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VIRUSES ISOLATED FROM *AEDEOMYIA SQUAMIPENNIS* MOSQUITOES COLLECTED IN PANAMA, ECUADOR, AND ARGENTINA: ESTABLISHMENT OF THE GAMBOA SEROGROUP

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Abstract. Twenty-four virus strains were isolated from *Aedeomyia squamipennis* mosquitoes collected in Ecuador. One additional strain each was isolated from this species from Panama and Argentina. All 26 isolates were shown to be related serologically to prototype Gamboa virus, originally isolated from *Ad. squamipennis* mosquitoes collected in Panama. Antigenic comparisons of eight strains, including prototype Gamboa virus, indicated the existence of four distinct viruses. Neutralization tests with sera from a variety of mammalian and avian species from Argentina provided further evidence that Gamboa serogroup viruses are transmitted between *Ad. squamipennis* and birds.

Gamboa virus was first isolated from *Aedeomyia squamipennis* (Lynch Arribalzaga) mosquitoes collected in Panama on 20 December 1962. The prototype strain, MARU-10962, was registered in the International Catalogue of Arboviruses as an ungrouped bunyavirus.[1] Four additional virus isolates closely related or identical to Gamboa virus were obtained from *Ad. squamipennis* in Panama. Of these isolates, MARU-11079 was tested by one of us (G.J.) and Dr. R. E. Shope at the Yale Arbovirus Research Unit, Yale University, New Haven, Connecticut, and was shown to be closely related but not identical to MARU-10962 by complement-fixation (CF), hemagglutination-inhibition, and neutralization tests. The two viruses, however, were not considered sufficiently distinct by these tests to warrant separate registration.

Between 1974 and 1978, field studies in Ecuador and Argentina resulted in the isolation of many virus strains from *Ad. squamipennis* mosquitoes. Preliminary testing indicated that these were all related to Gamboa prototype MARU-10962 or to strain MARU-11079.

This paper reports results of antigenic comparisons of these Gamboa-related viruses and provides evidence that MARU-11079, as well as two additional viruses, are distinct from the protytype. A classification scheme is suggested for what may now be considered the Gamboa serogroup of bunyaviruses.

Brief description of collection sites in Argentina and Ecuador

In Argentina, mosquito collections were made in November and December 1977 in Santa Fe Province. Three collection sites (Villa California, Santo Tome, and La Esmeralda) were located within a 10-mile radius of the city of Santa Fe, and one (Las Gamas) was situated 150 km north of Santa Fe. The area is at the transition between the pampa and espinal vegetational zones and is characterized by open, luxuriant grassland, pasture, cultivated fields of cereal crops, and a mosaic of open native forest of thorny trees and shrubs known as "*montes*." The collection sites were within 20 km of the Parana River. One site, Villa California, was located on a tributary of the Parana 1 mile from the river itself and was charac-

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* Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

terized by lagoons and partially flooded pastures. The climate is temperate; the collections were made in the antipodean springtime. Average rainfall is 500–1,000 mm annually, and the average annual temperature is 20°C.

Mosquito collections in Ecuador yielding Gamboa group virus strains were from the tropical monsoon region along the base of the western cordillera in the vicinity of the city of Babahoyo. The region is characterized by about 5 months of precipitation followed by several months of almost complete dryness. The native forest and vegetation is cut over in many areas to form small plantations or fincas. Crops grown in the area include banana, cacao, citrus fruits and sugar cane.

MATERIALS AND METHODS

Isolation of viruses from field-collected mosquitoes

Mosquito collections were made by using miniature CDC light traps supplemented with Dry Ice as an added attractant. Traps were operated from late afternoon until daybreak. Mosquitoes were anesthetized with CO_2 and placed in rubber-stoppered glass vials, which were held on Dry Ice or in a mechanical freezer at −60°C until they were returned to the Fort Collins laboratories. There the mosquitoes were thawed, identified with the aid of a stereoscopic microscope mounted on a chill table,[2] and pooled by species in groups of 100 or fewer. Pooled mosquitoes were triturated in chilled mortars in diluent consisting of Medium 199 supplemented with 20% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS) and antibiotics. After centrifugation, supernatant fluids were either inoculated directly or frozen and held at −70°C for later inoculation. Aliquots (0.2 ml) of each of the mosquito suspensions were inoculated onto monolayer cultures of primary Pekin duck embryo (DE) cells and a continuous cell line of African green monkey kidney (Vero) grown in 25-cm² (Corning®) plastic flasks. Monolayers were overlaid as previously described.[3] Cultures were observed for a minimum of 10 days for plaque formation. If plaques appeared, virus was passaged by removing the agar overlay and suspending the cells in 2 ml of the above diluent. Suspensions were reinoculated onto DE and Vero cell monolayers and also inoculated intracranially into 1- to 3-day-old suckling mice (SM). Mouse brain harvests from dead or moribund mice were used for virus identification[4] by CF tests.[5]

Identification of viruses

For preliminary identifications, CF tests[5] employed alkaline suspensions of SM brains as crude antigens of the isolates under study and a battery of immune reagents encompassing a large number of viruses, including Groups A, B, C, Bunyamwera, Guama, Capim, California, Patois, Simbu, Phlebotomus fever, Anopheles A, Anopheles B, Turlock, Changuinola, Vesicular stomatitis, Timbo, Hart Park, Hughes and Tacaribe and individual viruses Kwatta, Mirim, Aruac, Ieri, Triniti, and Gamboa.

Antisera were prepared according to standard methods for production of hyperimmune mouse ascitic fluids,[6] or by immunizing adult hamsters with 1,000 plaque-forming units (PFU) of virus given intraperitoneally at 4-weekly intervals.

For stock virus preparations in Vero cells, cultures grown in 25-cm² plastic flasks were inoculated with approximately 1,000 to 10,000 PFU of virus. When 3–4+ cytopathic effects occurred or at the 9th day after inoculation, whichever came first, heat inactivated FBS was added to a final concentration of 20%. Flasks were frozen and thawed, the contents were centrifuged at 900 × g for 10 min, and the supernate was aliquoted for storage at −60°C as stock virus. For production of virus in mice, SM were inoculated intracranially with 1,000–10,000 PFU of the isolate and frozen when they showed signs of illness. A 10% w/v suspension of infected SM brain in 4% bovine albumin phosphate-buffered saline, pH 7.2, was centrifuged at 900 × g for 15 min, and the supernate was aliquoted for storage at −60°C as stock virus. Virus titrations and serum dilution plaque reduction neutralization (N) tests were performed by the double agar overlay procedure described previously.[7] The second agar overlay, containing 1:25,000 neutral red, was added to each well on the 5th day after inoculation. Plaques were usually visible later that day and sufficiently distinct to enumerate by the next morning.

RESULTS

More than 770,000 mosquitoes from Ecuador and 44,464 from Argentina were collected during arbovirus field studies; detailed results of these studies will be reported elsewhere. There were

TABLE 1

History of Gamboa group virus strains isolated from Aedeomyia squamipennis *mosquitoes and used in comparative studies*

Strain	Date collected	Country of origin	Passage level*
MARU-10962	21 Dec 1962	Panama	SM 3
MARU-11079	9 Jan 1963	Panama	SM 8
E4-816	18 Jul 1974	Ecuador	V 3
75V-446	28 Aug 1974	Ecuador	V 3
65V-2374	27 Feb 1975	Ecuador	SM 4, V 1
75V-2621	29 Feb 1975	Ecuador	V 3
76V-4015	15 May 1975	Ecuador	V 3
78V-2441	30 Nov 1977	Argentina	V 2

* SM, suckling mice. V, Vero cells.

21,056 (2.7%) *Ad. squamipennis* in the Ecuadorian collection. From Argentina, 359 (0.8%) were *Ad. squamipennis*. The highest population of this species in Argentina, 322/14,247 (2.3%), was encountered at Villa California, in an area nearest the Parana River. Isolates from *Ad. squamipennis* were obtained from 22 of 469 pools from Ecuador and from three pools collected during the spring in Argentina (MIR 8.3:1,000). Two virus isolates were obtained from 322 *Ad. squamipennis* collected at Villa California (MIR 6.2:1,000) and one from 34 collected at Las Gamas (MIR 29:1,000).

Plaques appeared in Vero cells from 5–9 days after the cells were inoculated with mosquito suspensions. Subsequent passages in Vero cells reduced the time to appearance of plaques to a relatively uniform 5 days with titers of $10^{4.5}$ to $10^{6.7}$ plaque-forming units (PFU)/ml attained. Only 13 strains were reisolated from the same mosquito pools by inoculating SM. In addition, Vero cell culture harvests of some strains containing 2,000 to 20,000 PFU/0.02 ml passed to SM did not cause illness or death. In the case of mouse-pathogenic virus strains, SM showed signs of illness from 12–14 days after being inoculated with field-collected mosquitoes, with survival times reduced to 4–5 days after subsequent mouse passages.

Intracranial inoculations of suckling hamsters resulted in death only with Gamboa prototype strain (MARU-10962). No plaques were seen in duck embryo cells inoculated with any of the mosquito pools.

In CF tests, the Ecuadorian topotype (strain E4-816) reacted only with antibody to the Gamboa prototype strain. Attempts to confirm the identity of this virus as Gamboa by N tests indicated differences sufficient to suggest that strain E4-816 was a new virus. Subsequently, these two virus strains, as well as a second isolate from Panamanian *Ad. squamipennis* (MARU-11079), four additional isolates from Ecuador, and one of the isolates from Argentina were cross-tested by N in Vero cells. A history of the strains used in these comparative studies is presented in Table 1. Results of cross-testing these eight virus isolates by N tests in Vero cells are summarized in Table 2. All isolates were neutralized by at least two sera in addition to the homologous serum.

Only partial success was obtained when attempts were made to produce useable CF antigens for all eight strains by sucrose-acetone extraction[8] of infected Vero cell supernatant fluids. Both MARU-10962 (Gamboa prototype) and MARU-11079 yielded low titer (1:16) antigens, as evidenced by similarly low titer (1:8–1:64 reactions of antibody to the eight isolates. No reactions were obtained with supernates from Vero cells infected with strains from Ecuador (E4-816, 75V-446, 75V-2374, 75V-2621, 76V-4015) or Argentina (78V-2441). No differences were seen in CF antibody titers with antigens of the two Panamanian isolates.

TABLE 2

Results of cross-neutralization tests with prototype Gamboa virus (MARU-10962) and seven isolates from Aedeomyia squamipennis *mosquitoes from Panama, Ecuador and Argentina*

	Titer of antibody to:							
Strain	MARU-10962	75V-2621	E4-816	76V-4015	MARU-11079	78V-2441	75V-446	75V-2374
MARU-10962	640	40	80	20	40	10	10	<10
75V-2621	320	2,560	1,280	320	80	40	10	20
E4-816	80	>1,280	640	320	40	40	<20	<20
76V-4015	160	1,280	1,280	640	80	40	40	<20
MARU-11079	<40	20	<80	20	>1,280	20	<20	20
78V-2441	10	<20	40	<20	160	1,280	160	640
75V-446	20	<20	<20	<20	40	>640	640	>640
75V-2374	<10	<20	<10	<20	<40	160	80	320

TABLE 3

Prevalence of neutralizing antibody to strain 78V-2441 in birds and mammals of Santa Fe Province, Argentina, 1978

Species	No. pos./no. tested	% positive
BIRDS		
Family Columbidae	4/15	26.7
Furnaridae	9/87	10.3
Phytotomidae	1/12	8.3
Fringillidae	2/25	8.0
Ploceidae	1/120	0.8
Tyrranidae	0/24	
Icteridae	0/12	
Jacanidae	0/6	
Dendrocolapidae	0/4	
Formicaridae	0/2	
Piridae	0/2	
Thraupidae	0/1	
Caprimulgidae	0/1	
Subtotal	17/311	5.5
Domestic pigeon	7/15	46.7
Domestic goose	5/12	41.7
Domestic chicken	30/86	34.9
Domestic turkey	2/2	100.0
Domestic duck	1/2	50.0
Subtotal	45/117	38.5
Total	62/428	14.5
MAMMALS		
Order Rodentia		
Mus musculus brevirostris	0/31	
Cavia aperea pamparum	1/18	5.6
Akodon obscurus	1/14	7.1
Akodon azarae	0/4	
Rattus rattus frugivorus	0/4	
Calomys musculinus	0/1	
Calomys laucha	0/1	
Holochilus braziliensis	1/1	100.0
Rattus sp.	0/1	
Akodon sp.	0/1	
Order Marsupalia		
Lutreolina crassicaudata	0/12	
Didelphis albiventris	0/5	
Marmosa pusilla bruchi	0/1	
Order Lagomorpha		
Domestic rabbit	0/23	
Order Carnivora		
Domestic dog	1/1	100.0
Grand total	4/118	3.4

A serosurvey of birds and mammals collected in 1978 in Santa Fe Province, Argentina, showed a high prevalence rate of N antibody to Argentine strains 78V-2441 in birds, whereas few mammals were found to be immune (Table 3).

TABLE 4

Classification of Gamboa group viruses

Subgenus (group)	Complex	Species (prototype)	Subtype
Gamboa	Gamboa	Gamboa E4-816*	E4-816 75V-2621
	MARU-11079	MARU-11079† 75V-446‡	75V-446 78V-2441 75V-2374

* Provisional name, Pueblo Viejo virus.
† Provisional name, Alajuela virus.
‡ Provisional name, San Juan virus.

DISCUSSION

These results confirm the existence of a Gamboa serogroup comprised of two complexes, each composed of at least two serologically distinct viruses. The first complex, represented by prototype Gamboa virus MARU-10962, also includes the topotype Ecuadorian virus strain E4-816, for which we suggest the name Pueblo Viejo virus. Strains 75V-2621 and 76V-4015 isolated in Ecuador appear, by virtue of their reactivities with prototype Gamboa virus, to be variants of Pueblo Viejo virus. The second complex is represented by strain MARU-11079, for which we suggest the name Alajuela virus, and strain 75V-446, for which we suggest the name San Juan virus. All of these virus names are proposed according to the recommended procedure of naming a virus after the center of human habitation or prominent geographical site nearest the location at which the virus was isolated. Strains 75V-2374 and 78V-2441 appear to be variants of San Juan virus.

We consider the Gamboa group viruses to be properly classified as shown in Table 4.

The only species of mosquito from which Gamboa group viruses have been isolated is *Ad. squamipennis*. In addition, these are the only viruses which have been isolated from this species of mosquito. The distribution of *Ad. squamipennis* extends from Cuba to Argentina.[9] Since mosquitoes of this species are seldom abundant or troublesome, very little information is to be found in the literature concerning their biology. They are known to occur in permanent bodies of ground water with abundant vegetation, such as *Pistia* sp. and *Azolla* sp.[10–11] Transovarial transmission of Gamboa virus has not yet been demonstrated in *Ad. squamipennis* mosquitoes in Panama, but

workers at the Gorgas Memorial Laboratory have isolated Gamboa virus from mosquitoes collected throughout the year, from larval mosquitoes, and from both male and female *Ad. squamipennis*.[12] Isolation of the virus from *Ad. squamipennis* at high minimal infection rates in Argentina during the springtime also suggests the occurrence of transovarial transmission.

An additional 19 other viruses were identified by CF and/or N tests as belonging to the Gamboa serogroup. Preliminary results indicate that a number of Gamboa group viruses may exist. We consider it sufficient to define the group and illustrate the natural complexity of antigenic relationships among member viruses. Certain of these 19 isolates were neutralized by antisera to one or more of the eight strains intensively studied. Those which react to titer with more than one antiserum may represent natural recombinants or reassortants of these bunyaviruses. Had antisera been prepared with those which react to titer with only one antiserum, additional subtypes and variants might have been identified.

The vertebrate hosts of Gamboa virus are not known. The higher prevalence of N antibody to San Juan virus subtype 78V-2441 in birds than in mammals from Argentina suggests that the natural cycle of Gamboa serogroup viruses involves *Ad. squamipennis*-bird transmission. The high prevalence of infection in birds suggests that horizontal transmission and amplification of virus occurs. The role in viral maintenance of transovarial infection in the vector is uncertain, since *Ad. squamipennis* is active throughout the year. Inherited transmission may be an adaptation for assurance of virus survival during periods of low susceptible vertebrate-host density or low vector density at the temperate zone extreme of the distribution of *Ad. squamipennis* (e.g., Argentina).

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