The Venezuelan Equine Encephalomyelitis complex of group A arthropod-borne viruses, including Mucambo and Pixuna from the Amazon region of Brazil*

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Venezuelan Equine Encephalomyelitis (VEE) virus was first characterized in 1938 by Kubés and Rios[1], who isolated the virus from horse brain collected in Venezuela. Since then, other strains have been isolated and characterized in Colombia[2], Trinidad[3] and Ecuador[4]. As far as can be determined from the reports, there are no major antigenic differences among these strains[2-6] although Sanmartin et all.[7], comparing a VEE strain isolated in Colombia in 1952 with prototype VEE, found some minor differences by neutralization testing.

Causey et all.[8] in 1954 isolated the virus BeAn 8 from a sentinel Cebus monkey in Brazil. At the Rockefeller Foundation Virus Laboratories in New York, sera of a Rhesus monkey immunized with the Trinidad donkey nº 1 strain of VEE (one injection of live virus) neutralized 3.4 log LD_{50} of BeAn 8 virus and 4.8 log LD_{50} of homologous

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virus. On this basis, Causey et all. reported BeAn 8 as a strain of VEE. They also noted that some adult mice survived after intraperitoneal inoculation with about 10^8 baby mouse intracerebral LD_{50} of BeAn 8. Survival of adult mice after intraperitoneal inoculation with prototype VEE would have been unusual.

More detailed serological testing of BeAn 8 was prompted by the isolation in 1961 of BeAr 35645 virus, which was found to be separate from, but related to, both VEE and BeAn 8. Results of the studies of BeAr 35645 indicated that BeAn 8 also differs from VEE. The present report deals with these antigenic differences and gives a description of BeAr 35645. The three viruses form a complex in Casals' and Brown's group A of the arthropod-borne viruses.

It is proposed that BeAn 8 be named Mucambo after a section of forest near Belém, and that BeAr 35645 be called Pixuna after the small stream near which the mosquitoes yielding the prototype strain were collected.

MATERIALS AND METHODS

The techniques employed by this Laboratory for isolation of virus have been described by Causey et all.[8] Whenever possible, isolation was attempted from the vertebrate or invertebrate host brought alive to the laboratory. Blood and other tissues from trapped animals were kept at -60ºC until triturated for inoculation. For mosquitoes and viscera from dead animals, antibiotics were added to the bovine albumin phosphate-buffered diluent in the proportion of 2,000 units of penicillin and 0.5mg of streptomycin per ml of diluent.

Serological studies were carried out with the BeAn 8 and BeAn 10967 strains of Mucambo and the BeAr 35645 strain of Pixuna. These viruses, as well as the other group A viruses used with the exception of VEE, had all undergone less than 14 mouse passages. Of the two known VEE strains employed, the Trinidad donkey nº 1 strain

(American Type Culture Collection) had undergone 25 guinea pig and eight mouse brain passages, and the Beck and Wyckoff strain seven mouse passages.

Complement-fixation (CF) testing was done on plastic trays by a method described earlier[9], with overnight primary incubation at 4ºC and approximately two units of complement. Grid titrations were used.

Hemagglutination-inhibition (HI) testing was done by the techniques of Clarke and Casals[10], using antigens made by sucrose-acetone or acetone-ether extraction, goose red blood cells and lucite trays. The sucrose-acetone technique was modified by omitting the centrifugation steps directly after the first and second extractions. HI results were corrected to those expected with eight units of antigen to allow comparison of data. A hemagglutinating antigen was found in sucrose-acetone extracted brain of baby mice infected with Mucambo virus. This antigen agglutinated goose red cells at the 1:1600 dilution, pH 6.2, 37ºC. A similar antigen for Pixuna had a titer of 1:3200, pH 6.3, 37ºC. Some HI testing was also done with an antigen from acetone-extracted serum of baby mice infected with Pixuna. This antigen had a titer of 1:128 at pH 6.2, 37ºC.

Most of the neutralization testing was done in baby mice inoculated intracerebrally (i.c.), with mouse brain as virus source and a constant serum, varying ten-fold virus dilution technique. In the tests of horse sera, the mice were inoculated intraperitoneally (i.p.) and baby mouse serum served as virus source. All tests were incubated for one hour at 37ºC.

Immune sera for HI and CF testing were usually prepared in mice inoculated from three to six times, but in a few instances, for HI studies, single-injection guinea pig, mouse or rabbit sera were used. Immune fluids for neutralization testing were prepared in mice, using one inoculation of virus only and harvesting serum or ascitic fluid produced with mouse sarcoma 180[11].

ISOLATION AND CHARACTERIZATION OF VIRUSES

Mucambo

Prototype strain BeAn 8 was the first arborvirus obtained from sentinel Cebus monkeys in the Amazon region. It was isolated from the blood seven days after the sentinel entered the forest, and was the only one of 11 samples from similarly exposed animals that yielded a virus at this first bleeding in December, 1954[8]. The characteristics of this virus may be summarized as follows. The average survival time for baby mice inoculated i.c. or i.p. is one to two days. Adult mice inoculated i.c. die with an average survival time of about six days, and may present with hind-leg paralysis. Adult mice rarely succumb following i.p. injection. The titer in baby mice inoculated either i.c. or i.p. is 10^9 LD_{50}/ 0.02ml, and in adult mice inoculated i.c. is usually one or two log LD_{50} less. Guinea pigs survive i.p. inoculation. Mucambo virus caused complete destruction of HeLa cell monolayer cultures in from three to four days.

A homolog of BeAn 8, namely BeAn 10967, has also been extensively used in studies in this laboratory.

Pixuna

Prototype strain BeAr 35645 was obtained from a pool of 84 *Anopheles* (*Stethomyia*) *nimbus* collected in September, 1961, at ground level at kilometer 94 of the Belém-Brasilia highway and inoculated as a suspension i.c. into four day old mice. One infant was dead and one sick on the morning of the 4th day post inoculation, and another sickened that afternoon. Passages were made, and brain suspension inoculated i.c. in the 1:10 dilution killed four day old mice with an average survival time of 1.4 days. After i.p. inoculation the mice died with an average survival time of 2.0 days. In early passages, adult mice inoculated i.c. did not die, although they appeared lethargic, with ruffled hair, from about the 6th to the 15th day. Sodium desoxycholate inactivated more than four log LD_{50} of virus in a test performed in baby mice by the technique of Theiler[12]. Guinea pigs survived i.p. inoculation. The virus caused complete destruction of HeLa cell monolayer cultures by the 4th day.

A 2nd isolate was obtained in December, 1961, from the pooled viscera of a rat, *Proechimys guyannensis oris*, trapped in the Instituto Agronômico do Norte forest near Belém. A 3rd isolate was recovered in March, 1962, from a pool of 22 *Trichoprosopon digitatum* captured on human bait at ground level in the forest at kilometer 87 of the Belém-Brasilia highway. These two subsequently recognized strains of Pixuna showed the same isolation pattern as the original. Illness and death of mice were first manifested on the 4th day, and the average survival time was shortened to less than two days in the 1st passage.

EXPERIMENTAL INOCULATION OF HORSES

Mucambo and Pixuna viruses were inoculated into ten month old horses that had previously been shown to be free of HI antibody for Mucambo, Pixuna, Aura, Una, Eastern Equine Encephalomyelitis (EEE) and Mayaro viruses.

Horse UN 508 was inoculated intramuscularly with approximately $10^{9.5}$ baby mouse i.c. LD_{50} of Mucambo virus in the form of baby mouse serum. Circulating virus was present 24 hours ($10^{2.9}$ LD_{50}) and 48 hours ($10^{3.2}$ LD_{50}) after inoculation, and this virus was confirmed as Mucambo by CF testing. Four of six mice inoculated with 72 hour serum died. Temperature of 102.2ºF was recorded at 24 hours. Shortly afterward, the horse was found down on its side, but it continued to eat. At 48 hours the temperature had returned to the baseline of 98.8ºF and the horse recovered without sequelae. White blood cell counts performed daily showed a preinoculation count of 10,000 per cmm, a count of 5,700 per cmm on the 1st day, a further drop to 3,900 per cmm by the 3rd day and a gradual return to 8,700 per cmm on the 6th day.

Horse UN 513 was inoculated intramuscularly with approximately $10^{8.4}$ baby mouse i.c. LD_{50} of Pixuna-infected baby mouse serum. No circulating virus was detected during the 1st week, although the white blood cell count fell from a baseline of 10,200 per cmm to 5,200 per cmm on the 3rd day and gradually rose again to 8,100 per cmm by the 6th day. No fever or signs of illness were noted. On the 13th

day post inoculation, the serum had a log neutralization index of 4.9 for Pixuna virus and 2.1 for Mucambo virus. On day 21, the horse was challenged intramuscularly with approximately $10^{8.9}$ baby mouse i.c. LD_{50} of Mucambo virus. No circulating virus was detected during the subsequent five days, although the leukocyte count again dropped (5,400 per cmm) on the day after challenge. There was no fever or clinically evident abnormality in the horse and no sequelae were noted over a 60 day period of observation. Serum taken eight days after challenge had a log neutralization index of $\geq$6.3 for Mucambo virus.

IDENTIFICATION OF VIRUSES

Mucambo (BeAn 8) virus was recognized in 1955 as being a group A virus closely related to VEE on the basis of a neutralization test in adult mice. Subsequently, as stated in the introduction, a known VEE positive Rhesus monkey serum neutralized BeAn 8 virus, and the agent was therefore believed to be a strain of VEE.

Pixuna virus was shown to belong to group A primarily on the basis of HI testing. An antigen from the sera of baby mice infected with Pixuna was inhibited by homologous serum at the 1:1280 dilution and by Mucambo and Mayaro sera at the 1:40 dilution. Sera for Una, Aura and EEE viruses at the 1:20 dilution failed to inhibit Pixuna hemagglutinin. Neutralization testing (Table 1) confirmed the fact that Pixuna was related to, but different from, Mucambo virus, and that it also differed from the other group A viruses known in the Amazon region.

At this period of the study the impression still prevailed that Mucambo (BeAn 8) was a strain of VEE. In further HI testing with Pixuna and VEE antigens, sera for both BeAn 8 and Trinidad donkey nº 1 VEE were included. As shown in Table 2, Mucambo serum inhibited VEE only slightly. Additional HI testing of the same sera with antigens for VEE, Mucambo and Pixuna (Table 3) showed that the three viruses were distinct, although VEE and Mucambo cross-reacted to a greater degree with each other than either did with Pixuna. Neutralization testing i.c. in adult mice (Table 4) confirmed the fact that VEE and Mucambo

can be distinguished, although again considerable cross-reaction was demonstrated. In CF testing with hyper immune mouse sera (Table 5), Pixuna, VEE and Mucambo are once again seen to be distinct, in contrast to the similarity of the reactions between the Trinidad and the Beck and Wyckoff strains of VEE.

Table 1 – Cross-neutralization testing with Pixuna and the group A viruses of the Amazon region

Serum	Antigen					
	Pixuna	Mucambo	EEE	Mayaro	Una	Aura
Pixuna	3.0*	1.2	0	<0.4	<0.6	<1.5
Mucambo	1.5	3.0				
EEE	0		4.3			
Mayaro	0.6			>3.2		
Una	0.2				3.5	
Aura	0					>4.5

* Results expressed as log neutralization index.

Table 2 – HI testing of Pixuna with other group A viruses

Serum	Antigen			
	Pixuna	VEE (Trinidad)	Semliki	Homologous
Pixuna, 1 inj.	160*	20	0	–
Mucambo, guinea pig, 1 inj.	40	80	0	–
Mucambo, guinea pig, 1 inj.	0	20	0	–
Mucambo, guinea pig, 1 inj.	0	20	0	–
VEE (Trinidad), 6 inj.	160	?5120	?20	–
Semliki	0		?2560	–
EEE, rabbit	0			160
Sindbis	0			1280
Chikungunya	0			2560
AMM 2021	0			80
AMM 2354	0			?5120
Middelburg	0			1280
Mayaro	10			5120
Western equine encephalitis	0			5120
Aura	0			80
Una	0			160
O'nyong-nyong	0			1280

* Reciprocal of serum dilution inhibiting eight units of antigen.

Table 3 – Cross-HI testing with VEE, Mucambo and Pixuna viruses

Serum	Antigen		
	VEE (Trinidad)	Mucambo	Pixuna
VEE (Trinidad), mouse, 6 inj.	20480*	640	320
VEE (Trinidad), mouse, 1 inj.	40	10	0
Mucambo, guinea pig 291, 1 inj.	80	1280	40
Mucambo, guinea pig 292, 1 inj.	20	320	0
Mucambo, guinea pig 295, 1 inj.	20	160	0
Mucambo, mouse, 2 inj.	20	80	20
Pixuna, mouse, 1 inj.	20	20	160
Pixuna, mouse, 3 inj.	20	40	320

* Reciprocal of serum dilution inhibiting eight units of antigen.

Table 4 – Cross-neutralization test with VEE and Mucambo viruses

Virus	Mouse serum, 3 inj.	
	VEE (Trinidad)	Mucambo
VEE (Trinidad)	4.6*	2.3
Mucambo	3.1	$\geq$ 4.0

* Results expressed as log neutralization index.

Table 5 – Cross-CF test with VEE, Mucambo and Pixuna viruses

Antigen	Mouse			
	VEE (Beck & Wyckoff), 3 inj.	VEE (Trinidad), 3 inj.	Mucambo, 4 inj.	Pixuna, 5 inj.
VEE (Beck & Wyckoff)	256/$\geq$128*	64/$\geq$128	64/$\geq$128	16/$\geq$128
VEE (Trinidad)	256/$\geq$128	64/$\geq$128	32/$\geq$128	16/$\geq$128
Mucambo	64/32	16/32	256/128	16/32
Pixuna	8/8	0/0	16/$\geq$128	256/$\geq$128
Normal brain	0/0	0/0	0/0	0/0

* Reciprocal of the serum titer over the reciprocal of the antigen titer giving greater than 50% fixation of complement.

DISCUSSION

The three viruses, VEE, Mucambo and Pixuna, form an antigenic complex within group A of the arboviruses. VEE and Mucambo cross-react to a considerable degree in neutralization testing, a fact which accounts for the original belief that Mucambo was actually a strain of VEE. The two are easily distinguishable, however, by CF test, even with hyper immune sera. Pixuna is serologically the most distinct member of the complex, and less difficulty should be encountered in identifying it in HI, CF or neutralization testing.

Further study of the detailed relationships among strains of the VEE complex and a search for new strains that vary antigenically are important for an understanding of the geographical distribution and epidemiology of these viruses. In addition, the analysis of results of the use of live virus VEE vaccine[13] and, possibly, the future development of heterologous live virus vaccines for VEE depend on such studies.

Interpretation of serological survey results using either VEE or Mucambo virus or antigen may present difficulties. In discussing their survey studies of VEE in Trinidad, Tigertt et all.[14] state, "Any serological survey is open to question of specificity of the neutralizing antibody for the homologous virus" and "No instances have been found of VEE crossing with other viruses of this area in neutralization tests, although such studies are admittedly fragmentary". They used the Trinidad donkey nº 1 strain for their survey. This strain might not be the best one to use in another geographical area to detect activity of VEE or related agents. Causey and Theiler[15] found sera of human beings in the Belém area of Brazil which neutralized Trinidad VEE virus. These persons may have had previous experience with Mucambo or Pixuna, rather than with VEE virus.

Up to the end of 1963, 101 strains of virus of the VEE complex had been isolated in the Belém area. Four of these are Pixuna or very closely related to that virus. Reciprocal cross-testing for serological identity of the remaining agents has been accomplished to date only with two strains, both Mucambo. No strain homologous with the prototype VEE strain has yet been identified.

SUMMARY

Mucambo virus (BeAn 8), previously described as a strain of Venezuelan Equine Encephalomyelitis (VEE) virus from the Amazon region of Brazil, can be differentiated from VEE virus by hemagglutination-inhibition, complement-fixation and neutralization testing. Ipixuna virus, a hitherto undescribed agent isolated in the same region of Brazil in 1961, is related to both VEE and Mucambo, and the three viruses form an antigenic complex in arbovirus group A. VEE and Mucambo are more closely related to each other by all three tests than either is to Pixuna.

A horse inoculated with Mucambo virus developed fever, leukopenia and viremia. Inoculation of Pixuna virus in another horse failed to produce fever or circulating virus, and the horse did not develop viremia after challenge with Mucambo.

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