SOME EVIDENCE OF LOCAL ORIGIN OF EEE VIRUS IN FLORIDA [1]

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Several epidemiologically distinct features of eastern equine encephalomyelitis (EEE) are observed repeatedly during endemic and epidemic periods; (see Table 1). Major voids in the knowledge of various aspects of the etiology persist. Some of these are: The vector of the virus during endemic periods; the location of the virus during the winter season in the United States; and the method of virus activation during favorable seasons in the United States. In other words, is the virus transmitted during endemic periods by only one or two species of mosquitoes; and further, is the virus removed completely in the winter from its summer location to be reintroduced the following spring or summer by migratory birds, or does it persist in an arthropod or other animal host in a latent or masked stage from year to year in the United States?

While none of these highly complex problems was approached specifically in the study reported upon here, the study was comprehensive. It involved collections of arthropods and bird bloods, virus isolation studies, serological studies of birds and ecological studies in Georgia, South Carolina, and central Florida during 1957 and 1958. The major emphasis was placed on central Florida, in the Orlando area, during the spring and early summer of 1958, and this report covers only that aspect.

Florida is considered an especially fertile area for studying EEE. The Florida peninsula serves as a point of departure and arrival for the migratory birds, primary reservoirs of EEE virus, to and from Cuba, Central and South America, and Mexico (7). Furthermore, the reported incidence of equine cases of disease during the past 5 years has exceeded the reported incidence in other states by a considerable margin. For example: In Florida during 1956 there were 107 cases, in 1957 there

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TABLE 1.—Repeated observations during endemic and epidemic periods of EEE in the United States

Endemic period	Epidemic period
Low to moderate rate of serum neutralizing antibody in wild birds (1, 2).	High rate of serum neutralizing antibody among wild birds late in period (1, 2).
Infrequent indications of circulating virus in wild birds (1, 2).	Relatively frequent isolations of virus from wild birds (1, 2).
Localization of inapparent disease in large wading birds (3).	Inapparent disease in a wide variety of wild birds (3).
Few equine cases and rare human cases (4).	Numerous equine cases and outbreaks of human cases (4).
Biological vector uncertain.	Biological vectors incriminated most frequently, *Culiseta melanura* (5, 6).

were 386, and in 1958 there were 94 cases (8). Yet with this indication of virus activity, never has the virus of EEE been isolated from mosquitoes taken in Florida even though numerous workers have attempted to do so. Most of these attempts have not been published, presumably because of the negative results obtained.

FIELD AND LABORATORY STUDIES

ARTHROPODS. Adult mosquito collections were made from March to July at 11 different sites within a 50-mile radius of Orlando, Florida. Most of the sites were located immediately adjacent to, or within fresh water cypress and oak swamps. Both standard and modified New Jersey light traps, fitted with live-catch containers, were used for collection. Collections were removed at midnight and dawn of each day throughout the study. Specimens were identified while alive, sealed in screw-cap vials, placed on dry ice and held for subsequent virus isolation attempts. Within a week the specimens were transferred to an electric freezer, maintained at –60° C. Suspensions of high suspect vectors such as *Culiseta melanura* (Coq.), *Mansonia perturbans* (Walker) and *Aedes sollicitans* (Walker) were inoculated intracerebrally into three-week-old Swiss white mice within 10 days of collection. Other species had a storage period of 10 to 30 days. Preparation of mosquitoes into a suspension for intracerebral inoculation into three-week-old mice followed standard procedures except that 20 percent normal rabbit serum (phosphate buffered to pH 7.6) was used as a diluent.

A total of 5611 adult, unengorged female mosquitoes were retained for virus isolation attempts. Of this total, representing 8 genera and 24 species, there were 215 specimens of *Culiseta melanura*, 154 specimens of *Mansonia perturbans* and 8 specimens of *Aedes sollicitans*.

None of the mosquito pools inoculated into mice produced any lethal pattern which could be perpetuated or isolated.

BIRDS. Birds were collected by using Japanese Mist Nets and by shooting. Blood was collected from birds by several different methods, as dictated by the size of the bird. Small birds were difficult to bleed from veins or by cardiac puncture. Bending the head backwards and snipping the throat with iris scissors and allowing the blood to spurt into a sterile vial proved convenient for small birds. Larger birds were bled from veins or by cardiac puncture using a small hypodermic needle and suitably sized syringe. Blood was immediately transferred into sterile vaccine bottles and placed in refrigeration. When an adequate quantity of blood permitted, serum was removed and the residual blood was diluted with an equal volume of 20 percent normal rabbit serum. The blood was then frozen and held for virus isolation attempts. The serum was retained under refrigeration until serological studies

were performed. Frozen whole bloods were used both for virus isolation attempts and serological studies.

Three hundred and twenty birds, representing 44 species, were collected during the spring and early summer of 1958 in central Florida. Ninety-six specimens of serum or whole blood neutralized at least 1.5 logs of EEE virus. Four of these specimens also neutralized at least 1.5 logs of WEE virus, see Table 2.

Hemagglutination-inhibition (HI) test of whole bloods, reportedly satisfactory at some laboratories (10) gave equivocal results. HI test of 65 bird sera gave clear-cut results at a dilution of 1:20, using 8 units

TABLE 2.—Results of studies on birds collected in Florida, 1958

Species	Residence status (9)	Pos. EEE (WEE) *neut. test*[a] Total Coll.	EEE virus isolated
Water turkey	Perm.	1/4	0
Double-breasted cormorant	Perm.	0/1	0
Green heron	Perm.	0/2	0
Little blue heron	Perm.	1 (1)/2	0
American egret	Perm.	2/8	0
Snowy egret	Perm.	2/4	0
Osprey	Perm.	1 (1)/1	0
Bob-white	Perm.	1/3	0
Killdeer	Perm. & Winter	2/5	0
Spotted sandpiper	Winter	0/1	0
Ground dove	Perm. & Winter	0/3	0
Yellow-billed cuckoo	Summer	0/1	0
Chuck-will's widow	Summer	0/2	0
Red-headed woodpecker	Perm.	8/21	0
Red-bellied woodpecker	Perm.	4/12	0
Pileated woodpecker	Perm.	1/3	0
Red-cockaded woodpecker	Perm.	0/1	0
Yellow-shafted flicker	Perm.	0/4	0
Crested flycatcher	Summer	2/16	0
Eastern kingbird	Summer	0/6	0
Crow	Perm.	2/7	0
Bluejay	Perm.	2/7	0
Tufted titmouse	Perm.	1/4	1
Carolina wren	Perm.	0/5	1
Blue-gray gnatcatcher	Perm.	0/2	0
Bluebird	Perm. & Winter	6/16	1
Catbird	Perm. & Winter	1/2	0
Mockingbird	Perm.	2/4	0
Brown thrasher	Perm. & Winter	1/3	0
Loggerheaded shrike	Perm. & Winter	7/14	0
Yellow-throated warbler	Summer	0/1	0
Parula warbler	Perm. & Winter	0/3	0
Northern waterthrush	Winter	0/1	0
Blackpoll warbler	Winter	0/1	0
Meadowlark	Perm. & Winter	25 (2)/59	1
Red-wing blackbird	Perm. & Winter	3/16	0
Rusty blackbird (?)	Winter	2/6	1
Brewer's blackbird (?)	?	1/9	0
Boat-tailed grackle	Perm.	3/6	0
Summer tanager	Summer	1/1	0
Cardinal	Perm.	10/29	0
Towhee	Perm. & Winter	4/22	1
English sparrow	Perm.	0/1	0
Pine-woods sparrow	Perm.	0/1	0
Total		96 (4)/320	6

[a] Neutralized at least 1.5 logs of virus.

TABLE 3.—Comparison of EEE serum neutralization and hemagglutination-inhibition tests of 65 bird sera collected in Florida, 1958

Serum Neut. test	HI test	
	Positive [b]	Negative
Positive [a]	17	6
Negative	14	28
Total	65	

[a] Neutralized at least 1.5 logs of EEE virus.
[b] Inhibited hemagglutination at a dilution of 1:20 or greater using 8 units of antigen.

of antigen. Some specimens inhibited hemagglutination at dilutions as high as 1:160. A modification of the method of Clarke and Casals (11) was used. Table 3 shows the results of HI tests of 65 bird sera in comparison with the results of serum neutralization tests of the same sera.

Whole bloods and normal rabbit serum diluted bloods (from which most of the original serum had been removed) were inoculated intracerebrally into three-week-old mice for virus isolation attempts. Virus was isolated from six species of birds, three of which were collected on the same day from the same location. All six isolates were identified as EEE virus by serum neutralization tests, see Table 4. Hyperimmune serums to the viruses of EEE, WEE and SLE were prepared in rabbits. (Difficulty was experienced in maintaining a reasonably uniform infectivity titer with SLE virus, hence its homologous antiserum was unreliable in these tests.)

TABLE 4.—Identification of virus isolates obtained from birds collected in Florida, 1958

Blood source	Neutralization test results (Log LD_{50} of neutralization index)	
	EEE rabbit immune serum. (J-90B strain EEE virus*)	WEE rabbit immune serum. (L2-34a strain WEE virus*)
Rusty blackbird (?)	3.7	< 1.0
Meadowlark	3.2	1.0
Tufted titmouse	4.0	< 1.0
Carolina wren	3.0	< 1.0
Towhee	3.5	< 1.0
Bluebird	3.3	< 1.0

* Furnished by Dr. R. W. Chamberlain, Montgomery Laboratories, US DHEW, PHS, CDC.

DISCUSSION

The biological vector of EEE virus in Florida is still not established. Presumably the same species of mosquitoes which have been considered as vectors elsewhere, with the possible exception of Connecticut (12), would also be considered in Florida, since all the areas support populations of *Culiseta melanura* and *Mansonia perturbans*.

In the present study, of the 44 species of birds collected, 23 are classified as permanent residents, 10 as both permanent and winter residents, 5 as winter residents (two of which are of questionable identity) and 6 as summer residents (12).

The isolation and identification of EEE virus from six species of passerine birds indicates considerable virus activity in the area.

The laboratory finding of 30 percent "immunes" among the bird population by serum neutralization test is consistent with the findings of others following an epidemic cycle of EEE virus. The four specimens which showed immunity to WEE infection might well be instances of cross-reaction. However, WEE infection was diagnosed in horses in Flagler County, Florida in 1959 (13).

Little has been published in the literature pertaining to the use of the HI test on wild bird sera and a great deal of additional work is indicated. Assuming that the response observed in EEE experimentally infected chicks (wherein HI antibody is consistently demonstrable before serum neutralizing antibody, the latter appearing approximately 10 days following infection (14)) is analogous to the response in wild birds naturally infected with EEE, considerable speculation as to geographical origin of infection may be made. The results of these tests in this study lend strength to the opinion that there is a similarity between the response elicited from chicks following experimental infection with EEE virus and response of wild birds naturally infected with EEE virus.

Of the 17 birds found positive both by HI and serum neutralization tests, none were recognized as immatures. This is interpreted to mean that the birds in this group were infected in a previous year or sufficiently early in 1958 to allow for the development of serum neutralizing antibody by the time the survey was conducted.

The finding of 6 birds negative by HI test but positive by serum neutralization test is interpreted as being indicative of old infections or the result of individual variation among wild birds for the persistence of HI antibody. There is great need for studying the duration of HI antibody in EEE infected wild birds. Once these base-line data have been determined, the value of the HI test in this procedure can be established.

Fourteen birds were positive by HI test but negative by serum neutralization test. Additionally, 3 of the 14 birds were recognized as immature forms, suggesting local hatching. If these 14 specimens, particularly the three immatures, merely had not yet had time to develop serum neutralizing antibody, only two obvious conclusions are possible. Either they became infected just before arriving in the area or they became infected almost immediately after arriving in the area. The sparse information in the literature pertaining to the duration of serum neutralizing antibody in wild birds, experimentally and naturally infected with the virus of EEE, leaves a wide margin for ultimate contradiction. It has been shown in some experimentally infected wild birds that high levels of serum neutralizing antibody are obtained but their duration, as with HI antibody, also is unknown (3). From field and laboratory studies one can conclude that EEE serum neutralizing antibody persists at least one year in certain avian species (3), though to pursue the conclusion beyond this period is difficult to confirm.

Most present workers consider the bird to be the most important key to a full understanding of EEE in the United States. The problem of ascertaining whether the virus is reintroduced each year into the United States by northward migrating birds or whether the virus overwinters in the United States within its endemic range in an arthropod or other animal is a cardinal one. However, once the virus of EEE is found overwintering in the United States in a form which will allow its transmission under favorable conditions, the question of its annual reintroduction by migratory birds will be relegated to almost academic interest. In Florida, bird studies are more complex than in many other areas. Permanent resident species are diluted by northern members of the same species during the winter season. Some of the permanent residents, perhaps accompanied by northern migrants, emigrate to Cuba, Central and South America, Mexico, and probably to other Gulf coast areas of the United States; to immigrate again the following year. This situation serves to complicate field studies and point out the necessity for intimate ornithological assistance.

The study revealed that the virus of EEE was extremely active in localized foci and probably would have precipitated an equine outbreak were the populations of both vectors and susceptibles quantitatively adequate. This leads to the postulation that the virus of EEE in central Florida exists year-round in delimited fresh-water-swamp areas, becoming active during favorable seasons and held in check by the combined influences of susceptible hosts and density of efficient vectors.

SUMMARY

The data obtained during the 1958 Florida study reveal:

a. Absence of detectable virus in the mosquito population during a seasonal period when few equine cases attributable to the virus of EEE were reported in the immediate vicinity. Only 10 equine cases were reported from the study area during 1958 as compared with 67 cases the previous year.

b. The finding of 30 percent "EEE immunes" among the wild bird population by serum neutralization test during the spring and early summer of 1958 con-

firms a widespread exposure to the virus in a previous period, presumably 1957.

c. The detection of HI antibody to the virus of EEE in 14 birds, three of which were immature forms, without detectable serum neutralizing antibody gives rise to the opinion that the infection was recent and of local origin.

d. The relationship of the virological and serological findings to the epidemiology of EEE in the United States is discussed.

References

1. FEEMSTER, R. F., WHEELER, R. E., DANIELS, J. B., ROSE, H. D., KISSLING, R. E., HAYES, R. O., ALEXANDER, E. R., and MURRAY, W. A. 1958. Field and laboratory studies on equine encephalitis. New England Jour. Med. 259:107–113.

2. STAMM, D. D. 1958. Studies on the ecology of equine encephalomyelitis. Amer. Jour. Pub. Health 48:328–335.

3. KISSLING, R. E., CHAMBERLAIN, R. W., SIKES, R. K., and EIDSON, M. E. 1954. Studies on the North American arthropod-borne encephalitides. III. Eastern equine encephalitis in wild birds. Amer. Jour. Hyg. 60:251–265.

4. Surveillance Reports, Arthropod-borne encephalitis in the United States. 1955–1958. U. S. Dept. Health, Ed., and Welfare, PHS, Commun. Dis. Center.

5. CHAMBERLAIN, R. W., RUBIN, H., KISSLING, R. E., and EIDSON, M. E. 1951. Recovery of virus of eastern equine encephalomyelitis from a mosquito, *Culiseta melanura* (Coquillett). Proc. Soc. Exptl. Biol. and Med. 77:396–397.

6. BURBUTIS, P. P., and JOBBINS, D. M. 1957. *Culiseta melanura* (Coq.) and eastern equine encephalomyelitis in New Jersey. Proc. N. J. Mosq. Exterm. Assoc., pp. 68–78.

7. LINCOLN, F. C. 1950. Migration of birds. Dept. of the Interior, Fish and Wildlife Service, Circular 16. 102 pp.

8. Animal Morbidity Reports. 1956–1958. Florida State Board of Health.

9. AUSTIN, L. O. 1959. Curator of Birds, Florida State Museum. Personal Communication.

10. ANONYMOUS. 1957. Arthropod-borne encephalitis. Procedures for investigating outbreaks. Dept. of Health, Ed., and Welfare, CDC, Montgomery Lab. 22 pp.

11. CLARKE, D. H. and CASALS, J. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Amer. Jour. Trop. Med. and Hgy. 7:561–573.

12. WALLIS, ROBERT C. 1959. *Culiseta melanura* (Coquillett) and eastern equine encephalitis in Connecticut. Mosq. News 19:157–158.

13. SCATTERDAY, J. E. 1960. Fla. State Bd. of Health. Personal Communication.

14. BYRNE, R. J. and ROBBINS, M. L. 1960. Mortality patterns and antibody response in chicks inoculated with eastern equine encephalomyelitis virus. To be published.