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No Evidence of rVSV-Ebola Virus Vaccine Replication or Dissemination in the Sand Fly *Phlebotomus papatasi*

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Abstract. Following vaccination with the live attenuated, recombinant vesicular stomatitis virus Indiana serotype Ebola virus (rVSV-EBOV) vaccine, persons may exhibit a transient vaccine-associated viremia. To investigate the potential for Old World sand flies to transmit this vaccine following feeding on a viremic person, we fed laboratory-reared *Phlebotomus papatasi* an artificial blood meal containing 7.2 $\log_{10}$ plaque-forming units of rVSV-EBOV. Replication or dissemination was not detected in the body or legs of any *P. papatasi* collected at seven ($n = 75$) or 15 ($n = 75$) days post-feed. These results indicate a low potential for rVSV-EBOV to replicate and disseminate in *P. papatasi*, a species whose geographic distribution ranges from Morocco to southwest Asia and as far north as southern Europe.

The largest Ebola virus (EBOV: family *Filoviridae*, genus *Ebolavirus*) outbreak in recorded history began in West Africa in December 2014. This outbreak resulted in more than 11,000 deaths and highlighted the critical need for an Food and Drug Administration-approved vaccine to interrupt virus transmission. One such candidate is the live, attenuated recombinant vesicular stomatitis virus Indiana serotype EBOV (rVSV-EBOV) vaccine.[1] This live attenuated vaccine is a recombinant VSV (family *Rhabdoviridae*, genus *Vesiculovirus*) based on the Indiana serotype which has been genetically engineered to express the transmembrane glycoprotein of EBOV.[2–7] Clinical trials demonstrated that the rVSV-EBOV vaccine was immunogenic and well-tolerated in healthy adults,[8–10] and this vaccine was used during the 2014–2016 EBOV disease outbreak in West Arica,[11,12] as well as the recent and current EBOV outbreaks in the Democratic Republic of Congo.

Following vaccination with rVSV-EBOV, some persons may develop a transient vaccine-associated viremia with peak levels of approximately 3.0 $\log_{10}$ plaque-forming units (PFU)/mL.[8,13] A previous study found no evidence of rVSV-ZEBOV replication or dissemination in *Aedes aegypti* (Linnaeus) or *Culex quinquefasciatus* Say (Diptera: Culicidae) fed with a high-titer, artificial blood meal; or detected replication in mosquito (*Ae. albopictus* [C6/36] or *Anopheles gambiae* Giles [4a4b]), midge *Culicoides variipennis* (Coquillett) (CuVa), or sand fly *Lutzomyia longipalpis* (Lutz and Neivai) (LL-5) cell lines.[14] However, sand flies and blackflies are the considered primary vectors of VSV-Indiana in nature.[15–19] Therefore, we assessed the susceptibility of *Phlebotomus papatasi* Scopoli (Diptera: Psychodidae), an anthropophilic sand fly known to transmit viruses within the VSV serogroup,[19–22] to become infected with rVSV-EBOV following consumption of an artificial infectious blood meal.

Seven-day-old, laboratory-reared *P. papatasi* sand flies maintained in colony since 2004 (Origin: Akbuk, Turkey)[23] were sugar-starved for 24 hours and then allowed to feed for 1 hour on an artificial blood meal composed of one part rVSV-EBOV to six parts defibrinated rabbit blood (LAMPIRE Biological Laboratories, Pipersville, PA) contained in glass membrane feeders (Lillie Glassblowers, Atlanta, GA) covered in chicken skins. A portion of the artificial blood meal was frozen at −80°C to perform back titration by plaque assay. Because of their delicate peritrophic membrane, which is susceptible to rupture from handling, sand flies were sorted 24 hours post-blood feed, and only those sand flies which were blood-engorged were retained.[23] Sand flies were then maintained in a 12:12-hour light/dark cycle at 25°C, 80% relative humidity, in a 30% sucrose solution via cotton ball for the duration of the experiment. An exemplar DNA barcode (658 bp of the mitochondrial deoxyribonucleic acid cytochrome c oxidase I gene) was used to confirm and document species identity (GenBank accession MH780862). This sequence shares 99.2% homology with the full mitochondrial genome of *P. papatasi* (GenBank accession KR349298).

On days 7 and 15 post-exposure, 75 flies were collected to test for the presence of stable or replicating rVSV-EBOV. This sample size achieved an 80% power to detect a difference ($P_1 - P_0$) of 0.0593 using a one-sided binomial exact test with a target significance level of 0.05 for each time point, assuming the population proportion under the null hypothesis (P_0) is 0.013. The upper 95% confidence limit of the population proportion was estimated using Hanley's rule of three ($1 - \text{maximum population proportion} = \sqrt[n]{0.05}$) for sand flies collected at both time points.[24] Sand flies were chilled to immobilize, then dissected, and homogenized (legs and body separately) in tubes containing a 5 mm stainless steel bead (QIAGEN, Hilden, Germany) and 500 µL of Dulbecco's modified Eagle medium (DMEM: Corning Life Sciences, Tewksbury, MA), supplemented with 20% volume/volume (v/v) fetal bovine serum (HyClone, GE Healthcare, Logan, UT), 50.0 µg/mL gentamicin (Gibco, Carlsbad, CA), and 0.5 mg/mL amphotericin B (Sigma Aldrich, St. Louis, MO) and frozen at −80°C. Before virus titration by plaque assay, tubes were thawed and homogenized using a QIAGEN TissueLyser II (QIAGEN) for 5 minutes at 26 Hz, after which they were centrifuged at 13,000 revolutions per minute for 10 minutes at 4°C, and supernatant was collected.

Titration of vaccine and sand fly supernatants was performed on Vero cells (CCL-81, ATCC, Manassas, VA) by

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plaque assay. Briefly, duplicate cell wells were inoculated with serial 10-fold dilutions of the supernatant prepared in DMEM, supplemented with 50.0 μg/mL gentamicin, 1.0 mM sodium pyruvate (Sigma Aldrich), 1% v/v nonessential amino acids (Sigma Aldrich), and 0.5 mg/mL amphotericin B (Sigma Aldrich). We also included positive and negative controls. Vaccine or sand fly supernatant was allowed to absorb for 1 hour at 37°C and was overlaid with 3.0 mL of 1% weight/volume Sea-Plaque agarose (Cambrex Bio Science, East Rutherford, NJ) in DMEM. Cells were incubated at 37°C (5% CO_2) for 2 days (vaccine plaque assay) and 4 days (sand fly supernatant plaque assay). Following incubation, cells were fixed using 4% formaldehyde (Fisher Scientific, Waltham, MA) in phosphate-buffered saline (phosphate buffered saline; Corning Life Sciences). After 24 hours, the overlay was removed, and cells were stained using 2% crystal violet (Sigma Aldrich) in 70% methanol (Sigma Aldrich). Plaques were counted, and the results were reported as the number of PFU/mL. The limit of detection of the plaque assay was 1.0 $\log_{10}$ PFU/mL.

Although the ecology of VSV-Indiana is somewhat obscure,[18,25] New World sand flies of the genus *Lutzomyia* (Diptera: Psychodidae) are considered a primary virus vector.[16,17,19] As EBOV is known to circulate in Africa, we used *P. papatasi* to model the potential for Old World sand flies of the genus *Phlebotomus* to transmit rVSV-EBOV after feeding on a person with vaccine-associated viremia. Following the feeding of *P. papatasi* on an artificial blood meal containing 7.2 $\log_{10}$ PFU of rVSV-EBOV, we did not detect infectious rVSV-EBOV in the body or legs of any sand fly collected at seven ($n = 75$) or 15 ($n = 75$) days post-feed. Given that the observed proportion was 0.0, the true proportion can be estimated with 95% confidence to be no greater than 0.039 based on Hanley's rule of three for sand flies collected at both time points.

The refractoriness of *P. papatasi* to an estimated per sand fly rVSV-EBOV dose ranging from 4.0 to 4.1 $\log_{10}$ PFU indicates a low potential for rVSV-EBOV vaccine replication and dissemination following feeding on a person who developed a moderate, post-vaccination viremia.[8,13,26] However, the possibility of infection and dissemination in *P. papatasi* following the oral feeding of a higher dose of rVSV-EBOV cannot be ruled out. Furthermore, the sand flies used in this experiment had biologically unaltered intact midguts, whereas in nature, the possibility exists that parasitic or arboviral infections could result in damage to the midgut barrier.[27–29] Therefore, future experiments are needed to evaluate the risk for vaccine replication in the salivary glands of *P. papatasi*.

Nonetheless, our results indicate the rVSV vaccine platform could potentially be used to develop vaccines for other viruses of medical and veterinary importance that circulate in regions where *P. papatasi* has been reported—Morocco to southwest Asia and as far north as southern Europe.[20,30,31]

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