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VERTICAL AND VENEREAL TRANSMISSION OF CALIFORNIA GROUP VIRUSES BY *AEDES TRISERIATUS* AND *CULISETA INORNATA* MOSQUITOES

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Summary. - *Aedes triseriatus* and *Culiseta inornata* mosquitoes were compared in their ability to transmit vertically La Crosse (LAC) and snowshoe hare (SSH) viruses. LAC virus was transovarially transmitted by 53 % of *Ae. triseriatus,* the natural vector, and by 22 % of *Cs. inornata* mosquitoes. SSH virus was transovarially transmitted by 89 % of *Cs. inornata,* a proposed natural vector, and by 29 % of *Ae. triseriatus.* A genetic approach, using LAC, SSH, and LAC/SSH reassortant viruses was then used to elucidate viral genetic determinants of transovarial transmission of bunyaviruses by *Ae. triseriatus* mosquitoes. Viruses containing the LAC medium sized (M) RNA segment were most efficiently transovarially transmitted by *Ae. triseriatus* mosquitoes. LAC, SSH, and Ťahyňa (TAH) viruses were compared in their ability to be venerally transmitted. All three viruses replicated in the reproductive tract of male *Aedes triseriatus* and were venereally transmitted to female mosquitoes. LAC and TAH viruses infected previously blood fed (BF) but not non-blood fed (NBF) *Aedes triseriatus* female mosquitoes.

Key words: vertical transmission in vectors; California group viruses; veneral transmission in mosquitoes; reassortants

Introduction

Bunyaviridae is the largest family of arboviruses and consists of five genera: *Bunyavirus, Nairovirus, Phlebovirus, Uukuvirus,* and *Hantavirus.* The virus genome consists of three single stranded segments of negative sense RNA, which are labeled according to size: large (L), medium (M), and small (S). The S RNA codes for the nucleocapsid protein and a nonstructural protein NS_S, the M RNA codes for the surface glycoproteins G1 and G2 and a nonstructural protein NS_M, and the L RNA codes for the virion polymerase (Bishop, 1990; Elliott, 1990; Gonzalez-Scarano, 1990; Schmaljohn and Patterson, 1989).

The genus *Bunyavirus* is comprised of 16 different serogroups (Karabatsos, 1985). California serogroup viruses (principally LAC virus) are the major cause of mosquito-borne encephalitis in the United States, except during years of St. Louis encephalitis epidemics (Kappus *et al.*, 1983; Grimstad, 1988). The California serogroup includes La Crosse (LAC), snowshoe hare (SSH), and Ťahyňa (TAH) viruses.

The primary vector of LAC virus is *Aedes triseriatus* (LeDuc, 1979; Grimstad, 1988), and the primary vertebrate hosts are the gray squirrel (*Scurius carolinensis)* and the Eastern chipmunk (*Tamias striatus*) (Grimstad, 1988; Beaty and Calisher, 1991). LAC virus is vertically transmitted by *Ae. triseriatus* mosquitoes and overwinters within the diapaused egg (Watts *et al.*, 1973). Transovarially infected male progeny can further amplify the virus by venereally (horizontally) transmitting virus to previously uninfected female adults during mating (Thompson and Beaty, 1977; Beaty and Calisher, 1991). Interestingly, female mosquitoes that ingested a blood meal prior to or shortly after mating are more susceptible to venereal infection (Thompson, 1979; Patrican and DeFoliart, 1987). Females infected venereally are capable of transovarially infecting future progeny and transmitting the virus horizontally to vertebrate hosts by bite. Venereal transmission may be an important supplement to transovarial and oral transmission in the maintenance and evolution of bunyaviruses (Beaty and Bishop, 1988).

SSH virus was originally isolated from the blood of a snowshoe hare, *Lepus americanus,* and the principal vectors of the virus include *Aedes* spp. and *Culiseta inornata* mosquitoes (Grimstad, 1988; Turrell, 1988). SSH virus has been isolated from larvae of *Ae. canadensis, Ae. communis, Ae. hexadontus,* and *Ae. implicatus,* and the virus may also overwinter in adult *Cs. inornata* mosquitoes (Grimstad, 1988; Turrell, 1988). Unlike *Aedes,* this genus of mosquitoes overwinters in the adult stage, which may serve as a survival mechanism for the virus in transovarially infected mosquitoes.

A variety of *Aedes* mosquitoes have been postulated as the principal vectors of TAH virus (Hannoun *et al.*, 1969; Danielová *et al.*, 1976). The principle vertebrate host is the European hare (*Lepus europaeus)* (Bárdoš, 1975). TAH virus may overwinter in adult *Cs. annulata* (Danielová and Minár, 1969). TAH virus has also been isolated from *Cs. annulata* larvae, demonstrating the ability of this vector to transovarially transmit the virus (Bárdoš *et al.*, 1975). The virus can also be transovarially transmitted in *Aedes vexans* mosquitoes (Danielová and Ryba, 1979).

In these studies, we report the relative abilities of certain California group viruses to be vertically transmitted by vectors and demonstrate the role of the M RNA segment in transovarial transmission of bunyaviruses by *Ae. triseriatus* mosquitoes. In addition, studies were conducted to determine the venereal transmission efficiency of California group viruses to previously blood fed (BF) and non-blood fed (NBF) female *Ae. triseriatus* mosquitoes.

Materials and Methods

Mosquitoes. Aedes triseriatus were kindly provided by Dr. Wayne Thompson, University of Wisconsin, and originated from field materials collected near La Crosse, WI in 1981. Mosquitoes were maintained at 23 ^{0}C, 80 % relative humidity with a photoperiod of 16 hours light and 8 hours dark. Sugar cubes and water were provided *ad libitum*. Mosquitoes were deprived of sugar and water 12 hours before infection.

Culiseta inornata were field collected in Fort Collins, Colorado in 1987 and maintained at 80 % relative humidity, 20 ^{0}C, with a photoperiod of 15 hours light and 9 hours dark (Schopen, 1990).

Viruses. La Crosse (LAC) virus was originally isolated in 1965 from the brain of a four year old girl who died of encephalitis in La Crosse, Wisconsin (Thompson *et al.,* 1965). The virus was passed seven times in 2-day old mice and subsequently in BHK-21 cell culture.

Two snowshoe hare (SSH) virus isolates were used. SSH virus (76-Y-316) was isolated in the Yukon Territory and was kindly provided by Dr. Don McLean (University of British Columbia). The prototype SSH virus, which was originally isolated from a tick and had been passed in suckling mice and in BHK-21 cells, was used in the reassortant virus studies (Beaty and Bishop, 1988). LAC/SSH reassortant and parent viruses were obtained from Dr. David Bishop, NERC, Oxford. The passage histories of the parental viruses and the procedure used to derive the reassortant viruses are described elsewhere (Beaty and Bishop, 1988).

Prototype Ťahyňa (TAH) virus was obtained from the Yale Arbovirus Research Unit, New Haven, CT, and was passed twice in mice (Karabatsos, 1985).

Infection of mosquitoes. Male and female mosquitoes were cold anesthetized at 4 ^{0}C and parenterally infected via direct intrathoracic inoculation of virus. Virus was drawn into needles prepared from 50 μl capillary tubes and injected directly into thoraces through intersegmental membranes of mosquito thoraces (Thompson and Beaty, 1977). Approximately 0.5 μl of virus (5.0 $\log_{10}$TCID$_{50}$/ml) was inoculated per mosquito.

For vertical transmission studies, females were forced mated with noninfected male mosquitoes to ensure fertilization as described below. Individually mated females were held in cartons at 23 ^{0}C, 80 % RH with a 16 : 9 photoperiod. For venereal transmission studies, injected male mosquitoes were held in cartons at 23 ^{0}C, 80 % RH for a 7 day extrinsic incubation period.

Transovarial transmission. Following mating, females were provided meals composed of equal parts of washed human erythrocytes and 10 % fetal bovine serum with 10 % sucrose. Engorged females were then individually placed in cartons containing water cups for oviposition. Resultant eggs were recovered and stored at 23 ^{0}C, 80 % RH and 16 : 9 hour photoperiod for 10 days for embryonation. Following incubation, hatching was induced by placing eggs in pans containing 1 liter of deoxygenated water and 0.5 ml brain heart infusion (Difco). Resultant mosqito larvae were maintained on Tetramin until pupation. Pupae were placed in quart-sized cartons with organdy netting until adult emergence. Transovarial and filial infection rates were determined by direct immunofluorescence microscopy of head tissue (Beaty and Thompson, 1978). The transovarial transmission rate was the per cent of female mosquitoes transovarially transmitting virus, and the filial infection rate was the per cent of progeny infected by the females transovarially transmitting the virus.

Venereal transmission. Infected male mosquitoes were decapitated and induced to mate with noninfected 5–7 day old prevjously blood fed (BF) and non-blood fed (NBF) virgin females. Females were cold anesthetized and laid dorsally on the stage of a dissecting microscope. Claspers of decapitated males were placed directly on female genitalia until copulation occurred. Mating typically occurred from 30 to 90 seconds.

Immediately following mating, one group of paired mosquitoes was titrated to quantitate virus. An additional group of female mosquitoes was held for a 14 day extrinsic incubation period, and the corresponding males were stored at -70 ^{0}C. Following the incubation period, selected male and female mosquitoes were assayed by direct immunofluorescence for the presence of virus antigen or titrated to quantitate virus.

Viral antigen detection in mosquitoes. To determine infection rates, mosquitoes were cold anesthetized on ice, heads were severed, and squashed onto ethanol washed slides. Slides were then fixed in acetone (-20 ^{0}C) for ten minutes, and stained with anti-LAC antibodies conjugated with fluorescein isothiocyanate for 30 minutes at 37 ^{0}C (Beaty and Thompson, 1978). Evans blue (.005 %) was used for a counterstain. Following incubation, slides were washed twice in phosphate buffered saline for 10 minutes, dipped in distilled water, and air dried. Tissues were covered with phosphate buffered saline-glycerine and cover slips were added. All tissues were examined for the presence of viral antigen using an Olympus BH-2 microscope equipped with an vertical epifluorescence illuminator.

To investigate the anatomic basis of virus infection via venereal transmission, male and female mosquitoes from both incubation groups (0 and 14 days) were cold anesthetized and placed in a container on ice. Individual mosquitoes were dissected and reproductive organs of each sex were extracted from the exoskeleton, placed on poly-D-lysine coated slides, fixed in acetone, and examined for the presence of viral antigen by immunofluoresence.

Virus isolation and titration. Mosquitoes were individually triturated in small mortars containing 1.0 ml of medium consisting of L-15 (Sigma) medium with 20 % fetal bovine serum, fungizone (2.5 μg/ml), and penicillin (400 units/ml)/streptomycin (400 μg/ml, respectively). Triturated mosquito tissue was then centrifuged at 1500 rev/min to separate mosquito exoskeleton remnants. Serial ten fold dilutions of the supernatant (50 μl) were added to wells of a 96-well microtiter plate. One hundred and fifty μl of a cell suspension containing approximately 30, 000 BHK-21 cells in L-15 growth medium was added to each well. The microtiter plates were sealed, incubated at 37 ^{0}C for four days, and examined for cytopathic effect. The $\log_{10}TCID_{50}$ was then calculated using the Spearman-Karber technique (Spearman and Karber, 1964).

Results

Transovarial transmission of LAC and SSH viruses by Ae. triseriatus and Cs. inornata mosquitoes

Both species of mosquitoes transovarially transmitted the two viruses (Table 1). LAC virus appeared to be more efficiently transmitted by *Ae. triseriatus,* while SSH virus was most efficiently transmitted by *Cs. inornata* mosquitoes. LAC virus was transovarially transmitted by 53 % of *Ae. triseriatus* and 22 % of

Table 1. Transovarial transmission of LAC and SSH viruses

Virus	Transovarial transmission rate[1]			
	Ae. triseriatus			*Cs. inornata*
	OP 1[2]	OP 2[2]	Total[3]	OP 1
LAC	65 % (13/20)	33 % 4/12)	53 %	22 % (2/9)
SSH	29 % (10/34)	27 % (3/11)	29 %	89 % (8/9)

[1] Determined by immunofluorescence; per cent positive (number positive/number examined)
[2] OP-oviposition cycle. Transovarial transmission rates determined from progeny of first or second oviposition
[3] Combined transovarial transmission rate of OP 1 and OP 2

Table 2. Filial infection rates of LAC and SSH viruses

Virus	Filial infection rates[1] *Ae. triseriatus* OP 1	*Ae. triseriatus* OP 2	Total[2]	*Cs. inornata* OP 1
LAC	10 % (50/482)	30 % (24/86)	13 %	12 % (6/49)
SSH	8 % (48/620)	4 % (3/83)	7 %	16 % (33/206)

[1] Determined by immunofluorescence; per cent positive (number positive/number examined)
[2] Total filial infection rates from oviposition cycles 1 and 2

Cs. inornata mosquitoes (X^2=1.59, P=.206). In contrast, 89 % of *Cs. inornata* and 29 % of *Ae. triseriatus* mosquitoes transovarially transmitted SSH virus. The difference was statistically significant (X^2=8.97, P=.002).

Filial infection of Ae. triseriatus and Cs. inornata progeny by LAC and SSH viruses

Filial infection rates (infected progeny of infected females) with LAC and SSH viruses are summarized in Table 2. Filial infection rates of *Ae. triseriatus* progeny mosquitoes were 13 % (74/568) with LAC virus and 7 % (51/703) with SSH virus. LAC virus was more efficiently transmitted to progeny mosquitoes (X^2=11.8, P=<.001). Filial infection rates of *Cs. inornata* progeny mosquitoes

Table 3. Filial infection rates of LAC/SSH reassortant viruses in Aedes triseriatus mosquitoes

Virus[1]	Immunofluorescence[2]
LAC/LAC/LAC	**27 % (12/27)**
SSH/LAC/SSH	**13 % (10/75)**
SSH/LAC/LAC	**29 % (88/299)**
LAC/LAC/SSH	**30 % (9/30)**
TOTAL LAC M RNA	**28 % (119/431)**
SSH/SSH/SSH	**18 % (3/17)**
LAC/SSH/LAC	**15 % (10/63)**
SSH/SSH/LAC	**6 % (4/68)**
LAC/SSH/SSH	**3 % (2/60)**
TOTAL SSH M RNA	**11 % (21/188)**

[1] Virus genotype represented by parental origin of the L/M/S RNA segments
[2] Per cent positive (number positive/number examined); combined totals of progeny of three ovipositions

Table 4. Venereal transmission of LAC and TAH viruses to previously blood fed and non-blood fed Aedes triseriatus female mosquitoes assayed immediately following mating

Virus	Blood fed		Non-blood fed	
	Infection rate[1]	Titer[2]	Infection rate	Titer
LAC	53 % (8/15)	3.2	80 % (12/15)	3.5
TAH	47 % (7/15)	3.4	73 % (11/15)	3.2

[1] Per cent (number infected/number examined)
[2] Geometric mean titer (N>5), Log_{10} $TCID_{50}$/ml

were 16 % for SSH virus and 12 % for LAC virus. LAC virus filial infection rates were 13 % (74/568) in *Ae. triseriatus* and 12 % (6/49) in *Cs. inornata* (Table 2), but SSH virus more efficiently infected *Cs. inornata* progeny (X^2=14.59, P<.001).

Filial infection rates of reassortant viruses

Viruses containing a LAC M RNA segment (28 %, 119/431) were more efficiently transmitted to progeny *Ae. triseriatus* than viruses with a SSH M RNA segment (11 %, 21/188) (Table 3). This difference was statistically significant (X^2=20.21, P<.001). One LAC M RNA containing virus, SSH/LAC/SSH, exhibited a filial infection rate comparable to two viruses containing the SSH M RNA. The reason for this remains to be determined.

Infection of male mosquitoes with LAC and TAH viruses

All three viruses replicated in intrathoracically inoculated male mosquitoes. The geometric mean titers of males inoculated with LAC, SSH, and TAH viruses were 2.9, 2.6, and 3.2 $log_{10}TCID_{50}$, respectively. Viral antigen was detected in reproductive tract tissues of males infected with each of the viruses; thus most mosquitoes were theoretically capable of venereally transmitting virus.

Table 5. Isolation of LAC and TAH viruses from female Aedes triseriatus mosquitoes mated two weeks previously with infected males

Virus	Blood fed		Non-blood fed	
	Infection rate[1]	Titer[2]	Infection rate	Titer
LAC	30 % (6/20)	4.9	0 % (0/20)	0
TAH	15 % (3/20)	5.0	0 % (0/20)	0

[1] Infection rate (number yielding virus/number examined)
[2] Geometric mean titer (N>5), LOG_{10} $TCID_{50}$/ml

Table 6. Detection of La Crosse and Ťahyňa viral antigen in female Aedes triseriatus mosquitoes mated two weeks previously with infected male mosquitoes

Virus	Blood fed		Non-blood fed	
	Head[1]	Reproductive	Head	Reproductive
LAC	26 % (6/23)	26 % (6/23)	0 % (0/24)	0 % (0/24)
TAH	14 % (3/22)	14 % (3/22)	0 % (0/24)	0 % (0/24)

[1] Infection rate (number with detectable viral antigen by immunofluorescence in head or reproductive tract tissues/number examined)

Veneral transmission of Cal Group viruses to previously blood fed and non-blood fed Ae. triseriatus mosquitoes

LAC and TAH viruses were venereally transmitted by male *Ae. triseriatus* mosquitoes. There were no apparent differences between venereal transmission rates of LAC and TAH viruses to female mosquitoes. LAC virus was detected in 67 % (20/30) and TAH virus in 60 % (18/30) of females immediately after mating (Table 4). LAC virus was detected in 53 % of the BF mosquitoes and 80 % of the NBF mosquitoes (Table 4). TAH virus was detected in 47 % of BF and 73 % of NBF females. Although suggestive, these rates did not differ statistically (X^2=1.25, P=.26 and X^2=1.35, P=.24, respectively).

Infection of previously BF and NBF Aedes triseriatus mosquitoes following venereal transmission

Following 14 days incubation, LAC and TAH viruses could only be detected in previously BF mosquitoes. LAC virus was detected in 28 % (12/43) and TAH in 14 % (6/42) of mosquitoes examined (Tables 5 and 6). Although suggestive, the rates did not differ statistically (X^2=2.36, P=.12).

LAC virus was isolated from 30 % of mosquitoes assayed, and the geometric mean titer was 4.9 $\log_{10}TCID_{50}$ per mosquito. TAH virus was isolated from 15 % of mosquitoes, and the GMT was 5.0 $\log_{10}TCID_{50}$ (Table 5). LAC viral antigen was detected in 26 % of the mosquitoes examined, and TAH antigen was detected in 14 % (Table 6). In both instances, viral antigen disseminated from reproductive tract tissues to infect secondary target organs in all of the infected mosquitoes.

In a separate study, SSH virus was venereally transmitted to 32 % (8/25) of mated mosquitoes. Thus, all three CAL group viruses were venereally transmitted by *Aedes triseriatus.*

Venereal transmission of SSH virus by Cs. inornata

SSH virus was observed by immunofluorescence in head tissues of 1 of 6 female *Cs. inornata,* which had been mated 14 days previously with infected

males (data not shown). Thus, *Cs. inornata* can venereally transmit SSH virus, which can subsequently infect and disseminate in females.

Discussion

The success of arboviruses in nature can be attributed to the intricate relationship between the arthropod vector and the virus. The major determinants of arbovirus cycle integrity in nature remain to be elucidated. Biological and behavioral attributes of vector populations undoubtedly function in this regard. However, specific virus-vector interactions, such as infection and transmission, may function to preserve specificity of arbovirus cycle integrity.

These studies suggest that vertical transmission is important in the specific vector-virus interaction. LAC virus seemed to be more efficiently transmitted by *Ae. triseriatus,* while SSH virus was most efficiently transmitted by *Cs. inornata* mosquitoes (Tables 1 and 2). During vertical transmission, virus and vector are associated for long periods of time, which might be more revealing of an evolved host-parasite relationship. Viruses with long term co-evolution with the vector would probably be more likely not to exert untoward effects on developing embryos and would be more efficiently maintained within the host.

It is noteworthy that in these studies involving transovarial and filial infection rates of LAC and SSH viruses, *Ae. triseriatus* and *Cs. inornata* females were forced mated to ensure fertility. Thus, all females had been inseminated. Unfortunately, this requirement reduced the numbers of mosquitoes (especially *Cs. inornata)* to be examined.

Filial infection studies with LAC/SSH reassortant viruses suggest that the LAC M RNA segment is a determinant of transovarial transmission in *Ae. triseriatus* (Table 3). Unfortunately, limited numbers of *Cs. inornata* precluded doing the reciprocal experiment in a preferred vector for SSH virus. Such experiments will be necessary to unequivocally determine the role of the M RNA segment in vertical transmission of bunyaviruses in their natural vector species.

These studies suggest that a specific venereal infection receptor event may not contribute to the maintenance of bunyavirus cycle integrity (Tables 5 and 6). Although suggestive, the venereal infection rates for LAC and TAH (28 and 14 %, respectively) did not differ statistically. Thus, either California group viruses have not evolved specific venereal transmission mechanisms or the putative receptor is not sufficiently discriminatory to differentiate completely between the closely related viruses, LAC and TAH, used in these studies.

Interestingly, all of the mosquitoes that were venereally infected developed disseminated infections. No barrier to disseminated infection (Beaty and Bishop, 1988; Paulson, *et al.,* 1990) was apparent (Tables 5 and 6). However, it should be noted that the reproductive tracts examined frequently did not

include distal portions of the tract (i.e. - *bursa copulatrix*). Thus, it is possible that infection did occur in certain cells distal in the reproductive tract, but that virus was restricted to these cells and was not detectable by immunofluorescence (Table 6) or by virus isolation (Table 5).

Disseminated venereal infections were only detected in previously BF females. In previous studies, low rates of venereal transmission were detected in NBF females (Thompson and Beaty, 1977). Perhaps the relatively small sample size in these studies accounts for the lack of detection of venereal infection in NBF females. The importance of bloodmeals for efficient venereal infection of females has been demonstrated previously (Thompson, 1979; Patrican *et al.,* 1987); however, the anatomic or physiological basis of this phenomenon remains to be determined. Previous ingestion of a blood meal also makes *Aedes triseriatus* mosquitoes more susceptible to oral infection (Beaty and Thompson, 1977). Interestingly, in two studies, 45 % and 67 % of nulliparous *Aedes triseriatus* collected at human bait were not inseminated (Scholl, *et al.,* 1979; Porter and DeFoliart, 1985). A significant proportion of these mosquitoes would ingest a bloodmeal before mating, thereby enhancing the potential for venereal infection. In addition, enhanced venereal infection also occurs if the female blood feeds soon after mating (Patrican and DeFoliart, 1987). Thus, in nature, there would seem to be opportunity for enhancement of bunyavirus venereal infection of previously engorged female *Aedes triseriatus.*

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