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A Novel Bacterium-Free Method for Generation of Flavivirus Infectious DNA by Circular Polymerase Extension Reaction Allows Accurate Recapitulation of Viral Heterogeneity

Judith Edmonds,[a] Erinke van Grinsven,[a,b] Natalie Prow,[a] Angela Bosco-Lauth,[c] Aaron C. Brault,[c] Richard A. Bowen,[d] Roy A. Hall,[a] Alexander A. Khromykh[a]

Australian Infectious Disease Research Centre, School of Chemistry and Molecular Biosciences, University of Queensland, Brisbane, Queensland, Australia[a]; Graduate School of Life Sciences, Utrecht University, Utrecht, The Netherlands[b]; Division of Vector-Borne Diseases, Centers for Disease Prevention and Control, Fort Collins, Colorado, USA[c]; Colorado State University, Fort Collins, Colorado, USA[d]

A novel bacterium-free approach for rapid assembly of flavivirus infectious cDNAs using circular polymerase extension reaction was applied to generate infectious cDNA for the virulent New South Wales isolate of the Kunjin strain of West Nile virus (KUNV) that recently emerged in Australia. Recovered virus recapitulated the genetic heterogeneity present in the original isolate. The approach was utilized to generate viral mutants with designed phenotypic properties and to identify E protein glycosylation as one of the virulence determinants.

Generation of flavivirus infectious clones has been traditionally hindered by the toxicity of their full-length cDNAs in bacteria. Various approaches have been employed to overcome this problem, including the use of very-low-copy-number plasmids (1, 2), bacterial artificial chromosomes (3), cosmid vectors (4), specific *Escherichia coli* strains (5, 6), mutation of cryptic bacterial promoter sites in the flavivirus genome (7), separation of the genome into two plasmids followed by *in vitro* ligation and RNA transcription (8–12), and use of a yeast recombination system to assemble full-length clones (13, 14). All of these approaches require substantial efforts, are time-consuming, and are prone to introducing undesired mutations during amplification of plasmid DNA in bacteria and during *in vitro* RNA transcription by bacteriophage DNA-dependent RNA polymerases (T7 or SP6). A bacterium-free approach involving either *in vitro* ligation of two large overlapping cDNA fragments generated by reverse transcription and PCR (RT-PCR) or linking these two large cDNA fragments by fusion PCR was developed for tick-borne encephalitis virus (TBEV) (15). The resulting product containing the SP6 RNA polymerase promoter upstream of TBEV sequence was then used to produce RNA by *in vitro* transcription and virus was recovered by injecting *in vitro*-transcribed RNA into the brain of suckling mice. This approach allows rapid generation of infectious cDNA without a need for plasmid DNA amplification in bacteria; however, it still requires an *in vitro* RNA transcription step as well as the highly sensitive suckling mouse model to recover infectious virus. The former is prone to introduction of undesired mutations, and the latter is not applicable for flaviviruses that do not replicate well in mice (e.g., dengue viruses). We and others have previously developed infectious cDNA clones for West Nile virus (WNV) and Japanese encephalitis virus (JEV) in which the *in vitro* RNA transcription step is eliminated by replacing SP6 or T7 polymerase promoters with the cytomegalovirus (CMV) promoter, thus allowing transcription of viral RNA in cells directly from plasmid DNAs by the cellular RNA polymerase II (16–18). Although an infectious cDNA clone for the Kunjin strain of West Nile virus (KUNV) has been demonstrated to be relatively stable, further manipulations, including mutagenesis could render it less stable (A. Khromykh, unpublished results). To solve the problem of stability for CMV-based JEV and WNV infectious DNAs, the authors had to introduce introns into the viral genome (17, 18). An additional modification of the infectious cDNA approach to eliminate potential instability was recently developed for rapid generation of WNV mutants in which DNA fragments harboring mutated structural genes were ligated *in vitro* with the genome in which structural genes had been deleted under the control of a CMV promoter, and the *in vitro* ligation reaction mixture was then directly transfected into mammalian cells to recover infectious viruses (19). Despite bringing a substantial advance in rapid generation of flavivirus infectious clones, these CMV-based approaches still require cloning and amplification of at least one plasmid DNA containing a large portion of the flavivirus genome in bacteria.

Herein we describe a novel approach for rapid generation of flavivirus infectious cDNAs that completely eliminates the need for plasmid DNA propagation in bacteria and for *in vitro* RNA transcription; it also allows generation of viruses that accurately represent native viral heterogeneity. The approach is based on using a circular polymerase extension cloning (CPEC) reaction with Phusion high-fidelity DNA polymerase, which was successfully employed previously for assembly of complex gene libraries and pathways (20). The goal of CPEC is to assemble multiple RT-PCR amplicons in the right order in a vector. To this end, the RT-PCR fragments are designed to have overlapping ends and the first and the last RT-PCR amplicons also have overlapping ends with the vector fragment. When the fragments and the vector are mixed and denatured, the overlapping ends anneal to each other. DNA polymerase then extends the fragments, using the neighboring fragment as a primer. If the overlapping fragments are designed to have similar annealing temperatures, this reaction gen-

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Address correspondence to Alexander A. Khromykh, a.khromykh@uq.edu.au.

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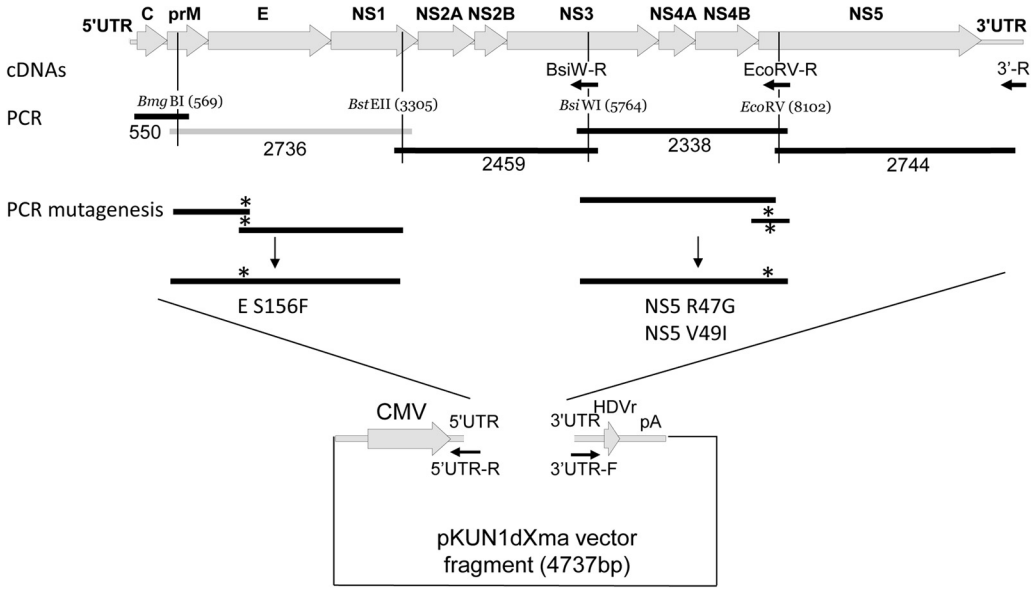

FIG 1 Assembly of infectious cDNA for the wild-type and mutant NSW2011 viruses by CPEC reaction. A schematic representation of the WNV genome is shown at the top of the figure, with individual genes and untranslated regions (UTR) indicated. Shown are locations of the unique restriction sites with numbers indicating nucleotide positions in the NSW2011 genome (GenBank accession no. JN887352). The primers used to generate cDNA fragments are shown by arrows and labeled by the designations of unique restriction sites. Lines show PCR fragments, with numbers representing their size in base pairs (bp). Asterisks show approximate locations of introduced mutations. The lightly shaded box for the BmgBI-BstEII fragment indicates that this fragment was cloned separately from other fragments in strategy 2. E S156F, PCR fragment contacting the S156F mutation in E protein; NS5 R47G and NS5 V49I, PCR fragments containing the corresponding mutations in the NS5 protein; CMV, cytomegalovirus promoter; HDVr, hepatitis delta virus ribosome; pA, SV40 poly(A) signal.

erates a circular end product. Depending on the number of RT-PCR fragments to be assembled, the reaction requires anywhere from 2 to 20 cycles to complete the assembly. The generated circular product can then be transformed directly into competent cells without any additional manipulations. The use of high-fidelity Phusion DNA polymerase for both RT-PCR amplification of fragments and for CPEC reaction ensures accurate amplification of required sequences.

To adapt this approach for generation of infectious DNA for the NSW2011 isolate of KUNV, we used the truncated pKUN1dXma vector containing the CMV promoter immediately upstream of the first nucleotide of KUNV sequence and the hepatitis delta virus ribozyme sequence followed by the simian virus 40 (SV40) poly(A) signal placed directly downstream of the last nucleotide of the KUNV sequence (Fig. 1) (16). The vector fragment was amplified using primers complementary to the 5′- and 3′-terminal KUNV sequences to contain the first 87 and the last 127 nucleotides of the KUNV genome, which are virtually identical to the corresponding NSW2011 sequences (21). To generate overlapping RT-PCR fragments, viral RNA isolated from a low-passage-number stock of the NSW2011 isolate was first converted to three cDNA fragments using the 3′-R, EcoRV-R, and BsiW-R primers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega) followed by PCR amplification of five DNA fragments using Phusion high-fidelity DNA polymerase (Thermo Scientific) and corresponding primers (Fig. 1). The primers were designed to contain overlapping ends for two neighboring fragments, similar annealing temperatures, and unique restriction sites in the NSW2011 genome to allow any future cloning if required. Two strategies were then employed to assemble infectious cDNA. The first strategy involved assembly of infectious full-length cDNA from all the PCR-amplified viral cDNA fragments and the vector fragment entirely by CPEC reaction without any intermediate steps in bacteria. The second (back-up) strategy involved production of two plasmid cDNAs, one (pNSW2011-1) containing most of the viral genome, except for the ~2.7-kb BmgBI-BstEII fragment in the pKUN1dXma vector assembled by CPEC, and the second plasmid (pUC-BmgB-BstE) containing the BmgBI-BstEII fragment in the pUC19 vector obtained by conventional cloning. CPEC reactions for both strategies were performed using 200 ng of vector fragment and equimolar amounts of each of the other PCR fragments. The total amount of DNA used in each reaction was approximately 700 ng. The CPEC reactions were initiated by denaturation for 45 s at 98°C, followed by addition of Phusion high-fidelity DNA polymerase and 20 cycles of denaturing for 15 s at 98°C, annealing for 30 s at 60°C, and extension for 7 min (strategy 1) or 6.5 min (strategy 2) at 72°C. The CPEC was completed by 15 min of extension at 72°C. The CPEC reaction mixture containing full-length cDNA was directly transfected into HEK293T cells using Lipofectamine LTX reagent (Invitrogen) as described by the manufacturer. In strategy 2, the CPEC reaction mixture for generation of the pNSW2011-1 plasmid was transformed into DH5α cells. Ten out of 10 bacterial colonies contained plasmid DNA of the expected size, and restriction digestion with HindIII resulted in appearance of DNA fragments of the expected molecular weights (not shown). A blunt-ended PCR-amplified fragment containing BmgBI and BstEII restriction sites was cloned into the SmaI-digested pUC19 vector to obtain a pUC-BmgB-BstE plasmid. To obtain the full-length infectious DNA, the BmgBI-BstEII fragment was excised from the pUC-BmgB-BstE plasmid (clone 11) and ligated with BmgBI- and BstEII-digested CPEC-generated vector plasmid pNSW2011-1 (clone 8).

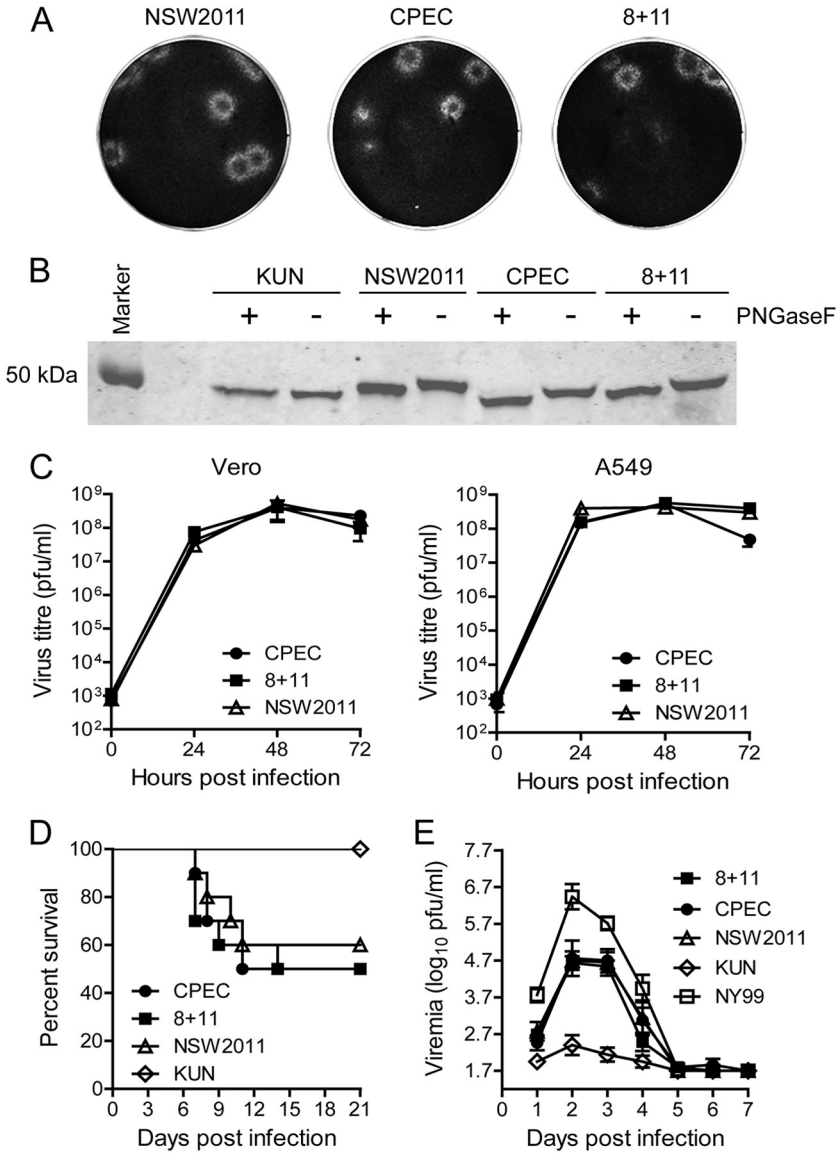

FIG 2 *In vitro* and *in vivo* properties of the recovered wild-type NSW2011 viruses. (A) Plaque morphology in Vero cells at 4 days after infection. Cells were fixed with 4% formaldehyde and stained with 0.2% crystal violet. (B) Western blot with anti-E antibodies. Lysates from viral culture supernatants were treated with PNGaseF (+) or mock treated (−). Samples were separated on 10% SDS-PAGE gels, transferred to nitrocellulose membrane, and probed with MAb 4G2. KUN, Kunjin virus. (C) Viral growth kinetics in Vero and A549 cells. Cells were infected at a multiplicity of infection (MOI) of 1, and culture supernatant was collected at the indicated time points. The viral titer was determined by plaque assay on Vero cells. Error bars represent the standard errors of the means of 2 samples. (D) Survival of 28-day-old CD-1 mice ($n = 10$) challenged intraperitoneally with 1,000 PFU of virus. Mice were monitored for 21 days and sacrificed when signs of encephalitis were evident. (E) Virulence in 4-day-old chickens ($n = 8$) following subcutaneous inoculation with ~1,000 PFU of the indicated viruses. Infected chickens were bled daily for the first 7 days after infection, and virus titers in the serum were determined by plaque assays on Vero cells.

The ligation reaction mixture (8 + 11) was then transfected directly into HEK293T cells using Lipofectamine LTX reagent. Culture fluid was harvested at 4 days after transfection with CPEC reaction mixture or ligation reaction mixture, at which point viruses reached titers of 8×10^7 PFU/ml for the virus generated by strategy 1 (CPEC virus) and 2×10^7 PFU/ml for the virus generated by strategy 2 (8 + 11 virus). These culture fluids were then amplified once in Vero cells to prepare high-titer stocks for further experiments; both viruses accumulated to the titers exceeding 10^8 PFU/ml by 3 days after infection (8 + 11, 8.8×10^8 PFU/ml; CPEC, 5.5×10^8 PFU/ml). Comparison of the plaque morphologies on Vero cells showed that viruses obtained using both strategy 1 (CPEC) and strategy 2 (8 + 11) formed plaques very similar in size to each other and to those of the parental NSW2011 isolate (Fig. 2A). The E proteins of both recovered viruses and the original NSW2011 isolates were glycosylated as expected (21), as shown by faster migration of E protein posttreatment with peptide:*N*-glycosidase F (PNGaseF) (Fig. 2B). Both recovered viruses also demonstrated indistinguishable growth kinetics in Vero and A549 cells (Fig. 2C) and virulence in 4 week-old mice (Fig. 2D) and 4-day-old chickens (Fig. 2E) compared to each other and to the original NSW2011 isolate. Notably, recovered viruses and the

TABLE 1 Sequence analysis of generated viruses

Virus	Mixed nucleotides or mutations in RNA	Corresponding amino acid position(s) in viral proteins	Polymorphism in original NSW2011 isolate
CPEC	8295 U/A	NS5 201 L/H	Yes
	9935 U/A	NS5 757 C/A	Yes
8 + 11	C3311G	NS1 L287V	No
	U5041C	NS3 L149 silent	No
	8295 U/A	NS5 201 L/H	Yes
	9935 U/A	NS5 757 C/A	Yes

original NSW2011 isolate were more virulent in chickens than the prototype KUNV strain MRM61C and less virulent than the NY99 strain of WNV, which correlates with our previous findings in mice (21).

Sequencing of RT-PCR products generated from the RNA of recovered viruses identified two sites in the NS5 gene showing mixed bases in the sequencing chromatograms (Table 1) indicating the presence of mixed virus populations. These were present in both viruses obtained by two different strategies (Table 1), suggesting that they may be present in the original viral RNA used to generate infectious DNAs. Retrospective sequencing of the original virus isolate indeed confirmed the presence of mixed bases at the same positions (Table 1). The result indicates that the CPEC-based strategy for generating infectious DNA may be used to accurately recapitulate natural viral heterogeneity. In addition to the mixed bases at the same positions in the NS5 gene, 8 + 11 virus obtained by strategy 2 contained two mutations, a codon change from leucine to valine at position 287 of the NS1 gene and a silent mutation in the NS3 gene, that were not present in the original viral isolate (Table 1). The results show that propagation in bacteria of the plasmids used to generate infectious DNA for 8 + 11 virus led to the appearance of mutations that can be avoided if infectious DNA is assembled entirely from PCR fragments by CPEC reaction without any bacterial propagation steps.

To demonstrate the utility of the CPEC method, three viral mutants were generated by site-directed PCR mutagenesis of corresponding cDNA fragments followed by CPEC. The locations of mutations were chosen so that the phenotype of mutants could be easily distinguished from that of the wild type: i.e., removal of glycosylation motif in E (E S156F) and change in the reactivity of the NS5 protein to the 5H1 monoclonal antibody (NS5 R47G and NS5 V49I) (22–25). To generate the E S156F mutant, two overlapping PCR fragments were generated using the BmgBI-BstEII fragment as the template (Fig. 1). To generate NS5 mutants, two sets of overlapping fragments were generated using the BsiWI-EcoRV fragment as the template (Fig. 1). These sets of overlapping fragments were then used to generate new mutated BmgBI-BstEII (E S156F) and BsiWI-EcoRV (NS5 R47G and NS5 V49I) frag-

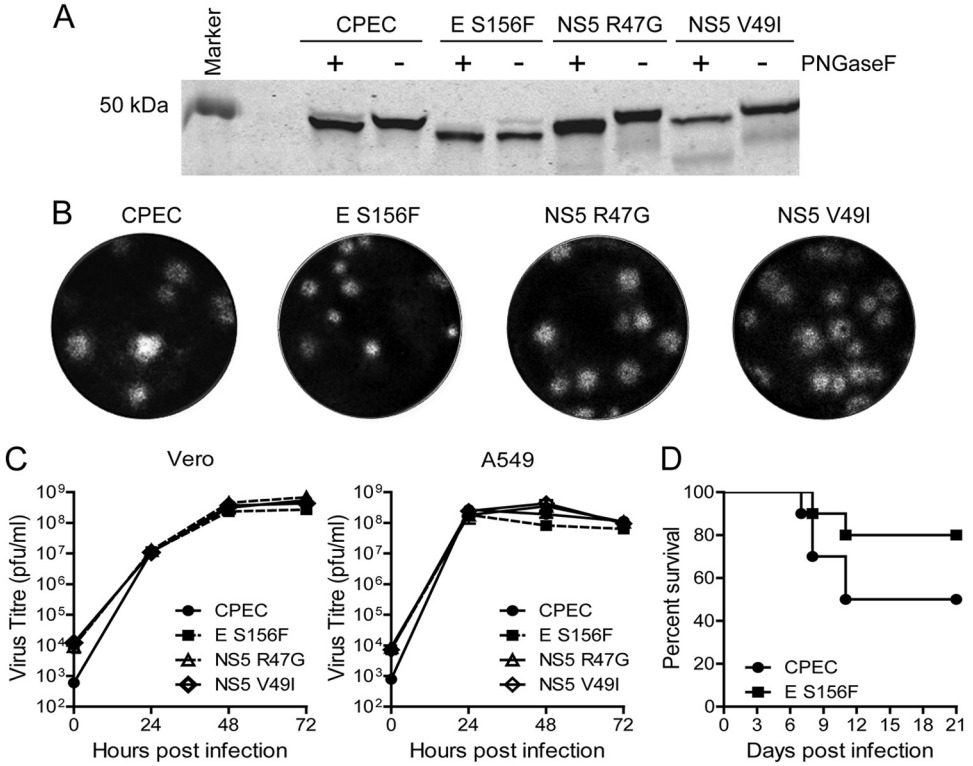

FIG 3 *In vitro* and *in vivo* properties of NSW2011 mutant viruses generated by CPEC. (A) Western blot with anti-E antibodies. Lysates from viral culture supernatants were treated with PNGaseF (+) or mock treated (−). Samples were separated on 10% SDS-PAGE gels, transferred to nitrocellulose membrane, and probed with anti-E MAb 4G2. (B) Plaque morphology in Vero cells at 4 days after infection. Cells were fixed with 4% formaldehyde and stained with 0.2% crystal violet. (C) Viral growth kinetics in Vero and A549 cells. Cells were infected at an MOI of 1, and culture supernatant was collected at the indicated time points. Viral titers were determined by plaque assay on Vero cells. Error bars represent the standard errors of the means of 3 independent experiments. (D) Survival of 28-day-old CD-1 mice ($n = 10$) challenged intraperitoneally with 1,000 PFU of the indicated viruses. Mice were monitored for 21 days and sacrificed when signs of encephalitis were evident.

TABLE 2 Reactivity of parental and generated viruses with monoclonal antibodies

Virus	Virus reactivity with MAb:		
	4G2 (glycosylated and nonglycosylated E)	5H1 (residue 49 in NS5)	3.101C (nonglycosylated E)
KUN	+	+	+
NSW2011	+	−	−
8 + 11	+	−	−
CPEC	+	−	−
E S156F	+	−	+
NS5 R47G	+	−	−
NS5 V49I	+	+	−

ments, respectively (Fig. 1). These mutated fragments were then used in combination with other original PCR fragments covering the rest of the genome for the CPEC reaction and recovery of mutant viruses as per the method described above. Secreted viruses were first harvested 3 days after transfection, when they reached titers of 8.8×10^5 PFU/ml for E S156F virus, 1.8×10^6 PFU/ml for NS5 R47G virus, and 9.8×10^5 PFU/ml for NS5 V49I virus. The viruses were then amplified once in Vero cells to prepare virus stocks for further studies: the titers were 8.3×10^7 PFU/ml for E S156F virus, 4×10^8 PFU/ml for NS5 R47G virus, and 3.8×10^8 PFU/ml for NS5 V49I virus. Sequencing of the corresponding RT-PCR products generated from RNA of recovered viruses confirmed the presence of introduced mutations. E S156F mutant virus lacked E glycosylation (Fig. 3A) and formed smaller plaques (Fig. 3B), as expected. A minor proportion of E protein appeared to be glycosylated (Fig. 3B), a phenomenon that may occur during virus passaging in cells (26). All mutant viruses replicated with similar efficiencies to each other and to the parental CPEC virus in Vero and A549 cells (Fig. 3C). As expected from previous studies, E S156F mutant virus showed decreased virulence in 4-week-old mice compared to the parent CPEC virus (Fig. 3D). The results demonstrate that glycosylation of E protein contributes to the virulence of the NSW2011 isolate.

Both NS5 mutants were glycosylated (Fig. 3A), formed similar plaques to those formed by CPEC virus (Fig. 3A), and had similar growth kinetics to the CPEC virus (Fig. 3C). Monoclonal antibody (MAb)-binding assays confirmed the lack of glycosylation for E S156F mutant, i.e., unglycosylated KUNV and the E S156F mutant bound to MAb 3.101C, which specifically recognizes unglycosylated E protein (J. Hobson-Peters and R. A. Hall, unpublished data), while the NSW2011, CPEC, and 8 + 11 viruses did not (Table 2). Reactivity to the 5H1 MAb, which recognizes an epitope containing residues 47 and 49 in KUNV NS5 protein (R47 and I49) but not in NY99 NS5 protein (G47 and V49) (24) showed that NSW2011 NS5 (R47 and V49) and the NS5 R47G mutant didn't bind 5H1 MAb, while KUNV and the NS5 V49I mutant did (Table 2). The results confirm our previous data distinguishing NSW2011 NS5 protein from KUNV NS5 protein in binding to 5H1 MAb (21) and showing the important role for residue 49 in this binding (24). Thus, all CPEC-generated mutant viruses behaved as expected in *in vitro* assays, while the E S156F mutant also behaved as expected *in vivo*. The results demonstrate the utility of the CPEC method for rapid generation of viral mutants. The ability to quickly exchange desired cDNA fragments makes the CPEC approach also very useful for generating chimeric viruses between different viral strains as well as for replacing regions containing an undesired polymorphism by well-characterized and stable sequences from cloned cDNA fragments.

Although our finding that the CPEC method allows accurate recapitulation of heterogeneous viral population is limited to only two loci in the genome that were identified to contain a mixed virus population in our NSW2011 virus preparation, the robustness, high fidelity, and the lack of bacterial amplification and *in vitro* RNA transcription steps afforded by the CPEC method should allow accurate recreation of more heterogeneous flavivirus populations. This in turn should facilitate studies investigating the potential role of viral heterogeneity in the adaptation of flaviviruses to different hosts.

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