

Bite Transmission of Vesicular Stomatitis Virus (New Jersey Serotype) to Laboratory Mice by *Simulium vittatum* (Diptera: Simuliidae)

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ABSTRACT Laboratory-reared female black flies (*Simulium vittatum* Zetterstedt) were infected experimentally with a 1997 vesicular stomatitis virus New Jersey serotype isolate and allowed to feed on susceptible laboratory mice. All mice exposed to black fly bite seroconverted by day 21 after infection, an indication of virus transmission. In addition, viral RNA was detected in the spleen of several mice. These findings are consistent with the hypothesis that black flies are involved in VSV-NJ transmission during epizootics in the western USA and represent the 1st confirmed example of biological transmission of an arbovirus by a member of the Simuliidae using an animal model.

KEY WORDS *Simulium*, black fly, vesicular stomatitis virus, biological transmission, vector, epizootic

VESICULAR STOMATITIS (VS) is a disease that primarily affects cattle, horses, sheep, goats, and swine, causing vesicular lesions on the mouth, coronary bands, and teats (Murphy et al. 1994). The causative agents of VS are a group of antigenically related, but distinct, viruses of the genus *Vesiculovirus*, family Rhabdoviridae. In the United States, both enzootic and epizootic forms of VS occur. Vesicular stomatitis virus New Jersey serotype (VSV-NJ) is enzootic on Ossabaw Island, GA (Comer et al. 1990 1992), and it is the serotype most often associated with the recurring and unpredictable epizootics in the western United States.

Despite many decades of research, the mechanism for VSV-NJ transmission during epizootics is not fully understood and remains controversial. This virus has been isolated from field-collected insects on at least 40 occasions, and insects are thought to play a role in virus dissemination. *Lutzomyia shannoni* Dyar has been identified as the vector of VSV-NJ on Ossabaw Island, GA (Comer et al. 1990 1992), but the insect vector(s) of VSV-NJ in the western United States, where the most recent epizootics have occurred, has not been identified unambiguously.

Barnett (1960) outlined the basic criteria for incriminating an arthropod species as a vector of the causal agents of disease. Briefly summarized, these include the following: (1) demonstration of feeding or other effective contact with the host under natural conditions, (2) a biological association in time or space of the suspected arthropod species and occurrence of clinical or subclinical infection in the host, (3) repeated demonstrations that the arthropod, under natural conditions, harbors the pathogen in the infective state, and (4) transmission of the pathogen under

controlled conditions (Barnett 1960, James and Harwood 1969).

Based on the above criteria, black flies are likely candidates for VSV-NJ transmission during epizootics. Many species of black flies are known pests of livestock (Crosskey 1981), and VS cases often occur near riverine habitats favored by black flies (Hanson 1952). Furthermore, black flies and black fly larval habitats were identified as risk factors associated with clinical VS on dairy farms in Costa Rica (VanLeeuwen et al. 1995). Vesicular stomatitis virus (New Jersey) has been isolated from black flies on 2 separate occasions. Theiler and Downs (1973) reported the isolation of VSV-NJ from *Simulium exiguum* Roubaud collected near Medellin, Colombia. More recently, VSV-NJ was isolated from 2 mixed pools of *Simulium vittatum* Zetterstedt and *S. bivittatum* Malloch collected in Colorado during the 1982–1983 VSV-NJ epizootic (Francy et al. 1988). Several laboratory studies corroborated these field findings by demonstrating that colonized and wild black flies are competent laboratory vectors of different isolates of VSV-NJ (Maré et al. 1991, Cupp et al. 1992, Mead et al. 1997).

The current study further examined the role of black flies in VSV-NJ transmission. The experimental design allowed black flies experimentally infected with VSV-NJ to feed on susceptible laboratory mice. The development of VSV-NJ neutralizing antibodies in the mice, in the presence or absence of illness, was considered to be evidence of virus transmission.

Materials and Methods

The VSV-NJ isolate used for this study was isolated by the authors from *Culicoides* sp. collected along the Rio Grande River near Belen, NM, before the 1st

The use of animals for this study was approved by the University of Arizona's Animal Care and Use Committee (control #97-096).

Table 1. Serum virus neutralizing antibody titers of mice fed upon by VSV-NJ infected black flies and of control mice

| Mouse no. | No. of flies feeding | Serum antibody level | | | | Viral RNA detection (organ) |
|-----------|----------------------|----------------------|----------------------|---------|----------|-----------------------------|
| | | 0 | Days after infection | | | |
| | | | 7 | 13 | 21 | |
| 1 | 1 | <1:4 | 1:1,024 | 1:2,048 | 1:2,048 | Yes (spleen) |
| 2 | 1 | <1:8 | 1:512 | 1:1,024 | 1:1,024 | Yes (spleen) |
| 3 | 2 | <1:4 | 1:32 | 1:64 | 1:128 | Yes (spleen) |
| 4 | 3 | <1:4 | 1:64 | 1:64 | 1:64 | No |
| 5 | 3 | 1:4 | 1:32 | 1:64 | 1:512 | No |
| 6 | 2 | <1:4 | 1:256 | 1:512 | 1:2,048 | Yes (spleen) |
| 7 | 2 | 1:2 | 1:32 | 1:512 | 1:1,024 | No |
| 8 | 2 | <1:2 | 1:64 | 1:256 | >1:2,048 | No |
| 9 | 1 | <1:8 | 1:256 | 1:1,024 | 1:1,024 | No |
| 10 | 2 | 1:4 | 1:512 | >1:2048 | >1:2048 | Yes (spleen) |
| 11 | 4 | <1:4 | 1:128 | 1:1,024 | 1:1,024 | Yes (spleen) |
| 12 | 2 | <1:2 | 1:64 | 1:256 | 1:256 | Yes (spleen) |
| 13 | NA | <1:8 | 1:256 | 1:512 | >1:2048 | Yes (spleen) |
| 14 | NA | <1:4 | 1:64 | 1:256 | 1:1,024 | Yes (spleen) |
| 15 | NA | <1:4 | <1:4 | <1:4 | <1:4 | No |
| 16 | NA | <1:4 | <1:4 | <1:4 | <1:4 | No |

Mice 1–12 were fed upon by VSV-NJ infected black flies, mice 13–14 were injected IM with 200 μ l VSV-NJ, and mice 15–16 were not infected. NA, not applicable.

reported 1997 animal case of VS. The virus was plaque purified and passed twice in Vero-M cells.

One- to 2-d-old *S. vittatum* females (IS-7 cytotyp) from the F₁₃₂ generation of a continuous laboratory colony (Bernardo et al. 1986) were used for this experiment. Previous studies have shown that a midgut barrier to virus dissemination does not exist in *S. vittatum* (Maré et al. 1991, Cupp et al. 1992). For this reason, female black flies were infected via intrathoracic inoculation with 1 μ l of a suspension containing 5.0×10^6 plaque forming units (pfu) /ml of VSV-NJ. Six flies were titrated immediately after injection to determine the baseline infection titer using a previously described plaquing method (Maré and Graham 1973). The remaining infected black flies were held in individual 5-ml snap cap tubes in an incubator at 30°C for 7 d.

Six-week-old female mice (ICR strain Harlan Sprague Dawley) were anesthetized (i.p. injection with a Ketamine/Xylazine mixture, Lloyd Labs, Shenandoah, IA) and the complete ventral side shaved. A baseline serum sample was collected, by tail-clip, from each mouse at day 0 and screened for neutralizing antibodies specific for VSV-NJ using microtiter serum neutralization. The mice were allocated randomly into 1 of 3 groups; group 1 consisted of 2 mice that were injected IM with 0.2 ml of a VSV-NJ suspension (4.5×10^6 pfu/ml), group 2 consisted of 2 mice that were not exposed to VSV-NJ, and group 3 consisted of 12 mice that were fed upon by VSV-NJ infected black flies.

After anesthesia, infected black flies (up to 2 at a time) were placed onto the shaved area of each mouse in group 3. When a fly fed to repletion or showed no interest in feeding, it was removed from the mouse and replaced with a new black fly.

Mice were housed in micro-isolation cages (4 mice per cage) at 22°C and were observed daily for the development of any clinical signs. Exposed and non-exposed mice were housed together to determine if

virus was transmitted from mouse to mouse during cage contact. Blood samples (25 μ l) were collected from each mouse for the first 4 d after infection to detect viremia by virus reisolation in tissue culture. In addition, serum samples were collected at 7, 13, and 21 d after infection and screened for the presence of VSV-NJ neutralizing antibodies using microtiter serum neutralization. Mice were euthanized on day 21 (cervical dislocation under anesthetics) and tissues (brain, spleen, kidney, liver, heart, and lung) were collected for virus reisolation in tissue culture or for viral RNA detection using RT-polymerase chain reaction (Rodriguez et al. 1993).

Results

Base-line infection titers in VSV-NJ inoculated black flies ranged from 2.8×10^2 to 5.6×10^3 pfu/fly (mean = 2.8×10^3). After the 7 d extrinsic incubation period, as many as 4 separate infected flies were allowed to feed on an individual mouse (mean, 2 flies per mouse; range, 1–4 flies per mouse) (Table 1). Fly feeding times varied from a few minutes to >30 min.

Base line titers (day 0) of VSV-NJ neutralizing antibodies present in each mouse ranged from 1:4 to <1:2 (Table 1). Neutralizing antibody titers rose with time in all of the mice except for those in group 2. These mice were not infected with VSV-NJ, but were housed in the same cages with mice from groups 1 and 3. At necropsy (day 21 after infection), neutralizing antibody levels in mice from groups 1 and 3 ranged from 1:64 to >1:2,048, whereas the neutralizing antibody titers in mice from group 2 remained at <1:4. None of the mice developed clinical signs during the 21-d observation period.

Viremia was not detected in blood collected from mice on days 1–4 after infection, nor was infectious virus recovered from any of the tissues harvested at necropsy. However, viral RNA (phosphoprotein gene RNA) was detected in the spleens of 7 group-3 mice

and in both group-1 mice, but not in either of the group-2 mice.

Discussion

To incriminate and demonstrate that an arthropod species may be a competent vector of a specific arbovirus, certain conditions promulgated by Barnett (1960) and later refined by the World Health Organization (1967) must be addressed.

Accumulating field and laboratory evidence indicates that black flies are involved in the transmission of VSV-NJ during epizootics in the western United States. During the 1995 and 1997 outbreaks, several hundred black flies could be collected as they fed off of individual horses or cows on premises with confirmed VS (D.G.M., unpublished data). In addition, black flies were collected in CO₂ traps from every premises investigated by the authors during the 1995 and 1997 epizootics. These observations, which confirm the association between black flies and livestock in epizootic areas, coupled with the previous association of VSV-NJ and black flies (Theiler and Downs 1973, Franczy et al. 1988) and with the previous laboratory studies demonstrating that black flies are competent laboratory vectors of VSV-NJ (Maré et al. 1991, Cupp et al. 1992, Mead et al. 1997), are further evidence for the role of black flies in VSV-NJ dissemination during epizootics. The combination of these field observations and laboratory studies satisfy the criteria set forth by Barnett (1960) to incriminate black flies as vectors of VSV-NJ.

Although the previous laboratory studies were necessary in determining the vector competence of black flies, they did not take into account specific and non-specific host defense factors that may affect pathogen transmission. In the current study, VSV-NJ infected black flies were allowed to feed on laboratory mice to determine if they were able to transmit VSV-NJ by bite to susceptible hosts. All of the mice fed upon by VSV-NJ infected black flies developed neutralizing antibodies specific for VSV-NJ. Neutralizing antibody titers increased from an average of 1:4 on day 0 to an average >1:1,024 on day 21 after infection in those mice exposed to black fly bite and from an average of <1:6 on day 0 to an average >1:1,500 in the mice injected with VSV-NJ. The presence of high titers of neutralizing antibodies in convalescent sera previously has been used as an indication of viral infection in experimental animals (Fultz et al. 1982, Fultz and Holland 1985, Barrera and Letchworth 1996). Viral RNA was detected in the spleen of several of the infected mice, another indication of virus transmission. Barrera and Letchworth (1996) suggest that this organ is involved in VSV-NJ clearance.

Neither neutralizing antibodies nor viral RNA was detected in the mice from group 2, indicating that virus was not transmitted from mouse to mouse by direct contact and that the results seen in the group 3 mice were caused by the bite or bites of infected black flies.

Three of the group 3 mice were bitten by a single infected black fly (Table 1). The neutralizing antibody titer in these mice on day 21 after infection was as high or higher than in the mice bitten by 2 or more flies, as well as in those mice injected IM with VSV-NJ. Therefore, a bite by a single VSV-NJ infected black fly can transfer sufficient virus to cause an infection.

Previous studies have shown that the amount of virus present in the saliva of a VSV-NJ infected black fly varies considerably. Virus titers between 3×10^1 and 1×10^4 pfu/ml and between $1 \times 10^{2-3}$ and 1×10^4 pfu/ml were found in the saliva of experimentally infected *S. vittatum* (Maré et al. 1991, Cupp et al. 1992) and *S. notatum* (Mead et al. 1997), respectively. Because of the in vitro method used to collect the saliva in these experiments, these numbers probably represent a low estimate of the total amount of virus actually present in the saliva.

The results of the current study confirm that *S. vittatum* is a competent biological vector of VSV-NJ by meeting the conditions addressed by Barnett (1960) and the World Health Organization (WHO 1967), and are the first confirmed example of biological transmission of an arbovirus by a member of the Simuliidae using an animal model.

Several questions remain regarding the epizootology of VSV-NJ in the western United States. For example, the WHO defines a vector as an arthropod (invertebrate host) that transmits the virus from one vertebrate host to another by bite. This has never been demonstrated for VSV-NJ. Although *S. vittatum* are capable of bite transmission, it still is not known where the virus is acquired in nature. Large gaps remain in our knowledge of the transmission and maintenance cycle(s) of VSV-NJ in the western United States.

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