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BRIEF REPORT

Construction of an infectious cDNA clone of Culex flavivirus, an insect-specific flavivirus from *Culex* mosquitoes

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Abstract Culex flavivirus (CxFV) is an insect-specific flavivirus that has recently been detected in various *Culex* spp. mosquitoes worldwide. Here, we report the successful construction of a full-length infectious cDNA clone of a Tokyo strain, CxFV-NIID21. The full-length CxFV-NIID21 cDNA was cloned into the low-copy-number plasmid pMW119, which was stably amplified in *Escherichia coli*. Transfection of a mosquito cell line with *in vitro*-transcribed RNA from the cDNA clone resulted in the production of recombinant progeny virus with growth properties, cytopathogenicity, and virion morphology similar to the parental virus.

Flaviviruses (family *Flaviviridae*, genus *Flavivirus*) are enveloped viruses with a 10–11-kb positive-strand RNA genome. The genome contains a single open reading frame encoding three structural (C, preM/M, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins flanked by 5′ and 3′ untranslated regions [16, 27]. Most flaviviruses are arthropod-borne viruses that are transmitted by mosquitoes or ticks, except for a group of vertebrate-infecting flaviviruses with unknown vectors or no vectors. A group of flaviviruses that lacks the capacity to replicate in vertebrates was found in a cell line and/or natural populations of mosquitoes [4–6, 8, 10–13, 23, 28]. These viruses are now recognized as "insect-specific flaviviruses" that are specifically adapted to their host mosquitoes and are probably maintained by vertical transmission in nature [2, 17, 22].

Culex flavivirus (CxFV) is an insect-specific flavivirus isolated for the first time from *Culex pipiens*, *Cx. tritaeniorhynchus*, and *Cx. quinquefasciatus* mosquitoes in Japan and Indonesia during 2003–2004 [10, 12]. Many strains of CxFV were subsequently detected in these and other *Culex* spp. mosquitoes (*e.g., Cx. restuans*, *Cx. tarsalis*, and *Cx. interrogator*) worldwide [1, 2, 7, 9, 15, 18, 21]. However, to date, CxFV has not been found in any other mosquito species, such as *Aedes* or *Anopheles* spp., suggesting that CxFV is strictly maintained in a host-genus-specific manner. Insect-specific flaviviruses, including CxFV, have often been detected in important vector species of mosquito-borne flaviviruses worldwide, although little is known of their own biological characteristics and their potential ecological effects on other viruses and host mosquitoes [14, 19].

Reverse genetics is a powerful molecular biology technique for analyzing the genetic determinants of viral growth and virulence in positive-strand RNA viruses, such as flaviviruses [3]. The construction of infectious cDNA clones and production of recombinant progeny viruses have been reported for multiple arthropod-borne flaviviruses, and these constructs have been utilized in molecular genetic studies or vaccine development. However, the production of infectious flavivirus cDNA clones is often difficult because of the genetic instability of the cloned

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viral cDNA in *Escherichia coli* [20, 24, 29]. To date, there have been no reports of the successful establishment of a system for the production of recombinant insect-specific flaviviruses, including CxFV. In this study, we report the first successful construction of a full-length infectious cDNA clone of CxFV.

A CxFV strain NIID21 (designated CxFV-NIID21; GenBank accession no. AB377213) isolated from *Cx. pipiens* mosquitoes in Tokyo in 2003 was used for the construction of a full-length CxFV cDNA clone. This viral strain was then propagated in C6/36 mosquito cells as described previously [10]. Viral RNA was extracted from the culture supernatant using a QIAamp Viral RNA kit (QIAGEN, Hiden, Germany) and used for viral cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Four regions of the virus genome were amplified by PCR using thermostable high-fidelity DNA polymerase KOD plus ver. 2.0 (Toyobo Co. Ltd., Tokyo, Japan) and serially ligated into the low-copy-number plasmid pMW119 (Nippon Gene Co., Tokyo, Japan) [25, 26], which was then introduced into *E. coli* DH5α or Stbl2 (Invitrogen) as follows (Fig. 1). The 5′ terminal-NS1 region of the CxFV genome was amplified using the primers T7-5T and CV3038r during the initial PCR and primers SI-AI-T7 and CV3038r during the second PCR (primers used for the construction of the clones are listed in Supplementary Table 1). Note that the primers T7-5T and SI-AI-T7 contained a complete T7 polymerase promoter sequence for *in vitro* transcription. The PCR product was subcloned into pMW119 at *Sph*I-*Sal*I sites (5′T-NS1/pMW119; Fig. 1). The NS1–NS4B region of the CxFV genome was amplified with the primers CV2426f and CV6956rBI, and the PCR product was subcloned into the *Nhe*I-*Bam*HI sites of 5′T-NS1/pMW119 (5′T-NS4B/pMW119; Fig. 1). The NS5-3′ terminal region of the CxFV genome was amplified with the primers NS5-GSP1 and KI-3T. The primer KI-3T contained an additional *Kpn*I site to produce run-off transcripts. The PCR product was subcloned into the *Bam*HI (blunt-ended)-*Kpn*I sites of 5′T-NS4B/pMW119 (5′T-NS4B-3′T/pMW119, Fig. 1). The NS3–NS5 region of the CxFV genome was amplified with the primers CV5925f and CV9925r, and the PCR product was subcloned into the *Eco*47III-*Eag*I sites of 5′T-NS4B-3′T/pMW119 to construct a complete recombinant CxFV clone (rCxFV/pMW119, Fig. 1). All transformants were incubated at low temperature (25–30°C) in the presence of low levels of antibiotic (25 μg mL^{-1} ampicillin) to minimize unexpected mutations in the viral sequence of the recombinant plasmid [24]. The nucleotide sequence of the viral genome region in the recombinant plasmid was confirmed after several passages in *E. coli*, and no sequence mutations were detected. This suggested that the full-length CxFV cDNA clone was stable in *E. coli* under the culture conditions.

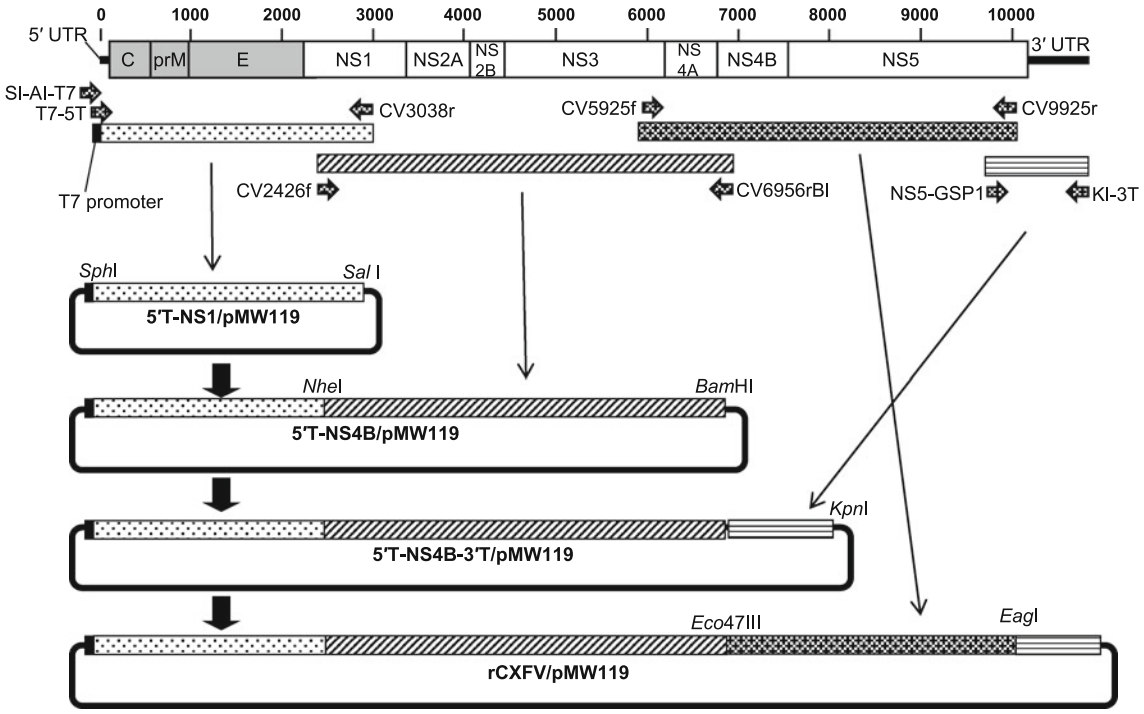

Fig. 1 Schematic representation of the construction of a full-length CxFV cDNA clone using the low-copy-number plasmid pMW119. The upper panel of the figure shows the genome organization of CxFV and the RT-PCR primers and cDNA fragments used for cloning. The T7 RNA polymerase promoter and restriction enzyme sites used for plasmid construction are indicated

To prepare a template for *in vitro* transcription, the rCxFV/pMW119 plasmid was digested at the 3′ end of the viral genome with *Kpn*I and purified using a GenElute PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, MO), and 1 μg of linearized DNA was transcribed using an mMESSAGE mMACHINE RNA transcription kit (Ambion, Austin, TX) containing $m^7G(5')ppp(5')G$ cap analog according to the manufacturer's instructions. After DNase I treatment, synthesized RNA was purified using an RNeasy MinElute Cleanup Kit (QIAGEN). An aliquot was electrophoresed on a 1% denaturing formaldehyde agarose gel to verify the efficient transcription of the full-length product (Supplementary Fig. 1). C6/36 cells (1×10^6 cells) were subsequently transfected with 3 μg of *in vitro*-transcribed RNA using Lipofectamine 2000 reagent (Invitrogen) as described previously [25, 26], and the cells were incubated at 28°C in an atmosphere of 5% CO_2 for 6 days. After two additional blind passages to increase the virus titer, culture supernatants were harvested and subjected to RNA extraction. Propagation of the recombinant progeny virus was confirmed by RT-PCR using a primer set specific for part of the E region of CxFV and sequencing the resultant RT-PCR product (data not shown). The recombinant progeny virus produced a mild cytopathic effect that was indistinguishable from the one caused by the parental virus at 5–6 days after infection (Fig. 2a-c). Negative-staining electron microscopy of infected culture supernatant confirmed the production of the recombinant virus particles, which were morphologically indistinguishable from the wild-type parent virus (Fig. 2d and e) [10].

Because plaque formation assays are not applicable to CxFV [10], the viral titer was measured by quantitative PCR (qPCR) to determine the copy number of viral genomic RNA. We initially prepared viral RNA standards. In brief, a 353-bp region within the E gene (sequence position 91–443) was initially amplified by RT-PCR. A second PCR was conducted to add a T7 promoter sequence at the 5′ terminus of the first PCR product, which was purified and subjected to *in vitro* transcription as described above (primer sequences are available upon request). The resultant transcripts were quantified and serially diluted 10-fold before being used as templates for standard curve analysis. To examine the growth properties of the recombinant progeny virus, C6/36 cells were seeded at a density of 0.5×10^6 cells per well in a 6-well plate and inoculated with 1.7×10^9 RNA copies of the parent or recombinant CxFV. The inoculum was removed after 1 h of virus adsorption, and cells were rinsed with phosphate-buffered saline before incubation at 28°C in 2 mL of minimum essential medium supplemented with 2% fetal bovine serum. A 140-μL aliquot of the culture supernatant was collected daily on days 0–8 after infection, and equal volumes of fresh culture medium were added to maintain the sample volume. RNA extracted from each culture supernatant was reverse-transcribed to cDNA using the PrimeScript RT Master Mix (Takara Bio, Shiga, Japan). qPCR was performed using 12 μL of reaction mixture

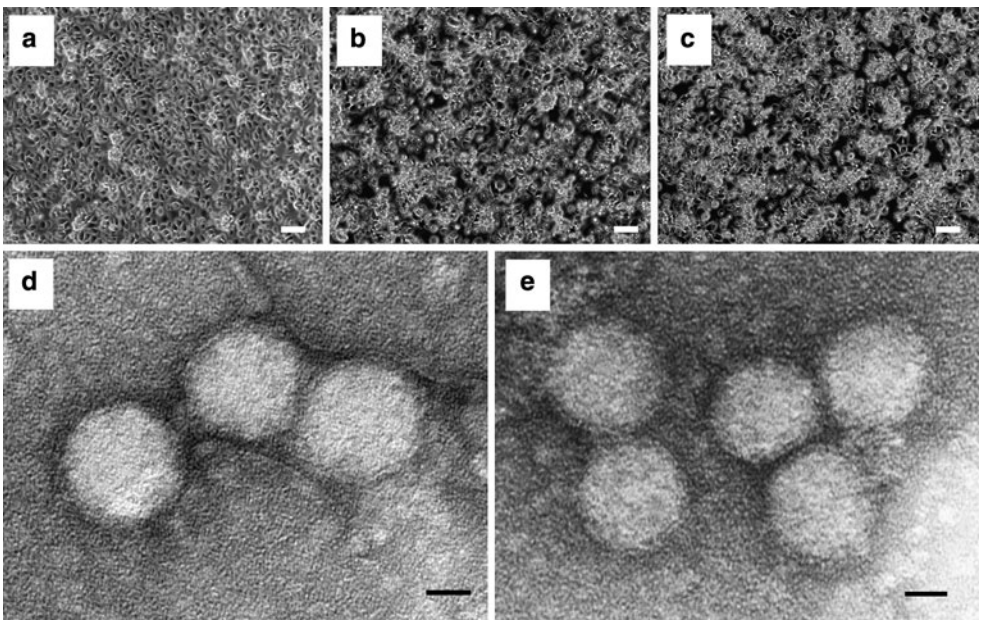

Fig. 2 a-c. Phase-contrast micrographs of control (mock-infected) C6/36 cells (**a**), parental CxFV-infected cells (**b**), and recombinant CxFV-infected cells (**c**) at 5 days post-infection. Scale bar, 100 μm. **d and e.** Negative-contrast electron micrographs of parental (**d**) and recombinant (**e**) CxFV particles from the culture supernatant at 5 days post-infection. Scale bar, 20 nm

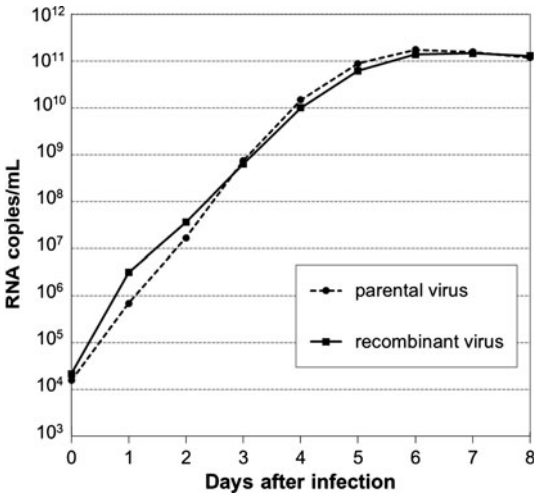

Fig. 3 Comparison of growth characteristics in C6/36 cells of the parental virus and recombinant progeny virus. Each point represents the mean titer of triplicate experiments

containing 1 μL of cDNA solution, 5 pmol of each forward and reverse primer for the partial E region (E129f and E280r, Supplementary Table 1), 0.25 μL of ROX Reference Dye II (Takara Bio), and 6 μL of SYBR premix Ex Taq II (Takara Bio) on the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). qPCR conditions were as follows: one cycle at 95°C for 30 s followed by 40 cycles at 95°C for 5 s and 60°C for 34 s. Viral RNA levels were estimated from the threshold cycle and standard curve. As shown in Fig. 3, growth properties of the recombinant progeny virus were similar to those of the parental virus. The yields of the parent and progeny virus reached peak titers within 5–6 days after infection.

In conclusion, we describe the construction of a stable full-length CxFV cDNA clone using a low-copy-number plasmid. RNA transcribed from the clone was infectious when introduced into susceptible mosquito cells. The recovered progeny virus was indistinguishable from the parental virus in terms of its growth kinetics, cytopathogenicity, and virion morphology. This is the first report of the production of an infectious insect-specific flavivirus clone. This infectious clone provides a useful basic tool for producing deletion, insertion, and amino acid substitution mutants or chimeric viruses to elucidate the molecular determinants of replication, virulence, and host range specificity of CxFV. The reverse genetics system for CxFV established in this study will offer new opportunities for molecular genetic studies directed at understanding the biological nature and evolution of insect-specific flaviviruses.

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References

1. Blitvich BJ, Lin M, Dorman KS, Soto V, Hovav E, Tucker BJ, Staley M, Platt KB, Bartholomay LC (2009) Genomic sequence and phylogenetic analysis of Culex flavivirus, an insect-specific flavivirus, isolated from *Culex pipiens* (Diptera: *Culicidae*) in Iowa. J Med Entomol 46:934–941
2. Bolling BG, Eisen L, Moore CG, Blair CD (2011) Insect-specific flaviviruses from *Culex* mosquitoes in Colorado, with evidence of vertical transmission. Am J Trop Med Hyg 85:169–177
3. Boyer JC, Haenni AL (1994) Infectious transcripts and cDNA clones of RNA viruses. Virology 198:415–426
4. Calzolari M, Bonilauri P, Bellini R, Caimi M, Defilippo F, Maioli G, Albieri A, Medici A, Veronesi R, Pilani R, Gelati A, Angelini P, Parco V, Fabbi M, Barbieri I, Lelli D, Lavazza A, Cordioli P, Dottori M (2010) Arboviral survey of mosquitoes in two northern Italian regions in 2007 and 2008. Vector Borne Zoonotic Dis 10:875–884
5. Cammisa-Parks H, Cisar LA, Kane A, Stollar V (1992) The complete nucleotide sequence of cell fusing agent (CFA): homology between the nonstructural proteins encoded by CFA and the nonstructural proteins encoded by arthropod-borne flaviviruses. Virology 189:511–524
6. Cook S, Bennett SN, Holmes EC, De Chesse R, Moureau G, de Lamballerie X (2006) Isolation of a new strain of the flavivirus cell fusing agent virus in a natural mosquito population from Puerto Rico. J Gen Virol 87:735–748
7. Cook S, Moureau G, Harbach RE, Mukwaya L, Goodger K, Ssenfuka F, Gould E, Holmes EC, de Lamballerie X (2009) Isolation of a novel species of flavivirus and a new strain of Culex flavivirus (*Flaviviridae*) from a natural mosquito population in Uganda. J Gen Virol 90:2669–2678
8. Crabtree MB, Nga PT, Miller BR (2009) Isolation and characterization of a new mosquito flavivirus, Quang Binh virus, from Vietnam. Arch Virol 154:857–860
9. Farfan-Ale JA, Loroño-Pino MA, Garcia-Rejon JE, Soto V, Lin M, Staley M, Dorman KS, Bartholomay LC, Hovav E, Blitvich BJ (2010) Detection of flaviviruses and orthobunyaviruses in mosquitoes in the Yucatan Peninsula of Mexico in 2008. Vector Borne Zoonotic Dis 10:777–783
10. Hoshino K, Isawa H, Tsuda Y, Yano K, Sasaki T, Yuda M, Takasaki T, Kobayashi M, Sawabe K (2007) Genetic characterization of a new insect flavivirus isolated from *Culex pipiens* mosquito in Japan. Virology 359:405–414
11. Hoshino K, Isawa H, Tsuda Y, Sawabe K, Kobayashi M (2009) Isolation and characterization of a new insect flavivirus from *Aedes albopictus* and *Aedes flavopictus* mosquitoes in Japan. Virology 391:119–129
12. Hoshino K, Takahashi-Nakaguchi A, Isawa H, Sasaki T, Higa Y, Kasai S, Tsuda Y, Sawabe K, Kobayashi M (2012) Entomological surveillance for flaviviruses at migratory bird stopover sites in Hokkaido, Japan, and a new insect flavivirus detected in *Aedes galloisi* (Diptera: *Culicidae*). J Med Entomol 49:175–182
13. Junglen S, Kopp A, Kurth A, Pauli G, Ellerbrok H, Leendertz FH (2009) A new flavivirus and a new vector: characterization of a novel flavivirus isolated from uranotaenia mosquitoes from a tropical rain forest. J Virol 83:4462–4468
14. Kent RJ, Crabtree MB, Miller BR (2010) Transmission of West Nile virus by *Culex quinquefasciatus* say infected with Culex Flavivirus Izabal. PLoS Negl Trop Dis 4:e671

15. Kim DY, Guzman H, Bueno R Jr, Dennett JA, Auguste AJ, Carrington CV, Popov VL, Weaver SC, Beasley DW, Tesh RB (2009) Characterization of Culex Flavivirus (*Flaviviridae*) strains isolated from mosquitoes in the United States and Trinidad. Virology 386:154–159
16. Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB (1998) Phylogeny of the genus *Flavivirus*. J Virol 72:73–83
17. Lutomiah JJ, Mwandawiro C, Magambo J, Sang RC (2007) Infection and vertical transmission of Kamiti river virus in laboratory bred *Aedes aegypti* mosquitoes. J Insect Sci 7:1–7
18. Morales-Betoulle ME, Monzón Pineda ML, Sosa SM, Panella N, López MR, Cordón-Rosales C, Komar N, Powers A, Johnson BW (2008) Culex flavivirus isolates from mosquitoes in Guatemala. J Med Entomol 45:1187–1190
19. Newman CM, Cerutti F, Anderson TK, Hamer GL, Walker ED, Kitron UD, Ruiz MO, Brawn JD, Goldberg TL (2011) Culex flavivirus and West Nile virus mosquito coinfection and positive ecological association in Chicago, United States. Vector Borne Zoonotic Dis 11:1099–1105
20. Rice CM, Grakoui A, Galler R, Chambers TJ (1989) Transcription of infectious yellow fever RNA from full-length cDNA templates produced by *in vitro* ligation. New Biol 1:285–296
21. Saiyasombat R, Dorman KS, Garcia-Rejon JE, Loroño-Pino MA, Farfan-Ale JA, Blitvich BJ (2010) Isolation and sequence analysis of Culex flavivirus from *Culex interrogator* and *Culex quinquefasciatus* in the Yucatan Peninsula of Mexico. Arch Virol 155:983–986
22. Saiyasombat R, Bolling BG, Brault AC, Bartholomay LC, Blitvich BJ (2011) Evidence of efficient transovarial transmission of Culex flavivirus by *Culex pipiens* (Diptera: Culicidae). J Med Entomol 48:1031–1038
23. Sang RC, Gichogo A, Gachoya J, Dunster MD, Ofula V, Hunt AR, Crabtree MB, Miller BR, Dunster LM (2003) Isolation of a new flavivirus related to cell fusing agent virus (CFAV) from field-collected flood-water *Aedes* mosquitoes sampled from a dambo in central Kenya. Arch Virol 148:1085–1093
24. Sriburi R, Keelapang P, Duangchinda T, Pruksakorn S, Maneekarn N, Malasit P, Sittisombut N (2001) Construction of infectious dengue 2 virus cDNA clones using high copy number plasmid. J Virol Methods 92:71–82
25. Tajima S, Nukui Y, Ito M, Takasaki T, Kurane I (2006) Nineteen nucleotides in the variable region of 3' non-translated region are dispensable for the replication of dengue type 1 virus *in vitro*. Virus Res 116:38–44
26. Tajima S, Nerome R, Nukui Y, Kato F, Takasaki T, Kurane I (2010) A single mutation in the Japanese encephalitis virus E protein (S123R) increases its growth rate in mouse neuroblastoma cells and its pathogenicity in mice. Virology 396:298–304
27. Thiel HJ, Collett MS, Gould EA, Heinz FX, Houghton M, Meyers G, Purcell RH, Rice CM (2005) Family *Flaviviridae*. In: Fauquet CM et al (eds) Virus Taxonomy, Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier, CA, pp 981–998
28. Tyler S, Bolling BG, Blair CD, Brault AC, Pabbaraju K, Armijos MV, Clark DC, Calisher CH, Drebot MA (2011) Distribution and phylogenetic comparisons of a novel mosquito flavivirus sequence present in *Culex tarsalis* mosquitoes from western Canada with viruses isolated in California and Colorado. Am J Trop Med Hyg 85:162–168
29. Yamshchikov V, Mishin V, Cominelli F (2001) A new strategy in design of (+)RNA virus infectious clones enabling their stable propagation in *E. coli*. Virology 281:272–280