JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1986, p. 155–159
0095-1137/86/010155-05$02.00/0

Vol. 23, No. 1

Complex-Specific Immunoglobulin M Antibody Patterns in Humans Infected with Alphaviruses

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Received 29 July 1985/Accepted 30 September 1985

Sera from humans with serologically confirmed eastern equine encephalitis, western equine encephalitis, Pogosta (Ockelbo), Mayaro, Ross River, and chikungunya virus infections were tested by immunoglobulin M (IgM) antibody capture enzyme immunoassay. Diagnostically useful IgM antibody titers were detected, and selected sera with high IgM antibody titers were tested for IgM antibody with nine heterologous alphaviruses. The results provide evidence for the complex specificity of IgM antibody and indicate the usefulness of this test in both individual cases and epidemic situations.

There are 26 viruses presently recognized as members of the genus *Alphavirus* (Table 1) (3); all are antigenically interrelated. These viruses have been separated into six antigenic complexes, each complex consisting of either a single species (virus) having no known close relatives, such as eastern equine encephalitis (EEE), Venezuelan equine encephalitis (VEE), Middelburg, and Ndumu viruses, or several species that are more closely related to each other than to other members of the genus, such as Semliki Forest (SF) and western equine encephalitis (WEE) virus. Certain members of all complexes except Middelburg and Ndumu are known to cause disease in humans (1, 8). Individual species belonging to the SF complex (chikungunya [CHIK], o'nyong nyong [ONN], Ross River [RR], and Mayaro [MAY] viruses) have been responsible for extensive outbreaks of fever, rash, and polyarthritis in humans. Subtypes of VEE virus are recognized to cause explosive and extensive periodic epidemics and epizootics in humans and equines in the Americas. Three members of the WEE complex (WEE, Sindbis [SIN], and Ockelbo [OCK] viruses) cause diseases (WEE virus causes encephalitis in humans and equines; SIN and OCK viruses cause fever, rash, and polyarthritis in humans). Because of their wide geographic distributions and the severity of the outbreaks caused by these viruses, adequate serodiagnostic methods are needed for clinical, epidemiologic, and virologic studies. Currently, hemagglutination-inhibition (HI), complement fixation (CF), and neutralization (N) tests are used in serodiagnostic tests for antibody in whole sera from patients with illnesses compatible with those recognized as caused by alphaviruses. Often, information concerning the known geographic distributions of these viruses, their antigenic relatedness, and the clinical picture assists the laboratory worker in selecting a proper battery of test antigens. However, the geographic overlap of certain closely related viruses, the absence of adequate travel history, and the need for paired sera to demonstrate significant antibody titer rises in whole sera make serologic tests by classical methods slow and the results often less than confirmatory.

Studies of rabbits infected with group B arboviruses by Westaway demonstrated the specificity of immunoglobulin M (IgM) in infections with closely related viruses, suggesting that such specificity might be applied to the serologic diagnosis of arbovirus infections in humans (15). Subsequently, studies of IgM antibodies in humans infected with dengue-2 (13), Japanese encephalitis (5), St. Louis encephalitis (9), and yellow fever (7) flaviviruses and the alphavirus, RR virus (11), demonstrated the relative specificity of this immunoglobulin. That is, IgM antibody titers were higher to the infecting virus than to closely related heterologous viruses. Enzyme-linked immunosorbent assays (ELISAs) have been applied to such diagnostic problems recently. Frazier and Shope (6) were able to detect antibodies to the alphavirus VEE virus in individuals vaccinated with that virus, but their ELISAs, while diagnostically useful, probably detected immunoglobulin G antibody, revealed low titer, and were not designed to test heterologous reactivity. We had obtained preliminary evidence that IgM antibody to WEE virus reacted with SIN virus, although to lower titer than with the homologous virus (2). However, there existed no published comprehensive comparison of homologous and heterologous reactivities of IgM antibodies produced by humans in response to infections with various alphaviruses. Therefore, we determined the specificities of IgM antibodies produced in humans after infection with EEE, WEE, Pogosta (OCK) disease (2), MAY, CHIK, RR, or VEE virus. The results demonstrate the sensitivity of IgM capture ELISAs in alphavirus infections and show conclusively that IgM antibody produced in response to alphavirus infections of humans is monotypic with regard to antigenic complex and homotypic with regard to viruses within such complexes. The ELISA for IgM antibody, therefore, can be used to

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TABLE 1. Classification of the *Alphavirus* genus based on antigenic relationships[a]

Complex	Species	Subtype	Variety
EEE	EEE		2
MID	MID		
NDU	NDU		
SF	SF		
	CHIK	CHIK	Several
		ONN	
	GET	GET	
		SAG	
		BEB	
		RR	
	MAY	MAY	
		UNA	
VEE	VEE	VEE	A and B
			C
			D
			E
			F
		EVE	
		MUC	A
			B
			C
		PIX	
		CAB	
WEE	WEE		
	Y-62-33		
	HJ		
	FM		
	SIN	SIN	
		WHA	
		KZL	
	AURA		

[a] Adapted from Calisher et al. (3). Virus abbreviations: EEE, eastern equine encephalitis; MID, Middelburg; NDU, Ndumu; SF, Semliki Forest; CHIK, chikungunya; ONN, o'nyong nyong; GET, Getah; SAG, Sagiyama; BEB, Bebaru; RR, Ross River; MAY, Mayaro; UNA, Una; VEE, Venezuelan equine encephalitis; EVE, Everglades; MUC, Mucambo; PIX, Pixuna; CAB, Cabassou; WEE, western equine encephalitis; HJ, Highlands J; FM, Fort Morgan; SIN, Sindbis; WHA, Whataroa; KZL, Kyzylagach; AURA, Aura.

make a rapid and specific serodiagnosis of alphavirus infections in humans.

MATERIALS AND METHODS

Human sera. Sera used in the tests reported here had been accumulated over a 10-year period and stored at −20°C since collection. Most were collected during outbreaks, but a few were from sporadic cases. All sera were from individuals shown originally to have fourfold or greater rises in HI, CF, or N titers or from whom the etiologic agent was isolated. We tested all sera at 1:100 and 1:1,000 dilutions for determination of IgM antibody by ELISA and then titrated the positive sera by testing serial twofold dilutions. A maximum of 20 sera from individuals with each disease were then used in subsequent tests. These 20 sera were selected for both high titer and volume. Approximately half of the sera from each group of sera had been obtained from patients of each sex and were reasonably distributed by age of the patients. Since we tested more sera in preliminary tests than in the final tests reported here, we also had an opportunity to assess IgM antibody titers by time after reported onset of illness.

Sera tested for heterologous reactivity by IgM ELISA were from patients with EEE in Massachusetts (n = 12), Florida (n = 6), and South Carolina (n = 2); patients with WEE in Minnesota (n = 15) North Dakota (n = 2) Colorado (n = 1), Kansas (n = 1), and New Mexico (n = 1); patients with Pogosta disease in Finland (n = 20); patients with MAY virus infection in Brazil (n = 14); patients with RR virus infection in American Samoa (n = 15), Fiji (n = 3), and Australia (n = 2); patients with CHIK in Indonesia (n = 17), Kenya (n = 2), an accidentally infected laboratory worker in New York (n = 1); and patients with VEE in Venezuela (n = 4).

Purification of viruses. Eleven viruses, representing four of the six recognized antigenic complexes of alphaviruses, were purified for use in detecting antibody by ELISA. Prototype strains of EEE (NJO), WEE (McMillan), Highlands J (B-230), SIN (EgAr 339), OCK (Edsbyn 5/82), VEE (vaccine strain TC-83), SF (original), CHIK (S-27), RR (T-48), MAY (TRVL-4675), and ONN (UgMP30) viruses were used to inoculate Vero cells grown to monolayer in plastic flasks (150 cm^2). Infected cell culture supernatant fluids were decanted on days 3 through 5, when cytopathic effects involved 75 to 100% of the cells. These fluids were clarified by centrifugation at 27,000 × g for 30 min, and the virus was precipitated with 7% polyethylene glycol 8,000 by a modification of the method of Roy and Bishop (12). Polyethylene glycol precipitates were suspended in a small volume of TNE (0.02 M Tris hydrochloride, 0.1 M NaCl, 1 mM EDTA; pH 7.8) buffer, layered onto a 12-ml glycerol-potassium tartrate gradient (10) and centrifuged at 150,000 × g for 4 h at 4°C in a swinging bucket rotor. Further purification was achieved by rebanding the virus overnight. The virus was then pelleted through 30% sucrose in TNE buffer at 110,000 × g for 4 h and resuspended in TNE buffer. Viral protein concentration was determined by using the

TABLE 2. Cross-reactivity with heterologous alphaviruses by IgM antibody capture ELISA: 20 sera from patients infected with EEE virus[a]

Serum sample	Days after onset	EEE titer			IgM ELISA titer with EEE antigen
		HI	CF	N	
E-1	6	320	<64	20	102,400
E-2	11	≥640	64	80	409,600
E-3	8	80	<8	10	6,400
E-4	15	≥640	64	80	102,400
E-5	23	≥640	64	40	102,400
E-6	29	320	64	40	102,400
E-7	9	≥640	32	80	102,400
E-8	12	NT[b]	128	80	1,638,400
E-9	11	≥640	64	80	409,600
E-10	4	80	8	20	6,400
E-11	29	≥640	256	80	102,400
E-12	16	≥640	64	≥80	25,600
E-13	1	40	<8	NT	25,600
E-14	26	160	128	NT	102,400
E-15	8	80	<8	1,280	6,400
E-16	22	320	32	10,240	6,400
E-17	9	40	<8	NT	25,600
E-18	18	80	8	NT	6,400
E-19	6	80	<8	NT	6,400
E-20	17	320	8	NT	102,400

[a] IgM ELISA titers of these 20 sera were <100 with the following alphaviruses: WEE, HJ, SIN, OCK, VEE, SF, RR, CHIK, and MAY (virus abbreviations are given in footnote *a* of Table 1).

[b] NT, Not tested.

TABLE 3. Cross-reactivity with heterologous alphaviruses by IgM antibody capture ELISA: 20 sera from patients infected with WEE virus[a]

Serum sample	Days after	WEE titer			IgM ELISA titer with the following antigen:			
		HI	CF	N	WEE	HJ	SIN	OCK
W-1	10	40	8	≥160	16,000	32,000	—[b]	1,000
W-2	38	160	8	NT[c]	16,000	16,000	—	—
W-3	18	160	32	160	8,000	16,000	—	1,000
W-4	0	<10	NT	1,280	16,000	8,000	—	1,000
W-5	16	160	64	320	32,000	32,000	—	1,000
W-6	9	NT	NT	1,280	64,000	32,000	—	1,000
W-7	13	NT	NT	640	32,000	16,000	100	4,000
W-8	17	NT	NT	1,280	16,000	16,000	100	4,000
W-9	5	NT	NT	320	8,000	2,000	—	
W-10	7	NT	NT	640	16,000	4,000	—	—
W-11	8	NT	NT	640	32,000	4,000	—	—
W-12	10	NT	NT	1,280	16,000	1,000	—	—
W-13	8	80	8	NT	16,000	8,000	—	—
W-14	17	160	<8	NT	64,000	4,000	—	—
W-15	28	320	32	NT	32,000	4,000	—	—
W-16	23	160	32	NT	16,000	8,000	—	100
W-17	7	20	<8	5,120	8,000	2,000	—	—
W-18	25	160	32	NT	6,400	2,000	—	—
W-19	25	320	<8	NT	32,000	16,000	—	100
W-20	13	160	<8	NT	8,000	8,000	—	100

[a] IgM ELISA titers of these 20 sera were <100 with the following alphaviruses: EEE, VEE, SF, RR, CHIK, and MAY (virus abbreviations are given in footnote *a* of Table 1).
[b] —, Titer of <100.
[c] NT, Not tested.

Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) with human globulin as the standard.

IgM antibody capture enzyme immunoassay. The procedure used was a modification of that published by Monath et al. (9). Briefly, Immulon 2 (Dynatech Laboratories, Inc., Alexandria, Va.) flat-bottomed polystyrene plates were coated with commercially available, anti-human mu chain antibody produced in goats (Cappel Laboratories, Cochranville, Pa.). The following reagents were then added sequentially in 100-μl volumes: human test serum, 200 ng of purified virus protein, hyperimmune mouse ascitic fluid (14), commercially available goat anti-mouse IgG conjugated with horse radish peroxidase (Jackson Immunoresearch Laboratories, Inc., Avondale, Pa.), and substrate (ABTS; Kirkegaard and Perry Laboratories, Gaithersburg, Md.). Diluent for all reagents except the anti-human mu chain (coating) antibody was phosphate-buffered saline (pH 7.2) containing 10% fetal bovine serum; diluent for coating antibody was carbonate-bicarbonate buffer, pH 9.3. Incubation periods and temperatures were 18 h and room temperature for coating, 1 h and 37°C for human serum, 3 h and 37°C for virus protein, 1 h and 37°C each for hyperimmune ascitic fluid and anti-mouse IgG conjugate, and 3 to 5 min and RT° for the substrate. Between each addition of reagent, plates were washed 10 times mechanically (Titertek Microplate washer; Flow Laboratories, McLean, Va.) with phosphate-buffered saline containing 0.05% Tween 20. Optical densities (OD) were measured at 409 nm in an automatic device (Titertek Multiskan, Flow Laboratories) and recorded by the same instrument. Controls included human test sera without virus protein and pooled normal human sera with virus protein. If control OD did not exceed 0.200 and test serum OD exceeded 0.350, results were accepted, and the ratio of the OD of the test serum to the OD of the negative control serum was calculated. When this ratio was ≥2.0, the test serum was considered positive for IgM antibody.

RESULTS

Although homologous IgM antibody titers were as high as 10^6 in sera from patients with EEE virus infections, heterologous titers were all < 100 (Table 2). Heterologous IgM antibody was not detected in sera from four individuals with confirmed VEE virus infections (data not shown).

IgM antibody in sera from individuals with WEE virus infections reacted only with HJ and OCK viruses and, in two individuals, SIN virus (Table 3). Likewise, sera from patients infected with OCK virus contained IgM antibody that reacted with WEE, HJ, and SIN viruses (Table 4).

IgM antibody reacted with CHIK, RR, and SF viruses in individuals with MAY virus infections (Table 5); it reacted with MAY, CHIK, and SF viruses in individuals with RR virus infections (Table 6); and it reacted with MAY, RR, and ONN viruses in those infected with CHIK virus (Table 7). One individual with CHIK virus infection had repeatable IgM heterologous reactivity to SF virus, and another had a repeatable IgM titer to HJ virus. Peak IgM titers were reached 2 to 3 weeks after the onset of illness in infections due to EEE, WEE, MAY, CHIK, and RR viruses and declined but persisted through at least the first 2 months in all but CHIK virus infections. Individuals infected with CHIK virus attained peak IgM titers 3 to 5 weeks after onset, and IgM titers declined somewhat but remained relatively high for at least 2 months after onset. Data for IgM antibody production in persons with Pogosta disease have been published previously (2).

DISCUSSION

IgM antibody detected as described here reacts only with heterologous viruses belonging to the same antigenic complex (3). Since only one virus is known to occur within the EEE and VEE complexes, IgM assays for antibody to these viruses should provide a rapid and specific diagnostic tool;

TABLE 4. Cross-reactivity with heterologous alphaviruses by IgM antibody capture ELISA: 20 sera from patients infected with Pogosta disease (OCK) virus[a]

Serum sample	Days after onset	SIN HI[b]	IgM ELISA titer with the following antigen:			
			WEE	HJ	SIN	OCK
P-1	12	40	—[c]	—	16,000	16,000
P-2	58	80	—	—	100	1,000
P-3	12	80	—	1,000	16,000	25,600
P-4	81	160	—	—	—	1,000
P-5	7	20	400	1,000	32,000	64,000
P-6	22	40	400	100	4,000	8,000
P-7	79	80	—	—	—	100
P-8	15	80	—	—	1,000	4,000
P-9	3	<10	—	—	—	400
P-10	15	40	400	100	16,000	8,000
P-11	80	80	—	—	—	1,600
P-12	17	40	1,000	100	64,000	32,000
P-13	17	40	—	100	1,000	16,000
P-14	15	10	—	—	—	1,000
P-15	34	160	—	—	32,000	8,000
P-16	95	160	—	—	100	1,000
P-17	19	40	400	—	64,000	16,000
P-18	70	80	—	—	100	1,600
P-19	20	20	100	—	32,000	32,000
P-20	77	80	—	—	—	1,000

[a] IgM ELISA titers of these 20 sera were <100 with the following alphaviruses: EEE, VEE, SF, RR, CHIK, and MAY (virus abbreviations are given in footnote *a* of Table 1).
[b] No virus has been isolated in association with Pogosta disease in Finland. OCK virus from mosquitoes collected in Sweden has been shown to react with sera from Pogosta disease patients and is closely ralated to SIN virus; antibodies in sera from Pogosta disease patients have been determined by using SIN virus as antigen (2).
[c] —, Titer of <100.

this will likely be the case in infections with Middelburg and Ndumu viruses, which are also single-species complexes. The utility of such tests in the differential serodiagnosis of human infections with SF and WEE complex viruses is not as clear-cut. Ratios of homologous to heterologous IgM titers were high in most of the individuals with infections due to SF complex viruses MAY, CHIK, and RR. On a highest titer basis, and certainly during epidemics, it would be possible to provide reasonably specific and rapid serodiagnostic results. One note of caution, however; OD values of sera with IgM antibody do not directly correspond to the dilution titer. This can be overcome by using 1:100, 1:1,000 and, perhaps, 1:10,000 dilutions of sera when screening for homologous and heterologous reactivities. It appears that IgM antibody capture ELISA is adequate for distinguishing infections due to the closely related CHIK and ONN viruses.

Similarly, infections caused by WEE and OCK viruses of the WEE complex produce IgM antibodies that cross-react significantly with other closely related viruses. Whereas IgM antibody titers to WEE and HJ and to OCK and SIN viruses may not clearly distinguish with certitude the infecting agent, clinical human infections caused by HJ virus are not known, and from the knowledge of the geographic distributions of OCK and SIN viruses, the latter could be excluded as the etiologic agent in OCK virus infections. The same can be said for CHIK (Africa and southeast Asia), RR (South Pacific and Australia), and MAY (northern and coastal South America) virus infections.

Cross-reactivity of IgM antibody in alphavirus infections occurs only with members of the same antigenic complex, probably because of the specificity of IgM antibody produced in response to specific viral envelope glycoproteins (4). Unlike monoclonal antibodies that are epitope specific, human IgM antibodies are undoubtedly polyclonal, hence the reactivity in HI, N, and ELISA. Later in the course of infection, IgG antibody is responsible for HI, CF, and N reactivity and is even less specific; the specificity of IgM antibody, as measured by ELISA, does not change with time after its onset. Thus, the IgM antibody-capture ELISA is a

TABLE 5. Cross-reactivity with heterologous alphaviruses by IgM antibody capture ELISA: 14 sera from patients infected with MAY virus[a]

Serum sample	Days after onset	MAY HI titer	IgM ELISA titer with the following antigen:			
			SF	RR	CHIK	MAY
M-1	14	320	—[b]	100	—	102,400
M-2	15	160	—	2,000	—	102,400
M-3	15	160	—	1,000	32,000	102,400
M-4	16	320	—	2,000	16,000	102,400
M-5	12	80	—	—	—	25,600
M-6	15	320	100	1,000	4,000	409,600
M-7	7	20	—	—	—	6,400
M-8	14	160	—	—	—	6,400
M-9	15	80	8,000	4,000	16,000	102,400
M-10	15	160	—	4,000	100	64,000
M-11	4	10	—	—	100	6,400
M-12	17	160	—	4,000	16,000	64,000
M-13	14	160	—	8,000	1,000	64,000
M-14	15	160	—	8,000	4,000	64,000

[a] IgM ELISA titers of these 14 sera were <100 with the following alphaviruses: EEE, WEE, HJ, SIN, OCK, and VEE (virus abbreviations are given in footnote *a* of Table 1).
[b] —, Titer of <100.

TABLE 6. Cross-reactivity with heterologous alphaviruses by IgM antibody capture ELISA: 20 sera from patients infected with RR virus[a]

Serum sample	Days after onset	RR titer			IgM ELISA titer with the following antigen:			
		HI	CF	N	SF	RR	CHIK	MAY
R-1	>20	80	16	2,560	—[b]	1,600	—	—
R-2	>120	320	32	5,120	—	400	—	—
R-3	106	160	8	1,280	—	1,600	—	—
R-4	16	80	8	640	—	6,400	—	—
R-5	37	160	32	1,280	—	1,600	—	—
R-6	>90	160	NT[c]	2,560	—	1,600	—	—
R-7	39	80	16	NT	—	25,600	100	—
R-8	43	20	<8	640	—	1,600	—	—
R-9	68	40	<8	640	—	400	—	—
R-10	17	160	16	NT	—	25,600	—	—
R-11	10	160	16	NT	—	25,600	—	—
R-12	17	640	64	NT	100	409,600	8,000	—
R-13	3	20	<8	<40	—	25,600	—	—
R-14	19	160	<8	1,280	8,000	102,400	8,000	—
R-15	16	80	<8	320	—	25,600	—	—
R-16	11	160	16	320	8,000	25,600	—	1,000
R-17	24	80	16	640	—	25,600	—	—
R-18	35	80	8	1,280	—	25,600	—	—
R-19	8	80	<8	80	—	25,600	—	—
R-20	22	80	<8	1,280	—	1,600	—	—

[a] IgM ELISA titers of these 20 sera were <100 with the following alphaviruses: EEE, WEE, HJ, SIN, OCK, and VEE (virus abbreviations are given in footnote *a* of Table 1).
[b] —, Titer of <100.
[c] NT, Not tested.

TABLE 7. Cross-reativity with heterologous alphaviruses by IgM antibody capture ELISA: 20 sera from patients infected with CHIK virus[a]

Serum sample	Days after onset	CHIK titer		IgM ELISA titer with the following antigen:					
		HI	CF	HJ	SF	RR	CHIK	ONN	MAY
C-1	38	80	256	—[b]	—	8,000	128,000	800	4,000
C-2	?	40	512	—	—	—	6,400	—	—
C-3	16	320	8	—	—	—	25,600		—
C-4	24	80	NT[c]	—	—	100	102,400	—	1,000
C-5	80	80	NT	—	—	—	25,600	—	—
C-6	24	80	NT	—	100	—	25,600	—	—
C-7	25	80	NT	—	—	500	128,000		1,000
C-8	23	80	NT	1,000	—	—	32,000	—	1,000
C-9	22	80	NT	—	—	—	25,600	3,200	—
C-10	22	40	NT	—	—	—	25,600	800	100
C-11	24	160	NT	—	—	—	25,600	—	—
C-12	23	80	NT	—	—	—	25,600	—	—
C-13	16	160	NT	—	—	—	25,600	3,200	—
C-14	58	80	NT	—	—	—	1,600	—	—
C-15	16	160	NT	—	—	—	25,600	—	800
C-16	18	40	NT	—	—		25,600	—	—
C-17	40	80	NT	—	—	100	25,600	—	—
C-18	8	20	NT	—	—	—	6,400	—	—
C-19	30	80	NT	—	—	—	102,400	—	4,000
C-20	31	160	NT	—	—	—	25,600	3,200	—

[a] IgM ELISA titers of these 20 sera were <100 with the following alphaviruses: EEE, WEE, SIN, OCK, and VEE (virus abbreviations are given in footnote *a* of Table 1).
[b] —, Titer of <100.
[c] NT, Not tested.

sensitive, specific, and rapid test for early differential serodiagnosis of alphavirus infections in humans. When appropriate, a second cross-reactive virus belonging to the WEE or SF complex could be included for comparison. Further investigations are needed to determine the cause of the apparent persistence of IgM antibody in CHIK virus infections and to determine whether it is a true indicator of antigenic persistence with continuing stimulation of IgM antibody or merely a sampling artifact.

Until such time as more sensitive antibody assays or antigen detection systems are devised, the IgM antibody capture ELISA can be applied in epidemic situations and in individual cases that could prove to be the indicator cases in such situations.

Adaptation of this test for the serodiagnosis of infections caused by EEE, WEE, VEE, RR, or Getah virus in equines could provide a rapid, epidemiologically relevant test in a veterinary setting. We have shown that the IgM antibody capture ELISA is applicable to rapid and specific serodiagnosis of WEE virus infections of equines (unpublished observations).

ACKNOWLEDGMENTS

We thank the many individuals who made these studies possible and fruitful by providing sera, data, and laboratory assistance. These include E. Buff, Florida Division of Health, Jacksonville, Fla.; V. Berardi and L. Farnham, Massachusetts State Diagnostic Laboratory, Boston, Mass.; R. Walder, Instituto Venezuelano Investigaciones Cientificas, Caracas, Venezuela; D. Shroyer, Florida Medical Entomology Laboratory, Vero Beach, Fla.; and J. Lazuick, D. Muth, and W. Vesely, Centers for Disease Control, Ft. Collins, Colo. The stimulating editorial comments and suggestions made by T. P. Monath are appreciated.

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