Am. J. Trop. Med. Hyg., 40(1), 1989, pp. 108–113 (8-189)

TRANSOVARIAL TRANSMISSION OF GAMBOA VIRUS IN A TROPICAL MOSQUITO, *AEDEOMYIA SQUAMIPENNIS*

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Abstract. We report transovarial transmission of Gamboa virus (Bunyavirus) in *Aedeomyia squamipennis*, a tropical mosquito which is active and bloodfeeding throughout the year. Gamboa virus was isolated during each of the 28 months of the study from every mosquito stage, including eggs, demonstrating that vertical transmission is a maintenance mechanism of this virus. The overall minimum infection rate was 5.1/1,000 mosquitoes. Identification of the 567 isolates by neutralization indicated that ≥ 2 serotypes or subtypes of Gamboa virus circulate at the study site.

Transovarial transmission of arboviruses in mosquitoes has been substantiated in temperate climates.[1] It has been postulated that transovarial transmission represents an adaptation by some arboviruses in the temperate zone to adverse climatic conditions (winter) when mosquitoes are inactive.[2] The arthropod then becomes the reservoir. We report Gamboa serogroup virus passing from one generation to the next through the egg of *Aedeomyia squamipennis*, a tropical mosquito which breeds and takes bloodmeals throughout the year.

The prototype strain of Gamboa virus (family Bunyaviridae, genus *Bunyavirus*) was first isolated in 1962 from adult *Ad. squamipennis* collected on the Chagres River in central Panama.[3] Five Gamboa serogroup virus isolates were recovered from 13 pools tested. No further isolates of this virus were obtained in Panama during the next 15 years, although several million mosquitoes were processed for virus isolation. These collections included >100 different species and 15 different genera of mosquitoes, yet they did not include any additional *Aedeomyia*.

In 1972, Gorgas Memorial Laboratory initiated a study to determine the changes in the ecology of arboviruses resulting from the construction of a hydroelectric dam on the Bayano River about 70 km east of the site of the 1962 isolations.[4] In February 1977, a year after the beginning of impoundment and 8 months after the first detection of *Ad. squamipennis*, Gamboa viruses began to be isolated and continued to be recovered throughout the remaining 9 months of the study. *Ad. squamipennis* was associated with a proliferation of *Pistia stratiotes* (water lettuce) during the impoundment period. Persistently high field infection rates of Gamboa viruses in *Ad. squamipennis* and isolations from male mosquitoes suggested that these viruses were transmitted transovarially.[5] We initiated this study to substantiate and extend these findings.

MATERIALS AND METHODS

Study site

Field studies were conducted at the Juan Mina Field Station (9°10′N, 79°39′W) on the Chagres River, 5 km northeast of the area where the prototype Gamboa virus was isolated in 1962. The area is tropical humid-dry transitional forest. Annual rainfall is 180–230 cm; January is the driest month and October the wettest. Mosquito collections were conducted in 4 areas within a 3 km radius (Fig. 1): Juan Mina Station (10 collection sites), Juan Mina Forest (15 sites), Chilibre River (10 sites), and Moja Pollo Creek (9 sites).

Mosquito collections

Larval and adult *Ad. squamipennis* were sampled 4 days a week for 28 months, April 1985–July 1987. Adult mosquitoes were collected with CDC light traps and chicken baited (Trinidad 10 and Bellamy-Reeves) traps. Larvae and pupae were collected individually from their breeding

Accepted 15 August 1988.

places in the river by dipping 2 L porcelain pans into the river, examining collected water and vegetation with a magnifying lens, and transferring *Aedeomyia* larvae and pupae to individual plastic cups. Eggs were collected from the leaves on which they had been oviposited.

All adults were frozen immediately in liquid nitrogen. Twenty-five percent of the pupae were allowed to emerge in a cage at the field station to provide male mosquitoes, which were frozen when they emerged. All other collections of immature stages were sent live to the central laboratory once a week. Larvae and pupae were frozen, as pools, within 3 days of arrival. Unhatched egg clutches were frozen 5 days after collection in the field. Species and sex of frozen adults were verified, and pools were prepared at the central laboratory.

Virus isolation

Pools of field collected mosquitoes contained 10 adult or 10 immature mosquitoes from the same site, date, and collection method, or an entire egg clutch containing ~160 ± 35 eggs. Egg clutches were washed with 70% ethanol and 6–7 changes of distilled water before sonification. To determine filial infection rate, egg clutches were allowed to hatch and the larvae were processed individually.

Mosquitoes were sonicated in 1 ml of medium containing 1 part 0.07 M phosphate buffer, pH 7.8, and 1 part stabilizing medium consisting of 4% hydrolyzed gelatin, 0.44 M sucrose and 0.01 M L-glutamic acid in 0.02 M phosphate buffer, pH 8.0. The final antibiotic concentration was 500 U penicillin and 800 μg streptomycin/ml. Suspensions were centrifuged, and 0.1 ml supernate inoculated, undiluted and at 1:10 dilution, into each of 2 Vero cell tube cultures which were maintained on Medium 199 (Earle's salts) containing 1% heated (56°C, 30') fetal bovine serum and antibiotics. Tubes were incubated in roller drums at 34°C with twice weekly fluid changes and observed for cytopathic effect for ≤14 days. Virus was stored frozen as cell culture fluid mixed with an equal volume of the above stabilizing medium.

Identification of virus isolates, usually at 2nd Vero passage, was done by fixed virus/antibody dilution plaque reduction neutralization test. A mouse hyperimmune ascitic fluid prepared in 1969 against the prototype Gamboa virus was used. Between 40 and 150 plaque forming units (PFU) of virus were tested against serial 4-fold dilutions (between 1:8 and 1:2,048) of ascitic fluid (heated at 56°C, 30') prepared in Dulbecco's phosphate-buffered saline solution with 0.5% gelatin, pH 7.3. Antibody-virus mixtures were incubated at 4°C overnight in microplates before inoculation on Vero cell monolayers in plastic 96-well (1.8 cm^2) trays and adsorption for 1 hr at 34°C. Cultures were then overlaid with nutrient medium containing 0.8% gum tragacanth[6] and incubated at 34°C and 3% CO_2. Medium was replaced 6 days later by a second overlay of 0.5

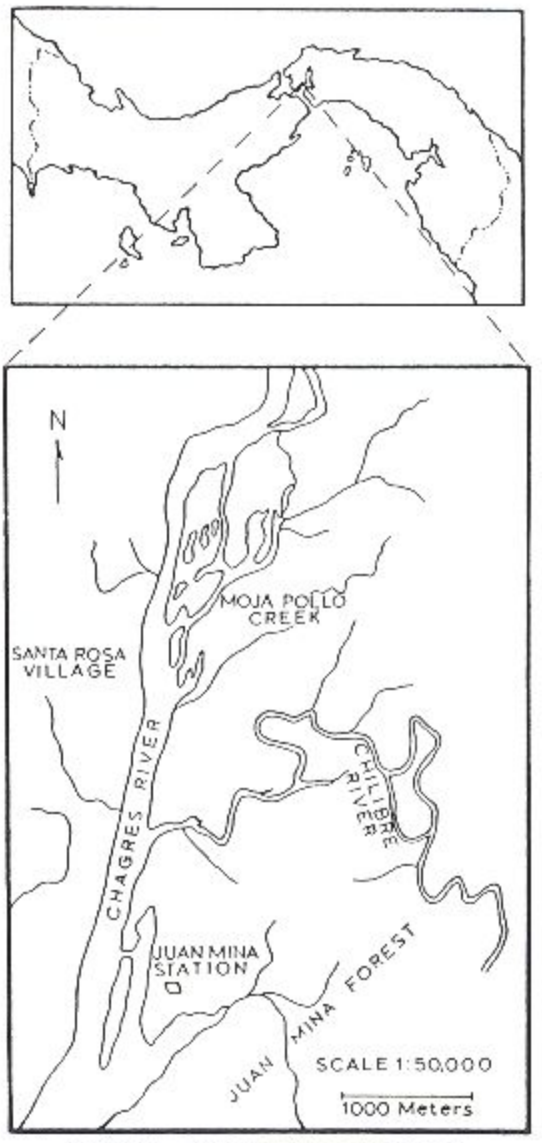

FIGURE 1. Map of the Juan Mina area.

ml nutrient medium containing 0.5% agar and neutral red (final 1:20,000); plaques were counted the following day. Neutralization titer was the highest dilution causing 90% plaque reduction.

Immunization for cross neutralization

Eight-week-old female white mice were injected intraperitoneally (ip) with 0.4 ml of an emulsion of equal volumes of 20% virus infected suckling mouse brain suspension in phosphate-buffered saline, pH 7.4, and Freund's complete adjuvant on days 0, 7, and 21. On day 22, sarcoma cells TG-180 were inoculated ip. Ascitic fluid obtained on day 35 (for the prototype strain) or day 44 (for the fresh isolates) was used in the neutralization tests.

RESULTS

Surveillance of Gamboa virus activity

Five hundred sixty-seven Gamboa virus isolates were recovered from field collected *Ad. squamipennis* during the 28 month study period: 549 from adults or immature forms and 18 from egg clutches. Gamboa virus was isolated from mosquitoes collected at all 4 sites and by all collection methods used. The most productive collection method for adult mosquitoes was the CDC light trap, which provided 59% of the specimens and 56% of the isolates.

Infection rate by month

Gamboa virus was isolated every month of the study (Table 1). The mean monthly minimum field infection rate (MFIR), for larval, pupal, and adult stages combined, was 4.9 ± 2.2/1,000 mosquitoes with a range of 1.6–7.3/1,000 in all but 3 months. Only during March 1986 and February–March 1987 was the MFIR >2 standard deviations above the mean, indicating a significant increase in virus circulation during the mid-dry season of both years.

Infection rate by stage

Virus was recovered at similar rates from all mosquito stages (Table 2). The overall MFIR for larval through adult stages was 5.1/1,000 mosquitoes. In the first 8 months of 1986, 1,371 egg clutches were processed, and virus was isolated from 18 of them, indicating a field infection rate of 13.1/1,000 clutches.

Filial infection rate

Individual larvae derived from egg clutches collected April–November 1986 were tested for virus. Only 1/150 egg clutches yielded positive larvae, and 18/31 (58%) surviving larvae from this family were infected, yielding an estimated infection rate of 6.7/1,000 clutches and 3.9/1,000 eggs.

Virus identification

More than 1 Gamboa virus serotype appeared to be present in the *Aedeomyia* population during the 28 month study period, as shown by a bimodal distribution of neutralization titers of prototype ascitic fluid against the isolates (Fig. 2). Thirty-eight percent of all isolates were neutralized by high dilutions of prototype mouse ascitic fluid (1:1,024–4,096), 46% were neutralized at dilutions of 1:128–512, and 16% at dilutions of 1:8–64. The distribution was similar for isolates from all developmental stages. There was no correlation between titer of typing ascitic fluid and mosquito stage yielding the isolate, or between titer and month of collection. Titers were reproducible within a 2-fold dilution for 126/164 isolates subjected to repeated neutralization tests on the same or next higher passage.

Cross neutralization tests on 2 representative isolates from Juan Mina (strains 046099 and 046737) and the prototype strain showed reciprocal ≥16-fold differences and suggested 2 different serotypes (Table 3).

DISCUSSION

We believe that this is the first demonstration that transovarial transmission is an important mechanism in the maintenance of an arbovirus in a tropical area. Evidence from virus isolation studies in various regions of the Americas, including Panama, indicates that *Ad. squamipennis* is the primary arthropod involved in Gamboa virus maintenance.[4,7,8] We believe that transovarial transmission is the most important mechanism for maintenance of Gamboa virus in this mosquito. Other mechanisms may operate since there appeared to be significant virus input

TABLE 1

Monthly isolation rates of Gamboa virus from Aedeomyia squamipennis

Year	Month	Total mosquitoes collected*	Positive pools	Minimum field infection rate†	Rate per 1,000 mosquitoes examined
1985	April	2,622	16	1:164	6.1
	May	3,925	17	1:231	4.3
	June	4,377	26	1:168	5.9
	July	3,331	20	1:167	6.0
	August	5,257	22	1:239	4.2
	September	2,698	9	1:300	3.3
	October	3,707	16	1:232	4.3
	November	6,822	35	1:195	5.1
	December	3,688	17	1:217	4.6
1986	January	13,329	35	1:381	2.6
	February	14,389	66	1:218	4.6
	March	6,414	71	1:90	11.1
	April	3,648	15	1:243	4.1
	May	2,851	13	1:219	4.6
	June	1,613	6	1:269	3.7
	July	603	2	1:301	3.3
	August	1,503	6	1:251	4.0
	September	3,317	12	1:276	3.6
	October	1,922	8	1:240	4.2
	November	2,303	7	1:329	3.0
	December	4,826	35	1:138	7.3
1987	January	3,620	19	1:191	5.2
	February	2,588	26	1:100	10.0
	March	2,832	27	1:105	9.5
	April	2,367	14	1:169	5.9
	May	1,517	6	1:253	4.0
	June	1,171	2	1:586	1.7
	July	622	1	1:622	1.6
Monthly mean					4.9 ± 2.2
All		107,862	549	1:196	5.1

* All stages, larvae through adults, combined.
† The minimum infection rate has been expressed as the reciprocal of the number of mosquitoes tested divided by positive pools.

into the cycle in the middle of the dry season each year.

There is a continuous association of Gamboa virus with *Ad. squamipennis* throughout the year, and the virus was equally distributed between the sexes and among all developmental stages from larvae to adults. We recognized *Ad. squamipennis* eggs infected with Gamboa sero-

TABLE 2

Gamboa virus minimum infection rates per stage of Aedeomyia squamipennis

Stage	Total number of mosquitoes	Number positive pools	Minimum infection rate	Infection rate per 1,000
Adult male	1,802	13	1:139	7.2
Adult female	67,169	349	1:192	5.2
All adults	68,971	362	1:191	5.2
Pupae	5,137	29	1:177	5.6
Larva—IV instar	22,138	109	1:203	4.9
Larva—III instar	4,894	20	1:245	4.1
Larva—II instar	5,726	23	1:249	4.0
Larva—I instar	996	6	1:166	6.0
All immatures	38,891	187	1:199	5.0
Above stages combined	107,862	549	1:196	5.1
Egg clutches*	1,371	18	—	—

* Each egg clutch has ~60 eggs.

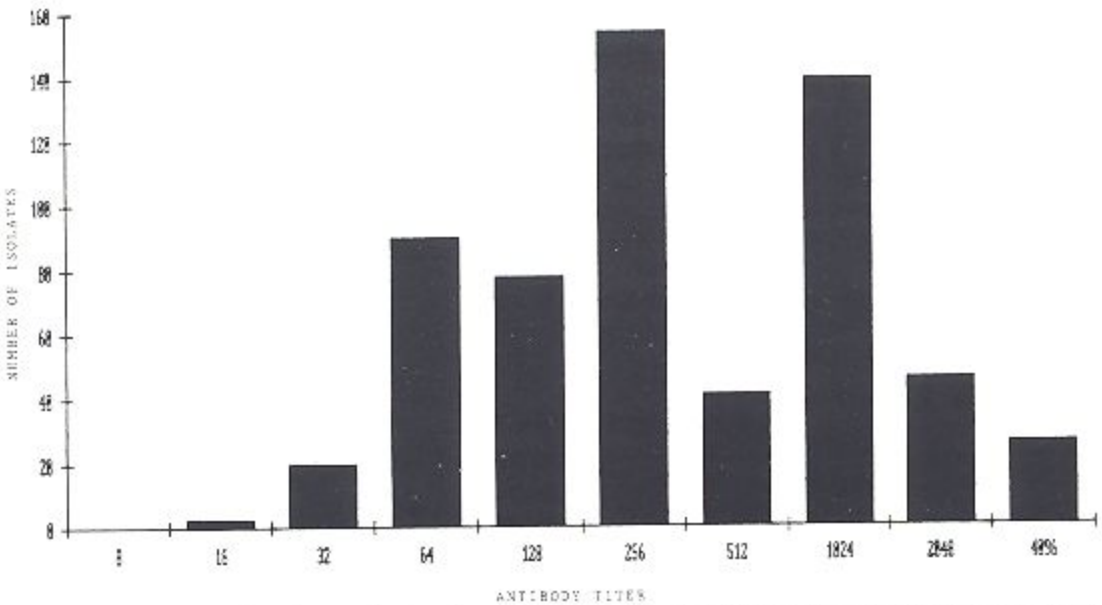

FIGURE 2. Distribution of neutralization titers of prototype Gamboa virus ascitic fluid vs. individual isolates from the Juan Mina study.

group virus. Since eggs were surface sterilized prior to isolation attempts, virus was ruled out as being on the surface of the eggs. The infection rates in egg clutches (13.1/1,000 clutches) could not be directly compared with that of other stages (mean 5.1/1,000 mosquitoes) since pool sizes were different. The pool size of stages other than eggs was 10, whereas the average number of eggs per clutch was ~160 (J. Petersen and B. Dutary, personal communication).

Since the MFIRs observed for each stage were similar, it appears that our system was not missing positives due to possible lower infectious titers in earlier stages. Processing of entire egg clutches as compared to pools of 10 individuals in later stages should favor the demonstration of positive clutches if the virus titer in the egg is lower than in larval stages. The infection rate in clutches, however, should not be higher than the MFIR for adult females, since the female of this species oviposits all her eggs in a single, discrete clutch; each egg clutch represents all offspring from a single ovipositing event. A possible explanation for the much higher infection rate observed in clutches than in adult females could be that our methodology incorporated a bias in favor of positive clutches. If virus infection prolongs eclosion time, our processing of eggs still unhatched 5 days after collection would have favored testing of virus-positive eggs, since the observed eclosion time is 3–6 days in this species (J. Petersen and B. Dutary, personal communication). The effect of virus infection on eclosion time requires further investigation.

TABLE 3

Results of cross neutralization tests with prototype Gamboa virus and other isolates from Panamanian Aedeomyia squamipennis *mosquitoes*

Virus strains*	Reciprocal titer of antibody to:		
	Prototype	Strain 046099	Strain 046737
Prototype	*4,096*	1,024	64
046099	256	*256*	16
046737	32	64	*1,024*

* Strains 046099 and 046737 from third and fourth instar larvae, respectively, March 1986, Chilibre River.

A separate estimate of the infection rate in clutches, obtained by testing individual surviving larvae from a smaller random sample of 150 clutches, provided a lower value of 6.7/1,000 clutches, which was compatible with the MFIR value for wild caught adult females.

Gamboa virus was isolated from *Ad. squamipennis* every month during the 28 month study. The monthly MFIR determined for combined mosquito stages (beyond the egg) showed a mean value of 4.9/1,000 and a range which remained within 2 standard deviations every month except in the middle of the dry season of February–March in 1986 and 1987, when significantly el-

evated peaks were noted. Virus amplification due to the introduction of an efficient intermediate host[7–9] into the cycle is the most probable explanation. The observed mosquito feeding habits,[9] the implication of the species as a vector of avian malaria,[7] and the high prevalence of Gamboa group antibodies in birds[4, 8] suggest that birds are the most likely intermediate hosts. Whether virus input from vertebrate hosts is necessary for long-term maintenance of Gamboa group virus remains to be studied.

Biologically, transovarial transmission of an arbovirus in its arthropod vector at an adequate rate would appear to be an extremely efficient mechanism to insure survival of the agent during adverse environmental conditions. Mathematical models of transovarial transmission[10, 11] suggest that if the filial infection rate is the same for all infected females, arboviruses could not persist in an insect population for more than a few generations by transovarial transmission alone. However, Turell and others[12, 13] provided evidence that filial infection rates might differ within a species depending upon the rate of maternal infection. They hypothesized 2 populations of females: 1 with nonstabilized viral infections acquired by ingesting virus from a viremic host, and the other consisting of transovarially infected females with stabilized infections in which the germ cells are persistently infected. The filial infection rate measured in females with stabilized infection of California encephalitis virus was 93%.[12] In our study, the 1 positive family showed a filial infection rate of 58%. Both of the hypothetical stabilized and nonstabilized mosquito infections could be present in the Juan Mina area.

ACKNOWLEDGMENTS

We offer our appreciation to Pedro Galindo, Director Emeritus, Gorgas Memorial Laboratory, for his advice. We thank Julio Cisneros, Leonardo Blake, Olga de Torres, Norberto Guerrero, Jose Montenegro, and Victor and Amable Herrera for technical assistance. This work was partially supported by NIH grant AI 22122.

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