California Group Arboviruses in Florida and Report of a New Strain, Keystone Virus

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THE PRESENCE of members of the California group of arboviruses was first demonstrated in Florida in 1963, when human serologic responses to the original California encephalitis virus (CEV) prototype antigen BFS-283 (*1*) were found by the University of Pittsburgh and the Encephalitis Research Center of the Florida State Board of Health. The isolation of members of this arbovirus group from mosquitoes collected during the 1962 epidemic of St. Louis encephalitis in the Tampa Bay area was reported subsequently by the Communicable Disease Center, Public Health Service (*2*).

Extensive studies by the authors and CDC investigators have shown that California group arboviruses are the most commonly recovered mosquito-borne virus agent in Florida. Others have shown that they also occur over a wide geographic area of the southeastern (*3*), central, midwestern (*4, 5*), far northwestern (*6*), and Pacific coastal (*7*) sections of the United States. Earlier reports of viral agents in the California group from Trinidad (*8*), Africa (*9*), Czechoslovakia (*10*), Yugoslavia (*11*), and Canada (*12*) give a worldwide significance to the small but rapidly growing serologically related group of arboviruses.

Our report summarizes virological, epidemiologic, and serologic studies of the California group of arboviruses in the Tampa Bay area of Florida from 1963 to 1965. In addition, a newly identified member of the group, the Keystone virus, is reported. The name Keystone was derived from a geographic location in the Tampa Bay area where mosquitoes were collected on the night of August 11, 1964. A report of the detailed serologic studies of this

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Epidemiologic studies were directed in part by Dr. Emily H. Gates, biological studies by Dr. William L. Jennings, entomological identification by Karen Meadows, serologic work by Florence Y. Lewis, and statistical analyses by Ingrid Baughman— all on the staff of the Encephalitis Research Center.

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and other known strains of the California group will appear in a separate publication. Data have been presented in part (*13*).

Methods

A comprehensive description of the methods used for studying these viruses by the Florida State Board of Health and the University of Pittsburgh has been published (*14*). Briefly, the 2- to 4-day-old suckling mouse has been the primary host system used for recovering these viruses from mosquitoes. Serologic studies for evidence of infection in man and mammals have been performed with sucrose-acetone-extracted (*15*) antigens prepared from the prototype BFS–283 strain (*1*). Standard techniques have been used for detecting hemagglutination-inhibition antibodies with the microtiter adaptation of the method described by Clarke and Casals (*15*). Serum-neutralization antibody studies have been performed in the 3- to 4-week-old weanling mouse by using the intracerebral route and the constant-serum, varying virus-dilution technique.

Identification of California Strains

During a 32-month period beginning January 1963, approximately 500,000 mosquitoes were collected and tested for viruses as a part of the routine surveillance program of the Encephalitis Research Center in the Tampa Bay area. In these mosquitoes, 41 pools were found positive for members of the California group. The pools were distributed among at least four different *Aedes* species of mosquitoes, representing 27,035 mosquitoes tested in 790 pools (table 1). The positive pools were collected throughout the entire Tampa Bay area where routine surveillance trapping is done, and they show the ubiquitous distribution of fresh floodwater *Aedes* mosquitoes. Positive pools were recovered every month of the year except September, November, and December. A definite relation between virus isolations and the abundance of *Aedes* mosquitoes is apparent. In turn, this is related to rainfall.

Mosquito-trapping techniques and the location of traps markedly influence the types of *Aedes* mosquitoes collected. *Aedes taeniorhynchus* comprise the most abundant species of mosquito in the Tampa Bay area; however, intensive efforts have not been made to trap it. The *Aedes atlanticus tormentor* species are more commonly attracted to lighted traps than to baited traps, but the latter were not used extensively in the surveillance program until 1964.

After initial isolation and identification of CEV strains by the Encephalitis Research Center laboratory in Tampa in 1963 and 1964, subsequent typing has been carried out at the University of Pittsburgh. We have summarized the following extensive studies, many still in process.

The eight viruses isolated in 1963 were identified as closely related to, or identical with, the trivittatus virus. All appeared to be similar (*14*). Ten of the viruses isolated in 1964, representing six isolations from *Aedes infirmatus* and four from *A. atlanticus tormentor* are currently being studied. Some tests are incomplete; others have progressed sufficiently to draw certain conclusions. Preliminary complement fixation tests with crude (pH 9.0 borate saline) antigens confirmed that all the 1964 strains belonged to the CEV group but indicated that all the isolates were not identical.

Mouse immune serums and sucrose-acetone-extracted antigens were prepared for each of the 10 isolates, and complement fixation cross comparisons are being made with representatives of the CEV group. Some resembled the earlier Florida prototype identified as trivittatus virus, but several appeared somewhat more closely related to the group prototype BFS–283 virus. BFS–283 antiserum reacted by comple-

Table 1. California group virus isolations in Florida mosquitoes, 1963–64

Year	Mosquito species	Number tested	Pools tested	Positive pools
1963	*Aedes infirmatus*	3, 882	234	8
1964	*Aedes infirmatus*	15, 534	382	10
1964	*Aedes atlanticus tormentor.*	2, 967	69	20
1964	*Aedes* species	1, 281	31	2
1964	*Aedes taeniorhynchus*	3, 371	74	1
	Total	27, 035	790	41

Table 2. Complement fixation cross comparison of two 1964 isolates with the California group BFS–283 and trivittatus viruses

Antigen	Antiserum			
	TA–28 [1]	TA–26 [2]	BFS–283	TB3–1 [3]
TA–28	[4] *256*	16	16	256
TA–26	16	*128*	64	16
BFS–283	16	16	*128*	16
TB3–1	256	16	32	*128*

[1] *Aedes infirmatus* isolate.
[2] *Aedes atlanticus tormentor* isolate, the Keystone virus.
[3] Florida prototype of trivittatus virus.
[4] Reciprocal of serum dilution showing complement fixation of more than 2.

ment fixation to higher titer with the Florida agents than with the trivitattus agents.

Two of the 1964 isolates have been compared by complement fixation (table 2). TA–28 (B64–2503.02) appears to be identical with the TB3–1 virus, the earlier Florida prototype resembling trivittatus. TA–26 (B64–5587.05) is distinctly different from TB3–1 and also differs significantly from BFS–283, the original CEV prototype from California.

At least 4 of the 10 viruses studied resemble the unique TA–26. Three of the four were isolated from the *A. atlanticus tormentor* and one from the *A. infirmatus* mosquitoes. Thus the mosquito species does not necessarily correlate with the type of virus isolated. By simultaneous cross-box titration, three of the TA–26-like agents, which have been completely compared, appear to be identical. The newer member of the CEV group from Florida, the Keystone, has been compared with other known isolates belonging in the group from the United States and abroad and is significantly different from all viruses so far available to us.

The Keystone virus was isolated from a pool of 55 engorged *A. atlanticus tormentor* mosquitoes collected in a stable trap, using an exposed donkey as bait. The agent produced death or sickness of an entire litter of eight suckling mice on the fifth day after inoculation. Subsequent second and third mouse passages reduced the incubation period to 3 to 5 days. The median lethal dose of the agent for weanling mice was $10^{-4.5}$ logs. In complement fixation identification studies at the Encephalitis Research Center, a BFS–283 antiserum titered 1 : 64 in the presence of a crude TA–26 antigen (pH 9.0 borate saline). Additional comparative studies at the University of Pittsburgh demonstrated that the virus was readily neutralized by immune serums to the CEV group (neutralization index=4.0), TB3–1 (neutralization index=3.6), trivittatus (neutralization index=3.4), La-Crosse (neutralization index=3.0), and Snowshoe hare (neutralization index=3.2). These confirmed the isolate as a member of the CEV group, and further demonstrated the advantages of the complement fixation test for typing.

These joint studies indicate the presence of at least two antigenic types of the CEV group in the Tampa Bay area during 1964: trivittatus and the newly identified Keystone strain. At the present time, the Keystone appears to represent another antigenic type not only for North America but for the world.

Evidence of Clinical Infection in Man

Since the major St. Louis encephalitis epidemic in the Tampa Bay area in 1962, routine surveillance for viral infections of the central nervous system in man has been carried out in the area. In a population of approximately 1 million, 832 persons have been referred to the center for viral diagnostic studies over a 32-month period beginning January 1963. Of these, 618 have received serologic tests for hemagglutination-inhibition antibodies to the California complex group, using the BFS–283 antigen (table 3). Paired serums from each

Table 3. Hemagglutination-inhibition antibodies to California group arboviruses in 618 central-nervous-system surveillance cases, Tampa Bay area, Fla., 1963–65

Surveillance cases	1963	1964	1965 [1]
Number of patients tested	56	369	193
Positive, no change in titer:			
1:10	[2] 13	1	2
1:20	[2] 6	2	1
Fourfold rise in titer	1	1	0

[1] August 1.
[2] Acetone-extracted serums.

suspected person have been tested simultaneously. The large number of persons with an unchanging titer in 1963 requires an explanation.

In the early part of the study, human serums were routinely extracted with acetone according to the standard technique (*4*). An unusually high number of such serums possessed hemagglutination inhibitors to the California antigens used in the test. In attempting to confirm this unexpectedly large number of positive serums, the laboratory at the University of Pittsburgh subjected the serums to both acetone and kaolin extraction. Both laboratories found that the inhibitor was frequently present after acetone extraction but not after kaolin extraction. Serum-neutralizing antibody studies of these serums, using the BFS–283 prototype virus, demonstrated that the inhibitor usually remaining after acetone extraction was most likely nonspecific. Serums with the inhibitor after kaolin extraction were also found to contain neutralizing antibody to the CEV group. All subsequent studies of human and mammalian serums at the Encephalitis Research Center were therefore carried out after kaolin extraction.

Three of the 369 surveillance patients tested in 1964 and three of the 193 tested so far in 1965 have had low unchanging hemagglutination-inhibition titers (table 3). However, in both 1963 and 1964, a single patient demonstrated a fourfold rise in antibody by the hemagglutination-inhibition test. One case has been reported in detail (*16*). The pertinent serologic findings for both patients are given in table 4. Both patients were children. S.H. manifested a relatively severe encephalitis syndrome from which she recovered within a period of 3 months without apparent sequelae. M.H. had a relatively mild encephalitis and apparent full recovery within 2 weeks. A fairly prompt rise and fall in hemagglutination-inhibition response occurred in both children within the first 3 months of illness. Complement fixation antibodies appeared more slowly and had not been detected in M.H. by the 94th day. Diagnostic rises in serum neutralization antibodies were detected in both patients. Extensive serologic tests for other arboviral agents and neurotropic viruses known to be present in the Tampa Bay area have been negative for both children.

Human Serologic Surveys

Another part of the continuing program of the Encephalitis Research Center in Tampa has been serologic surveys for inapparent human infection with various members of the arbovirus group. During the past 3 years, more than 1,000 such serums have been subjected to the hemagglutination-inhibition test, using the BFS–283 antigen (table 5). All serums tested and reported were kaolin extracted. The survey populations were predominantly healthy residents of households selected for serial blood samples after a major survey in 1962. The

Table 4. Two serologically confirmed cases of encephalitis due to a California group virus, Tampa Bay area, Fla., 1963–64

Patient	Onset	Serums collected [1]	Titer to California antigen BFS–283		
			Hemagglutination inhibition	Complement fixation	Neutralization index [2]
S.H., age 11, white, female	Aug. 20, 1963	3	<1:20	<1:4	<0.5
		16	1:80	<1:4	3.0
		30	1:640	1:16	3.5
		120	1:20	1:32	2.8
M.H., age 2, white, male	June 28, 1964	4	1:10	<1:4	<.2
		65	1:80	<1:4	2.7
		94	1:40	<1:4	2.6
		280	1:10	----------	----------

[1] Day after onset.
[2] Neutralization index $\log_{10}$, 3- or 4-week-old mice, intracerebral route.

Table 5. Human serologic surveys for California group arbovirus hemagglutination-inhibition antibodies,[1] Tampa Bay area, Fla., 1963–65

Year	Type of survey population	Total serums tested	Positive [2]		Range of titers			
			Number	Percent	<1:10	1:10	1:20	1:40
1963	Mentally retarded children	56	0					
1963	Hillsborough County	102	6	6	96	5	1	0
1964	Hillsborough, Manatee, and Pinellas Counties.	367	16	4	351	13	3	0
1965	Hillsborough, Manatee, and Pinellas Counties.	424	3	<1	421	2	1	0
1965	Migrant laborers	93	12	13	81	6	4	2

[1] Antigen BFS–283 prototype.
[2] Any titer of 1:10 or greater, kaolin-extracted serums.

Table 6. Animal serologic surveys for California group arbovirus hemagglutination-inhibition antibodies,[1] Tampa Bay area, Fla., 1964–65

Species	Total number tested	Positive [2]		Range of titers			
		Number	Percent	<1:10	1:10	1:20	1:40
Cotton rat	267	1	<1	266	0	1	0
Horse	149	6	4	143	5	1	0
Opossum	88	1	1	87	1	0	0
Rabbit	23	1	4	22	0	0	1
Raccoon	17	2	12	15	2	0	0
Squirrel	11	1	9	10	1	0	0
14 other	151	0		151			
Total	706	12	2	694	9	2	1

[1] Antigen BFS–283 prototype.
[2] Any titer of 1:10 or greater, kaolin-extracted serums.

results of studies of two special groups, mentally retarded children and migrant laborers, are also reported in table 5.

Using any titer of 1:10 or greater as a criterion for positives, the results show that 1 to 6 percent of the general population in the Tampa Bay area possess hemagglutination-inhibition antibody to the CEV group. In the special population surveys, none of the 56 mentally retarded children possessed hemagglutination-inhibition antibody; however, 2 had serum neutralization antibody. Thirteen percent of the migrant labor group studied in 1965 had hemagglutination-inhibition antibody, a not unexpected result due to their severe and prolonged exposure to mosquitoes in Florida and elsewhere.

Of the total group of survey serums with positive titers, 26 were at the level of 1:10, 9 at 1:20, and 2 at 1:40. Studies for confirmatory serum neutralization antibodies of 19 of these serums were carried out at the Encephalitis Research Center. Of 11 serums reacting at 1:10 by the hemagglutination-inhibition test, 9 were shown to have equal to or greater than 1.5 logs protection. Of six reacting at the 1:20 hemagglutination-inhibition titer level, all had equal to or greater than 1.5 logs protection. Of the two reacting at the 1:40 level, both were shown to possess serum neutralization antibodies equal to or greater than 2 logs protection. Similar results were found for a group of six other serums tested at the University of Pittsburgh laboratories.

Approximately 400 persons in the normal household survey group have submitted blood

samples annually for 3 years. No evidence of conversion from negative to positive hemagglutination-inhibition titers in any of the persons has been found over the 3-year period.

Animal Serologic Survey

Serologic tests have been performed on serums collected from 706 wild or domestic animals of 20 different species. Studies were performed as part of routine surveillance, as well as special studies of the vertebrate hosts of arboviruses known to be in the Tampa Bay area. The largest number of mammalian serums were collected from species of cotton rats, horses, opossums, rats, and the house mouse. A criterion of any hemagglutination-inhibition titer of 1:10 or greater on kaolin-extracted serums was applied to the BFS–283 antigen.

The species showing positive results are listed in table 6. One of 23 rabbits tested had a titer of 1:40. Titers of 1:20 were found in one each of 149 horses and 267 cotton rats. Low titers of 1:10 were also detected in nine serums from species of the squirrel, raccoon, opossum, and horse. Five of 12 reacting serums were subjected to the serum neutralization test, using the BFS–283 virus. Insufficient quantities did not permit the testing of all 12 serums. Only one of the five serums, that of the horse, with a hemagglutination-inhibition titer of 1:20, contained serum neutralization antibody at a level equal to or greater than 2 logs protection. The remaining four serums, all serum neutralization negative, had hemagglutination-inhibition antibody titers of 1:10.

Discussion

These studies clearly demonstrate the abundance of arboviruses belonging to the CEV group in the *Aedes* genus of mosquitoes in Florida. If appropriate trapping techniques are used when and where these mosquitoes are abundant, viruses of this group can be readily obtained throughout an entire area at any season of the year. These strains have demonstrated at least two distinct serotypes. One serotype, closely related if not identical to the trivittatus isolate (unpublished data of C. M. Eklund, director, Rocky Mountain Laboratory, Public Health Service) is most commonly recovered from *A. infirmatus*. The second serotype, the Keystone, appears to be distinct from other known members of the CEV group and was most frequently found during this study in the *A. atlanticus tormentor*.

Although our human and animal serologic studies have been carried out only with the BFS–283 antigen, detected infection with the CEV group in both man and animals is considerably less than that found in the Midwest and California, where similar extensive studies have been done (*4*, *7*). Only 2 clinical cases were found after examining 618 patients, and from 1 to 6 percent of the normal human population appear to have experienced inapparent infection with these viruses sometime in the past. The current level of transmission is less than 1 percent annually, based on serial observations of more than 400 persons. Serologic evidence of infection in mammals is extremely low by the hemagglutination-inhibition test and most of the hemagglutination-inhibition positives tested were not confirmed by the serum neutralization test. We do not know the significance of this. It is possible that the duration of hemagglutination-inhibition antibodies is too short for serologic survey work and that serum neutralization antibodies may reveal different prevalence rates in both man and animals.

Summary

The 1963–65 ecologic studies for arboviruses in the Tampa Bay area by the Encephalitis Research Center, Florida State Board of Health, have shown that the California encephalitis group arboviruses are the most commonly recovered viral agents from mosquitoes. Of 27,035 *Aedes* mosquitoes tested in 790 pools during the period January 1963 to December 1964, 41 pools were found to be positive. The trivittatus-like strain was recovered most frequently and predominantly from *Aedes infirmatus* mosquitoes. A newly identified strain, named the Keystone, was obtained from the *Aedes atlanticus tormentor*.

Human disease related to California viruses by serologic tests has been uncommon. Two patients with viral symptoms of the central nervous system were identified in 618 tested.

Human infection without history of central-nervous-system disease was found in 1 to 6 percent of the general population. The authors emphasized the importance of extracting human serums with kaolin rather than acetone to detect specific inhibitor to BFS–283 antigen.

The lower vertebrate source of the California encephalitis group arboviruses in Florida remains unknown. Extremely low rates of hemagglutination-inhibition antibody have been found in mammals. Preliminary serum neutralization tests suggest that most of these are nonspecific. The year-round recovery of the virus from mosquitoes, however, suggests its continual presence in a common host.

REFERENCES

(*1*) Hammon, W. McD., Reeves, W. C., and Sather, G.: California encephalitis virus, a newly described agent. II. Isolation and attempts to identify and characterize the agent. J Immunol 69: 493–510 (1952).

(*2*) Chamberlain, R. W., Sudia, W. D., Coleman, P. H., and Beadle, L. D.: Vector studies in the St. Louis encephalitis epidemic, Tampa Bay area, 1962. Amer J Trop Med 13: 456–461 (1964).

(*3*) U.S. Public Health Service: Summary of arbovirus encephalitis in the United States for the year 1964. Morbidity and Mortality Weekly Report, vol. 14, No. 29, Communicable Disease Center, Atlanta, Ga., July 24, 1965, pp. 246–248.

(*4*) Thompson, W. H., and Evans, A. S.: California encephalitis virus studies in Wisconsin. Amer J Epidem 81: 230–244 (1964).

(*5*) U.S. Public Health Service: Summary of California encephalitis cases in Wisconsin, Ohio and Indiana. Morbidity and Mortality Weekly Report, vol. 13, No. 47, Communicable Disease Center, Atlanta, Ga., Nov. 27, 1964, pp. 414–415.

(*6*) Newhouse, V. F., Burgdorfer, W., McKiel, J. A., and Gregson, J. D.: California encephalitis virus: Serologic survey of small wild mammals in northern United States and southern Canada and isolation of additional strains. Amer J Hyg 78: 123–129 (1963).

(*7*) Gresikova, M., Reeves, W. C., and Scrivani, R. P.: California encephalitis virus, an evaluation of its continued epidemic status in Kern County, California. Amer J Hyg 80: 229–234 (1964).

(*8*) Spence, L., Anderson, C. R., Aitken, T. H. G., and Downs, W. G.: Melao virus, a new agent isolated from Trinidadian mosquitoes. Amer J Trop Med 11: 687–690 (1962).

(*9*) Kokernot, R. M., et al.: Isolation of viruses from mosquitoes collected at Lumbo, Mozambique. I. Lumbo virus, a new virus isolated from *Aedes* (*Skusea*) *pembaensis* Theobald. Amer J Trop Med 11: 678–682 (1962).

(*10*) Bardos, V., and Danielova, V.: The Tahyna virus; a virus isolated from mosquitoes in Czechoslovakia. J Hyg Epidem (Praha) 3: 264–276 (1959).

(*11*) Likar, M., and Casals, J.: Isolations from man in Slovenia of a virus belonging to the California complex of arthropod-borne viruses. Nature (London) 197: 1131 (1963).

(*12*) McKiel, J. A., et al.: Viruses of California encephalitis complex in indicator rabbits. Amer J Trop Med 15: 98–102 (1966).

(*13*) Hammon, W. McD., and Sather, G. E.: History and recent reappearance of viruses in the California encephalitis group. Amer J Trop Med 15: 199–204 (1966).

(*14*) Lewis, A. L., et al.: Isolations of a California group arbovirus from Florida mosquitoes. Amer J Trop Med 14: 451–455 (1965).

(*15*) Clarke, D. H., and Casals, J.: Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Amer J Trop Med 7: 561–573 (1958).

(*16*) Quick, D. T., et al.: California encephalitis virus infection; a case report. Amer J Trop Med 14: 456–459 (1965).

Vaccine Against Rubella

THE FIRST EFFECTIVE experimental vaccine against rubella has been developed by Dr. Harry M. Meyer, Jr., and Dr. Paul D. Parkman of the Division of Biologics Standards, National Institutes of Health, Public Health Service.

Working with the Department of Pediatrics of the University of Arkansas Medical School and the Arkansas Children's Colony, near Conway, the pediatrician-virologists vaccinated eight rubella-susceptible children in the pilot clinical trial. The children developed immunity with no accompanying fever or rash and their eight rubella-susceptible playmates, with whom they shared a cottage, remained uninfected. The same results were obtained when an additional 26 children were inoculated and were in contact with 22 uninoculated playmates.

In searching for a strain of the rubella virus that would confer long-term immunity, Meyer and Parkman subjected the virus to 77 passages in primary African green monkey kidney cell cultures over a 2-year period. Their study of the biological characteristics of each virus passage level showed high-passage virus to be distinctly different from low-passage "virulent" virus. The high-passage material caused a rapid cytopathic effect and other distinctive changes in certain types of tissue cultures unaffected by the virulent virus. It also induced the production of increased amounts of interferon when propagated in tissue cultures. High-passage virus of the 77th passage level (HPV–77) immunized rhesus monkeys without viremia or spread to uninoculated contacts.

The HPV–77 strain was considered sufficiently modified to be used in a clinical trial, and the experimental vaccine was prepared, using the same standards and safety tests that apply to the production of live attenuated vaccines for measles and poliomyelitis. The attenuated rubella virus strain is being made available to university investigators and a number of pharmaceutical companies that are working toward a rubella vaccine.

Dr. Meyer (front) and Dr. Parkman (rear) with students of Arkansas Children's Colony and nurse Mrs. Bill Holloway