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Epitope-Blocking Enzyme-Linked Immunosorbent Assay for Detection of Antibodies to Ross River Virus in Vertebrate Sera

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We describe the development of an epitope-blocking enzyme-linked immunosorbent assay (ELISA) for the sensitive and rapid detection of antibodies to Ross River virus (RRV) in human sera and known vertebrate host species. This ELISA provides an alternative method for the serodiagnosis of RRV infections.

Ross River virus (RRV) is a mosquito-borne alphavirus that is endemic in Australia and Papua New Guinea. The primary hosts of the virus include humans, kangaroos, wallabies, and horses (2, 11, 12, 15). RRV is the major etiological agent of epidemic polyarthritis in humans, which is characterized by severe arthritis with possible development of a rash and mild fever or chills and for which there is no specific treatment. Effective surveillance of virus activity in its natural transmission cycle, with appropriate warnings to the public, is the best measure available for minimizing this disease. Traditionally, hemagglutination inhibition (HI), neutralization, and immunofluorescence assays (2, 7, 13) have been the means for detecting RRV antibodies in both human and animal sera. Enzyme-linked immunosorbent assay (ELISA) has also been used to specifically detect RRV immunoglobulin M in human sera (6, 17). Previously, epitope-blocking assays were developed for sensitive and specific detection of seroconversions to the medically important flaviviruses Murray Valley encephalitis virus (8, 9) and West Nile virus (3, 4, 10) in avian and mammalian sera. In this study, an epitope-blocking ELISA was developed for the rapid detection of RRV antibodies in both animal and human sera to improve the efficiency of seroepidemiological studies.

This study used seven isolates of RRV, obtained over 30 years from different regions in Australia, as well as the closely related alphaviruses Chikungunya virus, Getah virus, Barmah Forest virus (BFV), Semliki Forest virus, and Sindbis virus (Table 1). RRV ELISA antigen was produced by propagation of the prototype RRV strain T48 on Vero cells in serum-free medium. Virus supernatant was clarified at 4,000 × *g* for 15 min at 4°C and stored in 1-ml aliquots at −80°C. Polyclonal antisera were produced in New Zealand half-lop rabbits by intravenous inoculation with 50 μg purified virus/200 μl phosphate-buffered saline and bled at day 14 postinoculation (Table 1). Hyperimmune antisera were not used due to the enhanced cross-reactions observed after multiple immunizations. Nonreactive control sera were collected from nonim-

TABLE 1. Neutralization titer and percent inhibition of MAb binding in the epitope-blocking ELISA produced by rabbit antisera to reference RRV strains and other alphaviruses

Rabbit antiserum (location and yr of strain isolation)	Neutralization titer[b]	% Inhibition[a] of MAb:		
		3B2[c]	G8	B10
RRV strains				
T48 (North Queensland, 1959)	160	**56**	46	67
NB5092 (Eastern New South Wales, 1969)	160	**56.5**	62	64
Ch19575 (western Queensland, 1976)	640	**75.5**	54	0
K1503 (East Kimberly, WA, 1984)	320	**65**	17	0
WK20 (West Kimberley, WA, 1977)	320	**67**	18	0
SW876 (southwest WA, 1987)	160	**50**	82	61
SW2191 (southwest WA, 1988)	80	**76**	42	0
Other alphaviruses				
Getah virus	480	**6.5**	11	0
Sindbis virus	40	**0**	48	0
BFV	640	**0**	59	0
Semliki Forest virus	640	**4**	70	0
Chikungunya virus	40	**0**	70	0

[a] The percent inhibition of MAb binding was calculated as 100 − [OD (test)/OD (negative control) × 100].
[b] Against homologous virus.
[c] Boldface indicates that the RRV-specific MAb 3B2 produced the best results.

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TABLE 2. Comparison of the specificity and sensitivity of the epitope-blocking ELISA with virus neutralization to detect anti-RRV antibodies in kangaroo and horse sera

Animal species	% Inhibition of MAb 3B2[a]	Neutralization titer vs T48[b]
Kangaroo	95	80, 640
	86	80, 640
	76	40, 160
	84	640, 160
	91	40, 160
	79	80, 160
	94	<40, 320
	91	80, 80
	82	320, 160
	90	320, 160
	66	80, 160
	88	640, 160
	78	<40, 40
	89	320, 160
	84	320
	84	160
	80	160
	17	<40
	18	<40
	19	<40
	17	<40
	16	<40
	8	<40
Horse	22	<40, <40
	9	<40, <40
	12	<40
	59	640
	0	<40
	0	<40
	0	<40
	71	80
	0	<40
	42	80
	15	<40

[a] Mean inhibition calculated from two separate assays.
[b] A titer of 40 or greater was considered positive. Two entries represent titers from two separate assays.

mune animals. Clinical samples of human sera (PathCentre, Western Australia [WA] State Health Department, QE11 Medical Centre, Nedlands, Australia) and samples from kangaroos and horses were collected as part of an ongoing seroepidemiological study of RRV in parts of WA (13). These samples were previously tested for RRV antibodies by standard assays (1, 6, 7). Titers are presented as the reciprocal of the highest dilution of antibody to completely neutralize or inhibit RRV. In developing the epitope-blocking ELISA, the protocol described by Hall et al. (9) was adapted. U-bottomed 96-well polyvinyl chloride plates were coated with an optimal concentration of RRV ELISA antigen at 50 μl/well under appropriate biological containment and incubated overnight at 4°C in coating buffer (0.1 M carbonate/bicarbonate, pH 9.6). Antigen-coated plates were washed twice with wash buffer, and nonspecific sites were blocked with 100 μl blocking buffer (0.05 M Tris, 1 mM EDTA, 0.15 M NaCl, 0.05% [vol/vol] Tween 20, 0.2% [wt/vol] casein, pH 8.0) for 1 hour at room temperature (RT). Reference or test sera were added (50 μl/well) in duplicate at dilutions of 1/10 and 1/100 in blocking buffer and incubated for 2 hours at RT. Nonimmune chicken and rabbit sera were used as nonreactive controls. Without removal of serum, 50 μl of monoclonal antibody (MAb) (hybridoma culture supernatant diluted in blocking buffer) was added to each well, and after gentle agitation the plates were incubated at RT for 1 hour. Plates were washed four times and bound MAb detected by incubation with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Bio-Rad) diluted in block-

TABLE 3. Comparison of the RRV-specific epitope-blocking ELISA and standard diagnostic techniques for detection of antibodies to RRV in human sera[a]

Serum no.	% MAb inhibition	HI titer	Immunoglobulin M[b]	Serum no.	% MAb inhibition	HI titer	Immunoglobulin M[b]
5001237Z	0	<10	−	5020602Y	99	640	+
5001709Y	26	40	+	5020667R	96	160	+
5001710Z	5	<10	−	5020669T	77	80	+
5002634Z	25	160	+	5020680T	30	80	+
5003475S	86	80	+	5020958X	88	160	+
5005725Z	75	40	+	5023355W	90	80	+
5005726N	62	160	+	5023917X	95	160	+
5005732	73	40	+	5022402N	42	40	+
5005736	96	320	+	5024567Y	84	640	+
5005979N	42	160	+	5025726Q	0	<10	− (BFV+)
5002137X	4	10	−	5025727R	0	<10	− (BFV+)
5005721U	91	160	+	5025859R	38	80	+
5003914R	57	40	+	5025866P	87	320	+
5003734	73	160	+	5025872T	93	160	+
5006620R	78	160	+	5026087N	37	40	+
5007112N	**0**	**40**	**+**	5027036R	0	10	NT
5007587Q	**20**	**<10**	**−**	5027498R	88	40	− (RRV+)
5010454	84	>640	+	5027993R	89	640	+
5010813	98	160	+	5028013P	90	80	+
5010849	95	160	+	5028040U	81	640	NT
5012156U	65	80	+	**5028481W**	**10**	**80**	**+**
5012919Z	91	80	+	5029421P	88	80	+
5012941Z	81	80	+	5029446S	59	320	+
5013411W	87	640	+	5029423R	95	320	NT
5013914S	90	160	+	5030507Y	92	640	+
5013418	76	160	+	5030539X	91	80	+
5013430S	93	160	+	5030985R	89	320	+
5013438P	88	160	+	5031004N	34	80	+
5013919Y	82	160	+	5031017Q	85	160	+
5016123Q	90	80	+	5031453Y	96	320	+
5016283X	92	160	+	5032419W	96	40	− (RRV+)
5018282T	94	320	+	5032916Y	95	320	− (RRV+)
5018285X	90	80	+	5033430U	95	40	− (RRV+)
5019288Z	88	80	+	5033659S	91	80	− (RRV+)
5018296X	95	640	+	5034376U	91	40	− (RRV+)
5018292S	0	<10	− (BFV+)	5034746Q	96	>640	− (RRV+)
5019113N	36	40	+	5034773W	93	>640	− (RRV+)
5020529X	90	160	+	5035191W	58	40	− (RRV+)
5020658T	40	80	+				

[a] Boldface indicates discrepancies between ELISA, HI, and immunoglobulin M results.
[b] (RRV+), negative for immunoglobulin M but positive for immunoglobulin G; (BFV+), positive for antibodies to BFV; NT, not tested.

TABLE 4. Comparison of the epitope-blocking ELISA and standard diagnostic techniques for the detection of RRV antibodies in paired serum samples[a]

Serum no.	Serum pair no.	% Inhibition	HI titer	Immunoglobulin M
5001237Z	1	0	<10	−
5003914R		57	40	+
5001710Z	2	5	<10	−
5003734		73	160	+
5002137X	3	4	10	−
5006620R		78	160	+
5007112N	4	***0***	***40***	**+**
5013418		76	160	+
5007587Q	5	***20***	***<10***	**−**
5013919Y		82	160	+
5019113N	6	36	40	+
5023917X		95	160	+
5022402N	7	42	40	+
5028040U		81	640	NT[b]
5026087N	8	37	40	+
5030985R		89	320	+
5027036R	9	0	10	NT
5029446S		59	320	+

[a] Nine paired samples are listed sequentially as acute- and convalescent-phase samples. Boldface indicates discrepancies between blocking ELISA, HI, and immunoglobulin M results. The PathCentre Laboratory, which supplied these paired serum samples, requests that doctors obtain a convalescent-phase serum 14 days after the acute-phase specimen was taken. Although this is ideal, it is not always possible, and the convalescent-phase sera used in this study were taken between 5 and 18 days after the acute-phase sample.

[b] NT, not tested.

ing buffer for 1 hour at RT. Plates were washed six times and enzyme activity visualized by the addition of 100 μl substrate solution [1 mM 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid] (ABTS) and 3 mM H_2O_2 in a citrate/phosphate buffer, pH 4.2). Quantitative results were determined by measuring the optical density (OD) at 405 nm, and percent inhibitions were calculated as 100 − [OD (test)/OD (negative control) × 100]. A threshold of 20% inhibition by the test serum was considered "positive" for RRV antibodies (9).

Three MAbs (3B2, G8, and B10), produced to the E2 protein of reference RRV strains as previously described (5, 16), were assessed in the assay. The most sensitive and specific reactions were obtained using MAb 3B2, which was specifically inhibited from binding to RRV antigen in the presence of antisera to all seven reference RRV strains (Table 1). Thus, unless there is a major antigenic shift in circulating RRV strains, detection of RRV antibodies directed to this immunodominant epitope should be effective. Furthermore, using this MAb, there was no cross-reactivity (<20% MAb inhibition) with rabbit antisera to the closely related alphaviruses (Table 1). The results with field samples of kangaroo and horse sera showed ELISA to be as sensitive (100%) and specific (95%) as conventional neutralization for detecting RRV antibodies in these known vertebrate hosts (Table 2).

When used to test a panel of clinical RRV-positive human samples, the blocking ELISA exhibited 97% sensitivity and 98% specificity for the detection of antibody (Tables 3 and 4). This assay's ability to differentiate between seroconversion to BFV and RRV is also important, because both viruses are causative agents of epidemic polyarthritis and they circulate in the same geographical region (13). The acute-phase samples that were negative by ELISA (5007112N and 5028481W) (Table 3) may represent individuals who fail to mount a detectable immune response to the 3B2 epitope early in infection, as the convalescent-phase paired sample to 507112N (5013418) (Table 4) was subsequently shown to be positive by ELISA. The definitive criterion for confirming a recent viral infection is a fourfold or greater increase in the neutralization or HI titer of a paired serum sample (14). We propose that a twofold or greater increase in the percent inhibition of MAb by paired serum samples in the blocking ELISA be used as the criterion for serodiagnosis of recent RRV infection (Table 4). Past seroconversions to RRV could be identified by a greater than 20% inhibition by a single serum sample (Table 3) (9). In conclusion, the RRV epitope-blocking ELISA described in this paper provides a feasible alternative for the rapid diagnosis of clinical human RRV infection and for application in preclinical serosurveillance of susceptible vertebrate hosts (12, 15).

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