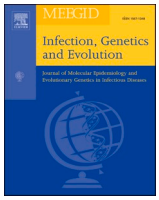
Infection, Genetics and Evolution 132 (2025) 105776

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid



Reverse genetics system for Tick-borne encephalitis virus using Circular Polymerase Extension Reaction

Saki Mitsunaga [a], Tomokazu Tamura [b,c,d,e,f], Samuel Nyampong [a], Takasuke Fukuhara [b,c,d,e,g,h], Hiroshi Shimoda [a,i,j], Daisuke Hayasaka [a,i,j,*]

[a] Laboratory of Veterinary Microbiology, Joint Graduate School of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan
[b] Department of Microbiology and Immunology, Faculty of Medicine, Hokkaido University, Sapporo, Japan
[c] Institute for Vaccine Research and Development (IVReD), Hokkaido University, Sapporo, Japan
[d] One Health Research Center, Hokkaido University, Sapporo, Japan
[e] Department of Virology, Faculty of Medicine, Kyushu University, Fukuoka, Japan
[f] Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan
[g] Laboratory of Virus Control, Center for Infectious Disease Education and Research, Osaka University, Suita, Japan
[h] AMED-CREST, Japan Agency for Medical Research and Development (AMED), Tokyo, Japan
[i] Laboratory of Veterinary Microbiology, Joint Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan
[j] Division of Pathogenic Microorganism, Research Center for Thermotolerant Microbial Resources, Yamaguchi University, Yamaguchi, Japan

ARTICLE INFO

Keywords:
Tick-borne encephalitis virus
Polymerase chain reaction-based Circular Polymerase Extension Reaction
Reverse genetics system

ABSTRACT

Tick-borne encephalitis virus (TBEV) belongs to the Family *Flaviviridae*, Genus *Orthoflavivirus*, and causes severe neurological diseases in humans. The reverse genetics system is a basic tool for viral research; however, cloning the genome of orthoflavivirus using bacterial plasmids is difficult because of their toxicity to *Escherichia coli.* Polymerase chain reaction-based Circular Polymerase Extension Reaction (CPER), an *E. coli*-free reverse genetics system, has been recently applied to several RNA viruses. However, there have been no reports on recombinant TBEV produced using CPER. In this study, we attempted to produce recombinant TBEV Oshima, Sofjin and Hypr strains by CPER. Genome sequencing and plaque-forming assays were performed to determine the properties of rescued TBEVs. In addition, infectivity and pathogenicity of rescued TBEVs was also monitored in C57BL/6 mice. Rescued TBEVs of Oshima, Sofjin and Hypr caused apparent cytopathic effect and efficient propagations in BHK cells, and showed intrinsic virulence in mice. The rescued TBEVs showed several nucleotide and amino acid substitutions compared to the original viral sequences. These results showed that infectious TBEVs from the Oshima, Sofjin, and Hypr strains were produced using CPER. We propose that future applications of this method will contribute to related research on TBEV.

1. Introduction

Tick-borne encephalitis (TBE) is a serious neurological disorder in humans and is an important public health concern. The typical symptoms of TBE include fever, myalgia, meningitis, ataxia, and paralysis with neurological sequelae (Pustijanac et al., 2023). TBE is currently distributed throughout Europe to far-east Asia and is related to the vectors of Ixodes ticks, such as *Ixodes ricinus*, *I. persalcatus,* and *I. ovatus* (Jaenson et al., 2012; Mansfield et al., 2009; Yoshii et al., 2017). Humans are mainly infected by tick bites, and unpasteurized milk from infected ruminants is a possible cause of infection (Holzmann et al., 2009; Hudopisk et al., 2013; Paulsen et al., 2019). Although there are no specific treatments for TBE, effective vaccines have been developed and introduced in European countries (Amicizia et al., 2013; Lehrer and Holbrook, 2011). Notably, the incidence of TBE has increased in some European countries in recent years (Kwasnik et al., 2023).

The causative agent of TBE is the TBE virus (TBEV) belonging to the Family Flaviviridae, the genus Orthoflavivirus, and the species *Orthoflavivirus encephalitidis*. Orthoflaviviruses are enveloped capsid viruses with a single-stranded, positive-sense RNA genome, and the viral genome is approximately 11 kb nucleotides with a single open reading frame (ORF) and 5′ and 3′ end untranslated regions (UTRs). The ORF encodes three structural proteins, capsid protein (C), a precursor to the membrane protein (PrM), envelope protein (E), and seven nonstructural

* Corresponding author at: Laboratory of Veterinary Microbiology, Joint Graduate School of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan.
E-mail address: dhaya@yamaguchi-u.ac.jp (D. Hayasaka).

https://doi.org/10.1016/j.meegid.2025.105776
Received 25 March 2025; Received in revised form 12 May 2025; Accepted 30 May 2025
Available online 1 June 2025

proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Chambers et al., 1990).

TBEV has been classified into three subtypes: far-eastern (FE), European (Eu) and Siberian (Sib) (Kovalev and Mukhacheva, 2017). In addition, Baikalian and Himalayan subtypes have recently been distinguished (Kwasnik et al., 2023). They have been suggested to cause more severe neurological diseases with higher fatality rates than the Eu and Sib subtypes (Ruzek et al., 2019).

The reverse genetics system is a basic tool used in viral research to elucidate the viral factors that determine biological properties, such as pathogenicity, infectivity, and immunogenicity. The generation of the first infectious cDNA clone of orthoflavivirus was reported for yellow fever virus (YFV) (Rice et al., 1989). These systems, including TBEV Eu and FE subtypes, have been developed (Hayasaka et al., 2004; C W Mandl et al., 1997), and bacterial plasmids harboring viral genomes have been used to clone and generate recombinant viruses as a conventional method (Lai et al., 1991; Christian W Mandl et al., 1997; Sumiyoshi et al., 1992; Yamshchikov et al., 2001). However, it has been shown that the recombinant plasmids, including some orthoflavivirus sequences such as YFV and TBEV are often not suitable for replication in *E. coli* due to their toxicity (Gritsun and Gould, 1998; Rice et al., 1989).

The PCR-based Circular Polymerase Extension Reaction (CPER) is a bacterial cloning-free reverse genetics system for generating infectious clones of viruses (Edmonds et al., 2013; Torii et al., 2021) that can generate recombinant viruses rapidly and simply without conventional plasmid cloning step in bacteria. CPER was first established to generate infectious clones of the Kunjin strain of the West Nile virus (Edmonds et al., 2013) and has since been applied to several orthoflaviviruses such as YFV, Binari virus, Dengue virus, Powassan virus and Japanese encephalitis virus, and other RNA viruses (Conde et al., 2023; Dong et al., 2023; Piyasena et al., 2019; Sanchez-Velazquez et al., 2020; Tamura et al., 2022; Torres et al., 2022). However, there have been no reports on recombinant TBEV produced using CPER. In this study, we generated infectious recombinant TBEV of FE and Eu subtypes using the CPER method.

2. Methods

2.1. Viruses and cells

Viral RNA of the TBEV Oshima and Sofjin strains used to produce infectious TBEVs was kindly provided by Dr. Hiroaki Kariwa of Hokkaido University. Plasmid DNA, including the genome of the TBEV Hypr strain (Wallner et al., 1996), was kindly provided by Dr. Daniel Ruzek of Masaryk University. Oshima strains were isolated from dog blood in Hokkaido in 1993 (Takashima et al., 1997). The Oshima and Sofjin strains were classified as FE subtype, and the Hypr strain was classified as an Eu subtype. All experiments using infectious TBEV were performed in a biosafety level 3 (BSL-3) facility at Yamaguchi University according to standard BSL-3 guidelines.

Baby hamster kidney (BHK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, NY, U.S.A) supplemented with 10 % heat inactivated fetal bovine serum (FBS; BIOSERA, France) and 1 % Penicillin Streptomycin (Pen/Strep; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and maintained at 37 °C in a 5 % CO_2 incubator.

2.2. Reverse transcription (RT)-PCR

Viral RNAs were extracted using ISOGEN-LS (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. Primers for the CPER of the Oshima, Sofjin, and Hypr strains were designed to divide each viral genome into seven fragments with 20 bp overlap regions referred to registered TBEV sequences (accession numbers AB062063.2 for Oshima, AB062064.1 for Sofjin and U39292.1 for Hypr), as shown in Table 1 and Fig. 1, according to previous studies (Tamura et al., 2022).

Table 1
Primers for PCR fragments of TBEV.

TBEV strain	Primer	Position	Sequence
Oshima	Os-F1-F	1–20	5′-ttgcttcggatagcaacag-3′
	Os-F1-R	1982–1998	5′-tcaccgggatcctacag-3′
	Os-F2-F	1982–1998	5′-ctgtaggatcccggtga-3′
	Os-F2-R	3993–4013	5′-caggtcttgctgtgtcatgag-3′
	Os-F3-F	3993–4013	5′-ctcatgacacagcaagacctg-3′
	Os-F3-R	6133–6152	5′-cattttgtcctgctctggtc-3′
	Os-F4-F	6133–6152	5′-gaccagagcaggacaaaatg-3′
	Os-F4-R	6836–6857	5′-cttgttgtcatcactgcttctc-3′
	Os-F5-F	6836–6857	5′-gagaagcagtgatgacaacaag-3′
	Os-F5-R	7960–7980	5′-caattgtgtaggctctgacac-3′
	Os-F6-F	7960–7980	5′-gtgtcagagcctacacaattg-3′
	Os-F6-R	10,049–10,068	5′-cgctagcatgaatgctccag-3′
	Os-F7-F	10,049–10,068	5′-ctggagcattcatgctagcg-3′
	Os-F7-R	11,081–11,100	5′-agcgggtgtttttccgagtc-3′
Sofjin	So-F1-F	1–20	5′- agattttcttgcacgtgcgt -3′
	So-F1-R	1974–1993	5′-gggatcctacagggtttggt-3′
	So-F2-F	1974–1993	5′-accaaaccctgtaggatccc-3′
	So-F2-R	3964–3983	5′-cagaagtccaacaccggtct-3′
	So-F3-F	3964–3983	5′-agaccggtgttggacttctg-3′
	So-F3-R	6062–6081	5′-tctgcgcttctttccactgc-3′
	So-F4-F	6062–6081	5′-gcagtggaaagaagcgcaga-3′
	So-F4-R	6795–6814	5′-agcaccgtcagcaacgtgta-3′
	So-F5-F	6795–6814	5′-tacacgttgctgacggtgct-3′
	So-F5-R	7928–7947	5′-gccgagaggctgcatagtat-3′
	So-F6-F	7928–7947	5′-atactatgcagcctctcggc-3′
	So-F6-R	10,044–10,063	5′-gcatgaatgctccaggtcgt-3′
	So-F7-F	10,044–10,063	5′-acgacctggagcattcatgc-3′
	So-F7-R	10,875–10,894	5′-agcgggtgtttttccgagtc-3′
Hypr	Hy-F1-F	1–20	5′- agattttcttgcgcgtgcgt −3′
	Hy-F1-R	1811–1830	5′-gccactcttcaggtggtact-3′
	Hy-F2-F	1811–1830	5′-agtaccacctgaagagtggc-3′
	Hy-F2-R	3637–3658	5′-tgacaagcaaagcgagaacgacg-3′
	Hy-F3-F	3545–3566	5′-gggtccccggaatagtggcatt-3′
	Hy-F3-R	5555–5574	5′-aggaggtgtcgctgtcatca-3′
	Hy-F4-F	5555–5574	5′-tgatgacagcgacacctcct-3′
	Hy-F4-R	6515–6534	5′-cagggcactgacgcatctgt-3′
	Hy-F5-F	6515–6534	5′-acagatgcgtcagtgccctg-3′
	Hy-F5-R	8019–8043	5′-aaatttgatcaagttccaacccagg-3′
	Hy-F6-F	7971–7993	5′-atacaccattggtggaagagggc-3′
	Hy-F6-R	10,009–10,028	5′-caatcgacaggcactgctga-3′
	Hy-F7-F	10,009–10,028	5′-tcagcagtgcctgtcgattg-3′
	Hy-F7-R	10,816–10,835	5′-agcgggtgtttttccgagtc-3′

RT reactions (65 °C for 5 min, 42 °C for 60 min, and 70 °C for 15 min) were performed using GeneAce cDNA Synthesis Kit (NIPPON GENE, Tokyo, Japan) with primers of Os-F7-R, So-F7-R, and Hy-F7-R. PCR was carried out using PrimeSTAR Max DNA Polymerase (Takara Bio, Shiga, Japan), and those conditions were 98 °C for 1 min, followed by 35 cycles of 10 s at 98 °C, 5 s at 55 °C, 30 s at 72 °C, and a final extension at 72 °C for 5 min. The linker fragment plasmid was designed to encode a hepatitis delta virus ribozyme, poly (A) signal, and cytomegalovirus promotor to transcribe viral RNA from CPER products in transfected cells. Each PCR product was purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

2.3. CPER

The PCR fragments of each virus and the linker fragment were used for CPER to generate circular DNA (Fig. 1). The DNA concentration was adjusted to 0.1 pmol. CPER was performed under the following conditions: 98 °C for 2 min, followed by 20 cycles of 10 s at 98 °C, 15 s at 55 °C, 12 min at 68 °C, and a final extension at 68 °C for 12 min using PrimeSTAR GXL DNA Polymerase (Takara Bio, Shiga, Japan).

2.4. Rescue of infectious viruses

CPER product (25 μL), X-tremeGENE™ HP DNA Transfection Reagent (12 μL; MilliporeSigma, Burlington, MA, USA), and Opti-MEM™ I

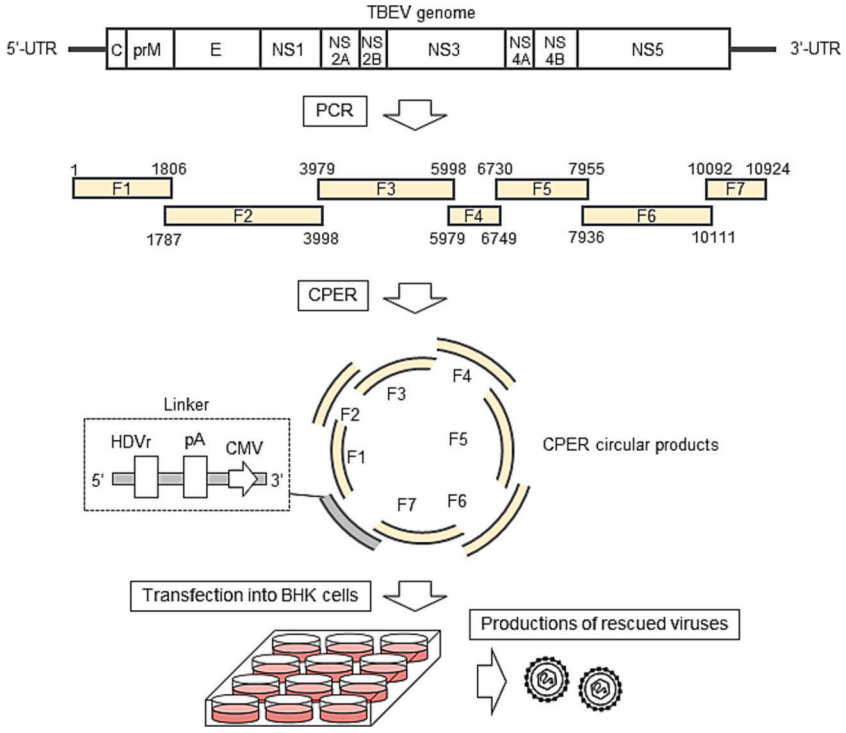


Fig. 1. A schematic presentation of CPER products from TBEV genome sequences. HDVr, pA and CMV stan for hepatitis delta virus ribozyme, poly (A) signal, and cytomegalovirus promoter, respectively.

Reduced Serum Medium (200 μL; GIBCO, NY, U.S.A) were mixed and incubated at room temperature for 15 min, and applied to BHK cells with 1 mL of 2 % FCS DMEM in 12 well plates. Following cytopathic effect (CPE) was observed, the supernatants of the inoculated cells were harvested and applied to fresh BHK cells to obtain stock viruses for further analysis (Fig. 1).

2.5. TBEV genome sequences

The original viral sequences of Oshima, Sofjin, and Hypr were determined by Sanger sequencing using RT-PCR products of each TBEV. Rescued viral genomic RNAs were extracted from the stock viruses and RT-PCR was performed on seven fragments from Oshima, Sofjin, and Hypr. The RT-PCR products were consigned to Eurofins (Tokyo, Japan) and analyzed by Sanger sequencing.

2.6. Plaque forming assay (PFA)

Serially diluted TBEV samples were inoculated into BHK cells. After incubation for 90 min at 37 °C in a 5 % CO_2, the cells were washed with DMEM with 0 % FBS twice, then overlaid with 0.3 % agarose (SeaPlaque™ Agarose, Lonza, Basel, Swiss) in DMEM containing 10 % FBS. The plate was incubated at 37 °C in a 5 % CO_2 for 3 days. The cells were then fixed with formalin for 30 min, stained with crystal violet, and the plaques were counted. Viral titers are indicated in pfu/mL.

2.7. Viral growth kinetics in cell cultures

The growth kinetics of the rescued TBEVs were examined in BHK cells at a multiplicity of infection (MOI) of 0.01 in 12 well-plates. After infection with each virus diluent with 2 % FBS DMEM, supernatant samples were collected at 0, 12, 24, 36, 48, and 60 h post-infection (hpi). The supernatants were centrifuged at 3500 rpm for 5 min to remove debris and stored at −80 °C. The viral titers of the supernatant samples were determined using PFA.

2.8. Mouse experiment

C57BL/6j mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and mated at the Yamaguchi University Animal Facility. Six-to eight-week-old mice were subcutaneously inoculated with rescued TBEVs (10^4 pfu of Oshima and Sofjin, and 10^5 pfu of Hypr) in 2 % FCS DMEM. The mice were monitored daily for clinical signs of disease, body weight, and survival for 21 days. Mice that exhibited *endo*-point (more than 30 % weight reductions or severe neurologic signs) were euthanized with overdose of isoflurane. The animal experiments were performed in accordance with the Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology. The Animal Care and Use Committee of Yamaguchi University approved all the experimental protocols (approval number: 05–30-564).

3. Results

3.1. Rescues of infectious TBEV by CPER

Seven fragments of each of the Oshima, Sofjin, and Hypr strains were successfully amplified using the designed primers (Fig. 2).

CPER was performed using RT-PCR fragments for each TBEV, and the products were transfected into BHK cells. Typical CPE such as rounding, detaching and floating was observed in cells transfected with Oshima, Sofjin, and Hypr strains at 3, 3, and 4 days post-infections (dpi), respectively (Fig. 3A). The production of infectious viruses was confirmed by plaque formation, and clear plaques were observed in the BHK cells transfected with the respective supernatants (Fig. 3B). The plaque size in Sofjin was larger than that in Oshima, as observed in a previous report (Goto et al., 2002, 2003), and Hypr had a mixture of large and small plaques (Fig. 3B). These results indicated that the infectious viruses of the Oshima, Sofjin, and Hypr strains were successfully produced by CPER in BHK cells transfected with CPER products.

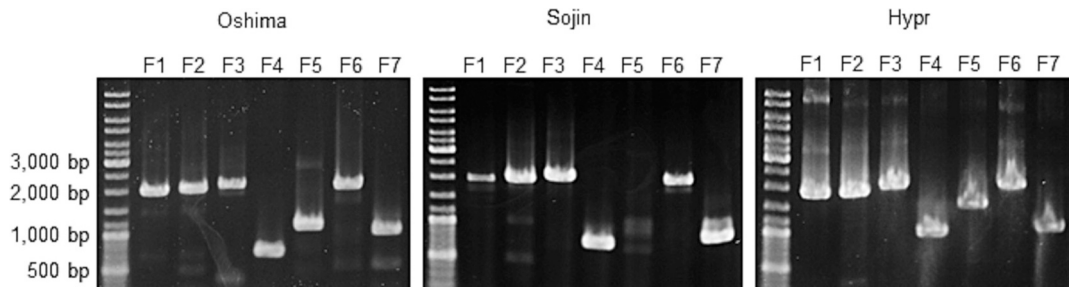


Fig. 2. RT-PCR amplification of overlapping TBEV genome fragments. Primers for CPER were designed to construct seven DNA fragments of each Oshima, Sofjin, and Hypr strain. Seven fragments of each virus were amplified using RT-PCR.

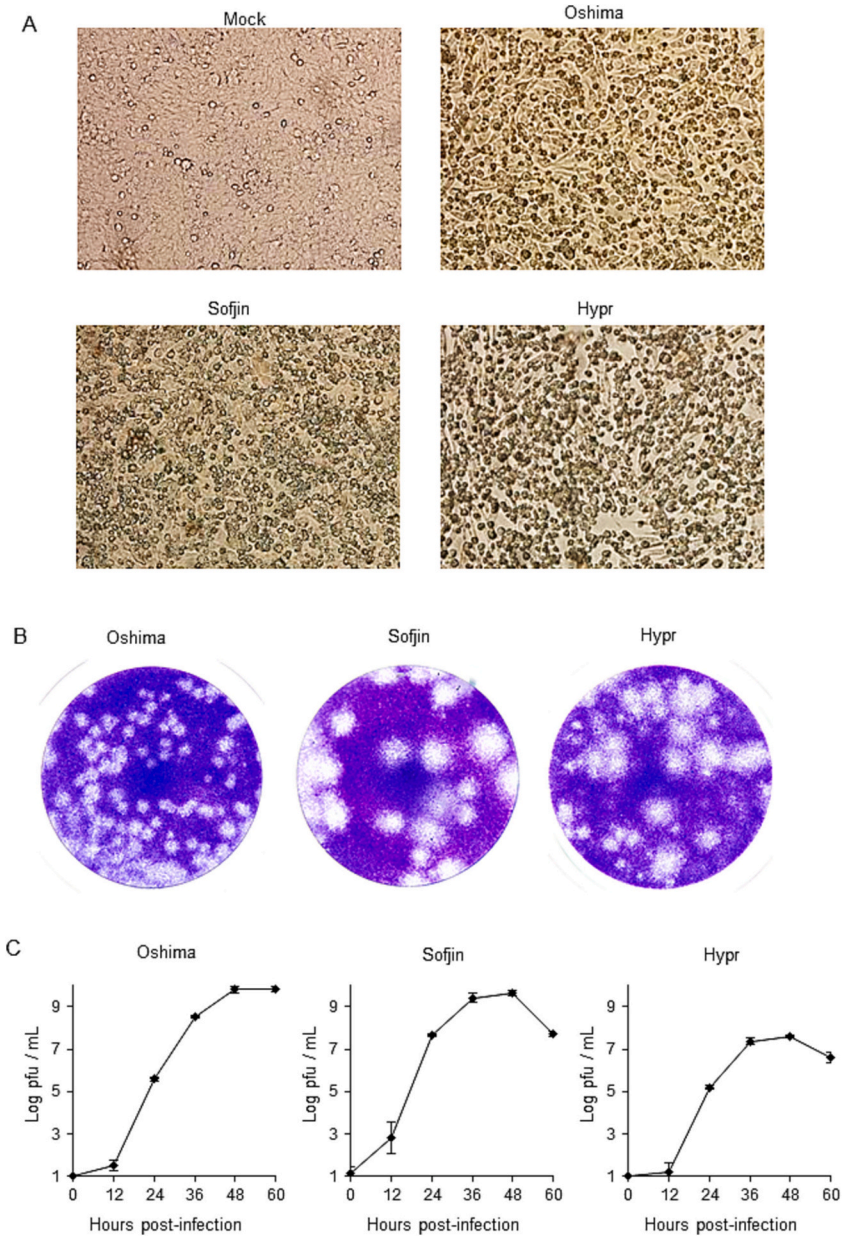


Fig. 3. Production of infectious TBEV. (A) CPEs observed in BHK cells transfected with CPER products of Oshima, Sofjin, and Hypr strains at 3, 3, and 4 dpi, respectively. Mock was inoculated with 2 % DMEM. (B) Plaque forming morphology in the BHK cells infected with rescued Oshima, Sofjin, and Hypr strains at 3 dpi. (C) Growth kinetics of infectious viral titers in the supernatants of BHK cells infected with rescued TBEV of Oshima, Sofjin and Hyper at MOI of 0.01 ($n = 3$). Error bars indicate standard deviations.

3.2. Growth kinetics of rescued TBEV in BHK cells

The supernatants of CPE cells were inoculated into fresh BHK cells to obtain a higher titer of stock viruses. Those viral titers of Oshima, Sofjin, and Hypr were 1.0×10^8 pfu/mL, 7.0×10^6 pfu/mL, and 2.7×10^7 pfu/mL, respectively.

We next examined growth kinetics of rescued TBEVs in BHK cells (Fig. 3C). Infectious viruses of rescued Oshima, Sofjin and Hypr strains were detected at 12 hpi and viral titers peaked at 48 hpi (Fig. 3C). These observations indicate that rescued TBEVs efficiently infect and propagate in susceptible BHK cells.

3.3. Viral sequences of rescued virus

The rescued virus of Oshima showed one nucleotide difference in 3′-NCR compared with the original viral sequences (Table 2). Rescued Sofjin possessed two mutations: A to G at nucleotide position 1579 and G to A at position 5995 in the region of E and NS3, respectively (Table 2). These mutations caused amino acid substitutions of alanine with threonine and asparagine with aspartate, respectively (Table 2). Rescue Hypr revealed five nucleotide mutations at positions 8322, 8381, 8404, 8409, and 8411 of NS5 (Table 2). Notably, these nucleotides were G or A, owing to overlapping nucleotide detection signals, and four amino acid substitutions were observed: arginine or lysine (position 8322), arginine or glutamine (position 8322), arginine or lysine (position 8409), and glutamic acid or lysine (position 8411).

Although the rescued TBEVs possessed several nucleotide and amino acid substitutions compared to the original viral sequences, these mutations were unlikely to influence the ability of replication and propagation in infected cells.

3.4. Pathogenicity of rescued TBEV in mice

Next, we examined the pathogenicity of the rescued TBEVs *in vivo* using a mouse model. The mice infected with the rescued Oshima showed 60 % mortality and died or reached the endpoint from 10 to 15 dpi (Fig. 4A), following a weight reduction from 6 dpi (Fig. 4B). These observations indicate that the rescued Oshima-infected mice exhibited late death, as observed in the original Oshima infection, as proposed in our previous study (Hayasaka et al., 2009). In contrast, mice infected with rescued Sofjin showed 100 % mortality (Fig. 4A), and mice died or reached the endpoint by 10 dpi (Fig. 4A), following an acute weight reduction from 6 dpi (Fig. 4B). These results suggest that the rescued Sofjin-infected mice died early, as proposed in our previous study (Tun et al., 2014). Hypr-infected mice showed an acute weight reduction from 6 dpi (Fig. 3B), and all mice died or reached the endpoint by 10 dpi (Fig. 3A).

These observations indicate that TBEVs rescued by CPER possess the intrinsic virulence in a mouse model. In addition, it has been suggested that nucleotide and amino acid substitutions observed in rescued viruses are unlikely to significantly alter their infectious and pathogenic properties in a mouse model.

Table 2
Nucleotide differences between original and CPER-rescued TBEVs.

Strain	Gene	Nucleotide			Amino acid	
		Position	Original	Rescued	Original	Rescued
Oshima	3′-NCR	10,634	T	A	–	–
Sofjin	E	1579	G	A	Ala	Thr
	NS3	5995	A	G	Asn	Asp
Hypr	NS5	8322	G	G/A	Arg	Arg/Lys
		8381	G	G/A	Arg	Arg/Gln
		8404	G	G/A	Glu	Glu
		8409	G	G/A	Arg	Arg/Lys
		8411	G	G/A	Glu	Glu/Lys

4. Discussion

This is the first report of CPER for TBEV. We successfully produced infectious TBEVs from the Oshima, Sofjin, and Hypr strains. The resulting TBEVs exhibited intrinsically infectious and pathogenic properties in cultured cells and experimental mice. The generation of infectious cDNA clones is an important tool for studying recombinant TBEVs, and bacterial plasmids have been commonly used. However, the cloning process is often difficult and time-consuming because of the toxicity of the viral genome to *E. coli* of competent cells (Dong et al., 2023; Liu and Gack, 2023). In contrast, the CPER method generates recombinant viruses in bacteria without a cloning step. Therefore, CPER is a useful method for generating recombinant TBEV.

In this study, rescued Oshima, Sofjin, and Hypr possessed one, two, and five nucleotide differences, respectively, compared to the original RNA sequences, although it was unclear whether these mutations were derived from CPER procedure or propagations in infected BHK cells from our current data. It is noteworthy that these mutations did not influence infectious virus production in susceptible BHK cells. In our previous study, Sofjin and Oshima viruses induced early death with high mortality and late death with lower mortality in mice, respectively (Hayasaka et al., 2009; Tun et al., 2014). In this study, rescued viruses of Sofjin and Oshima showed similar pathogenicity with early death and late death, although we could not compare with original parent.

Interestingly, Hypr showed five nucleotide differences compared with the original virus sequence within a specific range of the NS5 gene, suggesting that CPER may cause mutations in a specific gene site of a certain strain, such as Hypr. Although it is not clear whether these mutations occurred during CPER procedure or virus propagation in cells, further analyses may provide interesting insights into the possibility of mutations caused by CPER in certain TBEV strains.

TBEV is categorized as highly pathogenic agent, and biosafety level (BSL)-3 or BSL-4 facilities are required, depending on each country's rules of use and storage. Therefore, agent shipment is strictly restricted. In Japan, for biosecurity reasons under the Act on the Prevention of Infectious Diseases and Medical Care for Patients with Infectious Diseases (Infectious Diseases Control), shipment of infectious agents, including TBEV, is strictly restricted, and it was difficult and limited for our laboratory to obtain infectious TBEV strains for several reasons, including the high cost of shipment. Thus, we could not directly compare the infectious and pathogenic properties of the parent viruses. For further advancing the research on restricted infectious viruses, reverse genetics based on nucleic acid samples or nucleotide information is a useful approach for preparing infectious viruses, and CPER is a useful technique.

Recently, an experimental system has been established in order to generate reporter viruses that incorporate GFP by applying CPER, which has been applied to several viruses (Baker et al., 2020; Conde et al., 2023; Dong et al., 2021, 2023; Haviernik et al., 2021; Torres et al., 2022). The creation of viruses incorporating reporter genes is important for viral characterization and may provide fundamental information for the development of vaccines and antiviral drugs. CPER method would apply to generate a reporter virus for the TBEVs of Oshima, Sofjin, and Hypr strains to further our TBEV association studies.

5. Conclusion

In summary, we produced infectious TBEVs from the Oshima, Sofjin, and Hypr strains using CPER. The CPER method makes it possible to obtain infectious viruses without transporting highly hazardous pathogens, such as TBEV. This method is expected to contribute to further research on orthoflaviviruses.

CRediT authorship contribution statement

Saki Mitsunaga: Methodology, Investigation, Writing – original

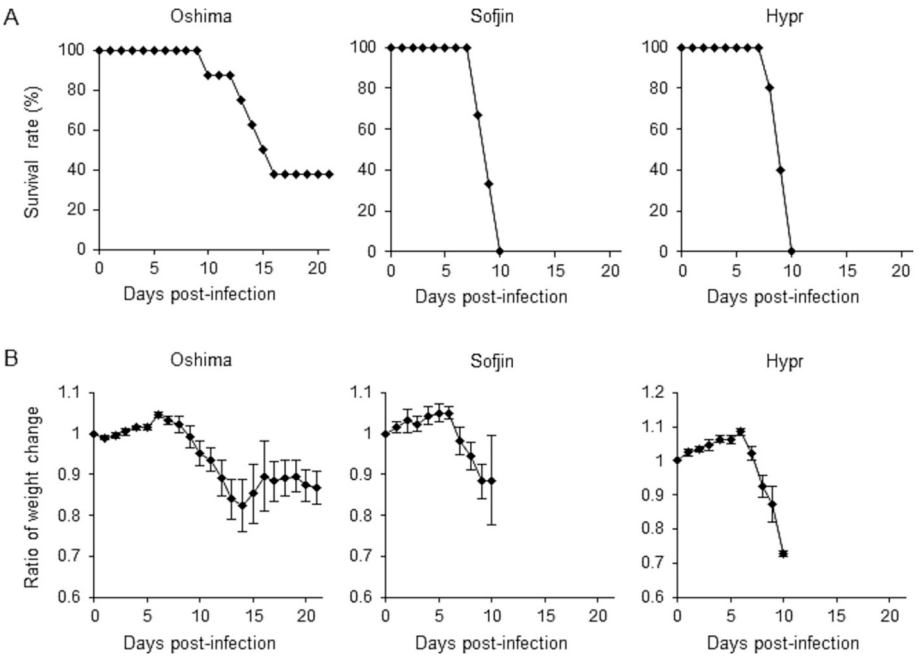


Fig. 4. Mortality and weight change of the C57BL/6 mice following subcutaneous infections with Oshima (10^4 pfu, $n = 8$), Sofjin (10^4 pfu, $n = 6$), and Hypr (10^5 pfu, $n = 5$). (A) Survival curves. (B) Weight changes. The weight ratio was calculated by comparing the weight of each mouse with its weight at 0 dpi. Mice were observed for 21 days. Error bars represent standard errors.

draft. **Tomokazu Tamura:** Supervision, Resources, Methodology, Funding acquisition, Writing – review & editing. **Samuel Nyampong:** Investigation, Data curation. **Takasuke Fukuhara:** Supervision, Methodology, Funding acquisition, Writing – review & editing. **Hiroshi Shimoda:** Supervision, Resources, Writing – review & editing. **Daisuke Hayasaka:** Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization, Writing – review & editing, Writing – original draft.

Funding sources

This work was supported by JSPS KAKENHI Grant Numbers 17H04661, 21H02736, 23K20041, 24K01930, 24K09259, the Research Center for Thermotolerant Microbial Resources (RCTMR), Yamaguchi University (Grant number 2023-19 and 2024-06), the Joint Usage/Research Center on Tropical Disease, Institute of Tropical Medicine, Nagasaki University (2022-Kyoten-03, 2023-Kyoten-02, and 2024-Kyoten-05), AMED Research Program on Emerging and Re-emerging Infectious Diseases (JP21fk0108617h0001, 22fk0108563h0001 and JP23fk0108598h0001), AMED Research Program on Interdisciplinary Cutting-edge Research (JP24wm0325073h0001), AMED SCARDA Hokkaido University Institute for Vaccine Research and Development (HU-IVReD) (JP223fa627005h0001), AMED CREST (JP22gm1610008h0001), Takeda Science of Foundation, Hokkaido University Support Program for Frontier Research, Hokuto Foundation for Bioscience, Oshimo Foundation, Akiyama Life Science Foundation for Young Scientist, Mochida Foundation, the Ichiro Kanehara Foundation for the Promotion of Medical Sciences and Medical Care, Grants-in-Aid for R&D of Young Scientist from Northern Advancement Center for Science &Technology (NOASTEC) of Hokkaido and Kuribayashi Scholarship Academic Foundation.

Declaration of competing interest

The authors declare no conflict of interest.
Data will be available by the corresponding author on request.

Acknowledgments

We thank, Dr. Hiroaki Kariwa of Hokkaido University for providing the viral RNA of TBEV Oshima and Sofjin strains, and Dr. Daniel Ruzek of Masaryk University, Czech Republic, for Plasmid DNA including TBEV Hypr strain.

Data availability

Data will be made available on request.

References

Amicizia, D., Domnich, A., Panatto, D., Lai, P.L., Cristina, M.L., Avio, U., Gasparini, R., 2013. Epidemiology of tick-borne encephalitis (TBE) in Europe and its prevention by available vaccines. Hum. Vaccin. Immunother. https://doi.org/10.4161/hv.23802.

Baker, C., Xie, X., Zou, J., Muruato, A., Fink, K., Shi, P.Y., 2020. Using recombination-dependent lethal mutations to stabilize reporter flaviviruses for rapid serodiagnosis and drug discovery. EBioMedicine 57. https://doi.org/10.1016/j.ebiom.2020.102838.

Chambers, T.J., Hahn, C.S., Galler, R., Rice, C.M., 1990. Flavivirus genome organization, expression, and replication. Annu Rev Microbiol 44, 649–688. https://doi.org/10.1146/annurev.mi.44.100190.003245.

Conde, J.N., Himmler, G.E., Mladinich, M.C., Setoh, Y.X., Amarilla, A.A., Schutt, W.R., Saladino, N., Gorbunova, E.E., Salamango, D.J., Benach, J., Kim, H.K., Mackow, E.R., 2023. Establishment of a CPER reverse genetics system for Powassan virus defines attenuating NS1 glycosylation sites and an infectious NS1-GFP11 reporter virus. mBio 14, e0138823. https://doi.org/10.1128/mbio.01388-23.

Dong, H.L., Wang, H.J., Liu, Z.Y., Ye, Q., Qin, X.L., Li, D., Deng, Y.Q., Jiang, T., Li, X.F., Qin, C.F., 2021. Visualization of yellow fever virus infection in mice using a bioluminescent reporter virus. Emerg. Microb. Infect. 10, 1739–1750. https://doi.org/10.1080/22221751.2021.1967705.

Dong, H.L., He, M.J., Wang, Q.Y., Cui, J.Z., Chen, Z.L., Xiong, X.H., Zhang, L.C., Cheng, H., Xiong, G.Q., Hu, A., Lu, Y.Y., Cheng, C.L., Meng, Z.X., Zhu, C., Zhao, G., Liu, G., Chen, H.P., 2023. Rapid generation of recombinant flaviviruses using circular polymerase extension reaction. Vaccines (Basel) 11. https://doi.org/10.3390/vaccines11071250.

Edmonds, J., van Grinsven, E., Prow, N., Bosco-Lauth, A., Brault, A.C., Bowen, R.A., Hall, R.A., Khromykh, A.A., 2013. A novel bacterium-free method for generation of flavivirus infectious DNA by circular polymerase extension reaction allows accurate recapitulation of viral heterogeneity. J. Virol. 87, 2367–2372. https://doi.org/10.1128/jvi.03162-12.

Goto, A., Hayasaka, D., Yoshii, K., Mizutani, T., Kariwa, H., Takashima, I., 2002. Genetic and biological comparision of tick-borne encephalitis viruses from Hokkaido and Far-Eastern Russia. Jpn. J. Vet. Res. 49.

Goto, A., Hayasaka, D., Yoshii, K., Mizutani, T., Kariwa, H., Takashima, I., 2003. A BHK-21 cell culture-adapted tick-borne encephalitis virus mutant is attenuated for neuroinvasiveness. Vaccine 21, 4043–4051. https://doi.org/10.1016/S0264-410X(03)00269-X.

Gritsun, T.S., Gould, E.A., 1998. Development and analysis of a tick-borne encephalitis virus infectious clone using a novel and rapid strategy. J. Virol. Methods. 76, 109–120. https://doi.org/10.1016/s0166-0934(98)00130-x.

Haviernik, J., Eyer, L., Yoshii, K., Kobayashi, S., Cerny, J., Nougairède, A., Driouich, J.S., Volf, J., Palus, M., de Lamballerie, X., Gould, E.A., Ruzek, D., 2021. Development and characterization of recombinant tick-borne encephalitis virus expressing mCherry reporter protein: a new tool for high-throughput screening of antiviral compounds, and neutralizing antibody assays. Antiviral Res. 185. https://doi.org/10.1016/j.antiviral.2020.104968.

Hayasaka, D., Gritsun, T.S., Yoshii, K., Ueki, T., Goto, A., Mizutani, T., Kariwa, H., Iwasaki, T., Gould, E.A., Takashima, I., 2004. Amino acid changes responsible for attenuation of virus neurovirulence in an infectious cDNA clone of the Oshima strain of Tick-borne encephalitis virus. J. Gen. Virol. https://doi.org/10.1099/vir.0.19668-0.

Hayasaka, D., Nagata, N., Fujii, Y., Hasegawa, H., Sata, T., Suzuki, R., Gould, E.A., Takashima, I., Koike, S., 2009. Mortality following peripheral infection with Tick-borne encephalitis virus results from a combination of central nervous system pathology, systemic inflammatory and stress responses. Virology 390, 139–150. https://doi.org/10.1016/j.virol.2009.04.026.

Holzmann, H., Aberle, S.W., Stiasny, K., Werner, P., Mischak, A., Zainer, B., Netzer, M., Koppi, S., Bechter, E., Heinz, F.X., 2009. Tick-borne encephalitis from eating goat cheese in a mountain region of Austria. Emerg. Infect. Dis. 15, 1671–1673. https://doi.org/10.3201/eid1510.090743.

Hudopisk, N., Korva, M., Janet, E., Simetinger, M., Grgič-Vitek, M., Gubenšek, J., Natek, V., Kraigher, A., Strle, F., Avšič-Županc, T., 2013. Tick-borne encephalitis associated with consumption of raw goat milk, Slovenia, 2012. Emerg. Infect. Dis. 19, 806–808. https://doi.org/10.3201/eid1905.121442.

Jaenson, T.G.T., Hjertqvist, M., Bergström, T., Lundkvist, Å., 2012. Why is tick-borne encephalitis increasing? A review of the key factors causing the increasing incidence of human TBE in Swedena. Parasit. Vectors. https://doi.org/10.1186/1756-3305-5-184.

Kovalev, S.Y., Mukhacheva, T.A., 2017. Reconsidering the classification of tick-borne encephalitis virus within the Siberian subtype gives new insights into its evolutionary history. Infect. Genet. Evol. 55, 159–165. https://doi.org/10.1016/j.meegid.2017.09.014.

Kwasnik, M., Rola, J., Rozek, W., 2023. Tick-borne encephalitis—review of the current status. J. Clin. Med. https://doi.org/10.3390/jcm12206603.

Lai, C.-J., Zhao, B., Hori, H., Bray, M., 1991. Medical Sciences Infectious RNA transcribed from stably cloned full-length cDNA of dengue type 4 virus (dengue virus/infectious transcripts/virus genetics). Proc. Nati. Acad. Sci. 88, 5139–5143. https://doi.org/10.1073/pnas.88.12.5139.

Lehrer, A.T., Holbrook, M.R., 2011. Tick-borne encephalitis vaccines. J. Bioterror. Biodef. 01. https://doi.org/10.4172/2157-2526.S1-003.

Liu, G.Q., Gack, M.U., 2023. An optimized circular polymerase extension reaction-based method for functional analysis of SARS-CoV-2. Virol. J. 20. https://doi.org/10.1186/s12985-023-02025-y.

Mandl, C.W., Ecker, M., Holzmann, H., Kunz, C., Heinz, F.X., 1997. Printed in Great Britain infectious cDNA clones of tick-borne encephalitis virus European subtype prototypic strain Neudoerfl and high virulence strain Hypr. J. Gen. 78, 1049–1057. https://doi.org/10.1099/0022-1317-78-5-1049.

Mansfield, K.L., Johnson, N., Phipps, L.P., Stephenson, J.R., Fooks, A.R., Solomon, T., 2009. Tick-borne encephalitis virus - a review of an emerging zoonosis. J. Gen. Virol. https://doi.org/10.1099/vir.0.011437-0.

Paulsen, K.M., Stuen, S., das Neves, C.G., Suhel, F., Gurung, D., Soleng, A., Stiasny, K., Vikse, R., Andreassen, Å.K., Granquist, E.G., 2019. Tick-borne encephalitis virus in cows and unpasteurized cow milk from Norway. Zoonoses Public Health 66, 216–222. https://doi.org/10.1111/zph.12554.

Piyasena, T.B.H., Newton, N.D., Hobson-Peters, J., Vet, L.J., Setoh, Y.X., Bielefeldt-Ohmann, H., Khromykh, A.A., Hall, R.A., 2019. Chimeric viruses of the insect-specific flavivirus Palm Creek with structural proteins of vertebrate-infecting flaviviruses identify barriers to replication of insect-specific flaviviruses in vertebrate cells. J. Gen. Virol. 100, 1580–1586. https://doi.org/10.1099/JGV.0.001326.

Pustijanac, E., Buršić, M., Talapko, J., Škrlec, I., Meštrović, T., Lišnjić, D., 2023. Tick-borne encephalitis virus: a comprehensive review of transmission, pathogenesis, epidemiology, clinical manifestations, diagnosis, and prevention. Microorganisms. https://doi.org/10.3390/microorganisms11071634.

Rice, C.M., Grakoui, A., Galler, R., Chambers, T.J., 1989. Transcription of infectious yellow fever RNA from full-length cDNA templates produced by in vitro ligation. New Biol. 1.

Ruzek, D., Avšič Županc, T., Borde, J., Chrdle, A., Eyer, L., Karganova, G., Kholodilov, I., Knap, N., Kozlovskaya, L., Matveev, A., Miller, A.D., Osolodkin, D.I., Överby, A.K., Tikunova, N., Tkachev, S., Zajkowska, J., 2019. Tick-borne encephalitis in Europe and Russia: review of pathogenesis, clinical features, therapy, and vaccines. Antiviral Res. https://doi.org/10.1016/j.antiviral.2019.01.014.

Sanchez-Velazquez, R., de Lorenzo, G., Tandavanitj, R., Setthapramote, C., Bredenbeek, P.J., Bozzacco, L., MacDonald, M.R., Clark, J.J., Rice, C.M., Patel, A.H., Kohl, A., Varjak, M., 2020. Generation of a reporter yellow fever virus for high throughput antiviral assays. Antiviral Res. 183. https://doi.org/10.1016/j.antiviral.2020.104939.

Sumiyoshi, H., Hoke, C.H., Trent, D.W., 1992. Infectious Japanese Encephalitis virus RNA can be synthesized from in vitro-ligated cDNA templates. J. Virol. 66, 5425–5431. https://doi.org/10.1128/JVI.66.9.5425-5431.1992.

Takashima, I., Morita, K., Chiba, M., Hayasaka, D., Sato, T., Takezawa, C., Igarashi, A., Kariwa, H., Yoshimatsu, K., Arikawa, J., Hashimoto, N., 1997. A case of Tick-borne encephalitis in Japan and isolation of the virus. J. Clin. 35, 1943–1947. https://doi.org/10.1128/jcm.35.8.1943-1947.1997.

Tamura, T., Zhang, J., Madan, V., Biswas, A., Schwoerer, M.P., Cafiero, T.R., Heller, B.L., Wang, W., Ploss, A., 2022. Generation and characterization of genetically and antigenically diverse infectious clones of dengue virus serotypes 1–4. Emerg. Microb. Infect. 11, 227–239. https://doi.org/10.1080/22221751.2021.2021808.

Torii, S., Ono, C., Suzuki, R., Morioka, Y., Anzai, I., Fauzyah, Y., Maeda, Y., Kamitani, W., Fukuhara, T., Matsuura, Y., 2021. Establishment of a reverse genetics system for SARS-CoV-2 using circular polymerase extension reaction. Cell Rep. 35. https://doi.org/10.1016/j.celrep.2021.109014.

Torres, F.J., Parry, R., Hugo, L.E., Slonchak, A., Newton, N.D., Vet, L.J., Modhiran, N., Pullinger, B., Wang, X., Potter, J., Winterford, C., Hobson-Peters, J., Hall, R.A., Khromykh, A.A., 2022. Reporter flaviviruses as tools to demonstrate homologous and heterologous superinfection exclusion. Viruses 14. https://doi.org/10.3390/v14071501.

Tun, M.M.N., Aoki, K., Senba, M., Buerano, C.C., Shirai, K., Suzuki, R., Morita, K., Hayasaka, D., 2014. Protective role of TNF-α, IL-10 and IL-2 in mice infected with the Oshima strain of Tick-borne encephalitis virus. Sci. Rep. 4. https://doi.org/10.1038/srep05344.

Wallner, G., Mandl, C.W., Ecker, M., Holzmann, H., Stiasny, K., Kunz, C., Heinz, F.X., 1996. Characterization and complete genome sequences of high-and low-virulence variants of tick-borne encephalitis virus. J. Gen. 77, 1035–1042. https://doi.org/10.1099/0022-1317-77-5-1035.

Yamshchikov, V.F., Wengler, G., Perelygin, A.A., Brinton, M.A., Compans, R.W., 2001. An infectious clone of the West Nile flavivirus. Virology 281, 294–304. https://doi.org/10.1006/viro.2000.0795.

Yoshii, K., Song, J.Y., Park, S.B., Yang, J., Schmitt, H.J., 2017. Tick-borne encephalitis in Japan, Republic of Korea and China. Emerg. Microb. Infect. https://doi.org/10.1038/emi.2017.69.