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Generation and characterization of West Nile pseudo-infectious reporter virus for antiviral screening

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ABSTRACT

West Nile virus (WNV), a mosquito-borne flavivirus, is an important neurotropic human pathogen. As a biosafety level-3 (BSL-3) agent, WNV is strictly to BSL-3 laboratories for experimentations, thus greatly hindering the development of vaccine and antiviral drug. Here, we developed a novel pseudo-infectious WNV reporter virus expressing the *Gaussia* luciferase (Gluc). A stable 293T$_{NS1}$ cell line expressing NS1 was selected for *trans*-supplying NS1 protein to support the replication of WNV-ΔNS1 virus and WNV-ΔNS1-Gluc reporter virus with large-fragment deletion of NS1. WNV-ΔNS1 virus and WNV-Gluc-ΔNS1 reporter virus were confined to complete their replication cycle in this 293T$_{NS1}$ cell line, displaying nearly identical growth kinetics to WT WNV although the viral titers were lower than those of WT WNV. The reporter gene was stably maintained in virus genome at least within three rounds of passage in 293T$_{NS1}$ cell line. Using a known flaviviruses inhibitor, NITD008, we demonstrated that the pseudo-infectious WNV-Gluc-ΔNS1 could be used for antiviral screening. Furthermore, a high-throughput screening (HTS) assay in a 96-well format was optimized and validated using several known WNV inhibitors, indicating that the optimized HTS assay was suitable for high-throughput screening WNV inhibitors. Our work provides a stable and safe tool to handle WNV outside of a BSL-3 facility and facilitates high throughput screening for anti-WNV drugs.

1. Introduction

West Nile virus (WNV) is a mosquito-borne flavivirus that causes encephalitis and viscerotropic disease in humans, especially in elderly persons or those with poor body conditions (Sejvar, 2014; Suthar et al., 2013a). WNV was first isolated from a febrile woman in the West Nile Province of Uganda in 1937. Prior to the mid-1990s, it only caused sporadic outbreaks in parts of Africa, the Middle East, Europe and Australia, and was considered a minor risk for humans (Anez et al., 2013; McMullen et al., 2013). In 1999, the WNV epidemic in New York City was responsible for severe human fatalities and a high rate of avian mortality. In subsequent years, this virus has rapidly spread westward through the entire continental United States and into Canada, Mexico, Central America and some Caribbean islands (Lim et al., 2014; Monaco et al., 2011; Patsoula et al., 2016; van den Hurk et al., 2014), and the number of cases reached the peak in 2002 and 2003 (Beasley et al., 2013; Roehrig, 2013). Recently, WNV has also been reported in East Asia including India and China, (Anukumar et al., 2014; Lu et al., 2014), indicating that this virus has become a global public health threat. Currently, no effective antiviral drug or vaccines are approved for use against WNV infection. Development of a reliable antiviral

Abbreviations: WNV, West Nile virus; DENV, dengue virus; YFV, yellow fever virus; JEV, Japanese encephalitis virus; BSL-3, biosafety level-3; VLP, virus like particle; MOI, multiplicity of infection; hpi, hours post-infection; hpt, hours post-transfection; Gluc, *Gaussia* luciferase; Rluc, *Renilla* luciferase; HTS, high-throughput screening; 2′-C-MeAdo, 2′-C-methyladenosine; MPA, mycophenolic acid.

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screening system is urgent for controlling this virus.

According to the extent of threats to human health, WNV is classified as a biosafety level-3 (BSL-3) pathogen. However, insufficient BSL-3 facilities have obviously hampered the studies of pathogenic mechanism and antiviral screening. To circumvent this limitation, researchers have developed a number of alternatives for WNV studies, such as noncytopathic replicons (Lo et al., 2003; Pierson et al., 2006; Shan et al., 2013) and pseudo-infectious viruses, namely viral like particles (VLPs) (Mason et al., 2006; Pierson et al., 2006; Scholle et al., 2004). The replicon is a self-replicating subgenomic RNA that is created by replacing structural protein genes with a reporter gene such as luciferase. VLPs are generated through packaging of luciferase-expressing replicon RNA with structural genes provided *in trans*. Transfection or infection of susceptible cells could lead to the expression of the reporter gene, which could be used to monitor anti-WNV activities of potential inhibitors. Thus, both systems have been widely used for high throughput screening (HTS) for WNV drug discovery (Ansarah-Sobrinho et al., 2008; Zou et al., 2011). In comparison with subgenomic replicon, VLPs-based HTS could cover the targets of the entire viral life cycle due to the structural features of VLPs mimicking natural viruses. However, it was reported that the replicon-encoded reporter gene in WNV VLPs is not stably retained when passaging in the packaging cell line. Thus, it is still urgent to develop a novel, stable antiviral screening system that could be manipulated at biosafety level 2 (BSL-2).

The WNV genome is a positive-sense single-stranded RNA containing about 11,000 nucleotides. The genome is translated into a single polyprotein, and subsequently processed by viral and host proteases into three structural (C, prM and E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins (Shi et al., 2002a; Suthar et al., 2013b; Zhang et al., 2008). The structural proteins have a significant role in virus entry, fusion, and assembly, and the non-structural proteins interact cooperatively to form the viral replication complexes that are necessary for viral RNA synthesis. Among them, glycoprotein NS1 functions at a very early stage of viral genome replication, possibly through interacting with NS4A and NS4B membrane proteins (Akey et al., 2014; Lindenbach and Rice, 1999; Youn et al., 2012). Prior studies showed that the replication defect caused by NS1 mutation could be compensated *in trans* by ectopic expression of NS1 although how it works remain unknown (Khromykh et al., 1999, 2000; Lindenbach and Rice, 1997; Muylaert et al., 1997; Youn et al., 2013).

In this study, a stable pseudo-infectious WNV reporter virus was developed through complementation of WNV-ΔNS1-Gluc with ectopically expressed NS1. Using an adenosine analog NITD008, a known inhibitor of flaviviruses, we demonstrated that the pseudo-infectious WNV reporter virus could be used for rapidly screening of anti-WNV compounds. A HTS assay in 96-well plate was also optimized and validated using several known WNV inhibitors and the corresponding Z' values were above 0.5, indicating a robust HTS assay was established for pseudo-infectious WNV reporter virus to screen the inhibitors. This novel pseudo-infectious virus resembles wild-type (WT) WNV but could be studied in non-BSL-3 laboratory, further facilitating the discovery of effective antiviral compounds.

2. Materials and methods

2.1. Cells, antibodies and compounds

HEK-293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 units penicillin ml^{-1} and 100 μg streptomycin ml^{-1} at 37°Cin a 5% CO_2 incubator. Anti-HA-tag (3724) was purchased from Cell Signaling Technology (CST, Boston, MA); Monoclonal antibody (mAb) 4G2 against the flavivirus envelope protein was kindly provided by Dr. Qin, cheng-feng (Beijing Institute of Microbiology and Epidemiology, China); Anti-WNV mC, anti-WNV NS1, anti-WNV E and anti-WNV NS5 polyclonal antibodies were home-made; FITC-conjugated goat anti-mouse IgG and Texas Red-conjugated goat anti-mouse IgG were purchased from Proteintech (China); Horseradish peroxidase (HRP) coupled goat anti-mouse antibody and goat anti-rabbit antibody were purchased from Proteintech (China). The known flavivirus antiviral compounds 2′-C-methyladenosine (2′-C-MeAdo) (Carroll et al., 2003) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nucleoside analogue inhibitor NITD008 (Yin et al., 2009) was synthesized in house. The inhibitors mycophenolic acid (MPA) (Diamond et al., 2002) and glycyrrhizin (Puig-Basagoiti et al., 2005) were purchased from Sigma (Sigma-Aldrich, MO, USA). Compounds were solubilized in 100% DMSO and further diluted in assay media to a final DMSO concentration of 0.1% for screening.

2.2. Plasmid construction

According to NS1 deletion strategy described previously (Khromykh et al., 2000), the truncated NS1 fragment with a deletion from residues 4 to 298 was generated by overlap PCR using the infectious cDNA clone pFLWNV (Shi et al., 2002b) (GenBank no. AF404756) as a template. The amplified product was then engineered into pFLWNV and WNV-Gluc constructs (Zhang et al., 2016) at the *Eco*NI and *Sph*I sites to produce pACYC-WNV-ΔNS1 and pACYC-WNV-Gluc-ΔNS1 mutants, respectively. The $_{Env}NS1_{HA}$ cassette encoding the full-length NS1 with an N-terminal signal peptide sequence (the last 24 amino acid of envelope protein) and a C-terminal HA-tag was generated by a standard PCR amplification using pFLWNV as a template. The resultant PCR products were then engineered into the pBABEpuro expression plasmid at the *Bam*HI and *Eco*RI sites. All the clones were sequenced prior to subsequent experiments.

2.3. Generation of 293T cell lines stably expressing recombinant NS1

The retroviral vector system (Morgenstern and Land, 1990) was used to establish the stable HEK-293T cell line expressing NS1 (named as $293T_{NS1}$). Briefly, the constructs pBABEpuro-NS1, m57 and VSV-G were co-transfected into 293T cells by using calcium phosphate-DNA precipitates. After 6 h post transfection (hpt), half of the culture medium was replaced with equal volume of fresh medium. The supernatants were harvested at 48 and 72 hpt followed by filtration through a 0.45 μm filter. Then the combined retroviral supernatants were used to infect naïve HEK-293T cells in the presence of 8 μg/ml of polybrene. At 2 h post infection (hpi), the medium was removed and replaced with fresh culture medium. The following day, the cells were selected with 2 μg/ml of puromycin. The resistant cell colonies were marked and trypsinized using cloning discs that dipped in trypsin solution. After complete trypsinization, the cloning discs adhered with cells were transferred into the 24-well culture plates. The confluence cells were further trypsinized as usual and transferred into larger culture plates for expansion of single stable clones.

2.4. RNA transcription and transfection

All the infectious cDNA plasmids of WNV were linearized by *Xba*I, followed by *in vitro* transcription using MEGAscript® T7 Kit (Ambion) according to the manufacturer's protocols. Transfection was performed in 35 mm dishes of 80% confluent $293T_{NS1}$ monolayers. 4 μL of DMRIE-C reagents (Invitrogen, USA) was diluted with