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

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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.

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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

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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