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A sensitive virus yield assay for evaluation of Antivirals against Zika Virus

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A B S T R A C T

Despite the rapid spread of Zika virus (ZIKV) infection and associated neurological complications in the America's, prophylactic or therapeutic countermeasures are not currently available. This is mostly due to the fact that until recently there was no presumed need for medical intervention since there was no association between ZIKV infection and significant human morbidity. Consequently, there are currently no tools due mostly to the lack of sensitive cell based assays amenable for identification of ZIKV inhibitors. To address this unmet need we have developed a cell based virus yield assay suitable for testing antivirals against Zika virus.

Using bioinformatics, several isolates of ZIKV from the Americas, Africa, and Asia were analyzed for sequence similarity. The alignment data were then used to design primers targeting a ZIKV genomic region that was highly conserved among all the ZIKV isolates. Subsequently, primers were used in a sensitive, quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay to detect ZIKV RNA. The qRT-PCR assay was found to be highly sensitive (lower limit of detection between-10-100 copies) and reproducible. Evaluation of the primers and probes used for ZIKV against another flavivirus (Dengue virus) demonstrated specificity of detection. To evaluate potential of qRT-PCR assay as an antiviral screening tool against ZIKV, Vero cells pretreated with Type I Interferons (IFN α) were infected with virus, followed by measurement of ZIKV RNA found in the cell culture supernatants using qRT-PCR assay. Dose-dependent antiviral activity of Type I Interferons and mycophenolic acid (MPA) against Zika virus in this cell culture system was confirmed using qRT-PCR. Due to reproducible assay performance, qPCR associated higher sensitivity and short duration of the assay time, this novel cell based assay will be very useful for confirming the activity of antivirals against ZIKV.

1. Introduction

Zika virus (ZIKV), a mosquito-borne flavivirus, is responsible for dengue like illness (fever, rash, joint pain), neurological symptoms (Guillain-Barre syndrome) and microcephaly in newborn babies. This ongoing outbreak has now spread to more than 50 countries around the world. Since the outbreak was first recognized, approximately 3132 travel-associated ZIKV cases, 43 locally acquired mosquito-borne cases and 731 cases of pregnant women with laboratory evidence of ZIKV infection have also been identified in the United States (www.CDC.gov). ZIKV is primarily transmitted by mosquitoes; however, sexual and perinatal transmission also have been demonstrated in a few cases(Hamel et al., 2016). The mosquito species that transmits ZIKV belong to the genus *Aedes*, and are widely distributed around the world including much of the Southern United States (www.cdc.gov). Epidemiological studies in Brazil have provided evidence of a strong causal link between ZIKV infection in pregnant women and serious neural developmental disorders such as microcephaly in fetuses and newborns (Nunes et al., 2016). Microcephaly is a neural developmental dis-

Abbreviations: ZIKV, Zika Virus; qRT-PCR, Quantitative Reverse Transcriptase-polymerase Chain reaction; CP assay, Cytoprotection assay; CPE, Cytopathic effect; IFA, Immunofluorescence Assay.

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order characterized by smaller than average head circumference in the affected babies. The underlying cause of microcephaly is malformation of cortical neurons leading to a significant diminution of the fetal cerebral cortex. Depending on the severity, the babies born with microcephaly can have severe and permanent impairments such as intellectual disability, developmental delay, motor skills defects, vision problems, and hearing loss. In addition to causing microcephaly in developing fetuses, ZIKV infection in adults has been linked to Guillain-Barré syndrome, an autoimmunue disorder that primarily affects the peripheral nervous system, and can be fatal (Lucchese and Kanduc, 2016). Due to the widespread nature of the ongoing outbreak, extensive geographic distribution of the mosquito vectors, and potentially grave outcome of infection, especially in the unborn fetus of pregnant women, the World Health Organization (WHO) has declared ZIKV to be a 'Public Health Threat of International Concern'.

Since its first isolation in 1947 from a sentinel Rhesus monkey in the Zika forest of Uganda, sporadic human infections of ZIKV have been recorded in several African and Asian countries (Weaver et al., 2016). However, it was not until 2007 when an outbreak occurred in the Pacific Island of Yap that the true epidemic potential of this virus was realized. It was estimated that 73% of Yap residents $\geq$ 3 years of age were infected with ZIKV during the outbreak. Subsequently, in 2013, a ZIKV outbreak was reported in French Polynesia with an estimated 19,000 cases (Cao-Lormeau et al., 2014). In May 2015, the WHO reported local transmission of ZIKV in Brazil, and in December, the Brazilian Ministry of Health estimated that 440,000–1,300,000 suspected cases of ZIKV associated disease had occurred in Brazil in 2015 (Hennessey et al., 2016). Phylogenetic analyses performed on isolates collected from various time periods and geographic locations indicated two distinct ZIKV lineages, the African lineage and the Asian lineage; the African lineage was further subdivided into East African and West African clades (Faye et al., 2014). The isolates within the Asian lineage share >99% nucleotide sequence identity, while the nucleotide sequence identity between members of Asian and African lineages is 89% (Lanciotti et al., 2016). The ZIKV strain responsible for the ongoing outbreak in the Americas belongs to the Asian lineage.

ZIKV is a member of the genus *Flavivirus*, family *Flaviviridae*, and is related to other human pathogenic flaviviruses such as dengue, St. Louis encephalitis and yellow fever viruses (Kuno et al., 1998). Like other flaviviruses, the ZIKV has an icosahedral nucleocapsid, which is surrounded by a host-cell derived lipid envelope. Embedded within the envelope are virally-encoded envelope glycoprotein spikes, which serve to bind virions to the host cell receptor(s). The ZIKV genome consists of an 11 kb single-stranded, positive-sense RNA, which contains a single open reading frame of 10,794 nucleotides flanked by two non-coding regions. The ORF is translated by host cell machinery shortly after nucleocapsid uncoating in the cytoplasm thus producing a precursor polyprotein. This polyprotein is co- and post-translationally cleaved into individual viral proteins that include three structural proteins (capsid, premembrane, and envelope) and seven nonstructural proteins (NS1, NS2, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Hamel et al., 2016).

Currently, no vaccine or specific therapy is available against ZIKV. In view of the global threat posed by ZIKV, effective therapies and a vaccine are urgently needed. This warrants accelerated efforts towards rapid discovery and confirmation of antiviral compounds that can readily be 'fed' into the drug development process A key requirement for the implementation of an efficient drug discovery and developmental program is the availability of an assay that allows detection and quantification of the intended action of the chemical compound(s) against the virus. Importantly, the assay should produce a robust signal, have a high sensitivity and specificity. Additionally, a cell-based confirmatory assay is preferred as they provide a better understanding of pharmacological interaction between the virus and the compound in a physiological environment. Moreover, with regards to evaluation of potential drug candidates, a cell-based system provides opportunity to eliminate compounds that are cell impermeable, cytotoxic, or rapidly degraded within cells; thus, allowing selection of scaffolds that are amenable to downstream applications in the drug development process.

Here, we describe the development and optimization of a cell-based Zika virus yield assay for assessing the antiviral activity of compounds against ZIKV. The assay is based on qRT-PCR detection and quantitation of ZIKV yield from infected cells treated with a candidate compound. To design the assay, we first aligned nucleotide sequences of several ZIKV isolates from the Americas, Asia, and Africa. The aligned sequences were subjected to bioinformatics analyses to identify regions that display high sequence identity among all ZIKV isolates, but not other flaviviruses. Specific primers and probe were then designed based on these conserved regions, and qRT-PCR assay was performed using the RNA from various ZIKV isolates or dengue virus-1/4 (DENV1/4) as a specificity control. The assay was found to be specific (signal detected in all ZIKV-infected, but not DENV1/4-infected cells), and highly sensitive (lower limit of detection- between 10 and 100 copies of ZIKV RNA). Furthermore, the assay has a high dynamic range (10^1–10^6 gene copies) and Z' factor >0.5, indicating that the assay is sufficiently robust for testing compounds. To provide proof-of-concept, we analyzed the dose response of antiviral activity of Type I Interferons (IFN α) and MPA against ZIKV, and confirmed that this assay could detect antivirals against ZIKV.

2. Materials and methods

2.1. Sequence analysis

Comparative ZIKV genomic sequence analysis and alignments were performed using the NCBI BLAST Suite (http://www.ncbi.nlm.nih.gov/pubmed). The "Megablastn" and "Primer-blast" programs were used for the genomic comparisons and sequence specific primer homology analysis, respectively.

2.2. Viruses, Cell Culture, and Antiviral compounds (IFN-α, MPA and Cyclosporine A)

Vero cells (CCL-81, ATCC, Manassa, VA) were grown in Dulbecco's Minimal Essential Medium (DMEM, Lonza, Walkersville, MD), supplemented with 10% Fetal Bovine Serum (FBS), NEAA and L-Glutamine according to standard culture conditions. A commercially available mixture of protease (Pronase, Roche Life sciences, Indianapolis, IN) was used to release viral RNA for qRT-PCR. IFN α was obtained from ProSpec East Brunswick, NJ. Mycophenolic acid (MPA) (Cat#S2487) and Cycloproine A (Cat# S2286) were purchased from Selleckchem (Houston, TX). ZIKV isolates were obtained from the following sources: DAK MR766 and IbH 30656 (BEI Resources, Manassas, VA), PRVABC59 (CDC, Atlanta, GA), FSS13025 (UTMB Arbovirus reference collection, Galveston, TX), and ZIKV East African Lineage (Zeptometrix, Buffalo, NY). In addition, the following Zika virus isolates were used for sequence analysis in this study: Brazil ZIKV2015 (Genbank Accession #KU497555.1) (Calvet et al., 2016), Brazilian isolate Natal RGN, Bahia, Brazil (Seq. Accession# KU527068.1) (Calvet et al., 2016) (Mlakar et al., 2016). Viral stocks of different isolates were amplified in Vero cells (ATCC, Manassas, VA) and quantitated according to the standard plaque assay methodology. Strain of virus used for each experiment is mentioned in the figure legend as appropriate.