

RAPID SEROLOGICAL DIAGNOSIS OF TICK-BORNE ENCEPHALITIS BY MEANS OF ENZYME IMMUNOASSAY

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Abstract. A system of IgM-capture EIA made up from Czechoslovak immunopreparations (SEVAC) was developed for a rapid serological diagnosis of tick-borne encephalitis (TBE). The method was tested on clinical material. The total IgM antibody titres were detected using pig antiserum and the selection of specific IgM antibodies was made with TBE antigen with following indirect way of detection. The antibody analysis made by means of this method is sensitive and fully conforms to the clinical picture of the disease.

A reliable rapid diagnosis of arboviral infections is a prerequisite for choosing the correct therapy of the patient. In Czechoslovakia, the clinically most important arbovirus is the virus of tick-borne encephalitis (TBE). The acute character of the disease with a distinct onset of the synthesis of specific antibodies of IgM isotype enables to use the detection of these antibodies for a rapid diagnosis. Various methods for a rapid antiviral diagnosis have been described (Flewett 1985). However, the methods of enzyme immunoassay (EIA) based on IgM-capture have been found to be most suitable in the clinical practice (Gadkari and Shaikh 1984). The technique of IgM-capture EIA best of all eliminates false negative results due to the excess of specific IgG antibodies and false positive results due to a rheumatoid factor in the sera of patients (Briantais et al. 1984).

The detection of specific antibodies against TBE virus in sera of patients has been based particularly on classical serological tests of haemagglutination inhibition (HIT), complement-fixation reaction (CFR) and exceptionally also neutralization test (NT) or indirect immunofluorescence (IIF). Kunz and Hofmann (1971) used for the semi-quantitative determination of specific IgM antibodies to TBE virus a modification of HIT with a reduction of IgM molecules by 2-mercaptoethanol, which had been used in clinical practice.

The method of indirect ELISA for the detection of specific antibody response to TBE virus was described for the first time by Hofmann et al. (1979). This procedure, however, similarly as IIF, gives false negative results (Cohen et al. 1967). Hofmann et al. (1983) therefore developed and verified a diagnostic system of IgM-capture EIA as a four-layer ELISA for the determination of specific antibodies of IgM isotype. Specific animal antiserum immobilized on the surface of a microtitration plate was used for the determination of total IgM human antibodies. The detection of specific IgM antibodies was performed using virion TBE antigen and rabbit antibody to TBE virus labelled peroxidase (Px). Hofmann et al. (1985) used the EIA capture system also for the determination of specific IgG antibodies and modified it for the determination of both antibody isotypes in the cerebrospinal fluid of patients. Also Bashkirtsev et al. (1986) have dealt with the use of EIA techniques for the detection of specific antibodies to TBE virus.

Individual variants of capture EIA differ in the arrangement of the detection systems. The haemagglutination activity of TBE virus may be used, in addition to EIA detection, also for a simplified haemadsorption method. This technique is termed SPIT (solid phase immunosorbent technique) and has successfully been used for many arboviruses (Gunasegaran et al. 1986).

In this paper, the first results of our own modification of IgM-capture EIA test for semiquantitative determination of specific antibodies to TBE virus are presented and compared with those obtained by conventional serological methods.

MATERIALS AND METHODS

Antigen. TBE virus, strain 414 (Institute of Parasitology, Czechoslovak Academy of Sciences, České Budějovice) was multiplied in CNS newborn mice (strain ICR, Velaz Praha). SA-TBE sucrose-acetone antigen was prepared from brain tissue after Clarke and Cassals (1958) and lyophilized. Optimum dilution of antigen was 1 : 100 (protein concentration 0.110 mg . ml⁻¹).

Sera. 1 — Mouse hyperimmune serum against TBE virus (MATBE): The serum was prepared by a repeated immunization of laboratory mice after Lennette and Schmidt (1969). The titre of specific Ig antibody response in indirect immunofluorescence (IIF) was 160. Optimum serum dilution was 1 : 500.

Table 1. Set of examined sera and their clinical characterization

Serum No.	Patient	Blood collection* (days)	Course**	Clinical picture
105	K. M.	35	1	of medium severeness
121	K. M.	27	2	of medium severeness
122	K. A.	45	1	light
128	P. J.	25	1	severe — paresis
131	V. M.	30—35	2	of medium severeness
136	N. S.	115	2	severe — paresis
141	M. F.	30	1	of medium severeness
144	K. Š.	30	3	of medium severeness
145	J. K.	21	2	of medium severeness
146	K. M.	35	1	of medium severeness

* Time after finding the tick on the patient.

** Number of phases of the disease.

2 — Sera of patients: Ten sera of patients (Table 1) from Infection Department of the District Hospital with Polyclinic at České Budějovice with the diagnosis of tick-borne meningitis or encephalitis and one control serum (negative) from a healthy donor were used. The sera of patients were usually obtained, if not otherwise stated, on the admittance of the patient to the hospital and examined for the presence of specific IgG, IgM or total specific Ig antibodies using the methods of HIT, CFR, NT (plaque-reducing test on PS cells), IIF (PS cells), and indirect ELISA. Serologic tests were performed by conventional procedures or their modifications (Lennette and Schmidt 1969). Indirect ELISA of IgG antibodies was performed using a modified method after Hofmann et al. (1979) with SA-TBE antigen adsorbed in microtitration plates Dynatech and with SwAHu/IgG, P_x conjugate (SEVAC Praha).

Protein assay. Protein content in SA-TBE antigen and Q-SwAHu/IgM antiserum (SEVAC Praha) was assayed after Lowry et al. (1951). The absorbance of colour change was measured at the wave length of 750 nm.

Analytical system of IgM-capture EIA (Fig. 1)

1 — Preparation of Q-SwAHu/IgM plates: Microtitration plates (KOH-I-NOOR "P", 96 wells) were activated with 150 µl of 2.5 % glutaraldehyde solution (Serva) in binding solution (0.05 M carbonate buffer, pH 9.5) for 120 min at laboratory temperature (Rozprimová 1987). After removal

of glutaraldehyde, 150 µl of 0.01 % solution of Q-SwAHu/IgM antiserum (SEVAC Praha) in binding solution (pH 9.5) was placed in the wells without previous washing. Then followed an incubation for 18 h at laboratory temperature. The plate was then washed twice with washing solution (PBS, 0.2 % Tween 20, 0.01 % thiomersal) and 150 µl of 0.5 M ethanolamine solution (pH 8.3) was put in the wells. After 90-min incubation at laboratory temperature the plate was washed 2× and immediately used or stored with the washing solution at 4 °C up to four weeks.

2 — Sera: The examined sera were diluted in the ratios of 1 : 400 up to 1 : 5200 with diluting solution (1 % BSA in PBS, 0.05 % Tween 20), placed in the wells (100 µl each) and incubated at laboratory temperature for 120 min. After incubation, the plate was washed 2× with washing solution. For the control of nonspecific adsorption, the diluting solution was applied instead of serum samples.

3 — SA-TBE antigen: Non-purified sucrose-acetone SA-TBE antigen in the dilution of 1 : 100 (0.110 mg . ml⁻¹) was placed in the wells (100 µl each) and incubated at laboratory temperature for 60 min. The incubation was terminated by repeated washing with washing solution (2×).

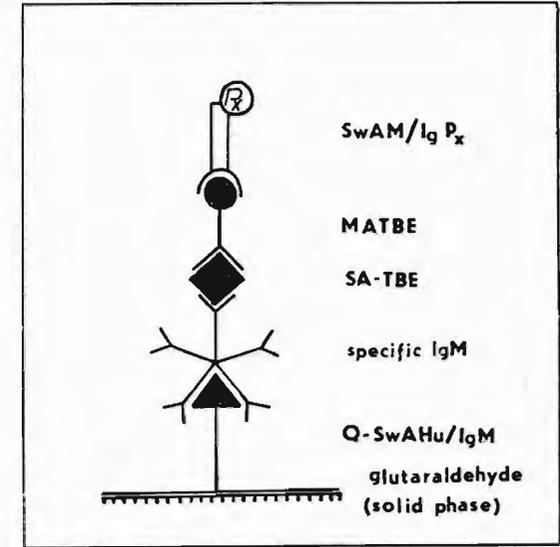


Fig. 1. The scheme of analytical system for determination of IgM antibodies.

4 — Detection system: Mouse hyperimmune serum against TBE (MATBE) was applied in the dilution of 1 : 500 (100 µl/well), incubated at laboratory temperature for 60 min, and washed in washing solution (2×). SwAM/Ig P_x conjugate (SEVAC Praha) was applied in the dilution of 1 : 500 (100 µl/well) and incubated at laboratory temperature for 60 min. After a thorough repeated washing (4× — 5×) in washing solution, 100 µl of H₂O₂ substrate with chromogen o-phenyldiamine (OPD) was added into each well. Colour reaction was stopped by 2 M H₂SO₄ after 10 min.

5 — Evaluation: Colour change of the reaction was measured by a Dynatech spectrophotometer at the wave length of 492 nm. The absorbance ratios of positive and negative P/N sera were used for semiquantitative evaluation of the levels of IgM specific antibodies, then the titre was determined as a reciprocal value of the highest serum solution for which holds a criterion P/N ≥ 2× supposing that absorbance N ≥ 0.1.

RESULTS

Serum solutions (Table 1) were tested with Q-SwAHu/IgM using various batches of titration plates. The results are the mean of three assays with the resulting value of variance coefficients (CV) 6—8 % in the "intra-assay" and 10—13 % in the "inter-assay". Individual assays are illustrated by dilution curves of the sera (Fig. 2). Table 2 summarizes in individual sera characteristic values for IgM assays of maximum index (P/N)_{max} in the whole interval of serum dilution, (P/N)_t values in the terminal titration

Table 2. Parameters for the detection of titres of IgM antibodies to TBE virus

Patient	(P/N) _{max}	(P/N) _t	Negative control A ₄₉₂	IgM titre
105 K. M.	3.4	2.2	0.146	3,200
121 K. M.	5.0	2.8	0.143	6,400
122 K. A.	3.9	2.5	0.143	6,400
128 P. J.	1.9	—	—	400
131 V. M.	4.4	2.8	0.143	6,400
136 N. S.	2.9	2.0	0.187	800
141 M. F.	4.6	2.2	0.143	6,400
144 K. Š.	4.1	2.6	0.146	3,200
145 J. K.	4.1	2.6	0.143	6,400
146 K. M.	4.5	3.4	0.143	3,200

point (see criterion of positivity), values of negative control and semiquantitatively determined levels of specific IgM antibodies. The value of negative control is the absorbance at the wave length of 492 nm of negative serum in the terminal titration point. The terminal titration point is the highest dilution of the examined serum which still does not answer the criterion of positivity. The mean value of nonspecific adsorption (A₄₉₂) was 0.290 under conditions of this test.

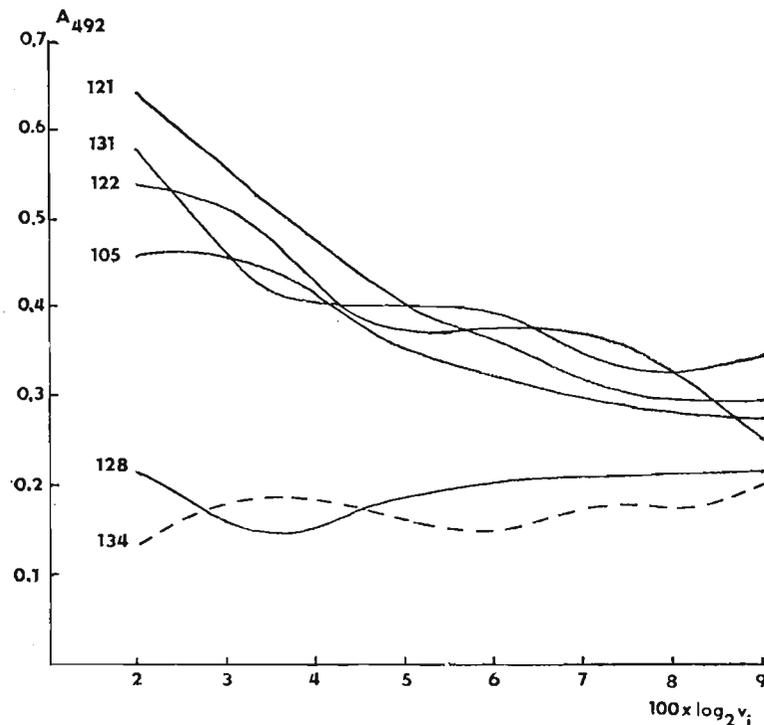


Fig. 2. The example of dilutive curves of sera 105—131.

The sera 105—146 were examined by a whole set of serologic methods. A comparison of the results obtained in the determination of total specific antibodies, IgG, and IgM with the results obtained by our capture EIA method is shown in Table 3.

Table 3. Serological examination of specific antibodies to TBE virus

Patient	Blood collection* (days)	IgM-capture EIA	IIF-IgM**	IIF-Ig**	NT	HIT	KFR	ELISA-IgG
105 K. M.	35	3,200	8	64	128	160	4	
121 K. M.	27	6,400	32	256	512	80	32	12,800
122 K. A.	45	6,400	8	256	1,024	320	64	3,200
128 P. J.	25	400	4	16	128	80	4	6,400
131 V. M.	30—35	6,400	32	128	2,048	640	ND	25,600
136 N. S.	115	800	8	512	4,096	640	8	25,600
141 M. F.	30	6,400	16	256	512	320	64	12,800
144 K. Š.	30	3,200	16	64	32	80	4	6,400
145 J. K.	21	6,400	16	128	256	320	4	12,800
146 K. M.	35	3,200	8	128	1,024	320	4	3,200

* Time after finding the tick on the patient.

** Indirect immunofluorescence of specific IgM antibodies or total antibodies to TBE virus.

DISCUSSION

The detection of specific IgM antibodies to TBE virus is the most suitable way of a rapid diagnosis of this arboviral infection. The method of indirect immunofluorescence (IIF-IgM), which has been used most widely, involves similar misleading factors as the direct ELISA. Moreover, negative effects may be produced by another factors, associated with the quality of the tissue culture of sensitive cells, or quality and quantity of synthesized viral antigens. According to the literary data, the method EIA is at least 30 × more sensitive than IIF (Meruman et al. 1982).

The capture-EIA method appears to be a very sensitive and reliable method for the detection of specific antibodies. The analytical system is universal and by a simple replacement of the antiisotype serum it enables to detect also the specific antibodies of IgG isotype. Similar analytical systems for the detection of specific IgM antibodies to some arboviruses have been described, but most of them use a shortened detection system and the viral antigen is demonstrated directly by a specific Px conjugate. For practical reasons we used an indirect system of the detection of viral antigen by mouse MATBE antiserum which enabled us to utilize the available conjugate SwAM/Ig Px (SEVAC Praha). A similar method for indirect detection of specific IgM antibodies to La Crosse virus has recently been described by Calisher et al. (1986).

In the construction of the IgM-capture EIA analytical system for the diagnosis of TBE virus infection we utilized our experience obtained in the analysis of the antibody response to the mumps virus (Grubhoffer et al. 1986). Most of the described IgM-capture EIA systems use the goat hyperimmune serum or its γ-globulin fraction for the detection of human IgM antibodies. The goat antisera seem to possess optimum properties for a hydrophobic adsorption to the surface of wells of the polystyrene plate. Since the goat antiisotype sera were not available to us, we used the Q-SwAHu/IgM

pig antiserum (SEVAC Praha). The pig antibody was found to be suitable as a capture antiisotype antibody only on the condition that it was immobilized on the surface of wells of KOH-I-NOOR "P" plates by means of glutaraldehyde (Grubhoffer et al. 1986, Grubhoffer 1987). The effectivity of a spontaneous adsorption of pig sera to the polystyrene plate is very low and the determination is very little sensitive. The described method of covalent immobilization of the pig serum affects favourably the homogeneity of the binding properties and decreases the coefficients of variation.

The verified analytical system sensitively differentiates the negative and positive sera and the values of IgM levels specific for TBE virus are in a good correlation with the individual character of sera, with the time profile of the disease, and with the general clinical picture (Table 1, 3; Fig. 2). Some of the sera (122, 136) in higher dilutions exhibit anomalies in the course of dilution curves. The serum 128 is most similar to the characteristic of a negative serum (Fig. 2). Its low level of IgM antibodies, together with a low total antibody response, indicates that the patient with the diagnosis of tick-borne encephalitis was strongly immunosuppressed due to a tumour disease and its therapy. Table 3 shows that the detection of specific IgM antibodies by the IgM-capture EIA method is 100—200× more sensitive than the detection by means of IIF-IgM, in spite of the fact that only a non-purified SA-TBE antigen was used. In the clinical practice, early specific antibodies (IgM) are usually detected by CFR. As it is evident from Table 3, this method, similarly as IIF-IgM, is less sensitive than IgM-capture EIA.

The results obtained indicate that the levels of IgM antibodies are very high (3,200 to 6,400) already 20 days after infection of the patient by the tick. As it is evident from the example of serum 136, our test showed the decrease in IgM antibody titre with simultaneous increase in the response of specific IgG antibodies or total specific antibodies. This serum illustrates well the structure of antibody response at the phase of convalescence. Specific IgM antibodies were detected still 3 months after the beginning of the disease. Hofmann et al. (1983) demonstrated specific IgM antibodies still 8 months after the beginning of the disease using their modification of the test, while the period of detection reached by indirect ELISA was only 6 months. The same authors used the highly sensitive IgM-capture EIA method also for the detection of specific IgM antibodies after vaccination against TBE. However, they note that the high sensitivity of detection is associated with a risk of false interpretation of the assay.

The IgM-capture EIA method for the detection of specific antibodies of the isotype IgM is a significant solution of the problem of an early clinical diagnosis. If we consider that the plates with the antiisotype serum in our modification can be stored without any loss of activity for 3—4 weeks, the time necessary for performing the test proper is reduced to 5 hours. It will certainly be possible to make this time still shorter and to use this test for an exact interpretation of the number of specific IgM antibodies.

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БЫСТРЫЙ СЕРОЛОГИЧЕСКИЙ ДИАГНОЗ КЛЕЩЕВОГО ЭНЦЕФАЛИТА ПРИ ПОМОЩИ ФЕРМЕНТАТИВНОГО ИММУНОАНАЛИЗА

Л. Грубхоффер, К. Крживанец, Й. Копсци и Е. Томкова

Резюме. Авторы разработали быстрый серологический метод для диагноза клещевого энцефалита с помощью ферментативного иммуноанализа, для которого использовали чехословацкий иммунопрепарат SEVAC. Метод проверили на клиническом материале. Для определения титров IgM антител применяли свиную антисыворотку и для отбора

специфических IgM антител - антиген клещевого энцефалита с последующим непрямым способом обнаружения. Этот метод достаточно чувствителен для обнаружения антител и их анализ вполне отвечает клинической картине заболевания.

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