passed twice in Vero cells (GenBank accession no. AF079456).⁶ CHIKV (37997) was originally isolated from the mosquito *Ae. furcifer* in Kadougou, Senegal in 1983 (GenBank accession no. AY726732). This isolate has been passed once in AP-61 (*Ae. pseudoscutellaris*) cells and twice in Vero cells. Stock virus was produced after a single passage in Vero cells maintained at 37°C in Leibovitz L-15 medium with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Cell supernatants were harvested when 75% of the cells showed a cytopathic effect (3 + CPE), aliquoted, and stored at –80°C for use in all experiments.

Construction of infectious clones. The full-length CHIKV (37997) infectious clone (pCHIKic) has been previously de-

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scribed.¹⁹ The full-length ONNV (SG650) infectious clone pONNnic-Foy, provided by K. E. Olson and B. D. Foy (Colorado State University, Fort Collins, CO), was derived from pONN.AP3, developed by Brault and others,²⁰ was modified by replacing the T7 promoter with a SP6 promoter and removing the second subgenomic promoter and restriction sites, and renamed pONNnic. The six chimeric viruses, shown in Figure 1, were constructed using standard polymerase chain reaction (PCR)-based cloning procedures. The PCR-amplified fragments of ONNV (SG650) or CHIKV (37997) were produced using high-fidelity *Pfu* polymerase (Stratagene, La Jolla, CA). Fragments were ligated either in tandem or singly with T4 DNA ligase (Stratagene) into pCHIKic or pONNnic. All plasmids were transformed into chemically competent *Escherichia coli* XL10-Gold cells (Stratagene), amplified, and DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Additional information, such as maps of the clones, is available from the authors upon request.

In vitro transcription and virus production. Infectious virus from the full-length and chimeric clones was produced by linearization with *Not* I, which was *in vitro* transcribed from the SP6 promoter using the mMESSAGe mMACHINE kit (Ambion, Austin, TX) following the manufacturer's instructions. The RNA was electroporated into baby hamster kidney-21 S cells as previously described.²¹ Cell culture supernatant containing virus was harvested, aliquoted, and stored at -80°C when cells showed 3 + CPE.

Mosquitoes. The Rexville D strain of *Ae. aegypti*, white-eyed Higgs variant, and the *An. gambiae* G3 strain were

reared at 27°C and a relative humidity of 80% under a 16-hour light: 8-hour dark photoperiod, as previously described.^{22–24} Adults were supplied with a cotton wool pad soaked in a 10% sucrose solution *ad libitum*.

Oral infections of mosquitoes. Four-day-old adult female *Ae. aegypti* or *An. gambiae* mosquitoes were fed a blood meal containing virus produced from pCHIKic, pONNnic, or one of the six chimeras to be analyzed. Vero cells were inoculated with stock virus and incubated at 37°C . Virus was harvested 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). Adenosine triphosphate at a final concentration of 2 mM was added to the blood meal as a phagostimulant. 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 for one hour (Discovery Workshops, Accrington, Lancashire, United Kingdom).²⁵ Fully engorged females were separated from un-fed females and were placed into new cartons. To compare replication of the chimeric viruses, six to eight mosquitoes were removed for titration on days 1, 2, 3, 7, and 14 post-infection (pi) for a total of approximately 40 mosquitoes per virus examined. Mosquitoes were stored at -80°C prior to titration on 96-well plates. Day 0 blood meal and three mosquito samples, collected immediately after feeding, were used to determine the titer of virus imbibed and to evaluate continuity between experiments. At days 7 and 14 pi, salivary glands were dissected from 10 mosquitoes and stained for viral antigen to evaluate dissemination properties of the viruses.

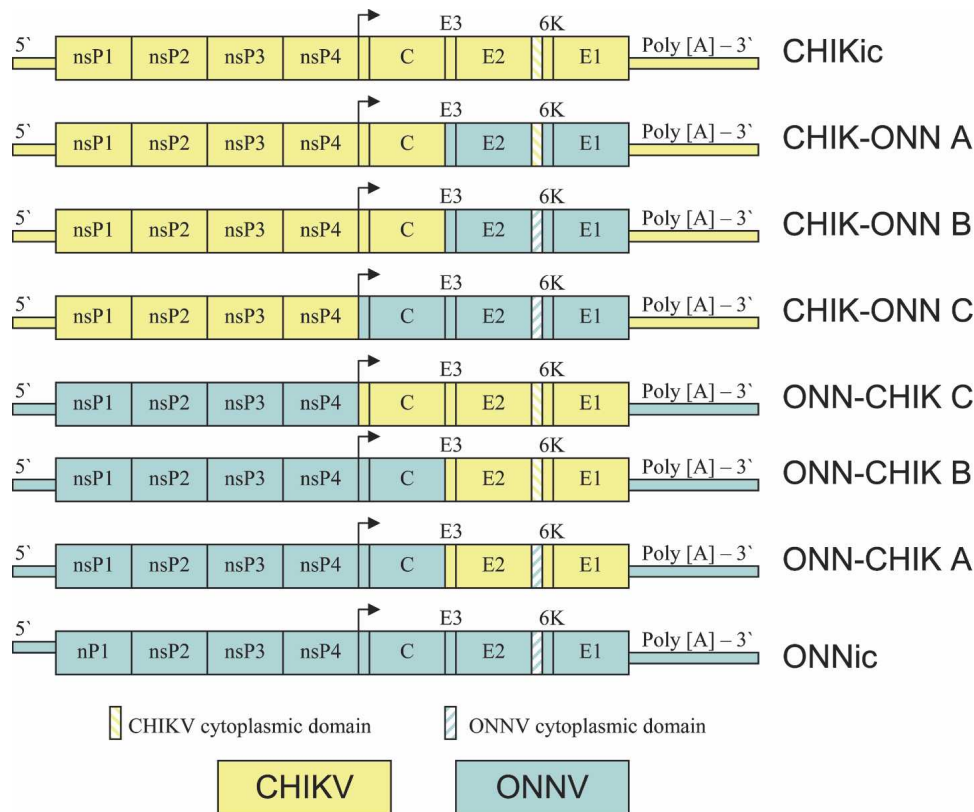


FIGURE 1. Schematic of full-length chikungunya virus infectious clone (pCHIKic), o'nyong nyong virus infectious clone (pONNnic), and chimeric clones produced. Reciprocal clones have the same letter (A, B, or C) for ease of comparison. The subgenomic promoter is indicated by an arrow. nsP = nonstructural protein; C = capsid; E = envelope. This figure appears in color at www.ajtmh.org.

Titration. The 50% tissue culture infectious dose endpoint titers (\log_{10} TCID₅₀/mL) were used to quantify titers of virus from cell culture and mosquito samples.²¹ Individual mosquitoes were triturated in 1 mL of L-15 medium and filtered through a 0.22- μ M syringe filter (Millipore, Carrigtwohill, Cork, Ireland), which was then 10-fold serially diluted in a 96-well plate seeded with Vero cells. Cells and virus were incubated at 37°C for seven days and then scored based on observed CPE. 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 (IFA). Salivary glands were dissected on glass microscope slides in phosphate-buffered saline, 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 IFA protocols previously described.¹⁸

RESULTS

Virus derived from pCHIKic and pONNc were compared with CHIKV (37997) and ONNV (SG650) virus in *Ae. aegypti* and *An. gambiae* mosquitoes (Figure 2). CHIKV (37997) and virus derived from pCHIKic replicated at a similar rate over time, with 100% of the *Ae. aegypti* mosquitoes infected on most days analyzed pi (Figure 3A). Although the infection rate on day 3 pi for pCHIKic was lower than for CHIKV (37997), no significant difference ($P > 0.05$) was found between these two viruses. As expected, on day 14 pi CHIKV

(37997) and the virus derived from pCHIKic was not detected in any of the *An. gambiae* tested. Viral titers decrease from 6.5 or 5.8 \log_{10} TCID₅₀/mL to 0 \log_{10} TCID₅₀/mL between days 0 and 3 pi for both CHIKV (37997) and virus derived from pCHIKic (Figure 2C). The viral titer of CHIKV (37997) in *An. gambiae* increased to an average of 1.4 \log_{10} TCID₅₀/mL with three of seven mosquitoes positive on day 7 pi. None of the *An. gambiae* mosquitoes examined on day 14 pi were infected (Figure 2C). Although the viral growth curves were similar, ONNV (SG650) virus infected significantly more ($P < 0.05$) *Ae. aegypti* than virus derived from pONNc on day 14 pi (Figure 2B). However, there was no significant difference between the titers of these two viruses on day 14 pi in *An. gambiae* (Figure 2D).

Three infectious clones containing all or part of the ONNV structural genes and CHIKV nonstructural genes and three reciprocal clones containing CHIKV structural genes and ONNV nonstructural genes were characterized *in vivo* in *Ae. aegypti* and *An. gambiae* mosquitoes (Figures 3 and 4). All chimeras produced were orally infectious to *Ae. aegypti* mosquitoes, with infection rates ranging from 56% to 100% on day 14 pi (Figure 3A and B). The infection rate in *Ae. aegypti* for each of the three chimeras containing the CHIKV nonstructural genes (CHIK-ONN A, B, and C) was 100% on day 14 pi (Figure 3A). The only chimera to infect *An. gambiae* on day 14 pi was CHIK-ONN C, which infected 38% of the *An. gambiae* at this time point (Figure 3C). A significant difference ($P < 0.05$) in infection rates was detected between two of the chimeras containing the nonstructural genes from ONNV, ONN-CHIK A, and ONN-CHIK C in *Ae. aegypti* on day 14 pi (Figure 3B). There was no significant difference ($P > 0.05$) in

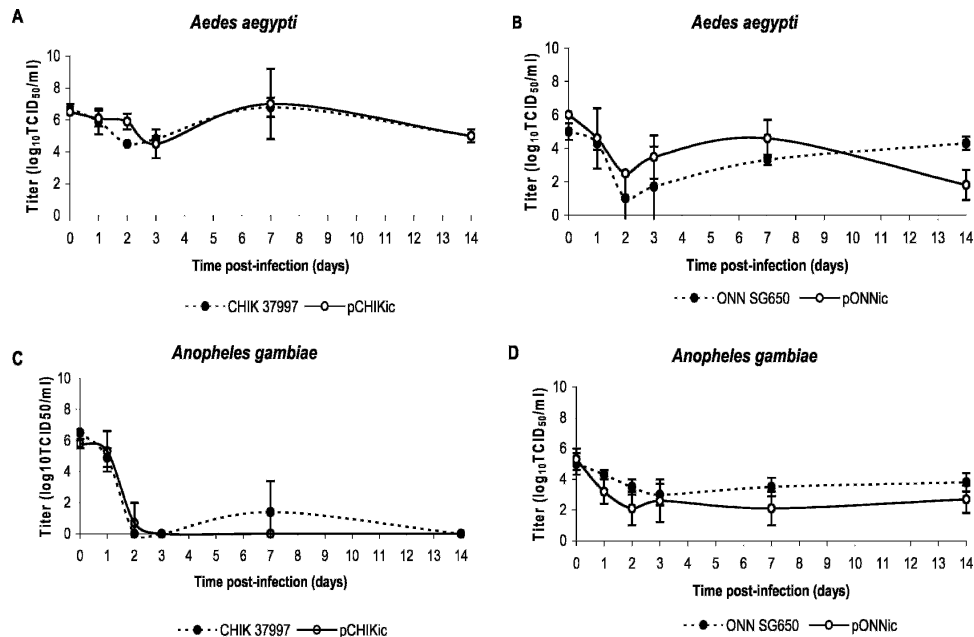


FIGURE 2. Viral titers and growth of viruses over time after a blood meal containing chikungunya virus (CHIKV) (37997), o'nyong nyong virus (ONNV) (SG650), or viruses derived from full-length pCHIKic or pONNc in *Aedes aegypti* (A and B) or *Anopheles gambiae* (C and D) mosquitoes. Bloodmeal titers: CHIKV (37997) 7.95 \log_{10} TCID₅₀/mL;* virus derived from pCHIKic 7.95 \log_{10} TCID₅₀/mL;† ONNV (SG650) 7.3 (± 0.6) \log_{10} TCID₅₀/mL;‡ virus derived from pONNc 6.52 \log_{10} TCID₅₀/mL. Error bars indicate variation between mosquitoes analyzed per time point: ≥ 3 mosquitoes on day 0 post-infection (pi); ≥ 8 mosquitoes on days 2 and 3 pi, except for CHIKV (37997)*; ≥ 8 mosquitoes on days 1, 7, and 14 pi, except for CHIKV (37997)*, pCHIKic.† Six or more mosquitoes were analyzed at each time point for virus derived from pONNc. TCID₅₀ = 50% tissue culture infectious dose. *Vanlandingham and others;¹⁸ †Vanlandingham and others;¹⁹ ‡Two replicates are shown for ONNV (SG650).

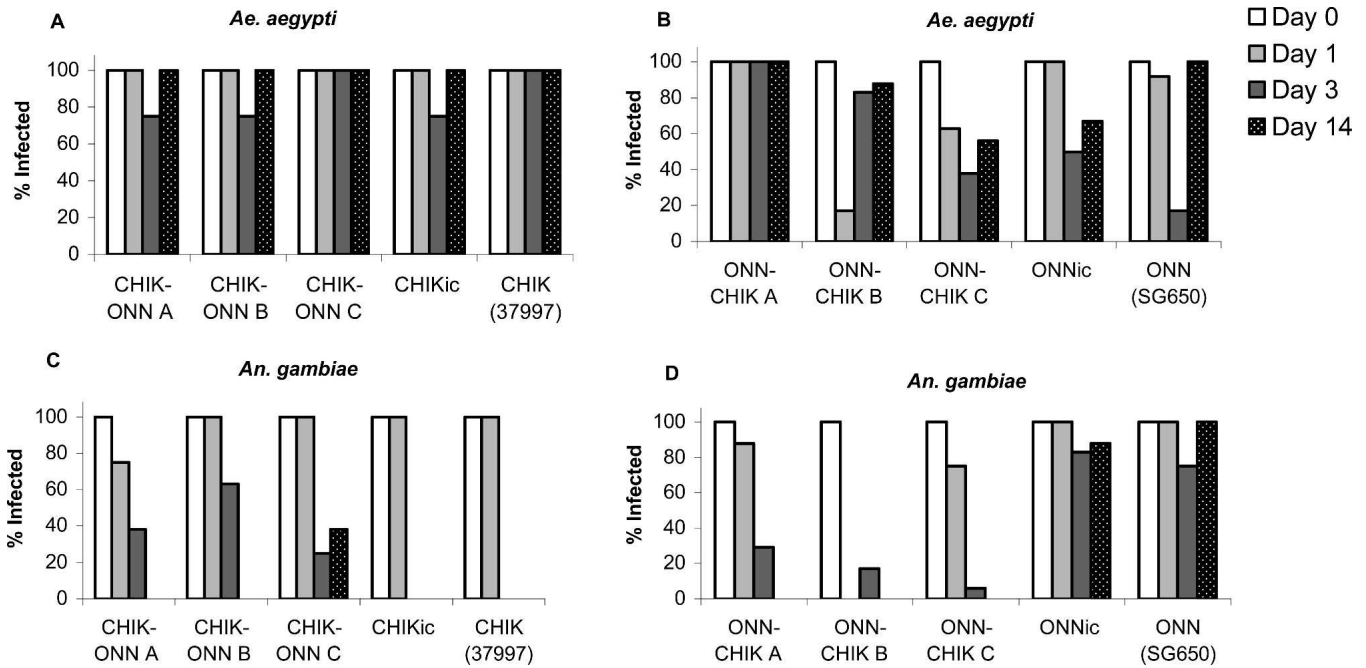


FIGURE 3. Percentage of orally infected *Anopheles gambiae* (A and B) and *Aedes aegypti* (C and D) mosquitoes analyzed by titration of whole mosquitoes on days 0, 1, 3 and 14 post-infection (pi). Bloodmeal titers: CHIK-ONN A $6.52 \log_{10}$ TCID₅₀/mL; CHIK-ONN B $6.52 \log_{10}$ TCID₅₀/mL; CHIK-ONN C $7.52 \log_{10}$ TCID₅₀/mL; ONN-CHIK A $7.52 \log_{10}$ TCID₅₀/mL; ONN-CHIK B $6.95 \log_{10}$ TCID₅₀/mL; ONN-CHIK C $6.95 \log_{10}$ TCID₅₀/mL. Mosquitoes analyzed per time point: three mosquitoes on day 0 pi; eight mosquitoes on days 1, 2, 3, 7, and 14 pi (except for ONN-CHIK B, which had six mosquitoes for days 2, 3, and 7 pi and ONN-CHIK A, which had seven mosquitoes on day 2 pi for *Ae. aegypti* and day 3 pi for *An. gambiae*). Titers and number of mosquitoes analyzed for pCHIKic, CHIKV (37997), pONNc, and ONNV (SG650) are shown in Figure 2. For definitions of other abbreviations, see Figures 1 and 2.

infection rates in *Ae. aegypti* between ONN-CHIK B and ONN-CHIK A or ONN-CHIK B and ONN-CHIK C (Figure 3B).

The interaction of the capsid and the E2 cytoplasmic domain, which is important during virus assembly, was evaluated using two sets of chimeras. The first set contained chimeric viruses that derived the capsid and the E2 cytoplasmic domain from the same virus, either CHIKV or ONNV (CHIK-ONN A and ONN-CHIK A) (Figure 1). The second set contained chimeric viruses containing the capsid and E2

cytoplasmic domain from different viruses (CHIK-ONN B and ONN-CHIK B) (Figure 1). No significant difference ($P > 0.05$) was found in day 14 pi infection rates in *Ae. aegypti* or *An. gambiae* infected with CHIKV nonstructural gene chimeras with both capsid and cytoplasmic domain genes from the same virus or from different viruses (Figure 3A and C). Infection rates in *Ae. aegypti* and *An. gambiae* with the reciprocal clones containing the nonstructural genes from ONNV also did not have a statistically different infection rates on day 14 pi (Figure 3B and D).

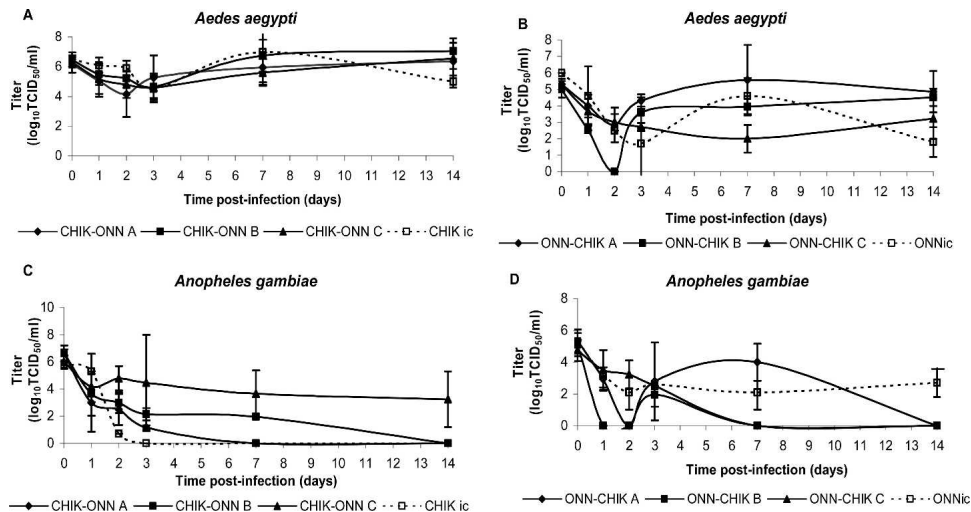


FIGURE 4. Viral titers and growth of viruses over time after a blood meal containing chimeric viruses and virus derived from the full-length pCHIKic or pONNc in *Aedes aegypti* (A and B) and *Anopheles gambiae* (C and D) mosquitoes. Blood meal titers and number of mosquitoes analyzed are shown in Figures 2 and 3. For definitions of abbreviations, see Figures 1 and 2.

The chimeric virus containing the CHIKV E1 and E2 structural genes with the ONNV capsid and nonstructural genes (ONN-CHIK A) (Figure 1) had a significantly increased infection rate ($P < 0.05$) in *Ae. aegypti* mosquitoes on day 14 pi compared with the chimera with the entire set of structural genes from CHIKV (ONN-CHIK C) (Figure 3B). The reciprocal chimeras containing the CHIKV nonstructural genes and either the capsid of CHIKV with the E1 and E2 of ONNV (CHIK-ONN A and B) or the complete ONNV structural genes (CHIK-ONN C) did not infect *Ae. aegypti* at a significantly different rate ($P > 0.05$) (Figure 3A).

The viral titers of the six chimeric viruses were compared *in vivo* in *Ae. aegypti* and *An. gambiae* mosquitoes (Figure 4). The chimeric viruses containing the CHIKV nonstructural genes (CHIK-ONN A, B, and C) produced growth curves that were similar to each other in *Ae. aegypti* (Figure 4A). The CHIK-ONN A and B chimeric viruses also showed similar growth characteristics in *An. gambiae* mosquitoes (Figure 4C). Virus derived from CHIK-ONN C was the only chimeric virus to replicate in *An. gambiae* to day 14 pi (Figure 4C). The reciprocal chimeric viruses, which contained the nonstructural genes from ONNV, were all able to infect *Ae. aegypti* mosquitoes (Figure 4B), but were unable to infect *An. gambiae* mosquitoes on day 14 pi (Figure 4D).

Dissemination rates of viruses in *Ae. aegypti* and *An. gambiae* were analyzed by IFA of dissected salivary glands on days 7 and 14 pi (Table 1). Viruses derived from the full-length infectious clones of CHIKV and ONNV and the six chimeric viruses were found to disseminate from 20% to 100% in *Ae. aegypti* mosquitoes on day 14 pi (Table 1). No significant differences were found between the three clones containing the CHIKV nonstructural genes with all or part of the ONNV structural genes (CHIK-ONN A, B, and C) in *Ae. aegypti*. Virus derived from these clones disseminated in 80–100% of *Ae. aegypti* on day 14 pi. The substitution of all structural genes from CHIKV (ONN-CHIK C) dramatically decreased the dissemination rate in *Ae. aegypti* to 20% on day 14 pi. Significantly fewer ($P > 0.05$) *Ae. aegypti* mosquitoes

were found to have a disseminated infection with virus derived from the ONN-CHIK C infectious clone compared with all other chimeras (Table 1). Only viruses containing the full structural genes of ONNV (virus derived from pONNVic and CHIK-ONN C) were found to disseminate in *An. gambiae* on day 14 pi. Virus derived from pONNVic disseminated in 100% of *An. gambiae* examined compared with CHIK-ONN C virus, which disseminated in 40% of the mosquitoes (Table 1).

DISCUSSION

The chimeric viruses containing ONNV and CHIKV genes that were produced for this project are valid tools to study the determinants of infection in *Ae. aegypti* and *An. gambiae* mosquitoes. The host specificities for the parental viruses and the viruses derived from full-length infectious clones were similar. Therefore, differences in host specificity of the chimeric viruses are due to the nucleotide substitutions within the structural genes. The full-length clones and the six chimeras were infectious to *Ae. aegypti* mosquitoes on day 14 pi, which indicates that all the viruses are viable and able to replicate to sufficient titers to produce infection and to disseminate in this mosquito species. Day 14 pi viral replication and dissemination data are used to standardize comparisons and to ensure that virus is replicating in the mosquitoes analyzed. Earlier time points may only indicate exposure to the virus and not viral replication.

The virus titers obtained from the full-length pONNVic and ONNV (SG650) were not statistically different in *An. gambiae* mosquitoes on day 14 pi (Figure 2D). Therefore, this infectious clone is suitable for analysis of ONNV genes that could potentially expand the host range of CHIKV to infect *An. gambiae* mosquitoes. The day 14 pi viral titer from pONNVic was not as high in *Ae. aegypti* mosquitoes as the ONNV (SG650) (Figure 2B); however, the infectious clone did infect most (67%) of mosquitoes examined at this time point (Figure 3B). Infection and dissemination rates for the virus derived from full-length pCHIKic were not statistically different from the parental virus at day 14 pi in both of the mosquito species examined, thereby enabling accurate comparisons of CHIK-ONN chimeras using this clone (Figure 2A and C). Three day 7 pi *An. gambiae* mosquitoes were infected with CHIKV, whereas none of the pCHIKic mosquitoes were infected past day 3 pi. This may indicate limited, low titer, replication of the parental virus in *An. gambiae*; however, neither virus was able to infect *An. gambiae* on day 14 pi (Figure 2C).

Chimeric viruses are not necessarily less infectious than virus derived from a full-length infectious clone. Comparison of the chimeric viruses with virus derived from full-length pONNVic or pCHIKic showed that there was no significant effect on the ability of the chimeric viruses to infect *Ae. aegypti* mosquitoes on day 14 pi (Figure 3A and B). The only chimeric virus that had a decreased infection rate compared with the viruses derived from either pONNVic or pCHIKic was the ONN-CHIK C chimera (Figure 3B). This chimera infected 56% of *Ae. aegypti* on day 14 pi compared with the virus derived from pONNVic and pCHIKic, which infected 67% and 100% of mosquitoes analyzed (Figure 3A and B). These data are in contrast to previous studies of chimeras based on Sindbis virus (SINV) and Ross River virus (RRV), which indicated that there is a reduced ability of chimeric

TABLE 1

Dissemination rates based on antigen detection in salivary glands by IFA from virus derived from full-length pCHIKic, pONNVic, and six chimeric viruses in *Aedes aegypti* and *Anopheles gambiae* mosquitoes*

Virus (blood meal titer)†	Day pi	<i>Ae. aegypti</i> disseminated Positive/total (%)	<i>An. gambiae</i> disseminated Positive/total (%)
CHIKic (7.95)	7	9/10 (90)	0/10 (0)
	14	5/10 (50)	0/10 (0)
CHIK-ONN A (6.52)	7	6/10 (60)	0/10 (0)
	14	9/10 (90)	0/10 (0)
CHIK-ONN B (6.52)	7	4/10 (40)	0/10 (0)
	14	10/10 (100)	0/10 (0)
CHIK-ONN C (7.52)	7	4/10 (40)	0/10 (0)
	14	8/10 (80)	4/10 (40)
ONN-CHIK C (6.95)	7	2/10 (20)	0/10 (0)
	14	2/10 (20)	0/10 (0)
ONN-CHIK B (6.95)	7	5/5 (100)	0/10 (0)
	14	9/10 (90)	0/10 (0)
ONN-CHIK A (7.52)	7	8/10 (80)	0/10 (0)
	14	8/10 (80)	0/10 (0)
ONNVic (6.52)	7	0/5 (0)	1/10 (10)
	14	3/5 (60)	10/10 (100)

* pCHIKic = chikungunya infectious clone; pONNVic = o'nyong nyong infectious clone; pi = postinfection.

† Blood meal titers are reported in log₁₀ 50% tissue culture infectious dose/mL.

alphaviruses to replicate in various cell lines.²⁶ The chimeric SIN-RR viruses did not replicate as well in cell culture as the parental viruses. It was concluded that the chimeras were defective in their interactions between the nonstructural and structural regions leading to inefficient RNA replication.²⁷ Our study suggests this to be a virus-specific phenomenon.

Four additional chimeras were produced to examine the role of the individual structural genes in determining host specificity. Chimeric viruses to evaluate the interaction between the E2 cytoplasmic domain and the capsid protein were examined in *Ae. aegypti* and *An. gambiae* (Figure 1). This interaction is important in the late stages of virus assembly within the cell.²⁷ A chimeric RRV in which the capsid sequence was replaced with the capsid of SINV had reduced virion production.²⁸ Virion production increased when seven amino acids located in the cytoplasmic domain of the E2 gene were replaced with SINV sequence.²⁸ Virus that was defective in the capsid and E2 glycoprotein interactions was deficient in budding.²⁸ In contrast, chimeras of SINV containing capsid sequence from either RRV or Simliki Forest virus did not have reduced virion production.²⁹ In our study, chimeric viruses of ONNV and CHIKV containing the capsid and the cytoplasmic domain of either the same virus or of a different virus (Figure 1) did not show a significant change in the infection rate of *Ae. aegypti* or *An. gambiae* on day 14 pi (Figure 3). The dissemination rates of these chimeras were similar, with CHIK-ONN A and B disseminating in 90% and 100% of the *Ae. aegypti*, respectively, and in 0% of the *An. gambiae* examined on day 14 pi (Table 1). The reciprocal chimeric viruses ONN-CHIK A and B had similar infection and dissemination rates in both the *Ae. aegypti* and *An. gambiae* mosquitoes analyzed (Figure 3B and D and Table 1). ONN-CHIK A and B disseminated in 80% and 90% of the *Ae. aegypti* analyzed and in 0% of the *An. gambiae* analyzed (Table 1). These results indicate that the capsid and cytoplasmic domain interactions in ONNV and CHIKV do not play a role in the ability of these viruses to infect different species of mosquitoes.

The primary molecular determinant of viral host range enabling infection of *An. gambiae* is found in all of the structural genes of ONNV. Virus derived from the full-length pCHIKic and virus from three chimeras containing the CHIKV nonstructural genes (CHIK-ONN A, B, and C) infected 100% of *Ae. aegypti* on day 14 pi (Figure 3A). A significant difference ($P < 0.05$) was found between the virus derived from pCHIKic and CHIK-ONN C on day 14 pi in *An. gambiae* (Figure 3C). None of the mosquitoes analyzed at this time point were infected with the full length pCHIKic virus whereas, 38% of *An. gambiae* were infected with virus derived from CHIK-ONN C (Figure 3C). The dissemination rates were high for virus derived from CHIK-ONN A, B, C and pCHIKic in *Ae. aegypti* (Table 1). Virus derived from CHIK-ONN C was the only chimeric virus to disseminate in *An. gambiae* (Table 1). The virus derived from full-length pONNVic was highly infectious and had a high dissemination rate in *An. gambiae* on day 14 pi (Figure 4D and Table 1). These data demonstrate that the ONNV structural genes are important in allowing infection and dissemination in *An. gambiae* mosquitoes and indicate that substitution of partial structural genes from ONNV do not change the host range of CHIKV.

The chimeric infectious clones produced for this research

represent useful tools for the investigation of the molecular basis of virus-vector relationships, and will facilitate further studies to identify the role of specific nucleotides in the infection and dissemination of arboviruses in mosquitoes. Additional chimeric viruses, such as ones examining the 5' and 3' NCRs and individual nonstructural genes, will enable further studies of viral host determinants of these viruses. The only chimeric virus produced from this study that was able to infect *An. gambiae* mosquitoes was the chimeric virus containing viral genes encoding all of the structural proteins of ONNV. The ONNV nonstructural genes enhance viral infection of *An. gambiae* mosquitoes, as indicated by the reduced infection rate of the chimeric virus containing the CHIKV nonstructural genes with the ONNV structural genes. However, the ONNV viral structural proteins alone enable the chimeric CHIK-ONN C virus to infect *An. gambiae* mosquitoes.

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