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Evaluation of the inactivation of *Venezuelan equine encephalitis virus* by several common methods

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ABSTRACT

Working with virological samples requires validated inactivation protocols for safe handling and disposal. Although many techniques exist to inactivate samples containing viruses, not all procedures have been properly validated or are compatible with subsequent assays. To aid in the development of inactivation protocols for Alphaviruses, and specifically *Venezuelan equine encephalitis virus* (VEEV), a variety of methods were evaluated for their ability to completely inactivate a high titer sample of the vaccine strain VEEV TC-83. The methods evaluated include reagents used in RNA extraction, fixation, treatment with a detergent, and heat inactivation. Most methods were successful at inactivating the sample; however, treatment with only Buffer AVL, SDS, and heat inactivation at 58 °C for one hour were not capable of complete inactivation of the virus in the sample. These results provide a substantial framework for identifying techniques that are safe for complete inactivation of Alphaviruses and to advise protocol implementation.

Working with infectious agents requires inactivation to safely perform downstream procedures. This is especially important when working with viruses that are categorized as Biosafety level 3 (BSL3) agents or above. Inactivation of BSL3 agents is essential for many techniques and has significant safety implications if these procedures are not validated. In addition, the recent Division of Select Agents and Toxins (DSAT) regulations for select agents have mandated that inactivation procedures must be verified (Centers for Disease Control and Prevention, 2017). Here we present data that validates the inactivation and provides a blueprint for successful inactivation of *Venezuelan equine encephalitis virus* (VEEV) for which some strains are classified as select agents, as well as for other important *Alphavirus* pathogens.

While there are many methods to inactivate a pathogen, not all protocols are appropriate for downstream uses. Techniques for diagnostics or research may require an intact genome or structural proteins. For this reason, specific reagents have become widely used in virology. RNA extraction is often performed using Qiagen kits (Blow et al., 2008) or TRIzol (Hofmann et al., 2000) and proteins are preserved by formalin (Mussgay and Weiland, 1973; Tiwari et al., 2009) or methanol-acetone (Fauvel and Ozanne, 1989). These reagents can inactivate a wide range of viruses, but these need to be validated for each group of viruses. Protocols for inactivation of VEEV have been previously reported for RNA extraction reagents (Blow et al., 2004; Kochel et al., 2017). Other methods for fixation, and heat inactivation have not been reported for VEEV and are also not widely reported for other Alphaviruses. VEEV is an enveloped, single-stranded RNA virus in the genus *Alphavirus*, family *Togaviridae* and are classified as BSL3 with the exception of the vaccine strain TC-83. VEEV TC-83, which was used here, is derived from the select agent strain Trinidad Donkey and is, therefore, an appropriate substitute for this important family of viruses. VEEV TC-83 differs from Trinidad Donkey strain by 12 mutations, including 8 in the structural genes (Kinney et al., 1993). The structure of TC-83 does not suggest a significant deviation from Trinidad Donkey strain (Zhang et al., 2011). The attenuating structural mutation at E2-120, a switch from a neutral to positive residue, likely improves infection in cell culture because of selection for heparin sulfate binding (Klimstra et al., 1998). The same mutation consequently attenuates *in vivo* because of binding negatively charged molecules that interfere with spread (Byrnes and Griffin, 2000). One additional benefit is that TC-83 typically grows to a log higher than most Alphaviruses, so this also increases the effectiveness of this strain as a surrogate. VEEV is responsible for several outbreaks that have occurred in central and South America. In addition, the recent outbreaks of *chikungunya virus* have brought the need for *Alphavirus* inactivation to the fore to facilitate the safe sharing of samples.

To test inactivation protocols, VEEV TC-83 rescued in baby hamster kidney cells and passaged twice in African green monkey kidney (Vero) (American Type Culture Collection) cells was used for infections and all experiments were performed in BSL2 facilities. Virus stock was diluted to infect Vero cells at an MOI of 1 in Dulbecco's modified Eagle medium (DMEM) (Gibco) with 10% fetal bovine serum (FBS) (Atlanta

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Biologicals) and 0.05 mg/mL gentamycin (Corning). Cells were incubated with inoculated media at 37 °C for 24 h. Media was aspirated from the cells and clarified by centrifugation at 1176 × *g* for 5 min. Cells were washed with PBS (Gibco), scraped from the surface, and added to a 1.5 mL microcentrifuge tube. Cells were pelleted by centrifugation at 188 × *g* for 5 min and the PBS was removed. Virus titers of the clarified media and washed cells were determined by plaque assay, using a previously described method (Beaty et al., 1995). Briefly, samples were serially diluted, and incubated with Vero cells for 1 h before overlay with DMEM containing 2% FBS, 0.4% agarose and gentamycin. Plaques were allowed to develop for 48 h at 37 °C. Samples were fixed with 10% formalin (Fisher Scientific), and stained with 0.25% crystal violet solution (Fisher Scientific).

Protocols regularly used during RNA isolation, fixation, or inactivation of contaminated or infected samples were selected to evaluate their ability to inactivate VEEV TC-83. Clarified media was used for inactivation steps of RNA extraction protocols with Qiagen (QIAamp Viral RNA Mini Kit, Qiagen), Clontech (NucleoSpin Virus, Clontech), and TRIzol (TRIzol Reagent, Ambion) reagents. The buffers from Qiagen and Clontech, Buffer AVL and Buffer VL, respectively, contain guanidine hydrochloride, a chaotrope often used for denaturing proteins. TRIzol also contains a chaotrope, guanidinium thiocyanate, for protein denaturation. Manufacturer's protocols were followed for incubation time, temperature, sample, and reagent amounts. Following the manufacturer's protocol, sample volume was adjusted to 1 mL with DMEM with 10% FBS, where possible. Brief protocols are described in Table 1. Washed cells from 1 well of a 6-well plate were resuspended in 500 µL of 0.1% sodium dodecyl sulfate (SDS) (Corning), 10% formalin (Fisher Scientific), or methanol-acetone (Fisher Scientific). Methanol-acetone was mixed at a 1:1 ratio and stored at −20 °C. Following the 30 min incubation, 500 µL of DMEM with 10% FBS was added to samples treated with 0.1% SDS, 10% formalin and methanol-acetone. Cells with 10% formalin and methanol-acetone were pelleted by centrifugation at 188 × *g* for 5 min. The supernatant was removed and cells were resuspended in 500 µL of DMEM with 10% FBS. Heat inactivation was performed with a heat block monitored with a thermometer on 500 µL stock samples that contained 9.75×10^8 pfu. After exposure to 58 °C (+/− 2 °C) or 80 °C (+/− 1 °C) for 1 h, 1×10^8 pfu was mixed with 5 mL of 2% FBS DMEM and added to Vero cells in T25 flasks. Protocols were performed in triplicate.

Samples inactivated in Qiagen Buffer AVL with or without ethanol, 0.1% SDS, 10% formalin and methanol-acetone were dialyzed (Float-A-Lyzer G2 MWCO: 3.5–5 kDa, Spectra/Por) in PBS to remove the toxic reagents. Due to the toxicity of the reagents used in chemical inactivation, inactivated samples cannot be applied directly to cells for analysis. Samples must be dialyzed or washed to maintain cell viability. Dialysis was performed overnight at room temperature with a complete buffer change. Additional positive control samples were dialyzed that were not treated with an inactivation procedure. These contained VEEV TC-83 in tenfold dilutions at titers of 1×10^5 pfu down to 1×10^1 pfu.

Due to the use of proteinase K in the protocol, Clontech Buffer VL treated samples were washed with 10 mL of PBS in centrifugal concentrators (Amicon Ultra MWCO: 100 kDa; Millipore). Due to the incompatibility of TRIzol with the dialysis membrane, TRIzol treated samples were washed with 25 mL of PBS in centrifugal concentrators (Amicon Ultra MWCO: 10 kDa, Millipore). All samples inactivated by Clontech Buffer VL and TRIzol were concentrated to a final volume of less than 1 mL.

All samples were added to 5 mL of DMEM with 2% FBS, applied to Vero cells in a T25 flask and incubated at 37 °C for 4 days. Flasks were monitored daily for cytopathic effect (CPE). Samples that showed no evidence of CPE were passaged a second time to confirm the absence of CPE and the full inactivation of the sample. The first passage of supernatant was clarified by centrifugation at 1176 × *g* for 5 min. Clarified media was mixed 1:1 with fresh DMEM with 2% FBS, and 5 mL was added to a second passage of Vero cells. Conditions suspected to be toxic to cells were confirmed for presence of virus by titration on Vero cells in standard plaque assays (Beaty et al., 1995). Observation of plaques would indicate active virus in the sample, while widespread CPE would signal the presence of toxic reagents that require further dilution or exchange.

Cells inoculated with the stock VEEV TC-83 had CPE following 24 h of incubation. The titer of the clarified media was 1.05×10^{10} pfu/mL, and the titer of the washed cells was 1.267×10^9 pfu/mL (Fig. 1A). Positive control samples (dialyzed, untreated virus samples) from 1×10^5 to 1×10^1 pfu, were all positive for CPE in the first passage on Vero cells. These samples were able to demonstrate that the dialysis process and level of detection are sensitive and appropriate for the titers used in the inactivation protocols.

Using media that contained a high titer of VEEV TC-83 to evaluate common methods used in RNA extraction inactivated virus in most samples. Samples inactivated only with Qiagen Buffer AVL showed CPE in one out of three replicates (Fig. 1B). A plaque assay confirmed the result. An additional step of the Qiagen protocol, which includes addition of ethanol, led to the inactivation of all replicates. A previous study reported the inactivation of VEEV 68U201, an endemic BSL3 strain, with Qiagen Buffer AVL only (Blow et al., 2004). The current study evaluated three experimental replicates, rather than technical replicates from a single sample, which revealed an inconsistency in the reagent's ability to inactivate VEEV strains. The use of a higher virus titer, and differences in strain may also cause the discrepancies for inactivation. Although the differences in experiments may lead to different outcomes, these results add to a growing list of viruses, including *Ebola virus*, *West Nile virus*, and *chikungunya virus*, which are not effectively inactivated with only Qiagen Buffer AVL (Ngo et al., 2017; Smither et al., 2015). For this reason, the addition of ethanol to Qiagen Buffer AVL for complete inactivation of virus is recommended for the safe use of the sample or removal from high containment.

Successful inactivation of the entire virus sample was seen with Clontech NucleoSpin Virus and TRIzol protocols. Cell lysis was seen in two of the samples inactivated using Clontech Buffer VL with proteinase K; however, these were due to remaining toxic reagents rather than active virus, as confirmed by plaque assay. Inactivation of VEEV by TRIzol has also been confirmed in multiple previous reports (Blow et al., 2004; Kochel et al., 2017).

Infected cells treated with reagents common for fixation, 10% formalin and methanol-acetone, were all completely inactivated. These included incubation temperatures of 4 °C, 21 °C, and 37 °C. Both reagents are widely used in preparation for microscopy, and their ability to successfully inactivate VEEV in cells was expected, although low concentrations of formaldehyde have been shown ineffective against *yellow fever virus* (Goodman et al., 2014). Glutaraldehyde is a more reactive crosslinker than formaldehyde, and may be used at concentrations as low as 0.1% to inactivate very high titers of *Sindbis virus*,

Table 1
Brief inactivation protocols for each condition by time, temperature, and virus titer.

Condition	Temperature (°C)	Time (min)	Sample (pfu)
Qiagen AVL	21	10	1.47×10^9
Qiagen AVL + ethanol	21	10	1.47×10^9
Clontech Nucleospin Virus	21	8 (5 min – Buffer VL, 3 min – ethanol)	2.10×10^9
TRIzol	21	5	1.000×10^8
0.1% SDS	21	30	2.53×10^9
10% Formalin	4	30	2.53×10^9
	21	30	2.53×10^9
	37	30	2.53×10^9
Methanol/Acetone	4	30	2.53×10^9
	21	30	2.53×10^9
	37	30	2.53×10^9
Heat inactivation	58	60	1.000×10^8
	80	60	1.00×10^8