Journal of Virological Methods 189 (2013) 184–188

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Inactivation of infectious virus and serological detection of virus antigen in Rift Valley fever virus-exposed mosquitoes fixed with paraformaldehyde

Rebekah Kading*, Mary Crabtree, Barry Miller

Centers for Disease Control and Prevention, Division of Vector-borne Diseases, Arbovirus Diseases Branch, 3156 Rampart Road, Mail Stop P02, Fort Collins, CO 80521, USA

Article history:
Received 22 October 2012
Received in revised form 11 January 2013
Accepted 28 January 2013
Available online 4 February 2013

Keywords:
Rift Valley fever virus
Paraformaldehyde fixation
Trypsin
Mosquito
Pathology

A B S T R A C T

Formaldehyde is routinely used to fix tissues in preparation for pathology studies, however concerns remain that treatment of tissues with cellular fixatives may not entirely inactivate infectious virus particles. This concern is of particular regulatory importance for research involving viruses that are classified as select agents such as Rift Valley fever virus (RVFV). Therefore, the specific aims of this study were to (1) assay RVFV-exposed *Aedes aegypti* mosquitoes fixed in 4% paraformaldehyde for the presence of infectious RVFV particles at various time points following infection and (2) demonstrate the utility of immunofluorescence assay (IFA) for the detection of RVFV antigen in various tissues of paraformaldehyde-fixed mosquitoes. Mosquitoes were administered an infectious blood meal containing one of two strains of RVFV, harvested at various time points following infection, intrathoracically inoculated with 4% paraformaldehyde, and fixed overnight at 4 °C. The infection status of a subset of mosquitoes was verified by IFA on leg tissues prior to fixation, and infectivity of RVFV in fixed mosquito carcasses was determined by Vero cell plaque assay. Paraformaldehyde-fixed mosquitoes harvested 14 days post infection were also paraffin-embedded and sectioned for detection of RVFV antigen to particular tissues by IFA. None of the RVFV-exposed mosquitoes tested by Vero cell plaque assay contained infectious RVFV after fixation. Furthermore, incubation of mosquito sections with trypsin prior to antibody staining is recommended for optimal visualization of RVFV antigen in infected mosquito tissues by IFA.

Published by Elsevier B.V.

1. Introduction

Formaldehyde is routinely used to fix tissues in preparation for pathology studies, as well as for inactivation of virus during vaccine development (Faran et al., 1986; Kistner et al., 2007; Tiwari et al., 2009; Fine et al., 2010). Treatment of tissues with formalin (37% formaldehyde) inhibits cellular processes, prevents tissue degradation, and preserves tissue architecture thereby allowing the detection of antigens in the context of natural cell morphology and/or histologic lesions (Eltoum et al., 2001; Ramos-Vara, 2005; Webster et al., 2009). Formalin also induces cross-linking of viral proteins leading to the loss of virus infectivity (Tiwari et al., 2009), a property of critical importance for the safe handling of infected materials. However, concerns remain that treatment of tissues with cellular fixatives may not entirely inactivate infectious virus particles, thereby putting researchers at risk of exposure to virus-infected material outside of proper containment (Fauvel and Ozanne, 1989).

Rift Valley fever virus (RVFV) (family *Bunyaviridae*, genus *Phlebovirus*) is an emerging zoonotic mosquito-borne virus endemic to Africa. RVFV poses a threat for introduction into new areas including the United States where it has the potential to cause significant economic losses to the livestock industry as well as substantial human morbidity and mortality (CDC, 2000; Kasari et al., 2008). RVFV is registered as a select agent with both the U.S. Department of Agriculture Animal and Plant Health Inspection Service and the Centers for Disease Control and Prevention (USDA-APHIS and CDC, 2010). Transmission studies of RVFV in North American mosquitoes have begun to evaluate the potential for the establishment and maintenance of RVFV transmission in the United States by examining the competence of various North American mosquito vectors to transmit RVFV (Turell et al., 1988, 2008, 2010). While these studies provide important foresight into our preparedness efforts, pathology studies can provide additional critical knowledge regarding the timing of dissemination and extent of RVFV infection in various mosquito tissues. Data generated from pathological studies has important application to determining the extrinsic incubation period for the virus in the mosquito vector, and examining the potential for vertical transmission.

A 2% formaldehyde solution has previously been used to fix mosquitoes infected with RVFV for pathology purposes (Faran

* Corresponding author. Tel.: +1 970 225 4210; fax: +1 970 494 6631.
E-mail address: fxk7@cdc.gov (R. Kading).

0166-0934/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jviromet.2013.01.014

et al., 1986), but this study provided no direct evidence that infectious RVFV particles in the mosquito tissues were destroyed following fixation. Furthermore, an avidin–biotin-peroxidase complex immunocytochemical procedure has been used successfully for the localization of RVFV antigen to mosquito tissues (Faran et al., 1986; Romoser et al., 1992), but such data are lacking regarding the applicability of immunofluorescence techniques to localize RVFV antigen to mosquito tissues. Therefore the specific aims of this study were to (1) assay RVFV-exposed *Aedes aegypti* L. mosquitoes fixed in 4% paraformaldehyde for the presence of infectious RVFV particles at various time points following infection and (2) demonstrate the utility of immunofluorescence assay (IFA) for the detection of RVFV antigen in various tissues of paraformaldehyde-fixed mosquitoes.

2. Materials and methods

2.1. Preparation of 4% paraformaldehyde

Paraformaldehyde crystals were dissolved into 1× PBS in a 60 °C water bath for 30 min to form a 4% solution. The 4% paraformaldehyde solution was then stored at 4 °C.

2.2. Paraformaldehyde fixation of mosquitoes

The protocol used for fixing and paraffin-embedding of mosquitoes was modified from Faran et al. (1986). Twenty uninfected mosquitoes were each briefly dipped in 70% ethanol to destroy hydrophobicity, then dipped in 1× PBS to wash off the ethanol. The legs and wings were removed and each mosquito was intrathoracically inoculated with 4% paraformaldehyde in PBS until the abdomen became completely distended with liquid. This procedure was performed to ensure that the paraformaldehyde penetrated the exoskeleton of the mosquito body, and that all tissues inside the mosquito became infused with fixative. Following inoculation, mosquitoes were placed in a 1.5 mL microcentrifuge tube containing 4% paraformaldehyde where they soaked overnight at 4 °C. Approximately 20–22 h after the inoculation of fixative, mosquitoes were removed from the paraformaldehyde and placed in PBS.

2.3. Assessment of the toxicity of paraformaldehyde-fixed mosquitoes to Vero cells

We first addressed the question of whether paraformaldehyde-fixed mosquitoes were toxic to Vero cells. If the cells were killed due to paraformaldehyde toxicity it would be impossible to assess the infectivity of mosquitoes following the fixation procedure. Six fixed, uninfected mosquitoes were washed for 15-min in PBS following fixation. Immediately following the PBS wash, mosquitoes were transferred individually to 2 mL tubes containing 1 mL DMEM and a single 4 mm copper BB and homogenized for 4 min in a TissueLyser (Qiagen, Valencia, CA) at 20 cycles/s. Homogenate was clarified by centrifugation for 8 min at 4 °C at 10,000 rpm. Supernatant was transferred to a clean microcentrifuge tube and frozen at −80 °C.

2.4. Paraformaldehyde fixation of RVFV-exposed mosquitoes

Reverse genetics-generated RVF viruses were used in this study (Bird et al., 2007, 2008). Freshly harvested wild type (rRVF-wt) and rRVF-ΔNSm strains were used in the infectious blood meal for maximum infectivity to mosquitoes. The rRVF-ΔNSm strain of RVFV is a deletion mutant that lacks the NSm virulence gene (Bird et al., 2008). Three days prior to the infectious blood-feed, one T-75 flask each of Vero cells was inoculated with either rRVF-wt or rRVF-ΔNSm at a multiplicity of infection (MOI) of 0.1. On day 3 post-infection, cell-culture supernatant was harvested and clarified for use in the infectious blood meal.

The infectious blood meal was prepared by mixing two parts washed defibrinated calf blood with two parts virus and one part FBS + 10% sucrose. A virus-negative blood meal contained cell culture media in place of virus-positive cell supernatant. Blood was warmed to 37 °C in a water bath. Eight–ten day-old *Ae. aegypti* mosquitoes starved for 27-h were administered an infectious RVFV blood meal containing either rRVF-wt or rRVF-ΔNSm on blood-soaked cotton balls. Screened pint cups containing 100–150 female *Ae. aegypti* were placed inside Tupperware bins inside the 28 °C environmental chamber. One blood-soaked cotton ball was placed on each carton for 25 min. Blood-soaked cotton balls were placed on the cartons inside the biosafety cabinet, and the Tupperware lid was secured on the bin before the bin was placed into the environmental chamber. Following the blood meal, mosquitoes were anesthetized by freezing at −20 °C for 1 min, and fully engorged mosquitoes were sorted over ice inside of a glove box; only fully engorged mosquitoes were used for the experiment. Engorged mosquitoes were placed into screened 3.8-L paperboard cartons and supplied with 5% sugar solution. Paperboard cartons were placed inside a 30-cm × 30-cm × 30-cm metal cage inside the environmental chamber for double containment. All work involving manipulations with infectious virus in cell culture and/or RVFV-exposed mosquitoes was performed under enhanced biosafety level 3 containment (U.S. Department of Health and Human Services, 2009). Additionally, 500 μL of each blood meal and 500 μL of virus seed brought to 20% FBS were frozen at −80 °C for later quantification.

Two experimental replicates were conducted to determine the infectivity of RVFV following fixation of mosquitoes in 4% paraformaldehyde. In the first experiment, three mosquitoes each from the rRVF-wt and rRVF-ΔNSm groups were harvested on day 0 and day 14 post infection and fixed in 4% paraformaldehyde as described above. One negative control mosquito (received an uninfected blood meal) was also tested. Because the infection rate of *Ae. aegypti* with either strain of RVFV at 14 days post infection (DPI) was not expected to be 100% (Crabtree et al., 2012), we could not be certain that all day 14 mosquitoes selected for paraformaldehyde fixation still contained infectious virus when harvested. Therefore, a second experiment was conducted using an additional three day 10 mosquitoes exposed to rRVFV-wt, three day 12 rRVF-wt mosquitoes, three day 12 rRVF-ΔNSm mosquitoes, and one day 14 negative control mosquito. The legs from each of these mosquitoes were acetone fixed onto microscope slides and examined by IFA for RVFV antigen to confirm whether or not each specimen had a disseminated infection prior to paraformaldehyde fixation.

Infectivity of RVFV in mosquito carcasses was determined by Vero cell plaque titration (Miller et al., 1989). The second overlay containing neutral red was added 3 DPI and plaques were read 4 and 5 DPI.

2.5. Immunofluorescence assay (IFA)

Immunofluorescence assay was performed using 12-well multispot slides (Thermo Electron Corp., Pittsburgh, PA) spotted with rRVF-wt- or rRVF-ΔNSm-infected Vero E6 cells as positive controls. For IFA on mosquito legs, legs were manually removed from mosquito specimens to be fixed, and squashed directly onto clean spot slides under a coverslip. Pieces of cuticle were manually removed with forceps. Slides were fixed in ice cold acetone for 10 min. Immunostaining of mosquito legs and control spot slides was performed as follows: Slides were incubated with a 1:2500 dilution of mouse anti-RVFV hyperimmune ascitic fluid for 30 min

at 37 °C in a humid box. Slides were washed twice for 10 min in PBS and air dried. Slides were then incubated for 30 min at 37 °C in a humid box with the secondary antibody conjugate AlexaFluor 488 goat anti-mouse IgG H+L (Invitrogen, Molecular Probes, Eugene, OR), diluted 1:2000 in PBS with 0.08% trypan blue. Again, slides were washed twice with PBS, rinsed briefly with distilled water, and air dried. Coverslips were mounted using SlowFade Gold mounting medium (Invitrogen, Molecular Probes, Eugene, OR) and visualized with a Zeiss AxioImager Z1 (Carl Zeiss Microimaging, Inc., Thornwood, NY).

IFA was also performed on sagittal sections of whole mosquitoes that were fixed in 4% paraformaldehyde to demonstrate that RVFV antigen could be still be detected in fixed specimens. Day 14 rRVF-wt and day 14 negative control mosquitoes were selected for this purpose. Paraformaldehyde-fixed mosquitoes were embedded in paraffin blocks with up to four mosquitoes per block and sectioned. Two sections were mounted on each slide. Slides were first heated to 40 °C for 10 min on a slide drying bench (ThermoFisher Scientific, Dubuque, IA) and allowed to dry overnight. Slides were deparaffinized by washing in xylenes (Fisher Scientific, Houston, TX) twice for 5 min each wash. Sections were rehydrated in a graded ethanol/PBS series of 5-min washes of 100%, 70%, 50%, and 30% ethanol. To reduce autofluorescence in mosquito tissues, sections were then subjected to dehydration/rehydration in a series of methanol/PBS washes consisting of 30%, 50%, 70%, 100%, 70%, 50%, and 30% methanol. Each wash was 5 min except for the 100% wash which was 15 min. Following the washes, a well was drawn around mosquito sections using a TechPen (Mark-Tex Corp., Englewood, NJ). Initially, RVFV antigen was barely detectable in mosquito sections, so the effect of trypsin on the antibody staining of paraformaldehyde-fixed mosquitoes was evaluated as an antigen retrieval technique (Swoveland and Johnson, 1979; Kurata et al., 1983). For this assessment, PBS was added to one well of each slide, and 0.05% trypsin (Gibco Cell Culture, Life Technologies, Grand Island, NY) was added to the other well such that different sections from the same mosquitoes were either digested with trypsin or undigested for comparison. Slides treated with trypsin and PBS were incubated at 37 °C in a humid box for 30 min and then washed in PBS for 5 min. Following the trypsin digest, slides were placed in blocking solution (PBS containing 10% nonfat milk and 0.1% Triton X-100 (Fisher Scientific, Houston, TX) at room temperature for 1 h. Milk was washed off the blocked slides by a brief immersion in PBS containing 0.1% Triton X-100. Next, slides were incubated with a 1:1600 dilution of mouse anti-RVFV hyperimmune ascitic fluid in PBS + 0.1% Triton X-100 in a humid box at 37 °C for 30 min. Incubation was followed by two 20-min washes in PBS containing 1% nonfat milk + 0.1% Triton X-100. Slides were then incubated for 30 min at 37 °C in a humid box with secondary antibody conjugate AlexaFluor 488 goat anti-mouse IgG H+ diluted 1:2000 in PBS with 0.08% trypan blue and washed as above. Coverslips were mounted using SlowFade Gold mounting medium and visualized with a Zeiss AxioImager Z1.

3. Results

3.1. Paraformaldehyde-fixed mosquitoes were not toxic to Vero cells

Uninfected mosquitoes fixed in 4% paraformaldehyde were washed in PBS following being fixed to determine if it was possible to effectively wash the paraformaldehyde out of the mosquitoes and eliminate any toxic effect on the cells. Vero cells remained viable, indicating that the amount of paraformaldehyde used to fix a single mosquito was not toxic to Vero cells following a single 15-min PBS wash.

3.2. Virus titers

The infectious blood meal titers were: rRVF-wt: 7.6 $\log_{10}$ plaque-forming units (pfu)/mL and rRVF-ΔNSm: 7.9 $\log_{10}$ pfu/mL. At the time day 0 mosquitoes were harvested for paraformaldehyde fixation, the average rRVF-wt titer was 5.3 $\log_{10}$ pfu/mosquito, and 5.4 $\log_{10}$ pfu/mosquito for rRVF-ΔNSm virus. On day 14, remaining mosquitoes infected with rRVF-wt contained an average of 5.5 $\log_{10}$ pfu of virus, and mosquitoes remaining infected with rRVF-ΔNSm RVFV contained an average of 2.7 $\log_{10}$ pfu of virus.

3.3. Effect of paraformaldehyde fixation on RVFV infection in mosquitoes

In total, 12 mosquitoes exposed to rRVF-wt ($n = 3$; day 0, 10, 12, 14), 9 mosquitoes exposed to rRVF-ΔNSm ($n = 3$; day 0, 12, 14), and two negative control mosquitoes were tested for infectious RVFV following fixation in 4% paraformaldehyde. None of the RVFV-exposed mosquitoes from either experiment contained infectious virus after fixation. Immunofluorescence assay performed on the legs of the mosquitoes fixed in paraformaldehyde in the second experiment demonstrated that 2/3 day 10 rRVF-wt and 3/3 day 12 rRVF-wt mosquitoes had disseminated infections prior to fixation (Fig. 1). None of the day 12 rRVF-ΔNSm mosquitoes had a disseminated infection.

3.4. Effect of trypsin on RVFV antigen detection in fixed mosquito tissues

When following our IFA protocol for staining fixed mosquito sections without performing a trypsin digest, RVFV antigen was barely detectable. Antigen detection was significantly improved by incubating sections in a 0.05% trypsin solution for 30 min at 37 °C (Fig. 1). Even though a higher primary antibody concentration was used for staining paraffin-embedded mosquito sections (1:1600) as opposed to mosquito leg squashes and control spot slides (1:2500), both trypsin-digested and -undigested sections were treated with the 1:1600 primary antibody concentration throughout the study. Therefore, the increased detectability of RVFV antigen was due to the trypsin treatment, not a higher primary antibody concentration.

4. Discussion

In this report we provide proof that fixation of RVFV-exposed mosquitoes with paraformaldehyde destroyed infectious virus present in the mosquito tissues. Contact of the paraformaldehyde with all tissues within the mosquito was ensured by removing the wings and legs prior to fixing, inoculating mosquitoes with enough fixative to completely distend their bodies, and soaking mosquitoes in fixative overnight. As a result, our fixation protocol utilizing 4% paraformaldehyde solution, and an incubation for 20–22 h at 4 °C was sufficient to inactivate greater than 5 $\log_{10}$ pfu of infectious RVFV particles within individual mosquitoes. This is a higher concentration of fixative than has previously been shown to inactivate viruses in other applications. Viruses being formalin-inactivated for vaccine preparation are typically inactivated in a low concentration of formalin (0.1–1%) over several days (Kistner et al., 2007; Tiwari et al., 2009; Fine et al., 2010), although in each of these three studies no infectious virus was detected by 24 h following the addition of fixative. Martin et al. (1987) noted a dose-dependent effect for cellular fixative treatments; >1% neutral buffered formalin or >0.5% paraformaldehyde was sufficient to inactivate human T-lymphocyte virus type III/lymphadenopathy-associated virus (HIV) in as little as 5 min. Kraus et al. (2005) evaluated the inactivation of Hantaan virus-containing cells treated with paraformaldehyde and

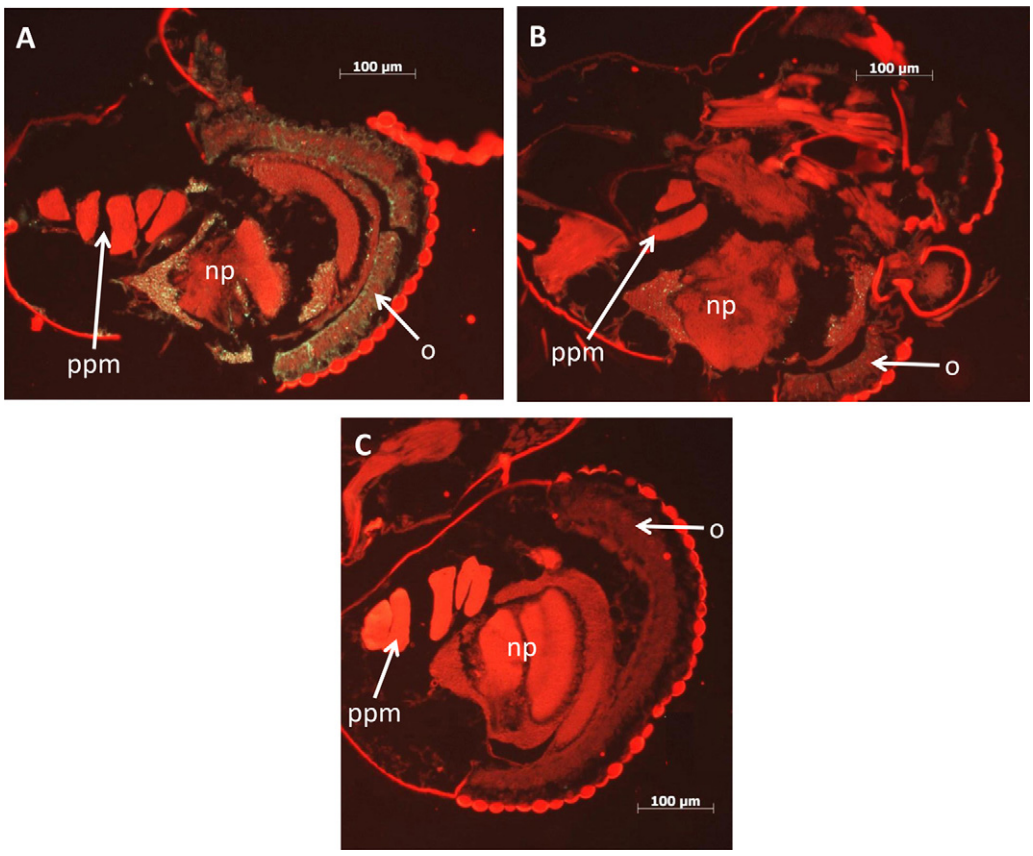

Fig. 1. Treatment of paraformaldehyde-fixed mosquito sections with trypsin greatly enhances the detectability of RVFV antigen. (A) Sagittal section of the head of *A. aegypti* treated with trypsin prior to antibody staining, demonstrating a disseminated infection with rRVF-wt 14 DPI. np = neuropile; o = ommatidia; ppm = pharyngeal pump musculature. (B) Head of *A. aegypti* not treated with trypsin prior to antibody staining. Panel B represents a different section from the same mosquito pictured in Panel A. (C) Head of an uninfected *A. aegypti* mosquito.

determined that treatment of infected cells with 1% paraformaldehyde for 20 min resulted in no infectious virus. Finally, infectious herpesviruses could not be recovered from any tissue harvested from animal carcasses soaked in neutral buffered formalin for 24 h (Ritchey et al., 2006) or from RVFV-infected calf and lamb tissues soaked in 10% buffered formalin for seven days (Drolet et al., 2012). These data collectively show that infectious virus can be ablated in minutes to hours using even small concentrations of formalin-based fixatives. We did not attempt to optimize the minimum concentration and time interval necessary to inactivate RVFV in mosquitoes.

While complete inactivation of the virus is of critical importance for biosafety concerns, using an inactivation procedure that preserves the integrity of viral antigens is essential for the success of subsequent diagnostics. Formalin is known to mask epitopes and result in decreased immunoreactivity (Arnold et al., 1996; Werner et al., 2000; Ramos-Vara, 2005). Arnold et al. (1996) found neutral buffered formalin to be the poorest fixative examined for maintaining antigen recognition, and that in formalin fixed archival tissues only a portion of antigen signal was detectable by routine immunohistologic methods. Formalin fixation is also a time-dependent process, in which the binding of formaldehyde groups to proteins increases to a point of equilibrium (Fox et al., 1985). The utility of formalin-fixed tissues for diagnostic purposes may necessitate the use of an antigen retrieval technique such as treatment of formalin-fixed tissues with an enzyme (Kurata et al., 1983) or ethylenediaminetetraacetic acid (EDTA)–Tween 20 (Meehan et al., 1989) to unmask antigens. Webster et al. (2009) evaluated the effects of prolonged formalin fixation on immunohistochemical detection of 61 different antigens and found that most antibodies could detect antigen following up to seven weeks of fixation, however the effects of formalin fixation were antibody and antigen dependent. Sagripanti et al. (2011) evaluated the efficacy of several liquid chemical reagents to inactivate microbial organisms and viruses while preserving immunological reactivity and nucleic acid detection. In their study, treatment of infectious material with 4–16% formaldehyde severely impaired virus detection by ELISA. In our study, we found the detection of RVFV antigen in paraformaldehyde-fixed tissues to be possible, but severely diminished (Fig. 1). Antigen detectability was not improved by increasing the concentration of primary antibody alone. However, treatment of the sections with trypsin prior to antibody staining successfully unmasked the viral antigen resulting in the successful detection of RVFV in mosquito tissues by IFA (Fig. 1). The treatment of formalin fixed tissues with trypsin has been successfully used as an antigen retrieval technique for RVFV and other viruses (Swoveland and Johnson, 1979; Kurata et al., 1983). In contrast, enzyme treatment was not necessary for the detection of RVFV in formalin-fixed vertebrate tissues by immunohistochemistry (Drolet et al., 2012).

5. Conclusion

Infectious RVFV particles in mosquitoes are effectively inactivated using our protocol, rendering specimens safe for preparation for pathology studies and successfully preserving viral antigens for immunological detection. Furthermore, incubation of mosquito sections with trypsin prior to antibody staining is recommended for optimal visualization of RVFV antigen in infected mosquito tissues by IFA.

Acknowledgements

Mouse anti-RVFV hyperimmune ascitic fluid was provided by Dr. Brian Bird and Dr. Stuart Nichol of the CDC Viral Special Pathogens Branch, Division of High Consequence Pathogens and Pathology. This work was supported by the CDC Division of Vector-borne Diseases, Arbovirus Diseases Branch.

References

Arnold, M.M., Srivastava, S., Fredenburgh, J., Stockard, C.R., Myers, R.B., Grizzle, W.E., 1996. Effects of fixation and tissue processing on immunohistochemical demonstration of specific antigens. Biotech. Histochem. 71, 224–230.

Bird, B.H., Albarino, C.G., Nichol, S., 2007. Rift Valley fever virus lacking NSm proteins retains high virulence in vivo and may provide a model of human delayed onset neurologic disease. Virology 362, 10–15.

Bird, B.H., Albarin, C.G., Hartman, A.L., et al., 2008. Rift Valley fever virus lacking the NSs and NSm genes is highly attenuated, confers protective immunity from virulent virus challenge, and allows for differential identification of infected and vaccinated animals. J. Virol. 82, 2681–2691.

Centers for Disease Control, 2000. Outbreak of Rift Valley fever – Yemen, August–October 2000. MMWR Morb. Mortal. Wkly Rep. 49, 1065–1066.

Crabtree, M.B., Crockett, R.J.K., Bird, B.H., et al., 2012. Infection and transmission of Rift Valley fever viruses lacking the NSs and/or NSm genes in mosquitoes: potential role for NSm in mosquito infection. PLoS Negl. Trop. Dis. 6, e1639.

Drolet, B.S., Weingartl, H.M., Jiang, J., et al., 2012. Development and evaluation of one-step rRT-PCR and immunohistochemical methods for detection of Rift Valley fever virus in biosafety level 2 diagnostic laboratories. J. Virol. Methods 179, 373–382.

Eltoum, I., Fredenburgh, J., Myers, R.B., Grizzle, W.E., 2001. Introduction to the theory and practice of fixation of tissues. J. Histotechnol. 24, 173–190.

Faran, M.E., Romoser, W.S., Routier, R.G., Bailey, C.L., 1986. Use of avidin–biotin-peroxidase complex immunocytochemical procedure for detection of Rift Valley Fever virus in paraffin sections of mosquitoes. Am. J. Trop. Med. Hyg. 35, 1061–1067.

Fauvel, M., Ozanne, G., 1989. Immunofluorescence assay for human immunodeficiency virus antibody: investigation of cell fixation for virus inactivation and antigen preservation. J. Clin. Microbiol. 27, 1810–1813.

Fine, D.L., Jenkins, E., Martin, S.S., et al., 2010. A multisystem approach for development and evaluation of inactivated vaccines for Venezuelan equine encephalitis virus (VEEV). J. Virol. Methods 163, 424–432.

Fox, C.H., Johnson, F.B., Whiting, J., Roller, P.P., 1985. Formaldehyde fixation. J. Histochem. Cytochem. 33, 845-–853-.

Kasari, T.R., Carr, D.A., Lynn, T.V., Weaver, J.T., 2008. Evaluation of pathways for release of Rift Valley fever virus into domestic ruminant livestock, ruminant wildlife, and human populations in the continental United States. J. Am. Vet. Med. Assoc. 232, 514–529.

Kistner, O., Barrett, N., Bruhmann, A., et al., 2007. The preclinical testing of a formaldehyde inactivated Ross River virus vaccine designed for use in humans. Vaccine 25, 4845–4852.

Kraus, A.A., Priemer, C., Heider, H., et al., 2005. Inactivation of Hantaan virus-containing samples for subsequent investigations outside biosafety level 3 facilities. Intervirology 48, 255–261.

Kurata, T., Hondo, R., Sato, S., et al., 1983. Detection of viral antigens in formalin-fixed specimens by enzyme treatment. Ann. N.Y. Acad. Sci. 420, 192–207.

Martin, L.S., Loskoski, S.L., McDougal, J.S., 1987. Inactivation of human T-lymphotropic virus type III/lymphadenopathy-associated virus by formaldehyde-based reagents. Appl. Environ. Microbiol. 53, 708–709.

Meehan, J.T., Cutlip, R.C., Lehmkuhl, H.D., 1989. Evaluation of ethylenediaminetetraacetic acid, tetrasodium salt dehydrate (EDTA)–Tween 20 treatment versus protease digestion of formalin-fixed tissue sections for detection of bovine respiratory syncytial virus antigen in infected ovine lung. Vet. Pathol. 26, 322–325.

Miller, B.R., Mitchell, C.J., Ballinger, M.E., 1989. Replication, tissue tropisms and transmission of yellow fever virus in *Aedes albopictus*. Trans. R. Soc. Trop. Med. Hyg. 83, 252–255.

Ramos-Vara, J.A., 2005. Technical aspects of immunohistochemistry. Vet. Pathol. 42, 405–426.

Ritchey, J.W., Black, D.H., Rogers, K.M., Eberle, R., 2006. In vivo experimentation with simian herpesviruses: assessment of biosafety and molecular contamination. J. Am. Assoc. Lab. Anim. Sci. 45, 7–12.

Romoser, W.S., Faran, M.E., Bailey, C.L., Lerdthusnee, K., 1992. An immunocytochemical study of the distribution of Rift Valley fever virus in the mosquito *Culex pipiens*. Am. J. Trop. Med. Hyg. 46, 489–501.

Sagripanti, J.-L., Hulseweh, B., Grote, G., et al., 2011. Microbial inactivation for safe and rapid diagnostics of infectious samples. Appl. Environ. Microbiol. 77, 7289–7295.

Swoveland, P.T., Johnson, K.P., 1979. Enhancement of fluorescent antibody staining of viral antigens in formalin-fixed tissues by trypsin digestion. J. Infect. Dis. 140, 758–764.

Tiwari, M., Parida, M., Santhosh, S.R., et al., 2009. Assessment of immunogenic potential of Vero adapted formalin inactivated vaccine derived from novel ECSA genotype of Chikungunya virus. Vaccine 27, 2513–2522.

Turell, M.J., Bailey, C.L., Beaman, J.R., 1988. Vector competence of a Houston, Texas strain of *Aedes albopictus* for Rift Valley fever virus. J. Am. Mosq. Control Assoc. 4, 94–95.

Turell, M.J., Dohm, D.J., Mores, C.N., et al., 2008. Potential for North American mosquitoes to transmit Rift Valley fever virus. J. Am. Mosq. Control Assoc. 24, 502–507.

Turell, M.J., Wilson, W.C., Bennett, K.E., 2010. Potential for North American mosquitoes (Diptera: Culicidae) to transmit Rift Valley fever virus. J. Med. Entomol. 47, 884–889.

USDA-APHIS, CDC, 2010. National Select Agent Registry, Available at: www.selectagents.gov (accessed 16.10.12).

U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Institutes of Health, 2009. Section IV – laboratory biosafety level criteria. In: Chosewood, L.C., Wilson, D.E. (Eds.), Biosafety in Microbiological and Biomedical Laboratories. , 5th ed. U.S. Government Printing Office, Washington, DC, pp. 30–59, Available at: www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm

Webster, J.D., Miller, M.A., DuSold, D., Ramos-Vara, J., 2009. Effects of prolonged formalin fixation on diagnostic immunohistochemistry in domestic animals. J. Histochem. Cytochem. 57, 753–761.

Werner, M., Chott, A., Fabiano, A., Battifora, H., 2000. Effect of formalin tissue fixation and processing on immunohistochemistry. Am. J. Surg. Pathol. 24, 1016–1019.