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STUDY OF TWO DIFFERENT ENZYME IMMUNOASSAYS FOR THE DETECTION OF MAYARO VIRUS ANTIBODIES

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This paper presents the evaluation of an enzyme immunoassay in which Mayaro virus-infected cultured cells are used as antigen (EIA-ICC) and an IgM antibody capture ELISA (MAC-ELISA) for Mayaro serologic diagnosis using 114 human sera obtained during a Mayaro outbreak occurred in Bolivia, in 1987. Results were compared with those obtained by haemagglutination-inhibition test (HAI). MAC-ELISA was the most sensitive technique for anti-Mayaro IgM detection. MAC-ELISA was twice as sensitive as IgM EIA-ICC. The data shows that MAC-ELISA is a practical and valid technique for diagnosis of recent Mayaro infection. IgG EIA-ICC showed high sensitivity and high specificity compared to HAI. The combination of anti-Mayaro IgG and IgM EIA-ICC results presented the highest sensitivity of the study. Anti-Mayaro IgG and IgM simultaneous detection by EIA-ICC can be used for recent infection diagnosis (in spite of a less sensitive IgM detection than by MAC-ELISA), for surveillance and sero-epidemiologic studies, and for studies of IgG and IgM responses to Mayaro infection.

Key words: Mayaro – alphavirus – arbovirus – enzyme immunoassay

The Mayaro virus was first isolated in Trinidad in 1954 (Anderson et al., 1957), and it is classified as an alphavirus (Togaviridae family) serologically related to the Semliki Forest complex (Karabatsos, 1985). The Mayaro virus causes a febrile exantematic disease associated with persistent joint pain (Pinheiro et al., 1981). Small outbreaks caused by the arbovirus Mayaro have been recorded in the Amazon areas of Brazil (Causey & Maroja, 1957), and Bolivia (Schaeffer et al., 1959) since 1954. Mayaro outbreaks occur in forest-associated communities and the members of maintenance cycle of this forest zoonosis are *haemagogus* mosquitoes, primates, and probably birds (Pinheiro, 1982). Serologic surveys carried out in the Brazilian Amazon area showed the prevalence of HAI antibodies to Mayaro to be as high as 62% (Pinheiro, 1982). Antibodies to Mayaro in South America have also been detected in Bolivia, Guyanna, Colombia, and Peru (Pinheiro, 1982).

Human sera obtained from 114 inhabitants of the Santa Cruz area, Bolivia, affected by a Mayaro outbreak were tested by an enzyme immunoassay in which Mayaro virus-infected cultured cells are used as antigen (EIA-ICC) (Figueiredo & Shope, 1987) and by an IgM antibody capture ELISA (MAC-ELISA) for Mayaro serologic diagnosis. Results were compared with those obtained by haemagglutination-inhibition test (HAI).

MATERIALS AND METHODS

Preparation of virus – Mayaro virus prototype TRVL4675 was obtained from Dr M. Rebello, Federal University of Rio de Janeiro and was propagated twice in C6/36 *Aedes albopictus* cells. Infected cell cultures were scraped, disrupted by freezing and thawing, centrifuged at 5000 g for 10 minutes and the supernatant aliquoted and stored at −70 °C for use as viral stocks. This procedure was performed at 4 days post-infection, during the second passage.

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Mayaro antiserum – Mayaro mouse immune ascitic fluid (MIAF) was prepared by the method of Brandt et al. (1967).

Mayaro antigens – Mayaro antigens from baby mouse brains were used in MAC-ELISA and mouse serum antigens were used in HAI (Shope & Sather, 1979).

Human sera – Sera from 114 inhabitants of a rural area of the Department of Santa Cruz, Bolivia, were collected during an outbreak of Mayaro virus infection in 1987 and supplied by Dr F. Mendoza of the Bolivian Ministry of Health. Blood was collected by veinpuncture and sera were kept at –18 °C until use.

Cell cultures – C6/36 cells were grown in Leibowitz L15 medium (GIBCO-New York, USA) containing 10% heat-inactivated fetal bovine serum, 10% triptose phosphate broth, non-essential amino-acids, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were incubated at 28 °C in a humidified atmosphere.

Viral infection and antigen processing for EIA-ICC – C6/36 cells were added to 96-well plastic plates (Falcon 9383-A10, Oxnard, CA, USA) in 150 μl of growth medium at a density of 5 x 10^4 cells per well. After 24 hours the cells were infected with 50 μl of 50 to 200 $TCID_{50}$ of Mayaro. Alternate columns of microplate wells contained uninfected cells. At 4 days post-infection, the wells received 100 μl of neutral buffered formalin pH 7 (37-40% formaldehyde 100 ml, sodium phosphate dibasic – anhydrous 6.5 g, sodium phosphate monobasic 4.0 g, and distilled water 900 ml) and were held overnight at 4 °C. Within 18-24 hours of fixation, the cells were washed twice with phosphate buffered saline (PBS). Some microplates were processed immediately for EIA-ICC. Others were air dried, put in plastic bags and stored at –70 °C (Figueiredo & Shope, 1987).

Adsorption of human serum with C6/36 cells for EIA-ICC – Ten μl of human sera were diluted 1:100 in 1% bovine serum albumin (BSA)-PBS and adsorbed 2 hours with 5 x 10^6 of previously pelleted and washed C6/36 cells in order to reduce background in EIA-ICC.

EIA-ICC – After 2 hours of blocking with 200 μl of 5% gelatin in PBS per well with infected or uninfected cells, the microplates were washed three times with .05% tween 20 in PBS. One hundred μl of human serum dilutions in BSA-PBS (1:100 to 1:8000) were added to infected and uninfected wells and microplates were incubated for 1 hour at 37 °C and washed 3 times. Depending on the test, 100 μl of goat antisera to either human IgG or human IgM conjugated with horseradish peroxidase (diluted 1/2000 in BSA-PBS; Institute Pasteur, Marnes, France) were added to all wells and the plates were incubated for 1 hour at 37 °C. Microplates were washed 5 times in PBS-tween and to each well were added 100 μl of ABTS substrate (Kirkegaard & Perry, Gaithersburg, MD, USA). Results were read visually by comparing colors (green +; colorless –) in virus infected and uninfected wells, 5 to 10 minutes after addition of the substrate.

Acetone extraction of normal human sera (NHS) for MAC-ELISA – Alphavirus antibody free human serum proteins were precipitated three times by incubation overnight with 50 volumes of acetone. The supernate acetone was decanted, the sediment was swirled around and allowed to seattle in a uniform depth over the botton of the flask. The remaining acetone was allowed to evaporate at 37 °C. The extracted serum proteins were added to 2 volumes of PBS and left to stand for one hour, clarified by centrifugation, diluted at 1/5 in PBS and stored at – 20 °C in aliquots for use as diluent of the MAC-ELISA as well as negative control.

MAC-ELISA – One hundred μ l of a μ chain specific goat anti-human IgM antibody (Kirkegaard & Perry, Gaithersburg, MD, USA) diluted 1/200 in carbonate buffer pH 9.6 (NA_2CO_3-1.59g, and $NaHCO_3$-2.39g in double distilled water – 1000 ml), were added to each well of Immulon II 96 – well, flat botton, polystirene microliter plates (Dynatech, Alexandria, USA). The plates were incubated overnight at 4 °C and then washed (5 times) with PBS pH 7.4. Two hundred μl of 4% bovine serum albumin (BSA) in PBS pH 7.4 were added to each well, the plates were left for 30 minutes at room temperature, washed in PBS, and then, 50 μl of serial dilutions of the human sera under test, in PBS containig 20% of NHS, were added to the wells. After incubation for 2 hours at room temperature, the microplates were washed, and 16 haemagglutination units of Mayaro antigen (50 μl) were added to the wells and incubated overnight at 4 °C. After

TABLE

Mayaro EIA-ICC, MAC-ELISA, and HAI antibody titers (reciprocal) obtained from 62 reacting sera

Serum	EIA-ICC		MAC-ELISA	HAI
	IgM	IgG		
7	N[1]	500	N	40
15	N	2000	N	80
16	N	500	N	40
18	N	2000	N	160
22	N	500	N	40
23	N	N	100	N
28	2000	100	8000	160
35	N	N	100	N
38	100	N	1000	N
39	2000	100	2000	80
42	500	2000	1000	80
46	500	N	1000	40
47	500	N	1000	40
52	N	2000	N	80
53	N	500	1000	40
54	500	500	500	80
57	100	2000	2000	160
58	100	2000	1000	80
62	100	2000	N	80
63	500	2000	500	80
64	N	2000	2000	160
65	100	2000	100	80
66	N	2000	500	80
68	N	100	N	40
69	N	500	N	40
70	N	2000	100	80
71	N	100	1000	80
72	N	2000	1000	80
73	100	2000	1000	40
74	N	N	1000	N
75	100	N	10	40
78	N	N	1000	40
79	N	2000	1000	160
80	N	2000	1000	160
81	500	2000	2000	160
82	8000	8000	8000	320
83	500	500	500	160
85	N	2000	2000	160
86	N	N	100	N
87	N	2000	1000	80
89	N	500	2000	160
90	N	N	2000	N
91	100	500	2000	80
92	100	500	500	80
95	500	500	2000	160
96	N	500	100	80
97	N	100	N	40
98	N	500	N	80
99	N	100	N	N
100	N	100	N	N
103	N	N	10	N
104	N	500	100	80
105	N	500	100	40
106	N	2000	10	40
107	N	2000	10	80
108	N	2000	100	80
109	N	500	100	40
110	N	500	N	40
111	N	500	N	40
112	N	500	100	40
113	N	500	10	80
114	500	2000	100	80

1) N – Negative test (Titers < 20 by HAI, < 100 by EIA-ICC, and < 10 by MAC-ELISA).

washing, 50 µl of a dilution of Mayaro MIAF 1/200 in PBS-NHS were added to the wells. The plates were incubated 1 hour at 37 °C, washed and 50 µl of a 1/1000 dilution of anti-mouse globulin labelled with peroxidase were added to the wells. The microplates were again incubated at 37 °C for one hour, washed and to each well were added 100 µl of ABTS substrate (Kirkegaard & Perry, Gaithersburg, MD, USA). Results were read spectrophotometrically (405 nm) 30 minutes after the addition of substrate. Optical density (OD) values were considered significant if they exceeded by 2.1 times the negative controls mean value.

HAI – Sera were extracted with acetone and tested as described by Shope & Sather (1979).

RESULTS

Of the 114 sera under study, 62 (54.4%) were positives by at least one of the tests and 39 (34.2%) by all the three tests (Table). Haemagglutination-inhibition antibodies to Mayaro were detected in 53 sera. Mayaro IgM antibodies were detected in 47 sera by MAC-ELISA and 21 sera by EIA-ICC. IgM EIA-ICC was estimated to have 42.6% sensitivity and 98.5% specificity by comparing its results with those of MAC-ELISA. Mayaro IgG antibodies were detected in 51 sera by EIA-ICC which was estimated to be 92.45% sensitivity and 93.65% specificity by comparison with HAI. Fifty five sera were positive either by IgM or IgG EIA-ICC. The total EIA-ICC results (IgM + IgG) showed a higher sensitivity than HAI or MAC-ELISA. Significant association was observed between positive and negative IgG EIA-ICC and HAI results (χ^2 91.201, $p \leqslant .001$). Mayaro antibodies were detected by MAC-ELISA only in 7 sera (sera 23,74, 86, 35, 38, 90, and 103) and by IgG EIA-ICC only in 2 sera (sera 99, and 100).

DISCUSSION

The HAI technique is considered a simple, rapid and sensitive classical technique for Mayaro and other alphavirus serological diagnosis (Theiler & Downs, 1973). Haemagglutination antigens are inhibited by IgG and IgM antibodies and recent infection diagnosis is done by comparison of HAI titers in paired acute – and convalescent – phase sera (Theiler & Downs, 1973). In our study HAI results

were compared with those obtained by the enzyme immunoassays.

IgM antibodies appear in the acute phase of disease and disappear a few months later in primary alphavirus infections (Shope, 1985; Calisher et al., 1986). IgM speficity for virus complexes in the alphavirus genus was determined by EIA in a study with 9 viruses including Mayaro (Calisher et al., 1986). IgG antibodies to alphaviruses are less specific than IgM and can be detected for many years after the infection by Neutralization Test (NT) or HAI (Karabatsos, 1985; Pinheiro, 1982). The diagnosis of recent infections by IgM specific antibody detection without the need of immunoglobulin separation procedures or of paired sera is an important advantage of enzyme immunoassays over HAI and NT.

MAC-ELISA was the most sensitive technique for anti-Mayaro IgM detection. Also serologic diagnostic studies with the Japanese Encephalitis flavivirus (Burke et al., 1985) and Ross River alphavirus (Carter et al., 1985) have shown a higher specificity and sensitivity of the antibody capture assay (MAC-ELISA) compared to HAI and indirect ELISA. In our study MAC-ELISA was shown to be twice as sensitive as IgM EIA-ICC. Mayaro antibodies detection by MAC-ELISA only, in 7 sera, also showed the high sensitivity of this test. Competition between Mayaro IgM and IgG antibodies for virus antigens in the fixed cell layer could explain the lower sensitivity of IgM EIA-ICC compared to MAC-ELISA. Based on data obtained in previous serologic studies with MAC-ELISA (Calisher et al., 1986), anti-Mayaro IgM cross-reactions could have occurred with Una virus, a South American member of Semlik Forest alphavirus complex (Karabatsos, 1985). MAC-ELISA false positive results are rare except in patients with high levels of serum rheumatoid factor (Burke et al., 1985).

IgG EIA-ICC results were well correlated with HAI and showed high sensitivity and specificity. A similar correlation between IgG antibody capture ELISA and HAI has been reported in a study of Japanese Encephalitis flavivirus (Burke et al., 1985). Extensive cross-reactions among IgG type antibodies to Mayaro and to endemic South American alphaviruses could make difficult the specific diagnosis of alphavirus infection diagnosis by EIA-ICC and HAI. The combination of anti-Mayaro EIA-ICC IgG and IgM results presented the highest sensitivity in our study. The specificity of IgM and IgG EIA-ICC were also very good.

CONCLUSIONS

Our results show that MAC-ELISA is a practical and valid technique for the diagnosis of recent infection by Mayaro virus. It has also been effectively used by us for the diagnosis of dengue virus with more than 1000 serum specimens (Nogueira et al., 1988). It permits screening of large number of sera in approximately 18 hours. The MAC-ELISA method can be shortened by 1 step, as we do for dengue (Nogueira et al., 1988), by doing anti-Mayaro immunoglobulin conjugation to peroxidase.

Our results confirm that EIA-ICC is a very useful technique for Mayaro IgG and IgM antibody detection. Anti-Mayaro IgG and IgM simultaneous detection by EIA-ICC can be used for the diagnosis of recent infection (in spite of IgM EIA-ICC to be less sensitive than MAC-ELISA), for surveillance and sero-epidemiologic studies, and for studies of IgG and IgM responses to Mayaro infection. EIA-ICC is simpler and faster than antibody capture ELISAs. It eliminates solid phase coating with Mayaro virus and laborius antigen preparation, it permits rapid screening of large numbers of sera in approximately 5 hours, allows the use of microplates with infected C6/36 cells fixed and stored for at least 2 months at −20 or − 70 °C, as described for dengue virus antibody assay (Figueiredo & Shope, 1987). More importantly, the EIA-ICC can be read visually, allowing its use in laboratories with moderate resources. Further studies with EIA-ICC are needed to get more information about the specificity and significance of IgM and IgG responses to primary and secondary Mayaro and ohter alphavirus infections.

RESUMO

Estudo com dois diferentes ensaios imuno-enzimáticos para a detecção de anticorpos contra o vírus Mayaro – Apresentamos a avaliação de um teste imuno-enzimático no qual células infectadas com o vírus Mayaro são usadas como antígeno (EIA-ICC) e a de um teste de captura de IgM (MAC-ELISA), no diagnóstico sorológico de infecções por Mayaro. Soros humanos em número de 114, obtidos durante uma epidemia ocorrida em 1987 na Bolívia, foram utilizados

neste estudo. Os resultados foram comparados com aqueles obtidos pelo teste de inibição da hemaglutinação (HAI). MAC-ELISA mostrou-se duas vezes mais sensível que EIA-ICC na detecção de anticorpos do tipo IgM para Mayaro. MAC-ELISA mostrou-se uma técnica válida e prática para o diagnóstico de infecções recentes por Mayaro. EIA-ICC para detecção de IgG mostrou-se mais sensível e também mais específico que HAI. Os resultados da combinação de EIA-ICC para a detecção de IgM e IgG de Mayaro apresentaram a maior sensibilidade dentre os testes estudados. A detecção simultânea de anticorpos IgG e IgM para Mayaro por EIA-ICC pode ser utilizada no diagnóstico de infecções recentes (a despeito de menos sensível para IgM que o MAC-ELISA), em inquéritos sorológicos e em estudos sobre a resposta imune de IgG e IgM às infecções por Mayaro.

Palavras-chave: Mayaro – alphavirus – arbovirus – teste imuno-enzimático

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