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Molecular Basis of Bunyavirus Transmission by Mosquitoes: Role of the Middle-Sized RNA Segment

Abstract. *In an examination of the molecular basis of oral transmission of bunyaviruses by mosquitoes, La Crosse (LAC), snowshoe hare (SSH), and LAC-SSH reassortant viruses were compared in their ability to be transmitted to laboratory mice by the natural mosquito vector of LAC virus,* Aedes triseriatus. *Both LAC virus and the reassortment viruses containing the middle-sized (M) segment from the LAC parent were efficiently transmitted. In contrast, SSH virus and reassortment viruses containing the M RNA from the SSH parent were inefficiently transmitted. Thus, the M RNA segment, which codes for the virion glycoproteins, may be a major determinant of oral transmission of bunyaviruses by mosquitoes.*

The tripartite genome of the California group of bunyaviruses (Bunyaviridae) is composed of a large, a middle-sized, and a small (L/M/S) RNA segment (*1*). The RNA segments of certain bunyaviruses have been reassorted in vitro (*2*), providing new genomes of known segment composition. Analyses of wild-type La Crosse (LAC) and snowshoe hare (SSH) viruses and certain LAC-SSH reassortant viruses have been used to determine gene-coding assignments (*3*); similar analyses were done previously with influenza viruses and reoviruses (*4*). In this study we report the use of reassortant viruses to delineate the molecular basis of oral transmission by *Aedes triseriatus* mosquitoes.

Serologically, LAC and SSH viruses are very closely related; they are distinguishable, however, by highly specific virus neutralization tests (*5*). The LAC virus was first isolated in 1964 from brain tissues obtained from a child who had died of meningoencephalitis in 1960 (*6*). Subsequently, the virus was shown to be transmitted by a mosquito vector, *A. triseriatus*, and the principal vertebrate hosts were shown to be chipmunks and squirrels (*7*). In spite of its serologic similarity to LAC virus, SSH virus is maintained in nature in different *Aedes* species and different vertebrate hosts (*7*).

We compared LAC, SSH, and six LAC-SSH reassortant viruses (Table 1) for their ability to be transmitted by *A. triseriatus*, the natural vector of LAC virus (*7*). The origins of the plaque-cloned LAC and SSH viruses and the reassortant viruses have been described (*1*, *2*). The six different LAC-SSH reassortants were originally produced by dual infection of BHK-21 cells with temperature-sensitive mutants of the parental viruses (*2*). The identities of the RNA segments of each reassortant virus were determined by oligonucleotide mapping (*2*). *Aedes triseriatus* were obtained from the colony at the Yale Arbovirus Research Unit. This colony originated from larval collections made in Connecticut during 1974, and it has been continuously maintained since then. Immunofluorescence (IF) was used to detect viral antigen in mosquito tissues (*8*). High titers of antibodies to LAC virus were prepared by hyperimmunization of mice. Ascitic fluids were collected by paracentesis. Antibodies were precipitated with $(NH_4)_2SO_4$ and conjugated with fluorescein isothiocyanate (*8*). The capacity of the conjugated antibodies to bind to wild-type LAC and SSH viruses in mosquito tissues and mouse brain impression smears was equal to that of the unconjugated antibodies. The high degree of cross-reactivity of the conjugate was expected because of the serologic similarities between the two viruses (*5*).

The oral transmission potential of the viruses was determined in four trials by inoculating *A. triseriatus* with ≤1000 plaque-forming units (pfu) of wild-type or reassortant virus. The viruses were inoculated intrathoracically in order to bypass the mesenteron, thereby precluding possible confounding variables asso-

Table 1. Transmission of bunyavirus reassortants by *Aedes triseriatus*. The data show the number of transmissions (T) compared to the number of attempted transmissions (A). N.D., not done.

Genome RNA segment composition	Trial 1		Trial 2		Trial 3		Trial 4		Total	
	T/A	Percentage	T/A	Percentage	T/A	Percentage	T/A	Percent age	T/A	Percentage
LAC/LAC/LAC	20/20	100	13/13	100	17/17	100	10/10	100	60/60	100
SSH/LAC/LAC	8/8	100	5/5	100	9/10	90	N.D.		22/23	96
SSH/LAC/SSH	13/15	87	9/10	90	13/14	93	N.D.		35/39	90
LAC/LAC/SSH	0/6	0	0/7	0	0/17	0	9/14	64	9/14	64*
Total LAC M RNA									126/136	93
LAC/SSH/LAC	N.D.		N.D.		N.D.		8/19	42	8/19	42
LAC/SSH/SSH	N.D.		N.D.		N.D.		5/14	36	5/14	36
SSH/SSH/LAC	6/17	35	2/5	40	3/13	23	N.D.		11/35	31
SSH/SSH/SSH	5/15	33	3/11	27	4/10	40	N.D.		12/36	33
Total SSH M RNA									36/104	35

*Two different LAC/LAC/SSH viruses were studied. The virus used in trials 1, 2 and 3 was replaced with an alternate LAC/LAC/SSH virus in trial 4. Since the first virus was defective (see text), only the results obtained with the alternative virus are included in the total.

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ciated with passage through the midgut. The three new reassortant viruses used in trial 4 were not yet available when trials 1, 2, and 3 were conducted. After extrinsic incubation periods of 5 to 7 days and 12 to 14 days in trial 1, and 10 days in all subsequent trials, each mosquito was permitted to engorge on a suckling mouse; the mouse was then observed for 10 days. Suckling mice are extremely susceptible to infection by the California group of viruses and are commonly used for isolating these viruses (*7*). The LAC and SSH viruses seem to have almost equal virulence in mice, with the SSH virus sometimes causing death more rapidly than the LAC virus (*9*). Thus the mouse appears to be a satisfactory substitute for the natural vertebrate hosts of the two viruses in the assay for virus transmission.

The presence of viral antigens in the mice that died was confirmed by IF examination of brain impression smears. Selected brain preparations were used to reisolate virus which was analyzed by oligonucleotide mapping in order to reconfirm the identity of the genome RNA segment of the respective isolates. In trial 1, the serum of surviving mice was examined by a neutralization test (80 percent plaque reduction) for the presence of viral antibodies; all were negative.

The mosquitoes that had engorged on suckling mice were examined by IF for the presence of viral antigen. Heads and abdomens were severed and squashed on slides. The smears were fixed in acetone and stained with the conjugated antibodies for 30 minutes at 37°C. Slides were then examined for the presence of viral antigen by using a Leitz-Wetzlar fluorescence microscope equipped with an HBO Osram mercury-vapor light with a KP 490 interference plate-K510 barrier filter. Salivary glands were dissected from selected mosquitoes and also processed for IF examination.

We found a strong correlation between the presence of the LAC M RNA segment and transmission of the virus to suckling mice (Table 1). More than 90 percent of the mosquitoes infected with viruses containing the LAC M RNA segment, but only 35 percent of the mosquitoes infected with viruses containing the SSH M RNA segment, transmitted the virus (Table 1).

Two different LAC/LAC/SSH reassortant viruses were examined for transmission potential. The first virus, used in trials 1, 2, and 3, was not transmitted and was subsequently shown to be nonpathogenic for mice (*10*) and chipmunks (*11*). The reassortant (derived by 5-fluorouracil mutagenesis) contained a silent mutation in the L RNA and possibly in one or more of the other RNA segments which resulted in attenuation of the virus (*10*). The second virus, used in trial 4, derived the M and S RNA segments from the original LAC/LAC/SSH reassortant, but the L RNA was obtained from an alternative LAC temperature-sensitive mutant (clone 20) which was detected as a spontaneous temperature-sensitive mutant in wild-type virus (*10*). This reassortant virus was transmitted, albeit at a lower rate than the other viruses containing the LAC M RNA segment (Table 1).

Table 2. Number of mosquitoes transmitting virus to suckling mice compared to number with detectable virus antigen in the salivary glands.

Genome RNA segment composition	Mosquitos (No.)		
	Transmitting virus	With antigen in salivary glands	Percentage
LAC/LAC/LAC	13	13	100
SSH/LAC/LAC	3	3	100
SSH/LAC/SSH	4	4	100
SSH/SSH/LAC	2	8	25
SSH/SSH/SSH	4	8	50

The difference in transmission rates between those viruses containing the LAC M RNA segment, excluding the results obtained in trials 1, 2, and 3 with the initial LAC/LAC/SSH virus (Table 1), and those viruses containing the SSH M RNA segment was statistically significant ($\chi^2 = 90.5$, $P < .001$). Excluding the parent viruses, those reassortant viruses containing the LAC M RNA (66 of 76) were transmitted significantly more frequently than those reassortant viruses containing the SSH M RNA segment (24 of 68) ($\chi^2 = 40.7$, $P < .001$).

These results show that the LAC M RNA segment is associated with efficient oral transmission of the virus by *A. triseriatus*. To determine the anatomic basis for this phenomenon, we dissected the salivary glands from selected mosquitoes that had successfully fed on suckling mice. The glands were examined by IF for the presence of viral antigen. When the mosquitoes were infected with viruses containing the LAC M RNA segment, the presence of viral antigen in salivary gland cells was uniformly correlated with virus transmission (Table 2). In contrast, only 38 percent of the mosquitoes with demonstrable SSH/SSH/LAC or SSH/SSH/SSH viral antigen in the salivary gland cells transmitted the virus ($\chi^2 = 17.3$, $P < .001$).

The M RNA segment of bunyaviruses codes for two glycoproteins that function in receptor and maturational events in the replicative cycle (*1*). Since viral antigen can be detected in salivary gland cells of nontransmitting mosquitoes, it seems logical that differences in transmission rates may be caused by differences in maturational efficiences of the two viruses. Those containing the SSH M RNA may simply be less capable of maturation or escape from the salivary gland cell; thus less infective virus is transmitted. Alternatively, there may be a difference in the rate of replication of the two viruses in salivary gland cells. Extrinsic incubation periods longer than the 12 days used in trial 1 may result in increased transmission rates for the viruses containing the SSH M RNA segment.

The applicability of these findings to arbovirus cycles in nature has not been ascertained. Transmission rates determined by infection and subsequent mortality of suckling mice may have little relation to conditions in nature. It is possible that the snowshoe hare is susceptible to doses of SSH M RNA viruses that are too small to infect laboratory mice. Thus if the differences in transmission rates observed in these studies were due only to the quantity of virus egested, such differences would be of little significance in nature. It is therefore necessary to study the transmission of these viruses in their natural vertebrate hosts and to determine if the rates of transmission of the LAC and SSH M RNA viruses are reversed when the mosquito used is a natural vector of SSH virus.

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Non-Mendelian Inheritance of Mosquito Susceptibility to Infection with *Brugia malayi* and *Brugia pahangi*

Abstract. *The mode of inheritance of susceptibility or refractoriness of insect vectors to medically important pathogens such as those causing malaria or filariasis is usually believed to follow normal Mendelian laws and to involve a single pair of alleles. In this report, experiments are described that demonstrate another mode of inheritance of mosquito susceptibility to filarial parasites. Crosses were made between susceptible and refractory species of the* Aedes scutellaris *complex, and the hybrid and backcross progeny were tested for susceptibility to infection by* Brugia malayi *and* Brugia pahangi. *The data indicate that inheritance follows a non-Mendelian pattern indicative of extrachromosomal factors inherited through the maternal parent.*

The interaction between medically important pathogens and their vector hosts is vital to the survival of both. The vector host must possess certain defense mechanisms against the parasite and the parasite must cope with the insect host defenses. Through coevolution, pathogens become increasingly specialized with respect to their vector hosts. Variations in vector susceptibility to infection with either filarial worms or malaria parasites have been observed among diverse populations of vectors, as well as among individuals of the same population. The genetic basis of the relation between vectors and pathogens is poorly understood. Most reports on the genetics of vector susceptibility to organisms pathogenic to man or domestic animals deal with susceptibility of *Culex pipiens* or *Aedes aegypti* to *Plasmodium cathemerium* (*1*, *2*), *P. gallinaceum* (*3*), *Dirofilaria immitis*(*4*-*7*), *Brugia malayi* (*8*), *B. pahangi* (*9*, *10*), and *Waltonella flexicanda* (*11*). In all of these studies, the susceptibility of the vectors to the pathogens appeared to be controlled by a single gene system.

In this report we demonstrate another mode of inheritance of mosquito susceptibility to filarial parasites. It is maternal, non-Mendelian inheritance controlled by cytoplasmic factors. Our genetic studies were done on species of the *Aedes scutellaris* complex of mosquitoes. The *A. scutellaris* complex consists of about 30 species that are widely distributed throughout the Pacific and Oriental regions. The Oriental and the western Pacific groups of the *A. scutellaris* complex that we have tested (*A. alcasidi*, *A. riversi*, *A. seatoi*, and *A. malayensis*) are refractory to infection with both *B. malayi* and *B. pahangi* (*12*). In contrast, all species of the Polynesian group (*A. polynesiensis*, *A. cooki*, *A. tabu*, *A. kesseli*, and *A. pseudoscutellaris*) are highly susceptible (*13*-*15*). All species of the Polynesian group are vectors of subperiodic *W. bancrofti* in the South Pacific (*16*). Backhouse and Woodhill (*17*) tested *A. scutellaris* and *A. pseudoscutellaris* for susceptibility to infection with the New Caledonian strain of *W. bancrofti* and found that *A. scutellaris* was refractory and *A. pseudoscutellaris* was susceptible. Since all tests for susceptibility of different members of the *A. scutellaris* complex to infection with *Brugia* were in agreement with susceptibility or refractoriness to the subperiodic *W. bancrofti*, we could use *B. malayi* and *B. pahangi* as laboratory models for vector transmission studies.

In our experiments, mosquito larvae were reared in an insectary at 27° ± 1°C with a relative humidity of 80 ± 5 percent. Larvae were reared in white enamel pans, approximately 100 larvae per pan containing 2 liters of water. Larvae were fed with 2 ml of a suspension of liver powder in water daily. Pupae were collected from the pans, their sexes were determined, and those of the same sex were placed together in 50-ml cups until emergence of the adults. The sexes of the newly emerged adults were rechecked and appropriate crosses were made by placing the adults in cylindrical cages (180 mm in diameter and 180 mm high). All adult mosquitoes were provided with a cup of water and with honey mixed with cellulose fibers. After 5 days the females were fed on gerbils infected with *B. malayi* or *B. pahangi*. The blood-fed mosquitoes were individually collected from the feeding cage and placed into a new cage provided with honey food and a 50-ml paper cup lined with paper towel and filled with water for oviposition. Ten days after the infective blood meal, all surviving females were checked for infective third-stage larvae of *B. malayi* or *B. pahangi*. Individual females were placed on a microscope slide with four drops of insect saline and dissected into four parts: the proboscis, head, thorax, and abdomen. These parts were opened and the tissue teased in order to release active *Brugia* larvae into the saline. The number of larvae found in each body part was recorded.

Table 1. Inheritance of mosquito susceptibility to infection with *B. malayi* in crosses of susceptible females with refractory males. Po = *A. polynesiensis*, which is susceptible; Al = *A. alcasidi*, which is refractory.

Cross	Parents ♀	Parents ♂	Number dissected	Susceptible Number	Susceptible Percentage	Refractory Number	Refractory Percentage
P	Po/Po	Po/Po	87	87	100	0	0
P	Al/Al	Al/Al	92	0	0	92	100
F_1	Po/Po	Al/Al	42	42	100	0	0
F_1	Al/Al	Po/Po*					
F_2	Po/Al	Po/Al	22	22	100	0	0
F_3	Po/Al	Po/Al	38	38	100	0	0
F_6	Po/Al	Po/Al	68	68	100	0	0
Bx_1	Po/Al	Po/Po	17	17	100	0	0
	Po/Po	Po/Al	73	73	100	0	0
Bx_2	Po/Al	Al/Al	8	8	100	0	0
	Al/Al	Po/Al*					

*Incompatible cross.

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