A STUDY OF EQUINE VIRULENCE OF NATURALLY OCCURRING VENEZUELAN ENCEPHALITIS VIRUS IN VERACRUZ WITH DESCRIPTION OF ANTIBODY RESPONSES [1]

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Venezuelan encephalitis virus strains cycling in nature at Sontecomapan, Veracruz, Mexico, during July-August 1965 usually produced inapparent infection of horses and burros. Therefore, the absence of previous equine epizootics does not necessarily indicate absence of virus from that region.

Introduction

Venezuelan encephalitis (VE) virus was first recognized during 1936-1938, when it caused equine encephalitis in Venezuela (*1*). The word *equine* is therefore often included in the name of the virus, even though it also causes human epidemics of encephalitis and systemic disease. Discoveries of the virus in Mexico and Florida in 1962 and 1963 (*2-4*) have prompted inquiry into the possibilities that the virus is moving northward from South America and that it might become a public health hazard to man and/or equines in Mexico and the United States. Prior to that time, there was no recognition of equine epizootic disease in southeastern tropical Mexico (*5*), and the only equine encephalitis known along the Gulf coast of Mexico was a case in Tamaulipas in 1941, from which eastern encephalitis virus was isolated (*6*).

A study was therefore carried out during July-August 1965 to assess the equine pathogenicity of VE virus in southeastern tropical Mexico. This was done by exposure of sentinel equines (burros and horses) to mosquitoes in nature and by inoculation of a horse with a local strain of virus. This article describes the virologic and clinical observations of these animals and their antibody responses following infection by VE virus.[5]

Material and Methods

Study sites. The endemic site of VE virus in Mexico where these studies were done was near the village of Sontecomapan, Veracruz, approximately 160 km southeast of Veracruz city. This village is located at sea level on a lagoon off the Gulf of Mexico in a narrow strip of land north of the San Andres mountains, and had about 1,000 inhabitants in 1965; domestic animals included a few dogs, pigs, chickens and horses with cattle grazing in surrounding pastures. The San Andres mountains begin to the south of the village, and the altitude rises abruptly to about 300 meters at Lago Catemaco and higher in the surrounding hills. To the north of the village along a road constructed during 1963 and 1964, by cutting through fields and patches of forest, were various habitats such as open pasture,

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[5] Since this study in 1965, a VE equine epizootic occurred in the tropical coastal region of Tampico and Altamira, Tamaulipas, and nearby Panuco, Veracruz. At least 300 horses were diseased and 60 died during summer 1966 (*7*).

Photographs of study sites at Sontecomapan, Veracruz, Mexico.

FIGURE 1—North edge of Sontecomapan village.

FIGURE 2—Pasture between village and forest in distance. Horses were kept in this pasture September 1965 to April 1966.

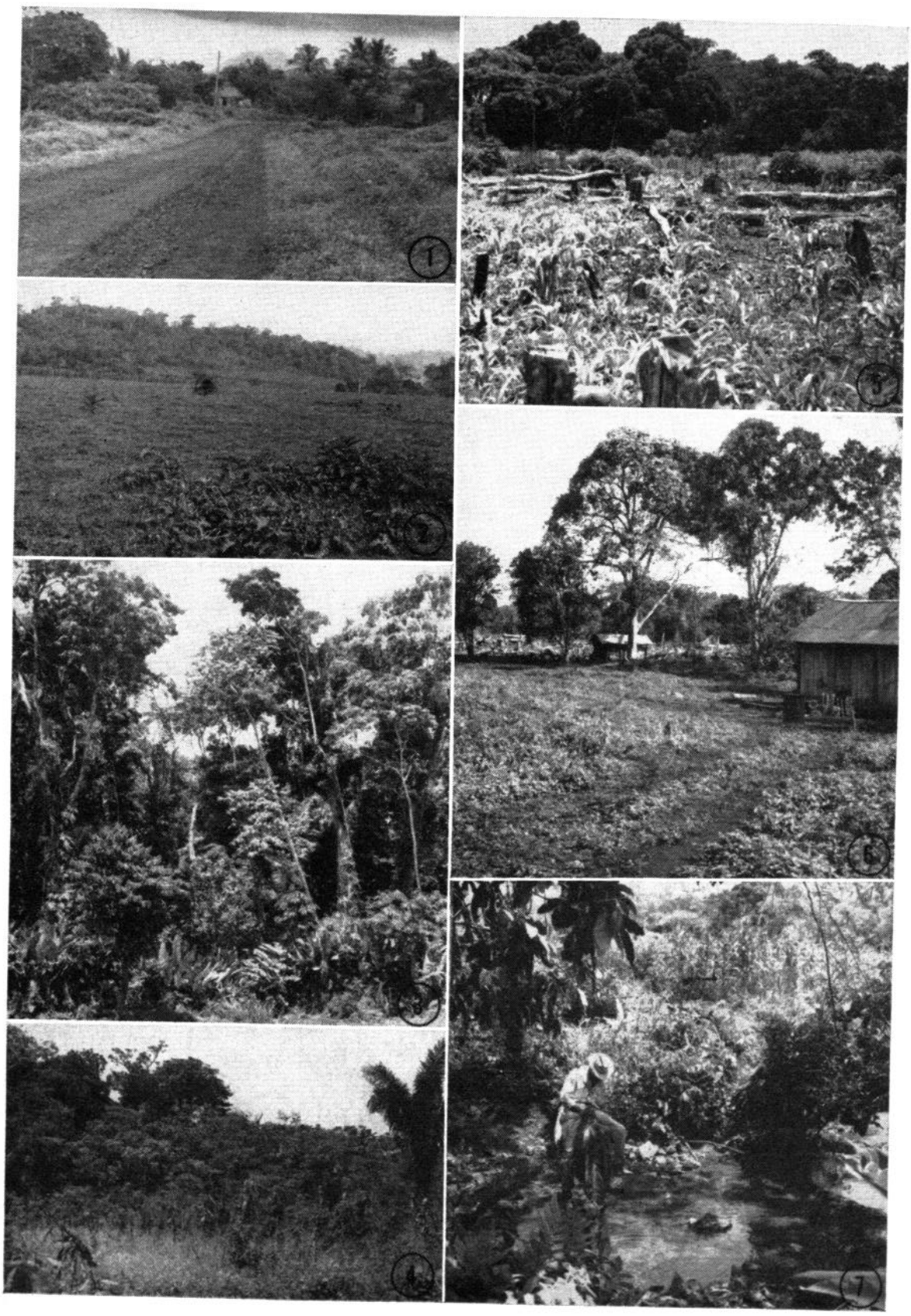
FIGURE 3—Cross section view of tropical wet forest from roadside. One sentinel animal was tethered in low vegetation in foreground.

FIGURE 4—Brushy growth adjacent to forest where a second animal was exposed. Note cornfield in foreground.

FIGURE 5—First planting of corn on newly cleared forest behind house.

FIGURE 6—House constructed by settlers in 1964.

FIGURE 7—Fresh water spring near house is watering stop for farmers returning to village from fields farther down the road.



tropical wet forest, dominated by canopy-forming trees, secondary forest, recently cleared and cultivated fields, and yards near houses (figures 1-6). In addition mangroves bordered the lagoon and there were frequent springs which served as sources of water for villagers and domestic animals (figure 7).

Exposure of sentinel equines. The sexes and original source localities of sentinel horses and burros, and dates and ages when exposure began at Sontecomapan are given in tables 1 and 2. Horses nos. 1 and 2 and burros nos. 2 and 3 were adults purchased at a slaughter house in Mexico City in June 1965. Horses nos. 3 and 4 and burro no. 1 were young animals purchased in June 1965 at Catemaco, Veracruz, from farmers who had kept them in open fields at about 300 meters altitude in the San Andres mountains; each was still suckling when purchased, even the nine-month old horse no. 4.

Equines were exposed to VE virus 1-2 km north of Sontecomapan and less than 100 meters from the road. The total area of exposure was about 12,000 square meters and was shaped like a trapezoid with sides about 200×600 meters. Sentinel equines were tied to stakes at the edge of tropical wet forest in grass (figure 8) or allowed to roam freely in a small corral (figure 9) near a spring (figure 7). Sentinel burro no. 1 was placed in a modified Magoon trap as mosquito bait on nights 0, 1, 5, 7, 9, 13, 15, 19, 21, and 23, and horse no. 3 on nights 0, 4, 6, 10, 12, 14, 18, and 20 (figure 10). The screened pen to prevent mosquito contact with horse no. 4 during the week before and after inoculation with VE virus, was near the spring (figure 7) among the trees and brush (figure 11) at the end of a pasture (figure 2). The animals were fed by grazing within these habitats and were taken to the spring for water. In early September 1965, all surviving sentinel equines were moved to an open pasture (figure 2) within 1 km of their previous habitats, and in late September horse no. 4 was put at 350 meters altitude in open fields at Dos Amates, a small village between Sontecomapan and Catemaco where vector mosquito populations were known to be low and there was no evidence of VE virus activity. Equines were thereafter in these locations until June 1966 when horses nos. 1, 3 and 4 and burro no. 2 were moved by truck to the National Communicable Disease Center, Atlanta, Georgia, for further study.

Inoculation of horse no. 4 with VE virus. VE virus, strain 65U64, recovered from heart tissue of a sentinel hamster exposed at the spring (figure 7) near Sontecomapan during July 1965, was passed once in brains of suckling mice and identified as VE virus at the field laboratory by neutralization test in mice. Horse no. 4 was kept in a field at Playa Azul on Lago Catemaco, Veracruz, until day −7 before inoculation when it was placed at Sontecomapan in a screened pen to prevent mosquito contact (figure 11). On August 15, 1965, it was inoculated subcutaneously over the right hip region, with 20,000 SM ic LD_{50} of strain 65U64 of VE virus in 1.0 ml of first passage mouse brain suspension.

Viremia and VE virus antibody tests. At least semi-weekly during the first three to four weeks of exposure, equines were bled from the jugular vein with sterile equipment. Blood was collected in test tubes as it dripped from the needle or in a syringe (figures 12 and 13). Ten ml of blood was mixed with about 0.2 ml of heparin, 2 mg/ml, for immediate inoculation in the field of suckling mice intracranially and subcutaneously, 0.01 ml and 0.02 ml, respectively. The remainder of the heparinized blood was kept cool until returned to the laboratory truck at Playa Azul within several hours, and then aliquots of blood were placed in sterile sealed glass ampules and stored on dry ice at −60°C. If serum was also desired for antibody tests, additional blood was collected in a dry test tube, allowed to clot, centrifuged, and serum stored in sterile plastic test tubes on dry ice

Photographs of study sites at Sontecomapan, Veracruz, Mexico.

FIGURE 8—Horse colt tethered at edge of tropical wet forest (figure 3).

FIGURE 9—Corral in dense secondary growth at north end of pasture (figure 2). Outside corral is a pig from nearby house of immigrant farmers.

FIGURE 10—Modified Magoon mosquito trap in forest where burro colt and horse colt were used as bait.

FIGURE 11—Screened shelter for housing the inoculated horse, located in dense brush near corral (figure 9).

FIGURE 12—Bleeding horse colt by jugular venipuncture. After heparinized blood was collected, syringe was removed and additional blood collected for serum (figure 13).

or later after transportation to New York, at −20°C.

If mice died within 14 days after inoculation of blood, they were frozen on dry ice; the brains were then harvested either at the field or New York Laboratories, and brain suspensions passed to tube cultures of primary chicken embryonic cell cultures (CEC) with liquid medium, prepared as described elsewhere (*8*). An interpretation that viremia existed was made only when mice from the inoculated litter yielded a virus that killed CEC and was neutralized in CEC by specific VE virus rabbit antiserum.

VE virus hemagglutination-inhibition (HI) and complement-fixation (CF) antibodies were detected in serum by methods described elsewhere (*9, 10*), except that some tests were done in microtiter plastic wells with volumes of 0.025 ml antigen, 0.025 ml serum and 0.05 ml goose erythrocyte suspension or 0.05 ml complement and 0.05 ml sensitized sheep erythrocyte suspension. Neutralization (N) antibodies were detected either by virus-dilution tests in weanling mice inoculated intraperitoneally employing usually two or three virus dilutions containing 5, 50, 500, or 5000 $WMipLD_{50}$ for admixture with undiluted, unheated serum and incubation at 37°C for one hour before mouse inoculation, or by serum-dilution tests in which serum was diluted in Hanks' solution, admixed with 32-120 50% well-destructive units of virus, incubated for one hour at 37°C and inoculated onto 1.3 cm^2 sheets of CEC in wells of plexiglass plates as previously described (*11*). Log_{10} neutralization indices (LNI) and serum-dilution, 50% end points, were then calculated (*12*).

Results

Viremia, clinical manifestations and antibody responses of sentinel equines following natural infection from VE virus. At the beginning of this investigation, it was assumed that adult horses or burros from the lowlands of Veracruz or other parts of the southeastern tropics of Mexico might have experienced previous natural immunizing infections by VE virus and therefore could not be used as sentinels to determine pathogenicity of the virus at Sontecomapan, Veracruz. To circumvent this possibility, susceptible equines were obtained from two sources that were thought to make previous VE virus infection unlikely: 1) Two horses from Jalisco and San Luis Potosi, and two burros alledgedly from the Central Plateau, all from elevations of 1,500-2,000 meters, were purchased at a slaughter house in Mexico City, and transported over 1 day period to Sontecomapan, and 2) Two young horses and one burro were purchased in June 1965 near Catemaco, Veracruz, and were weaned immediately thereafter. Six of the seven equines proved to be free of pre-existing VE virus neutralizing antibody. One horse, no. 3, 1.5 months old from Veracruz had pre-existing antibody detectable at significant levels by virus-dilution and serum-dilution neutralization tests (table 1). This antibody was evidently maternal in origin and passively acquired because its titer declined after weaning; it was not actively acquired VE antibody since such antibody would have prevented the VE viremia which developed in this horse after natural infection (table 1).

When three horses and three burros were exposed in field and forest habitats at Sontecomapan, during July and August 1965, each became infected and developed detectable HI, CF and N antibodies to VE virus 6-22 days after initiation of exposure; these infections occurred between 21 July and 5 August (tables 1 and 2). By bleeding animals semi-weekly and inoculating blood into suckling mice in the field, immediately after bleeding, VE viremia was detected in the two young animals, horse no. 3 and burro no. 1, but only on one day (*i.e.*, days 7 and 4, after placement at Sontecomapan) (tables 1 and 2). The concentration of virus in blood of horse no. 3 was about 1

TABLE 1—Venezuelan encephalitis viremia and antibody responses of 2 horses without preexisting antibody and one horse with maternal antibody, following natural exposure to vector mosquitoes in Mexico.

Sentinel equine number, sex, original source, locality, date and age when exposure began in 1965	VE virus test [1]	Results by days before or after exposure in nature [1]																								
		—43	0	4	5	7	10	11	12	13	14	16	17	18	19	20	21	22	23	26	29	67	75	136	144	212	219
Horse 1 female Jalisco 14 July about 5 yrs.	Viremia					–			–			–			–			–		–	–						
	HI		–			–			–			–			10			320		640	320		80 40		80		160
	CF		–			–			–			–			–			4		32	32		4				
	SDN		3			8						3			8			16			40		320		25		12
	VDN		1.4 1.1						0.9			1.2 <1.2						>3.7			>4.0						
Horse 2 male San Luis, Potosi 14 July about 2 yrs.	Viremia				–	–	–			–			–		–				–	Died on day 23							
	HI		–		–	80	1280			1280			2560		80				80								
	CF		–		–	–	32			64			128		128				32								
	SDN		2		3	5	63						≧8000		≧1000				400								
	VDN		<1.0																								
Horse 3 female Catemaco, Veracruz 22 July 1.5 months	Viremia				–	+ (5/8)		–		–			–		–		–										
	HI	–	–	–		–		320		80			40				40					40 80		160		80	
	CF	–	–	–		–		32		8			8		8							–					
	SDN	8	2	1		–		80									12					32		12		12	
	VDN	2.7 3.6	2.6																								

[1] Viremia: ( ) signifies titer as fraction of suckling mice dying; – signifies no viremia detectable in suckling mice.
Hemagglutination-inhibition (HI), complement fixation (CF) and serum dilution neutralization (SDN) test antibody titers of serum expressed as reciprocals of serum dilutions; – signifies HI < 10, CF < 4 and SDN < 1.
Virus dilution neutralization (VDN) test antibody titers are $\log_{10}$ neutralization indices in weanling mice inoculated ip or in primary chicken embryonic cell microcultures.

TABLE 2—Venezuelan encephalitis viremia and antibody responses of 3 burros without preexisting antibody following natural exposure to vector mosquitoes in Mexico.

Sentinel equine number, sex, original source, locality, date and age when exposure began in 1965		Results by days before or after exposure in nature [1]																										
		−41	0	4	5	6	7	9	10	12	13	14	15	16	17	19	20	21	22	23	24	26	27	29	30	69	75	144	219
Burro 1 female Catemaco, Veracruz 20 July 2 months	Viremia			+ (3.2)		−	−		−			−			−			−											
	HI	−	−	−		−	20					320			160			320			160					20			
	CF	−	−	−		−	4					128			128			128			64					16			
	SDN	−		−		25	16					125									125					≧320			
	VDN	<1.1																											
Burro 2 male Central plateau 14 July about 2.5 yrs.	Viremia				−		−		−		−			−			−						−		−			
	HI		−		−		−		320		5120			5120			5120			1280			1280		2560		160 40	10	10
	CF		−		−		−		−		−			−			128			256			64		64		64		
	SDN		2		3		3		125		200														80		63	11	11
	VDN		0.9 0.9		0.9		0.8		>3.7		>4.0						>4.0												
Burro 3 female Central plateau 14 July about 8 yrs.	Viremia					−	−			−			−			−			−			−		−				
	HI		−			−	−			−			−			−			2560			2560		2560		40		
	CF		−			−	−			−			−			−			128			64		64		8		
	SDN		−				2						8			−			1600					400		80		
	VDN		1.0 0.9							1.1									>4.0					>3.7				

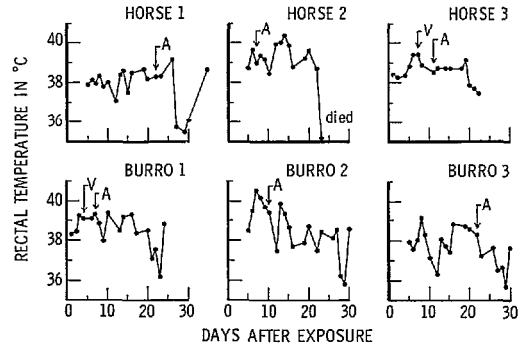
[1] Viremia: ( ) signifies titer as $SMicLD_{50}$/0.01 ml of blood; − signifies no viremia detectable in suckling mice.
Hemagglutination-inhibition (HI), complement fixation (CF) and serum dilution neutralization (SDN) test antibody titers of serum are expressed as reciprocals of serum dilutions; − signifies HI < 10, CF < 4 and SDN < 1.
Virus dilution neutralization (VDN) test antibody titers are $\log_{10}$ neutralization indices in weanling mice inoculated ip or in primary chicken embryonic cell microcultures.

SM ic LD_{50}/0.01 ml (table 1), and in burro no. 1, $10^{-3.2}$ (table 2). Antibodies were detectable in serum simultaneously by all three methods in horse no. 3 and burro no. 3 (tables 1 and 2). In horses nos. 1 and 2, HI antibody was detected three days before CF and N antibodies, in burro no. 1, N antibody was found one day before HI and CF antibodies, and in burro no. 2, CF antibody was not found until 10 days after HI and N antibodies (tables 1 and 2). Thus, with the exception of burro no. 2, antibodies were first detected in serum at about the same time with all three tests employed. The maternal N antibody in horse no. 3, decreased in titer during the 43 day interval between weaning and exposure in nature, and did not prevent infection, since viremia occurred seven days after exposure and HI, CF and N antibodies appeared by 11 days (table 1).

All animals except horse no. 2 appeared healthy throughout the periods of exposure, viremia and antibody development. Rectal temperatures during the first 3-5 weeks of exposure remained below 40°C except for horse no. 2 and burro no. 2 (figure 14). In burro no. 2, definite fever occurred seven and eight days after exposure, just a few days before VE HI, and N antibodies appeared in serum (day 10). Even though viremia was not demonstrable on days 5 or 7 in this burro, it seems likely that the fever was due to VE virus infection. A sharp, transient but low elevation in temperature also occurred in horse no. 3 concurrent with viremia (figure 14).

Horse no. 2 had a temperature of 40°C, 12-14 days after exposure and after VE virus antibody had developed. By day 19, the animal appeared depressed and was surrounded by flies, though there were no flies on a nearby healthy burro. On day 20 maggot infestations were noted, one-half inch deep on the dorsa of both front and hind hoofs, and the horse could no longer stand by day 22, though the pulse was strong

FIGURE 14—Rectal temperatures of sentinel equines exposed at Sontecomapan, Veracruz, Mexico, July-August 1965.



V = VE viremia; A = first detectable VE antibody in serum

at 115/minute, and it drank water well. However, on day 23, the hoof infections were more purulent, pupils were dilated, limbs were extended though not stiff, the pulse was slow, irregular and weak, and the horse died after withdrawing 150 ml of blood from the jugular vein. The animal was under-nourished when originally purchased at the slaughter house in Mexico City in June, and since it had no detectable viremia upon tests at the field laboratory, there was no way of knowing at the time whether its illness and death were due to VE virus infection (antibody tests were done subsequently in New York) or to malnutrition, hoof infections and possible bacteremia. Of course once antibody tests were performed, it became apparent that the horse had been infected by VE virus promptly after exposure at Sontecomapan (table 1). In retrospect, it therefore is possible that this horse died of VE virus encephalitis with depressive syndrome. It is unfortunate that isolation of virus from brain was not attempted even though it might well have been unsuccessful, since death was delayed until about 20 days after infection (*14*).

Inapparent infection of a horse following subcutaneous inoculation with VE virus. After inoculation sc of 20,000 SM ic LD_{50} of a strain of VE virus, freshly isolated in July 1965 from a sentinel hamster, horse

no. 4, an 11 month-old female purchased at Catemaco, Veracruz, developed no signs of illness, except for rectal temperatures of 39.7°C on days 2 and 3, and 38.7 and 38.2 on days 4 and 5; these were in contrast to daily temperatures that ranged between 37.2°C and 38.0 on days −6 through 1, and between 36.4 and 37.5 on days 6 through 13.

Viremia with virus titers about $10^{-1.5}$ $SMicLD_{50}$/0.01 ml of blood was present on days 2 through 5. HI and N antibodies appeared in serum by day 5 and were still present on days 65, 179 and 255 (table 3). VE virus CF antibodies were found on days 65, 179 and 255 (table 3).

VE virus antibody in sera of healthy army horses in Veracruz. In addition to finding VE virus maternal antibody in horse no. 3, evidence that VE virus infected horses in Mexico probably prior to 1965 and in other regions than Sontecomapan, was obtained when HI and N antibodies were found in sera of Mexican army horses bled during August 1965 at San Andres Tuxtla, Veracruz (table 4). The absence of VE virus antibody in a few army horses in the northern, arid, upland state of Coahuila served as a negative control (table 4). Since army horses are sometimes moved from one part of Mexico to another, it cannot be concluded that VE virus infections occurred only in Veracruz. Nevertheless, the presence of antibody in serum indicated that VE virus caused infections of Mexican horses that

TABLE 4—Prevalences of Venezuelan encephalitis HI and N antibodies in sera of healthy Army horses from the southeastern tropics (Veracruz) and upland plateau (Coahuila) of Mexico.

Location and dates of bleeding	No. of horses with detectable VE virus antibody [1]/No. tested HI	N-CEC
Veracruz, Aug. 1965	9/48 (.19)[2]	16/49 (.33)[3]
Coahuila, June 1964	0/7 [4]	0/7 [4]

[1] Detectable antibody means HI titer ≧1:10 serum dilution and LNI > 1.9.
[2] Reciprocals of HI titers for 9 positives were: 10, 10, 10, 10, 20, 20, 40, 40, 80. Eight were < 10 with western encephalitis antigen; one 10 with VE was also 10 with WE antigen.
[3] Twelve of the 16 positives for VE N antibody in CEC were also tested with VE virus in weanling mice inoculated ip and 10 were positive (LNI > 2.2). Three of the 16 were positive (LNI > 1.7) and six were negative (LNI < 1.0) for western encephalitis N antibody upon test in weanling mice inoculated ic; three negative for VE N antibody were positive for WE antibody.
[4] These 7 sera also had western encephalitis virus HI antibody titers <10 and LNI < 1.4.

TABLE 3—Viremia and antibody responses during inapparent infection of horse no. 4 inoculated subcutaneously on 15 Aug. 1965 with Venezuelan encephalitis virus.

Tests for viremia [1] and antibodies in serum	Results by days before or after sc inoculation of VE virus														
	−56	−7	−4	−3	−2	−1	0	1	2	3	4	5	65	179	255
VE viremia			−	−	−	−		−	+ (1.5)	+ (1.5)	+ (1.6)	+ (8/8)			
VE virus antibodies															
HI	−	−	10	−	−	−			−	−	−	20	40 80	40	160
CF	−	−	−	−	−	−			−	−	−	−	16	8	16
SDN	−	−				−					3	16	12 −	5 63	≧200 200
VDN	1.4 1.5	<0.8													
Antibody tests with western encephalitis virus															
HI	160 40	40 20	20 20	20 20		− −			− −		− −	− −	− −	80 20	10 −
CF	−	4		−		−					−		−	−	−
SDN	<2.5	<2.5	<2.5	−	−	−			−	−	−	−	−	−	2
VDN	<0.4	<0.4												<0.4	<0.4

[1] Viremia: ( ) signifies titer as $SMicLD_{50}$/0.01 ml of blood or fraction of suckling mice dying: − signifies no viremia detectable in suckling mice.
Hemagglutination-inhibition (HI), complement fixation (CF) and serum dilution neutralization (SDN) test antibody titers of serum are expressed as reciprocals of serum dilutions; − signifies HI < 10, CF < 4 and SDN < 1.
Virus dilution neutralization (VDN) test antibody titers are $\log_{10}$ neutralization indices in weanling mice inoculated ip or in primary chicken embryonic cell microcultures.

were not lethal and did not leave crippling sequelae of encephalitis.

Other arbovirus antibodies in sera of sentinel and inoculated equines. Sera used for VE virus antibody tests (table 1-3) were also tested for antibodies to the following viruses (and strains): Nepuyo (63U11), Patois (63A49), Tlacotalpan (61D240), western encephalitis (Rocky Mountain Laboratory 1985-60), eastern encephalitis (Riche), St. Louis encephalitis (Tr 9464), California (BFS-283), Anopheles A (original) and Bwamba (M 459). No antibodies developed to Nepuyo (HI and CF tests), Tlacotalpan (CF), eastern and St. Louis encephalitis (HI), California, Anopheles A and Bwamba (CF). Only burro no. 2 developed antibody to Patois virus; HI titers were <10 until days 27, 30 and 75 when they rose to 10, 80 and 20 (repeat 20, 40, 20) and CF titers became <4, 4 and 8 respectively.

With the strain of western encephalitis virus employed, there were negative results with horses nos. 1 and 3, and burro no. 2 by HI and N tests, and burro no. 3 by HI test. In horse no. 2, HI titers were <10 until days 16, 20 and 23 when they were 40, 40 and 40 vs. 8 units of hemagglutinin (repeat with 2 units: 160, 160, 160); however serum-dilution neutralization titers were <1, <1 and <5, respectively. In the inoculated horse, WE hemagglutinin reacted with preinoculation sera collected on days -56, -7, -4, and -3, though there were no positive reactions by CF or N tests (table 3). Upon infection by VE virus, WE HI titers rose slightly, probably indicating cross reaction with VE virus antibodies, since CF and N tests failed to detect WE virus antibody (table 3). In burro no. 1, HI titers were <10 on days 0 and 21, but were 10 and 20 (repeat 80) on days 24 and 69 respectively; WE virus serum-dilution N antibody titers were <1 and CF titers <4 on days 17, 21, 24, and 69. Six sera from Mexican Army horses in Veracruz were positive by WE N test (LNI>1.7); only 3 of these were also positive for VE N antibody (footnote, table 4).

Discussion

Since its original discovery in Venezuela, VE virus has continued to cause equine epizootics and human epidemics in northern South America, the latest in 1967 in Colombia (*13*). The discovery of VE virus antibody in southeastern tropical Mexico in 1962 (*2*) and isolation of the virus in 1963 (*3*), together with the absence at that time of equine epizootics or human epidemics in that region (*5*), suggested either a) that VE virus had only recently come to the tropical Gulf coast of Mexico and when sufficiently established and disseminated in nature would cause recognizable disease, or b) that the virus had been there for many years, but was an equine and human avirulent strain. The studies reported here were done to learn the pathogenicity of VE virus in an endemic focus in Veracruz, Mexico.

The results of these investigations showed clearly that burros and horses were regularly and promptly, but usually *inapparently* infected by VE virus strains cycling at Sontecomapan, Veracruz, during July-August, 1965. Although these horses and burros were the first to be observed in detail from the virologic and clinical viewpoints following natural infection by VE virus, their disease responses and patterns of viremia and antibody production closely resembled those described previously in horses and burros experimentally inoculated or infected by colonized mosquitoes (*14, 15*). In one study of 16 horses given the Trinidad donkey strain of VE virus (after 13 passages in embryonated chicken eggs and 2 in guinea pig brains) in various doses by subcutaneous or intranasal routes or by contact with other horses or mosquito bite, 10 of 16 horses became infected, 9 developed fever, 8 clinical disease and 5 died 5-9 days after infection

(*14*).[6] Viremias were within 1-5 days of inoculation, and N antibody appeared in 4 of 5 survivors by day 7 and in 1 by day 14, though it was not present in 3 horses on day 5 or in 2 on day 6 after inoculation. Virus was recovered from brains of horses dying less than 24 and 40 hours after the end of viremia, but not in 2 dying at 75 and 95 hours. Thus, since viremia terminated 5 days after inoculation, deaths 8 days beyond inoculation did not yield virus from brain or from numerous other tissues tested. This makes it unlikely that virus would have been recovered from the brain or other tissues of horse no. 2 at Sontecomapan, since death was not recorded until about 20 days after infection (table 1). In another study, Trinidad donkey strain of VE virus (13th embryonated chicken egg and 4 guinea pig brain passage) and a Colombian strain from human serum (4th mouse brain passage) were given intramuscularly to burros in large doses ($10^{9.1}$ $WMicLD_{50}$ and 10^{8} $WMipLD_{50}$ respectively) (*15*). The Trinidad strain produced rectal temperatures 40°C or above in 3 of 3 animals and killed 1 of 3 within 6 days; HI antibody was first detected on day 5 and CF antibody on day 6 in the 2 survivors. All 3 burros receiving Colombian strain had fever and 2 died on days 7 and 12; HI but not CF antibodies were found in sera on day 6 and both antibodies were found by day 12 and 16. Thus, the patterns of VE virus antibody development in experimentally and naturally infected horses and burros seem to be similar, with appearance of N and HI antibodies within 7 days of infection and delay in appearance of CF antibodies in serum in some animals for an additional few days to a week.

In contrast, viremias were of longer duration in inoculated horses (*14*) (table 3) or burros (*15*) given large doses of virus than in sentinel horses infected by mosquito bite. Levels of virus attained in horse no. 4 inoculated with 20,000 $SMicLD_{50}$ of a 1965 Mexican strain (table 3) were somewhat lower ($10^{-1.5}$ $SMicLD_{50}$/ml) than observed by Kissling in horses receiving similar size doses of Trinidad strain of virus (usually about 10^{-4} to 10^{-7} $WMipLD_{50}$/ml) (14). However, in burros with the Trinidad strain, viremia levels were often 10^{-1} to 10^{-3} $WMipLD_{50}$/ml on days 1-4 except in the lethal infection where they were $10^{-5.5}$ to $10^{-8.1}$ (*15*). With a Colombian strain, viremias ranged from undetectable on days 1-6 to titers of $10^{-2.5}$ to $10^{-4.4}$ $WMipLD_{50}$/ml on days 1 and 2 after inoculation. Thus evidently the Mexican strains of VE virus as inoculated by mosquitoes in nature or the freshly isolated, first mouse passage strain given by needle produced shorter-lived and lower-titered viremia in horses and burros than higher passaged Trinidad strain. Apparently the shorter viremia or its absence in burros surviving inoculation with 4th mouse passaged Colombian strain more closely resembled the viremias in sentinel equines following natural infection.

The results of these studies could indicate that both equine avirulent and virulent strains of VE virus existed at Sontecomapan, Mexico, in 1965, and/or that the host responses of individual equines varied sufficiently so that disease and death sometimes occurred. The latter phenomenon has been clearly established as a possibility by the findings of others with inoculated horses and burros (*14, 15*). The fact that at Sontecomapan 5 of 6 naturally infected and 1 experimentally inoculated equine were inapparently infected (except for mild fever in

[6] With a subcutaneous inoculum of 32 50%-weanling mouse-intraperitoneal LD_{50} ($WMipLD_{50}$)/0.25 ml or more, 2 developed rectal temperatures to 40°C and 1 of these died; with 4,270 $WMipLD_{50}$, 2 were infected but only 1 developed fever, and with 42,700 $WMipLD_{50}$, 1 horse died; 1.4 $WMipLD_{50}$/0.25 ml failed to infect 2 horses. Intranasally, 94,000 and 188,000 $WMipLD_{50}$ infected and produced fever in individual horses and killed 1 horse; 250 and 25 $WMipLD_{50}$ did not infect by this route. One of 2 horses became infected and died after contact with a subcutaneously inoculated horse during the viremic period, but the other did not become infected after contact with an intranasally inoculated horse. Lastly, 2 horses bitten by infected *Aedes triseriatus* became infected, one developed only fever without clinical manifestations of disease, but the other died; another horse bitten by infected *Mansonia indubitans* did not become infected (*14*).

2) plus the poor health and nutritional status of the horse that died, strongly suggested that numerous if not all, VE virus strains there during July-August 1965, were avirulent for equines. However, the subsequent occurrence of a VE virus equine epizootic in Tamaulipas and northern Veracruz in summer 1966 (7), clearly established that equine virulent strains of VE virus exist along the Gulf coast of Mexico. Thus, the conclusion seems evident that both equine avirulent and equine virulent strains of VE virus are now present in Mexico.

The fact that the 1966 horse epizootic was the first recognized occurrence of equine disease caused by VE virus in Mexico can be cited as evidence to support either of two theories: 1) that VE virus has only recently come to the Gulf coast of Mexico and that it has just now reached sufficient density in nature to cause recognizable disease, or 2) either that virus has been endemic there for years and has been either a relatively avirulent strain or a virulent virus transmitted by mosquitoes only rarely to equines or in small, non-disease-producing doses. This second theory could explain an epizootic by postulating 1) that an unusually virulent mutant arose, and was selected perhaps by rapid passage between vector and amplifying hosts, 2) that populations of ordinary mosquito vectors increased sufficiently to transmit virus to enough equines to cause disease in some or 3) that virus came in contact with a mosquito vector capable of transmitting larger, pathogenic doses. In any case, the second theory requires for genesis of an epizootic, some ecologic change in the virus habitat, as mentioned by de Mucha Macias (16). Obviously further studies will be required to differentiate among these possibilities. Nevertheless the first human VE cases were recognized only in 1962 in Campeche (2) and the first human VE virus fatality with encephalitis was found in Acayucan, Veracruz, in 1965 (17). Thus, as physicians and veterinarians become aware of the presence of VE virus in Mexico and laboratory facilities become available to establish diagnoses, the recognized incidence of VE virus disease of man and equines may well increase, eventually to reach a point of major public health importance.

Also noteworthy to future public health considerations in Mexico are: 1) the inapparent asymptomatic infection of a burro with Patois virus (burro no. 2, table 2) and 2) the finding of equine antibody reacting by HI but not CF or N tests with western encephalitis virus. Whether Patois virus causes equine disease remains to be determined. The antibody reacting by HI test with WE virus was found as maternal antibody in a horse from Catemaco, Veracruz, and as actively produced antibody in a sentinel burro at Sontecomapan. This HI antibody reacting with western encephalitis virus probably represented equine infection by a group A arbovirus other than VE or WE since its presence in serum did not correlate with VE antibody, and it did not react with WE virus by CF or N tests. The identity and significance of these HI inhibitory substances remain to be determined. The WE N antibody found in Mexican Army horses in Veracruz was possibly due to previous inoculation with WE virus vaccines.

Summary

To evaluate equine virulence of Venezuelan encephalitis (VE) virus at an endemic site in Veracruz, Mexico, during July-August, 1965, 3 horses and 3 burros were exposed in nature. These sentinel equines soon became infected by VE virus, but only 1 horse developed clinically apparent disease and this disease could not unequivocally be related to VE virus infection. Otherwise, only transient fever was detected in 2 animals, and VE viremia occurred in a horse while febrile and in an afebrile burro. VE virus hemagglutination-inhibiting, complement-fixing and neutralizing antibodies were found in serum 6-22 days after initiation

of exposure; all 3 antibodies appeared essentially simultaneously in 5 equines, but CF antibody was delayed 10 days in 1 burro. One horse inoculated subcutaneously with a freshly isolated virus developed only mild fever, viremia on days 2-5, and antibodies thereafter.

Since VE virus strains in Veracruz during summer, 1965, usually caused asymptomatic equine infection, the historical absence of equine epizootics along the Atlantic coastal lowlands of Mexico prior to that time could be explained by low virus virulence infrequent transmission of ordinary virus to equines, transmission of virus at low, non-disease-producing doses or previous absence of virus in this region. Future equine epizootics may occur if virulent mutants arise, if mosquito populations increase to cause numerous equine infections some of which are clinically apparent, if virus is introduced into areas where mosquitos transmit larger, pathogenic doses, or if virus moves into new regions where there are susceptible equines. □

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Un estudio de la actividad del virus de la encefalitis equina venezolana natural, realizado en Veracruz, con una descripción de la respuesta de anticuerpos (*Resumen*)

A fin de evaluar la virulencia equina de la encefalitis venezolana (EV) en un lugar endémico de Veracruz, México, durante los meses de julio y agosto de 1965, tres caballos y tres asnos fueron expuestos en un medio natural. Estos equinos centinela pronto quedaron infectados por virus de EV, pero sólo uno de los caballos contrajo la enfermedad clínicamente manifiesta, enfermedad que no pudo relacionarse inequívocamente con la infección por virus EV. Por lo demás, únicamente se observó fiebre transitoria en dos de los animales, y se dio viremia específica en un caballo, durante el período febril, y en un burro no febril. Se observaron en el suero anticuerpos de inhibición de la hemaglutinación, fijación del complemento y neutralizantes del virus de la enfermedad de 6 a 22 días después del comienzo de la exposición; esencialmente, los tres anticuerpos aparecieron al mismo tiempo en 5 equinos, pero el anticuerpo de fijación del complemento se retrasó 10 días en un asno. Un caballo inoculado por vía subcutánea con un virus recién aislado presentó únicamente fiebre moderada, viremia en un plazo de 2 a 5 días y, con posterioridad, anticuerpos.

Como en el verano de 1965 las cepas de virus de EV causaron comúnmente en Veracruz infección equina asintomática, la inexistencia hasta esa fecha de epizootias equinas en las tierras bajas de la costa mexicana del Atlántico podría obedecer a la reducida actividad del virus, a la transmisión poco frecuente del virus ordinario a equinos, a la transmisión del virus en dosis bajas que no originan la enfermedad o a la inexistencia previa de este en esta región. Pueden ocurrir futuras epizootias equinas si surgen mutantes virulentos, si las poblaciones de mosquitos aumentan hasta causar numerosas infecciones en equinos, algunas clínicamente manifiestas, si el virus se introduce en zonas donde los mosquitos transmiten dosis mayores y patógenas o si el virus circula en nuevas regiones en que haya equinos susceptibles.

Estudo de Virulência do Vírus da Encefalite Venezuelana Natural nos Eqüinos em Veracruz com uma Descripção da Resposta de Anticorpos (*Resumo*)

A fim de avaliar a virulência do vírus da encefalite venezuelana (EV) nos eqüinos numa área endêmica em Veracruz, México, durante julho-agôsto de 1965, 3 cavalos e 3 burros foram expostos em condições naturais. Êsses animais logo se infectaram com o vírus EV, porém apenas 1 cavalo apresentou sintomas clínicos da doença, os quais não foi possível, porém, atribuir com certeza a infecção com o vírus EV; fora êsse caso, só se registrou febre passageira em 2 animais e viremia EV num cavalo acometido de febre e num burro afebril. As provas de hemo-aglutinação, fixação do complemento e de anticorpos neutralizantes foram positivas em soros examinados 6-22 dias após o início da exposição; todos os 3 anticorpos apareceram mais ou menos simultâneamente nos 5 eqüinos, porém a prova de fixação do complemento atrasou-se 10 dias em 1 burro. Um cavalo inoculado subcutâneamente

com vírus recém-isolado manifestou apenas febre benigna, viremia nos dias 2-5 e anticorpos a partir de então.

Como as raças de vírus EV em Veracruz durante o verão de 1965 geralmente causaram infecção eqüina assintomática, a ausência histórica de epizootias eqüinas nas baixadas da costa atlântica do México, até aquela ocasião, pode ser explicada pela baixa virulência do virus, transmissão pouco freqüente do vírus comum aos eqüinos, transmissão do vírus em doses pequenas, insuficientes para a manifestação de sintomas ou pela ausência do vírus, até então, na região. No futuro, poderão ocorrer epizootias eqüinas se o vírus passar por mutação, se a população dos mosquitos aumentar de modo a causar numerosas infecções em eqüinos e, entre elas, casos clínicos, se o vírus fôr introduzido em áreas onde os mosquitos transmitem doses mais altas e patogénicas ou se o vírus penetrar em novas regiões onde existam eqüinos susceptíveis.

Etude sur la virulence équine du virus vénézuélien naturel de l'encéphalite dans l'Etat de Veracruz avec une description de la réponse des anticorps (*Résumé*)

En vue d'évaluer la virulence équine du virus vénézuélien de l'encéphalite (VE) dans une zone endémique de l'Etat de Veracruz (Mexique), pendant la période juillet/août 1965, 3 chevaux et 3 ânes ont été exposés dans la nature. Ces sentinelles équines furent rapidement infectées par le virus VE, mais un seul cheval contracta une maladie cliniquement apparente et cette maladie n'a pas pu être nettement rattachée à l'infection par le virus VE. Par ailleurs, une fièvre temporaire a été seulement décelée chez 2 animaux, et une virémie VE chez un cheval pendant qu'il était fébrile, et chez un âne non fébrile. Des anticorps inhibiteurs d'hémoagglutination, de fixation du complément et neutralisants du virus VE ont été constatés dans le sérum 6 à 22 jours après le début de l'exposition; tous les 3 anticorps apparurent presque en même temps chez 5 équidés mais l'anticorps CF est apparu chez un âne avec un retard de 10 jours. Un cheval auquel a été inoculé par voie sous-cutanée un virus fraîchement isolé contracta une fièvre bénigne, une virémie entre le deuxième et cinquième jours et des anticorps ensuite.

Etant donné que les souches du virus VE ont causé au cours de l'été 1965, dans l'Etat de Veracruz, une infection équine asymptomatique, l'absence d'épizooties équines le long des terres basses mexicaines de la côte de l'Atlantique avant cette époque pourrait s'expliquer par la faible virulence du virus, la transmission peu fréquente du virus ordinaire aux équidées, la transmission du virus à de faibles doses ne produisant pas de maladie, ou l'absence anterieure du virus dans cette region. Des épizooties équines pourraient se produire dans l'avenir si des mutants virulents apparaissent si les populations de moustiques augmentent de façon à causer de nombreuses infections équines dont certaines sont cliniquement apparentes, si le virus est introduit dans d'autres régions où les moustiques transmettent de plus fortes doses pathogènes, ou si le virus envahit de nouvelles régions où il y a des équidés susceptibles.