ORAL TRANSMISSION OF JAMESTOWN CANYON VIRUS BY *AEDES PROVOCANS* MOSQUITOES FROM NORTHEASTERN NEW YORK

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ABSTRACT. *Aedes provocans* were allowed to feed on a bloodmeal containing 5.6 $\log_{10}$ $TCID_{50}$/ml of Jamestown Canyon (JC) virus. After 14 days of incubation at 21°C and 80% RH, 100% (36/36) were midgut infected, 50% (18/36) developed disseminated infections and 50% (9/18) of the latter specimens transmitted virus to capillary tubes. When mosquitoes were intrathoracically inoculated with 6.1 $\log_{10}$ $TCID_{50}$/ml of JC virus, 100% (40/40) became disseminated infected and 95% (38/40) transmitted virus after 12 days of incubation. A midgut escape barrier was recognized as the major barrier to JC virus transmission in orally infected *Ae. provocans*.

INTRODUCTION

During the past 20 years, annual surveys of human and mosquito populations for arboviral infections have revealed that Jamestown Canyon (JC) virus, a member of the California serogroup, is the most widespread arbovirus in New York (Grayson et al. 1983). This virus was isolated most frequently from the *Aedes communis* complex, a group of closely related, morphologically similar mosquito species.

A recent study from New York reported 17 JC virus isolates and a minimum field infection rate (MFIR) as high as 1:219 from *Aedes provocans* (Walker), a member of the *Ae. communis* group; MFIRs as high as 1:38 were observed on individual days from human-baited field collections (Boromisa and Grayson 1990). Evidence of transovarial transmission (TOT) of JC virus from 2 pools of insectary-reared adult male *Ae. provocans* was also obtained. A study conducted in northern Michigan revealed 14 isolates of JC virus from *Ae. provocans* (MFIR 1:27–1:714), and a number of JC virus isolates were also obtained from sentinel deer blood as early as 1 wk after the first *Ae. provocans* were collected (Heard et al. 1990). Collectively, these findings implicated *Ae. provocans* as a potential vector of JC virus.

This study was done to determine if orally infected and intrathoracically inoculated *Ae. provocans* from northeastern New York could transmit JC virus. This paper also includes a discussion of barriers to JC virus infection in *Ae. provocans*.

MATERIALS AND METHODS

Field and insectary techniques: Mosquitoes were collected at a study site in Saratoga County, NY, where JC virus-infected adult *Ae. provocans* were previously collected (Boromisa and Grayson 1990). Pupae were collected on May 4 and May 9, 1989, and reared to the adult stage as previously described (Boromisa and Grayson 1990). Adult female mosquitoes were chilled on wet ice, placed in a wet ice chilled glass staining dish and anesthetized by placing a very small piece of dry ice on a 5-mm-thick pad of paper toweling inside the dish. Female *Ae. provocans* were identified (Means 1979) and transferred to small aluminum cages (15.3 cm) provisioned with 10% sucrose.

Adult female mosquitoes were collected with a battery-powered aspirator (Nasci 1981) at the study site on May 25 and June 1, 1989, held on wet ice and transported to the insectary. The mosquitoes were anesthetized, identified and handled as described above.

Infection of mosquitoes: Bloodfeeding was done using a membrane feeding apparatus (Rutledge et al. 1964) with a Baudruche membrane (Joseph Long Co., Belleville, NJ) containing 15 ml of a warm (37°C) mixture of defibrinated rabbit blood and JC virus. The JC virus strain (8835024) used in these experiments was obtained from adult female *Ae. provocans* mosquitoes collected at the study site (Boromisa and Grayson 1990) and had been passaged once in African green monkey kidney (Vero) cell culture (original isolate). A sample of each blood-virus mixture was taken before and after each bloodfeeding, frozen at −70°C, and later titrated in 16 × 125 mm Vero cell culture tubes.

There were 2 groups of insectary-reared 5 to 7-day-old mosquitoes collected as pupae on May 9 that were orally infected by feeding on viremic blood meals; there were 191 mosquitoes in group ORAL-A and 147 mosquitoes in group ORAL-B. One week later, 161 field-collected mosquitoes (ORAL-C), captured on May 25 (>10 days old), were allowed to feed on a viremic blood meal. To obtain the maximum number of infected specimens, the nonbloodfed mosquitoes from viremic bloodfeedings were used as control mosquitoes.

The fully engorged and control mosquitoes were transferred to clean small cages (15.3 cm) provisioned with 10% sucrose and a plastic petri dish containing a water-soaked cotton pad. No cage contained more than 52 specimens and the sucrose was changed every 2 days. The cotton pad was kept wet throughout the incubation period. All cages were covered with plastic wrapping, leaving only the fronts of the cages uncovered. The cages were held in an environmental cabinet (Forma Scientific, Inc., Model 3940, Marietta, OH) at 21°C and 80% RH for 12 or 14 days.

Intrathoracic inoculation of 0.34 μl of JC virus diluted in 7.5% bovine plasma albumin (BPA) into adult female *Ae. provocans* was done through the pleural membranes of the thorax (Rosen and Gubler 1974). Two groups of mosquitoes were inoculated: the first (INOC-A) consisted of 100 insectary-reared 5 to 7-day-old mosquitoes, collected as pupae on May 4, and the second (INOC-B) consisted of 98 field-collected 10 to 14-day-old mosquitoes collected on June 1. The mosquitoes were chilled and anesthetized with dry ice vapor before intrathoracic inoculation. A small sample of the inoculum was taken at the beginning of the procedure, stored at −70°C, and later titrated in Vero cell culture. To serve as uninfected control specimens, mosquitoes from the May 4 and June 1 field collections were inoculated with 0.34 μl of BPA. After inoculation, the mosquitoes were caged, held as described above and incubated in the environmental cabinet for 12 days.

For each of the groups of mosquitoes infected, a field-cohort was processed for virus isolation to determine if there was an initial level of JC virus infection. A description of each field-cohort is as follows: May 4 (INOC-A), 253 specimens (14 pools); May 9 (ORAL-A & B), 94 specimens (4 pools); May 25 (ORAL-C), 243 specimens (10 pools); and June 1 (INOC-B), 140 specimens (10 pools). A detailed description of laboratory procedures for the isolation and identification of JC virus was presented by Boromisa and Grayson (1990).

Oral transmission: An *in vitro* method for demonstrating virus transmission in capillary tubes was developed by modifying several earlier techniques (Boromisa et al. 1987, Cornel and Jupp 1989). The end of the capillary tube containing the salivation medium (50% fetal bovine serum (FBS) diluted in 10% sucrose) was dipped into mineral oil and 1 mm of oil was allowed to rise up into the tube, directly underneath the layer of salivation medium. The proboscis of a mosquito was inserted into the capillary tube so that the mouthparts were in the salivation medium and the head was firmly secured at the end of the capillary tube by the viscosity of the mineral oil. The mosquitoes were removed from the capillary tubes after 45 min or until ingestion of the salivation medium was observed and stored at −70°C until assayed for virus infection. The capillary tubes were rinsed by pipetting up and down 3 times in 0.15 ml of BPA with antibiotics (100 units penicillin and 100 μg streptomycin/ml) in 12 × 75 mm glass tubes using a 1-ml syringe and latex rubber hose. The glass tubes containing the salivation media were frozen and stored at −70°C until assayed for virus.

Virus assays: Disseminated infection (DI), i.e., infection beyond the midgut, was determined by an indirect immunofluorescent antibody (IFA) head squash technique using JC virus immune mouse ascitic fluid produced in the laboratory and a goat anti-mouse IgG conjugate as previously described (Boromisa and Grayson 1990). Midgut infection (MI), i.e., nondisseminated infection, was determined by triturating each IFA head squash negative mosquito body in a 1-dram glass vial with 5 glass beads (4 mm) and 1 ml of BPA with antibiotics using a vortex mixer. The contents were transferred to small sterile plastic tubes and centrifuged at 1,500 rpm for 30 min at 4°C. The supernatants were transferred to fresh tubes and frozen at −70°C for virus isolation attempts. Mosquitoes with midgut escape barriers (MEB) were those midgut infected mosquitoes with nondisseminated infections as demonstrated by negative IFA head squashes.

Oral transmission was demonstrated by inoculating Vero cell culture tubes with 0.1 ml of salivation media from capillary tubes (diluted in BPA) and testing for the presence of virus. Virus infected Vero cell cultures from both nondisseminated infected mosquitoes and oral transmission assays were harvested and tested for JC virus using an IFA identification method (Boromisa and Grayson 1990).

Salivation media were titrated to determine how much JC virus was transmitted to the capillary tubes during oral transmission. Equal numbers of JC virus transmitting ORAL-C, INOC-A and INOC-B mosquitoes were selected at random for comparison. All specimens to be titrated were diluted in cell culture maintenance media (Eagle's minimum essential media (MEM) with 2% FBS and antibiotics (100 units of penicillin, 100 μg of streptomycin and 50 units of mycostatin/ml) and 0.1 ml of each 10-fold dilution (4 replications per dilution) was inoculated into each Vero cell culture tube. The culture tubes were incubated for 1 h at 37°C before 1.5 ml of cell culture medium was added to each tube. The culture tubes, which were incubated for 7 days, were examined daily for cytopathic effects (CPE). The $TCID_{50}$ endpoints were es-

timated using the method of Reed and Muench (1938).

Statistics: A chi-square (χ^2) contingency table analysis with 1 d.f. and Yates' correction for continuity was used to determine if there were significant differences between rates of infection and transmission. A one-way analysis of variance (ANOVA) was used to determine if there were significant differences between salivation medium titers (Steel and Torrie 1980).

RESULTS

Bloodfed mosquitoes: The results of JC virus infection and oral transmission experiments with orally infected (ORAL) *Ae. provocans* are shown in Table 1. Due to the high rates of mosquito mortality observed on the 12th day of incubation in both bloodfed and control specimens, the ORAL-A and B mosquitoes could not be incubated for a longer period of time. The ORAL-C mosquitoes were able to be incubated an additional 2 days before high rates of mosquito mortality were observed.

The survival rates of the bloodfed and control ORAL-A mosquitoes were 40% (36/91) and 32% (13/40), respectively. After feeding on a blood meal containing 4.2 $\log_{10}$ $TCID_{50}$/ml of JC virus, 11% (4/36) developed a MI with 25% (1/4) of them developing DIs. Oral transmission was not attempted with ORAL-A mosquitoes.

The survival rates of the bloodfed and control ORAL-B mosquitoes were 19% (20/103) and 25% (10/40), respectively. The surviving mosquitoes were lethargic, unresponsive and showed a lack of interest in refeeding when tempted with a human host. Twelve days after feeding on a blood meal containing 5.4 $\log_{10}$ $TCID_{50}$/ml of JC virus, 85% (17/20) of the ORAL-B mosquitoes had a MI with 47% (8/17) of them developing a DI. The mosquito head squashes, used in determining the rate of DI, showed that only 2 of the 8 specimens had a fully developed DI, as demonstrated by strong (4+) IFA reactions; the 6 weakly positive specimens were recorded as 1+ IFA reactions. None of the ORAL-B mosquitoes with a DI transmitted JC virus to the capillary tubes (0%, 0/8). The rates of MI in the ORAL-B mosquitoes were significantly different ($\chi^2 = 26.88$; $P < 0.001$) from those in the ORAL-A mosquitoes. None of the 94 adult female *Ae. provocans* field-cohorts (4 pools) from ORAL-A and ORAL-B mosquitoes were found to be naturally infected with JC virus when processed for virus isolation.

The survival rates of the ORAL-C bloodfed and control mosquitoes were 38% (36/96) and 40% (16/40), respectively. Fourteen days after feeding on a blood meal containing 5.6 $\log_{10}$ $TCID_{50}$/ml of JC virus, 100% (36/36) of the ORAL-C mosquitoes had a MI, 50% (18/36) developed a DI, and 50% (9/18) of the latter specimens transmitted JC virus to the capillary tubes. The IFA head squashes showed that 16 of the 18 positive specimens had strong (4+) IFA reactions. This demonstrated that the ORAL-C mosquitoes had a higher rate of fully developed DIs than that observed for the ORAL-B mosquitoes; the 2 moderately positive specimens were recorded as 2+ IFA reactions. The rates of MI between the ORAL-A and ORAL-C mosquitoes were significantly different ($\chi^2 = 54.06$; $P < 0.001$) as were the rates of oral transmission between ORAL-B and ORAL-C mosquitoes ($\chi^2 = 4.76$; $P < 0.05$). No other comparisons between ORAL-A, B, and C mosquitoes were significant ($P > 0.05$). Jamestown Canyon virus was not isolated from an ORAL-C field-cohort of 243 adult female *Ae. provocans* (10 pools).

Inoculated mosquitoes: The incubation period was ended at 12 days when high rates of mosquito mortality were observed for the inoculated mosquitoes. The surviving 32 specimens (32%, 32/100) of INOC-A mosquitoes were allowed to feed from capillary tubes containing salivation medium. The INOC-A control specimens had a similar survival rate of 28% (7/25). These mosquitoes were similar to the ORAL-B mosquitoes in that they may have been too physiologically

Table 1. Results of Jamestown Canyon (JC) virus transmission trials with orally infected (ORAL) and intrathoracically inoculated (INOC) *Aedes provocans*.

Group	Titer**	Percent infection and transmission*		
		MI	DI	TRANS
ORAL-A	4.2	11(4/36)	25(1/4)	ND
ORAL-B	5.4	85(17/20)	47(8/17)	0(0/8)
ORAL-C+	5.6	100(36/36)	50(18/36)	50(9/18)
INOC-A	6.0	—	100(32/32)	75(24/32)
INOC-B	6.1	—	100(40/40)	95(38/40)

* Midgut infection (MI); disseminated infection (DI); transmission (TRANS); not done (ND).
** $\log_{10}$ $TCID_{50}$/ml of JC virus blood meal or inoculum.
\+ Incubated for 14 days; all other groups incubated for 12 days.

stressed for demonstrating accurate oral transmission results. All of the INOC-A mosquitoes (100%, 32/32) had a DI as demonstrated by the IFA head squash technique and 75% (24/32) transmitted JC virus to the capillary tubes (Table 1). None of the 253 adult female *Ae. provocans* (14 pools) in the INOC-A field-cohort was found infected with JC virus.

The INOC-B mosquitoes experienced a 41% (40/98) survival rate through 12 days of incubation. The INOC-B control specimens had a survival rate of 48% (12/25). Identical results to that of the INOC-A mosquitoes were noted for the INOC-B specimens except that 95% (38/40) transmitted virus to capillary tubes (Table 1). The rates of oral transmission between the INOC-A and INOC-B mosquitoes were significantly different ($\chi^2 = 4.39$; $P < 0.05$). There were 2 JC virus isolates from the INOC-B field-cohort of 140 adult female *Ae. provocans* (10 pools).

Salivation medium titers: There was no significant difference in mean salivation medium titer between the JC virus transmitting ORAL-C ($\bar{x} = 4.8 \pm 1.9$ $\log_{10}$ $TCID_{50}$/ml; range = 2.0–8.0 $\log_{10}$ $TCID_{50}$/ml; n = 9), INOC-A ($\bar{x} = 3.8 \pm 0.9$ $\log_{10}$ $TCID_{50}$/ml; range = 3.0–5.5 $\log_{10}$ $TCID_{50}$/ml; n = 9), and INOC-B ($\bar{x} = 5.4 \pm 1.4$ $\log_{10}$ $TCID_{50}$/ml; range = 4.0–8.0 $\log_{10}$ $TCID_{50}$/ml; n = 9) mosquitoes ($F = 2.84$; $P > 0.05$).

DISCUSSION

Aedes provocans has been shown to become infected with and orally transmit JC virus (Table 1). Midgut infection of *Ae. provocans* was shown to be dependent upon the dose of JC virus given in viremic blood meals and length of incubation. All *Ae. provocans* developed a MI within 14 days after consuming a blood meal containing 5.6 $\log_{10}$ $TCID_{50}$/ml of JC virus.

A midgut escape barrier (MEB) played a significant role in preventing the development of disseminated infections. There were no significant differences in the rates of DI between orally infected mosquitoes by either increasing the JC virus titer in the blood meals or lengthening the incubation time to 14 days (Table 1). After 12 days of incubation, only 2 of 8 JC virus positive mosquito head squashes, used in determining the rate of DI, for the ORAL-B mosquitoes had strong (4+) IFA reactions. This suggested that 12 days of incubation was insufficient for the complete development of DI and that this, rather than the physiologically stressed condition of the ORAL-B mosquitoes, was most likely responsible for their lack of oral transmission. In contrast, the head squashes used in determining DI for the ORAL-C mosquitoes showed 16 of the 18 positive specimens to have very strong (4+) IFA reactions after 14 days of incubation. If the development of DIs was incomplete in the ORAL-C mosquitoes, we should have observed a number of weak (≤1+) IFA reactions. We feel that the 50% rate of DI observed in the ORAL-C mosquitoes may have been representative of *Ae. provocans* and that an incubation period greater than 14 days would not have changed this rate significantly.

The inoculated *Ae. provocans* demonstrated that once the MEB was bypassed, 100% of the INOC-A and B mosquitoes developed disseminated infections. We believe that the significant difference in the rate of JC virus transmission between the INOC-A and B mosquitoes was most likely the result of poor refeeding on the salivation medium by the physiologically stressed INOC-A mosquitoes and not the presence of salivary gland infection or escape barriers; the INOC-B mosquitoes transmitted JC virus at a rate of 95% (38/40). Although there were 2 JC virus isolates from the INOC-B mosquito field-cohort, it was not thought that this level of natural infection would have made any difference in the results considering the very high rates of virus infection and transmission observed. The intrathoracic inoculation data of the INOC-B mosquitoes do suggest that if there were any barriers to virus transmission in specimens with DIs, that the rates of such barriers were probably low and that once *Ae. provocans* developed a fully DI, it was capable of transmitting JC virus.

Once an ORAL-C mosquito transmitted JC virus, it seemed to be able to transmit as much virus as the INOC-A and B mosquitoes based on the similarities in mean salivation medium titers between these 3 groups of mosquitoes ($P > 0.05$). The ORAL-C transmitting mosquitoes were capable of transmitting as much JC virus as the INOC-B mosquitoes (8.0 $\log_{10}$ $TCID_{50}$/ml) to capillary tubes. However, the ORAL-C mosquitoes did have a much lower range of salivation medium titers (2.0 $\log_{10}$ $TCID_{50}$/ml) than the INOC-B mosquitoes (4.0 $\log_{10}$ $TCID_{50}$/ml), which may have been indicative of an insufficient incubation period.

Due to poor survival rates, we were unable to incubate mosquitoes for 21 days. Heard et al. (1991) also reported a high mortality in *Ae. provocans*, which shortened the incubation period in their study to 14 days. The bionomics of *Ae. provocans* (as *trichurus* Dyar in the older literature) has been described as having a typical Northern *Aedes* life cycle, is univoltine, and appears to be a relatively short-lived species (Means 1979). There is no information regarding mosquito longevity in captivity because this

species has not been colonized and there are no previous longevity records for field-collected specimens.

Aedes stimulans (Walker) has been shown to be a vector of JC virus in northern Indiana (Boromisa and Grimstad 1986, Heard et al. 1991). This species not only exhibited a MEB but also had a substantially lower rate of MI than that observed for *Ae. provocans*. The rates of DI and oral transmission between these 2 vector species were similar. We concluded that the New York strain of *Ae. provocans* was a better vector of JC virus based on a higher rate of MI than that observed for the northern Indiana strains of *Ae. stimulans* as well as similar rates of DI and virus transmission. Higher rates of JC virus transmission may have been anticipated from *Ae. provocans*, in our study, if the incubation time could have been extended to 21 days. In contrast, Heard et al. (1991) were able to show that Michigan strains of *Ae. provocans* could become infected with but not transmit an Indiana strain of JC virus. The variations in vector and virus competence of *Ae. provocans*, *Ae. stimulans* and JC virus strains from different geographical locations may play a role in understanding these conflicting results (Hardy et al. 1983).

Aedes stimulans was found along with *Ae. excrucians* (Walker) and *Ae. fitchii* (Felt and Young), both members of the *Ae. stimulans* group, in northeastern New York (Boromisa and Grayson, unpublished data). It has not been demonstrated whether New York strains of *Ae. stimulans* are vectors of JC virus; however, it may be suspected to be so based on the numerous JC virus isolates from *Ae. stimulans* group mosquitoes from New York. *Aedes stimulans* group mosquitoes (68,536 specimens) accounted for 16% of JC virus isolates (14) from New York in a retrospective study conducted from 1972 to 1980 (Grayson et al. 1983). However, there were 17 JC virus isolates from 4,287 *Ae. provocans* and none from 4,161 *Ae. stimulans* group mosquitoes collected at our study site in 1988 and 1989 (Boromisa and Grayson 1990). It is not clear why this lack of JC virus infection in *Ae. stimulans* group mosquitoes would occur in such a highly enzootic JC virus focus. Because of the high rates of JC virus infection in *Ae. provocans* observed at our study site, some virus may have been expected to "spill over" to other potential vectors, such as *Ae. stimulans* group mosquitoes. This "spill over" may have occurred instead with other members of the *Ae. communis* group mosquitoes, namely *Ae. communis* (De Geer), *Ae. intrudens* (Dyar) and *Ae. punctor* (Kirby) (Boromisa and Grayson 1990). Jamestown Canyon virus has also been isolated from *Ae. abserratus* (Felt and Young), another *Ae. communis* group mosquito, from Connecticut (Main et al. 1979). It has recently been shown that *Ae. abserratus/punctor*, *Ae. cinereus* and *Ae. intrudens* can become infected with JC virus, however, virus transmission by these species was not demonstrated (Heard et al. 1991). Further investigations into the vector competence of these and other *Ae. communis* group mosquitoes for JC virus are warranted. It is not inconceivable that more than one vector or that different vectors in different locations may exist for JC virus.

The high field infection rates in *Ae. provocans* in northeastern New York first suggested that this species of mosquito may have considerable importance as a vector of JC virus (Boromisa and Grayson 1990). It was also determined that JC virus can overwinter in the eggs of *Ae. provocans* by the mechanism of transovarial transmission and that this species of mosquito was attracted to and would attempt to feed on a human host. In this study, it was demonstrated that *Ae. provocans* can become infected with and orally transmit JC virus; therefore, we concluded that it is a vector of JC virus in northeastern New York.

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