Construction of a Full-length Infectious Clone of Zika Virus Stably Expressing EGFP Marker in a Eukaryotic Expression System

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Abstract

Background: Zika virus is among the most widely transmitted arboviruses in the world and closely associated with diseases, such as encephalitis, fetal microcephaly, and Guillain–Barré syndrome. The pathogenic mechanism of the virus has not been fully elucidated, and there are no vaccines or specific drugs targeting the virus. To address these issues, the application of reverse genetics is needed for viral reconstruction and reproduction.

Methods: Polymerase chain reaction (PCR) was used to merge the full-length Zika virus genome, CMV promoter, intron, EGFP, hepatitis delta virus ribozyme, and SV40 terminator sequence for cloning into a pBAC11 vector through recombination to produce recombinant pBAC-ZIKA-EGFP. The ZIKA–EGFP was rescued by transfection of 293T cells with pBAC-ZIKA-EGFP, and at 7-days post-transfection, the supernatant (P0 generation) was passed through a 0.45-μm membrane and used to infect Vero cells (to produce the P1 generation). Fluorescence-based quantitative PCR, 50% tissue culture infectious dose, and plaque assays were used to measure differences in replication ability and pathogenicity relative to the rescue virus (ZIKA–WT), the sequence of which is consistent with that of the wild-type Zika virus. Additionally, caffeic acid phenethyl ester (CAPE), a nuclear factor kappaB (NF-kB) inhibitor, was used to examine its effect on viral replication.

Results: The results showed that ZIKA–EGFP could effectively infect Vero cells, SH-SY5Y cells and C6/36 cells, and cause cytopathic effects on them. ZIKA–EGFP exhibited stable replication and EGFP expression during cell passage for at least six generations, with no significant difference in replication ability relative to the ZIKA–WT. Fluorescent cell foci were observed in the plaque assay while the ZIKA–EGFP was in the absence of phage plaque formation. The inhibition of NF-kB inhibitor on ZIKA-EGFP was observed by fluorescence microscopy, which was consistent with the results of fluorescence quantitative PCR.

Conclusions: We constructed an infectious clone of the full-length genome of Zika virus which could replicate with stable EGFP expression in eukaryotic cells during passage. The infectious clone, remaining main characteristics of wild type ZIKA virus could be appied on the studies of reverse genetics, drug screening and gene function of ZIKA virus.

Background

Zika virus is among the most widely transmitted arboviruses in the world. The virus was first discovered in the Zika Forest in Uganda in 1947, and an outbreak of Zika virus in the Americas in 2015 caused a global public health emergency [1–4]. Approximately 80% of Zika infections are asymptomatic, with the most common symptoms including fever, arthralgia, rash, myalgia, edema, vomiting, and non-purulent conjunctivitis [5]. The virus can infect the placenta and blood–brain barrier endothelial cells, neurons, and neural stem cells and is closely associated with diseases, such as encephalitis, fetal microcephaly, and Guillain–Barré syndrome [6–11]. Similar to other flaviviruses, the Zika genome encodes an open reading

frame containing three structural proteins (C: capsid; prM: precursor of membrane; and E: envelope) that comprise the viral particles and seven genes encoding non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) that participate in RNA replication [12]. Recent studies show that some mutations of the Zika virus are related to host adaptation[13–16]; however, the pathogenic mechanism of the virus has not been fully elucidated, and there are no vaccines or specific drugs targeting the virus. To address these issues, the application of reverse genetics is needed for viral reconstruction and reproduction [13, 17].

This study describes a method for constructing a full-length infectious clone of the Zika virus in a eukaryotic expression system that can be used to produce the virus along with stable expression of the enhanced green fluorescence protein (EGFP) marker. We found that the replication ability of ZIKA-EGFP did not significantly differ from that of ZIKA-WT. These results provide a foundation for future studies on Zika virus mutations, pathogenic mechanisms, and drug screening.

Methods

Construction of a Full-length Infectious Clone of Zika Virus

As shown in Fig. 1, the CMV promoter sequence synthesized by Guangzhou IGE Biotechnology Co., Ltd. (Guangzhou, China) was fused to the Zika viral sequence of (nt 1–5908) using polymerase chain reaction (PCR) in order to generate the 5' half sequence of the Zika virus infectious clone (ZF). The hepatitis delta virus ribozyme and SV40polyA sequences synthesized by Guangzhou IGE Biotechnology Co., Ltd. were then ligated to the Zika viral sequence (nt 5735–10,807) using PCR to generate the 3' half sequence of the clone (ZR). Recombination was then performed using a tan Infusion kit (Takara, Dalian, China). Two fragments were ligated into the pBAC11 vector (Addgene, Watertown, MA, USA) previously digested with restriction enzymes (*Sfo*I and *Pac*I; New England Biolabs, Ipswich, MA, USA) to generate recombinant pBAC-ZIKA (Fig. 1b).

The intron sequence (derived from the pCI-neo vector plasmid) synthesized by Guangzhou IGE Biotechnology Co., Ltd. was inserted into the ZF sequence of the Zika virus (between nt 3128 and 3129) to generate the sequence of the ZFi fragment (the G-to-A mutation introduced at nt 3128 was used as a marker for subsequent experiments). The ZFi and ZR fragments were recombined using the described restriction-digested vector pBAC11 to generate recombinant pBAC-ZIKAi (Fig. 1c).

The *EGFP* gene was inserted downstream of the 37^{th} codon of the zika virus using the described methods, and the full-length coding-region sequence of the Zika virus was inserted to construct pBAC–ZIKA–EGFP (Fig. 1d). Amplification of the constructed plasmids was conducted using DH10B competent cells.

Virus Rescue

293T cells (held in our laboratory) were seeded in 6-well plates and transfected with 5 μg of the pBAC–ZIKA–EGFP plasmid using the transfection reagent Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA). The supernatant containing the P0 generation of the Zika virus was collected 7 days later, passed through a 0.45-μm membrane filter (Pall Corporation, Port Washington, NY, USA), and aliquoted and stored at −20°C until use for infecting Vero cells (held in our laboratory). At 3- to 6-days post-infection, supernatant containing the P1 generation of the virus was collected, and Vero cells were re-infected with the P1 virus in order to produce viral generations P2, P3, P4, and P5.

Total RNA from Vero cells infected with ZIKA–EGFP was extracted, and *EGFP* expression was examined by PCR using primer 1 (5’ AGTTGTTGATCTGTGTGAATCAGACTG 3’) targeting the Zika sequence and primer 2 (5’CTTGTACAGCTCGTCCATGCCGAGAGTG3’) targeting the EGFP sequence. Cytopathic changes and expression of green fluorescence were monitored. Western blot was then used to measure levels of EGFP, NS1 and E protein in Vero cells infected with ZIKA–EGFP, ZIKA-WT and wide-type Zika virus.

Viral Replication Ability

ZIKA–WT and ZIKA–EGFP, each at a multiplicity of infection (MOI) of 1, were used to infect Vero cells. Supernatants and cells were collected after 12 h, 24 h, and 48 h, respectively, and total RNA was extracted and reverse transcribed. The replication of viral RNA in cells was measured using fluorescence-based quantitative PCR (qPCR) [18] under the following conditions: one cycle at 95 °C for 2 min, followed by a three-step procedure comprising 30 s at 95 °C, 15 s at 55 °C, and 15 s at 72 °C for 40 cycles (with data collection at the end of the 72 °C step at each cycle) and cooling at 37 °C for 10 min. The sequences of primers and probe are provided in Table 1. An assay to determine the 50% tissue culture infectious dose ($TCID_{50}$) was used to determine viral titer in the supernatant, with $TCID_{50}$ values calculated according to the Reed–Muench method.

Viral Pathogenicity

ZIKA-WT and ZIKA-EGFP, each at a MOI of 1, were used to infect SH-SY5Y cells (KG217, KeyGEN BioTECH, Jiangsu, China), respectively. After 48 h and 72 h, we measured the cytotoxic effects using a Cell Counting Kit-8 (CCK-8; Dojindo, Shanghai, China) assay. Cytopathic changes were observed using a microscope (Eclipse E200, Nikon, Japan).

Plaque assays was used to determine the virulence of ZIKA-WT and ZIKA–EGFP [19]. Vero cells were seeded in 24-well plates at 5×10^4 cells/well, and the following day, an appropriate viral titer was used to infect the cells. At 2-h post-infection, 2% carboxymethylcellulose was used to overlay the cells, and after 6 days, Crystal Violet staining was performed to observe plaque sizes.

Inhibitory Effect of Caffeic Acid Phenethyl Ester (CAPE; Selleck, Shanghai, China) on ZIKA–EGFP Viral Replication

ZIKA–EGFP and ZIKA–WT (MOI: 0.1) were used to infect Vero cells, and at 2-h post-infection, the supernatant was discarded and replaced with a media containing 10 μM CAPE. After a 48-h incubation, relative expression levels of the viruses were measured using fluorescence-based qPCR. Additionally, fluorescence intensity was observed under a fluorescence microscope (ECLIPAS Ti2, Nikon).

Results

Production of Rescued Virus

The pBAC–ZIKA–EGFP clone generated as described in Fig. 1 was used to transfect 293T cells, supernatant from which was collected at 7-days post-transfection and used to infect Vero cells. The cells were passaged every 4 to 6 days for a total of five consecutive passages. We extracted total RNA from the cells after infection and identified *EGFP* expression by PCR, demonstrating the stability of the ZIKA–EGFP viral sequence following cell passage (Fig. 2a). At 48-h post-infection with ZIKA–EGFP (generation P5), green fluorescence was clearly visible under a fluorescence microscope (Fig. 2b), and western blot revealed the presence of EGFP in Vero cells at 48-h post-infection (Fig. 2c). We then infected C6/36 cells (held in our laboratory) with the virus and observed ZIKA–EGFP proliferation (Fig. 2d), with cytopathic changes and cell death prominent after 48 h.

Viral Replication Ability

Levels of viral RNA in Vero cells infected with ZIKA–EGFP (MOI: 0.1) for 12 h, 24 h, and 48 h were measured using fluorescence-based qPCR. The results showed that ZIKA–EGFP viral RNA could be amplified in Vero cells within a time period comparable with that of the ZIKA–WT (Fig. 3a). Cell-culture supernatants were then collected and subjected to $TCID_{50}$ assay, which showed similar titers of ZIKA–EGFP and ZIKA–WT (Fig. 3b). These findings indicated similar replication abilities between the ZIKA–EGFP and ZIKA–WT.

Viral Pathogenicity

After infection of SH-SY5Y cells with the ZIKA–EGFP and ZIKA–WT (MOI: 1) for 48 h, we observed prominent cytopathic changes, with dead cells seen floating in the culture media. Additionally, CCK-8 assays showed that the 72-h viability of ZIKA–EGFP-infected cells decreased significantly, and that virulence was only slightly weaker than that of the ZIKA–WT (Fig. 4a).

Plaque assay results showed that infection with the ZIKA-WT led to the formation of prominent, perfectly round, and uniform plaques after 6 days (Fig. 4b). By contrast, infection with the ZIKA–EGFP did not result in plaque formation, although pronounced cell clusters were observed by fluorescence microscopy (Fig. 4c).

Inhibitory Effect of CAPE on Viral Replication

Vero cells infected with the ZIKA–EGFP and ZIKA–WT, respectively, were treated with 10 μM CAPE for 48 h, with fluorescence-based qPCR results revealing that CAPE significantly inhibited viral replication (Fig. 5a). These findings were verified by fluorescence microscopy showing no fluorescence, which agreed with the results of fluorescence-based qPCR (Fig. 5b).

Statistics Analysis

All experiments were repeated at least three times. Data were presented as mean ± SEM. Results were analyzed using Graphpad Prism (GraphPad Software Inc., La Jolla, CA). Comparison between experimental groups was made by Student's t-test and one-way ANOVA. A value of $P<0.05$ was considered statistically significant.

Discussion

The reverse genetics approach is an important method for studying viral gene function, virulence variation, and drug screening. The presence of cryptic bacterial promoters in flaviviruses has increased the challenges associated with construction of infectious clones [17, 20]. The common solution to this issue is mutation of the cryptic promoter or utilization of a single-copy plasmid-based bacterial artificial chromosome. In this study, we used a bacterial artificial chromosome to reduce the toxicity of the viral DNA in order to obtain a full-length clone of Zika virus.

Infectious clones can be constructed in three types of systems. The first is the in vitro transcription system[21–23], where a full-length DNA clone is used as a template to transcribe viral mRNA molecules using T7 or SP6 RNA polymerase. The purified viral RNA is then used to transfect cells via electrotransfection or other methods to produce rescued viruses. This method requires highly pure mRNA and often expensive reagents and longer experimental times, because suitable transfection methods and conditions need to be optimized. The second approach involves use of eukaryotic systems[1, 24, 25], where a clone containing the full-length viral sequence is constructed and placed under the control of a eukaryotic promoter, such as CMV or SV40. Upon transfection of target cells with the plasmid, viral mRNA can be generated using the transcription machinery of the eukaryotic cells, enabling subsequent rescue of the viruses produced in the cells. This method generally does not require expensive reagents or instruments, is simple to perform, and the conditions are easily optimizable. However, the limitation of this method involves the need for transcription inside the nucleus during generation of viral mRNA. Chen Y et al. used this method to construct infectious clones and did not observe incorrect pre-mRNA splicing, which could be due to sequence differences in different viral strains [24]. The third approach is using recombination systems with overlapping fragments[18, 26]. In this system, the full-length DNA clone and the transcription-control elements are amplified as three fragments, which are then transfected into target cells via electrotransfection. The complete viral genome sequence is then generated in the cells by homologous recombination to subsequently produce the rescued viruses. This method can avoid the toxic effect of the cryptic bacterial promoter, however, the transfection conditions need to be optimized, and the efficiency of homologous recombination can greatly affect viral production.

To facilitate investigations of Zika virus pathogenesis, previous studies attempted to include a marker, such as EGFP or luciferase at C25, C33, or other positions, in the Zika viral genome[20, 27]. However, most attempts did not lead to stable expression of the markers. Upon analysis of the secondary structure of the viral RNA, we designed the marker-insertion site at the C37 position, which was in the middle of a stem. Our results showed that insertion at this site led to a ZIKA–EGFP viral sequence that remained stable in the genome for at least five generations, with no adverse effect on the range of cells that could be infected (the ZIKA–EGFP was able to infect Vero, SH-SY5Y, C6/36, and endothelial cells). Additionally, we found that the viral-replication speed did not differ significantly from that of the ZIKA–WT. Moreover, ZIKA–EGFP was cytopathogenic and led to cytopathic changes but showed weaker virulence than the ZIKA–WT and no plaque formation. These findings indicated the effectiveness of constructing a full-length infectious clone of the ZIKA–EGFP in a eukaryotic system. Furthermore, we noted that EGFP insertion at positions C36 and C38 also produced stable expression clones, and that the vector constructed using this method contained two cyclization sequences (CS) sites. Introduction of a mutation into the second CS site resulted in no change in viral titer, and insertion of luciferase at position C37 resulted in stable expression for at least three generations (data not shown).

To validate the use of ZIKA–EGFP for monitoring drug effectiveness, we performed an experiment using CAPE, shown to inhibit Zika viral replication in our previous experiments. The results showed that treatment with the drug significantly suppressed fluorescence associated with EGFP levels, which was consistent with results observed following inhibition of RNA replication. These findings suggested that ZIKA–EGFP could be applicable for use in drug screening.

Conclusions

In summary, we successfully constructed an infectious clone of the full-length genome of Zika virus stably expressing an EGFP marker in a eukaryotic expression system. The results presented here provide a foundation for future studies on ZIKA viral gene mutation, function, and drug screening.

Abbreviations

EGFP: green fluorescence protein; MOI: multiplicity of infection; qPCR: quantitative PCR; TCID50: 50% tissue culture infectious dose; CAPE: Caffeic Acid Phenethyl Ester; CS: cyclization sequences

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

JG, LJS, and BZ designed the experiments. JG, LJS, JYC, and JTC carried out the experiments. ZWL and JG analyzed the data. JG and LJS were Co-first author and wrote the paper. BZ and WZ approved the final version of the paper. All authors read and approved the final manuscript.

References

1. Shan C, Xie X, Muruato AE, Rossi SL, Roundy CM, Azar SR, et al. An Infectious cDNA Clone of Zika Virus to Study Viral Virulence, Mosquito Transmission, and Antiviral Inhibitors. Cell Host Microbe. 2016; 19(6):891-900.
2. Dick GW, Kitchen SF, Haddow AJ. Zika virus. I. Isolations and serological specificity. Trans R Soc Trop Med Hyg. 1952; 46(5):509-20.
3. Dick GW. Zika virus. II. Pathogenicity and physical properties. Trans R Soc Trop Med Hyg. 1952; 46(5):521-34.
4. World Health Organization. Zika virus outbreaks in the Americas. Wkly Epidemiol Rec. 2015; 90(45):609-10.
5. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis. 2008; 14(8):1232-9.
6. Mlakar J, Korva M, Tul N, Popovic M, Poljsak-Prijatelj M, Mraz J, et al. Zika Virus Associated with Microcephaly. N Engl J Med. 2016; 374(10):951-8.
7. De Carvalho NS, De Carvalho BF, Fugaca CA, Doris B, Biscaia ES. Zika virus infection during pregnancy and microcephaly occurrence: a review of literature and Brazilian data. Braz J Infect Dis. 2016; 20(3):282-9.

8. Calvet G, Aguiar RS, Melo ASO, Sampaio SA, de Filippis I, Fabri A, et al. Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study. Lancet Infect Dis. 2016; 16(6):653-60.
9. Himmelsbach K, Hildt E. Identification of various cell culture models for the study of Zika virus. World J Virol. 2018; 7(1):10-20.
10. Mladinich MC, Schwedes J, Mackow ER. Zika Virus Persistently Infects and Is Basolaterally Released from Primary Human Brain Microvascular Endothelial Cells. mBio. 2017; 8(4).
11. Rolfe AJ, Bosco DB, Wang J, Nowakowski RS, Fan J, Ren Y. Bioinformatic analysis reveals the expression of unique transcriptomic signatures in Zika virus infected human neural stem cells. Cell & Bioscience. 2016; 6.
12. Kuno G, Chang GJ. Full-length sequencing and genomic characterization of Bagaza, Kedougou, and Zika viruses. Archives of virology. 2007; 152(4):687-96.
13. Duggal NK, McDonald EM, Weger-Lucarelli J, Hawks SA, Ritter JM, Romo H, et al. Mutations present in a low-passage Zika virus isolate result in attenuated pathogenesis in mice. Virol J. 2019; 530:19-26.
14. Rossi SL, Ebel GD, Shan C, Shi PY, Vasilakis N. Did Zika Virus Mutate to Cause Severe Outbreaks? Trends in microbiology. 2018; 26(10):877-85.
15. Lin D, Li L, Xie T, Yin Q, Saksena N, Wu R, et al. Codon usage variation of Zika virus: The potential roles of NS2B and NS4A in its global pandemic. Virus Res. 2018; 247:71-83.
16. Xia H, Luo H, Shan C, Muruato AE, Nunes BTD, Medeiros DBA, et al. An evolutionary NS1 mutation enhances Zika virus evasion of host interferon induction. Nature communications. 2018; 9(1):414.
17. Avila-Perez G, Park JG, Nogales A, Almazan F, Martinez-Sobrido L. Rescue of Recombinant Zika Virus from a Bacterial Artificial Chromosome cDNA Clone. J Vis Exp. 2019(148).
18. Usme-Ciro JA, Lopera JA, Enjuanes L, Almazan F, Gallego-Gomez JC. Development of a novel DNA-launched dengue virus type 2 infectious clone assembled in a bacterial artificial chromosome. Virus Res. 2014; 180:12-22.
19. Yu J, Liu X, Ke C, Wu Q, Lu W, Qin Z, et al. Effective Suckling C57BL/6, Kunming, and BALB/c Mouse Models with Remarkable Neurological Manifestation for Zika Virus Infection. Viruses. 2017; 9(7).
20. Avila-Perez G, Nogales A, Martin V, Almazan F, Martinez-Sobrido L. Reverse Genetic Approaches for the Generation of Recombinant Zika Virus. Viruses. 2018; 10(11).
21. Zhao F, Xu Y, Lavillette D, Zhong J, Zou G, Long G. Negligible contribution of M2634V substitution to ZIKV pathogenesis in AG6 mice revealed by a bacterial promoter activity reduced infectious clone. Sci Rep. 2018; 8(1):10491.
22. Marquez-Jurado S, Nogales A, Avila-Perez G, Iborra FJ, Martinez-Sobrido L, Almazan F. An Alanine-to-Valine Substitution in the Residue 175 of Zika Virus NS2A Protein Affects Viral RNA Synthesis and Attenuates the Virus In Vivo. Viruses. 2018; 10(10).

23. Liu ZY, Yu JY, Huang XY, Fan H, Li XF, Deng YQ, et al. Characterization of cis-Acting RNA Elements of Zika Virus by Using a Self-Splicing Ribozyme-Dependent Infectious Clone. J Virol. 2017; 91(21).
24. Chen Y, Liu T, Zhang Z, Chen M, Rong L, Ma L, et al. Novel genetically stable infectious clone for a Zika virus clinical isolate and identification of RNA elements essential for virus production. Virus Res. 2018; 257:14-24.
25. Schwarz MC, Sourisseau M, Espino MM, Gray ES, Chambers MT, Tortorella D, et al. Rescue of the 1947 Zika Virus Prototype Strain with a Cytomegalovirus Promoter-Driven cDNA Clone. mSphere. 2016; 1(5).
26. Tsetsarkin KA, Kenney H, Chen R, Liu G, Manukyan H, Whitehead SS, et al. A Full-Length Infectious cDNA Clone of Zika Virus from the 2015 Epidemic in Brazil as a Genetic Platform for Studies of Virus-Host Interactions and Vaccine Development. mBio. 2016; 7(4).
27. Gadea G, Bos S, Krejbich-Trotot P, Clain E, Viranaicken W, El-Kalamouni C, et al. A robust method for the rapid generation of recombinant Zika virus expressing the GFP reporter gene. Virology. 2016; 497:157-62.

Tables

Table 1. Primers used to detect Zika virus genes.

Primer	Sequence (5'→3')
ZIKV E-Forward	CVGACATGGCTTCGGACAGY
ZIKV E-Reverse	CCCARCCTCTGTCCACYAAYG
ZIKV E-probe	AGGTGAAGCCTACCTTGACAAGCARTCA

Figures

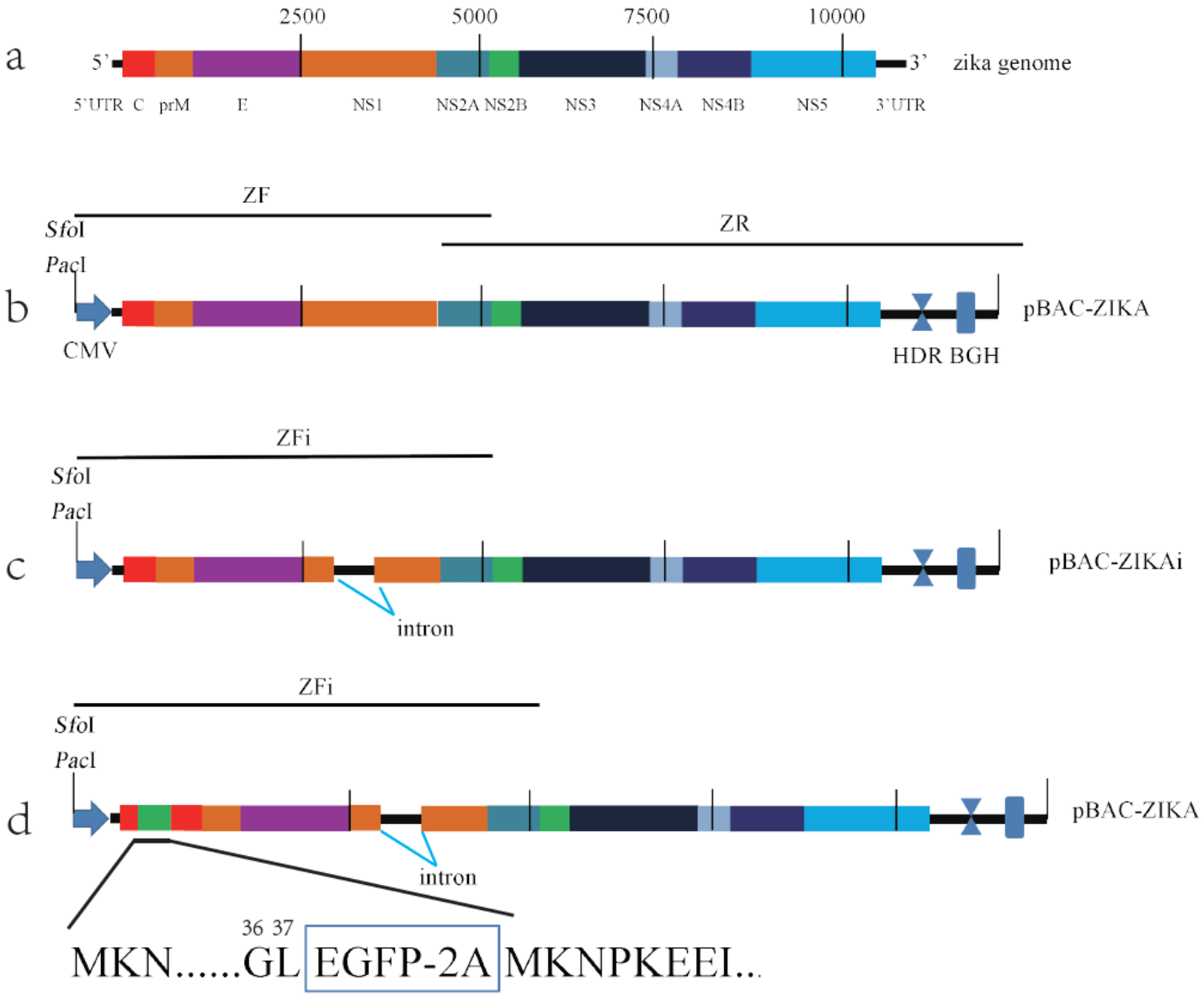

Figure 1

a Genomic structure of the zika virus. b Schematic diagram showing the structure of pBAC-ZIKA. CMV, HDV, and BGH denote the CMV promoter sequences, the hepatitis delta virus ribozyme sequence, and the bovine growth hormone terminator sequence, respectively. SfoI and PacI are restriction endonucleases. pBAC-ZIKA was constructed by recombination of the ZF and ZR fragments using the restriction-digested vector. c Schematic diagram showing the structure of pBAC-ZIKAi. The ZFi fragment was generated by inserting the intron sequence into the genomic sequence of the Zika virus downstream of nt 3128, followed by its recombination with ZR with the restriction-digested vector to generate pBAC-ZIKAi. d pBAC–ZIKA–EGFP was constructed by inserting the EGFP gene downstream of the 37th codon of the Zika virus sequence

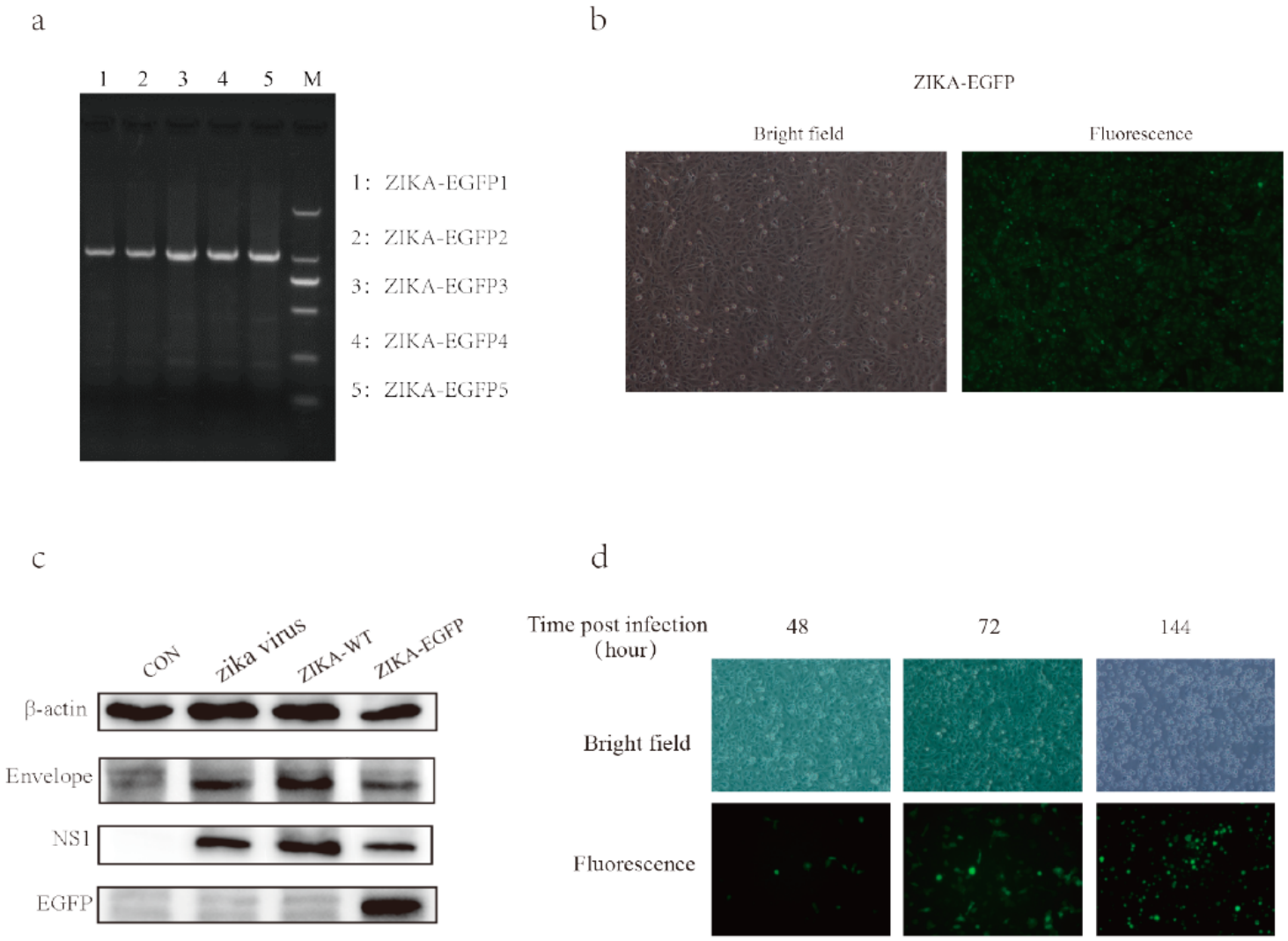

Figure 2

Rescue of recombinant ZIKA–EGFP. a Detection of EGFP by electrophoresis. b Fluorescence of Vero cells infected with the ZIKA–EGFP (generation P5) for 48 h. C Western blot detection of EGFP levels in Vero cells infected with the ZIKA–EGFP (generation P5) for 48 h. D Fluorescence of C6/36 cells infected with ZIKA–EGFP at different time points. CON, control

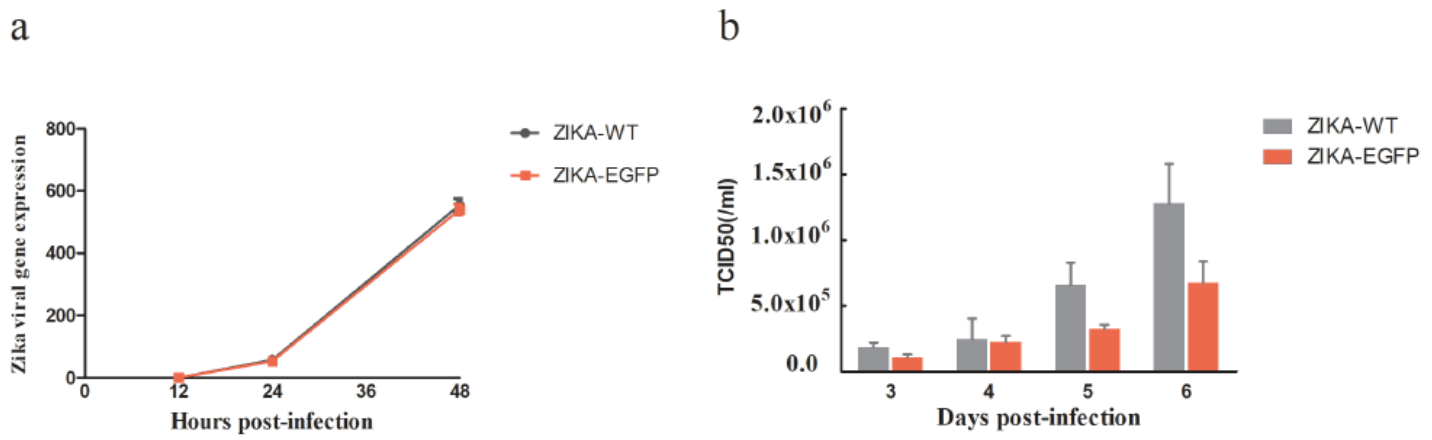

Figure 3

Viral kinetics of ZIKA–WT and ZIKA–EGFP in Vero cells. Vero cells were infected with ZIKA–WT and ZIKA–EGFP, and a intracellular viral RNA expression was detected at various time points by fluorescence qPCR. b Viral titer in culture supernatant was determined by TCID50

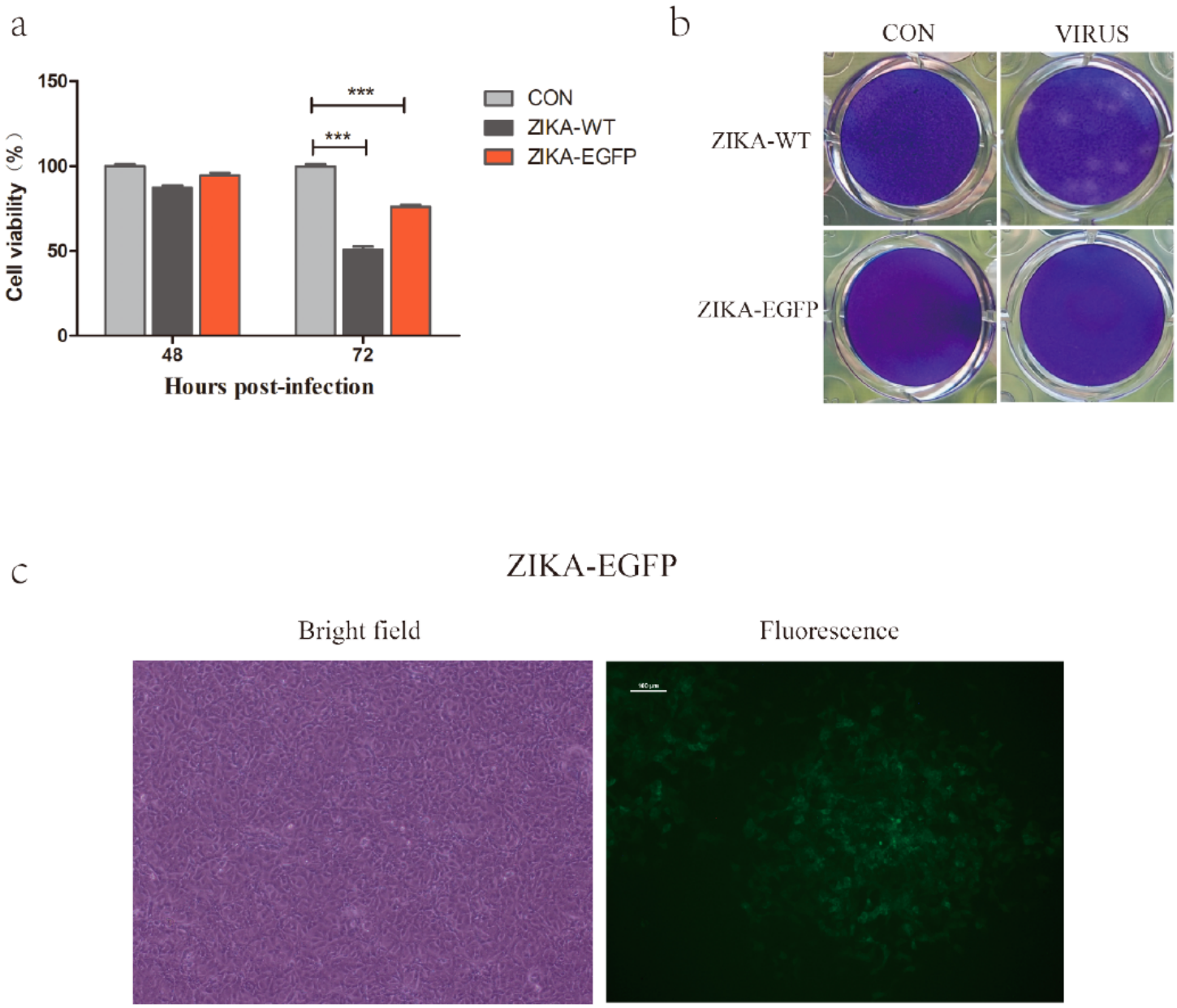

Figure 4

Comparison of ZIKA–EGFP and ZIKA–WT virulence. a SH-SY5Y cells were incubated with ZIKA–WT and ZIKA–EGFP (MOI = 1.0), and cell viability was determined by CCK-8 assay at 48- and 72-h post-infection. b Virulence was compared by plaque assays. C Fluorescent cells aggregated due to the cytopathic effects associated with ZIKA–EGFP. ***p < 0.001. CON, control

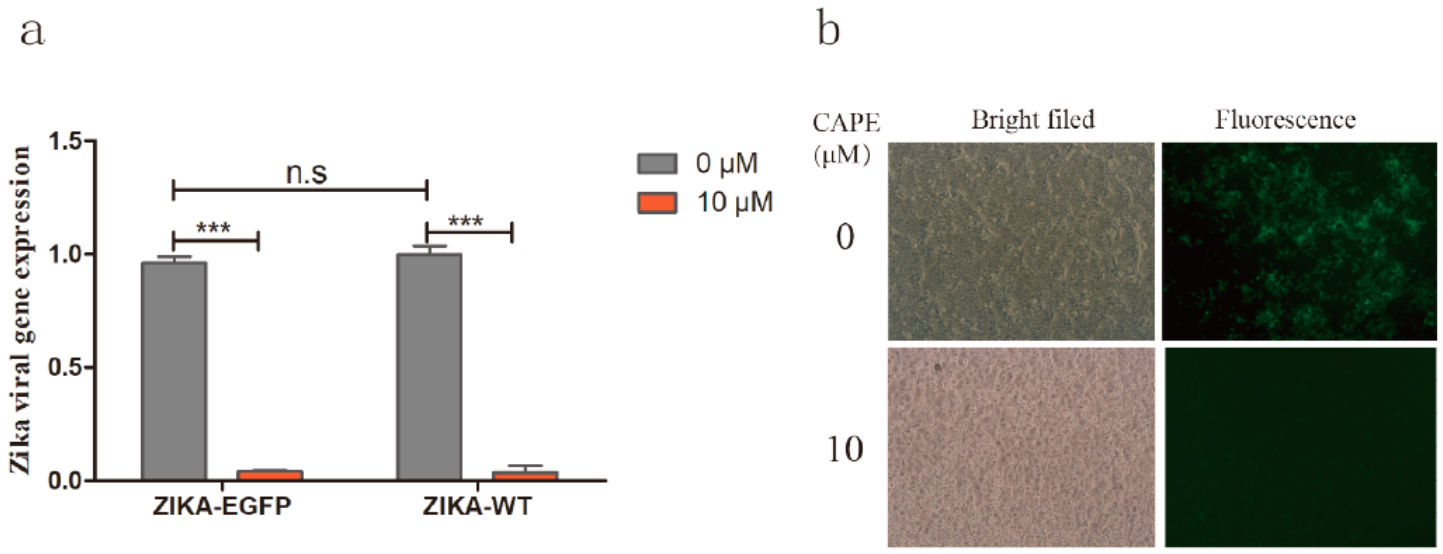

Figure 5

CAPE inhibition of ZIKA–EGFP infection. a Vero cells infected with ZIKA–WT and ZIKA–EGFP at a MOI of 0.1 were cultured in the presence and absence of 10 μM CAPE for 48 h, followed by detection of Zika viral gene expression by fluorescence qPCR. b Fluorescent cells were observed under a fluorescence microscope. ***$p < 0.001$. n.s., not significant