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Inactivation of West Nile virus in serum with heat, ionic detergent, and reducing agent for proteomic applications

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ABSTRACT

Research involving biosafety level 3 pathogens such as West Nile virus (WNV) is often limited by the limited space and technical constraints of these environments. To conduct complex analytical studies outside of high containment, robust and reliable inactivation methods are needed that maintain compatibility with downstream assays. Here we report the inactivation of WNV in spiked serum samples using a commercially available SDS-PAGE sample buffer for proteomic studies. Using this method, we demonstrate its utility by identification proteins differentially expressed in the serum of mice experimentally infected with WNV.

1. Introduction

West Nile virus (WNV; family *Flaviviridae*, genus *Flavivirus)* has a ~11-kb single-stranded positive-sense RNA genome and an icosahedral virion with a diameter of approximately 50 nm that is derived from host cell membranes (Mukhopadhyay et al., 2003; Selisko et al., 2014). WNV is primarily transmitted to humans and other mammals by mosquitoes of the genus *Culex* (Colpitts et al., 2012), with birds serving as amplifying hosts for the virus and enabling successive cycles of mosquito infection (Hollidge et al., 2010). The majority of human WNV infections result in subclinical or asymptomatic diseases; however, approximately 20% of infected individuals present with an acute febrile illness with less than 1% of infected persons developing neuroinvasive forms of the disease (Nasci et al., 2013). Due to the potential risk for laboratory-associated infections, WNV must be handled in biosafety level 3 (BSL-3) containment (U. S. Department Human Services et al., 2013), thus limiting studies due to regulatory and logistical constraints. Therefore, samples must first be rendered noninfectious prior to removal from BSL-3 containment for detailed studies or other applications.

To safely conduct proteomic experiments with WNV-infected animal sera outside of BSL-3 containment, a validated protocol is needed for virus inactivation in a compatible buffer system. Protein denaturation is one strategy for virus inactivation, and therefore, we investigated the use of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffers for virus inactivation. SDS-PAGE buffers are commonly used to denature protein samples for downstream assays. These samples can be used directly for SDS-PAGE and/or western blotting, or filter-aided sample preparation (FASP) prior to mass spectrometry-based peptide identification (Wisniewski et al., 2009).

NuPAGE® LDS Sample Buffer and NuPAGE® Sample Reducing Agent are manufactured by ThermoFisher Scientific for the purpose of preparing protein-containing samples for denaturing SDS-PAGE. The active component of the sample buffer is lithium dodecyl sulfate (LDS), which is an ionic detergent. Such detergents can effectively disrupt host-derived lipid membranes and integral membrane proteins (such as those of enveloped viruses), and also have a denaturing effect on intra- and intermolecular protein interactions (Seddon et al., 2004). The reducing agent, dithiothreitol (DTT), breaks disulfide bonds formed between cysteine residues of proteins, further denaturing tertiary and quaternary folding of proteins (Singh et al., 1995). Finally, the addition of high heat (95 °C) accelerates the previous two processes as well as effecting protein denaturation on its own. The combination of ionic detergent,

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reducing agent, and incubation at 95 °C destabilizes protein and lipid membrane structure thereby promoting enveloped virus disassembly and loss of infectivity. In this report, we describe a protocol for inactivation of WNV infectivity in serum by addition of NuPAGE® LDS Sample Buffer, NuPAGE® Sample Reducing Agent, and heat. We verified virus inactivation by a combination of blind passage and mammalian cell cultures, RT-qPCR, and plaque assays. The inactivated serum was then used for a proteomic strategy employing LC–MS/MS and mass tags to characterize the serum proteomic response of mice to WNV infection.

2. Materials and methods

2.1. Cells and viruses

African green monkey kidney epithelium Vero cells were grown in Minimum Essential Medium (MEM; Corning) supplemented with 10% [vol/vol] heat-inactivated fetal bovine serum (FBS; HyClone), 100 units/ml penicillin and 100 µg/ml streptomycin (Corning). West Nile virus (WNV) strain WN-USAMRIID99 was originally isolated from a crow at the Bronx Zoo in New York City (Lanciotti et al., 1999) and then subsequently passaged three times on Vero cells. All procedures described herein that involved potentially infectious material were conducted within a biological safety cabinet (BSC) in a biosafety level 3 (BSL-3) suite at USAMRIID.

2.2. Inactivation method

WNV NY-99 was added to heat-inactivated FBS to simulate infectious sera. These serum samples were combined with 4 x NuPAGE® LDS Sample Buffer and 10 x NuPAGE® Reducing Agent according to the manufacturer's recommendations to a 1 x final mixture. For each sample to be inactivated, 65 µl serum containing 6.5×10^5 plaque forming units (PFU) WNV was combined with 25 µl 4 x NuPAGE® LDS Sample Buffer and 10 µl NuPAGE® Reducing Agent in a screw-cap microcentrifuge tube with O-ring (Sarstedt AG & Co). Sample tubes were incubated at 95 °C and vigorously agitated in a heated vortexer (Eppendorf AG) for 10 min. Sample tubes were removed, inverted, and then briefly vortexed (Vortex-Genie 2, Scientific Industries, Inc.) on their caps to ensure complete coverage of the inner surfaces of the tubes. Finally, samples were returned to the heated vortexer and vigorously agitated for an additional 5 min at 95 °C.

2.3. Serial passage of control and inactivated samples

To demonstrate non-viability inactivation, test samples were ten-fold serially diluted from 10^{-1} to 10^{-6} in complete MEM (to mitigate potential cytotoxic effects of the inactivating reagents) and then 200 µl of each dilution was used to inoculate a single well of confluent Vero cells within a six-well plate and then adsorbed on the cell monolayers for 1 h at 37 °C/5% CO_2. In parallel, 200 µl of ten-fold serial dilutions of WNV (1×10^7 to 1×10^2 PFU/ml) in heat-inactivated FBS were also used as positive infection controls on Vero cells. After initial adsorption, each well was supplemented with an additional 3 ml of complete MEM and incubated for seven days at 37 °C/5% CO_2. At the end of this first cell passage, 1.5 ml of culture medium from each well was collected, added to individual wells of fresh, confluent Vero monolayers with 1.5 ml complete MEM, and then cultured for an additional seven days. At the conclusion of the second passage, two aliquots of 500 µl each were collected at stored at −80 °C for plaque titration assays and a single 100 µl aliquot was combined with 300 µl TRIzol® LS (ThermoFisher Scientific) for use in reverse transcription real-time PCR (RT-qPCR) assays.

2.4. RNA extraction and RT-qPCR assays

Total RNA was extracted from TRIzol® LS-treated cell culture supernatants using an EZ1® Advanced XL sample processor and the EZ1® Virus Mini Kit v2.0 (Qiagen). Total RNA was eluted in a volume of 60 µl. Viral RNA was reverse transcribed using SuperScript™ II Reverse Transcriptase and them amplified with Platinum® *Taq* DNA polymerase (ThermoFisher Scientific) and 3 mM $MgSO_4$. The following primers (1 µM each) and TaqMan® MGB probe (0.2 µM) were used: panWNV-F10541, 5′-TAG ACG GTG CTG CCT GCG-3′; panWNV-R10627, 5′-CGA GAC GGT TCT GAG GGC TTA-3′; panWNV-p10560S, 6FAM-CTC AAC CCC AGG AGG A-MGBNFQ. The RT-qPCR assay was performed on a LightCycler® 96 (Roche Diagnostics) using the following thermocycling conditions: reverse transcription, 50 °C for 15 min; *Taq* polymerase activation, 95 °C for 5 min; amplification, 45 cycles of denaturation at 95 °C for 1 s and annealing/extension at 60 °C for 20 s. Data was analyzed using LightCycler® 96 desktop software. Positive and negative samples were identified using the "Qualitative Detection" analysis function using the default settings.

2.5. Plaque assays

To determine the infectivity of control and inactivated samples, confluent monolayers of Vero cells in six-well cluster plates were used. In brief, samples were ten-fold serially diluted in complete MEM and then 100 µl of each dilution was adsorbed onto cells from which the liquid medium had been removed. Each sample was analyzed in triplicate wells in each iteration of the inactivation method validation. After a 1 h incubation at 37 °C/5% CO_2, a primary overlay consisting of EBME supplemented 10% [vol/vol] heat-inactivated FBS (HyClone), 100 units/ml penicillin and 100 µg/ml streptomycin (Corning), 2 mM L-glutamine (HyClone), MEM non-essential amino acids, and 0.6% (wt/vol) SeaKem ME agarose (Lonza) was added to the cells and then returned to the incubator. After 2 days, a secondary overlay (same composition as primary overlay) containing 5% [vol/vol] neutral red solution was added. Plaques were observed approximately 24 h following addition of the secondary overlay.

2.6. Infection of mice with WNV

Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

Female BALB/c mice (6–8 weeks old) were obtained from Charles River Laboratories, and then house in microisolator cages in an ABSL-3 laboratory. Prior to infection, mice were anesthetized with 0.1 ml of mouse K-A-X (Ketamine-acepromazine-xylazine). Once mice were sufficiently sedated, an area of about 0.75 in^2 was shaved on the lower back using hair clippers. Each mouse was exposed to 1×10^4 PFU WNV NY-99 by the intradermal route. Following exposure, mice were placed back in their respective cages and monitored to confirm recovery from anesthesia. At predetermined study endpoints (6, 24, 48, and 72 h post-exposure), groups of ten mice were processed as follows: Each mouse was anesthetized with 0.2 ml of mouse K-A-X. When mice were fully sedated, terminal intracardiac exsanguination was performed using a 1 ml syringe and a 25-gauge needle. Whole blood (0.5 ml) was placed in a tube containing 3.5 ml TRIzol LS and 0.5 ml H_2O. Any remaining blood was transferred to a serum separator mini collection tube. Euthanasia was confirmed by cervical dislocation of each animal. Samples were immediately flash frozen in a dry ice/ethanol bath and then stored at −80 °C for subsequent analyses. During these procedures, the amount and time of mouse K-A-X administered was recorded; along with the amount of blood collected, the time of euthanasia, and the times at which samples were flash frozen.