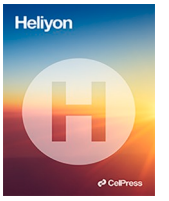
Heliyon 10 (2024) e33142

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Generation of rescued Japanese encephalitis virus genotype 1 from infectious full-size clone using reverse genetics

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ARTICLE INFO

Keywords:
Japanese encephalitis virus
Genotype 1
Reverse genetics
Rescued virus

ABSTRACT

Japanese encephalitis virus (JEV) is a pathogen responsible for high mortality and morbidity rates among children with encephalitis. Since JEV genotype 1 (GI) is the most prevalent strain in South Korea these days, corresponding research and vaccine development is urgently required. Molecular genetic studies on JEV vaccines can be boosted by obtaining genetically stable full-length infectious JEV complementary DNA (cDNA) clones. Furthermore, the significance of the reverse genetics system in facilitating molecular biological analyses of JEV properties has been demonstrated. This study constructed a recombinant JEV-GI strain using a reverse genetics system based on a Korean wild-type GI isolate (K05GS). RNA extracted from JEV-GI was used to synthesize cDNA, a recombinant full-length JEV clone, pTRE-JEVGI, was generated from the DNA fragment, and the virus was rescued. We performed *in vitro* and *in vivo* experiments to analyze the rescued JEV-GI virus. The rescued JEV-GI exhibited similar characteristics to wild-type JEV. These results suggest that our reverse genetics system can generate full-length infectious clones that can be used to analyze molecular biological factors that influence viral properties and immunogenicity. Additionally, it may be useful as a heterologous gene expression vector and help develop new strains for JEV vaccines.

1. Introduction

Japanese encephalitis (JE) caused by the mosquito-borne Japanese encephalitis virus (JEV) is highly prevalent in Asian and Western Pacific regions [1–3]. According to the World Health Organization (WHO) report, more than 68,000 cases of JE infection are reported annually, of which 10–15 % are fatal. Up to 50 % of patients report serious neurological and psychiatric sequelae [4–6].

JEV is a flavivirus belonging to the family *Flaviviridae*, which demonstrates a positive-sense single-stranded RNA genome approximately 11 kb in length and includes dengue, Zika, yellow fever, and tick-borne encephalitis viruses [7,8]. This large polyprotein is cleaved into three structural and nonstructural proteins [9]. The three structural proteins include capsid protein C, membrane protein prM, and envelope protein E [10]. The seven nonstructural proteins include NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [11]. Based on the nucleotide sequence of the viral envelope (*E*) gene, JEV is phylogenetically classified into five genotypes (I–V).

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https://doi.org/10.1016/j.heliyon.2024.e33142
Received 5 June 2024; Received in revised form 14 June 2024; Accepted 14 June 2024
Available online 17 June 2024

Abbreviations

JE	Japanese encephalitis
JEV	Japanese encephalitis virus
IFLC	infectious full-length clone
WHO	world health organization
FBS	fetal bovine serum
EMEM	Eagle's Minimum Essential Medium
WT	wild-type
KCDC	Korean Disease Control and Prevention Agency

Most isolates are characterized as GI or GIII [12,13].

The JEV GIII strain has been the dominant genotype in most Asian countries since its first isolation in 1935 until the end of the 20th century. GI strains were first isolated in 1967. GI has gradually replaced GIII as the dominant genotype over the past 20 years; since then, co-circulation of GI and GIII viruses has been observed [6,14]. No clinically approved antiviral agents are available for treating JEV infections, and vaccination is the most effective strategy for preventing JE [15]. Additionally, all currently licensed JE vaccines derived from the JEV GIII strain have been reported to cover GI strain infections but cannot provide complete protection [16–18]. In South Korea, as in many other Asian JEV-endemic countries, JEV-GIII was widespread before 1990. Since 2010, JEV-GI has been the most prevalent virus among JE patients [17–20]. The emergence of GI strains as the dominant genotype represents an urgent need for specific or broad-spectrum antiviral drugs or vaccines to treat JEV infections caused by both GI and GIII genotypes [6,21].

Reverse genetics is a powerful tool for the development of effective vaccines and reliable antiviral screening systems. To generate an infectious full-sized JEV clone, a stable full-length cDNA clone of the virus must be developed [22]. Traditionally, reverse genetics experiments for JEV have incorporated plasmid-based full-length cDNA clones that can be amplified in *Escherichia coli* [23]. In another study, researchers developed a stable full-length cDNA clone of JEV genotype 3 or JEV SA14-14-2 and produced viral RNA using an

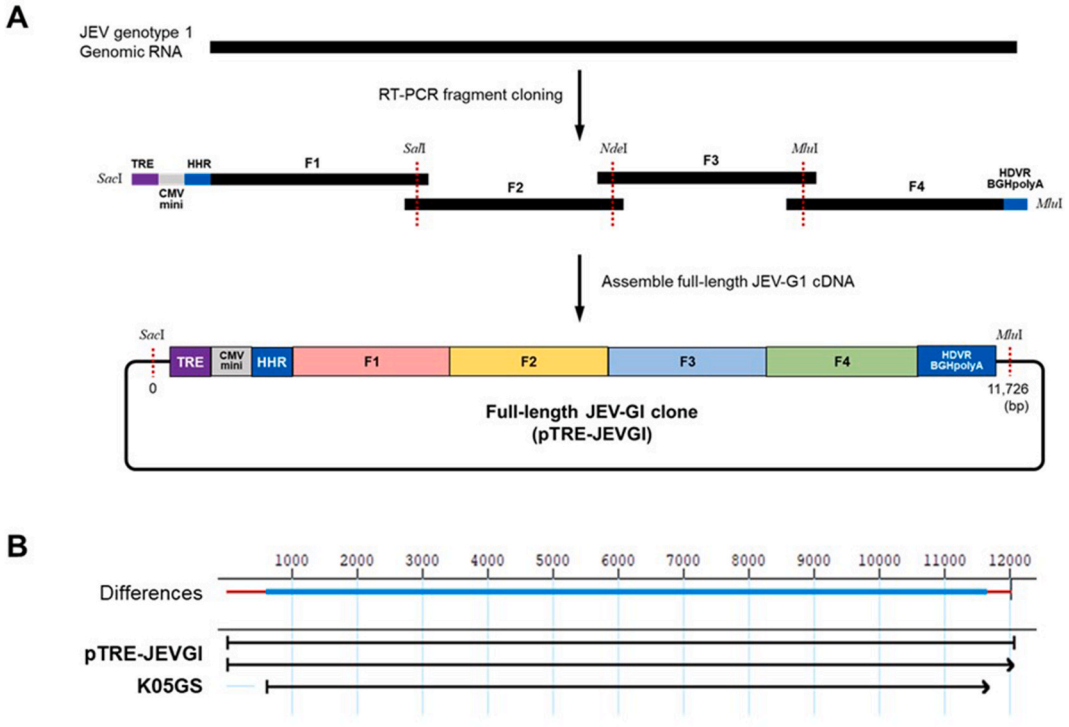

Fig. 1. Construction of the full-length clone of JEV genotype 1 (pTRE-JEVGI). (A) Four overlapping cDNA fragments (F1, F2, F3, and F4) were cloned from JEV-GI genomic RNA. A tetracycline response element (TRE) linked to a minimal cytomegalovirus (CMV) promoter was introduced before the 5′ UTR of the F1. At the 3′ end, the hepatitis D virus ribozyme (HDVR) and the bovine growth hormone transcription terminator (BGH poly A) were introduced immediately after the 3′ UTR. The full-length JEV-GI clone was synthesized by assembling four cDNA fragment clones. (B) Analysis of the full-length sequences between pTRE-JEVG1 and K05GS, the backbone sequence used for making the recombinant plasmid. The blue region in 'Differences' refers to the identical sequence, and the red regions refer to unmatched sequences. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

RNA production system to rescue the virus [18,23,24]. Other researchers have used PCR to amplify overlapping fragments of the viral genome and then assembled them into full-length cDNA clones using homologous recombination in yeast cells [25]. They also developed an efficient transfection protocol to rescue infectious viruses from cloned cDNA. Furthermore, in 2019, reverse genetics was used to characterize the commercial vaccine strain SA14-14-2 based on recombinant viruses [22].

Most previously reported JEV reverse genetics systems have been established for GIII, and the use of reverse genetics systems for JEV-GI is not widely reported. Therefore, to develop new vaccines and antiviral drugs against JEV infection, it is necessary to establish a stable reverse genetics platform for JEV-GI strains.

In this study, we aimed to develop recombinant JEV-GI strains using a reverse genetics system based on a Korean wild-type GI isolate. We also intended to compare the properties of JEVs rescued by the parent virus *in vitro*.

2. Results

2.1. Full-length clone of JEV genotype 1

The pTRE-JEVGI plasmid was subcloned using overlapping PCR. pTRE-JEVGI carried the full-length cDNA of the wild-type JEV (*K05GS*) gene under a CMVmini promoter harboring seven repeats of a tetracycline-response element (7xTRE) (Fig. 1A).

Nucleotide sequence analysis confirmed that pTRE-JEVGI was identical to K05GS (GenBank no. KR908702). As shown in Fig. 1B, the sequence of pTRE-JEVGI appears different from that of K05GS, as it contains the TRE-CMVmini-HHR cassette and the poly A region (red line indicates the difference). The remaining overlapping region matched the K05GS nucleotide sequence (blue line).

2.2. Generation and characterization of the rescued JEV genotype 1

The full-length JEV-GI clone pTRE-JEVGI was transfected into BHK-21 cells to produce viruses. BHK-21 cells transfected with pTRE-JEVGI showed CPE at 4 days post-infection (dpi). To confirm the generation of infectious viral clones, BHK-21, Vero, and C6/36 cells were infected with the harvested virus. CPE were also observed in BHK-21, Vero, and C6/36 cells infected with rescued JEV genotype 1 (rJEV-GI). Thus, the subcultured culture supernatant contained infectious rJEV-GI. Both viruses showed similar CPEs at 4 dpi in BHK-21 and Vero cells. Furthermore, similar CPE was observed at 5 dpi in C6/36 cells (Fig. 2A). rJEV-GI showed a CPE pattern similar to that of the parent virus K05GS in the three cell lines.rJEV-GI harvested from pTRE-JEVGI-transfected BHK-21 cells were defined as P1 viruses and serially passaged in C6/36 cells. The rescued viruses from each passage were defined as those corresponding to passages P1 to P5 and were used to infect cells to observe CPE and plaques (data not shown). Because the virus remained stable for five passages, we selected the P4 virus for subsequent studies.

The patterns and morphologies of both viral plaques were observed in all three cell lines. Plaques appeared after 5 days in BHK-21 and Vero cells and 7 days in C6/36 cells. Although the shape and size of plaques varied across the three cell types, both viruses demonstrated similar plaque morphologies (Fig. 2B).

To compare the protein expression of rJEV-GI and K05GS, SDS-PAGE and Western blot analyses were performed. Both virus samples showed similar protein expression patterns after Coomassie brilliant blue staining (Fig. 3A). Additionally, protein expression

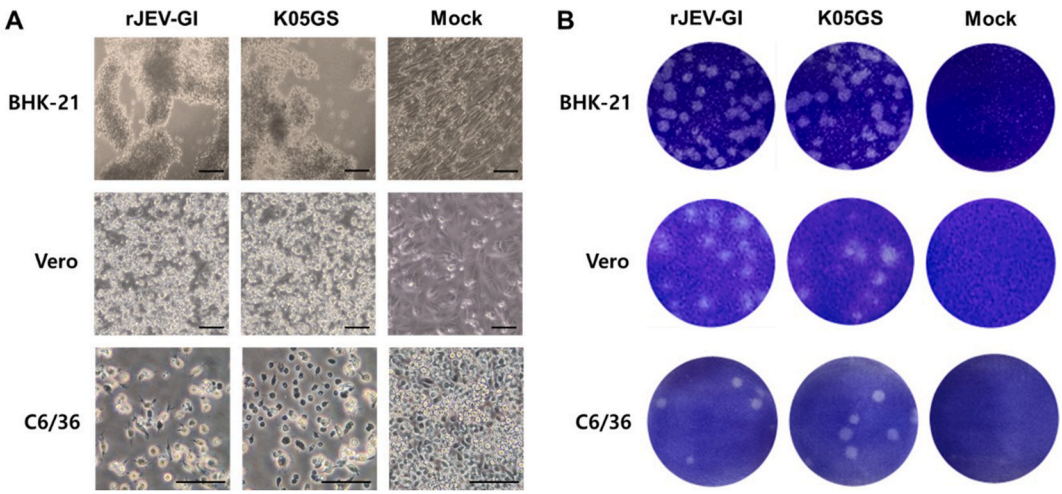

Fig. 2. Cytopathic effect (CPE) and plaques of rJEV-GI or K05GS in each cell. (A) BHK-21, Vero, and C6/36 cells were infected with rJEV-GI or K05GS at a multiplicity of infection (MOI) of 5 each. The morphology of each cell was observed under a microscope (KI-450, Korea Labtech) at 4 days post-infection (dpi) for BHK-21 and Vero cells and 5 dpi for C6/36 cells. Scale bars represent 100 μm in this figure. (B) Cells were infected with 10-fold serially diluted rJEV-GI or K05GS and overlaid with agar. Plaques appeared 5–7 dpi and were stained with crystal violet solution. This experiment was repeated twice, and no significant differences were found. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of JEV E− and NS1-specific antibodies against rJEV-GI and K05GS showed similar patterns and intensities (Fig. 3B).

To test the genetic stability of rJEV-GI, RNA was isolated from rJEV-GI (P4), corresponding cDNAs were synthesized, and full-length nucleotide sequences were analyzed by Sanger sequencing after PCR amplification. The results showed that the entire gene sequence was similar, except for seven substitutions in the envelope gene and one substitution in the prM gene. The substitutions in the envelope gene were silent mutations that did not change the amino acid sequence. However, a single mutation in the prM region on the 731th site from A to G led to an amino acid substitution from glutamic acid to glycine (Table 3). Although this amino acid was substituted in rJEV-GI, it did not appear to affect CPEs, plaques, or cellular protein expression.

2.3. Growth characteristics of the rescued JEV genotype 1

To compare the growth curves of the rescued viruses with those of the parent strain, BHK-21, Vero, and C6/36 cells were infected with rJEV-GI and K05GS at an MOI of 0.1. Viruses were harvested at 12-h intervals, and growth curves were tested using JEV *NS2* gene-specific qRT-PCR. At 96 hpi, no noticeable difference was observed in the growth curves between the two viruses (Fig. 4).

The copy numbers of the two viruses were similar in all three cell types. In BHK-21 cells, $4.88x10^9$ copies/μL ($\pm 2.37 \times 10^8$) of rJEV-GI and 5.26×10^9 copies/μL ($\pm 1.14 \times 10^9$) of K05GS were noted at 96 hpi. In Vero cells, 1.38×10^{10} copies/μL ($\pm 2.21 \times 10^9$) and 1.17×10^{10} copies/μL ($\pm 2.22 \times 10^8$) of rJEV-GI and K05GS, respectively, were noted. The copy numbers of rescued or parental viruses in C6/36 cells were $2.61x10^8$ copies/μL ($\pm 1.41 \times 10^7$) or 2.47×10^8 copies/μL ($\pm 2.74 \times 10^7$), respectively.

Upon comparing the infected cell lines, the fastest and slowest growth of both viruses was observed in BHK-21 cells and c6/36 cells, respectively. Each virus was harvested at 3 hpi, and the titer of rJEV-GI isolated from BHK-21 cells was 4.17×10^7 $TCID_{50}$/mL, while that of K05GS was 1.61×10^7 $TCID_{50}$/mL. The titer of rJEV-GI from Vero cells obtained was 1.33×10^7 $TCID_{50}$/mL and that of K05GS was 3.16×10^7 $TCID_{50}$/mL. The viral titer of C6/36 cells, which had the slowest growth, was 7.50×10^5 $TCID_{50}$/mL. The titer of K05GS was 1.61×10^6 $TCID_{50}$/mL, which was also the lowest. Statistical analysis shows that the two viruses have no significant difference in virus growth rate in the cell lines.

Taken together, via *in vitro* experiments, we confirmed that the rescued JEV-GI exhibited characteristics similar to those of the wild-type JEV, including CPEs, plaque morphology, protein expression, and growth kinetics.

2.4. Pathogenicity of the rescued JEV genotype 1 in mice

To determine the neurovirulence of rJEV-GI, 5-week-old mice were inoculated intracerebrally with 10-fold serial dilutions of rJEV-GI. The mice were monitored for 12 d after virus inoculation (Fig. 5). In the groups inoculated with 1×10^5 and 1×10^4 $TCID_{50}$/mL, all mice died at 8–9 dpi. In the groups administered 1×10^3 $TCID_{50}$/mL of the viruses, all mice inoculated with K05GS died, and only one out of eight mice inoculated with rJEV-GI survived. Both rJEV-GI and K05GS showed similar survival rates in the groups inoculated with 1×10^2 $TCID_{50}$/mL, two out of eight mice survived until 12 dpi at the end of the experiment. All mice survived in the groups inoculated with 10 $TCID_{50}$/mL of rJEV-GI and K05GS. Statistical analysis shows that the two viruses have no significant difference in neurovirulence *in vivo*.

These results showed that both the rescued virus and the parent virus could infect and replicate in mice and had similar pathogenicity.

3. Discussion

Several reverse genetics systems have been developed to rescue recombinant flavivirus. However, the inherent toxicity of flavivirus genome sequences when introduced into *E. coli* remains a challenge. Prokaryotic promoter activity of the CMV promoter in bacteria leads to the cryptic expression of harmful viral proteins [26–29]. Pu et al. developed a convenient and practical novel method that places the 7X TRE-CMVmini tandem repeat sequence upstream of the JEV genome [30,31]. However, there are currently no reports of

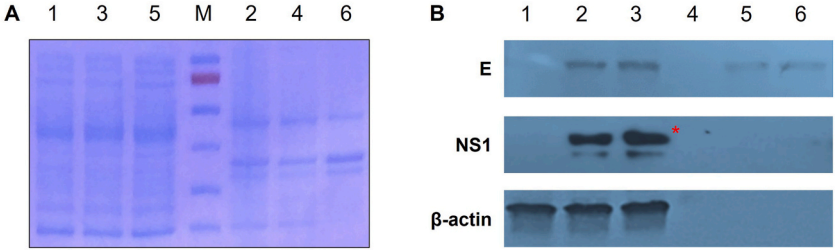

Fig. 3. Expression of JEV E and NS1 proteins. Vero cells were infected with rJEV-GI or-K05GS at an MOI of 0.1, and the cell culture supernatant and lysates were harvested at 5 dpi. (A) SDS-PAGE followed by Coomassie Brilliant Blue G-250 staining (B) Western blot analysis with specific antibodies against JEV E and NS1 protein. M, protein size marker; 1: mock-infected cell lysate; 2: rJEV-GI-infected cell lysate; 3: K05GS-infected cell lysate; 4: mock-infected cell supernatant; 5: rJEV-GI-infected cell supernatant; 6: K05GS-infected cell supernatant. Asterisks indicate a possible cleavage fragment of the JEV NS1 protein (50 kDa) following the instructions from Abcam (ab41651). This experiment was repeated twice, and no significant differences were found. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

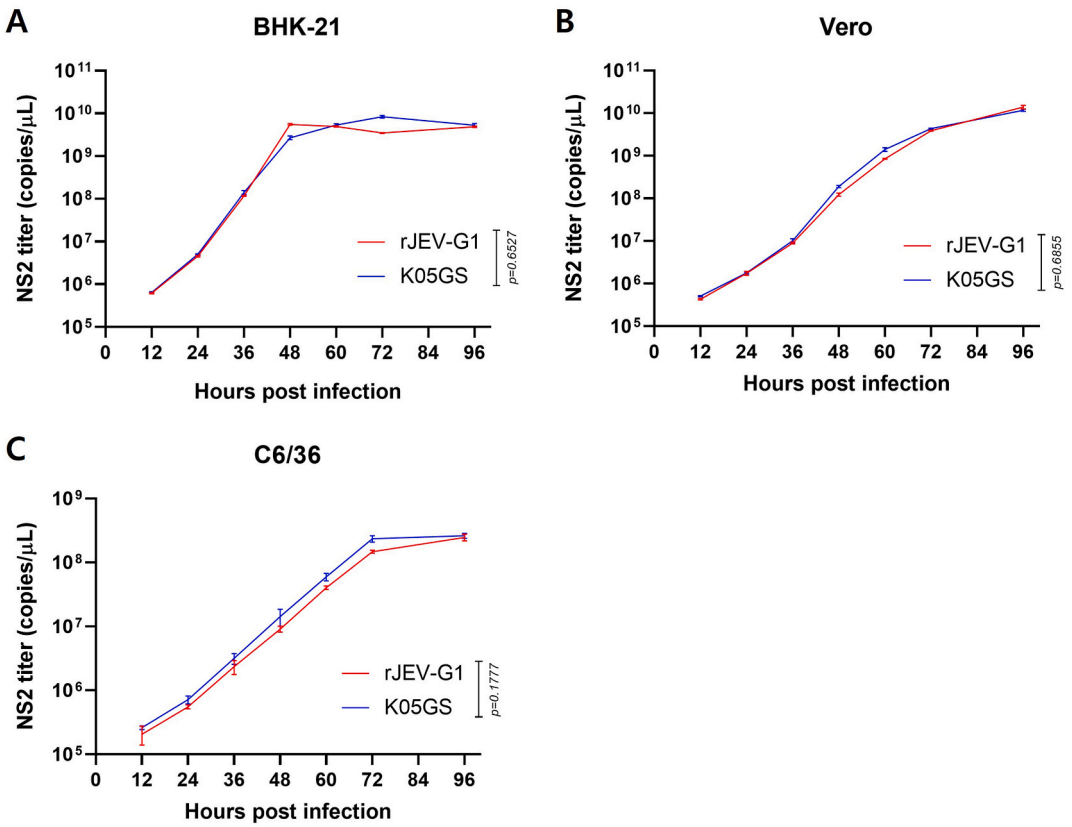

Fig. 4. Growth comparison of three different cell types infected with rJEV-GI and K05GS infected. BHK-21 (A), Vero (B), and C6/36 cells (C) were infected with two JEVs at 0.1 MOI. The culture supernatant samples were collected after 12, 24, 36, 48, 72, and 96 h. Viral RNAs were isolated, and viral cDNAs were synthesized and subjected to qRT-PCR. The data represent the mean ± standard deviation (SD) obtained from three independent experiments. A paired *t*-test was performed for statistical analysis to determine whether the overall growth curves of each virus showed significant differences, and no significant differences were shown at all three cell types (p = 0.6527, 0.6855, 0.1777, respectively).

its application to JEV-GI driven by a CMV promoter system.

In this study, we improved the stability of JEV cDNA and provided precise control over gene expression by strategically placing seven repeated tetracycline response elements upstream of the CMVmini promoter sequence. This represents a promising approach to potentially overcome the problems associated with the use of CMV promoters in bacterial systems. We constructed a vector using the CMV promoter and transfected the constructed plasmid DNA into cells to produce a recombinant virus. This method is much more economical than *in vitro* transcription processes and can be universally applied through a variety of genetic manipulations.

Full-sized clones produce these recombinant viruses through homologous recombination between plasmid DNA containing mutated genes and viral genomic DNA. This technique has been successfully used to generate infectious JEV mutant strains with desired genetic modifications, such as deletions or insertions of foreign gene sequences into the genome [24,25,32,33]. The full-size clone system, specifically, the infectious full-length clone (IFLC) system, is an important tool for studying the entire viral replication cycle and generating infectious recombinant viruses [34,35]. This system consists of expression plasmids encoding viral proteins and transcription plasmids expressing the entire viral genome, thereby enabling the generation of infectious wild-type or recombinant viruses. IFLC systems have been successfully developed for various viral families, including *Arenaviridae, Hantaviridae, Nairoviridae, Peribunyaviridae,* and *Phenuiviridae* [36]. It also allows researchers to examine the impact of specific mutations on the virulence, immunogenicity, and other properties associated with this virus without going through the laborious cloning steps required for traditional reverse genetics approaches using cDNA-based constructs or minigenomes [37–41].

This approach allows for the modification of viral genomes to generate new vaccine candidates. Reverse genetics has been instrumental in the development of vaccines, particularly against influenza viruses [42]. Moreover, this technology opens up the possibility of generating new live attenuated vaccines that could potentially be more effective than current commercial products [25].

Researchers have used reverse genetics to develop new vaccines against JEV. One example is JEV SA14-14-2, which belongs to genotype 3. The JEV SA14-14-2 reverse genetics system has been used to insert or replace foreign genes, point mutations, and gene deletions in vaccine screening and pathogenesis studies [22,24,43]. All current JEV vaccines are GIII-based, inactivated, or live-attenuated whole-virus vaccines. These live attenuated SA14-14-2 vaccines protect mice against lethal challenges caused by various JEV-GI strains [44], and similar cross-genotype protection has been reported in human serum samples [45].

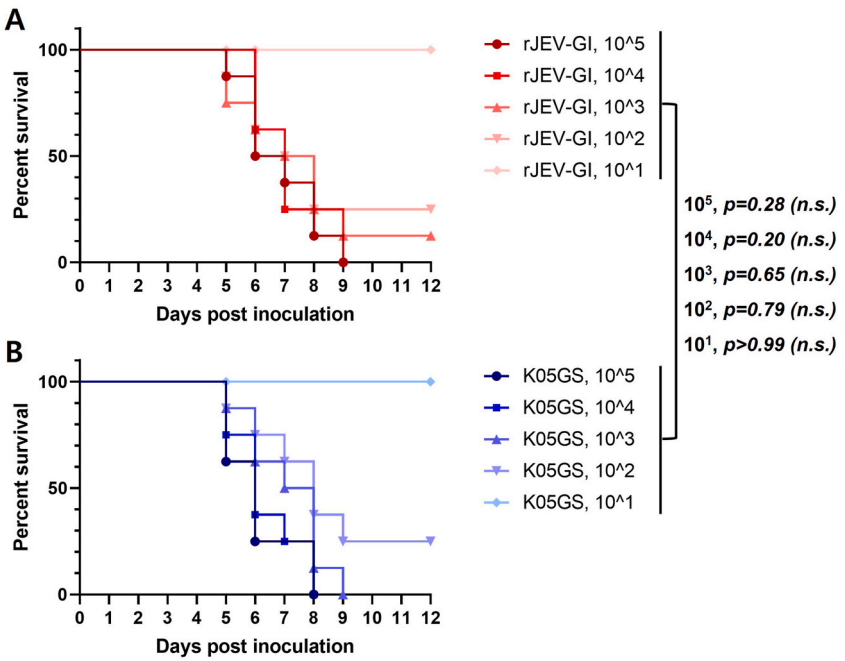

Fig. 5. Comparison of pathogenicity of rJEV-GI and K05GS. K05GS and rJEV were serially diluted up to 10-fold and injected intracerebrally to 5-week-old female BALB/c at 40 μL/dose (eight mice/group). After viral injection, the survival rate of rJEV-GI-injected mice (A) and K05GS-injected mice (B) was recorded every 24 h for 12 days. Log-rank (Mantel-Cox) test was performed to compare each dilution factor of viruses, respectively, using GraphPad Prism.

The first case of JE in South Korea was reported in 1947. Although the incidence of JE has decreased significantly since the implementation of a nationwide vaccination program in 1984, infection with JE continues to be reported in humans [46]. Similar to other Asian JEV-endemic countries, JEV-GIII was widespread in South Korea. The most recent JEV study conducted in 2020 isolated JEV-GVs from mosquitoes. Overall, the epidemiological data from South Korea have shown a transition from JEV-GI to JEV-GV in mosquitoes as the source of infection [6]. Additionally, genotype shifts from JEV-GIII to GI and GV have occurred frequently in South Korea. Hence, there is a need to evaluate the protective effects of JEV-GIII-based vaccines for different genotypes as well as the need to develop a vaccine against JEV-GV [19].

Assuming that JEV-GV will become the dominant strain, the development of GV strain-based vaccines or multivalent vaccines is the need of the hour. Therefore, novel vaccines can be expected to provide effective protection against JEV-GV outbreaks. To develop a JEV-GV-based vaccine, further studies using various recombinant viruses, including in situ isolation, are required to elucidate the molecular mechanisms underlying self-replication, neurovirulence, and pathogenesis of JEV.

4. Conclusions

We developed pTRE-JEVGI, an infectious cDNA clone of the Korean JEV K05GS strain, based on four cDNA fragments and transfected it to generate infectious JEV. It is novel that a recombinant virus for Korean JEV-GI was rescued using a reverse genetics system. We found that the rescued JEV-GI displayed identical viral properties and growth characteristics *in vitro* and similar neurovirulence *in vivo* as the parent strain K05GS.

Through the reverse genetics system, we propose that the development of noncytopathogenic JEV cloning vectors could serve as a valuable tool for studying the biology of JEV and stable vaccine production in cell lines. It is also expected to be useful in developing antiviral drugs or JE vaccines to counter the emergence of new genetic variants of JEV.

5. Materials and methods

5.1. Cells and viruses

Vero (Korean Cell Line Bank, No. 10081) and BHK-21 (Korean Cell Line Bank, No. 10010) cells were maintained in Dulbecco's Minimal Essential Medium (DMEM; Gibco) containing 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin (P/S) at 37 °C in an atmosphere containing 5 % CO_2. C6/36 cells (JCRB cell bank, No. IFO50010) were maintained in Eagle's Minimum Essential Medium (EMEM; ATCC) supplemented with 10 % FBS and 1 % antibiotics at 28 °C, in an atmosphere containing 5 % CO_2. We confirmed that the cell lines used in this experiment have been authenticated within the last three years. These cell lines were tested

using a mycoplasma detection kit (Takara PCR Mycoplasma Detection Set, Cat. No. 6601, Takara) and confirmed mycoplasma free. The wild-type (WT) strain of JEV genotype 1, K05GS (GenBank accession No. KR908702), was obtained from a Korean isolate and purchased from the Korean Disease Control and Prevention Agency (KCDC).

5.2. Construction of full-length complementary DNA (cDNA) clone of JEV

The plasmid harboring the full-length cDNA of the JEV genotype I genome was constructed via a multi-step cloning process. The sequences and locations of the oligonucleotide primers are presented in Table 1 and Fig. 1, respectively. Total RNA was extracted from BHK-21 cells infected with JEV genotype I K05GS using the Direct-zol RNA Miniprep kit (Zymo Research). First-strand cDNA was synthesized using 1 μg of RNA and the reverse transcriptase master premix (Promega) with random primer according to the manufacturer's instructions. To amplify JEV cDNA fragments, PCR was conducted in a reaction volume of 20 μL containing 5x HS Plus PCR Premix (ELPIS Biotech), 1 μL of the cDNA template, and 1 μL of specific primers for each fragment. Particularly, to enhance the stability of JEV cDNA in bacteria, we placed seven repeat tetracycline-response elements (7xTRE) upstream of the CMV mini promoter sequence [30,31]. Therefore, we first amplified the CMVmini promoter region harboring 7x TRE using pTRE-Bi-SG-T (Addgene, Cat. No. 26084) as a template. Then, PCR amplification was conducted on the JEV fragment 1 containing hammerhead ribozyme (HHR), ensuring 15 bp of overlapping sequences with 7xTRE-CMVmini. To provide a precise end, JEV fragment 4 was amplified hepatitis D virus ribozyme (HDVR) and bovine growth hormone transcription terminator (BGH poly A) at the 3′ end using overlapping PCR. Thermal cycling conditions were as follows: 1 cycle of 3 min at 95 °C (initial denaturation) followed by 30 cycles of 30 s at 95 °C, 30 s at 60 °C, and 3 min at 72 °C, with a final extension step of 7 min at 72 °C. PCR products were purified using a Gel/PCR Purification Kit (ELPIS Biotech) and subcloned into the pGEM T-easy vector (Promega). Several clones were sequenced using an ABI 3730xl DNA analyzer (Applied Biosystems). A plasmid containing the full-length JEV cDNA was constructed by assembling each fragment into a pGEM T-easy vector backbone using unique restriction sites. Finally, all artificially created restriction sites in the viral genome were exchanged with the original viral sequences using site-directed mutagenesis. The constructed vector was named pTRE-JEVGI.

5.3. Generation of the recombinant virus

To generate the rescued virus (rJEV), pJEV-GI was transfected into BHK-21 cells using Lipofectamine 2000 (Invitrogen). BHK-21 cells were seeded on a 6-well cell culture plate at a density of $0.8x10^6$ cells/well. The following day, 2.5 μg of plasmid and 8 μL of Lipofectamine 2000 in 100 μL serum-free DMEM were mixed and then left at room temperature for 30 min. The cells were incubated for 3 days at 37 °C in an atmosphere containing 5 % CO_2, and the culture supernatants were collected by centrifugation and passaged into Vero cells to obtain fresh rJEVs.

5.4. Western blot analysis

Vero cells seeded on T75 flasks at a density of $6.0x10^6$ cells/flask were infected with rJEV and K05GS at an MOI of 0.1. After 5 days

Table 1
Primers used in cDNA construction.

Name of primer[a]	Sequence (5' to 3')[b]
For construction of T-TRE-JEV GI vector	
TRE-SacI-F	GTCGACAAGCGCATGAACTCTTTGATGACCT
CMVmini-HHR-R	ACTCATCAGAGAAGTAGCTCTGCTTATATAGGCCTCCC
HHR-F1-1	CGAAACGAGTAAGCTCGTCAGAAGTTTATCTGTGTGAACTTCTTG
HHR-F1-2	GAGTCCGTGAGGACGAAACGAGTAAGCTCG
HHR-F1-3	ACTTCTCTGATGAGTCCGTGAGG
F1-SalI-R	GTCGACCCCCATGCTTTCCAGCCCATC
F2-SalI-F	GTCGACCATTCTCTTCGCCCCCGAATTGG
F2-NdeI-R	CATATGCCCGCATAGTCCGTGATCCATTC
F3-NdeI-F	CATATGTGTGTGGTTCGTGGCAAGTGTGA
F3-MluI-R	ACGCGTGCTATGGACCTCTCCCTTCCCCAC
F4-MluI-F	ACGCGTGAAAAAATTAGGAAGAGAATCCAGAAGCTCAAAG
polyA-OC-R	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGATCCTGTGTTCTTCC
PolyA-HDVR-OC-F	AAAAAAAAAAAAAAAAAAAAAAAAAAGGGTCGGCATGGCATCTCCACCT
BGHpA-MluI-R	ACGCGTCCATAGAGCCCACCGCATCCCC
For site-directed mutagenesis	
F1-F2 SDM-F	GAGATGGGCTGGAAAGCATGGGGAAAAAGCATTCTCTTCGCCCCCG
F1-F2 SDM-R	CGGGGGCGAAGAGAATGCTTTTTCCCCATGCTTTCCAGCCCATCTC
F2-F3 SDM-F	GAATGGATCACGGACTATGCGGGAAAAACTGTGTGGTTCGTGGCAAGTG
F2-F3 SDM-R	CACTTGCCACGAACCACACAGTTTTTCCCGCATAGTCCGTGATCCATTC
F3-F4 SDM-F	GGGGAAGGGAGAGGTCCATAGCAACCAGGAAAAAATTAGGAAGAGAATC
F3-F4 SDM-R	GATTCTCTTCCTAATTTTTTCCTGGTTGCTATGGACCTCTCCCTTCCCC

[a] F and R indicate forward and reverse primers.
[b] Restriction endonuclease sites are underlined.

of infection, each infected cell was collected by repetitive pipetting, centrifuged, and separated into cell debris and supernatant. The cell debris samples were diluted by ten-fold using 1X PBS (phosphate-buffered saline). Each infection sample was boiled at 99 °C for 15 min using a 5X protein sample buffer (Elpis Biotech). The sample was separated in 10 % SDS-PAGE gel and transferred onto the nitrocellulose membrane (Bio-rad). The membranes were blocked with 5 % skim milk in Tris-buffered saline (TBS). *Anti*-JEV envelope (E) antibody (GeneTex; 1:1000 dilution) and anti-JEV non-structural protein 1 (NS1) antibody (Abcam; 1:1000 dilution) were reacted with the membrane. After repeated washing, the membranes were bound with goat anti-rabbit IgG-HRP (Abcam; 1:5000 dilution) and goat anti-mouse IgG-HRP (Abcam; 1:5000 dilution) to detect specific JEV E and NS1-specific antibodies, respectively. Chemiluminescence was observed using a ChemiDoc Imaging System (Bio-Rad) after washing and reacting the membranes with Clarity Western ECL substrate (Bio-Rad). To normalize the concentration of the samples, β-actin was detected using an anti-β-actin antibody (Santa Cruz Biotechnology) and goat anti-mouse IgG-HRP.

5.5. Plaque observation and titration of the rescued virus

To visually observe cytopathic effects (CPE) in Vero or BHK-21 cells, each cell at 70 % confluence was infected with 10-fold diluted rJEV-GI or wild-type JEV-GI in 6-well plates. For C6/36 cells, diluted virus was used to infect C6/36 cells at 90 % confluency. To measure the titer of the viruses, each virus-infected cell in 96 well plates was stained after 5–7 days of infection with 0.5 % crystal violet solution and washed with tap water. The absorbance was measured using an Epoch microplate reader (BioTek) at 570 nm.

$TCID_{50}$ (50 % tissue culture infectious dose) was calculated by referring to the Reed–Muench method [45]. For plaque assay, Vero or BHK-21 cells in a 6-well plate were infected at a density of $0.8x10^6$/well with 10-fold diluted rJEV-GI or wild-type JEV-GI and incubated for 2 h at 37 °C. The cells were washed and cultured in 3 mL of 1.5–2 % SeaPlaque® agarose (Lonza) melted in virus growth media (VG-DMEM; DMEM with 1 % FBS and 1 % P/S) for 6 days. C6/36 cells, which were cultured and infected at 28 °C, were incubated with 3 mL of 1.5 % agarose melted in virus growth media (VG-EMEM; EMEM with 1 % FBS and 1 % P/S). The cells were fixed and stained with 0.5 % crystal violet solution to visualize plaques.

5.6. Quantitative real-time RT-PCR for viral growth curve

Multiple sequence alignments of the gene encoding nonstructural protein 2 (NS2) in the JEV sequence available in GenBank were performed using the SeqMan program (Lasergen, Inc.), and conserved regions were identified. Primers specific to the target region were designed and synthesized by Macrogen Inc. Information on the primers and their amplicon sizes are shown in Table 2. WT JEV-G1 (K05GS) and rJEV were used to infect the three cell lines at an MOI of 0.1, and culture supernatants were collected at 12, 24, 36, 48, 72, and 96 h post-infection (hpi). The viral RNAs were isolated from each infected culture supernatant using a NucleoSpin Virus kit (Macherey-Nagel), and then real-time RT-PCR (qRT-PCR) was performed using Step One Plus real-time PCR system (Applied Biosciences) with One Step TB Green® PrimeScript™ PLUS RT-PCR kit (TaKaRa) following the manufacturer's instructions.

To establish a standard curve for the quantification of viral RNA copies, a 113-bp-long JEV partial *NS2* gene-containing plasmid was constructed. Briefly, viral RNA was isolated from K05GS as described above, and JEV cDNA was synthesized by RT-PCR using SuperScript II RTase. JEV NS2 region-specific PCR was performed using cDNA and NS2-specific primers. After insertion of the PCR product into pGEM®-T easy vector (Promega) by TA cloning technique, the cloned plasmid was serially diluted up to 10 folds and used for setting the quantitative standard for NS2-specific qRT-PCR.

5.7. Neurovirulence test

Four-week-old BALB/c mice were purchased from Orient Bio Inc. and housed in animal isolator cages with ad libitum access to laboratory-grade feed and water supplements. rJEV and K05GS were serially diluted up to 10 folds from $1x10^5$ $TCID_{50}$ to 10 $TCID_{50}$ in PBS. Under the influence of anesthesia, eight mice per group were inoculated intracerebrally with 40 μL of either rJEV and K05GS and monitored for 12 days, twice a day. This animal study was approved by the Institutional Animal Care and Use Committee of Konkuk University (approval number: KU23105) and was carried out following the guidelines of animal welfare and standards for animal tests.

5.8. Statistical analysis

GraphPad Prism software Version 8 (GraphPad Prism Version 8, GraphPad Software) was used in this study. The various treatments were compared by a paired *t*-test and Log-rank (Mantel-Cox) test assuming unequal variance.

Ethics approval and consent to participate

Not applicable.

Data availability

Data will be made available on request.

Table 2
Primers for JEV-specific quantitative PCR.

Name of primer	Sequence (5′ to 3′)
JEV_NS2–F	CCCAAGCATCAGCACAAG
JEV_NS2-R	AGCTGGGCCTTCTGGT

Table 3
Nucleotide and amino acid substitutions in the infectious cDNA clone pTRE-JEVGI, rescued JEV-GI (P4), and the wild-type virus K05GS.

Nucleotide position within K05GS genome	Nucleotides			Amino acid substitutions	Gene position
	K05GS	pTRE-JEVGI	rJEV-GI		
15	A	A	G	No	E[a]
234	A	A	C	No	E
399	G	G	A	No	E
435	A	A	G	No	E
438	C	C	G	No	E
731	A	A	G	Glu[b]→ Gly[c]	prM[d]
1362	G	G	A	No	E
1380	A	A	G	No	E

[a] E, envelop.
[b] G, glutamic acid.
[c] Gly, glycine.
[d] prM, premembrane.

CRediT authorship contribution statement

Sehyun Kim: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation. **Min Sun Kim:** Writing – review & editing, Writing – original draft, Methodology. **Aleksandra Nowakowska:** Validation, Methodology, Investigation. **Heejae Choi:** Methodology, Investigation. **Hee Won Bang:** Methodology, Investigation. **Young Bong Kim:** Supervision, Data curation. **Hee-Jung Lee:** Writing – review & editing, Writing – original draft, Validation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that may have influenced the work reported in this study.

Acknowledgments

This study was supported by the Korean Health Technology Research and Development Project (No. HV23C0074 and HV22C0263) and the Research of the Korea Centers for Disease Control and Prevention (2020-ER5301-01).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33142.

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