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RAPID IDENTIFICATION OF VENEZUELAN AND EASTERN EQUINE ENCEPHALITIS VIRUSES USING INFECTED SUCKLING MOUSE SERUM AS THE HEMAGGLUTINATING ANTIGEN

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In the routine identification of an arbovirus isolate, various serological tests are required for the final determination of its identity.[1] Of the basic serological tests, hemagglutination-inhibition (HI) is the simplest and least time-consuming technique. Many of the recognized arboviruses have been shown to produce a hemagglutinin by one or another of the standard methods.[2] Infected suckling mouse brain is the usual source of hemagglutinating (HA) antigen which is obtained either by crude preparation or by various laborious extraction techniques. An alternative source of HA antigen, at least for group C arboviruses, is infected suckling mouse serum.[3] Our experience has shown that crude brain antigens of some arboviruses prepared from early mouse passage material were either negative or of low HA activity; therefore we have attempted to apply the method previously used for the preparation of group C hemagglutinins to certain group A arboviruses which are prevalent in Panama.

This report describes the production of specific high titered hemagglutinins of Venezuelan and Eastern equine encephalitis (VEE, EEE, respectively) viruses in infected suckling mouse serum both from type strains and from early passage material of 23 newly-isolated strains of these viruses. This paper also demonstrates the use of these serum HA antigens in the rapid identification of VEE and EEE isolates.

MATERIALS AND METHODS

Virus strains. VEE and EEE viruses used as references for typing of the new isolates in this study were isolated in Panama; VEE (#3880)[4] had been through six suckling mouse brain passages, and EEE (#3847)[5] had undergone eight mouse passages. An additional VEE isolate (TRVL 28215-2) from Trinidad was kindly supplied by Dr. Thomas Aitken and had been passed four times in suckling mice. Other group A arboviruses used were: Mayaro (BeAr 20290), Pixuna (BeAr 35645), Mucambo (BeAn 8) and Aura (BeAr 10315) kindly supplied by Dr. Robert Shope. Western equine encephalitis (WEE, Fleming) was obtained from the Communicable Disease Center. Una (BT 1495-3) isolated at the Middle America Research Unit (MARU)[6] was also used.

Sixteen newly-isolated strains of VEE from various sources in our laboratory, four EEE isolates from MARU[6] and three EEE isolates from New Jersey (N.J.)[7] were used in this study.

Antigens. All HA antigens from suckling mouse serum were prepared by acetone extraction by a modification of the method of Clarke and Casals.[2] The blood of the infected suckling mice, most of which were sick, was harvested by cutting through the anterior chest wall and allowing the blood to drop into a small tube held in an ice-bath. After the blood clotted, 0.1 ml of normal saline per mouse was added, mixed, and centrifuged under refrigerated conditions. The diluted serum (approximately 1:5) was drawn off by a syringe with a 25G needle, measured and added to five volumes of chilled acetone held in an ice-bath, while being stirred constantly with a glass rod. Within five to ten minutes, the chilled mixture was centrifuged lightly and the sediment re-extracted with the same amount of acetone. After the second extraction and centrifugation, the sediment was dried and rehydrated to twice the volume of the diluted serum with borate-saline, pH 9.0. Approximately 0.2 ml of antigen (final dilution 1:10) was obtained per suckling mouse. This antigen was then tested for HA activity at room temperature, using goose erythrocytes in adjusting diluent,[2] to obtain a final pH of 5.75 to 6.0.

Other antigens were prepared as follows:

a) For test strains, crude brain antigens consisted of 20% infected mouse brain suspension in borate-saline, pH 9.0. The mixture was held overnight at 4°C and centrifuged at 10,000 r.p.m. for 30 minutes. Aliquots of the supernatant fluid were stored in glass ampules at −65°C to be used as crude antigen.

b) For all group A type strains available, HA and complement-fixing (CF) antigens were prepared by sucrose-acetone extraction[2] of infected suckling mouse brain.

Immune sera. VEE and EEE immune sera were prepared in adult mice by an intraperitoneal (i.p.) injection of 0.2 ml of a mixture of 10% virus suspension and an equal volume of 0.6% formalin after 1 hour incubation at 37°C. The mice were bled 10 days later. Homologous antibody titers in sera of mice given one injection of VEE virus were sufficiently high to be used in HI tests. VEE and EEE immune sera for CF and neutralization (N) tests, as well as EEE immune serum for HI tests, were obtained 10 days after reinoculation of mice with 0.2 ml of a 10% suspension of infected suckling mouse brains.

Preparation of immune sera for test strains of suspected VEE and EEE isolates was essentially the same as for type strains.

Serological tests. In the HI test, serial twofold dilutions of kaolin-treated immune serum were mixed with four units of HA antigen. Primary incubation of the mixture was done at 37°C for one hour or 4°C overnight. After addition of goose cells, the test was held at room temperature. Occasionally the tests were held at 37°C for one hour because this appeared to be optimal for some EEE isolates.

CF titrations were performed by a microtechnique modified from Fulton and Dumbell,[8] using two units of complement and overnight primary incubation at 4°C. The N test was performed in weanling or young adult mice using equal volumes of serum and serial tenfold dilutions of mouse-brain virus suspensions inoculated intracerebrally (i.c.). The serum-virus mixture was held in a 37°C water bath for one hour before inoculation and the titers were calculated by the method of Reed and Muench.[9]

RESULTS

Hemagglutinin Production

1. *Type strains.* With the simple technique of extraction described above, HA antigens from sera of infected suckling mice inoculated with VEE and EEE type strains yielded titers of 1:2560 and 1:1280 respectively. The VEE strain from Trinidad also produced hemagglutinin with a titer of 1:1280. Since Mucambo and Pixuna viruses are closely related to VEE,[10] serum antigens of these two viruses were tested for their HA activities. Mucambo produced HA titer of 1:80 while Pixuna was negative under our test conditions. HA activity of WEE was positive but with a low titer (1:40). When VEE serum antigen was tested for CF activity, only a low titer (1:8) was obtained.

TABLE 1

Hemagglutination titers of VEE antigens from newly isolated strains prepared from brain and serum

Source	Isolate no.	Mouse passage level	HA titer*	
			Crude brain antigen	Serum antigen
Man	1	2	0	2,560
	2	1	40	2,560
	3	2	20	2,560
	4	2	0	640
	5	2	80	1,280
	6	2	0	1,280
Sentinel mice	7	1	40	1,280
	8	2	20	2,560
	9	2	40	1,280
	10	2	80	320
Rodents	11	1	80	640
	12	1	40	1,280
	13	1	0	5,120
	14	2	160	5,120
Mosquitoes	15	2	160	5,120
	16	1	40	1,280

* Reciprocal titer, 0 = less than 1:20, tests done at 22°C.

2. *New isolates.* The preparation of HA serum antigens for all 16 newly-isolated strains of VEE virus, obtained in Panama during the last half of 1964, was successful even at the first or second mouse passage level. Crude brain antigens prepared from the same litters of infected suckling mice were either negative or had low HA titers (Table 1). Despite the small yield of suckling mouse blood, the high HA titers of these serum antigens make it unnecessary to use more than a few baby mice for HI testing of isolates during the early passages.

Results of HA titrations of serum antigens of new EEE isolates are shown in Table 2. Positive results were obtained in the first suckling mouse brain passage for all three N.J. EEE strains. These had been isolated and passed twice in day-old chicks. Four EEE isolates from Panama also produced serum antigens with high HA titers.

TABLE 2

Hemagglutination titers of EEE antigens from newly isolated strains prepared from brain and serum

Isolates obtained from:	Source	Isolate no.	Mouse passage level	HA titer*	
				Crude brain antigen	Serum antigen
New Jersey†	Bird	1	1	0	2,560
	Pheasant	2	1	0	320
	Horse	3	2	20	320
Panama	Horse	4	3	0	2,560
		5	3	10	1,280
	Sentinel mice	6	2	0	2,560
		7	2	640	2,560

* Reciprocal titer, 0 = less than 1:10. HA test of pheasant isolate was done at 37°C, others at 22°C.

† Isolates from New Jersey had been passed 2 times in day old chicks.

Stability of Hemagglutinins

A limited number of experiments have been performed periodically to check the stability of some VEE serum antigens. Tests in the form of four-unit working dilutions of antigens of 10 test strains maintained at 4°C showed no drop in HA titers for a period of five to seven months. Titration of VEE serum antigen from undiluted specimens stored in sealed glass ampules at −70°C showed the same amount of hemagglutinin for at least seven months.

Specificity of Typing Immune Sera

VEE and EEE immune sera were tested by HI technique against different group A antigens. As shown in Table 3, VEE immune serum (1-injection) and EEE immune serum (2-injections) showed specific reactions. CF testing was performed with VEE and EEE immune sera (2-injections) against antigens of eight group A arboviruses. A fourfold or greater difference was demonstrated between the homologous and heterologous viruses in both HI and CF tests.

Identification Technique

In the course of a three-year arbovirus survey conducted in Almirante, Panama in 1959–62, most of the virus isolates with short incubation periods in suckling mice were identified as VEE,[11] or as various group C arboviruses.[12] In the present studies, after hemagglutinins were demonstrated in the sera of suckling mice infected with VEE and EEE type strains, all isolates with incubation periods of less than three days in suckling mice were routinely screened for HA activity in acetone-extracted serum antigen. Since group C arboviruses are known to produce HA in suckling mouse serum, HA-positive serum antigens were, therefore, tested by HI technique with both VEE and polyvalent group C immune sera. Those HA antigens which were inhibited by VEE immune serum were then titrated simultaneously with the standard VEE antigen. Results were considered positive when comparable HI titers (not more than twofold difference) were observed. In this report, six isolates from man, four from sentinel mice, four from wild rodents and two from mosquitoes were identified as VEE virus by this technique.

HA serum antigens which exhibited negative results for both VEE and group C viruses were then retested with immune sera prepared from EEE or other viruses. Three isolates from horses, two from sentinel mice, one from an unidentified bird and one from a pheasant were identified as EEE virus by this HI test.

In order to determine if any new VEE or EEE isolates were missed by this technique, 28 unidentified isolates obtained during the same period were tested by CF technique using crude brain antigens at dilutions of 1:4 and 1:16 against

TABLE 3

Titration of VEE and EEE type immune sera against certain group A arboviruses

Antigen	Serum			
	HI titer		CF titer	
	VEE 1 inj.	EEE 2 inj.	VEE 2 inj.	EEE 2 inj.
VEE	320*	0†	*256*	4
EEE	20	*320*	4	*128*
WEE	0	0	8	0
Mayaro	0	0	4	0
Pixuna	80	20	16	0
Aura	0	0	8	0
Una	0	40	4	4
Mucambo	80	0	64	0

* Reciprocal of serum titer.

† 0 = titer less than 1:20 in HI, 1:4 in CF.

reference VEE and EEE immune sera. No positive reactions were encountered.

Confirmation of Identity

1. *Antibody survey.* Forty human sera from VEE endemic areas of Panama were tested against both type strain brain antigens and test strain serum antigens. Twenty of these sera gave HI positive reactions with type strain antigens and duplicate titers were obtained with the four VEE and two EEE test strain serum antigens used. The sera nonreactive with type strain antigens likewise gave negative reactions with these test antigens.

2. *Cross-HI test.* Immune sera prepared for eight VEE and seven EEE isolates had comparable HI titers, when reacted with homologous and type strain antigens. Homologous HI titers of immune sera of newly-isolated VEE viruses ranged from 1:80 to 1:320 with single-dose, formalin-killed virus immunization. Titers of 1:40 to 1:80 were observed in immune sera prepared for EEE isolates by the same technique. After another injection of live virus, homologous HI titers for EEE isolates ranged from 1:80 to 1:320.

3. *CF test.* All 16 isolates of VEE virus were tested by CF technique using immune serum prepared in adult mice by two injections of the VEE type strain. EEE isolates were also tested in the same manner. Comparable serum titers, of no more than a fourfold difference, were observed with test and type strain antigens.

4. *N test.* Four VEE isolates, one each from man, sentinel mice, wild rodents and mosquitoes, were tested with 2-injection VEE immune serum by the *in vivo* technique. Neutralization index of more than five log LD_{50}'s was obtained in each test (Table 4). A 2-injection immune serum prepared for one of the EEE isolates (#1) from New Jersey was used in the N test with the other two N.J. strains. The latter isolates, previously typed by HI, were demonstrated to be closely related to EEE isolate #1 which, in turn, was shown to be indistinguishable from the EEE type strain by the N test (Table 4).

5. *Rise of antibody titers in human cases.* In the six persons from whom isolates of VEE virus were obtained, rises in HI antibody titers of more than 32-fold against both homologous serum antigen and type strain antigen were demonstrated between the acute- and convalescent-phase sera. Significant rises in CF antibody titers and in neutralization indices were also obtained in the three cases tested by these techniques.

TABLE 4

*Results of neutralization test of VEE and EEE isolates**

Virus	Log$_{10}$ neutralization index of immune serum†		
	VEE (#3880)	EEE isolate #1	EEE (#3847)
VEE (#3880)	*5.5*	—	—
VEE isolate #1	6.9	—	—
#8	5.0	—	—
#14	6.8	—	—
#15	6.6	—	—
EEE (#3847)	—	3.3	*3.3*
EEE isolate #1	—	*4.9*	—
#2	—	4.1	—
#3	—	3.8	—

* Tests done in young adult mice inoculated intracerebrally.

† All antisera obtained from adult mice after 2 injections of 1:10 dil. of infected brain suspension, the first inoculum with formalin-killed virus.

DISCUSSION

In areas where VEE and EEE viruses are indigenous, the technique of rapid identification described in this communication can be of great value. First, the findings in this study indicate that the HI test alone may be used for the final identification of VEE and EEE isolates obtained from field specimens. Second, the presence of hemagglutinins in the infected suckling mouse sera, during the early passages, makes possible the performance of HI tests within a few days after processing field specimens. Third, the number of experimental animals and working hours may be reduced by this technique because one or two litters of suckling mice are sufficient for processing and identifying a positive field specimen. Finally, the danger of handling VEE virus during primary isolation may be minimized. The infected mouse serum, considered as a by-product, can be used for HA antigen, while the infected brains may serve as the stock virus for further reference work. Since none of the virus isolates that were negative for HA serum antigens were subsequently identified as VEE or EEE virus by other techniques, it is probable that all VEE and

EEE virus strains produce HA in suckling mouse sera during the early passages.

The simplicity of the HI test results in a saving of the time actually spent in identification of new isolates and in the quantities of immune typing sera used. Reading time and handling of the test are not critical as in the case of the CF test. If desired, the identification of many strains of VEE or EEE viruses can be confirmed simultaneously by the CF technique.

SUMMARY

Specific high-titered Venezuelan equine encephalitis and Eastern equine encephalitis hemagglutinins were demonstrated in infected suckling mouse sera following a simple acetone extraction procedure, for both prototype strains and all of the 16 VEE and 7 EEE isolates tested during the early passages. Rapid identification of these isolates by hemagglutination-inhibition test was possible and their identities were confirmed by several different methods.

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