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**A Novel Reverse Genetics System for Production of Infectious West Nile Virus using Homologous
Recombination in Mammalian Cells**

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Running title: *Novel reverse genetics system for WNV*

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Abstract

Reverse genetics systems facilitate investigation of many aspects of the life cycle and pathogenesis of viruses. However, genetic instability in *Escherichia coli* has hampered development of a reverse genetics system for West Nile virus (WNV). In this study, we developed a novel reverse genetics system for WNV based on homologous recombination in mammalian cells. Introduction of the DNA fragment coding for the WNV structural protein together with a DNA-based replicon resulted in the release of infectious WNV. The growth rate and plaque size of the recombinant virus were almost identical to those of the parent WNV. Furthermore, chimeric WNV was produced by introducing the DNA fragment coding for the structural protein and replicon plasmid derived from various strains. Here, we report development of a novel system that will facilitate research into WNV infection.

Abbreviations

WNV, West Nile virus; HDV, hepatitis delta virus

Keywords

Reverse genetics, West Nile virus, homologous recombination

1. Introduction

West Nile virus (WNV) belongs to the family *Flaviviridae* and is the etiological agent of West Nile fever and West Nile encephalitis in horses and humans. WNV is classified phylogenetically into two major genetic lineages (Lanciotti et al., 2002). Recently, there are several reports that WNV can be grouped into five distinct genetic lineages (Bondre et al., 2007; Zaayman, Human, and Venter, 2009). In a mouse model of WNV neuroinvasion, the incidence of encephalitis was associated with viral strains (Beasley et al., 2005), and various molecular determinants of WNV pathogenesis have been reported (Hasebe et al., 2010; Shirato et al., 2004a).

The WNV genome consists of a positive-sense single-stranded RNA of approximately 11 kb, which encodes three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Subgenomic WNV replicon RNA, which lacks most of the sequence coding for the structural proteins, has been reported to replicate efficiently in cells (Kobayashi et al., 2014; Scholle et al., 2004; Shi, Tilgner, and Lo, 2002).

Reverse genetics systems facilitate investigation of many aspects of the life cycle and pathogenesis of viruses. In many flavivirus reverse genetics systems, the full-length cDNA of the viral genome is cloned into a plasmid under the transcriptional control of an appropriate promoter, and the synthesized viral RNAs produce infectious recombinant viruses in susceptible cells (Pavitrakar et al., 2015; Pierson et al., 2005; Yamshchikov, Mishin, and Cominelli, 2001a; Yamshchikov et al., 2001b). However, establishment of a reverse genetics system for WNV has been hampered by the instability of plasmids harboring WNV full-length cDNA during propagation in bacteria. This instability is likely caused by the production of undesired proteins toxic to *E. coli* due to cryptic promoter activity of WNV sequences (Pu et al., 2011; Zheng et al., 2016). To overcome the genetic instability of flaviviral genomes in bacteria, an *in vitro* ligation method, PCR-based protocol, infectious-subgenomic-amplicons method and systems using a bacterial artificial chromosome vector have been developed for preparation of full-length flaviviral RNA (Aubry et al., 2014; Maeda et al., 2009; Suzuki et al., 2007).

Homologous recombination is a DNA repair mechanism, a feature of which is an exchange of DNA strands between a pair of homologous duplex DNA sequences (Alberts et al., 2015). Homologous recombination is a useful means of inserting a particular DNA sequence into a specific genetic locus (Hall, Limaye, and Kulkarni, 2009). In this study, homologous recombination was applied to the production of recombinant WNV in mammalian cells.

2. Materials and Methods

2.1 Cells and viruses

HEK-293T cells were grown in high-glucose Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Vero cells were grown in minimal essential medium (MEM) supplemented with 10% FBS and 2 mM L-glutamine (Wako). *Aedes albopictus* C6/36 cells were grown in MEM supplemented with 10% FBS and 200 μ M MEM non-essential amino acids solution (Wako). The WNV 6-LP strain was established from the WNV NY99-6922 strain isolated from mosquitoes in 1999 by plaque purification (Shirato et al., 2004b). The WNV Eg101 strain was kindly provided by Dr. Duane Gubler of the Centers for Disease Control and Prevention (CDC, Fort Collins, CO, USA). All experiments with WNV were performed at the Biosafety Level-3 facility at Hokkaido University in accordance with institutional guidelines.

2.2 Plasmid construction

The region coding the structural proteins of the 6-LP and Eg101 strains was cloned into pCXS_N, which was generated from pCMV-myc (Clontech, Mountain View, CA, USA) by replacing the sequence of the myc tag with a multicloning sequence containing sites for the restriction enzymes *Xho*I, *Sall*, and *Not*I (Kobayashi et al., 2013; Kobayashi et al., 2016). The resultant plasmids were named pCXS_N-NY99CME and pCXS_N-Eg101CME, respectively. For the construction of the pCMV-WNrep-DsRed plasmid expressing WNV replicon RNA in mammalian cells, the sequence of the SP6 promoter of pWNrep-DsRed (Maeda et al., 2008) was replaced with that of the CMV promoter, and the hepatitis delta virus (HDV) ribozyme sequence for self-catalytic cleavage at its 5'-end and a polyadenylation signal was fused to the 3'-end of the WNV genome.

2.3 Virus production

The DNA fragments encoding the structural proteins were amplified by PCR or excised from the pCXS_N-NY99CME plasmid using *Xho*I and *Not*I. The DNA fragments electrophoresed in agarose gel were stained with a Gel Indicator Kit (BioDynamics Laboratory, Tokyo, Japan) to visualize the DNA and were purified with a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The purified DNA and pCMV-WNrep-DsRed were transfected into 293T cells using Polyethylenimine Max (Polysciences, Warrington, PA, USA). After 3 days, supernatant containing infectious virus was collected and centrifuged at 1,300 rpm at room temperature for 3 min. C6/36 cells were inoculated with the cleared culture supernatant from 293T cells and

incubated for 5 days. After incubation, the supernatant of C6/36 cell cultures was centrifuged at 1,300 rpm at room temperature for 3 min and stored at -80°C until use.

2.4 Titration and growth curve assay

Diluted culture supernatant from transfected 293T or infected C6/36 cells was inoculated onto monolayers of Vero cells. After 1 h of incubation at 37°C with rocking, the inoculum was removed, overlay medium (MEM containing 5% FBS and 1.25% methyl cellulose) was added, and the cells were incubated for 4 days. Plaques were visualized by staining with a 0.25% crystal violet solution in 10% formalin.

Subconfluent Vero cells cultured in 24-well plates were inoculated with WNV at a multiplicity of infection (MOI) of 0.001 plaque-forming units (Pfu)/cell and cultured for the indicated times. The supernatants of WNV-infected cell cultures were collected and stored at -80°C until use for determination of viral titers.

2.5 Immunocytochemistry

Vero cells inoculated with supernatant containing viruses from transfected cells were fixed in 4% paraformaldehyde for 10 min. The cells were permeabilized in 0.1% Triton X-100 for 5 min, blocked with 1% bovine serum albumin (BSA)-PBS, and probed with anti-Japanese encephalitis virus serum produced as described previously (Kimura et al., 1994; Kobayashi et al., 2012) in 1% BSA-PBS overnight at 4°C . Immune complexes were visualized by incubation with an Alexa-Fluor-488-conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA). The cells were visualized using an inverted fluorescence microscope (IX71; Olympus, Tokyo, Japan), and images were processed using DP Manager software (Olympus).

2.6 Viral RNA analysis

For RNA extraction, C6/36 cells were collected 5 days after inoculation with supernatant containing viruses from transfected cells. Total RNA was isolated from the inoculated cells using Isogen II (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol and reverse-transcribed with random primers using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific).

To detect the WNV genome, the sequence coding for the structural protein of WNV was amplified using GoTaq DNA polymerase (Promega) and a pair of primers (5'-AGTTCGCCTGTGTGAGCTGAC-3'

1 and 5'-CGTCCAAGCCTCCACATCATTG-3'). To confirm the sequence of the produced WNV, the
2 complete coding sequence was amplified using Platinum Taq polymerase (Thermo Fisher Scientific) and
3 three sets of primers (5'-AGTTCGCCTGTGTGAGCTGAC-3' and
4 5'-CGTCCAAGCCTCCACATCATTG-3', 5'-AGGTCCATAGCTCTCACGTTTCTCGCAG-3' and
5 5'-CAGGGCCATTCTGTGAGCTCTTCCTC-3' or 5'-AGATGCCTGAGCACTTCATGGGGAAG-3' and
6 5'-ACTGTGCCGTGTGGCTGGTTGTGCAG-3'). The nucleic acid sequences of the complete coding
7 sequence were determined by direct sequencing. The cycle sequencing reactions were performed using a
8 BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific), and the sequences were determined
9 on a 3130 Genetic Analyzer (Thermo Fisher Scientific). The primers used for sequencing were shown in
10 Supplementary Table 1.

3. Results

3.1 Production of infectious WNV using a DNA-based replicon and DNA fragment encoding the structural proteins

To apply homologous recombination to the production of recombinant WNV, we first constructed a DNA-based replicon system (pCMV-WNrep-DsRed). As shown in Fig. 1A, the WNV replicon sequences were cloned into the pUC19 plasmid under the transcriptional control of the CMV promoter, and the ribozyme sequence from HDV and the polyA signal from SV40 were fused to the 3'-end of the WNV genome. In the replicon sequences, the DsRed gene was replaced with WNV nucleotides sequence 190–2379, corresponding to the majority of the WNV C, prM and E genes. Transfection of pCMV-WNrep-DsRed resulted in production of the DsRed protein (Fig. 1B). These results suggested that the DNA-based replicon produced the WNV replicon RNA.

To examine production of infectious WNV by homologous recombination, 293T cells were transfected with the pCMV-WNrep-DsRed plasmid and the PCR product of the viral structural protein gene, which had overlapping sequences at the 5'-end of the C gene and 3'-end of the E gene with the pCMV-WNrep-DsRed plasmid (Fig. 1C). At 3 days post-transfection, 4,800 Pfu/mL of infectious virus was detected in the supernatant of the cells transfected with both the DNA-based replicon and the PCR product, while no virus was detected in the supernatant of cells transfected with only the PCR product (Fig. 2A). The collected supernatant from the cells transfected with only the PCR product, only the DNA-based replicon or both the DNA-based replicon and the PCR product was transferred to Vero cells or C6/36 cells. At 5 days post-inoculation, WNV antigen was detected in Vero cells inoculated with the supernatant of cells transfected with the DNA-based replicon and PCR product similarly to the cells inoculated with parental NY99 (Fig. 2B), and a WNV-specific DNA band was amplified by RT-PCR from C6/36 cells inoculated with the culture supernatant of the transfected cells (Fig. 2C). Furthermore, no mutation was detected in the complete coding region of WNV produced from C6/36 cells. These results indicate production of infectious WNV by homologous recombination between the DNA-based replicon and the DNA fragment encoding the structural protein gene.

To prevent the introduction of mutations due to PCR error, a DNA fragment comprising the whole structural protein gene was prepared from the pCXS-NY99CME plasmid by cutting with appropriate restriction enzymes. The resultant fragment (RE fragment) contained additional recognition sequences for *XhoI* and Kozak sequences at the 5'-end, and a stop codon and sequences for *NotI* at the 3'-end. The RE fragment and pCMV-WNrep-DsRed were transfected directly into 293T cells. At 3 days post-transfection, the viral titer in the culture supernatant of cells

transfected with both DNAs was 1,200 Pfu/mL, while no virus was detected in the culture supernatant of cells transfected with only the RE fragment (Fig. 2D). The culture supernatant of co-transfected cells was inoculated into Vero cells, in which the WNV antigen was detected after 5 days (Fig. 2E). These results indicated that recombinant WNV could be produced using a DNA fragment excised from the plasmid by cutting with restriction enzymes.

The relationship between viral production and the length of the overlap region was next investigated. The DNA fragment of the whole structural protein gene contained about 90 bases of consensus sequence with the pCMV-WNrep-DsRed at the 5'- and 3'-ends. A DNA fragment with 60, 30 or 15 bases of overlap sequences at both ends was prepared and transfected with pCMV-WNrep-DsRed. The viral titers in culture supernatant of the transfected cells decreased with decreasing overlap sequence length (Fig. 3). This result indicated that viral production by the homologous recombination method was dependent on the length of the overlap region.

3.2 Production of chimeric WNV between different strains

Next, generation of chimeric WNV was examined using the reverse genetics system and the homologous recombination method. The DNA fragment of the whole structural protein gene of the Eg101 strain was amplified by PCR. Four nucleotides differ between the NY99 and Eg101 strains at the 3'-end of the overlap sequences in the fragment. The DNA fragment and pCMV-WNrep-DsRed were transfected into 293T cells, and at 3 days post-transfection the supernatant was inoculated onto Vero and C6/36 cells. The viral titer in culture supernatant of cells transfected with both DNAs was below the detection limit (data not shown). However, the WNV antigen was detected in Vero cells inoculated with the culture supernatant of co-transfected cells (Fig. 4A). Sequence analysis of the chimeric WNV confirmed that the virus had the structural protein gene of the Eg101 strain, while its other genes were from the NY99 strain. This result indicated successful generation of a chimeric WNV by the homologous recombination method.

To assess the biological properties of the recombinant and the chimeric WNV, termed NY99-HR and NY99-CMEEg101, respectively, its growth kinetics were compared with those of the parental NY99 and Eg101 strains. The viral titer of NY99-HR was similar to that of parental NY99 strain at all time points, with peak levels at 48 h post-infection (Fig. 4B). The plaque size in monolayers of Vero cells infected with NY99-HR was identical to that of monolayers infected with the parental NY99 strain (Fig. 4C). The viral titer of NY99-CMEEg101 was lower than that of the parental Eg101 and NY99 strains at 24 and 48 h post-inoculation (Fig. 4B). The plaques in Vero cell monolayers infected with NY99-CMEEg101 were identical in size to those of monolayers infected with the parental

1 Eg101 strain, but smaller than those of monolayers infected with the parental NY99 strain (Fig. 4C). These results
2 indicated that NY99-HR had growth properties and plaque morphologies similar to those of the parental strain, while
3 NY99-CMEEg101 had growth properties different from those of the parental strains and produced plaques with
4 morphology similar to those generated by the Eg101 strains.

4. Discussion

In this study, we established a novel reverse genetics system using homologous recombination for production of infectious WNV using homologous recombination. Since the system relied on cloning of full-length flavivirus cDNA is hampered by genetic instability, various reverse genetics systems for flavivirus have been developed. Plasmids containing the full-length flavivirus cDNA sequence are unstable in bacteria due to the presence of cryptic promoter sequences within the 5'-UTR and the sequences coding for the E-NS1 proteins (Pu et al., 2011; Zheng et al., 2016). Furthermore, full-length flaviviral cDNA generated using a PCR-based protocol and infectious-subgenomic-amplions method could contain mutations caused by PCR error. In our system, full-length flaviviral cDNA was divided into the sequence coding for the structural protein and a replicon harboring homologous sequences. These constructs were stable in bacteria and no mutation was detected in the recombinant WNV sequence. This system prevents production of undesired, or toxic proteins in *E. coli* due to cryptic promoter activity in the WNV sequence. Furthermore, cloned recombinant WNV could be produced by homologous recombination between the DNA-based replicon plasmid and the DNA fragment of the structural proteins excised from the cloned plasmid. However, the viral titer in the culture supernatant of the transfected 293T cells using our system was lower than that produced using a fusion PCR protocol or infectious molecular clone protocol (Maeda et al., 2009; Pierson et al., 2005), suggesting that the homologous recombination rate to generate complete genome is not high. In our system, the rate of recombinant WNV production depends on the length of the homologous sequence and the efficacy of homologous recombination in the cells. Overexpression of a recombinase in mammalian cells has been reported to promote spontaneous homologous recombination (Park, 1995; Stéphane Vispé, 1998), and so may enhance the production of recombinant WNV in our homologous recombination method.

Our system was applied to the production of chimeric WNV, the structural protein gene of which was from the Eg101 strain, and the other genes from the NY99 strain. The NY99 and Eg101 strains have been reported to exhibit different degrees of neuroinvasiveness in a mouse model (Shirato et al., 2004a). Analysis of the properties of chimeric WNV between two strains may facilitate identification of molecular determinants of pathogenesis.

5. Conclusion

In conclusion, this paper reports development of a novel method that will facilitate further research on WNV infection. This method can be applied for production of recombinant virus of other flaviviruses. Genetic modification of the viral genome using this system enables to produce chimeric virus leading to identification of the virulence determinants and intracellular dynamics of flaviviruses, and can be applied for discovery of novel antiviral agents.

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7. References

- Alberts, B., Wilson, J.H., Hunt, T., Johnson, A.D., Lewis, J., Morgan, D., Raff, M.C., Roberts, K. and Walther, P., 2015. Molecular biology of the cell. Garland Science, New York, NY.
- Aubry, F., Nougairede, A., de Fabritus, L., Querat, G., Gould, E.A. and de Lamballerie, X., 2014. Single-stranded positive-sense RNA viruses generated in days using infectious subgenomic amplicons. *The Journal of general virology* 95, 2462-7.
- Beasley, D.W., Whiteman, M.C., Zhang, S., Huang, C.Y., Schneider, B.S., Smith, D.R., Gromowski, G.D., Higgs, S., Kinney, R.M. and Barrett, A.D., 2005. Envelope protein glycosylation status influences mouse neuroinvasion phenotype of genetic lineage 1 West Nile virus strains. *J Virol* 79, 8339-47.
- Bondre, V.P., Jadi, R.S., Mishra, A.C., Yergolkar, P.N. and Arankalle, V.A., 2007. West Nile virus isolates from India: evidence for a distinct genetic lineage. *The Journal of general virology* 88, 875-84.
- Hall, B., Limaye, A. and Kulkarni, A.B., 2009. Overview: generation of gene knockout mice. *Current protocols in cell biology*, 19.12. 1-19.12. 17.
- Hasebe, R., Suzuki, T., Makino, Y., Igarashi, M., Yamanouchi, S., Maeda, A., Horiuchi, M., Sawa, H. and Kimura, T., 2010. Transcellular transport of West Nile virus-like particles across human endothelial cells depends on residues 156 and 159 of envelope protein. *BMC Microbiol* 10, 165.
- Kimura, T., Kimura-Kuroda, J., Nagashima, K. and Yasui, K., 1994. Analysis of virus-cell binding characteristics on the determination of Japanese encephalitis virus susceptibility. *Archives of virology* 139, 239-51.
- Kobayashi, S., Orba, Y., Yamaguchi, H., Kimura, T. and Sawa, H., 2012. Accumulation of ubiquitinated proteins is related to West Nile virus-induced neuronal apoptosis. *Neuropathology* 32, 398-405.
- Kobayashi, S., Orba, Y., Yamaguchi, H., Takahashi, K., Sasaki, M., Hasebe, R., Kimura, T. and Sawa, H., 2014. Autophagy inhibits viral genome replication and gene expression stages in West Nile virus infection. *Virus Res* 191, 83-91.
- Kobayashi, S., Suzuki, T., Igarashi, M., Orba, Y., Ohtake, N., Nagakawa, K., Niikura, K., Kimura, T., Kasamatsu, H. and Sawa, H., 2013. Cysteine residues in the major capsid protein, Vp1, of the JC virus are important for protein stability and oligomer formation. *PLoS One* 8, e76668.
- Kobayashi, S., Suzuki, T., Kawaguchi, A., Phongphaew, W., Yoshii, K., Iwano, T., Harada, A., Kariwa, H., Orba, Y. and Sawa, H., 2016. Rab8b Regulates Transport of West Nile Virus Particles from Recycling Endosomes. *J Biol Chem* 291, 6559-68.
- Lanciotti, R.S., Ebel, G.D., Deubel, V., Kerst, A.J., Murri, S., Meyer, R., Bowen, M., McKinney, N., Morrill, W.E., Crabtree, M.B., Kramer, L.D. and Roehrig, J.T., 2002. Complete Genome Sequences and Phylogenetic Analysis of West Nile Virus Strains Isolated from the United States, Europe, and the Middle East. *Virology* 298, 96-105.
- Maeda, A., Murata, R., Akiyama, M., Takashima, I., Kariwa, H., Watanabe, T., Kurane, I. and Maeda, J., 2009. A PCR-based protocol for the generation of a recombinant West Nile virus. *Virus Res* 144, 35-43.

- 1 Maeda, J., Takagi, H., Hashimoto, S., Kurane, I. and Maeda, A., 2008. A PCR-based protocol for generating West
2 Nile virus replicons. *J Virol Methods* 148, 244-52.
- 3 Park, M.S., 1995. Expression of human RAD52 confers resistance to ionizing radiation in mammalian cells. *Journal*
4 *of Biological Chemistry* 270, 15467-15470.
- 5 Pavitrakar, D.V., Ayachit, V.M., Mundhra, S. and Bondre, V.P., 2015. Development and characterization of reverse
6 genetics system for the Indian West Nile virus lineage 1 strain 68856. *J Virol Methods* 226, 31-9.
- 7 Pierson, T.C., Diamond, M.S., Ahmed, A.A., Valentine, L.E., Davis, C.W., Samuel, M.A., Hanna, S.L., Puffer, B.A.
8 and Doms, R.W., 2005. An infectious West Nile virus that expresses a GFP reporter gene. *Virology* 334,
9 28-40.
- 10 Pu, S.Y., Wu, R.H., Yang, C.C., Jao, T.M., Tsai, M.H., Wang, J.C., Lin, H.M., Chao, Y.S. and Yueh, A., 2011.
11 Successful propagation of flavivirus infectious cDNAs by a novel method to reduce the cryptic bacterial
12 promoter activity of virus genomes. *J Virol* 85, 2927-41.
- 13 Scholle, F., Girard, Y.A., Zhao, Q., Higgs, S. and Mason, P.W., 2004. trans-Packaged West Nile virus-like particles:
14 infectious properties in vitro and in infected mosquito vectors. *J Virol* 78, 11605-14.
- 15 Shi, P.Y., Tilgner, M. and Lo, M.K., 2002. Construction and characterization of subgenomic replicons of New York
16 strain of West Nile virus. *Virology* 296, 219-33.
- 17 Shirato, K., Kimura, T., Mizutani, T., Kariwa, H. and Takashima, I., 2004a. Different chemokine expression in lethal
18 and non-lethal murine West Nile virus infection. *J Med Virol* 74, 507-13.
- 19 Shirato, K., Miyoshi, H., Goto, A., Ako, Y., Ueki, T., Kariwa, H. and Takashima, I., 2004b. Viral envelope protein
20 glycosylation is a molecular determinant of the neuroinvasiveness of the New York strain of West Nile virus.
21 *The Journal of general virology* 85, 3637-45.
- 22 Stéphane Vispé, C.C., Claire Lesca and Martine Defais, 1998. Overexpression of Rad51 protein stimulates
23 homologous recombination and increases resistance of mammalian cells to ionizing radiation. *Nucleic*
24 *Acids Research*.
- 25 Suzuki, R., de Borja, L., Duarte dos Santos, C.N. and Mason, P.W., 2007. Construction of an infectious cDNA clone
26 for a Brazilian prototype strain of dengue virus type 1: characterization of a temperature-sensitive mutation
27 in NS1. *Virology* 362, 374-83.
- 28 Yamshchikov, V., Mishin, V. and Cominelli, F., 2001a. A new strategy in design of +RNA virus infectious clones
29 enabling their stable propagation in *E. coli*. *Virology* 281, 272-80.
- 30 Yamshchikov, V.F., Wengler, G., Perelygin, A.A., Brinton, M.A. and Compans, R.W., 2001b. An infectious clone of
31 the West Nile flavivirus. *Virology* 281, 294-304.
- 32 Zaayman, D., Human, S. and Venter, M., 2009. A highly sensitive method for the detection and genotyping of West
33 Nile virus by real-time PCR. *J Virol Methods* 157, 155-60.
- 34 Zheng, X., Tong, W., Liu, F., Liang, C., Gao, F., Li, G., Tong, G. and Zheng, H., 2016. Genetic instability of Japanese
35 encephalitis virus cDNA clones propagated in *Escherichia coli*. *Virus Genes* 52, 195-203.
- 36

Figure Legends

Fig. 1. A method for the production of recombinant WNV. (A) Schematic representation of the WNV genome and constructs used in this study. pCMV-WNrep-DsRed (DNA-based replicon) was constructed from pWNrep-DsRed. Coding sequences for the structural protein of the 6-LP and Eg101 strains were cloned into the pCXSN plasmid. (B) 293T cells were transfected with empty vector (Mock) or pCMV-WNrep-DsRed, the signal of which was visualized by fluorescence microscopy at 48 h post-transfection. Cell nuclei were counterstained with Hoechst (blue). (C) A DNA fragment coding for the WNV structural proteins was obtained by PCR or by cutting with appropriate restriction enzymes. The DNA fragment and the pCMV-WNrep-DsRed plasmid were then transfected into 293T cells.

Fig. 2. Production of recombinant WNV. (A) 293T cells were transfected with only the PCR product, or the PCR product plus pCMV-WNrep-DsRed (replicon). The culture supernatant from the transfected cells was harvested and the viral titer was determined by plaque assay. Data represent mean values $\pm$ SD of three independent experiments. N. D., not detected. (B) Vero cells were inoculated with 100 μ l of culture supernatant of the transfected 293T cells or parental NY99 strain (MOI = 0.001). The cells were fixed at 5 dpi and probed for WNV antigen (green); cell nuclei were counterstained with DAPI (blue). Scale bars: 50 μ m. (C) C6/36 cells were inoculated with the culture supernatant of transfected 293T cells or parental NY99 strain. The cells were harvested at 5 dpi and the WNV genome was detected by RT-PCR. Arrowhead indicates the WNV-specific band. (D) 293T cells were transfected with only the RE fragment, or with the RE fragment plus pCMV-WNrep-DsRed (replicon). The culture supernatant of transfected cells was harvested and viral titer was determined by plaque assay. Data represent mean values $\pm$ SD of three independent experiments. N. D., not detected. (E) Vero cells were inoculated with 100 μ l of the culture supernatant of the transfected 293T cells or parental NY99 strain (MOI = 0.001). The cells were harvested at 5 dpi and probed for WNV antigen (green); cell nuclei were counterstained with DAPI (blue). Scale bars: 50 μ m.

Fig. 3. Relationship between viral production and the length of the overlap region. DNA fragments comprising the full-length (90), 60, 30 or 15 bases of overlap sequences at both ends plus pCMV-WNrep-DsRed were transfected into 293T cells. The culture supernatant of the transfected cells was harvested and the viral titer was determined by plaque assay. Bars represent mean values of three independent experiments.

1 **Fig. 4.** Production of chimeric WNV and biological properties of the recombinant WNV. (A) 293T cells were
2 transfected with only the PCR product of the whole structural protein gene of the Eg101 strain [PCR product
3 (CME-Eg)], or this PCR product (CME-Eg) plus pCMV-WNrep-DsRed (replicon). The culture supernatant of
4 transfected cells was harvested and inoculated onto Vero cells. The cells were fixed at 5 days post inoculation and
5 probed for WNV antigen (green); cell nuclei were counterstained with DAPI (blue). Scale bars: 100 μ m. A
6 representative result of three independent experiments is shown. (B) Vero cells were inoculated with the parental
7 NY99, parental Eg101, NY99-HR or NY99-CMEEg101 at MOI = 0.001, and the culture supernatants were
8 harvested at the indicated time points. Viral titers were determined by plaque assay. Data represent mean values $\pm$ SD
9 of three independent experiments. (C) Vero cells were inoculated with the parental NY99, parental Eg101,
10 NY99-HR or NY99-CMEEg101 and incubated with overlay medium for 5 days, and plaques were visualized by
11 crystal violet staining.