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DETECTION OF TICK-BORNE ENCEPHALITIS VIRUS IN IXODID TICKS COLLECTED IN NATURAL FOCI BY TIME-RESOLVED FLUOROIMMUNOASSAY

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Summary. - Time-resolved fluoroimmunoassay (TR-FIA) was used for the first time for evaluation of infestation of ixodid ticks with tick-borne encephalitis virus. Comparison of TR-FIA results with those obtained in enzyme immunoassay and by virus isolation confirmed the high efficacy of the method in question. Positive results of TR-FIA coincided with the data of virus isolation in 83.6 % cases, the level of false-negative results did not exceed 1.2 %, the overall time consumption amounted to about 1.2 hr.

Key words: tick-borne encephalitis virus; infectivity of tick-vector; time-resolved fluoroimmunoassay; enzyme immunoassay

Introduction

Tick-borne encephalitis (TBE) is still an urgent problem of the C. I. S. health service so that effective methods for evaluation of ticks infestation are demanded. Until recently, the only way of evaluating tick infectivity was the isolation of virus by inoculation to mice or tissue cultures. Due to its significant labour-consuming nature and large numbers of animals required, pools of 10-12 ticks are usually employed. This method reflects the tick infestation relative extent only. At present, solid phase enzyme immunoassay (EIA) has been widely introduced for TBE virus detection in individual ticks. However, this assay has frequently proved an inadequate sensitivity and, therefore, virus--specific antigens were detected only in 50-55 % infected ticks (Navolokin and Subbotina, 1985; Kokorev and Subbotina, 1989; Schipakin *et al.*, 1989). Consequently, enhancement of sensitivity and specificity of current short-term assays as well as the development of new assays is still urgent.

TR-FIA, time-resolved fluoroimmunoassay (Soini and Hemmilä, 1979) has recently received the attention of virologists. It is a solid phase immunoassay based on the application of lanthanide ion-labelled antibodies such as euro-

pium, and on the specific way of the monitoring of luminescence (Soini and Kojola, 1983). This assay has been very helpful for rapid diagnosis of a number of virus infections in particular, of respiratory viruses in the nasopharyngeal secretion of patients.

The present research was preceded by investigations laying the basic of TR-FIA application for arbovirus detection in cell cultures. Here we present the results of virus assay in ticks collected in natural foci by TR-FIA as compared with EIA.

Materials and Methods

Ixodes persulcatus ticks (749 individuals) were collected from the plants in the natural focus of TBE virus, Permsky region, in May-June 1989. Before the initiation of studies, the ticks were kept alive for 1 month at 4 °C in a humidified chamber.

Preparation of tick suspension. The ticks were washed once in 70 % ethanol and two times in Hank's solution containing antibiotics. Each tick was placed in a separate plastic tube which was cooled in liquid nitrogen for 10-15 sec. Thereafter, single ticks were homogenized with a stainless pestle after adding 0.2 ml Hank's solution containing antibiotics and 2 % normal bovine serum to each tube. From these suspensions 50 μl were used for TR-FIA and EIA, the remaining material was stored at -20 °C for no longer than 1-2 weeks prior to virus isolation.

Biological isolation of TBE virus (bioprobe) was carried out in newborn white mice or continuous SPEV cell lines. Two-day-old mice were inoculated intracerebrally with 0.01-0.02 ml tick suspension. For each bioprobe a litter of 4-7 newborn mice was used. The animals were followed up for 14 days. Brain suspensions of sick mice were assayed in EIA for confirmation of virus presence. SPEV cells were grown in 96-well plates (Costar), 0.02 ml of tick suspension was inoculated into 2 wells and 0.18 ml of maintanance medium 199 was added to each well. The plates were incubated at 37 °C in CO_2 incubator and 48 hr later the culture fluid was assayed in EIA for TBE virus antigens.

Antibodies. Polyclonal immunoglobulins (poly IgG) were fractionated from ascitic fluid of mice immunized with TBE virus (4072 strain) by ammonium sulfate sedimentation with subsequent purification of IgG fraction using Sephadex G-200. Monoclonal antibodies (MoAb) to TBE virus (KEN 46-8) were prepared at the Arbovirus Department, Institute of Virology of the U. S. S. R. AMS. MoAb from the immune ascitic fluid were isolated by means of two-fold sedimentation with 20 % polyethylenglycol 6,000; the latter was removed from the immunoglobulin solution with the use of chlorophorm.

Enzyme or lanthanide labelled conjugates were prepared by standard procedures (Hemmilä *et al.*, 1984; Nakane and Kawaoi, 1974) at the Department of Virology, Turku University, Finland.

Sensibilization of plates. As primary antibodies, MoAb KEN 46-8 at the concentration of 0.5 μg/well (TR-FIA) or 1 μg/well (EIA) were used. The MoAbs 100 μl in 0.05 mol/l carbonate-bicarbonate buffer (pH 9.6) were incubated at 4 °C for 18 hr. After washing the wells were treated with 1 % bovine serum albumin (BSA) solution at 37 °C for 1 hr.

Enzyme immunoàssay. A "double sandwich" procedure was adjusted for arbovirus testing (Lavrova and Navolokin, 1986) as follows: 50 μl of tick suspension was placed in each well of presensitized plates and incubated at 4 °C for 18 hr. After washing, 100 μl of peroxidase conjugate diluted 1:400 was added (0.4 μg/ well). Orthophenylendiamine ("Sigma") was used as substrate.

Time-resolved fluoroimmunoassay. For analysis 50 μl of tick suspension and 50 μl of conjugate (50 ng/well) were placed into each well of presensitized microplate strips ("LKB-Wallac") and incubated at 37 °C for 1 hr. After washing 6 times, 200 μl of enhancement solution was added per well and the luminescent signal was recorded by the Arcus 1230 time-resolved fluorometer ("LKB-Wallac"). The wash buffer contained: 0.05 mol/l Tris-HCl pH 7.75, 9 g/l NaCl, 0.5 g/l

Tween 20. The buffer for antigen and conjugate dilution contained: 0.05 mol/l Tris-HCl pH 7.75, 0.15 mol/l NaCl, 0.01 % Tween 20, 20 μl diethylentriaminopentaacetic acid and 0.5 % BSA.

Evaluation of results. The result was considered positive if the signal exceeded the cut-off level of luminescence (TR-FIA) or extinction (EIA) of the negative control. The minimal cut-off level was defined as 2-fold signal of the negative control. The results were statistically evaluated using the method of correlation analysis and χ^2 criterium (Bessmertny, 1967).

Results

The major problem of evaluating the results while analysing a great number of samples containing an unknown amount of antigen was to determine an adequate cut-off level. As it is evident from the Fig. 1, the minimal cut-off level for evaluation of TR-FIA results appeared to be 4,000 cps. False negative results (negative TR-FIA but positive virus isolation) were absent, however, false positive level was markedly high (positive TR-FIA and negative bioprobe). No false positive results were noted when the cut-off level was 10,000 cps, but the efficacy of TR-FIA was lowered when compared to that of

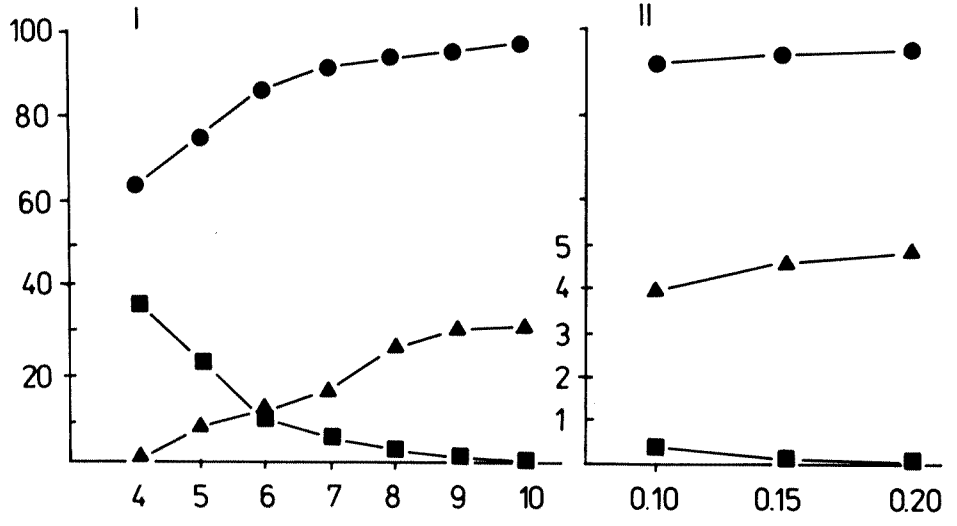

Fig. 1

The effect of the cut-off level value on TR-FIA (I) and EIA (II) results

Circles denote coincidence with the results of a bioprobe; squares - the level of false positive results; triangles - false negative results.

Ordinate: on the left - coincidence with the results of a bioprobe and hyperdiagnosis level; on the right - false negative results (expressed in %); abscissa: cut-off level, cps x 10^3 (TR-FIA) or absorbance units (EIA).

bioprobe (false negative results). Therefore, the cut-off level of 6,000 cps was considered optimal and allowed to evaluate the TR-FIA results in case of low level of false negative diagnosis (less than 1.2 %) and in good accordance with virus isolation. EIA results were evaluated in relation to cut-off level 0.150, and both increase and decrease of cut-off level did not affect significantly the level of hypodiagnosis and the extent of correlation with virus isolation.

In accordance with TR-FIA and EIA cut-off levels all the samples were distributed into 4 tentative groups (Table 1). In each group the antigen level was defined as negative, low, medium or high.

By the 2 described methods the quantitative composition of groups was different (Table 2). According to the data of EIA 726 out of 749 samples were free from TBE virus antigen. Positive samples comprised 3.1 % of the total and were evenly detected in the groups with low, medium and high levels of antigen. According to the TR-FIA data, the number of positive samples was signifi-

Table 1. Preliminary grouping of tick suspension studied according to the concentration of virus-specific antigen

Sample	TR-FIA	EIA
	Fluorescence cps x 10^3	Extinction, absorbance units
Positive control in dilutions:		
1 : 500	214.8	1.780
1 : 5 000	28.1	0.320
1 : 50 000	3.9	0.051
Negative control:		
No 1	1.7	0.067
No 2	2.5	0.092
No 3	3.9	0.075
Cut-off level	6.0	0.150
Ticks (antigen level):		
negative	< 6.0	< 0.150
low	6–10	0.150–0.300
medium	10–20	0.300–0.600
high	> 20	> 0.600

Footnote: Positive control – sucrose acetone TBE virus antigen in respective dilutions; negative control: No 1 – buffer; No 2 – normal antigen from uninfected mouse brain tissue diluted 1:50; No 3 – tick suspensions mixed with normal globulin as primary antibodies.

cantly higher (18.3 %) and predominantly found in the group with low antigen content. Comparative analysis of the values of TBE virus antigen levels and the data on biological isolation of the infectious virus showed that the latter is detected in 100 % of samples only in case of medium or high antigen levels in ticks both by TR-FIA and EIA data. In the case of low antigen level the number of positive findings in EIA (10 out of 749) and particularly, in TR-FIA (104 out of 749) was significantly superior to the number of those containing infectious virus. However, a certain amount of false negative results was noted: 35 samples in EIA and 9 in TR-FIA. The correlation between virus isolation and positive TR-FIA and EIA results was statistically significant ($\varkappa^2 = 899.1$; $p < 0.001$ and $\varkappa^2 = 242.3$; $p < 0.001$, respectively).

The results obtained in TR-FIA and EIA during examination of 749 tick suspensions are summarized in Table 3. Negative results coincided almost completely. The rate of positive coincidence depended on the amount of the antigen in ticks: 100 % rate of coincidence was recorded only in case of high antigen level and decreased to 42.9 % in case of medium values. 103 out of 104 samples containing low levels of antigen as shown in TR-FIA proved negative in EIA. Consequently, high correlation ($r = 0.86$; $p < 0.001$) between the number of positive results in TR-FIA and EIA and the amount of virus-specific antigens in samples was registered only in the case of medium and high antigen levels, i.e., when the value of the luminescent signal exceeded 10,000 cps. The total number of positive findings in TR-FIA was nearly 6-fold higher as compared to the similar index in EIA.

Table 2. Correlation of TR-FIA and EIA results with those obtained during direct isolation of the infectious TBE virus

Assay	Antigen level	Number of samples	Positive results of a bioprobe	
			Total	%
TR-FIA	Negative	613	9	1.5
	Low	104	14	13.5
	Medium	21	21	100
	High	11	11	100
	Total	749	55	7.3
EIA	Negative	726	35	4.8
	Low	10	7	70*
	Medium	7	7	100
	High	6	6	100
	Total	749	55	7.3

Footnote: *the value cannot be considered statistically significant owing to a small number of samples in the group under study.

Table 3. Distribution of TR-FIA and EIA results obtained in the studies of 749 tick suspensions

Antigen level in TR-FIA	Total number of ticks examined	Positive results of EIA				
		Total		Antigen level		
		General number	%	Low	Medium	High
Negative	613	2	0.3	2	0	0
Low	104	1	1.0	1	0	0
Medium	21	9	42.9	6	3	0
High	11	11	100	1	4	6
Total	749	23	3.1	10	7	6

The comparative efficacy of 3 methods (TR-FIA, EIA and bioprobe) used for TBE virus antigen detection in ticks is shown in Table 4. Positive results of TR-FIA and bioprobe coincided in 83.6 % cases, EIA and bioprobe - in 33.3 %, TR-FIA and EIA only in 15.4 % cases. This indicates hypodiagnosis to a certain extent. According to TR-FIA the rate of false negative results (9 out of 749) was 1.2 % whereas in EIA it was significantly higher (35 out of 749) and amounted to 4.7 %. The total coincidence of results obtained in 3 methods was, however, sufficiently high owing to almost complete coincidence of negative results.

Table 4. Comparative efficacy of TR-FIA, EIA and virus isolation in detecting TBE virus in tick suspension

Result	Number of samples	TR-FIA		EIA	
		+	−	+	−
Bioprobe:					
positive	55	46	9	20	35
negative	694	90	604	3	691
Total	749	136	613	23	726
x^2		174.5		338.5	
p		< 0.001		< 0.001	
TR-FIA:					
negative	613			2	611
positive	136			21	115
Total	749			23	726
x^2				84.6	
p				< 0.001	

Discussion

Comparative examination of ticks for the presence of TBE virus by TR-FIA and EIA showed the different efficacy of these methods. The results of TR-FIA and EIA completely matched the data of direct virus isolation in the case of medium and high antigen levels only. At low antigen levels in ticks false positive diagnosis was noted, particularly by TR-FIA. It is in agreement with the results of many authors using other serological methods (Klisenko *et al.*, 1982; Goldfarb and Zaklinskaya, 1969) this can be accounted for the low cut-off level when evaluating TR-FIA results on the one hand and lower accuracy of assays in the case of low antigen concentrations (Suopää *et al.*, 1985), on the other. In addition, a number of biological factors should be considered. From these should be mentioned the lower pathogenicity for white mice and reduced capacity for reproduction in cell cultures of warm-blooded animals as a result of TBE virus attenuation during its persistance in ticks (Dzhivanjan *et al.*, 1988; Chunikhin *et al.*, 1986). One should also take into account the detection of inactivated virus, or virions with altered biological properties as well as of structural and non-structural virus-specific proteins (Timofeev *et al.*, 1987; Beljavskaya *et al.*, 1987; Heinz and Kunz, 1982). Besides, the amount of the infectious virus in ticks with low antigen concentration can be lower than the infecting dose cut-off for the given biosystem.

By means of varying cut-off level when evaluating TR-FIA results, it was possible to reduce to nil the number of false negative probes although the level of hyperdiagnosis in this case rose markedly. In EIA even a significant decrease in the cut-off level resulted in negligible alterations of the number of hypodiagnosis cases. This agrees with the data (Kokorev and Subbotina, 1989; Schipakin *et al.*, 1989) on lower sensitivity of EIA as compared to that of bioprobe and results in detection of infectious virus in 50–60 % of infected ticks only. On the other hand, a number of investigators (Navolokin *et al.*, 1989; Mansurov *et al.*, 1989) consider EIA to be more effective than bioprobe as it is possible not only to evaluate virus infectivity of individual ticks but also to prognose the risk of human infection following tick bite (Penjevskaya *et al.*, 1989).

According to the data of several investigators, TR-FIA sensitivity is usually higher or comparable with that of EIA (Hemmilä *et al.*, 1986; Meurman *et al.*, 1982). We demonstrated the indisputable advantages of TR-FIA. First of all, this assay was characterized by higher sensitivity. The total number of positive findings in TR-FIA was 6 times higher than in EIA; the correlation with positive results of bioprobe was 83.6 % in TR-FIA compared to 33.3 % in EIA; the level of hypodiagnosis in TR-FIA (1.2 %) was significantly lower than that in EIA (4.7 %).

Second using one-stage mode TR-FIA the overall duration of the test (without plate sensibilization) did not exceed 1.2 hr which is particularly important when a rapid analysis of the epidemiological situation is required. All this

allows one to recommend the application of TR-FIA to determine the infectivity of ticks in TBE virus natural foci.

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