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EPIDEMIOLOGICAL STUDIES ON EASTERN EQUINE ENCEPHALITIS VIRUS IN SÃO PAULO, BRAZIL

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SUMMARY

An arbovirus surveillance is being undertaken in the State of São Paulo, Brazil, and several isolations of EEE obtained during the program showed that this virus is not endemic in spite of being responsible for horse epizootics in the region. A silent forest cycle was detected without any horse involvement, since EEE virus was isolated from mammals, birds, mosquitoes and sentinel mice in three different field stations, from 1969 to 1971. The role of wild birds and mammals in EEE cycle was evidenced and gave rise to several questions regarding the duration, mechanism of introduction, maintenance of the virus in nature and its importance to Public Health in South Brazil.

INTRODUCTION

Eastern Equine Encephalitis (EEE) virus has been isolated regularly in the Americas since 1933 [3], causing epidemics of encephalitis and horse epizootics. In Brazil it has been isolated from different areas along the Atlantic Coast. In the State of São Paulo, EEE virus has caused sporadic horse epizootics since 1937, when it was first recognized and all isolations were obtained from horses [4, 11, 12]. As a study on ecology of arbovirus in forested areas around the City of São Paulo is being undertaken since 1961, several isolations of EEE virus were made from mammals, birds and mosquitoes. This paper deals with these isolations as well as with serological and epidemiological data obtained.

MATERIALS AND METHODS

The field stations used in this study were located in Itapetininga, Casa Grande and Rio Guaratuba.

Itapetininga (23°40′ SL and 48°05′WL) is located in the interior of São Paulo State, in the beginning of the Brazilian central plateau. The region is formed by natural fields where rivers and creeks are surrounded by gallery forests which act as a concentration place for the local fauna.

The second field station, Casa Grande (23°40′SL and 45°55′WL) is located on a mountain range, called "Serra do Mar", which borders the Atlantic Ocean. The region has an altitude of about 800 meters and is covered by an extensive very humid primary forest. Immediately under Casa Grande but at sea level there is the third field station called Rio Guaratuba (23°45′SL and 45°55′WL), near a river of the same name. The mountains form a kind of a sharp wall and the forest which covers Casa Grande spreads over Rio Guaratuba without solution of continuity. In these field stations we collected birds, mammals, mosquitoes and exposed sentinel animals.

Birds were netted using Japanese mist nets (NEBBA type A with 36 mm mesh). The birds were bled by cardiac or jugular puncture, after which they were identified, banded and released. The blood obtained in a solution of heparin was brought to the laboratory, under refrigeration. Part was inoculated in suckling mice and the remaning was

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used for serological testing. Mammals were trapped using metal traps and brought alive to the laboratory. They were sacrified by exanguination and the carcasses were identified tentativelly by us and sent to the Mammals Identification Service from the U.S. National Museum to confirm or correct the identification. The blood was used for serological purposes and a pool of heart and kidney was inoculated in infant Swiss mice. Sentinel animals were exposed under metal hoods inside the forests and mosquitoes were collected using human baits for diurnal and light traps for nocturnal collections.

The viruses were identified by complement-fixation (CF) tests as described by CASALS [2]. Immune ascitic fluids (AF) were prepared using methods similar to those described by TIKASINGH et al.[15] and immune AF and sucrose-acetone extracted mouse brains reacted in CF tests in a grid fashion. Hemagglutination-inhibition (HI) tests were done according to the methods described by CLARKE & CASALS [6] using a microtechnique with an initial serum dilution of 1/40. Sera were treated with acetone to avoid non-specific inhibitors. Neutralization (NT) testing was done in VERO cells using 50-100 TCD 50/ml. A serum found to protect against the virus cytopathogenic effect was considered as positive.

We used two known strains of EEE to identify our isolates. Tatui was the first strain of the virus isolated in São Paulo. It has undergone an unknown number of passages in guinea-pigs intracerebrally at the Instituto Biológico, São Paulo, from where it came, and three passages in suckling mice in our laboratory. TRVL 24443, the second EEE strain, was isolated in Trinidad in 1959 and supplied by the Rockfeller Foundation Virus Laboratories. It was used in its tenth mouse passage.

RESULTS

The two initial isolations of EEE virus came from two *Oryzomys* spp (*) captured at Itapetininga field station in the last quarter of 1969. They were very fast agents, killing mice rapidly and behaving differently from all viruses isolated from the studied areas till that time. Both viruses were identical in serological testing and the strain with laboratory number SPAn 14723 was chosen as prototype. An antigen from infant mouse brains which reacted by CF tests with homologous serum was tested with sera of arbovirus Groups A, B, C, Bunyamwera and Guama as well as several other grouped and ungrouped viruses. A strong cross-reaction was observed with EEE virus and further CF and HI tests confirmed this finding. It was shown that SPAn 14723 reacted to titer with both strains of EEE virus to which it was serologically compared (Table I). Fourteen other strains of EEE virus were isolated by us and identified by CF testing only, antigens reacting against immune AF prepared with our prototype strain, SPAn 14723 (Table II).

The serological comparison of SPAn 14723 and other members of arbovirus Group A as Mayaro, Una, Mucambo, Western Equine Encephalitis viruses, gave the same results usually obtained in Group A serological testing [1, 14], and therefore were not described.

SERUM SURVEY

Before 1969 we have not observed any human or animal sera with antibodies for EEE virus in the samples coming from above mentioned field stations. In 1971, of 1896 sera of wild birds and mammals captured and examined in HI tests we observed that some of them were able to inhibit the viral agglutination with titers from 1/40 to 1/160 (Table III). The positive sera were submitted to confirmatory NT tests when the quantity of serum was enough. Sera from 208 Itapetininga field station inhabitants were tested in NT tests and 39 were positive, but none of 284 inhabitants of Casa Grande showed neutralizing antibodies.

DISCUSSION

EEE virus has been isolated along the Atlantic side of the Americas, from Northeastern United States to Argentine [14]. Epi-

(*) Some of the *Oryzomys* sent to the U.S. National Museum were not classified to species since it seems that apparently they belong to some species previously unrecorded in São Paulo, Brazil. (Dr. Ronald H. Pine, Mammal Identification Service, U.S. National Museum, personal communication)

TABLE I

Serological tests for identification of SPAn 14723 as a Eastern Equine Encephalitis virus strain

	SPAn 14723 Brain antigen titer (*)		SPAn 14723 AF titer		
AF Fluid	HI H_t/H_o	CF H_t/H_o	Antigen	HI H_t/H_o	CF H_t/H_o
An 14723	640	1024	An 14723	640	256
EEE Tr 24443	640/640	512/1024	EEE Tr 24443	640/1280	128/128
EEE Tatui	320/640	512/1024	EEE Tatui	640/1280	64/64

(*) Titer expressed as a reciprocal of the heterologous AF titer/homologous AF titer.
H_t: heterologous titer
H_o: homologous titer
HItests with 8 units of antigen

TABLE II

Strains of EEE virus isolated from various sources in São Paulo, Brazil

Strain no.	Date of collection	Place	Source
An 14536	31-10-69	Itapetininga	*Oryzomys* sp
An 14723	5-12-69	"	*Oryzomys* sp
An 14771	11-12-69	Casa Grande	*Didelphis marsupialis*
An 15875	1- 5-70	Itapetininga	*Chiroxiphia caudata*
An 15877	1- 5-70	"	*Chiroxiphia caudata*
An 15879	1- 5-70	"	*Pipromorpha rufiventris*
An 16138	14- 6-70	Rio Guaratuba	*Turdus albicollis*
An 16158	14- 6-70	" "	*Turdus albicollis*
An 16192	14- 6-70	" "	*Dendrocincla fuliginosa*
An 16250	14- 7-70	Casa Grande	*Oryzomys nigripes*
An 16259	16- 7-70	" "	*Oryzomys nigripes*
An 16360	16- 8-70	" "	*Grallaria varia*
An 16738	10- 9-70	Rio Guaratuba	Sentinel mice (mother)
An 17249	30-11-70	Cotia	*Phoniomyia pilicauda*
An 18629	28- 4-71	Rio Guaratuba	Sentinel mice (mother)
An 18716	5- 5-71	" "	Sentinel mice (infant)

demics of encephalitis have occurred in the United States, Dominican Republic, Jamaica and epizootics are regularly observed in the United States and sporadically in Central and South Americas [3]. In São Paulo, Brazil, this virus was isolated in 1937 by CARNEIRO [4] and it causes sporadic epizootics in horses. No disease in human beings due to EEE infection has been reported in this region and the serological surveys carried out on sera from farmers living around the places where two different epizootics occurred did not show detectable antibodies [11, 12].

We started our surveillance program on arbovirus in several forested areas around the Capital of the State of São Paulo and in 1966 we established the Itapetininga field station with the special aim to study EEE

TABLE III

Wild vertebrates exhibiting hemagglutination-inhibition antibodies for Eastern Equine Encephalitis Virus in São Paulo, Brazil, during 1971

Name	Procedence	Number
Vireo chivi	Rio Guaratuba	1/24 (*)
Turdus albicollis	" "	3/22
Turdus rufiventris	" "	3/19
Formicarius ruficeps	" "	1/3
Tachyphonus coronatus	" "	2/66
Phylidor atricapillus	" "	1/9
Phyllomyias fasciatus	" "	1/18
Elaenia sp	Itapetininga	1/113
Didelphis marsupialis	"	1/5

(*) Number of positives/number of captured.

virus. Recently, horse epizootics occurred in São Paulo, very near to that station, as Conchas [11], located at about 65 kilometers North and Itaporanga [12], at about 150 kilometers West, both places being very similar to Itapetininga in their ecological features.

Until the end of 1969 we had not observed any evidence of EEE activity in the study areas. Initially, we noted that SPAn 14723 was identical to the Tatui strain isolated in São Paulo in 1937 and to the strain TRVL 24443 isolated in Trinidad in 1959. So we may suppose that our strain falls in the Central-South American subtype described by CASALS [1] but we have not included in the identification any strain belonging to the North American subtype.

Since EEE virus was isolated, antibodies could be demonstrated in the wild vertebrates, showing that they could play a role in the maintenance of the virus in the field stations. EEE was isolated from birds classified as "permanent residents" in our bird banding studies in relation to arbovirus ecology [8]. Serologically, we found that the major part of the bird sera reacting with SPAn 14723 antigen is among the permanent residents. Besides, we have also obtained isolations from small mammals from genus *Oryzomys,* and from an opossum, *Didelphis marsupialis,* animals which have smaller home ranges. We have not observed positive sera within any other mammal species, except for a unique marsupial. We suppose that EEE should be able to kill the infected small rodents and this could explain the absence of antibodies in 245 mammal sera examined in 1971, in spite of the four isolations from rodents. These findings showed that birds and mammals were supporting a virus transmission in the study areas.

We must admit that, according to our data, since 1966, there was a quiescence in EEE activity in our State because no other horse epizootics was reported or diagnosed by the Instituto Biológico de São Paulo, from the Secretary of Agriculture in which assistance is granted for domestic animal diseases [10]. We were able to isolate EEE from the end of 1969 through 1971, showing a spreading of this virus in the State. Remarkably, no horse epizootics was detected in spite of the silent cycle being in course, as demonstrated by our surveillance program. After May 1971 no further isolations of EEE virus were made, indicating that the virus could have disappeared from the study areas. For these reasons, we suppose that EEE virus is not endemic in this region.

Furthermore, it is interesting to note that the isolations of EEE virus were made during all year round, which, according to our observations, is unusual in the region. São Paulo, being under the Tropic of Capricorn, has two definite weather seasons. From Spring to Fall (from September to April) there is

a hot-wet season, with high average temperature and abundant rains. From May through September (from Fall to beginning of Spring) we have a cold-dry season, with temperature decrease which never falls below freezing, and the rains are scarse. Since the beginning of our surveillance program on arbovirus, in 1961, it has been very infrequent to isolate a virus during the cold-dry season, probably due to the very low density of mosquitoes [7]. We might suppose that the survival of EEE virus in our region during the period of studies was probably due to a mechanism that the data obtained by our methods of collection were unable to uncover. Moreover, we did not isolate EEE virus from 223 pools of mosquitoes processed in 1970 except once from a pool of *Phoniomyia pilicauda* which is the first isolation of EEE from mosquitoes in South Brazil. This isolation came from a place called Cotia which is a state forest reserve (23°40'SL and 46°55'WL), from where we received only live mosquitoes for our studies. However, being a mosquito which lives inside the forests [7], it seemed to us that this species is not a primary vector in the area, as the epizootics of EEE virus in this State occur in natural open fields and pastures, which are the usual places for horse breeding.

There are many problems to be solved in relation to the ecology of EEE virus in South Brazil. We do not know how it is introduced in the region, as we have observed a quiescence period of several years and we could detect a silent cycle of EEE virus without any reported epizootics in São Paulo. We may consider an eventual role that wild birds might play in its epidemiological cycle, as described in the United States [8], because we were able to isolate virus from our birds. However, we have recovered the virus only from those species classified as residents, according to our banding program. From the "Summer or Winter residents" or from the "transients" which might be incriminated as virus circulators, we did not isolate any EEE strain and their role on its ecology remains to be seen. The hypothesis that birds are responsible for the introduction and circulation of EEE virus in the United States, as pointed out by STAMM & NEWMAN [13] and by LORD & CALISHER [9], should be kept in mind and only by further studies we will be able to clear this point.

We have isolated EEE virus only once from mosquitoes; however, we obtained three isolations from sentinel mice exposed to the flying mosquitoes in the field stations, fact that could incriminate these arthropods as partakers of the virus cycle. Also the maintenance of EEE virus in the study areas, in a different behavior from the usual pattern observed by us, i.e., the disappearance of the arboviruses during the cold-dry season, should be fully investigated, as well as the eventual role of any other blood-sucking insects as vectors.

The absence of human positive sera for EEE virus as found in other surveys carried out in São Paulo [11, 12], are in disagreement with the data obtained by others in the Americas [3], in North Brazil [5] and by us during this study. In spite of the positive human sera found by us, we could not discover any case of disease which could be attributed to EEE virus among the inhabitants of Casa Grande and Itapetininga (in Rio Guaratuba field station area there are no human dwellings at all).

For this reason, we can assume that EEE virus is not a cause of overt human illnesses, probably causing inaparent infections detected only by serological surveys.

RESUMO

Estudos epidemiológicos sobre o vírus da Encefalite Equina Leste em São Paulo, Brasil

Durante vigilância epidemiológica sobre arbovírus executada em certas áreas florestais do Estado de São Paulo, foram isoladas dezesseis amostras do vírus da Encefalite Equina Leste, a partir de mosquitos, animais sentinelas, mamíferos e aves silvestres. As amostras foram isoladas por inoculação em camundongos recém-nascidos e identificadas por provas de fixação de complemento e de inibição de hemaglutinação. Os isolamentos de vírus e os dados sorológicos obtidos com soros humanos e de vertebrados silvestres

demonstraram que o vírus da Encefalite Equina Leste não é endêmico na região.

A transmissão do vírus em questão ocorreu em ciclo silencioso, pois não foi detectada qualquer doença nos cavalos. Porém, puderam ser demonstrados anticorpos em seres humanos, cujas infecções, provavelmente, foram inaparentes.

Além disso, o papel de aves e mamíferos silvestres no ciclo viral foi discutido, tendo em vista o significado do vírus da Encefalite Equina Leste em Saúde Pública no Sul do Brasil.

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