Laboratory Vector Competence of Black Flies (Diptera: Simuliidæ) for the Indiana Serotype of Vesicular Stomatitis Virus

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ABSTRACT: In previous experiments we have demonstrated that colonized and wild black flies are competent laboratory vectors of different Mexican and Western USA isolates of vesicular stomatitis virus, serotype New Jersey (VSV-NJ). We have recently demonstrated biological VSV-NJ transmission by black flies using animal models. In the study described here, we tested the vector competence of colonized and wild black flies for the vesicular stomatitis virus, serotype Indiana (VSV-IN). A 1998 equine isolate was used. After a 10 day incubation period, saliva from experimentally infected *Simulium vittatum* and *S. notatum* was individually collected and tested for the presence of infectious virus. Virus was detected in the saliva of both species following oral infection, indicating that they are competent laboratory vectors of VSV-IN. In addition, the results suggest that the black fly gut may exert evolutionary pressures on the virus.

INTRODUCTION

Vesicular stomatitis (VS) is primarily a disease of cattle, horses, and swine caused by related viruses in the genus *Vesiculovirus*, family Rhabdoviridæ.[1] Two vesicular stomatitis virus (VSV) serotypes occur in Central and North America, VSV-Indiana (VSV-IN) and VSV-New Jersey (VSV-NJ). Both serotypes are enzootic in Mexico, Colombia, Venezuela, Panama, and Costa Rica.[2,3] The New Jersey serotype is enzootic on Ossabaw Island, Georgia and has been the predominant serotype associated with *recent* epizootics in the Western United States, with recent epizootics occurring in 1982–1983, 1984–1986, 1995, and 1997. Following 35 years of quiescence in the United States, epizootics of VSV-IN occurred in 1997 and 1998.

Despite years of intensive study, large portions of the epidemiology of VSV, including specific insect vectors, remain unclear. Ecological studies of VSV in Panama,[4,5] Costa Rica,[6] and other Central America countries[5] suggest that VSV-NJ and VSV-IN have similar but not identical transmission cycles in nature.

In Costa Rica, the prevalence in cattle of antibodies to the Indiana and New Jersey serotypes are 21 and 46%, respectively.[6] Vesicular stomatitis is regularly diagnosed in livestock at the end of the rainy season and transmission continues into the

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dry season. Older, lactating cows are at higher risk of disease than nonlactating old cows or calves.[6–8] Farm factors associated with an increased risk of VS include the presence of poultry and a long calving interval.[8] Variables related to breeding sites for sand flies and black flies were found to be associated with occurrence of VS in livestock. In addition, the risk of disease caused by the New Jersey serotype was found to be greater for cattle pastured in or near lower mountain moist forests (500–1,500 meters above sea level) and tropical dry forests (below two meters of annual rainfall and between sea level and 500 meters above sea level) than for cattle pastured at other regions.[6] No environmental factors were found to be associated with the Indiana serotype.

Several field and laboratory studies confirm that sand flies are enzootic vectors of both VSV serotypes. In Panama, VSV-IN has been isolated repeatedly from pools of mixed species of sand flies, *Lutzomyia spp.*, and from three pools of *L. trapidoi.*[9,10] Virus multiplication in and bite transmission by *L. trapidoi* has been demonstrated.[11] Transovarial transmission of VSV-IN in *L. trapidoi* and *L. ylephilator* has also been demonstrated.[12] On Ossabaw Island, Georgia, *L. shannoni* was shown to be the enzootic vector for VSV-NJ.[13] Experimental infection of *L. shannoni* by oral and intrathoracic inoculation resulted in virus replication by both routes.[14] Both orally and parenterally infected *L. shannoni* transmitted VSV-NJ by bite to susceptible rodents. In addition, field evidence[15] coupled with laboratory confirmation[14] demonstrated that transovarial transmission of VSV-NJ occurs in *L. shannoni.*

Information regarding the transmission of VSV during epizootics is based largely on limited observational and entomological studies conducted during the sporadic epizootics in the Western US. During epizootics, the New Jersey serotype has been isolated from biting Culicoides midges,[16,–18] mosquitoes,[19] black flies,[20–22] eye gnats,[23] and other non-biting diptera (mainly Muscid and Anthomyiid flies).[21] The Indiana serotype was isolated from mosquitoes (*Aedes* spp.) collected during the 1965 epizootic in New Mexico.[24]

Laboratory based studies investigating epizootic transmission of VSV are limited. The conclusions of experimental infections of *Aedes aegypti* with VSV were that *A. aegypti* is a competent laboratory vector of the Indiana serotype[25,26] but not of the New Jersey serotype.[26] Previous laboratory studies have shown that colonized[27,28] and wild[29] black flies are competent laboratory vectors of different isolates of VSV-NJ. Virus was recovered from the saliva of colonized *Simulium vittatum* and wild *S. notatum* following experimental infection with the Camp Verde and Oaxaca isolates of VSV-NJ. More recently, a mouse model was used to confirm the vectorial competence of *S. vittatum* for VSV-NJ.[18] The vector competence of black flies for VSV-IN has never been investigated.

The purpose of this study was to investigate the role of black flies in VSV-IN transmission. Experiments were designed to (1) determine the presence or absence of a salivary gland barrier (SGB) to VSV-IN in the black fly species used, and (2) determine if virus is present in the saliva of orally infected black flies following extrinsic incubation (EI).

MATERIALS AND METHODS

Virus Tested

The VSV-IN isolate (98-22488) used in this study was isolated from a horse during the 1998 epizootic. This isolate was obtained from the U.S. Department of Agriculture, National Veterinary Services Laboratory, Ames, Iowa and passed twice in Vero-M cells.

Black Flies Tested

Colonized *Simulium vittatum* (IS-7 cytotype, F_{130} generation) and field collected *S. notatum,* reared from pupae collected from Arivaipa Creek and the Gila River (Pinal Co., Arizona), were used in these experiments. Two- to three-day-old females were used.

Intrathoracic Inoculation

To determine if a salivary gland barrier existed to VSV-IN, flies were intrathoracically inoculated[30] with one microliter (µl) of a suspension containing $10^{6.3}$ to 10^7 plaque-forming units of VSV-IN (98-22488) per milliliter (pfu/ml). Infected black flies were held in individual snap cap tubes at 27°C for six days. After 10 days EI, saliva was collected by allowing individually tethered flies to feed into capillary pipettes containing 10 µl RPMI-1640 cell culture medium with 20% newborn calf serum and antibiotics.[30] Infected flies were then placed in individual 2-ml cryovials containing 1.0 ml of cell culture medium and antibiotics for virus isolation attempts. Individual saliva samples were diluted in 0.5 ml cell culture medium and antibiotics in 2-ml cryovials. Saliva and fly samples were frozen (−90°C) until evaluated for presence of virus.

Oral Infection

The next experiments were designed to determine if selected black fly species could be infected with VSV-IN (98-22488) *per os,* if the virus was maintained or replicated in the fly, and if the virus reached the salivary glands and was present in the saliva. *Simulium vittatum* females were infected using an *in vitro* feeding system.[31] *Simulium notatum* females were infected by first tethering them and then allowing each fly to individually feed into a capillary pipette containing a $10^{6.5}$ pfu VSV-IN (98-22488) per ml suspension. Infected flies were held for at least 10 days post feeding before saliva was collected (as previously described). Individual fly and saliva samples were then stored (as above) and evaluated for presence of virus.

Virus Reisolation and Titration

Three to six infected flies were used for virus titration immediately after intrathoracic inoculation or *per os* infection to obtain a baseline infection titer using a plaquing method described elsewhere.[32] Whole flies were ground in sterile glass grinders in 1.0 ml of cell culture medium containing an antibiotic solution of penicillin, streptomycin, and amphotericin B. The mixture was clarified by light centrifugation and

tenfold serial dilutions of the supernatant fluid were then used as the cell culture inoculum. Two 0.5-ml. portions of each dilution were aliquoted onto each of two wells of two-day-old confluent Vero-M cell culture monolayers in 24-well cell culture plates and incubated at 37°C for one hour. After removal of the inocula, the cells were overlaid with 2× Iscoves medium containing 2% newborn calf serum, 2% antibiotics, and 1% gum tragacanth. After incubating for six days at 37°C, cells were fixed by adding 20% formalin to the overlay, and then stained with 5% crystal violet. Plaques were counted and the virus titers expressed as plaque-forming units per fly (pfu/fly). This method reflects the amount of virus present per ml in the original fly suspension.

Saliva samples were evaluated for presence of virus by titration (as above) or by direct observation of cytopathic effects (CPE) of confluent Vero-M monolayers or both. These results are not a quantitative measure of the amount of virus in the saliva because the *in vitro* method for collecting saliva results in dilution and probably considerable swallowing of saliva and virus by the tethered fly. Thus, this method probably provides a low estimate of the total amount of virus actually in the saliva.[28]

RESULTS

Intrathoracic Inoculation of Black Flies

In the first trial, 45 *S. vittatum* and 36 *S. notatum* females were inoculated intrathoracically with VSV-IN (98-22488). Average baseline (T = 0) titers were $10^{3.72}$ pfu/fly (n = 6) for *S. vittatum* and $10^{2.85}$ pfu/fly (n = 3) for *S. notatum.* After 10 days EI, saliva was collected. Saliva samples and infected flies were then individually evaluated for presence of virus. Of the 30 surviving *S. vittatum,* all were positive for virus. The day 10 average virus titer was $10^{4.39}$ pfu/fly. Eight of 30 (26%) saliva samples were also positive. Titers ranged from 10 to $10^{3.2}$ pfu/ml. All 21 of the surviving *S. notatum* tested positive and the average virus titer was $10^{2.5}$ pfu/fly. None of the 21 (0%) saliva samples contained virus.

Twenty-five *S. notatum* were infected in a second trial. The average baseline titer was $10^{4.36}$ pfu/fly (n = 3). Of the 16 flies surviving to day 10 PI, all were positive for virus. The average virus titer was $10^{3.78}$ pfu/fly (n = 16). None (0/16) of the saliva samples contained virus.

Oral Infection of Black Flies

Simulium vittatum and *S. notatum* females were orally infected with VSV-IN (98-22488). Infected black flies were either immediately evaluated for baseline infection rates or held for at least 10 days before being evaluated for virus content.

Baseline infection titers were $10^{3.6}$ and $10^{2.85}$ pfu/fly for *S. vittatum* (n = 3) and *S. notatum* (n = 3), respectively. Twelve of 15 (80%) *S. vittatum* were positive for virus on day 10 PI. The average virus titer was $10^{3.27}$ pfu/fly. Four of 12 (33.3%) saliva samples contained virus. Six of 35 (17%) *S. notatum* contained virus on day 10 PI, the average virus titer was $10^{2.70}$ pfu/fly. Three of six (50%) saliva samples contained virus.

DISCUSSION

Ecological studies in VSV enzootic areas suggest that different transmission cycles exist for VSV-IN and VSV-NJ. Although it is generally accepted that sand flies, *Lutzomyia* sp., are important enzootic vectors of both virus serotypes, the epizootic vector(s) have not been fully identified. Virus isolations from field collected insects have identified several candidate vector species, however, experimental transmission studies are limited.

To date, vector competence studies have been reported for colonized and wild black flies for enzootic and epizootic strains of VSV-NJ, and for *Aedes aegypti* for VSV-NJ[26] and VSV-IN.[25,26] Virus was recovered from the saliva of colonized *Simulium vittatum*[27,28] and wild *S. notatum*[29] following experimental infection with the Camp Verde and Oaxaca isolates of VSV-NJ. More recently, a mouse model was used to confirm the vectorial competence of *S. vittatum* to VSV-NJ.[18] The objective of this present study was to investigate the vector competence of colonized and wild black flies to VSV-IN.

The results demonstrate that black flies are competent vectors of VSV-IN under laboratory conditions. Virus was detected in the saliva of a high percentage of *S. vittatum* following intrathoracic and oral infection, and in the saliva of *S. notatum* following oral infection but not following intrathoracic inoculation.

Barriers to virus infection in arthropods have been described by Hardy *et al.*,[33] and include the mesenteronal infection barrier, the mesenteronal escape barrier, and the salivary-gland infection barrier. A fourth barrier, the salivary-gland escape barrier, is described as one that prevents a virus infected arthropod from transmitting the virus when blood feeding.

The absence of VSV-IN (98-22488) in the saliva of *S. notatum* following intrathoracic inoculation indicates the presence of a salivary-gland escape (SGE) or an infection (SGI) barrier in this black fly to VSV-IN. However, *S. notatum* was shown to be susceptible to oral infection of VSV-IN (98-22488). Seventeen percent of black flies contained virus after 10 days EI and virus was detected in the saliva of a high percentage (50%) of those black flies. This suggests that viral adaptation to selection pressures within the gut may occur. Changes in VSV fitness due to different environmental conditions provided in insect and mammalian cell culture systems have been reported.[34] The black fly midgut may act in a similar manner and may drive virus evolution.

A higher proportion of *S. vittatum* were susceptible to VSV-IN oral infection compared to *S. notatum.* Eighty percent of *S. vittatum* were contained virus 10 days after oral infection, whereas only 17% of orally infected *S. notatum* contained virus. These differences are similar to those seen in black fly infection with VSV-NJ, and are consistent with the differences in infection susceptibility reported for different insect species of the same genus experimentally infected with the same arbovirus.[35]

In summary, this study demonstrates that wild and colonized black flies readily ingest VSV-IN and that virus is present in the saliva in a large percentage of susceptible black flies. This study also adds to the growing body of knowledge that suggests that insects, including black flies, are responsible for transmitting VSV to livestock during epizootics.

ACKNOWLEDGMENTS

This research was supported through funds from a cooperative agreement with the United States Department of Agriculture, Cooperative State Research, Education, and Extension Service.

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