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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.

2.7. Mouse serum sample preparation for LC–MS/MS

Samples were processed using isobaric tagging and a filter assisted sample prep (iFASP) protocol (McDowell et al., 2013). Briefly, 5 µl of each inactivated serum sample was added to 200 µl 8 M urea/0.1 M Tris-HCl pH 8.5 and filtered through a Microcon–30 kDa centrifugal filter unit with Ultracel-30 membrane (Millipore) at 14,000 xg for 15 min. Following several washing steps, proteins were alkylated with 55 mM iodoacetamide and digested with 4 µg Trypsin/Lys-C Mix (Promega) overnight at 37 °C. TMT 5-Plex labelling (ThermoFisher) was performed directly on the FASP filters per the manufacturer's instructions. TMT-labeled digests were then purified by strong cation exchange spin column, dried to completion by SpeedVac, and stored at −20 °C until analyzed by LC MS/MS.

2.8. LC–MS/MS tandem mass tag analysis

Sample digests were suspended in 240 µl of 0.1% [vol/vol] formic acid in HPLC grade water. A Dionex 3000 RSLCnano system (ThermoFisher Scientific) injected 5 µl of each digest onto a pre-column (C18 PepMap 100, 5 µm particle size, 5 mm length x 0.3 mm internal diameter) housed in a 10-port nano switching valve using a flow rate of 10 µl/minute. The loading solvent was 0.1% [vol/vol] formic acid in HPLC grade water. The pre-column eluent was directed to waste. After 5 min, the switching valve changed to backflush the trapped peptides from the pre-column onto an Easy-Spray analytical column (15 cm × 75 µm) packed with PepMap C18, 3 µm particle size, 100 Å porosity particles (ThermoFisher Scientific, Inc.). A 2–38% B gradient elution in 160 min was formed using pump-A (0.1% [vol/vol] formic acid) and pump-B (85% [vol/vol] acetonitrile in 0.1% [vol/vol] formic acid) at a flow rate of 300 nl/minute. The column eluent was connected to an Easy-Spray nanospray source (ThermoFisher Scientific) with an electrospray ionization voltage of 2.2 kV. An Orbitrap Elite mass spectrometer (ThermoFisher Scientific, Inc.) with an ion transfer tube temperature of 3000 °C and an S-lens setting of 55% was used to focus the peptides into the mass spectrometer. A top ten data-dependent MS/MS method was used to detect and characterize the tryptic peptides. The top ten most abundant ions were selected in a 400–1600 Da survey scan (120,000 resolution FWHM at *m/z* 400) with a full automatic gain control (AGC) target value of 1×10^6 ions and a maximum injection time of 200 ms. Higher energy collisional dissociation (HCD) ms/ms spectra were acquired at a resolution of 30,000 FWHM (at *m/z* 400) with an AGC target value of 5×10^4 ions and a maximum injection time of 200 ms. The isolation width for ms/ms HCD fragmentation was set to 2 Da. The normalized HCD collision energy was 40% with an activation time of 0.1 ms. The dynamic exclusion duration was 30 s.

Peptide database searches were performed with ProteomeDiscoverer 2.1 (ThermoFisher Scientific) using a *Mus musculus* (mouse) subset of the SwissProt_2016_11-30 database which contains 24,961 sequences. Variable modifications used were as follows: TMT 5-plex (N-terminal, K), carbamyl (KMR), methyl (DE), acetyl (K), deamidated (NQ), and oxidation (M). Cysteine carbamidomethylation was specified as a constant modification. The false discovery rate (FDR) was set at 0.1% using posterior error probability validation. Normalization by total peptide amount was used with a control channel average scaling mode. Mass tolerances were 10 ppm for the MS1 scan and 100 ppm for all ms/ms scans. Quantitation results were filtered such that only high-confidence/unambiguous peptide spectral matches (PSM) having MS2 isolation interference values less than 30% were used.

3. Results

3.1. Determination of viability test sensitivity

To simulate sera collected from experimentally-infected animals, we tested WNV (strain WN-USAMRIID99) serially diluted in heat-inactivated FBS from 1×10^7 to 1×10^2 PFU/ml with two subsequent passages on Vero cells (see methods). At the end of the second passage, TRIzol® LS-extracted cell culture supernatant showed the presence of WNV in each culture by RT-qPCR assays. In fact, cell cultures inoculated with as little as 20 PFU of WNV in serum could be robustly detected by RT-qPCR (average Cq = 13.05) of culture supernatants after two passages on Vero cells (Table 1).

Table 1
Sensitivity of WNV Vero cell viability test.

	Cq, WNV RT-qPCR		
	Experimental Replicate		
PFU	1	2	3
2.00E + 06	13.74	13.22	12.98
2.00E + 05	13.61	13.23	12.13
2.00E + 04	13.08	13.67	12.38
2.00E + 03	13.81	13.81	11.91
2.00E + 02	12.93	13.18	11.43
2.00E + 01	13.18	13.37	12.60
FBS only	negative	negative	negative

3.2. Inactivation method performance

Heat-inactivated FBS spiked with WNV (1×10^7 PFU/ml) was treated with NuPAGE® reagents and heat, and then serially diluted in cell culture medium to mitigate potential cytotoxic effects of the inactivating agents. Each dilution was used to infect Vero cells (final inocula: 1.3×10^5 to 1.3 PFU/well) and then passaged twice as previously described, resulting in undetectable levels of WNV by RT-qPCR (Fig. 1). Given the lowest infectivity tested as a positive control in the viability test was 20 PFU, we estimate the reduction factor for method was $\geq 6.5 \times 10^3$ ($\geq$ 99.98%).

RT-qPCR assays do not address the infectivity of test samples. Therefore, we also performed plaque assays of the most concentrated inactivated WNV samples (1.3×10^5 PFU), along with appropriate positive and negative controls. No plaques were observed in any of the inactivated samples (Fig. 2).

3.3. Proteomic evaluation of serum protein abundance changes in mice during WNV infection

In this study, we used serially collected (pre-infection, 6, 24, 48 and 72 hpi) serum from mice infected with WNV. The serum samples were

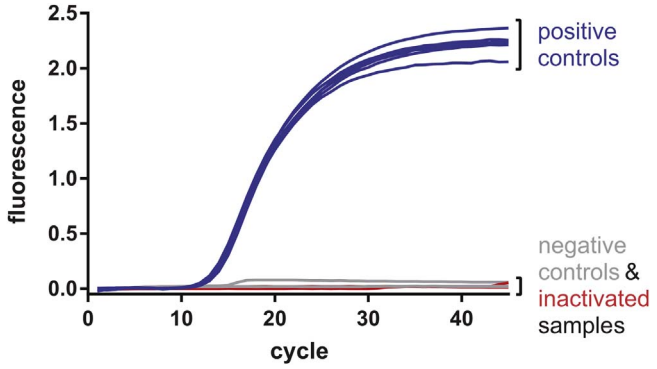

Fig. 1. WNV RNA is not detected in inactivated serum samples. WNV-spiked FBS was treated with NuPAGE® reagents and heat, and then passaged twice for one-week intervals on Vero cell cultures. Total RNA was extracted from aliquots of culture medium collected after the second cell culture passage, and then analyzed using a WNV-specific RT-qPCR assay. Representative amplification curves from one of three replicate inactivation experiments are shown.

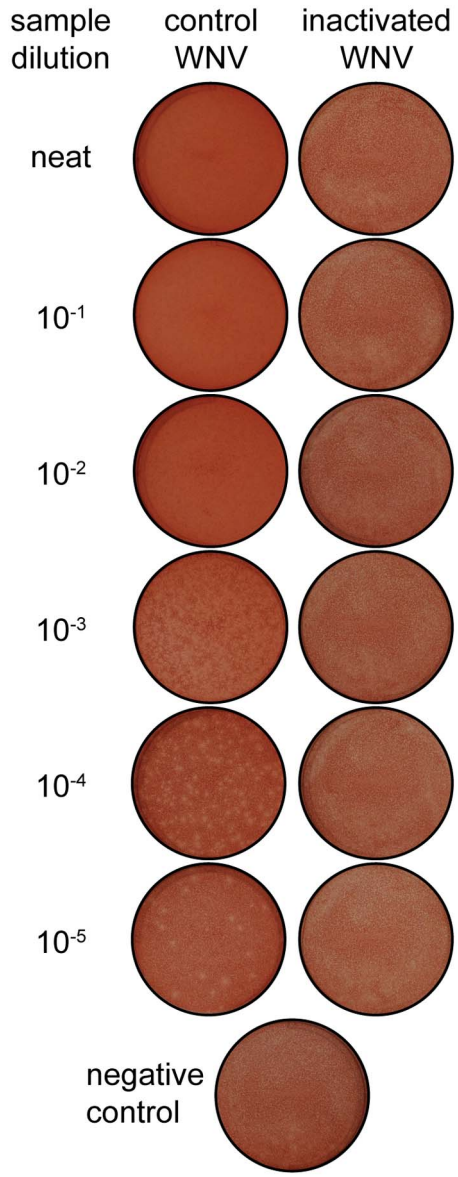

Fig. 2. Inactivated serum samples do not contain infectious WNV. Aliquots of culture medium collected from positive control of inactivated WNV samples after two seven-day passages on Vero E6 cells were used for plaque assays to detect infectious virus. A representative well of a single plaque assay is shown for each dilution of these test samples. Note: The absence of visible plaques in the control WNV wells for neat, 10^{-1}, and 10^{-2} dilutions is due to complete destruction of the cell monolayers.

inactivated using our developed method, removed from BSL-3 containment, and subjected to proteomic quantitation to evaluate changes to protein content during infection with WNV. For this evaluation, serum samples were pooled from a total of seven mice at each time point after inactivation, and no depletion was performed for the removal of high abundant serum proteins. We employed an analytical workflow using 5 µl of inactivated serum, filter assisted sample prep (FASP), and peptide tagging (TMT 5-plex) followed by LC–MS/MS using high resolution mass spectrometry. FASP allows the removal of SDS and other components in the inactivation buffer which may interfere with this downstream analysis. In addition, quantification with TMTs offers multiplexing that significantly reduces the overall analysis time and, because the labelling occurs early in the analytical workflow, the experimental variance for the quantification is reduced.

A total of 131 proteins were identified and quantitated. Relative abundance of serum proteins to pre-infection levels was determined at each post-infection time-point. Using a cut-off of at least 1.8 fold

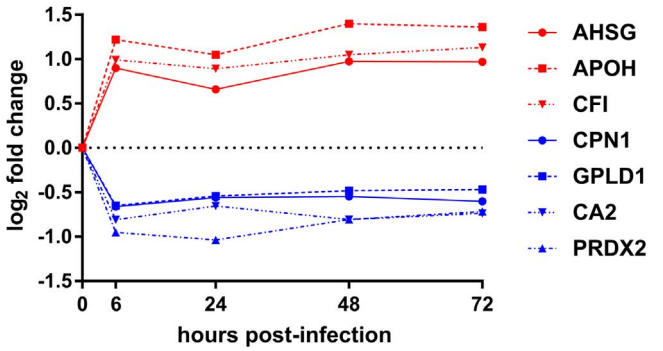

Fig. 3. Relative quantitation of mouse serum proteins using LC–MS/MS. Inactivated pooled serum samples from mice at each time point post-infection were subjected to FASP, trypsin digestion and TMT tagging) of peptides followed by LC–MS/MS analysis. Protein abundances were calculated using Proteome Discoverer 2.1 and serum proteins with significant increased (red lines) or decreased (blue lines) abundance changes detected during WNV infection were plotted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

increase or a 0.7 fold decrease in abundance which represents a significant change ($p \leq 0.01$) compared to the pre-infection level, three proteins showed increased abundance and five proteins displayed decreased abundance. As shown in Fig. 3, beta-2 glycoprotein 1 (APOH), alpha-2-HS glycoprotein (AHSG), and complement factor I (CFI) were all increased in abundance beginning at 6 h post-infection. Maximum levels of all three proteins were reached at 48–72 hpi. In contrast, caboxypeptidase N subunit 1 (CPN1), glycosylphosphatidylinositol specific phospholipase D1 (GPLD1), carbonic anhydrase 2 (CA2), and peroxiredoxin 2 (PRDX2) displayed decreased abundance during WNV infection in mice at all post-infection time points measured.

4. Discussion

For pathogens that pose a significant risk to human and animal health, research must balance the needs of scientific programs with biosafety and biosecurity requirements. Risk mitigation for these requirements is addressed by conducting research in high containment (BSL-3 and BSL-4) laboratories engineered to minimize unwanted release of these agents into the environment while also limiting unauthorized access. While effective in the control and safe usage of these agents, limited access and laboratory space may limit the implementation of complex, sensitive instrumentation with high maintenance burdens, such as proteomic or genomic studies. Thus, there is an unmet need to develop robust and well-characterized pathogen inactivation methods to remove potentially infectious samples from space-constrained containment laboratories for less constrained BSL-2 environments. Moreover, transfer and/or shipment of samples between research entities often requires verified inactivation in order to maintain compliance with various local and federal statutes.

To date, numerous approaches have been developed in inactivate WNV. Given the potential risk of transfusion-related infections, several studies have examined the utility of photochemical treatments of blood products such as riboflavin-UV (Singh et al., 2006), methylene blue-white light (Mohr et al., 2004; Ruane et al., 2004), and amotosalen-UV (Mohr et al., 2004; Singh et al., 2006), with the latter effecting a 5–6 $\log_{10}$ reduction in WNV infectivity. To produce safe, inactivated WNV vaccines, hydrogen peroxide (H_2O_2) treatment resulted in up to a 10 $\log_{10}$ reduction in infectivity (Amanna et al., 2012; Pinto et al., 2013). Similarly, β-propiolactone and formalin treatments have also been used for producing inactivated WNV vaccines, but the reduction in infectivity was not described (Chowdhury et al., 2015). For diagnostic applications, both use of heat and detergents have proven effective to reduce infectivity. Heating WNV-spiked samples containing 15% [vol/vol] animal serum at 56 °C for 45 min decreased infectivity by 6 $\log_{10}$

(Fang et al., 2009), whereas incubating WNV for 30 min at 37 °C in ELISA wash buffer containing 0.05% Tween-20 reduced infectivity by 8 $\log_{10}$ (Mayo and Beckwith Iii, 2002). Treatment of WNV with the nonionic detergent Triton X-100 yielded a 6.2 $\log_{10}$ infectivity reduction, but was not complete (Colavita et al., 2017).

In this report, we describe an effective method for inactivating WNV-containing animal serum samples for use in proteomic applications using commercially available reagents. Ongoing host biomarker discovery efforts for WNV and other pathogens warrant the need to profile the abundance of viral and host proteins in the serum and/or plasma of experimentally infected animals. This method meets these needs by enabling technical staff to perform SDS-PAGE/western blotting and liquid chromatography-tandem mass spectrometry-based peptide identification of acute phase host response proteins without the burdens of BSL-3 containment. The ability to conduct these proteomic analyses may provide opportunities for greater understanding of WNV-host interactions, as well as identifying novel targets for diagnostic applications.

The titer of our WNV stock and the design of our experiments limited the upper bound of inactivation testing to 1.3×10^5 PFU, from which we observed a 6.5×10^3-fold reduction to the limit of detection for our viability test (20 PFU). These data do not preclude the possibility that this method would also be effective for samples with higher infectivity. Indeed, experimental infectious of crows and robins can result in serum viremia titers approaching 10^9 and 10^7 PFU/ml, respectively (Brault et al., 2004; VanDalen et al., 2013). However, our primary application of this method will be to perform proteomic studies of WNV-infected mice, which typically peaks at 1×10^4 PFU/ml in serum at 48 h post-exposure (Caraballo et al., 2015), therefore the data presented here meets that objective. We were unable to find any reports describing the infectivity of serum from WNV-infected human patients, most likely because quantitative infectivity assays are not the standard for clinical diagnosis and also because peak viremia is expected to occur several days prior to onset of symptoms. Without such data, we cannot determine the suitability of this method for processing human diagnostic specimens. Further inactivation studies using WNV samples of higher infectivity would help to decrease margins of risk.

Although this study has focused on serum as the sample matrix, it would most likely be applicable to plasma and other clarified or low complexity sample types such as urine, oral fluid, cerebrospinal fluid, swab material resuspended in viral transport media, cell culture media and/or aqueous buffers. In contrast, treatment of more complex sample types such as whole blood or clarified tissue homogenates warrant further investigation before employing this approach. Investigators should consult with their institutional biosafety officers prior to implementing these procedures in their own laboratories.

Given the significant similarity in structural and biochemical properties of the *Flaviviridae*, we expect that this protocol would be applicable to all viruses in this family. More broadly, our method should also hold true for most lipid enveloped viruses, but should be verified with representative type species as appropriate.

Using our method, we were able to identify seven differentially expressed proteins in the serum of mice experimentally infected with WNV. To our knowledge, none of these proteins have been shown to be differentially expressed in the context of WNV infection. The proteins AHSG (39 kDa), APOH (38 kDa), and CFI (66 kDa) were all upregulated following infection. These proteins are primarily secreted by the liver, and play roles in regulating innate immune and inflammatory responses to infection (Bennett et al., 2017; de Groot and Meijers, 2011; Wang and Sama, 2012). In contrast, we identified CPN1 (52 kDa), GPLD1 (92 kDa), CA2 (29 kDa), PRDX2 (22 kDa) as being downregulated following infection. We are currently investigating the roles that all of these proteins may play during WNV infection and pathogenesis.

These results confirm that inactivated serum samples are compatible with downstream proteomic analysis using LC–MS/MS techniques, facilitating the in-depth study of proteomic profiles resulting from WNV infection of laboratory animals or humans outside of BSL-3 containment.

5. Conclusions

These studies demonstrate the complete inactivation of WNV in serum using NuPAGE® LDS Sample Buffer, NuPAGE® Reducing Agent, and heat. This approach will enable future proteomic studies to examine viral and host proteins present in the sera of WNV-infected animals and humans. Although it is very likely that other members of the family *Flaviviridae* would also be rendered noninfectious by this method in similar biological matrices, investigators should follow the guidance of their institutional biosafety officers and also consider independent verification of this and other inactivation methods before removing potentially infectious samples from biological containment.

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Disclaimer

Opinions, interpretations, conclusions, and recommendations contained herein are solely those of the authors and are not necessarily endorsed by the US Army, DTRA-CB, or the US Department of Defense.

References

Amanna, I.J., Raue, H.P., Slifka, M.K., 2012. Development of a new hydrogen peroxide-based vaccine platform. Nat. Med. 18, 974–979.
Bennett, K.M., Rooijakkers, S.H., Gorham Jr., R.D., 2017. Let's tie the knot: marriage of complement and adaptive immunity in pathogen evasion, for better or worse. Front. Microbiol. 8, 89.
Brault, A.C., Langevin, S.A., Bowen, R.A., Panella, N.A., Biggerstaff, B.J., Miller, B.R., Komar, N., 2004. Differential virulence of West Nile strains for American crows. Emerg. Infect. Dis. 10, 2161–2168.
Caraballo, E.V., Hunsperger, E., Martinez, I., 2015. Characterization of puerto rican West Nile virus isolates in mice. Virol. J. 12, 137.
Chowdhury, P., Topno, R., Khan, S.A., Mahanta, J., 2015. Comparison of beta-Propiolactone and formalin inactivation on antigenicity and immune response of West Nile virus. Adv Virol 2015, 616898.
Colavita, F., Quartu, S., Lalle, E., Bordi, L., Lapa, D., Meschi, S., Vulcano, A., Toffoletti, A., Bordi, E., Paglia, M.G., Di Caro, A., Ippolito, G., Capobianchi, M.R., Castilletti, C., 2017. Evaluation of the inactivation effect of Triton X-100 on Ebola virus infectivity. J. Clin. Virol. 86, 27–30.
Colpitts, T.M., Conway, M.J., Montgomery, R.R., Fikrig, E., 2012. West Nile virus: biology, transmission, and human infection. Clin. Microbiol. Rev. 25, 635–648.
Fang, Y., Brault, A.C., Reisen, W.K., 2009. Short report: comparative thermostability of west nile St. louis encephalitis, and western equine encephalomyelitis viruses during heat inactivation for serologic diagnostics. Am. J. Trop. Med. Hyg. 80, 862–863.
Hollidge, B.S., Gonzalez-Scarano, F., Soldan, S.S., 2010. Arboviral encephalitides: transmission, emergence, and pathogenesis. J. Neuroimmune Pharmacol. 5, 428–442.
Lanciotti, R.S., Roehrig, J.T., Deubel, V., Smith, J., Parker, M., Steele, K., Crise, K., Volpe, E., Crabtree, M.B., Scherret, J.H., Hall, R.A., MacKenzie, J.S., Cropp, C.B., Panigrahy, B., Ostlund, E., Schmitt, B., Malkinson, M., Banet, C., Weissman, J., Komar, N., Savage, H.M., Stone, W., McNamara, T., Gubler, D.J., 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 286, 2333–2337.
Mayo, D.R., Beckwith Iii, W.H., 2002. Inactivation of West Nile virus during serologic testing and transport. J. Clin. Microbiol. 40, 3044–3046.
McDowell, G.S., Gaun, A., Steen, H., 2013. iFASP: combining isobaric mass tagging with filter-aided sample preparation. J. Proteome Res. 12, 3809–3812.
Mohr, H., Knuver-Hopf, J., Gravemann, U., Redecker-Klein, A., Muller, T.A., 2004. West Nile virus in plasma is highly sensitive to methylene blue–light treatment. Transfusion (Paris) 44, 886–890.
Mukhopadhyay, S., Kim, B.S., Chipman, P.R., Rossmann, M.G., Kuhn, R.J., 2003. Structure of West Nile virus. Science 302, 248.
Nasci, R.S., Fischer, M., Lindsey, N.P., Lanciotti, R.S., Savage, H.M., Komar, N., McAllister, J.C., Mutebi, J.-P., Lavelle, J.M., Zielinski-Gutierrez, E., Petersen, L.R., 2013. West Nile virus in the United States. Guidelines for Surveillance, Prevention, and Control, 4th ed. Revision.
Pinto, A.K., Richner, J.M., Poore, E.A., Patil, P.P., Amanna, I.J., Slifka, M.K., Diamond, M.S., 2013. A hydrogen peroxide-inactivated virus vaccine elicits humoral and cellular immunity and protects against lethal West Nile virus infection in aged mice. J.

Virol. 87, 1926–1936.
Ruane, P.H., Edrich, R., Gampp, D., Keil, S.D., Leonard, R.L., Goodrich, R.P., 2004. Photochemical inactivation of selected viruses and bacteria in platelet concentrates using riboflavin and light. Transfion 44, 877–885.
Seddon, A.M., Curnow, P., Booth, P.J., 2004. Membrane proteins, lipids and detergents: not just a soap opera. Biochim. Biophys. Acta 1666, 105–117.
Selisko, B., Wang, C., Harris, E., Canard, B., 2014. Regulation of Flavivirus RNA synthesis and replication. Curr. Opin. Virol. 9, 74–83.
Singh, R., Lamoureux, G.V., Lees, W.J., Whitesides, G.M., 1995. Reagents for rapid reduction of disulfide bonds. Methods Enzymol. 251, 167–173.
Singh, Y., Sawyer, L.S., Pinkoski, L.S., Dupuis, K.W., Hsu, J.C., Lin, L., Corash, L., 2006. Photochemical treatment of plasma with amotosalen and long-wavelength ultraviolet light inactivates pathogens while retaining coagulation function. Transfusion (Paris) 46, 1168–1177.
U. S. Department Human Services, Department of Health and Human Services, Centers for Disease Control and Prevention and National Institutes of Health, 2013. Biosafety in Microbiological and Biomedical Laboratories, 5th edition. CreateSpace Independent Publishing Platform (pp. 438).
VanDalen, K.K., Hall, J.S., Clark, L., McLean, R.G., Smeraski, C., 2013. West Nile virus infection in American Robins: new insights on dose response. PLoS One 8, e68537.
Wang, H., Sama, A.E., 2012. Anti-inflammatory role of fetuin-A in injury and infection. Curr. Mol. Med. 12, 625–633.
Wisniewski, J.R., Zougman, A., Nagaraj, N., Mann, M., 2009. Universal sample preparation method for proteome analysis. Nat. Methods 6, 359–362.
de Groot, P.G., Meijers, J.C., 2011. beta(2) −Glycoprotein I: evolution, structure and function. J. Thromb. Haemost. 9, 1275–1284.