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Detection of Immunoglobulin M Antibody to Epstein-Barr Virus by Use of an Enzyme-Labeled Antigen

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Immunoglobulin M (IgM) antibodies to Epstein-Barr virus were detected by using microtiter plates coated with anti-μ-chain antiserum and enzyme-labeled Epstein-Barr virus antigen. Optimum conditions for labeling were determined. The addition of unlabeled control antigen to the enzyme-labeled antigen was effective in reducing background reactions. Rheumatoid factor no longer interfered. Blocking of specific IgM antibody by IgG also was no longer observed. Four different methods for the detection of acute Epstein-Barr virus infections were compared. A combination of the enzyme-labeled antigen-IgM test with the detection of antibodies to either Epstein-Barr nuclear antigen or heterophile antigen was highly sensitive and specific. By changing the solid phase, IgA antibodies to Epstein-Barr virus could be detected.

During the last decade, the detection of immunoglobulin M (IgM) antibody to Epstein-Barr (EB) virus by indirect immunofluorescence (IF) has become a routine procedure for the diagnosis of acute EB virus infections (10, 14). With optimum test conditions and properly fixed cell smears, very low specific IgM antibody concentrations are detected, and positive results can be obtained in almost all infectious mononucleosis (IM) cases (7, 11, 14). Even in IM cases in which heterophile antibody is absent, i.e., in young children or patients above 30 years of age, IgM antibody to EB virus is frequently present and helps to confirm the suspected diagnosis (10, 15).

On the other hand, certain nonspecific reactions may interfere with the detection by indirect IF of IgM antibody to EB virus. Anticellular antibodies belonging to the IgM immunoglobulin class may occasionally produce IF staining which makes accurate reading of the slides impossible (11, 16). More problems arise from human serum specimens containing rheumatoid-factor (RF), which accounts for a considerable number of false-positive results (6, 14). Problems also may arise from competition by specific IgG antibody, especially in newborns (13), in whom low specific IgM titers have to be detected in the presence of high maternal IgG levels. In addition, the reading of the immunofluorescent slides differs from person to person and is influenced by properties of the microscope.

Thus, a more specific and less time-consuming method would be of value for the detection of IgM antibodies to EB virus. Recently, an enzyme-labeled antigen (ELA) test for IgM antibodies to human cytomegalovirus (CMV) was reported (12, 16). With this test system, RF does not interfere. Blocking by IgG antibody also does not play a role, as IgG is eliminated before IgM antibody reacts with the ELA. This test system has also been shown to be of value for the detection of IgM antibody to *Toxoplasma* (4), parainfluenza virus (3), influenza A virus (H. Schmitz, B. Flehmig, and A. Vallbracht, Abstr. Int. Cong. Virol. 5th, Strasbourg, France, abstr. no. W13108, 1981, p. 165), and herpes simplex virus (A. M. van Loon, A. M. Heesen, J. van der Logt, and J. van der Veen, Abstr. Int. Cong. Virol. 5th, Strasbourg, France, abstr. no. P13120, 1981, p. 171). I therefore tried to develop a similar test for improved detection of EB virus IgM antibody. The relatively low specific IgM titers to the EB virus obviously require a highly sensitive test. Moreover, the low ratio of viral material to cellular material in EB virus-producing cells makes the production of a specific EB virus ELA more difficult. Thus, several problems had to be solved so that a sensitive and specific EB virus ELA-IgM test could be developed. Three alternative methods were included in this study to clearly evaluate the efficiency of the new method for the diagnosis of acute EB virus infections. First, the results obtained with the ELA-IgM test were compared with the detection of EB virus-specific IgM antibody by indirect IF. Second, the serodiagnosis of acute EB virus infections was further confirmed by simultaneous detection of IgG antibody to the EB viral capsid antigen (EB-VCA) and to the EB nuclear antigen (EBNA) (VCA-EBNA combination). In a serum specimen, the presence of anti-

EB-VCA IgG antibody and the absence of anti-EBNA antibody strongly suggests acute IM (5). Third, heterophile antibody was sought.

Finally, some preliminary studies were carried out to adopt the test to specific IgA antibody detection by simply using anti-α-chain antibody-coated plates.

MATERIALS AND METHODS

Antigen purification. P3HR1 EB virus-producing cells were grown in RPMI 1640 containing 10% fetal calf serum (FCS) as described previously (14) and were stimulated to increased production of EB-VCA according to the method of zur Hausen et al. (18). In brief, aged cells (50 ml) were suspended in 100 ml of fresh medium. Then, 0.1 ml of a solution of 0.02 mg of TPA (phorbol-12 myristate-13-acetate, 10 mg prediluted in 50 ml of dimethyl sulfoxide; Sigma Chemical Co., St. Louis, Mo.) per ml in RPMI 1640 was added. After 10 days of incubation at 37°C, the cells were sedimented and suspended in 0.5 ml of Tris-EDTA buffer (0.01 M Tris-hydrochloride [pH 8.5], 1 mM EDTA) containing 1% Nonidet P-40 (nonionogenic detergent; Shell, Hamburg, West Germany). Cells were disrupted in an ice bath (Microtip, interval pulse with a Branson sonifier; Branson Instruments Co., Danbury, Conn.) for 2 min, and cell debris was removed by centrifugation (3,000 × *g*, 5 min). A 0.5-ml portion of the supernatant fluid was layered onto 10 ml of a solution of 30% dextran T10 (Pharmacia Fine Chemicals, Uppsala, Sweden) in Tris-EDTA and centrifuged for 1 h at 180,000 × *g* at 4°C (Beckman SW41 rotor; Beckman Instruments, Inc., Fullerton, Calif.). The sediment was suspended in 0.5 ml of carbonate buffer (0.05 M; pH 9) by brief sonication (10 s). The protein concentration was determined by the Fluram method (1).

Antigen labeling. A 4-mg amount of horseradish peroxidase (type IV; Sigma) in 1 ml of distilled water was activated by the addition of 0.2 ml of 0.1 M $NaIO_4$. After a 20-min incubation, overnight dialysis at pH 4.4 (1 mM sodium acetate) was performed (17).

A 0.5-ml sample of viral antigen (0.5 mg of protein) in carbonate buffer (0.05 M; pH 9) was mixed with 0.25 ml of activated peroxidase. After incubation overnight at 4°C, 0.1 M $NaBH_4$ was added (1:40 [vol/vol]), and the mixture was incubated for another 2 h.

The ELA (without further purification) was diluted (1:100 [vol/vol]) in FCS which had been dialyzed against distilled water. Then, 10% unlabeled control antigen was added. The ELA was stored frozen at −70°C and lyophilized.

Control antigen. An unlabeled control antigen from uninfected human fibroblast cells was prepared according to the method described above for the ELA. However, the control antigen was not purified by centrifugation through dextran T10.

Coating of the plates. Flat-bottomed Microelisa plates (Dynatech, Plochingen, West Germany) were coated with antiserum to human IgM or IgA (μ- or α-chain specific, IgG fraction; Dako Immunoglobulins, Copenhagen, Denmark) at a dilution of 1:500 in phosphate-buffered saline containing 1 mg of NaN_3 and 0.01 mg of phenol red per ml. After 2 days of incubation at 4°C, the plates were vacuum sealed.

ELA-IgM and -IgA tests. The plates were washed three times with washing buffer (0.1 M Tris, 0.15 M NaCl, 0.05% Tween 20 [pH 7.5]; Serva, Heidelberg, West Germany). During the second washing, the buffer was incubated on the plates for 2 min. Fifty microliters of each serum dilution (1:100 and 1:1,000 in phosphate-buffered saline) was incubated on the plates for 2 h at room temperature. After another washing, the lyophilized ELA was dissolved in washing buffer (final dilutions: ELA, 1:200; FCS, 1:2; control antigen, 1:20), and 50 μl per well was incubated for another 2 h at room temperature. After a final washing, the substrate (1,2-phenylene diamine, 1 mg/ml in 0.1 M phosphate buffer [pH 6]–0.01% H_2O_2) was added, and the reaction was stopped with 2 M H_2SO_4 after 10 min. The tests were read with a spectrophotometer at 495 nm (Microelisa Reader, Dynatech).

IF tests. (i) Test for IgM antibody to EB-VCA (IF-IgM test). Smears of EB virus-producing cells (P3HR1) were prepared as described previously (14). They were not dried after fixation and were stored in 10% glycerol–phosphate-buffered saline (11, 14). Sera were incubated for 3 h on the smears, and finally the specific IgM antibody was detected with fluorescein isothiocyanate-labeled anti-human IgM (Wellcome Reagents Ltd., Beckenham, England).

(ii) Test for IgG antibody to EB-VCA (IF-IgG test). In contrast to the IF-IgM test, the sera were incubated for only 1 h. FITC fluorescein isothiocyanate-labeled anti-human IgG (Behringwerke, Marburg, West Germany) was applied.

(iii) Test for antibody to EBNA (EBNA test). The anticomplement IF technique was applied by using cell smears of Raji cells (5), human complement, and fluorescein isothiocyanate-labeled anti-human complement immunoglobulin (Hyland Laboratories, Inc., Costa Mesa, Calif.). Cell smears of EBNA-negative Molt 4 cells were used to exclude nonspecific staining by antinuclear antibodies.

Detection of heterophile antibody (heterophile test). The test kit of Gamma Diagnostics, Houston, Tex., was applied according to the protocol of the producer.

Serum specimens. Sera from 548 suspected IM cases were included in this study.

RESULTS

First, optimum test conditions for the ELA-IgM test were investigated. I explored test modifications that would give maximum values for the P/N ratio (ratio of the optical density (OD) of a highly positive standard serum to that of a negative standard serum pool). With a high ratio, when the OD of the positive control exceeded that of the negative control by 10-fold, even sera with low specific IgM antibody titers of 32 by the IF-IgM test showed a P/N ratio of at least 3. The P/N ratio could be increased by several general modifications of the test system such as additional washing or prevention of bacterial contamination in the reagents. The composition of the ELA also was of great importance. A suitable ELA giving P/N ratios of >2 could be obtained only from TPA-stimulated lymphoid cells which showed at least 30% EB-VCA-positive cells. As can be seen in Table 1, the composition (ionic strength) of the buffer in

TABLE 1. Influence of the antigen dilution and the diluent composition on the P/N ratio of the ELA-IgM test

Antigen dilution	Diluent	P/N ratio
1:100	0.9% NaCl	2.1
1:200	0.9% NaCl	3.3
1:200	0.9% NaCl, 50% FCS	5.3
1:200	0.9% NaCl, 50% FCS, 0.1 M Tris (pH 8)	8.2
1:200	0.9% NaCl, 50% FCS, 0.1 M Tris, 0.05% Tween 20	10.1

which the ELA was dissolved, as well as the concentration of FCS and the presence of Tween 20, influenced the P/N ratio of the test.

Nonspecific reactions. In my first experiments, 10% FCS was added to the anti-IgM-coated plates to prevent or reduce nonspecific binding of human IgG to the plates. Even without additional protein, only very small amounts of specific IgG were demonstrated on the plates when sera with very high IgG antibody titers to EB-VCA were examined in the ELA-IgM test (Table 2, row 3). A serum (HE, see Table 2) with an IgG antibody titer of >10,000 but without specific IgM had an OD of 0.03 when it was examined in the ELA-IgM test at a dilution of 1:100 compared to an OD of 0.03 of the arithmetic mean of the negative pool. Moreover, the addition of RF-positive serum (16) to the high-titer serum did not significantly increase the OD in the test (Table 2, row 4). On the other hand, a serum with antinuclear IgM antibody produced a false-positive reaction which was no longer observed when a control antigen prepared from noninfected human fibroblasts was added to the ELA (Table 2, rows 5 through 7). Therefore, the addition of unlabeled control antigen to the ELA seemed to be helpful in reducing nonspecific anticellular background reactions.

CMV-EB virus cross-reactivity. As reported previously, about 30% of all sera derived from patients with acute IM and with specific IgM antibody to EB virus also show IgM antibody to CMV in the indirect IF test (15) or in an enzyme immunoassay (13). This reactivity of IM sera is also seen in an ELA-IgM test for CMV (16). To look for the reverse reaction, 42 sera taken from adults with high levels of specific IgM antibody to CMV during acute CMV hepatitis were tested for IgM antibody to EB virus by the ELA-IgM test. Surprisingly, positive reactions were not found. Therefore, the production of IgM antibody to CMV during an acute EB virus infection seems to be a one-way phenomenon.

Dose-response curve of IgM antibody to EB virus in the ELA-IgM test. The shape of the dose-response curves differed depending on whether sera contained low or high amounts of specific IgM antibody (Fig. 1). Moreover, low serum dilutions of less than 1:50 did not show markedly increased OD values in the ELA-IgM test. With these low dilutions, a saturation of the coated solid phase for IgM binding was reached, which was indicated by the flattening of the dose-response curve. With some enzyme immunoassay systems, antibody titers are calculated from a standard curve (2); the titers are defined as the reciprocal of the dilution at which the OD reaches the positive-negative cutoff level ($x + 3s$; see Fig. 1). When I applied this formula to my test system, titers of <1,000 were very rarely found, as the curve declined slowly with progressive dilution. Titers of >10,000 also were infrequent.

Comparison of different methods. Of 548 patients with IgG antibody to EB virus, 46 showed serological evidence of acute IM by all four tests, whereas acute IM could be excluded in 459 patients (Table 3). Of special interest were the sera from which divergent results were obtained. A total of 14 sera were positive in the IF-IgM test only (Table 3), but in 12 of these a nonspecific interference by RF could be demonstrated. In contrast to the results in the ELA-IgM test, four sera were not positive in the IF-

TABLE 2. Nonspecific reactions in the ELA-IgM test

Serum specimen	Dilution	OD
1. Negative control sera (n = 100) (mean value)	1:10	0.03
	1:100	0.03
2. Positive control serum	1:100	0.55
	1:1,000	0.46
3. Serum HE[a]	1:10	0.06
	1:100	0.03
4. Serum HE + RF[b]	1:10	0.05
5. Antinuclear antigen-containing serum[c]	1:100	0.22
6. Antinuclear antigen-containing serum + 5% control antigen[c]	1:100	0.03
7. Positive control serum + 5% control antigen	1:100	0.52[d]

[a] Serum HE was from a patient with prolonged IM and contained a high concentration of EB-VCA-specific IgG (IgG titer >10,000; no specific IgM antibody determined by IF-IgM test).

[b] RF serum was added to serum HE (RF titer >100 determined by latex agglutination test).

[c] Serum contained IgM antibody to antinuclear antigen.

[d] The addition of control antigen did not significantly affect the OD of positive control serum.

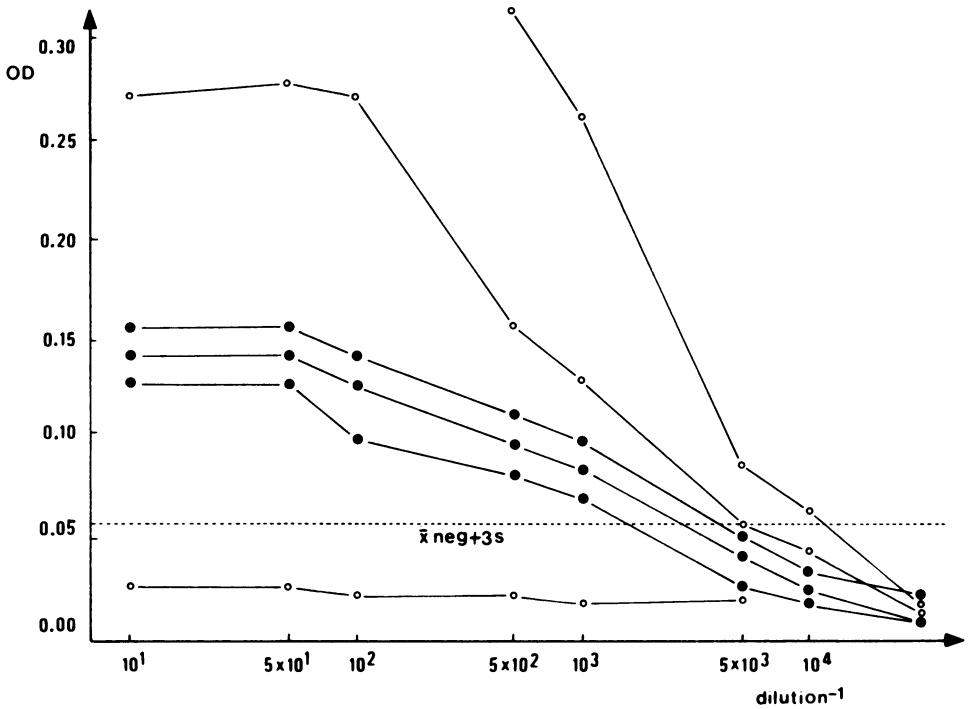

FIG. 1. Dose-response curve for the ELA-IgM test. Symbols: ○, two sera with high (>512) IF-IgM titers and one negative serum; ●, sera with low IF-IgM titers (<128). The OD values are the arithmetic mean of at least two independent tests. The positive-negative cutoff line (x̄ neg × 3s) was calculated from 100 negative sera.

IgM test (Table 3). This may be a result of IgG blocking, as all four sera had high (>1,024) IgG titers to EB-VCA by indirect IF. Two sera were not positive in the ELA-IgM test but were positive at a dilution of 1:16 in the IF-IgM test. Furthermore, the presence of both anti-EB-VCA and anti-EBNA antibodies (negative VCA-EBNA test; Table 3) did not rule out acute IM, as 10 patients already had antibody to EBNA, and the other three tests supported the diagnosis of IM. On the other hand, the absence of anti-EBNA antibody (positive VCA-EBNA test) in a serum containing IgG antibody to EB virus gave a strong indication of an acute EB virus infection. Practically all IM patients develop anti-EBNA antibodies within a few months (5). One patient (Table 3) had no antibody to EBNA in an initial serum specimen but was positive for it in a second specimen taken 6 weeks later, although all other tests were negative. In eight patients (Table 3) heterophile antibody could not be detected, although the VCA-EBNA, IF-IgM, and ELA-IgM tests were positive.

TABLE 3. Comparison of the results for 548 sera in the ELA-IgM, IF-IgM, heterophile antibody, and VCA-EBNA tests

Test reaction		No. of sera with indicated test reaction			
ELA-IgM	IF-IgM	VCA-EBNA+		VCA-EBNA−	
		HA +	HA −	HA +	HA −
+	+	**46**	**4**	**8**	**2**
+	−	**2**	**4**	**1**	1
−	+	0	**2**	**1**	14
−	−	**2**	**1**	1	459

[a] All sera contained antibody to EB-VCA. Positive reactions (+) indicate acute IM. Negative reactions are indicated by (−). True-positive cases are indicated in boldface type.

To obtain more information on the sensitivity and specificity of the different tests, I divided the sera into true-positive sera and true-negative sera. With 46 sera positive in all four tests (Table 3) the statistical (polynomial) distribution of positive results in three, two and, only one test can be estimated by $(p + q)^4$ ($p^4 = 0.52$; $4p^3q = 0.37$; $6p^2q^2 = 0.1$; $4pq^3 = 0.01$; $q^4 = 0.001$), if a mean sensitivity of 90% ($P = 0.9$) in all four tests is assumed (5, 7–10, 14). True positive results in two tests must be relatively frequent (nine by calculation), but true-positive results in only one test must be very rare (only one by calculation). Therefore, all sera listed in Table 3 were designated as true-positive sera if they were positive in at least two of the tests applied. All sera which were negative in at least three tests were consid-

ered to be true-negative sera. Moreover, all sera with a clear seroconversion to EBNA were considered to be true-positive sera. Therefore, there were 46 + 4 + 8 + 2 + 2 + 4 + 1 + 0 + 2 + 1 + 2 + 1 = 73 true-positive sera (boldface type in Table 3) and 1 + 14 + 1 + 459 = 475 true-negative sera (Table 3). From the data listed in Table 3, the sensitivity and specificity of the individual tests were calculated (Table 4). The VCA-EBNA, heterophile antibody, and ELA-IgM tests all showed a high specificity, whereas the sensitivity of the VCA-EBNA and heterophile antibody tests was relatively low. Different combinations of two tests showed the highest sensitivity as well as a good specificity (Table 4), if a positive reaction in one of the tests was sufficient for the diagnosis of an acute EB virus infection.

Detection of IgA antibody to EB virus. By simply changing the antiserum on the solid-phase microtiter plates the IgM-ELA test could be adapted to specific IgA detection. In sera from 22 out of 46 IM patients and in sera from all 6 patients with nasopharyngeal carcinoma, specific IgA antibodies were found.

DISCUSSION

As described above, several alternative methods can be applied to diagnose acute EB virus infections. Unfortunately, a single test with optimum sensitivity and specificity is not available. All tests described so far have their limitations. For optimum results, a combination of different methods is required, and discrepancies among different test results need careful analysis. In this respect, there are some additional methods for the detection of acute EB virus infections which were not included in this study as they are too complicated (neutralization test) or are less specific and sensitive (test for antibody to early antigen [7]). Nevertheless, these methods may occasionally help to confirm a suspected diagnosis.

For routine purposes, a combination of only two simple tests is desirable. As shown in this report, a combination of either the heterophile antibody test or the ELA-IgM test with detection of antibody to EBNA (including detection of IgG antibody to EB-VCA) gives both high sensitivity and specificity, and the absence of anti-EBNA antibody in patients with IgG antibody to EB-VCA seems to be a reliable sign for acute IM. Practically all patients with IgG antibody to EB-VCA but without anti-EBNA antibody had an acute EB virus infection, and in questionable cases a suspected diagnosis could be further supported by a seroconversion to EBNA, which frequently takes place only 3 months after the onset of the disease. On the other hand, the sensitivity of the VCA-EBNA combination was low, as about 16% of the IM patients already had low anti-EBNA antibody titers. This is in good agreement with earlier findings showing that anti-EBNA antibody was present in sera from patients with acute IM as early as week 2 after the onset of the disease (5). The sensitivity of the VCA-EBNA test is reduced further if it is not controlled for interfering antinuclear antibodies. The test also requires careful standardization of the anticomplement IF technique by using EBNA-positive and -negative lymphoid cells grown in tissue culture. The detection of anti-EBNA antibody should be left for the diagnosis of the rare complicated clinical cases for which other techniques do not give reliable results.

Alternatively, the combination of the ELA-IgM test with the heterophile antibody test rarely produced false-positive or false-negative results. This combination was very effective, since IgM antibody to EB virus can be found in cases in which heterophile antibody is missing (10, 15).

TABLE 4. Sensitivity and specificity of the four single tests (ELA-IgM, IF-IgM, heterophile antibody, EB-VCA-EBNA) and of the combination of two tests[a]

Test	No. of false-positive results	% Specificity	No. of false-negative results	% Sensitivity
ELA-IgM	1	99.8	6	91.7
IF-IgM	14	96.8	10	86.3
Heterophile antibody	1	99.8	13	82.1
EB-VCA-EBNA	1	100.0	12	83.5
EB-VCA-EBNA + ELA-IgM	1	99.8	1	98.6
EB-VCA-EBNA + heterophile antibody	1	99.8	2	97.2
ELA-IgM + heterophile antibody	2	99.5	3	95.8

[a] Calculations were from data presented in Table 3; 475 true-negative sera were used to calculate the specificity, and 73 true-positive sera were used to calculate the sensitivity.

The easy performance of these tests compensates for a possible slight reduction in sensitivity as compared with the combination of the ELA-IgM and VCA-EBNA tests. Also, by using more sophisticated techniques (8, 9), the sensitivity of the heterophile antibody test can be increased.

The combination of the heterophile antibody test with the IF-IgM test is less promising because of the problems with RF. Since RF does not interfere with the ELA-IgM test, this test seems to be the method of choice. Moreover, in contrast to indirect IF, the ELA-IgM test reliably detects IgM titers in the presence of high concentrations of IgG antibody to EB virus, and it is easier to read and standardize. As a further advantage, EB virus-specific IgA antibody can be detected by simply modifying the solid-phase plates.

On the other hand, two of the very low specific IgM titers (<32 by indirect IF) could not be detected by the ELA-IgM test, which might be slightly less sensitive than the IF-IgM test, especially when high total IgM concentrations in a serum specimen compete with a low amount of virus-specific IgM. Obviously, the ELA-IgM test depends not only on the amount of specific IgM antibody but also on the ratio of virus-specific IgM to total IgM in a serum specimen (16). Increased binding of IgM antibody to the solid phase might further improve the sensitivity of the test. This would seem to be possible by using both monoclonal anti-IgM antibody combinations and improved solid-phase plates with increased capacity for protein binding.

The relatively mild labeling conditions described here may permit labeling of additional microbial antigens and the development of new specific IgM antibody tests for rapid clinical diagnosis.

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