Chikungunya Virus Fidelity Variants Exhibit Differential Attenuation and Population Diversity in Cell Culture and Adult Mice

Kasen K. Riemersma,* Cody Steiner,* Anil Singapuri,* Lark L. Coffey*

*Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, California, USA

ABSTRACT  Chikungunya virus (CHIKV) is a reemerging global health threat that produces debilitating arthritis in people. Like other RNA viruses with high mutation rates, CHIKV produces populations of genetically diverse genomes within a host. While several known CHIKV mutations influence disease severity in vertebrates and transmission by mosquitoes, the role of intrahost diversity in chikungunya arthritic disease has not been studied. In this study, high- and low-fidelity CHIKV variants, previously characterized by altered in vitro population mutation frequencies, were used to evaluate how intrahost diversity influences clinical disease, CHIKV replication, and antibody neutralization in immunocompetent adult mice inoculated in the rear footpads. Both high- and low-fidelity mutations were hypothesized to attenuate CHIKV arthritic disease, replication, and neutralizing antibody levels compared to wild-type (WT) CHIKV. Unexpectedly, high-fidelity mutants elicited more severe arthritic disease than the WT despite comparable CHIKV replication, whereas a low-fidelity mutant produced attenuated disease and replication. Serum antibody developed against both high- and low-fidelity CHIKV exhibited reduced neutralization of WT CHIKV. Using next-generation sequencing (NGS), the high-fidelity mutations were demonstrated to be genetically stable but produced more genetically diverse populations than WT CHIKV in mice. This enhanced diversification was subsequently reproduced after serial in vitro passage. The NGS results contrast with previously reported population diversities for fidelity variants, which focused mainly on part of the E1 gene, and highlight the need for direct measurements of mutation rates to clarify CHIKV fidelity phenotypes.

IMPORTANCE  CHIKV is a reemerging global health threat that elicits debilitating arthritis in humans. There are currently no commercially available CHIKV vaccines. Like other RNA viruses, CHIKV has a high mutation rate and is capable of rapid intrahost diversification during an infection. In other RNA viruses, virus population diversity associates with disease progression; however, potential impacts of intrahost viral diversity on CHIKV arthritic disease have not been studied. Using previously characterized CHIKV fidelity variants, we addressed whether CHIKV population diversity influences the severity of arthritis and host antibody response in an arthritic mouse model. Our findings show that CHIKV populations with greater genetic diversity can cause more severe disease and stimulate antibody responses with reduced neutralization of low-diversity virus populations in vitro. The discordant high-fidelity phenotypes in this study highlight the complexity of inferring replication fidelity indirectly from population diversity.

KEYWORDS  RNA virus, arbovirus, cell culture, chikungunya virus, deep sequencing, fidelity variant, genetic diversity, intrahost virus evolution, mouse, sequencing

The global health risk of mosquito-borne chikungunya virus (Togaviridae, Alphavirus; CHIKV), which causes a severely debilitating febrile illness marked by polyarthralgia (1), has been highlighted by recent explosive epidemics in the tropics and subtropics...
While the severity and chronicity of CHIKV-induced polyarthralgia varies, approximately 25% of affected individuals remain symptomatic for two or more months (3). Host and viral factors that drive the severity and duration of disease are not well understood, although autoimmunity (4, 5) and antigen persistence (6, 7) have been implicated. For other RNA viruses, including other arthropod-borne viruses (arboviruses), genetic diversity of the intrahost viral population has been associated with both disease progression (8–11) and tissue tropism (12, 13). However, the role of intrahost CHIKV genetic diversity in chikungunya arthritic disease is unknown.

CHIKV encodes a viral polymerase incapable of proofreading that, coupled with exponential population growth, generates genetically diverse viral populations in hosts (14). RNA viruses like CHIKV are presumed to converge on a replication fidelity that optimizes either the trade-off between adaptability through genetic diversity and the accumulation of deleterious mutations (14, 15) or between replication speed and replication fidelity (16–18). In support of this premise of optimized fidelity, laboratory-generated fidelity-variant viruses replicating with either increased or decreased mutation rates compared to their wild-type (WT) progenitors typically exhibit reduced fitness and virulence (12, 19–32), although counterexamples have been reported. A high-fidelity variant of foot-and-mouth-disease virus was reported to exhibit enhanced fitness in vitro (33), and a low-fidelity variant of Venezuelan equine encephalitis virus exhibited virulence comparable to that of WT virus in mice (34). Fidelity variant viruses allow for manipulation of intrahost diversity and can be harnessed to study phenotypic effects of intrahost diversity. For CHIKV, point mutations that arose during in vitro mutagen treatment in 2 viral nonstructural genes, nsP2 and nsP4, were shown to confer mutagen resistance and alter mutation frequencies of in vitro CHIKV populations in standard arbovirus cell lines (20, 21, 35). High-fidelity CHIKV resulted from substitutions in nsP2 G641D and nsP4 C483Y (here termed double mutant high fidelity, or DM HiFi) (35) or nsP4 C483Y alone (high fidelity, or HiFi) (21), while a low-fidelity phenotype was observed with nsP4 C483G (LoFi) (20).

Previous studies characterized in vitro growth kinetics and mutation frequencies for fidelity-variant CHIKV (20, 21, 35). Both DM HiFi (35) and HiFi CHIKV (21) exhibited replication kinetics similar to those of the WT, while LoFi produced higher levels of viral RNA than the WT but similar levels of infectious virions (20). Mutation frequencies, measured by bacterial cloning methods, of viral populations 24 h postinoculation of hamster cells were reduced for DM HiFi (35) and HiFi (21) and elevated for LoFi (20) relative to the WT, leading to their designation as fidelity variants. CHIKV fidelity variant fitness was also assessed in neonatal C57BL/6 mice, in which HiFi CHIKV generated lower infectious virus levels in the blood and liver than WT CHIKV (21) and LoFi CHIKV generated lower CHIKV RNA levels in muscle, blood, brain, and liver than WT CHIKV (20). DM HiFi has not been studied in vivo. Despite reversion in mosquitoes, the stability of the LoFi mutation in neonatal mice was not reported (20). Together, these three studies provide evidence that intrahost CHIKV diversity can affect viral fitness in mice. However, since CHIKV-induced polyarthralgia in adult humans is of great public health relevance, evaluation of fidelity-driven modification of disease in an arthritogenic adult mouse model is needed. Additionally, the role of the genome-wide CHIKV mutant spectrum on infection dynamics and disease severity has yet to be defined; previous studies relied on Sanger sequencing of a portion of the CHIKV genome, which produces a lower breadth and depth of sequencing coverage than next-generation sequencing (NGS) approaches (20).

In this study, we describe experimental infection of immunocompetent adult mice with fidelity-variant or WT CHIKV to assess the effects of intrahost CHIKV population diversity on arthritic disease and neutralizing antibody production. We used NGS to compare CHIKV populations between fidelity variants and tissues in infected mice. Inoculation of CHIKV in the footpads of adult mice produces localized arthritis and foot swelling, representing the best murine model of human arthritic disease (36). An immunocompetent adult mouse model also allows for testing neutralizing antibody development, an important protective measure with implications for vaccine develop-
ment. The high-fidelity mutations used in this study have been proposed as safety enhancers for CHIKV live-attenuated vaccines (LAV) based on the premise that lower mutability reduces the likelihood of reversion to virulence (37).

Based on the rationale that WT CHIKV replication fidelity has evolved to maximize viral fitness, we hypothesized that both high- and low-fidelity CHIKV variants would exhibit reduced fitness, in the form of attenuated replication kinetics and restricted tissue tropism, and would elicit milder arthritic disease. Furthermore, we anticipated that both high- and low-fidelity CHIKV would stimulate lower serum-neutralizing antibody titers than the WT in adult mice. Surprisingly, our results show that the high-fidelity CHIKV variants replicate to titers comparable to those of the WT in adult mice and elicit more severe foot swelling, whereas low-fidelity CHIKV exhibits attenuated replication and foot swelling. NGS revealed that high-fidelity CHIKV populations are more diverse than WT populations in mice, an outcome which we then recapitulated by serial in vitro passage. We also found that mouse sera developed against both high- and low-fidelity CHIKV exhibit a diversity-dependent reduction in neutralization of WT CHIKV in vitro. Taken together, our findings suggest that the observed diversity of CHIKV populations depends on the cell or host environment they infect and highlight the complexity of inferring fidelity phenotypes from population diversity.

RESULTS

CHIKV fidelity variant phenotypes are supported in vitro by growth curves and bacterial cloning. The CHIKV fidelity variants (Fig. 1A) used for this study were generated and characterized previously (20, 21, 35). We first sought to confirm the established phenotypes in both BHK-21 and C6/36 cells. Both high-fidelity variants replicated to higher titers than the WT (P < 0.0001 by repeated-measures analysis of variance [ANOVA]), with the greatest differences observed 6 (P < 0.001 by Dunnett’s post hoc test) and 12 (P < 0.01) h postinfection (hpi) (Fig. 1B and C). In both cell types, the specific infectivities (ratio of genome equivalents to PFU) (Fig. 1D) were lower for both high-fidelity variants and higher for LoFi than for the WT (P < 0.01 by Tukey’s post hoc test). Mutation frequencies of each fidelity-variant virus and WT were quantified by bacterial cloning and Sanger sequencing, similar to methods originally used to establish these CHIKV mutants as fidelity variants. In BHK-21 cells, HiFi and DM HiFi CHIKV populations had 10% and 40% lower mutation frequencies, respectively, than the WT, and the LoFi CHIKV population had a 40% higher mutation frequency than the WT (Fig. 1E). Similar relationships were observed in C6/36 cells, except for the DM HiFi mutant. Because this was unexpected, we measured the mutation frequencies of DM HiFi and the WT in C6/36 cells in 3 additional biological replicates, comprising approximately an additional 168,000 nucleotides (nt) sequenced. Each of the additional analyses showed that DM HiFi generated a lower mutation frequency than parallel WT replicates. A Grubbs’ outlier test determined the initial elevated DM HiFi mutation frequency value was an outlier (P < 0.05), although no methodological differences across replicates explain the outlier. Given that the fidelity genotypes and phenotypes measured here were similar to those previously observed, we proceeded with an infection experiment in adult mice.

High-fidelity, but not low-fidelity, CHIKV elicits more severe foot swelling than WT CHIKV. Adult C57BL/6 mice were inoculated with 10^3 PFU WT or fidelity-variant CHIKV in the rear footpads to test effects of CHIKV fidelity on clinical disease, as determined by rear foot swelling (36, 38–40). Mice infected with LoFi CHIKV exhibited significantly less foot swelling than mice infected with the WT (Fig. 2A) (P < 0.05 by one-way ANOVA). Conversely, mice infected with either HiFi or DM HiFi exhibited more severe early footpad swelling than the WT at 3 and 4 days postinfection (dpi) (P < 0.01 by one-way ANOVA). CHIKV HiFi-infected mice also exhibited greater peak disease severity than those infected with the WT 7 dpi (P = 0.003 by one-way ANOVA). We next evaluated the relationship between clinical disease and viremia (Fig. 2B). Mean viremia titers were significantly reduced in LoFi-infected mice 1 and 3 dpi relative to the WT (P < 0.0001 by one-way ANOVA). Lower viremia titers were also observed for DM
HiFi-infected mice 1 dpi ($P < 0.001$ by one-way ANOVA) (Fig. 2B), despite elevated clinical disease at later time points. Mean viremias 5 and 9 dpi were not different across groups. These results demonstrate that the high- and low-fidelity mutations elicit more and less severe arthritic disease in adult mice, respectively, and the magnitude of peak CHIKV viremia correlates with disease severity.

**Tissue CHIKV levels are attenuated in LoFi- but not HiFi-infected adult mice.** Infectious CHIKV titers and CHIKV RNA levels in primary target tissues, muscle and ankle, and secondary tissues, brain and liver, were measured to determine whether clinical disease severity was associated with differential viral loads. Similar to viremia kinetics, LoFi CHIKV RNA and infectious virus titers in brain, liver, and muscle were significantly reduced relative to those of the WT 3 dpi ($P < 0.05$ by two-way ANOVA). In contrast, HiFi and DM HiFi CHIKV RNA and infectious virus levels were not different from that of the WT in any tissue ($P > 0.05$ by two-way ANOVA). At 9 dpi, low titers ($< 10^3$
PFU/g) were detected in at least one mouse ankle for all variants, with significantly lower titers in DM HiFi-infected mice than WT-infected mice \((P = 0.04)\) by two-way ANOVA. These results indicate that the low-fidelity mutation reduces CHIKV RNA and infectious virus levels in mice but that the high-fidelity mutations do not.

**Intrahost CHIKV mutant spectra vary by individual mouse and tissue.** CHIKV populations from inocula and ankles of 3 mice per treatment group were whole-genome sequenced using Illumina NGS, while sequencing of calf muscle isolates was

**FIG 2** High-fidelity CHIKV produces more severe clinical disease than the WT in adult mice. Adult female C57BL/6J mice were bilaterally inoculated subcutaneously in the rear footpads with 10^3 PFU of either WT CHIKV, HiFi CHIKV, DM HiFi CHIKV, LoFi CHIKV, or virus-free cell culture supernatant (mock). (A) Bilateral foot swelling was measured as percent increase in dorsoplantar diameter of hind feet from day 0 preinoculation. Numbers of feet per group were the following: for CHIKV cohorts 1 to 3 dpi, \(n = 32\); 4 to 9 dpi, \(n = 20\); 10 to 11 dpi, \(n = 8\); for the mock-inoculated group, 1 to 3 dpi, \(n = 16\); 4 to 9 dpi, \(n = 10\); 10 to 11 dpi, \(n = 4\). (B) Adult mouse viremia titers were determined by qRT-PCR of CHIKV RNA in whole blood. Each symbol represents an individual mouse. LOD is the limit of detection. Error bars represent standard deviations. \(P\) values for both graphs were calculated by one-way ANOVA. a, \(P < 0.05\); b, \(P < 0.01\); c, \(P < 0.001\); d, \(P < 0.0001\); all other cases, \(P > 0.05\).

**FIG 3** Tissue viral RNA (A and C) and infectious virus (B and D) levels of low-fidelity CHIKV are lower than those of the WT 3 and 9 dpi. CHIKV RNA was assessed by measuring genome equivalents (eq) per gram of tissue, and infectious viruses were measured in PFU per gram of tissue. Each symbol represents an individual mouse, \(n = 6\) per group. LOD, limit of detection. LOD varies by tissue based on the mass of tissues tested. \(P\) values were calculated by two-way ANOVA for all graphs. a, \(P < 0.05\); b, \(P < 0.01\); c, \(P < 0.001\); d, \(P < 0.0001\); all other cases, \(P > 0.05\).
limited to the whole genome for 1 mouse and partial genome for the remaining 2 mice per group. Sequencing from LoFi-infected muscle was not possible due to poor PCR amplification. For all isolates, the mean depth of coverage postprocessing ranged from 1,126 to 2,622 (Table 1). Comparing mutant spectra of CHIKV isolates from ankles by specific nucleotide substitution frequencies, the only significant differences from the WT were greater frequencies of A/H11022C and G/H11022U substitutions in LoFi CHIKV populations (P < 0.01 and P < 0.0001, respectively, by two-way ANOVA with Dunnett’s post hoc test) (see Fig. 5B).

Shared SNPs detected in more than one mouse at >1% frequency were identified to characterize tissue- and variant-specific mutations (Table 2). Four trends in the tissue distribution of shared SNPs were observed: (i) SNPs present in inocula and in both ankle and muscle, (ii) SNPs present in inocula and the ankle but not muscle, (iii) SNPs detected in only the ankle or muscle, and (iv) SNPs detected in both the ankle and muscle (Fig. 4). None of the shared SNPs were consensus changes (>50% frequency). The only SNP restricted to a single CHIKV variant was a revertant SNP at nsP4 483, where the LoFi mutant reverted to WT. By 3 dpi in the ankles of LoFi-infected mice, nsP4 483G mutated to 93% G483C in one mouse, 48% G483C and 43% G483V in the second mouse, and 92% G483C and 6% G483V in the third mouse. Valine at nsP4 483 has been previously reported to confer WT fidelity (20).

TABLE 1 Descriptive statistics for CHIKV NGS from infected adult mice

| Virus  | Tissue | dpi | Mouse ID | Region of genome | % Covered | Mean depth | Mut freq per 10K | RMSD | Shannon entropy |
|--------|--------|-----|----------|------------------|-----------|------------|----------------|------|----------------|
| WT     | Inoculum | 0   | NA       | Whole            | 99.4      | 2,464      | 4.01          | 0.0010 | 0.0032         |
| HiFi   | Inoculum | 0   | NA       | Whole            | 99.4      | 2,587      | 3.92          | 0.00094 | 0.0032       |
| DM HiFi | Inoculum | 0   | NA       | Whole            | 99.4      | 2,915      | 2.03          | 0.00094 | 0.0016       |
| LoFi   | Inoculum | 0   | NA       | Whole            | 99.4      | 2,494      | 3.27          | 0.00090 | 0.0027       |
| WT     | Tarsus  | 3   | 21N      | Whole            | 99.4      | 2,622      | 1.74          | 0.0018  | 0.0012       |
| WT     | Tarsus  | 3   | 35N      | Whole            | 99.4      | 2,380      | 1.98          | 0.0012  | 0.0015       |
| WT     | Tarsus  | 3   | 35R      | Whole            | 99.4      | 2,155      | 2.03          | 0.00094 | 0.0016       |
| HiFi   | Tarsus  | 3   | 25N      | Whole            | 99.4      | 2,293      | 2.94          | 0.0030  | 0.0019       |
| HiFi   | Tarsus  | 3   | 25R      | Whole            | 99.4      | 2,370      | 2.64          | 0.0019  | 0.0019       |
| HiFi   | Tarsus  | 3   | 31R      | Whole            | 99.4      | 2,384      | 2.50          | 0.0032  | 0.0017       |
| DM HiFi| Tarsus  | 3   | 19L      | Whole            | 99.4      | 2,479      | 2.31          | 0.00085 | 0.0019       |
| DM HiFi| Tarsus  | 3   | 19R      | Whole            | 99.4      | 2,424      | 2.42          | 0.0017  | 0.0018       |
| DM HiFi| Tarsus  | 3   | 33R      | Whole            | 99.3      | 2,156      | 2.90          | 0.0033  | 0.0020       |
| LoFi   | Tarsus  | 3   | 23R      | Whole            | 99.2      | 1,984      | 4.97          | 0.017   | 0.0015       |
| LoFi   | Tarsus  | 3   | 29N      | Whole            | 99.3      | 2,468      | 3.81          | 0.0077  | 0.0020       |
| LoFi   | Tarsus  | 3   | 29R      | Whole            | 99.4      | 2,388      | 3.51          | 0.010   | 0.0018       |
| WT     | Muscle  | 3   | 21N      | Whole            | 99.2      | 1,323      | 2.20          | 0.0012  | 0.0016       |
| WT     | Muscle  | 3   | 35N      | 26S-E2           | 18.3      | 2,489      | 2.23          | 0.0010  | 0.0017       |
| WT     | Muscle  | 3   | 35R      | 26S-E2           | 18.3      | 2,555      | 2.22          | 0.00085 | 0.0017       |
| HiFi   | Muscle  | 3   | 31R      | Whole            | 99.2      | 1,126      | 2.42          | 0.0014  | 0.0018       |
| HiFi   | Muscle  | 3   | 25L      | 26S-E2           | 18.2      | 2,213      | 2.34          | 0.00083 | 0.0018       |
| HiFi   | Muscle  | 3   | 25N      | 26S-E2           | 18.3      | 2,550      | 2.42          | 0.0011  | 0.0018       |
| DM HiFi| Muscle  | 3   | 33R      | Whole            | 99.3      | 2,185      | 2.53          | 0.00089 | 0.0020       |
| DM HiFi| Muscle  | 3   | 19L      | 26S-E2           | 18.3      | 2,556      | 2.11          | 0.0011  | 0.0016       |
| DM HiFi| Muscle  | 3   | 19R      | 26S-E2           | 18.1      | 1,770      | 2.60          | 0.00079 | 0.0020       |

Percent covered, percentage of genome with >300× coverage after read processing; mean depth, mean depth of coverage after read processing; Mut Freq per 10K, mutation frequency per 10,000 nucleotides sequenced. NA, not applicable.

High- and low-fidelity CHIKV diversify more than the wild type in adult mice.
In addition to comparing mutant spectra, we also compared overall population diversity by number of SNPs (Fig. 5A) and by two proportional diversity metrics, Shannon entropy (Fig. 5C) and root mean square deviations (RMSD) (Fig. 5D). As a metric of virus diversity.
population genetic variance, RMSD is skewed by high-frequency variants and therefore is useful for comparing high-frequency variants between groups. In contrast, Shannon entropy is maximized at a variant frequency of 0.5 and less biased by variant frequency, so it is better for comparing low-frequency variants between groups. More high-frequency (>5%) SNPs were detected in ankles of HiFi-, DM HiFi-, and LoFi-infected mice, although the differences were not statistically significant ($P > 0.05$ by chi-squared test) (Fig. 5A). Unexpectedly, HiFi and DM HiFi populations in ankles and muscles were comparably or more diverse than WT populations. In ankles, the diversities of HiFi and DM HiFi populations were significantly elevated relative to that of the WT by Shannon entropy ($P < 0.05$ by one-way ANOVA) (Fig. 5C) but not RMSD ($P > 0.05$ by one-way ANOVA) (Fig. 5D). Diversity of LoFi populations was significantly higher than that of the WT by RMSD ($P < 0.001$ by one-way ANOVA) (Fig. 5C) but not Shannon entropy ($P > 0.05$ by one-way ANOVA) (Fig. 5D). In muscle, no differences in population diversities were observed between HiFi and DM HiFi populations relative to the WT by Shannon entropy or RMSD ($P > 0.05$ by one-way ANOVA) (Fig. 5C and D). The pCHIK and pCHIK-PCR sequencing controls had no SNPs at more than 1% frequency (Fig. 5A) and low Shannon entropy and RMSD values (Fig. 5C and D), indicating that false-positive mutations derived from library preparation and NGS had minimal effects on the diversity metrics of CHIKV populations. These results show that relative in vitro diversity calculated from mutation frequencies by bacterial cloning and Sanger sequencing does not parallel relative CHIKV population diversities in adult mice, as measured by NGS, or that mutation frequencies detected by these methods change as a function of host environment.

High-fidelity CHIKV populations diversify more than the WT, as measured by NGS after single and serial passage in vertebrate cells. Despite corroborating HiFi and DM HiFi as high fidelity in vitro by bacterial cloning, the unexpectedly expanded diversity of HiFi and DM HiFi populations in vivo warranted further investigation. NGS was applied to measure the diversity of CHIKV populations after a single 24-h passage in BHK cells, as was done for bacterial cloning. Mutation frequencies were calculated by NGS across the whole genome and across the same E1 region used for bacterial cloning. We found that the differences in mutation frequencies established by bacterial cloning

| TABLE 2 Shared SNPs in mouse tissues and inocula$^a$ |
|-----------------------------------------------|
| **Ankle** | **Muscle** | **Inoculum** |
| Gene | Amino Acid Sub | WT | HiFi | DM HiFi | LoFi | WT | HiFi | DM HiFi | WT | HiFi | DM HiFi |
| nsP3 | A135S | 21N | 35N | 35R | 25N | 25R | 31R | 19L | 19R | 33R | 23N | 29N | 29R |
| nsP1 | A515T | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| nsP1 | E530D | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| nsP2 | V516E | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| nsP4 | V122A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| nsP4 | A123T | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| nsP4 | G483C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| nsP4 | G488V | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| nsP4 | C125N | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C | 233G (syn) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E2 | Q146R | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E2 | K221R | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E2 | R272S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E1 | V281M | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3' UTR | 11133B(T>G) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Legend
- **Found in both tissues**
- **Tarsus only**
- **Muscle only**
- **nsP4 G483s reversion**
- $\star$ **Found in at least one inoculum**

$^a$Highlighted SNPs detected with greater than 1% frequency in at least 2 mice. Mouse identification codes (e.g., 21N), treatment group, and tissue type are reported for each mouse sample. syn, synonymous mutations. The legend details the color coding scheme.
were not paralleled by our NGS observations (Fig. 6). Differences in mutation frequencies measured by NGS between both high-fidelity variants and the WT were not statistically significant ($P = 0.48$ by one-way ANOVA) (Fig. 6B). Only small differences were observed between NGS whole-genome and partial E1 mutation frequencies. The discordance in mutation frequencies measured by bacterial cloning and NGS called into question the attribution of these variants as fidelity variants and prompted further examination of mutation frequencies after serial passage in cell culture.

We reasoned that 5 serial passages on BHK-21 cells would amplify real differences in population mutation frequencies. Over serial passages, the viral titers did not vary significantly between passages or virus variants ($P = 0.95$ by two-way ANOVA) (Fig. 7A). The populations from the first passage (p1) and fifth passage (p5) were sequenced by NGS and compared (Fig. 7B to E). The mean depth of coverage for each sample ranged from 2,084 to 2,645 (Table 3). Over five passages, WT CHIKV developed more low-frequency SNPs, while HiFi and DM HiFi developed more high-frequency SNPs (Fig. 7B). RMSDs at p5 were marginally elevated for HiFi and significantly elevated for DM HiFi ($P = 0.0003$ by two-way ANOVA with Tukey's post hoc test) (Fig. 7D). In contrast, Shannon entropy was not significantly different across CHIKV variants at p1 or p5 (Fig. 7C). The elevation in RMSD and accumulation of high-frequency SNPs in both high-fidelity variants relative to WT CHIKV further indicate that HiFi and DM HiFi do not always produce less genetically diverse populations than WT CHIKV. Furthermore, there were no specific nucleotide substitutions in which HiFi and DM HiFi populations had significantly lower frequencies than the WT after p1 or p5 (by two-way ANOVA with Dunnett's post hoc test) (Fig. 8A). After p1, the only significant difference from the WT was a greater G>A frequency for DM HiFi ($P < 0.05$). After p5, HiFi populations exhibited greater frequencies of A>G, G>U, and U>C substitutions ($P < 0.05$, $P < 0.0001$, and $P < 0.01$), and DM HiFi populations had greater frequencies of C>A.
C, U, G, A, G, U, and U>C substitutions (P < 0.0001, P < 0.01, P < 0.0001, P < 0.01, and P < 0.0001). For p5 populations of HiFi and DM HiFi, a trend of GC>AU substitutions at a higher frequency than reciprocal AU>GC substitutions was observed (Fig. 8A and C). For both HiFi and DM HiFi, the G>U/U>G substitution ratios were significantly greater than those for the WT (P < 0.0001 and P = 0.02, respectively, by two-way ANOVA with Dunnett’s post hoc test) (Fig. 8C), while the differences in G>A/A>G and C>A/A>C ratios were not statistically significant. To determine if the GC>AU trend was genome wide, we evaluated the mutant spectra and GC<>AU ratios by frequency of mutated sites (Fig. 8B and D). A mutated site was defined as any nucleotide position with evidence of a substitution regardless of frequency. By mutated site frequency, the observed GC>AU trend is no longer evident (Fig. 8B), and the GC<>AU ratios of p5 populations are similar between HiFi, DM HiFi, and the WT (P > 0.05 by two-way ANOVA with Dunnett’s post hoc test) (Fig. 8D). These results indicate that the CHIKV fidelity variants in this study did not show differences in mutation bias after 5 serial BHK-21 cell passages.

**Fidelity mutant CHIKV impair serum neutralization of less diverse WT CHIKV in vitro.** Since these high-fidelity mutations are being investigated as safety enhancers for CHIKV LAVs in our other projects, we tested the effect of CHIKV fidelity on neutralization of WT CHIKV populations of low and high relative population diversity. Sera from the four mice in each treatment group at 30 dpi were serially diluted and tested for neutralization of passage zero (p0) and p5 WT CHIKV by PRNT. Sera from WT-inoculated mice neutralized low-diversity p0 WT CHIKV inocula better than any of the fidelity-
variant sera ($P < 0.01$ by one-way ANOVA with Dunnett’s multiple-comparison test) (Fig. 9A). In contrast, no differences in neutralization of high-diversity p5 WT CHIKV were observed between any groups ($P = 0.62$ by one-way ANOVA) (Fig. 9A). The RMSD for the low-diversity p0 and high-diversity p5 inocula approximated the minimum and

**FIG 6** In vitro mutation frequencies measured by bacterial cloning and NGS do not align. Mutation frequencies of CHIKV populations were measured after 24 hpi on BHK cells (MOI, 1). (A) Mutation frequencies for WT, Hifi, and DM Hifi populations ($n = 1$) measured by bacterial cloning of 750-nt fragment of E1. (Data are the same as those presented in Fig. 1E). (B) Mutation frequencies for WT, Hifi, and DM Hifi populations ($n = 3$) measured by NGS across the whole genome (left) or across the same 750-nt fragment of E1 as that for bacterial cloning (right). Mutation frequencies of pCHIK and pCHIK-PCR sequencing controls ($n = 1$) are included. Error bars in panel B represent standard deviations. $P$ values were calculated by one-way ANOVA for panel B. $ns$, $P > 0.05$. pCHIK-PCR is CHIKV cDNA with PCR at library preparation (control for PCR error), pCHIK is CHIKV cDNA without PCR as a control for sequencing error.

**FIG 7** High-fidelity CHIKV variants diversify more than the WT following serial passage. WT, Hifi, and DM Hifi CHIKV were passaged in BHK-21 cells for five serial passages (p1 to p5). p1 and p5 populations were sequenced by whole-genome NGS. (A) CHIKV RNA genome equivalents per ml of cell culture supernatant after each serial passage. (B) Mean number of SNPs across CHIKV genome. (C) Diversity of CHIKV populations as measured by Shannon entropy. (D) Diversity of CHIKV populations as measured by RMSD. Error bars in panels A, C, and D show standard deviations from the geometric mean. $P$ values were calculated by repeated-measures ANOVA (A), chi-squared test (B), or one-way ANOVA (C and D). $c$, $P < 0.001$; absence of letter or $ns$, $P > 0.05$. Three replicates were passaged, sequenced, and compared per group. One replicate was sequenced for each inoculum.
maximum RMSD for WT CHIKV isolated from mouse ankles at 3 dpi (Fig. 9B). The differential neutralization of low- and high-diversity WT CHIKV by sera from fidelity variant-infected mice suggests that serum antibody developed against more diverse CHIKV populations impairs virus neutralization in a diversity-dependent manner.

**DISCUSSION**

This is the first study to address effects of CHIKV fidelity mutations on CHIKV-induced arthritic disease. We demonstrate that HiFi and DM HiFi CHIKV replicate faster in vitro and elicit more severe arthritic disease in adult mice than the WT while generating viral loads in tissues comparable to those of the WT. Furthermore, we show using NGS that HiFi and DM HiFi CHIKV produce populations with greater diversity than the WT in adult mice, and we reproduce this finding in cell culture. These findings contrast with previous studies reporting comparable in vitro replication, reduced mutation frequencies, and attenuated titers of HiFi CHIKV in neonatal mice subcutaneously inoculated in the dorsum (21), suggesting that attenuation of HiFi CHIKV is not host age dependent. Additionally, our observation of genotypic reversion of LoFi to WT in mice, along with the previous report of reversion in mosquitoes (20), further indicates the strong selective pressure against the nsP4 LoFi mutation.

Our initial measures of mutation frequencies using bacterial cloning of in vitro populations aligned with the expectation of altered diversity based on the fidelity

| TABLE 3 Descriptive statistics for CHIKV NGS of cell culture-derived samples<sup>a</sup> |
|---------------------------------------------------------------|
| CHIKV plasmid or variant | CHIKV amplicon enrichment | Overlap error correction | Cell type | Passage no. | Replicate no. | % Covered | Mean depth | Mut freq per 10K | RMSD | Shannon entropy |
|--------------------------|---------------------------|--------------------------|-----------|-------------|---------------|-----------|------------|----------------|------|-----------------|
| pCHIKV                   | No                        | NA                       | NA        | NA          | 1             | 99.7      | 2,648      | 0.72           | 0.00021 | 0.00062       |
| pCHIKV                   | Yes                       | NA                       | NA        | NA          | 1             | 99.4      | 2,487      | 0.95           | 0.00024 | 0.00083       |
| pCHIKV                   | No                        | No                       | NA        | NA          | 1             | 100       | 28,004     | 2.06           | 0.00029 | 0.002        |
| HiFi                     | Yes                       | Yes                      | BHK       | Inoculum    | 1             | 99.8      | 58,639     | 2.03           | 0.0012  | 0.0021        |
| HiFi                     | Yes                       | Yes                      | BHK       | Inoculum    | 1             | 99.4      | 2,452      | 4.54           | 0.0011  | 0.0036        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | Inoculum    | 1             | 99.4      | 2,628      | 2.57           | 0.0014  | 0.0020        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 1           | 2             | 99.4      | 2,452      | 1.84           | 0.00046 | 0.0016        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 1           | 3             | 99.4      | 2,615      | 1.68           | 0.00045 | 0.0014        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 5           | 1             | 99.4      | 2,369      | 3.47           | 0.0019  | 0.0026        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 5           | 2             | 99.4      | 2,528      | 2.50           | 0.0037  | 0.0017        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 5           | 3             | 99.4      | 2,559      | 2.55           | 0.0046  | 0.0016        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 1           | 1             | 99.4      | 2,513      | 3.28           | 0.0011  | 0.0027        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 1           | 2             | 99.4      | 2,369      | 2.34           | 0.00051 | 0.002        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 1           | 3             | 99.4      | 2,641      | 2.14           | 0.00051 | 0.0018        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 5           | 1             | 99.4      | 2,103      | 4.88           | 0.0046  | 0.0030        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 5           | 2             | 99.4      | 2,626      | 3.50           | 0.006   | 0.0019        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 5           | 3             | 99.4      | 2,468      | 3.82           | 0.0062  | 0.0022        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 1           | 1             | 99.4      | 2,274      | 2.17           | 0.0010  | 0.0025        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 1           | 2             | 99.4      | 2,084      | 2.51           | 0.00057 | 0.0021        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 1           | 3             | 99.4      | 2,632      | 1.89           | 0.00046 | 0.0016        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 5           | 1             | 99.4      | 2,259      | 4.82           | 0.0061  | 0.0029        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 5           | 2             | 99.4      | 2,508      | 4.10           | 0.011   | 0.0019        |
| DM HiFi                  | Yes                       | Yes                      | BHK       | 5           | 3             | 99.4      | 2,598      | 4.38           | 0.01    | 0.0020        |

<sup>a</sup>Percent covered, percentage of genome with >300× coverage after read processing; mean depth, mean depth of coverage after read processing; Mut Freq per 10K, mutation frequency per 10,000 nucleotides sequenced. NA, not applicable.
characterizations, but surprisingly, when we employed NGS, the mutation frequencies for HiFi, DM HiFi, and WT CHIKV were not statistically different after one cell culture passage. To clarify this discrepancy, we serially passaged the CHIKV variants in cell culture to amplify differences in population diversity. After 1 passage, decreased diversity was observed in all CHIKV populations relative to their highly diverse inocula, likely due to purifying selection. After 5 passages, HiFi and DM HiFi populations were more diverse than those of the WT. Furthermore, in vivo populations of HiFi and DM HiFi were also found to be more diverse than those of the WT. The increase in diversification of high-fidelity CHIKV populations observed here was counterintuitive but similar to a recent study reporting increased in vitro mutation frequencies in populations of Venezuelan equine encephalitis virus, a related alphavirus, bearing an nsP4 mutation analogous to CHIKV C483Y (HiFi) (34).

When measuring intrahost viral diversity, systematic errors (41) and host antiviral deaminases (42, 43) can alter the observed population diversity. To limit systematic errors, we equalized input titers prior to library preparation, prepared libraries in parallel, included CHIKV DNA plasmid libraries as controls for sequencing and reverse transcription-PCR (RT-PCR) errors, and used a conservative quality filter with overlapping read error correction. The absence of called SNPs and relatively low

![Graphs showing mutation frequency and site frequency](image-url)

**FIG 8** Serially passaged high-fidelity CHIKV populations exhibit mutational biases in total mutations but not mutated sites. Mutant spectra for WT, HiFi, and DM HiFi CHIKV passaged in BHK-21 cells for five serial passages (p1 to p5). Mutational spectra of inocula and passage 1 and 5 CHIKV populations by total mutation frequency (A) and mutated site frequency (B). C and D) Ratios of GC to AU substitutions by total mutations (C) and mutated sites (D). A mutated site was defined as any nucleotide position with evidence of a substitution regardless of frequency. Error bars in all panels show standard deviations from the geometric means. P values were calculated by two-way ANOVA. a, P < 0.05; b, P < 0.01; c, P < 0.001; d, P < 0.0001; absence of letter, P > 0.05. Three replicates were passaged, sequenced, and compared per group. One replicate was sequenced for each inoculum.
The diversity observed in our plasmid controls indicate that the contribution of false-positive mutations from library preparation and sequencer error was minimal. To assess the potential contribution of genetic diversification by antiviral host deaminases, we evaluated mutant spectra for evidence of mutational biases. The mutant spectra of in vitro and in vivo CHIKV populations in this study lack evidence of specific mutational biases from APOBEC (C->U) (43) or ADAR (A->I) (42) deaminases that could explain the observed differences in population diversity. Instead, the spectra for the in vitro p5 populations exhibit an increased frequency of GC->AU substitutions for HiFi and DM HiFi CHIKV. Host adaptation via matched virus-host nucleotide bias has been suggested for RNA viruses (44), including CHIKV (45). To explore this further, we examined the frequency of GC->AU substitution at a nucleotide site level across the genome with the expectation that a shift in GC->AU frequency would result in more G and C nucleotide sites being mutated. Instead, we observed a loss of the GC->AU trend at the nucleotide site level, indicating there was no genome-wide trend but instead that measures of nucleotide bias were skewed by a few sites with high-frequency GC->AU substitutions. In both in vitro and in vivo CHIKV populations, we observed a higher frequency of transition nucleotide substitutions than transversion substitutions, aside from G->U transversions, as expected (46). The elevated frequency of G->U transversions may be explained in part by oxidative damage during NGS library preparation (47). To assess whether G->U substitutions biased our diversity metrics, we excluded all G->U substitutions, recalculated the metrics, and found the relationships were maintained (data not shown). From these analyses, we conclude that our observed population diversity measurements are unlikely to have been biased by systematic errors, host deaminases, or differential host adaptation. While host adaptation via matched virus-host nucleotide bias is unlikely, we are unable to rule out other intrahost selective pressures that could have affected the observed CHIKV population diversity.

The initial attribution of fidelity phenotypes to the CHIKV nsP2 and nsP4 mutations relied on comparative analysis of mutation frequencies measured by Sanger sequencing and NGS approaches (20, 21, 35). The incongruency in population diversity of high-fidelity CHIKV between this and previous studies highlights the difficulty of inferring replication fidelity from population diversity. To better evaluate potential fidelity-modifying effects of these mutations, future studies using cell-based Luria-Delbruck fluctuation tests (48, 49) and cell-free biochemical assays (50–52) are required to directly measure mutation rates. An additional advantage of these assays is the ability to study effects of the cellular or biochem-
ical environment on fidelity. The type of cell or host has been shown to affect the mutation rate of vesicular stomatitis virus (48) and cucumber mosaic virus (53, 54) and the mutant spectra of human immunodeficiency virus type 1 (HIV-1) (55). Further, the balance or availability of intracellular deoxynucleoside triphosphate (dNTP) pools affects the mutation rate of HIV-1 (56) and spleen necrosis and murine leukemia viruses (57). In this and previous studies (20, 35), cell type has been shown to alter mutation frequencies of CHIKV populations; Stapleford et al. specifically demonstrated that HiFi and DM HiFi replication complexes isolated from cell culture can adjust their replication speed to a greater degree than the WT and utilize low-concentration dNTP pools more efficiently. Whether CHIKV fidelity is determined, as suggested for poliovirus and HIV (18, 58), by the kinetic proofreading model (59–62), which proposes a trade-off between replication speed and accuracy such that accuracy decreases as speed increases, has not been studied. Further studies into kinetic proofreading for CHIKV replication complexes and the effects of dNTP availability on the fidelity phenotype for the nsP2 and nsP4 mutations are warranted. While the incongruency in high-fidelity CHIKV population diversity casts uncertainty on the fidelity phenotypes, we clearly demonstrate that the nsP2 G641D and nsP4 C483Y mutations enhance CHIKV virulence in adult mice.

For a high-fidelity variant of poliovirus, replication speed was suggested to drive attenuation of virulence more so than increased replicase fidelity (18). It is possible that faster replication is driving the enhanced virulence observed here in HiFi- and DM HiFi-infected mice. Stapleford et al. previously showed that isolated HiFi and DM HiFi CHIKV replication complexes synthesize CHIKV subgenomic RNA faster than the WT (35). Here, we demonstrate faster replication of HiFi and DM HiFi CHIKV than of the WT in BHK and C6/36 cells. Furthermore, attenuation of LoFi CHIKV in spite of early reversion to the WT genotype (which we first detected 3 dpi) suggests that robust replication early in infection (<3 dpi) is essential for maximizing peak viral titers and pathogenesis. In the HiFi- or DM HiFi-infected mice, elevated titers in blood early in the course of infection were not observed, but we are unable to compare early CHIKV replication near the inoculation site, as tissues were not collected prior to reaching peak CHIKV titers at 3 dpi. An alternative explanation for the increased virulence not addressed in this study is that the high-fidelity nsP4 C483Y mutation exerts phenotypic effects beyond altered replication speed or fidelity. Unlike CHIKV nsP2 and nsP3, evidence for extensive interactions of nsP4 with host proteins is limited (63), although interactions with proteins of the unfolded protein response within the endoplasmic reticulum have been suggested to promote CHIKV replication (64, 65). Whether nsP4 mutations can modulate the effects of these interactions on viral replication has not been studied. In addition to faster in vitro replication, the high-fidelity CHIKV variants counterintuitively produced populations with greater diversity than did the WT. Although the mechanism driving the enhancement in virulence of HiFi and DM HiFi CHIKV remains unclear, the high-fidelity CHIKV variants produce populations with altered diversity relative to those of the WT, a feature that maintains their utility for evaluating intrahost CHIKV evolution.

Our novel characterization of CHIKV population diversity in different tissues by NGS highlights that intrahost CHIKV evolution can be tissue specific. Eight SNPs shared by at least two mice were restricted to ankle or muscle tissues at 3 dpi, with most (6/8) detected in the ankle. Three of the tissue-restricted SNPs arose de novo, 2 were nonsynonymous mutations in E2, and 1 mutation was detected in the 3′ untranslated region (UTR). Phenotypic characterization of these mutations by reverse genetics is warranted to elucidate their fitness effects. These findings emphasize the value in sequencing from multiple tissues to get a full picture of intrahost populations, as well as the importance of performing NGS on virus inocula to discern de novo and preexisting mutations. Our results parallel tissue-specific evolution of poliovirus (66), indicating that tissue microenvironment as a driver of viral evolution is common across RNA virus families.

Use of an immunocompetent adult mouse model also serves as a platform for
understanding how the fidelity mutations alter neutralizing antibody responses. Here, we demonstrate a diversity-dependent reduction in serum neutralization in vitro, in that CHIKV high-fidelity mutants impair serum neutralization of low-diversity WT CHIKV populations but not high-diversity populations of WT CHIKV. This observation suggests that serum neutralization of CHIKV is driven more by the depth than the breadth of the antibody response, although studies of the antibody repertoire would be required to confirm this idea. Additionally, the presence of specific neutralization-susceptible variants in the p5 WT CHIKV populations biasing serum neutralization titers cannot be ruled out, although the lack of consensus mutations in those populations suggests this is unlikely. Studies are ongoing to address whether the diversity-dependent impairment in neutralization observed here will limit the capacity of anti-high-fidelity CHIKV sera to protect against challenge with WT CHIKV in vivo, a better proxy than in vitro neutralization. Although the high-fidelity CHIKV populations accumulated greater genetic diversity in serial cell culture, the genetic stability of the nsP2 and nsP4 high-fidelity mutants in vivo, in contrast to the unstable low-fidelity nsP4 mutation, suggests they lower the risk of reversion for attenuating mutations, as has been proposed in the context of vaccine development (37).

In summary, we show that the nsP2 and nsP4 high-fidelity mutations induce more severe arthritic disease in adult mice than WT CHIKV while producing more diverse virus populations and serum antibodies less able to neutralize low-diversity inocula in vitro. Furthermore, we demonstrate that intrahost CHIKV evolution can be tissue specific. Importantly, our findings highlight the need for direct measurement of replication fidelity to clarify the fidelity phenotype of the nsP2 and nsP4 mutations under different cellular contexts.

**MATERIALS AND METHODS**

**Viruses, cells, and viral titration.** Infectious cDNA clones of WT, HiFi, and LoFi 2005 La Réunion CHIKV outbreak strain (06-049; GenBank accession number AM258994.1), generously provided by Marco Vignuzzi, Institut Pasteur, were previously described (20, 21). The nsP2 G641D substitution was introduced by site-directed mutagenesis (QuickChange II site-directed mutagenesis kit; Agilent) in the HiFi CHIKV clone with a single point mutation (GGC→GAC) to generate DM HiFi CHIKV. Genotypic integrity was verified by whole-genome Sanger sequencing for all clones. Infectious CHIKV was rescued from cDNA clones as previously described (15). For rescued virus stocks and experiments described below, viral RNA and infectious viros were titrated in triplicate by quantitative RT-PCR (qRT-PCR) (CHIKV primers 6856, 6981, and 6919-FAM) and Veroplaque assays, respectively, as previously described (67, 68). Baby hamster kidney cells (BHK-21; ATCC CCL-10) and African green monkey kidney cells (Vero; ATCC CCL-81) were maintained in high-glucose Dulbecco’s modified Eagle medium (DMEM; Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific) and 1% penicillin-streptomycin (P/S; Gibco, Thermo Fisher Scientific) at 37°C and 5% CO2. The Aedes albopictus cell line C6/36 (ATCC CRL-1660) was maintained in Schneider’s insect medium (Caisson Labs) supplemented with 20% FBS and 1% P/S at 28°C and atmospheric CO2.

**In vitro growth assays and serial passage.** BHK-21 and C6/36 cells were inoculated with rescued stocks (p0) of each CHIKV fidelity variant or WT in triplicate at a multiplicity of infection (MOI) of 1. Cell culture supernatants were harvested (1/20 total volume) and replenished after a 1-h absorption period and 6, 12, and 24 h postinoculation (hpi). To amplify differences in mutation frequencies, the WT and the high-fidelity CHIKV variants were inoculated and passaged 5 times in BHK-21 cells in triplicate at an MOI of 1. After 24 hpi, cell culture supernatant was collected and CHIKV RNA titers were used to estimate the PFU titer based on measured genome/PFU ratios at 24 hpi for each variant in the CHIKV clone with a single point mutation (GGC→GAC) to generate DM HiFi CHIKV. Genotypic integrity was verified by whole-genome Sanger sequencing for all clones. Infectious CHIKV was rescued from cDNA clones as previously described (15). For rescued virus stocks and experiments described below, viral RNA and infectious viros were titrated in triplicate by quantitative RT-PCR (qRT-PCR) (CHIKV primers 6856, 6981, and 6919-FAM) and Veroplaque assays, respectively, as previously described (67, 68). Baby hamster kidney cells (BHK-21; ATCC CCL-10) and African green monkey kidney cells (Vero; ATCC CCL-81) were maintained in high-glucose Dulbecco’s modified Eagle medium (DMEM; Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific) and 1% penicillin-streptomycin (P/S; Gibco, Thermo Fisher Scientific) at 37°C and 5% CO2. The Aedes albopictus cell line C6/36 (ATCC CRL-1660) was maintained in Schneider’s insect medium (Caisson Labs) supplemented with 20% FBS and 1% P/S at 28°C and atmospheric CO2.

**Mutation frequencies by bacterial cloning.** Mutation frequencies were measured from CHIKV populations harvested after the first 24-h passage described above. Viral RNA was extracted (Qiagen QiaAMP viral RNA minikit), and a 750-nt region (nt 10019 to 10768) of the envelope gene was amplified by high-fidelity RT-PCR (Agilent Accuscript PfuUltra II RT-PCR kit). Bacterial cloning of E1 amplicon cDNA, Sanger sequencing, and mutation frequency calculations were performed as previously described (21). At least 80 amplicons, representing >60,000 nt, were sequenced for each virus from both BHK-21 and C6/36 cells. Since the goal of this step was to validate established genotypes, mutation frequencies were measured by bacterial cloning for one biological replicate per virus in BHK-21 and C6/36 cells, except for WT and DM HiFi in C6/36 cells, where four biological replicates were used.

**Mouse infections.** All research animals were housed at animal biosafety level 3, and procedures were performed in accordance with University of California (UC) Davis IACUC protocol 19108. Six-week-old female C57BL/6J mice (The Jackson Laboratory) were used in this study. Only female mice were used, since the immunocompetent, adult mouse footpad model was described in female C57BL/6 mice only (36), and no sex bias in clinical disease or pathology was reported following footpad inoculation of immunodeficient C57BL/6 adult mice (69). Sixteen mice in each group were bilaterally inoculated with treated and untreated CHIKV, with input titers corrected to 10^5 PFU/mL. Viruses were administered into the footpads of mice using a 100-μL aliquot per footpad for each challenge inoculum.
subcutaneously in the rear footpads with 10^3 PFU CHIKV per footpad in 10 μl of sterile 0.9% NaCl solution. Mock-treated mice were inoculated with virus-free cell culture supernatant diluted in 0.9% NaCl solution. Blinded hind foot height measurements were recorded daily by digital caliper operated by the same person. Blood was collected on 1, 3, 5, 9, and 30 dpi, with 30-dpi blood processed to harvest serum. Mice from each treatment group were euthanized on 3, 9, or 30 dpi, and the brain, liver, calf muscles, and ankle joints were collected. Tissues for virus titration by plaque assay were homogenized and stored at −80°C. Tissues for NGS and qRT-PCR were immersed in RNAlater (Thermo Fisher Scientific) at 4°C for 1 day prior to homogenization. RNA was extracted by a MagMax-96 viral isolation kit (AM1836; Thermo Fisher Scientific) on a MagMax Express-96 particle processor (Thermo Fisher Scientific).

PRNT. Neutralizing antibody levels from 30 dpi mouse sera were determined by plaque reduction neutralization tests (PRNT) using low-diversity p0 and high-diversity p5 BHK-21 cells with WT CHIKV. Mouse sera were heat inactivated at 56°C for 30 min. Neutralization assays with 2-fold dilutions of mouse sera (1:20 to 1:2,560) were performed using Vero cells as previously described (70). The reciprocals of the highest dilution of sera that caused >80% reduction in plaque formation are reported.

**Amplicon library preparation and next-generation sequencing.** For *in vitro* serial passage isolates, all replicates were sequenced. For CHIKV from mouse tissues, 3 mice with tissue RNA levels at or near the median for each treatment group were selected for NGS. Target enrichment on equivalent quantities of viral RNA was performed by high-fidelity RT-PCR (Accuscript PfuUltra II) of nine cDNA amplicons spanning the CHIKV 5′ to 3′ UTRs (Table 4). For calf muscle, sufficient whole-genome RT-PCR amplification was achieved for just one mouse in the WT, HiFi, and DM HiFi cohorts. For 2 mice in those cohorts, amplification was only achieved with primer set 7, which covered the 26S promoter to the 3′ end of the envelope protein 2 gene (E2). Due to poor RT-PCR amplification, NGS was not performed for any LoFi-infected muscle isolates. Amplicons were fragmentated with double-stranded DNA fragmentase (New England Biolabs), followed by KAPA pure bead (Kapa Biosystems) size selection targeting a mean length of 150 bp. Library preparations were performed with a NEBNext Ultra DNA library preparation kit and NEBNext multiplex oligonucleotides (New England Biolabs). Libraries generated from CHIKV infectious clone DNA with p(CHIK-PCR) or without (pCHIK) PCR amplification prior to library preparation were incorporated as controls for PCR and sequencing errors. Libraries were sequenced on a single flow cell lane using paired-end 150 Illumina HiSeq 4000 technologies at the UC Davis DNA Technologies Core.

**Bioinformatics.** Demultiplexed paired-end reads were quality (>Q35), adapter, and primer trimmed with Trimmomatic (v0.36) (71). Overlapping paired-end reads were merged, and mismatched base calls were resolved by highest quality score with FLASH (v1.2.11) (72). Reference-guided alignment was performed with the Burrows Wheeler alignment tool (bwa mem, v0.7.5) (73). To control for variance in within-genome and between-sample coverage depth, the aligned reads were downsampled with BBTools (v33.48; Joint Genome Institute) to ca. 2,500× coverage. SNPs were called by LoFreq* (v2.1.2) (74) and annotated with SNPdat (v1.0.5) (75). Shannon entropy was calculated in R (v3.4.3) via the diverse package (76), while RMSD (77) and specific nucleotide substitution frequencies were calculated via in-house R scripts. A minimum coverage cut-off of 300 was used for all analyses to eliminate bias of low-coverage positions. These NGS and bioinformatics methods capture nucleotide substitutions but are biased against deletions and recombination events.

**Statistical analysis.** All statistical analyses were performed with GraphPad Prism 7 software (GraphPad Software, CA, USA). Statistical significance was ascribed to *P* values of less than 0.05.

**Accession number(s).** Raw NGS data are available from the NCBI Sequence Read Archive under BioProject entry PRJNA453810. Pipeline and in-house scripts are available at https://github.com/kasenriemersma/CHIKV-NGS-diversity.

**ACKNOWLEDGMENTS**

We thank Marco Vignuzzi for providing the infectious DNA clones used in this study, Chris Barker for his helpful guidance on statistical analyses, and Chris Weiss and Hongwei Liu for providing helpful critiques of drafts of the manuscript.

K.K.R. and L.L.C. conceptualized and designed experiments. K.K.R., C.S., and A.S. conducted experiments and data collection. K.K.R. performed data analyses and drafted the manuscript and figures. K.K.R., C.S., A.S., and L.L.C. edited and reviewed the manuscript.
This study was supported by a National Institutes of Health Ruth L. Kirschstein National Research Service Award (T32 OD 011147 to K.K.R.), R01 AI125902 to L.L.C., and start-up funds provided by the UC Davis School of Veterinary Medicine, Department of Pathology, Microbiology and Immunology, to L.L.C. Funding sources played no part in experiment design, data analysis or interpretation, or decision to publish.

REFERENCES

1. Thiberville S-D, Moyen N, Dupuis-Maguiraja L, Noaugerede A, Gould EA, Rques P, de Lamballerie X. 2013. Chikungunya fever: epidemiology, clinical syndrome, pathogenesis and therapy. Antiviral Res 99:345–370. https://doi.org/10.1016/j.antiviral.2013.06.009.

2. Lo Presti A, Cellè E, Angeletti S, Ciccozzi M. 2016. Molecular epidemiology, evolution and phylogeny of chikungunya virus: an updating review. Infect Genet Evol 41:270–278. https://doi.org/10.1016/j.meegid.2016.04.006.

3. Rodríguez-Morales AJ, Cardona-Ospina JA, Urbano-Garzón SF, Hurtado-Zapata JP. 2016. Prevalence of post-chikungunya chronic inflammatory rheumatism: a systematic review and meta-analysis. Arthritis Care Res 68:1849–1858. https://doi.org/10.1002/acr.22900.

4. Maek-A-Nantawat W, Silachamroon U. 2009. Presence of autoimmune antibody in chikungunya infection. Case Rep Med 2009:840183. https://doi.org/10.1155/2009/840183.

5. Hogeboom C. 2015. Peptide motif analysis predicts alphaviruses as triggers for rheumatoid arthritis. Mol Immunol 68:465–475. https://doi.org/10.1016/j.molimm.2015.09.010.

6. Hoarau J-J, Jaffar Bandjee M-C, Krejbich Trotot P, Das T, Li-Pat-Yuen G, Dassa B, Denizot M, Guichard E, Ribera A, Henni T, Tallet F, Moiton MP, Gauzère BA, Bruniquet S, Jaffar Bandjee Z, Morbidelli P, Martigny G, Jolivet M, Gay F, Grandadam M, Tolou H, Vivillard E, Debré P, Autran B, Gasque P. 2010. Persistent chronic inflammation and infection by chikungunya arthropigenic alphavirus in spite of a robust host immune response. J Immunol 184:5914–5927. https://doi.org/10.4049/jimmunol.0900255.

7. Poo YS, Rudd PA, Gardner J, Wilson JAC, Larcher T, Collé-M-A, Le TT, Nakaya HI, Palliwol D, Alloire C, Bielefeldt-Ohmann H, Schroder WA, Kromyak AA, Lopez JA, Suhbier A. 2014. Multiple immune factors are involved in controlling acute and chronic chikungunya virus infection. PLoS Negl Trop Dis 8:e3354. https://doi.org/10.1371/journal.pntd.0003354.

8. Farci P, Shimoda A, Coiana A, Diaz G, Peddiss G, Melpolder JC, Strazzera A, Chien DY, Munoz SJ, Balestri A, Purcell RH, Alter HJ. 2000. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. Science 288:339–344.

9. Lada O, Benhamou Y, Poynard T, Thibault V. 2006. Coexistence of hepatitis B surface antigen (HBs Ag) and anti-HBs antibodies in chronic hepatitis C virus: influence of “a” determinant variants. J Virol 80:2968–2975. https://doi.org/10.1128/JVI.80.6.2968-2975.2006.

10. Dekking B, Lindevall LF, Ferris MT, Swanstrom J, Lalli P, Beltrèmo M, Corti M. 2016. Differential Attenuation of CHIKV Fidelity Variants Journal of Virology 93:1855–1858.

11. Norström MM, Buggert M, Tauriainen J, Hartogensis W, Prosperi MC, Meloni F, Mäkinen K, Savolainen M, Aallonkoski M, Korlin J, Vihko R, Pieskä P, Salminen A, Rehman S, Rhoades M, Savolainen M, Uronen H, Vahter M, Färkkilä M, Hietala S, Virolainen E, Riekkinen P, Leinonen J. 2017. Proofreading-deficient coronaviruses adapt for increased fitness over long-term passage without reversion of exonuclease-inactivating mutations. mBio 8:e01503-17.

12. Tonnelle M, Diaz-San Segundo F, Campagnola G, Keith A, Schafer EA, Kloc A, de Los Santos T, Peersen O, Rieder E. 2017. Attenuation of foot-and-mouth disease virus by engineering viral polymerase fidelity. J Virol 91:e00081-17.

13. Griesemer SB, Kramer LD, Van Slyke GA, Pata JD, Gohara DW, Cameron CE, Ciota AT. 2017. Mutagen resistance and mutation restriction of St. Louis encephalitis virus. J Gen Virol 98:201–211. https://doi.org/10.1099/jgv.0.006626.0.

14. Van Slyke GA, Arnold JJ, Lugo AJ, Griesemer SB, Moustafa IM, Kramer LD, Cameron CE, Ciota AT. 2017. Sequence-specific fidelity alterations associated with West Nile virus attenuation in mosquitoes. PLoS Pathog 11:e1005009. https://doi.org/10.1371/journal.ppat.1005009.

15. Zeng J, Wang H, Xie X, Li C, Zhou G, Yang D, Yu L. 2014. Ribavirin-resistant variants of foot-and-mouth disease virus: the effect of restricted quasispecies diversity on viral virulence. J Virol 88:4006–4020. https://doi.org/10.1128/JVI.03594-13.

16. Graham RL, Becker MM, Eckerle LD, Bolles M, Denison MR, Baric RS. 2012. Mutagen resistance and mutation restriction of St. Louis encephalitis virus. J Gen Virol 93:1855–1858. https://doi.org/10.1099/jgv.0.006626.0.

17. Griesemer SB, Kramer LD, Van Slyke GA, Pata JD, Gohara DW, Cameron CE, Ciota AT. 2017. Mutagen resistance and mutation restriction of St. Louis encephalitis virus. J Gen Virol 98:201–211. https://doi.org/10.1099/jgv.0.006626.0.

18. Van Slyke GA, Arnold JJ, Lugo AJ, Griesemer SB, Moustafa IM, Kramer LD, Cameron CE, Ciota AT. 2017. Sequence-specific fidelity alterations associated with West Nile virus attenuation in mosquitoes. PLoS Pathog 11:e1005009. https://doi.org/10.1371/journal.ppat.1005009.

19. Zeng J, Wang H, Xie X, Li C, Zhou G, Yang D, Yu L. 2014. Ribavirin-resistant variants of foot-and-mouth disease virus: the effect of restricted quasispecies diversity on viral virulence. J Virol 88:4006–4020. https://doi.org/10.1128/JVI.03594-13.

20. Graham RL, Becker MM, Eckerle LD, Bolles M, Denison MR, Baric RS. 2012. Live, impaired-fidelity coronavirus vaccine protects in an aged, immunocompromised mouse model of lethal disease. Nat Med 18:1820–1826. https://doi.org/10.1038/nm.2972.

21. Sierra S, Dávila M, Lunenstern PR, Domingo E. 2000. Response of foot-and-mouth disease virus to increased mutagenesis: influence of viral load and fitness in loss of infectivity. J Virol 74:8316–8323.

22. Pfeffer JK, Kirkegaard K. 2005. Increased fidelity reduces poliovirus fitness and virulence under selective pressure in mice. PLoS Pathog 1:e11. https://doi.org/10.1371/journal.ppat.0010011.
ultra-sensitive variant caller for uncovering cell-population heterogeneity from high-throughput sequencing datasets. Nucleic Acids Res 40: 11189–11201. https://doi.org/10.1093/nar/gks918.

75. Doran AG, Creevey CJ. 2013. Snpdat: easy and rapid annotation of results from de novo snp discovery projects for model and non-model organisms. BMC Bioinformatics 14:45. https://doi.org/10.1186/1471-2105-14-45.

76. Guevara MR, Hartmann D, Mendoza M. 2016. diverse: an R Package to analyze diversity in complex systems. R J 8:60–78.

77. Li K, Venter E, Yooseph S, Stockwell TB, Eckerle LD, Denison MR, Spiro DJ, Methé BA. 2010. ANDES: statistical tools for the ANalyses of DEep Sequencing. BMC Res Notes 3:199. https://doi.org/10.1186/1756-0500-3-199.