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Generation of a recombinant West Nile virus stably expressing the *Gaussia* luciferase for neutralization assay

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ABSTRACT

West Nile virus (WNV) is a neurotropic human pathogen that has caused increasing infected cases over recent years. There is currently no licensed vaccine or effective drug for prevention and treatment of WNV infection in humans. To facilitate antiviral drug discovery and neutralizing antibody detection, a WNV cDNA clone containing a luciferase reporter gene was constructed through incorporating *Gaussia* luciferase (Gluc) gene within the capsid-coding region of WNV genome. Transfection of BHK-21 cells with the cDNA clone-derived RNA generated luciferase reporter WNV (WNV-Gluc) and the stable WNV-Gluc with high titers (>10^7 PFU/ml) was obtained through plaque purification. Luciferase activity was used to effectively quantify the viral production of WNV-Gluc. Using the reporter virus WNV-Gluc, we developed a luciferase based assay in a 12-well format for evaluating neutralizing antibodies. The reporter virus could be a powerful tool for epidemiological investigation of WNV, vaccine evaluation, antiviral drug screening, and the study of WNV replication and pathogenesis.

1. Introduction

West Nile virus (WNV) is an enveloped, single-stranded, positive-sense RNA virus belonging to the genus *Flavivirus* within the family Flaviviridae. The majority of WNV infections are asymptomatic or show merely mild flu-like symptoms, while about 1% of human cases, especially older people (>50 years old) and patients with immunosuppressed or chronic diseases, may develop severe central nervous system diseases including meningitis, encephalitis and acute flaccid paralysis or even death (Gyure, 2009; Petersen and Marfin, 2002). The viral genome is approximately 11 kb, containing a single open reading frame (ORF) and two untranslated regions (UTRs) at the 5′ and 3′ ends. The ORF encodes a polyprotein that is subsequently cleaved into three structural proteins (capsid, envelope, and pre-membrane) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by cellular and viral proteases (Shi et al., 2002). The viral replication occurs in the cytoplasm and the nonstructural proteins play critical roles in RNA synthesis.

WNV was discovered in the West Nile District of Uganda in 1937 and only sporadic cases have been reported during the subsequent 60 years (Kramer et al., 2008). Since it was recognized in New York City in 1999, WNV has rapidly spread across the North American continent, resulting in continuous epidemics in the United States, Canada, and Mexico (Chung et al., 2013; Deardorff et al., 2006; Lindsey et al., 2010; Mann et al., 2013; Rodriguez Mde et al., 2010). With the recent identification of WNV-infected cases (Li et al., 2013) and the subsequent isolation of this virus from mosquitoes in Kashi region, Xinjiang, China (Lu et al., 2014), WNV has become an important emerging virus in China.

Currently, several serological assays are available for detection of WNV infections: enzyme-linked immunosorbent assay (ELISA) (Hogrefe et al., 2004; Niedrig et al., 2007), immunofluorescence assay (IFA), and plaque reduction neutralization test (PRNT). Due to the serological cross-reactivity within the Flaviviridae family (*e.g.*, Japanese encephalitis virus, dengue virus, or yellow fever virus), PRNT is still considered as the most specific diagnostic test so far. However, with the time-consuming and technically difficult of

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PRNT, a more reliable and rapid method for detecting neutralization antibodies of WNV is urgently needed.

In this study, we firstly developed a WNV reporter virus (WNV-Gluc) stably expressing the *Gaussia* luciferase (Gluc). Furthermore, a simple, rapid and reliable neutralization assay for screening WNV neutralizing antibodies was established based on WNV-Gluc reporter virus. This reporter virus would greatly facilitate the epidemiological survey of WNV, drug discovery and vaccine development.

2. Materials and methods

2.1. Cells, viruses and antibodies

BHK-21 (Baby Hamster kidney fibroblast) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and maintained in 5% CO_2 at 37 °C. Confluent cell monolayers and virus-inoculated cultures were maintained in DMEM with 2% FBS. Wild type WNV was generated from an infectious cDNA clone pACYC-FLWNV by electroporation of BHK-21 cells with *in vitro* transcribed RNAs (Shi et al., 2002). WNV mouse serum and control negative serum were from State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China. The serum samples were inactivated at 56 °C for 30 min before the assay. Humanized monoclonal antibodies (mAbs) against WNV were used to estimate the performance of Gluc-based assay in this study.

2.2. Construction of a full-length WNV cDNA clone containing the Gaussia *luciferase gene*

As shown in Fig. 1A, an infectious cDNA clone of WNV (designated as pACYC-FLWNV) was used to construct the cDNA clone of *Gaussia* luciferase WNV reporter virus (WNV-Gluc). Two-step cloning strategy was used to construct WNV-Gluc plasmid. First, a standard overlap PCR was used to create a cassette containing "BamHI-T7 promoter-5′UTR-N-terminal 33 amino acids of capsid protein–Gluc gene-FMDV 2A-authentic initiation codon of capsid protein to the unique EcoNI site in E protein". Fragment covering "BamHI to N-terminal 33 amino acids of capsid protein" was amplified with the primers pACYC BamHI-F and Capsid 33 Gluc-R, using the pACYC-FLWNV as a template. Fragment of Gluc-FMDV 2A without the signal peptide sequence of Gluc was amplified with the primers Capsid 33 Gluc-F and FMDV 2A Capsid-R, using the Gluc-WNV-Rep (Shan et al., 2013) as a template. Fragment spanning "the authentic initiation codon of capsid protein to EcoNI unique site in E protein" (located at nucleotide position 1127 of the viral genome) was amplified with the primers FMDV 2A Capsid-F and WNV-E-EcoNI-R using the pACYC-FLWNV as a template. Next, the fragment from BamHI to EcoNI, which was fused by overlapping PCR using the three fragments mentioned above, was engineered into pACYC-FLWNV at the corresponding sites, resulting in plasmid pACYC-WNV-Gluc. The detailed primer sequences are presented in Table 1.

pACYC-WNV-Gluc contained an extra fragment (representing the first 33 amino acids of Capsid protein–Gluc gene-FMDV 2A) between the 5′UTR and the complete ORF of the viral genome when compared with the wild-type pACYC-FLWNV. The duplication of the N-terminal region nucleotide sequences of capsid protein (capsid 33) is to maintain RNA elements that are required for genome cyclization. The insertion of the FMDV 2A sequence is to ensure correct processing of Gluc protein. All the constructs were verified by DNA sequencing.

Table 1
Primer sequences in this study.

Primer	Sequence 5′–3′
pACYC BamHI-F	CGCGGATCCTAATACGACTCACTA
Capsid 33 Gluc-R	TCGGTGGGCTTAGCCCTCTTCAGTCCAAT
Capsid 33 Gluc-F	TGAAGAGGGCTAAGCCCACCGAGAACAAC
FMDV2A Capsid-R	TTCTTAGACATTGGCCCAGGGTTGGACTC
FMDV2A Capsid-F	ACCCTGGGCCAATGTCTAAGAAACCAGGA
WNV-E-EcoNI-R	CTCGCCTCTGCCAGGTTGGCCGC
WNV-5′UTR-F	AGTAGTTCGCCTGTGTGAGCTGA
WNV1218-R	TTTGTCATTGTGAGCTTCTCCCAT

F, forward; R, reverse.

2.3. Rescue of recombinant virus

The RNAs of WNV-Gluc were *in vitro* transcribed from the corresponding cDNA plasmids that were linearized with XbaI. The mMESSAGE MEGAscript® T7 Kit (Ambion) was used for RNAs synthesis according to the manufacturer's protocols. The 8×10^6 BHK-21 cells were electroporated with five micrograms WNV-Gluc RNAs as previously described (Zhang et al., 2010). The transfected cells were resuspended in 25 ml of DMEM containing 10% FBS and seeded into 12-well plates. At 24, 48, 72 and 84 h post transfection (hpt), the cell culture supernatants were harvested and immediately stored at −80 °C until used for Gluc assay.

2.4. Luciferase activity assay

The infected cells or supernatant were disposed by lysates (Promega) and subjected to Gluc activity assay using a Multimode Microplate Reader (Varioskan Flash) according to the manufacturer's instruction. The relative light units (Rlus) were recorded for each sample. All luciferase assays were performed in triplicate.

2.5. Plaque assay and purification of WNV-Gluc virus

The titers of WNV-Gluc were determined by plaque assay as described previously (Shang et al., 2013). Briefly, 100 μl of 10-fold diluted virus samples were seeded to monolayers of BHK-21 cells in 24-well plates (1.2×10^5 cells per well, plated one day in advance). The cells were incubated with diluted virus for 1 h and covered by the layer of 2% methyl cellulose. After 3 days of incubation at 37 °C with 5% CO_2, the cells were fixed by 3.7% formaldehyde containing 1% crystal violet. Plaque numbers were counted after washing the plates with tap water.

The WNV-Gluc virus was purified through double plaque assay. Briefly, the 10-fold diluted virus samples (100 μl for each well) were prepared to inoculate 90% confluent BHK-21 cells (4×10^5 cells per well were plated one day in advance) in a 6-well plate. The infected cells were incubated at 37 °C with 5% CO_2 for 1 h with rocking every 15 min until 3 ml DMEM containing 0.6% agarose and 2% FBS was added. The second layer of agarose containing 0.33% neutral red was added after 3 days of incubation at 37 °C with 5% CO_2. Plaque morphology and numbers were recorded after an additional 12 to 24 h incubation (Zhang et al., 2010).

Plaque purification was performed as the following steps (Fig. 2A): (1) The P0 virus was used for double plaque assay as mentioned above, and small and medium individual plaques were picked at 5 days post infection (dpi). (2) The plaques were placed in 2 ml of DMEM containing 2% FBS. And then the plaque-containing medium was inoculated in BHK-21 monolayers in 6-well plates. (3) Culture supernatants were collected for measuring Gluc signal at 3 dpi as mentioned above, and the virus stocks, which have higher Gluc signal, were selected for repeating this process until all the plaques have uniform-sized morphology.