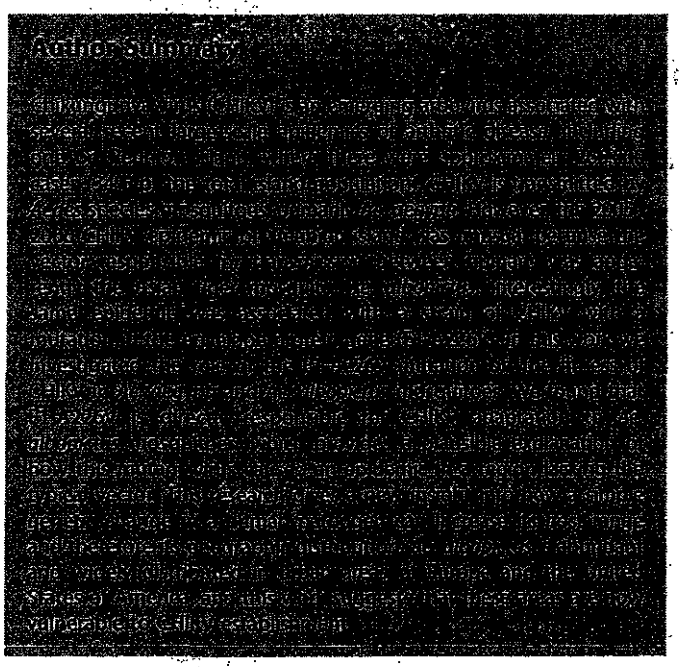

outbreak [32]. The reason for this was unclear but it was hypothesized that the E1-A226V mutation might influence infectivity of CHIKV for mosquito vectors [11,32]. Interestingly, earlier studies have identified that a P→S mutation in the same position of the E1 glycoprotein is responsible for the modulation of Semliki Forest virus's (SFV, a member of the alphavirus family) requirements for cholesterol in the target membrane [33]. It also has been shown that the presence of this mutation results in more efficient growth of SFV in *Ae. albopictus* mosquitoes [34]. However, no evidence has been presented to directly correlate the release from the cholesterol dependence, associated with the E1-P226S mutation in SFV, with a growth advantage in *Ae. albopictus*. It is unknown if dependence on cholesterol for growth in mosquito cells is a requirement of all alphaviruses.

To test the hypothesis that the E1-A226V mutation might influence the fitness of CHIKV in mosquito vectors, we compared the effect of this mutation on CHIKV mosquito infectivity, the ability to disseminate into heads and salivary glands, and the relative fitness in competition assays for transmission by *Ae. albopictus* and *Ae. aegypti* to suckling mice. We also analyzed the effect of the E1-A226V mutation on CHIKV cholesterol dependence for growth in mosquito C6/36 (*Ae. albopictus*) cells. Here we report findings that a single nucleotide change, which arose during the epidemic, significantly increases fitness of the virus for *Ae. albopictus* mosquitoes and was associated with CHIKV dependence on cholesterol in the mosquito cell membrane. This change likely enhanced CHIKV transmission by an atypical vector and contributed to the maintenance and scale of the epidemic.

Results

Effect of E1 A226V Mutation on Fitness of CHIKV in *Ae. albopictus* Mosquitoes

To test the hypothesis that the E1-A226V mutation altered CHIKV infectivity for *Ae. albopictus* mosquitoes, CHIKV infectious clones derived from an epidemic Reunion island human isolate were used [15], including one clone (LR-GFP-226V) expressing enhanced green fluorescent protein (eGFP). Clones were further engineered to express E1 protein containing an alanine at position E1–226 (LR-GFP-226A) representing the CHIKV genotype prevalent prior to the outbreak gaining momentum (Figure S1). RNAs produced from both clones (LR-GFP-226V and LR-GFP-226A) have comparable specific infectivity values, produced similar viral titers following transfection into BHK-21 cells (Table S1) and have similar growth kinetics in mosquito (C6/36) and mammalian (BHK-21) cells lines (Figure S2A and S2B).

The relative infectivity of LR-GFP-226V and LR-GFP-226A viruses was analyzed in female *Ae. albopictus* mosquitoes orally exposed to serial 10-fold dilutions of CHIKV (LR-GFP-226 V or A). To determine whether infection rates correlate with blood meal titer, midguts dissected from mosquitoes at 7 days post-infection (dpi) were analyzed for foci of eGFP-expressing cells by fluorescence microscopy (Figure 1A; Table 1). In two independent experiments, LR-GFP-226V virus was found to be approximately 100-fold more infectious to *Ae. albopictus* than LR-GFP-226A virus ($p<0.01$). To test if the infectivity phenotype was directly linked to the mutation, the complementary reverse mutation, E1-A226V, was introduced into an infectious clone of a West African CHIKV strain, 37997-GFP (37997-GFP-226A) (Figure S1). The Reunion and 37997 strains of CHIKV are distantly related, with only 85% nucleotide sequence identity. The parental 37997-GFP-226A and the 37997-GFP-226V viruses were indistinguishable in cell culture experiments (Table S1; Figure S2C and S2D); however, *in vivo* experiments in *Ae. albopictus* mosquitoes revealed that the E1-A226V mutation significantly decreases the oral infectious dose 50 (OID_{50}) value for the 37997-GFP-226V virus ($p<0.01$) to an extent similar to that observed for LR-GFP-226V virus (Figure 1B; Table 1). These data conclusively demonstrate that the single E1-A226V point mutation is therefore sufficient to significantly reduce the OID_{50} of the 37997-GFP virus ($p<0.01$) in *Ae. albopictus* mosquitoes equivalent to that observed for the LR-GFP-226V virus (Figure 1A; Table 1).

To further evaluate viral fitness of the epidemic CHIKV E1-A226V mutation in *Ae. albopictus*, viral competition experiments were performed. Although our CHIKV eGFP-expressing infectious clones, have similar infection properties in mosquitoes as wild-type viruses [15,35], to address potential concerns that eGFP expression might influence OID_{50} values, we constructed LR-226A and LR-ApaI-226V viruses without eGFP and employed them in viral competition experiments (Figures 2A and S1). LR-ApaI-226V was derived from previously described CHIK-LR ic, by the introduction of a silent marker mutation, A6454C, in order to add an *ApaI* restriction site into the coding sequence. It was shown that the A6454C mutation does not affect the specific infectivity value (Table S1), the viral titer after RNA transfection into BHK-21 cells value (Table S1), the viral growth kinetics in BHK-21 and C6/36 cells (Figure S3), infectivity for and viral titers in *Ae. aegypti* and *Ae. albopictus* mosquitoes (Table S2), or viral fitness for growth in BHK-21 and C6/36 cells as determined by competition assay (Figure S4). These data indicate that the introduced mutation is indeed silent and does not affect the fitness of LR-ApaI-226V.

For viral competition experiments LR-ApaI-226V virus (10^7 plaque-forming units (pfu)) was mixed with an equal

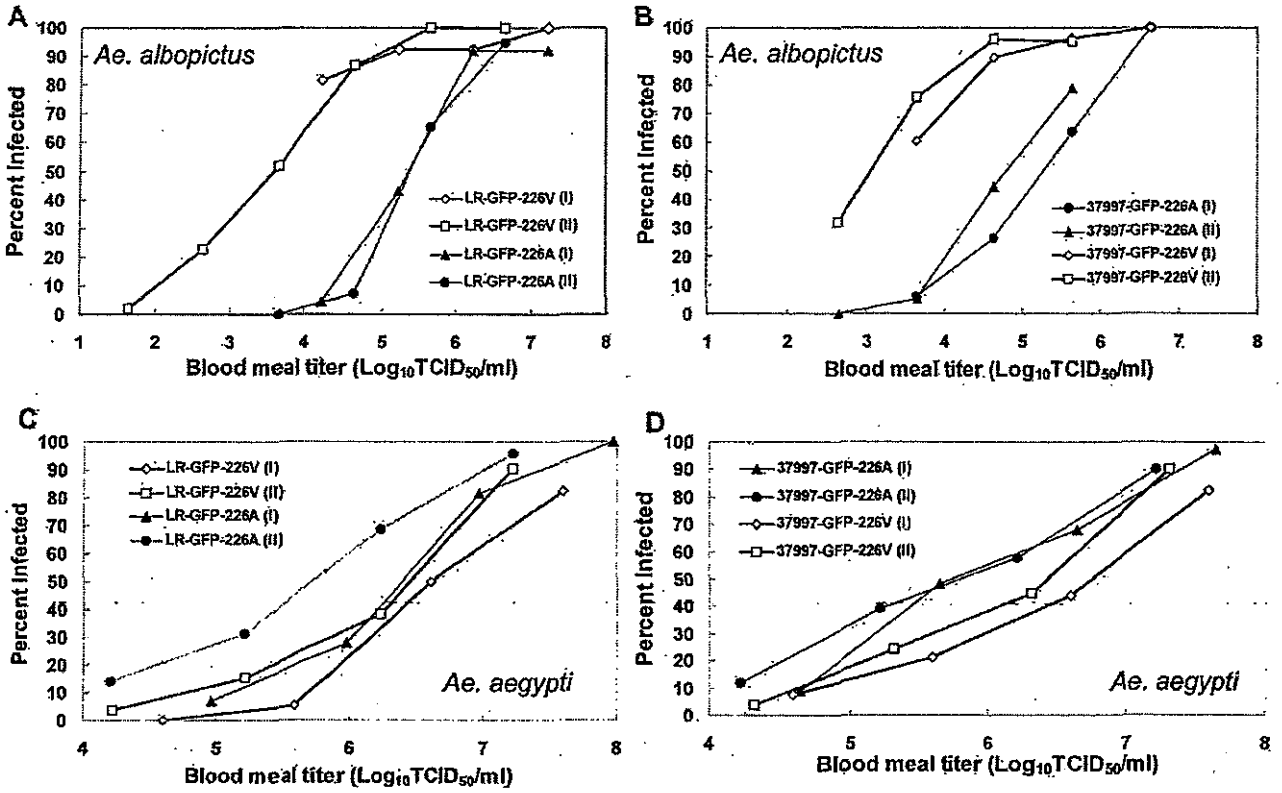

Figure 1. Effect of E1-A226V Mutation on CHIKV-GFP Viruses *Ae. albopictus* and *Ae. aegypti* Midgut Infectivity

Percent of orally infected *Ae. albopictus* (A, B) and *Ae. aegypti* (C, D) mosquitoes presented with blood meals containing various concentration of eGFP-expressing CHIK viruses. Serial 10-fold dilutions of viruses in the backbone of Reunion (LR-GFP-226V and LR-GFP-226A) (A, C) and 37997 (37997-GFP-226A and 37997-GFP-226V) (B, D) strains of CHIKV were made in L-15 medium followed by mixing the samples with defibrinated sheep blood. Mosquitoes were dissected at 7 dpi and eGFP expression in infected midguts was analyzed by fluorescence microscopy. A mosquito was considered infected if at least one foci of eGFP-expressing cells was present in the midgut. The experiments were performed twice for each virus (I and II).

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amount of LR-226A virus. LR-ApaI-226V and LR-226A viruses are indistinguishable in cell culture experiments (Figure S3). Mixtures of LR-ApaI-226V and LR-226A viruses were orally presented to *Ae. albopictus* mosquitoes in a blood meal, and midguts were examined at 7 dpi. The relative amount of RNA derived from LR-ApaI-226V in the midgut cells increased 5.7±0.6 times as compared to the initial relative amount of LR-ApaI-226V RNA in the blood meal sample (Figure 2B). These data support our observation that the E1-A226V mutation enhances infectivity of CHIKV for *Ae. albopictus* mosquitoes and furthermore demonstrate that the mutation could provide an evolutionary advantage over E1-226A viruses in an atypical vector and may have perpetuated the outbreak in a region where *Ae. albopictus* was the predominant anthropophilic mosquito species.

To determine if the enhanced midgut infectivity associated with the E1-A226V mutation may result in more efficient viral dissemination into secondary tissues, the kinetics of viral dissemination by LR-GFP-226V and LR-GFP-226A into salivary glands, and competition between LR-ApaI-226V and LR-226A for dissemination into mosquito heads were analyzed (Figure 3A and 3B). LR-GFP-226V virus disseminated more rapidly into *Ae. albopictus* salivary glands at all time points, with a significant difference at 7 dpi ($p=0.044$, Fisher's exact test). Similarly, in three of four replicates of competition experiments, RNA from LR-ApaI-226V virus was dramatically more abundant in the heads of *Ae. albopictus* mosquitoes as compared to RNA from LR-226A (Figure 3B, lines 1, 3, 4), although in one replica LR-ApaI-226V RNA was only slightly more abundant as compared to the initial viral RNA ratio (Figure 3B, line 2). This variability of the results may be due to random pooling of mosquito heads. Thus, replicate two may have included more heads negative for LR-

Table 1. $Log_{10}OID_{50}$/ml for CHIKV in *Ae. albopictus* Mosquitoes

Backbone	Exp[a]	Virus	Mosquitoes Analyzed[b]	Log_{10} $OID_{50}\pm CI_{95}$[c]	p Value
CHIK Reunion	1	LR-GFP-226V	98	<4.22	p<0.01
		LR-GFP-226A	101	5.42±0.29	
	2	LR-GFP-226V	171	3.52±0.28	p<0.01
		LR-GFP-226A	93	5.48±0.23	
CHIK 37997	1	37997-GFP-226A	131	5.20±0.22	p<0.01
		37997-GFP-226V	138	3.31±0.42	
	2	37997-GFP-226A	129	4.90±0.25	p<0.01
		37997-GFP-226V	136	3.06±0.32	

OID_{50} values and confidence intervals were calculated using PriProbit (version 1.63).

[a]Experiment number.

[b]Number of mosquitoes used to estimate $Log_{10}OID_{50}$/ml.

[c]95% confidence intervals.

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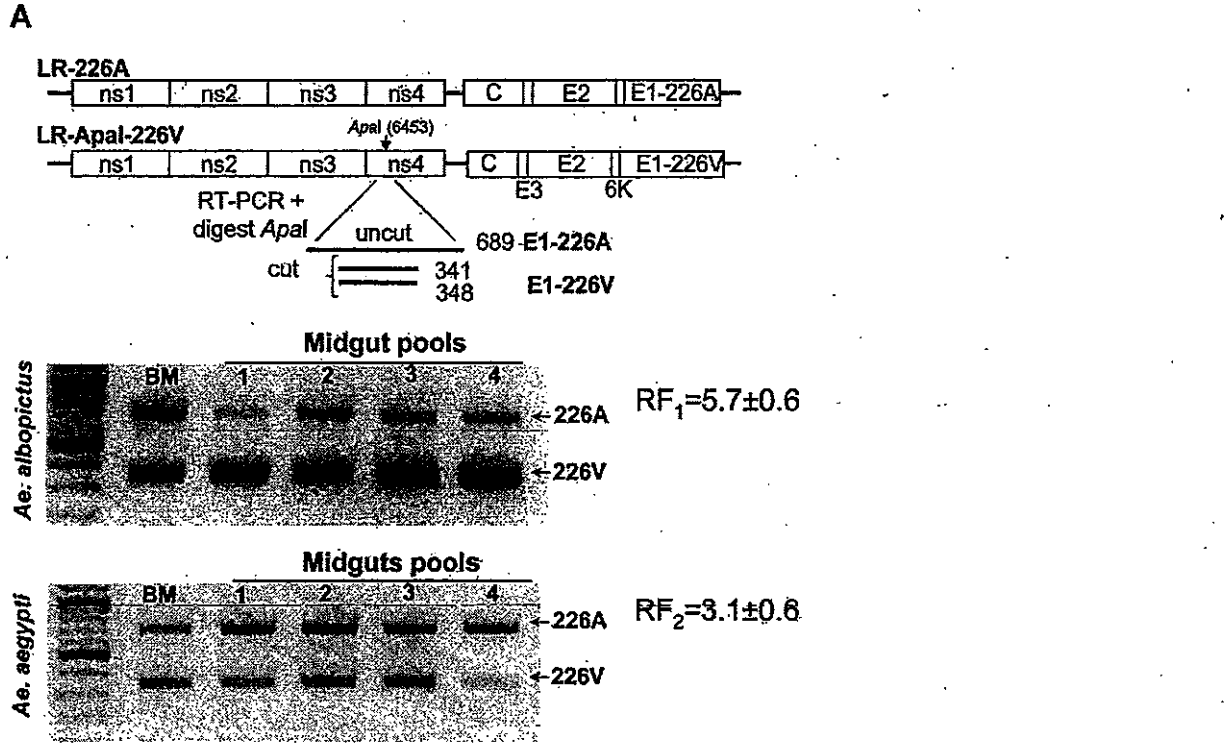

Figure 2. Schematic Representation of Competition Experiments (A) and Competition between LR-ApaI-226V and LR-226A Viruses for Colonization of Midgut cells of *Ae. albopictus* (B) and *Ae. aegypti* (C) Mosquitoes

10^7 pfu of LR-ApaI-226V and LR-226A were mixed and orally presented to *Ae. albopictus* (B) and *Ae. aegypti* (C). Viral RNAs were extracted from four pools of eight to ten midguts at 7 dpi. RT-PCR products were digested with *ApaI*, separated in 2% agarose gel, and gels were stained using ethidium bromide.

BM - initial ratio of LR-ApaI-226V and LR-226A in blood meal samples. 1–4 ratio of LR-ApaI-226V and LR-226A RNA in four independent replicas of the eight to ten midguts per replica.

Relative fitness (RF_1) of LR-Apa-226V to LR-226A was calculated as a ratio between 226V and 226A bands in the sample, divided by the control ratio between 226V and 226A in the blood meal.

Relative fitness (RF_2) of LR-226A to LR-Apa-226V was calculated as a ratio between 226A and 226V bands in the sample, divided to the control ratio between 226A and 226V in the blood meal.

Results expressed as the average of four replicas ± standard deviation (SD).

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ApaI-226V relative to heads positive for LR-226A RNA. Another possibility is that at some point during viral dissemination from the midguts into mosquito heads, LR-226A may replicate more rapidly than LR-ApaI-226V. To further investigate this relationship, *Ae. albopictus* mosquitoes were orally presented with either LR-ApaI-226V or LR-226A and whole mosquito body viral titers were compared at different time points pi. Surprisingly, no significant differences between viral titers were found, with the exception of 1 dpi, where the LR-ApaI-226V titer was 0.5 Log_{10} tissues culture infectious dose 50 percent end point titer (Log_{10} $TCID_{50}$/mosquito) higher than of the LR-226A titer (Figure 4A). This may be due to more efficient colonization of *Ae. albopictus* midguts by LR-ApaI-226V. The absence of significant differences in viral titers at later time points may be due to variation in viral titers among individual mosquitoes. Competition between LR-ApaI-226V and LR-226A was analyzed at different time points in order to investigate the relationship between replication of LR-ApaI-226V and LR-226A viruses in *Ae. albopictus* mosquitoes (Figure 4B). As expected, the viral RNA from LR-ApaI-226V was predominant at the early time points of 1 and 3 dpi. Interestingly, between 3 and 5 dpi the viral RNA ratio shifted toward LR-226A virus indicating that at these time points, LR-226A replicates more efficiently in some mosquito tissues (Figure 4B). This short period of time may have a slight effect on the overall outcome of competition for dissemination into salivary glands because there is a reverse shift in the RNA ratio between days 5 and 7 toward LR-ApaI-226V virus, which continues through 14 dpi. These data indicate that the E1-A226V mutation not only increases midgut infectivity but also is associated with more efficient viral dissemination from the midgut into secondary organs, suggesting that the E1-A226V mutation would increase transmissibility of CHIKV by *Ae. albopictus* mosquitoes.

A competition assay between LR-ApaI-226V and LR-226A viruses was used to examine transmission by *Ae. albopictus* to suckling mice to assess the potential for the E1-A226V mutation to influence virus transmission. *Ae. albopictus* mosquitoes were orally presented with a mixture of LR-ApaI-226V and LR-226A viruses and at 14 dpi were allowed to feed on suckling mice. Mice were sacrificed and bled on day 3 following exposure and the presence of CHIKV RNA in the blood was analyzed by RT-PCR followed by restriction digestion with *ApaI* (Figure 5B). Blood obtained from 100% of experimental mice contained detectible amounts of viral

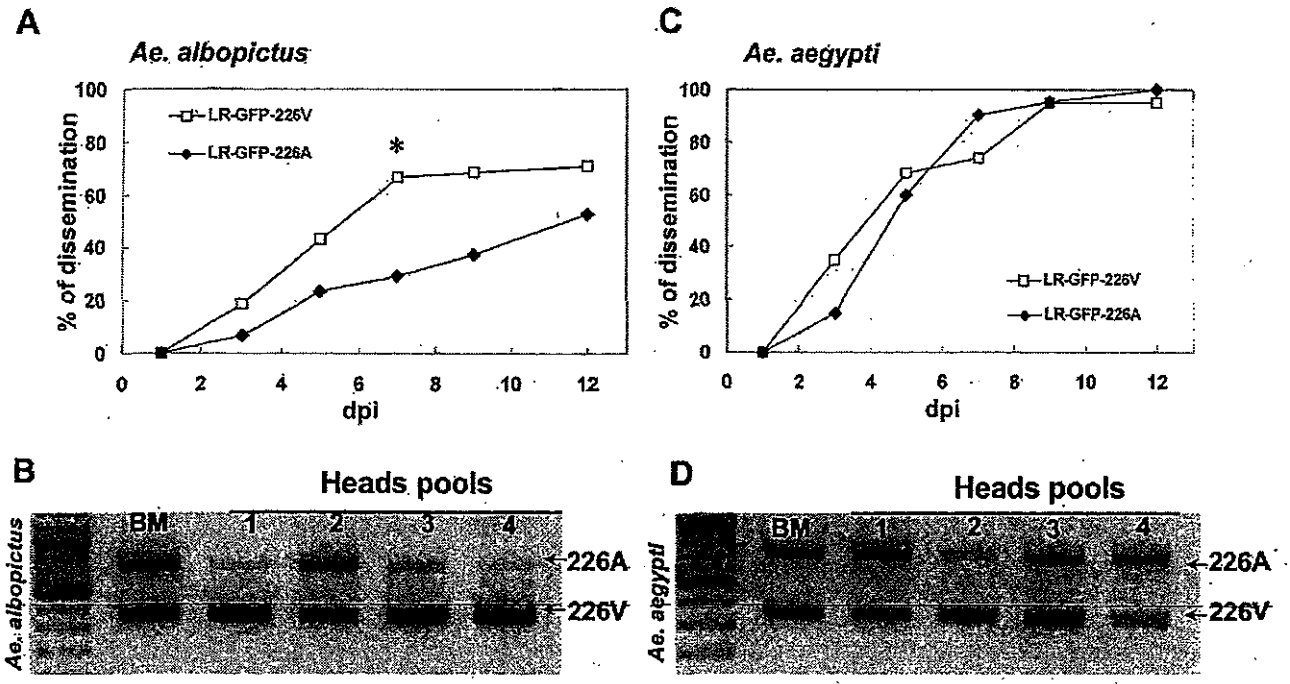

Figure 3. Effect of E1-A226V Mutation on CHIKV Dissemination into Salivary Glands and Heads of *Ae. albopictus* and *Ae. aegypti* Mosquitoes

Ae. albopictus (A) and *Ae. aegypti* (C) mosquitoes were orally infected with LR-GFP-226V and LR-GFP-226A. At the indicated time points, 16–21 mosquitoes were dissected and salivary glands were analyzed for eGFP expression. Percent of dissemination was estimated as a ratio of the number of mosquitoes with eGFP-positive salivary glands to the number of mosquitoes with eGFP-positive midguts. For *Ae. albopictus*, infectious blood meal titers were 5.95 and 6.52 $Log_{10}TCID_{50}$/ml for LR-GFP-226V and LR-GFP-226A, respectively. For *Ae. aegypti*, the infectious blood meal titer was 6.95 $Log_{10}TCID_{50}$/ml for both LR-GFP-226V and LR-GFP-226A viruses. Dissemination rates were compared statistically by Fisher's exact test using SPSS version 11.5. Asterisk indicates $p < 0.05$.

(B and D) Competition between LR-ApaI-226V and LR-226A for dissemination into heads of *Ae. albopictus* and *Ae. aegypti* mosquitoes. 10^7 pfu of LR-ApaI-226V and LR-226A were mixed and orally presented to *Ae. albopictus* (B) and *Ae. aegypti* (D). Viral RNAs were extracted from four pools of five heads collected at 12 dpi. RT-PCR products were digested with *ApaI*, separated in 2% agarose gel, and gels were stained using ethidium bromide. BM - initial ratio of LR-ApaI-226V and LR-226A in blood meal samples. 1–4 ratio of LR-ApaI-226V and LR-226A RNA in four independent replicas of the five pooled heads per replica.

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RNA, indicating that virus was transmitted by *Ae. albopictus* mosquitoes to suckling mice. More importantly, in all six mice analyzed, RNA derived from LR-ApaI-226V was the predominant viral RNA species, indicating that under the conditions of competition for transmission, the E1-A226V mutation directly increases CHIKV transmission by *Ae. albopictus* mosquitoes. Interestingly, in the control experiment in which mice were subcutaneously inoculated with ≈ 50 pfu of 1:1 mixture of LR-ApaI-226V and LR-226A viruses, RNAs from both viruses were readily detected and no difference was observed in the viral RNA ratio 3 dpi (Figure 5A) indicating that at least in mice, E1-A226V is not associated with changes in viral fitness.

Effect of E1 A226V Mutation on Fitness of CHIKV in *Ae. aegypti* Mosquitoes

Since the E1-A226V mutation confers a fitness advantage in *Ae. albopictus*, it is unknown why this mutation had not been observed previously. It is possible that this change might have a deleterious effect on viral fitness in the vertebrate host, although our data of direct competition of LR-ApaI-226V and LR-226A viruses in suckling mice (Figure 5A) and analysis of CHIKV cellular tropism of four clinical isolates from Reunion (which have either A or V at position E1–226) [14], suggest that this is unlikely. An alternative hypothesis is that the E1-A226V mutation might compromise the fitness of CHIKV or have neutral fitness effects in the mosquito species which served as a vector for CHIKV prior to its emergence on Reunion island. Since *Ae. aegypti* has generally been regarded as the main vector for CHIKV prior to the emergence on Reunion island, we analyzed the effect of the E1-A226V mutation on fitness of CHIKV in *Ae. aegypti*.

In contrast to the results obtained in *Ae. albopictus* mosquitoes, OID_{50} values of viruses containing the E1-226V in the backbone of the Reunion and 37997 strains of CHIKV were approximately 0.5 $Log_{10}OID_{50}$/ml higher than the OID_{50} values of E1-226A viruses in all experiments using *Ae. aegypti*. These differences were statistically significant for one out of two replicates for each virus pair (Figure 1C and 1D; Table 2). A competition assay examining LR-ApaI-226V and LR-226A virus infection in *Ae. aegypti* midguts, demonstrated that LR-226A virus out-competed LR-ApaI-226V virus at 7 dpi in all four replicates using ten midguts per replicate and that the amount of LR-226A RNA increased on average 3.1 times as compared to the initial blood meal RNA ratio (Figure 2C). These data suggest that the E1-A226V mutation has a slight negative effect on CHIKV infectivity of *Ae. aegypti* midguts.

The effect of the E1-A226V mutation on the ability of CHIKV to disseminate into *Ae. aegypti* secondary organs was also analyzed (Figure 3C and 3D). LR-GFP-226V and LR-GFP-

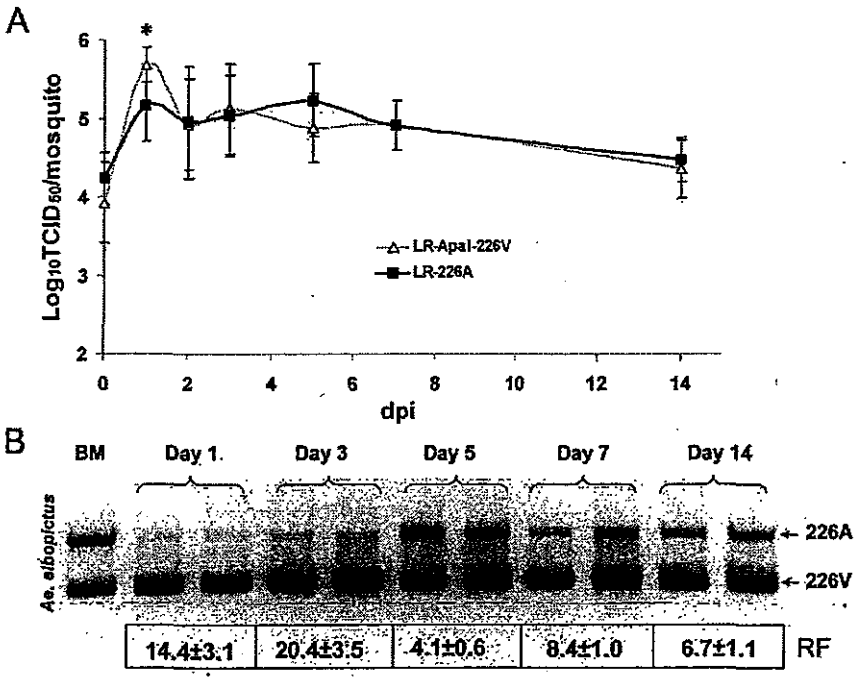

Figure 4. Effect of E1-A226V Mutation on CHIKV Kinetics of Viral Growth in Bodies of *Ae. albopictus* Mosquitoes

(A) Virus production in orally infected *Ae. albopictus* mosquitoes. Infected mosquitoes were sampled at 0, 1, 2, 3, 5, 7, and 14 dpi and titrated on Vero cells to estimate average titer ± standard deviation of eight whole mosquitoes. Differences in viral titers were analyzed by pairwise t-tests. Asterisk indicates $p < 0.05$.

(B) Kinetics of competition between LR-ApaI-226V and LR-226A in bodies of *Ae. albopictus* mosquitoes. 10^7 pfu of LR-ApaI-226V and LR-226A were mixed and orally presented to *Ae. albopictus*. Infected mosquitoes were sampled at 1, 3, 5, 7, and 14 dpi. For each time point, viral RNA was extracted from two pools of ten mosquitoes.

BM - initial ratio of LR-ApaI-226V and LR-226A in blood meal samples.

RF - relative fitness of LR-Apa-226V to LR-226A was calculated as a ratio between 226V and 226A bands in the sample, divided to the control ratio between 226V and 226A in the blood meal. Results expressed as average of two replicas ± standard deviation.

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226A viruses both have similar kinetics of dissemination into salivary glands following oral infection using titers 1–2 $Log_{10}TCID_{50}$ higher than their OID_{50} value in *Ae. aegypti* (Figure 3C). In a competition assay, both LR-ApaI-226V and LR-226A viruses disseminated similarly into the heads of *Ae. aegypti*. In two of four replicas, there was a slight increase in the relative amount of LR-226A RNA (Figure 3D, lines 1, 4); whereas the other two replicas showed a decrease in LR-226A RNA (Figure 2D, lines 2, 3), relative to the initial ratio of the RNA of LR-ApaI-226V and LR-226A viruses in the blood meal. A competition of LR-ApaI-226V and LR-226A viruses for transmission by *Ae. aegypti* to suckling mice was also analyzed (Figure 5C). In contrast to transmission by *Ae. albopictus* mosquitoes, five out of six mice fed upon by *Ae. aegypti* contained comparable amounts of RNA derived from both viruses and only one out of six mice contained RNA derived exclusively from LR-ApaI-226V.

E1-A226V Mutation Modulates Cholesterol Dependence of CHIKV

It has been previously shown that a P→S mutation in the same E1–226 position of SFV releases cholesterol dependence of the virus in C6/36 cells [33] and results in significantly more rapid growth of SFV in *Ae. albopictus* mosquitoes after intrathoracic inoculation [34]. To determine if a requirement for cholesterol in the cell membrane is important for CHIKV, we analyzed cholesterol dependence of CHIKV E1-226A and E1-226V viruses (Figure 6). Growth curves of E1-226A and E1-226V viruses in the background of Indian Ocean and West African strains of CHIKV were almost indistinguishable when grown in C6/36 cells maintained in L-15 supplied with standard 10% FBS (Figure 6A). However, when the cells were depleted of cholesterol, LR-226A and 37997-226A viruses replicated significantly more rapidly than LR-226V and 37997-226V viruses, reaching 3 $Log_{10}TCID_{50}$/ml higher titer at 1, 2 and 3 dpi (Figure 6B). These data indicate that adaptation of CHIKV to *Ae. albopictus* mosquitoes coincides with CHIKV dependence on cholesterol in the target cell membrane.

Discussion

The CHIKV outbreak in Reunion is unique because it is the first well-documented report of an alphavirus outbreak for which *Ae. albopictus* was the main vector. Interestingly, this was also the first Chikungunya epidemic during which fatal infections were reported. Our data clearly indicate that an E1-A226V mutation in CHIKV results in increased fitness of CHIKV in *Ae. albopictus* mosquitoes with respect to midgut infectivity, dissemination to the salivary glands, and transmission to a vertebrate species. These data demonstrate that a single E1-A226V mutation is sufficient to dramatically increase the ability of different strains of CHIKV to infect *Ae. albopictus* mosquitoes and that this substitution requires no

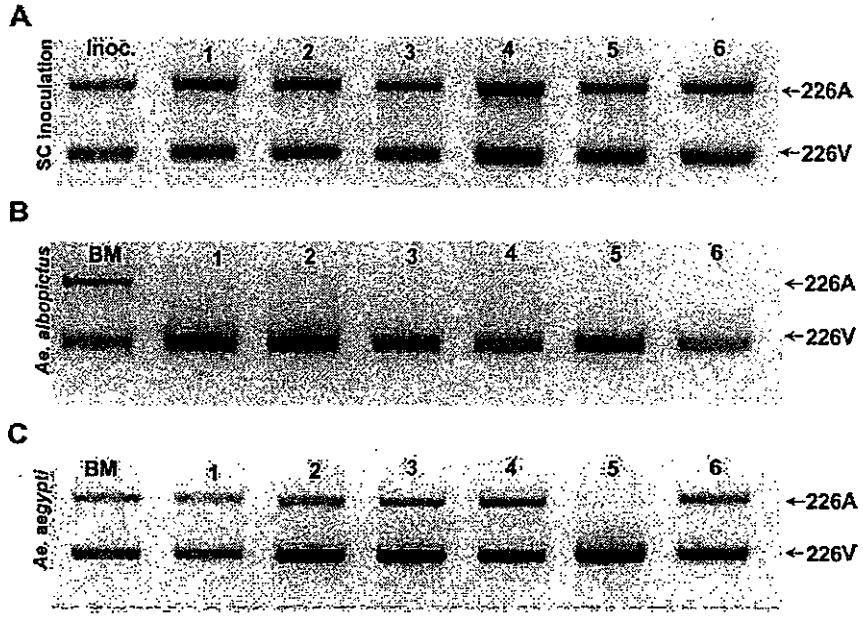

Figure 5. Effect of E1-A226V Mutation on CHIKV Transmission by *Ae. albopictus* and *Ae. aegypti* Mosquitoes

(A) Six 2- to 3-day-old suckling mice (Swiss Webster) were subcutaneously infected with a 20-μl mixture of ≈ 25 pfu LR-Apa-226V and ≈ 25 pfu of LR-226A viruses.

(B and C) *Ae. aegypti* and *Ae. albopictus* mosquitoes were presented with a blood meal containing 10^7 pfu/ml of LR-Apa-226V and 10^7 pfu/ml of LR-226A viruses. At 13 dpi, ten to 15 mosquitoes were placed in separate paper cartons and starved for 24 h. The next day, the mosquitoes in each carton were presented with a 2- to 3-day-old suckling mouse (Swiss Webster).

Mice were returned to their cage and sacrificed on day 3 post-exposure. Blood from each individual mouse (≈ 50 μl) was collected and immediately mixed with 450 μl of TRIzol reagent for RNA extraction.

BM and inoc. - initial ratio of LR-Apal-226V and LR-226A in blood meal samples and inoculum for subcutaneous infection. 1–6 ratio of LR-Apal-226V and LR-226A RNA in six individual mice.

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additional adaptive mutations to gain intermolecular compatibility. These complimentary experimental data demonstrate that a single mutation is sufficient to modify viral infectivity for a specific vector species and as a consequence, can fuel an epidemic in a region that lacks the typical vector. These observations provide the basis for an explanation of the observed rapid shift among CHIKV genotypes to viruses containing the E1-A226V mutation during the Reunion outbreak [32].

Interestingly, our data and data from previous studies [36,37] indicate that prior to acquiring the E1-A226V mutation, CHIKV is capable of producing high enough viremia in humans to efficiently infect *Ae. albopictus* mosquitoes. One explanation of the evolutionary force which allowed CHIKV to be selected so rapidly into a CHIKV strain which is adapted to *Ae. albopictus*, is that the increased infectivity (lower OID_{50}) of CHIKV E1-A226V mutants for *Ae. albopictus* means that the human viremic thresholds required for *Ae. albopictus* infection would likely occur earlier and be sustained for longer. Several recent studies indicate that during the course of human viremia, which last up to 6 days, CHIKV loads can reach up to $3.3x10^9$ RNA copies per ml of the blood [38,39], which corresponds to 6–7 $Log_{10}TCID_{50}$/ml [39]. Earlier studies that utilized a suckling mouse brain titration protocol, which is more sensitive than titration on Vero cells, also found that human viremia often exceeded 6 $Log_{10}SMICLD_{50}$/0.02 ml [40]. Based on viremia studies in rhesus monkeys that can develop up to 7.5 Log/ml if assayed by suckling mice brain titration [41] and a maximum viremia of only 5.5 Log_{10}/ml based on Vero cell titration [42], we believe that viremias in humans would correlate to 6–7 $Log_{10}TCID_{50}$/ml. From these data we calculate that the maximum virus load which can be achieved in human blood is 1–2 $Log_{10}TCID_{50}$/ml higher than the $Log_{10}OID_{50}$/ml for E1-226A viruses but 3–4 $Log_{10}TCID_{50}$/ml higher than the $Log_{10}OID_{50}$/ml for E1-226V viruses. During the course of viremia there should therefore be a substantial time frame in which CHIKV blood load is high enough for E1-226V viruses to infect *Ae. albopictus* but below the threshold for infection

Table 2. $Log_{10}OID_{50}$/ml for CHIKV in *Ae. aegypti* Mosquitoes

Backbone	Exp[a]	Virus	Mosquitoes Analyzed[b]	Log_{10} $OID_{50}\pm CI_{95}$[c]	p Value
CHIK Reunion	1	LR-GFP-226V	65	6.77±0.40	p<0.1
		LR-GFP-226A	103	6.12±0.28	
	2	LR-GFP-226V	107	6.26±0.30	p<0.05
		LR-GFP-226A	53	5.62±0.33	
CHIK 37997	1	37997-GFP-226A	161	5.77±0.25	p<0.01
		37997-GFP-226V	162	6.59±0.34	
	2	37997-GFP-226A	136	5.83±0.30	p<0.1
		37997-GFP-226V	127	6.34±0.29	

OID_{50} values and confidence intervals were calculated using PriProbit (version 1.63).

[a]Experiment number.

[b]Number of mosquitoes used to estimate $Log_{10}OID_{50}$/ml.

[c]95% confidence intervals.

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