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EXAMINATION OF THE RELATIONSHIP BETWEEN NET EXTINCTION IN ELISA AND VIRUS NEUTRALIZATION METHODS FOR ESTIMATING THE TITER OF NEUTRALIZING ANTIBODIES AGAINST BOVINE HERPESVIRUS 1.

S. LAZIĆ*, G.PEROVIĆ**, RUŽICA AŠANIN***, L. DRAŽIĆ****, DUBRAVKA MILANOV* and BRANKA VIDIĆ*

Scientific Institute for Veterinary Medicine, Novi Sad, Yugoslavia **Faculty of Civil Engineering, Beograd, Yugoslavia ***Faculty of Veterinary Medicine, Beograd, Yugoslavia **"Geokarta", Beograd, Yugoslavia*

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Specific antibodies (Ab's) against bovine herpesvirus-1 (BHV-1) were detected by parallel examination of blood samples obtained from cows and calves, utilizing the ELISA-test and the method of micro virus neutralization. An adequate regression relationship between net extinction and the values of neutralizing antibody titer was determined by statistical analysis of the results obtained.

However, values for antibody titers could not be accurately predicted on the on the basis of net extinction. It was determined that the accuracy rate was 30% for the majority of tested titers However, accuracy approched 80% for particular values of neutralizing antibody titer.

Key words: antibodies against BHV-1, ELISA, micro virus neutralization, regression analysis, analysis of variance, hypothesis testing.

INTRODUCTION

Several methods are used for determination of specific antibodies (Ab's) against bovine herpesvirus-1 (BHV-1; IBR/IPV). Besides the virus neutralization (VN)-test, which is the most widely applied technique, assays such aspassive haemagglutination, immunofluorescence, gel-diffusion and Enzyme Linked Immunosorbent Assay (ELISA) are also frequently used. ELISA is becoming increasingly implemented in scientific and diagnostic laboratories world wide. Due to it's excellent performance (rapid and easy to carry out this method has been recommended by numerous authors (Edwards, 1988; Bommeli and Khim 1982; Krause et al., 1989) for routine laboratory examination of large numbers of blood and milk samples.

In that respect the ELISA method has been subjected to numerous snsitivity and specificity tests. Several authors (Nakajima et al., 1989; Riegel Sunthia et al., 1987; Edwards et al., 1986) have compared ELISA and the virus neutralization test during a period of 1 and 24 hours. Different, yet remarkably high correlation coefficients, were obtained, particularly when the results were obtained for virus neutralization after 24 hours. In comparison to the method of virus neutralization, ELISA revealed a higher rate of specificity and sensitivity, especi ally for early detection of low antibody levels, either after infection or vaccination. It is impor-

tant to emphasize the observations of Edwards et al. (1986) who pointed out some disadvantages of ELISA, Namely the, authors emphasised the wide variation of the ELISA results and the difficulties during standardization of this method among laboratories. Interpretation of results obtained by ELISA also presents a problem which is not negligible.

The results obtained by ELISA are designated as net extinction values, on which basis the presence or not of specific antibodies against BHV-1 in the investigated sera can be determined, However, a positive result does not confirm the antibody level (titer value).

The aim of this study was to investigate the statistical relationship between net extinction and values for neutralizing antibody titers in order to obtain a functional model which enables the net extinction values to be expressd as a function of antibody titer values.

MATERIALS AND METHODS

Test samples

Vaccination and revaccination of randomly selected highly pregnant cows on a cattle farm in Vojvodina was carried out, using different anti-BHV-1 vaccines (Iffavax, Rhone Merieux - France; Borinak, Mevak - Slovakia and Iberol-plus, Vet. Institute Zemun - Yugoslavia). The cows were distributed in three groups according to the vaccine applied. Blood was sampled three times: at the moment of vaccination, at revaccination (three weeks post-vaccination) and at parturition. A total of 233 blood samples were obtained from these cows.

Blood samples were also collected from calves originating from the vaccinated cows. Sampling was performed periodically at 15- day-intervals, beginning at calving and carried on to the age of three months. A total of 329 blood samples were obtained from the calves.

Blood samples were obtained by punction of v. jugularis. Serum was separated, after spontaneous coagulation at room temperature, by centrifugation at 3.000 rpm for 10 minutes. Separated samples of blood sera were stored at-20 ^{0}C until assayed.

Determination of specific antibodies against BHV-1

Specific antibodies against BHV-1 in blood sera were detected by the method of micro virus neutralization and ELISA.

Micro virus neutralization method

Duplicates of sera dilutions were prepared in microtiter plates ("Nunc"). Aliquots of 25 μl BHV-1 suspension containing 100 CCID-50/25 μl were added to each serum dilution. Microtiter plates were incubated for 60 minutes at 37^{0}C. After incubation, 50 μl of Madin Darby bovine kidny (MDBK) cell suspension were added.Eagle MEM with 10% fetal bovine serum (FBS) was used as the culture medium. Each plate was subjected to serum-toxicity testing, as well as virus- and cell-control. Plates were incubated for 5 days at 37^{0}C with 5% CO_2. Plates were observed daily over a period of 5 days. The results were read on Day 5, when cytopathogenic effects (CFE) were completely visualized. The cytopathogenicity of the virus was determined in wells containing a mixture of virus and cells.

ELISA

Detection of specific antibodies against BHV-1 by an immunoenzyme method was conducted using commercial SANOTEST anti IBR/IPV set kits obtained from Werft Chemie (Vienna, Austria; Code: BR-96; Art. No: 303). The ELISA was performed according to the manufacturer's instructions.

Statistical methods for analysis of results

The following statistical methods were used for of the analysis of the results regression relationship, test of model-adequacy, Pope's outliers test, Bartlet's test of variance.

RESULTS

The statistical relationship between net extinction (NE) and values for neutralizing antibody titers (TNA) in the examined sera was determined. (Table 1) The response variable, designated Y, corresponds to net extinction values, while the predictor variable, designated X, corresponds to the values for neutralizing antibody titers, that is X = (2; 4; 8; 16; 32; 64; 128; 256; 512; 1024). Each group of cows or calves was examined separately or combined together as a whole.

Table 1. Basic data for examination of the regression function between NE and TNA in blood serum of cows.

	Model (Group)		TITER OF NEUTRALIZING ANTIBODIES (TNA)									
			1:2 x=2	1:4 x=4	1:8 x=8	1:16 x=16	1:32 x=32	1:64 x=64	1:128 x=128	1:256 x=256	1:512 x=512	1:1024 x=1024
1.	Cows and calves all vaccines	n	37	56	72	101	105	78	64	37	11	1
		$\bar{y}$	0.453	0.948	1.115	1.677	1.990	2.211	2.381	2.442	2.202	3.486
	(f_1 = 541)	s_1	0.278	0.433	0.518	0.410	0.287	0.328	0.356	0.340	0.668	–
2.	Cows vaccinated Iffavax vak.	n	2	9	8	15	26	6	3			
		$\bar{y}$	0.940	1.343	1.364	1.988	2.051	2.171	2.290			
	(f_1 = 62)	s_1	0.050	0.418	0.323	0.307	0.248	0.342	0.471			
3.	Cows vaccinated Borinak vak.	n		4	6	14	12	17	14	9	4	1
		$\bar{y}$		1.280	1.564	1.933	2.106	2.420	2.592	2.836	2.683	3.486
	(f_1 = 72)	s_1		0.406	0.254	0.249	0.309	0.231	0.222	0.225	0.185	–
4.	Cows vaccinated Iberol-plus	n	4	8	10	10	13	15	16	6	1	
		$\bar{y}$	0.514	0.977	1.183	1.549	2.114	2.251	2.363	2377	2.473	
	(f_1 = 74)	s_1	0.0354	0.349	0.166	0.249	0.245	0.393	0.237	0.147	–	
5.	Calves orig. from cows Iffavax vakcine	n	13	13	15	16	11	12	5	3	2	
		$\bar{y}$	0.401	0.944	1.256	1.652	2.074	2.427	2.503	2.791	2.411	
	(f1 = 81)	s_1	0.094	0.371	0.508	0.444	0.315	0.358	0.361	0.320	0.393	
6.	Calves orig. from cows Borinak vakcine	n	10	9	19	25	24	13	13	9		
		$\bar{y}$	0.552	0.691	1.341	1.512	2.035	2.177	2.567	2.628		
	(f1 = 114)	s_1	0.459	0.282	0.579	0.435	0.216	0.293	0.553	0.176		
7.	Calves orig. from cows Iberol-plus	n	8	13	14	21	19	15	13	10	4	
		y	0.437	0.936	1.392	1.796	1.969	2.373	2.551	2.661	2.873	
	(f1 = 108)	s_1	0.135	0.433	0.373	0.354	0.356	0.235	0.204	0.266	0.636	

Mean values of Y and variance s^2_1 were calculated for each value of X. Homogenety of within the system was assessed calculated by applying Bartlet's variance test.

The results of the model-adequacy test are summarized in Table 2. The adequacy test was accepted for groups 1, 2, 3, 5, 6 and 7 without application of Pope's outliers test, while for group 4 (cows vaccinated with Iberol-plus vaccine) the adequacy test was accepted after application of Pope's outliers test, rejecting only one result Yi.

Table 2. Results of the regression model adequacy test

Model (Group)	Mean square of deviation From model S_2^2 (f_2)	Within the system S_1^2 (f_1)	Statistic $F(LF)=\frac{S_2^2}{S_1^2}$	Critical statistical value $za\ \alpha = 0.05$	Adequacy acceptance YES / NO
1. Cows and calves (all vaccines)	0.1581 (7)	0.1259 (541)	1.256	2.027	yes
2. Cows vaccinated with Iffavax vaccine	0.2041 (4)	0.0970 (62)	2.104	2.520	yes
3. Cows vaccinated with Borinak vaccine	0.0870 (6)	0.0668 (62)	1.302	2.249	yes
4. Cows vaccinated with Iberol-plus vaccine	0.1193 (6)	0.0745 (73)	1.601	2.226	yes
5. Calves orig. from cows with Iffvax vaccine	0.1379 (6)	0.1446 (81)	0.953	2.216	yes
6. Calves orig. from cows with Borinak vaccine	0.1840 (5)	0.1683 (114)	1.093	2.210	yes
7. Calves orig. from cows with Iberol-pl. vaccine	0.04364 (6)	0.1104 (108)	0.395	2.090	yes

DISCUSSION

The results of ELISA are expressed as net extinction values (optical density of color developed during the immunoenzyme reaction with a cut-off value determining the ELISA reaction as positive or negative. This implies the presence or absence of specific antibodies against BHV-1. However, such positive results obtained by ELISA indicate only the presence, but not the level of specific antibodies against BHV-1, which represents a significant disadvantage of the method (Edwards et al., 1986). Our investigation revealed differences between net extinction values in the investigated sera.

Analysis of exclusively ELISA-positive reactions demonstrated a positive correlation between net extinctions and values of the neutralizing antibody titers, namely higher net extinction is associated with higher values of neutralizing antibody titers. This observation is in accordance with the mechanism of im-

munoenzyme reaction, that is net extinction, i. e.optical density of the developed color increases with higher level of antibodies in the investigated serum.

Analysis of Y and X variables raises two questions: firstly, which kind of inter-relationship, if there is any, exists between variables Y and X (Aivazyan et al., 1985) and secondly, is it possible to estimate corresponding X-values on the basis of Y-results, and with which reliability.

The results presented in Table 1 partially explain the first question, i. e. it is possible to investigate the familiy of regression relationships $F = (f(X,O))$, where Θ represents the vector of unknown parameters, which is to be investigated on the basis of NE Y. We have investigated all seven models (for seven defined groups) and we have determined the family of regression relationships if $(X,\Theta))$ which corresponds with the investigated data. This has been confirmed by the model-adequacy test, using the F - statistic (Hald, 1957; Draper and Smith, 1966; Seber, 1977; Searle, 1971; Perović, 1997),

$$F(LF) = \frac{S_2^2}{S_1^2}$$

which under the null hypothesis H_0 - "model f (X,Θ) is adequate", has a central F-distribution with f_2 and f_1 degreee of freedom, where S_1^2 presents the "pure error" (dispersion) within the system (after the acceptance of the hypothesis of the equality of internal variances - Bartlet's variance test), and S_1^2 presents the mean square of the model deviation. This may present the answer to the first question, i. e. a regression function $E(Y) = f(X,\Theta)$ has been established, where E is the operator of mathematical expectation, which is well in accordance with experimental data. This correspondence can be determined using other methods, e. g. determination coefficient R^2, which has been derived by Perović (1997) applying different numbers of Y-values for different X-values.

The second question, i. e. to estimate TNA on the basis of NE-values, was negatively answered. Only a rough estimation can be made, indicating an accuracy rate of ca.30%, although higher rates were obtained for some TNA values, e. g. for TNA 1:2, 1:4, 1:16 and 1:32 the rates were 86%, 50%, 37% and 50%, respectively.

On the basis of the results obtained, we may presume that an adequate regression relationship between NE and TNA values in blood serum of cows and calves was established.

Further investigations will be aimed towards obtaining results with lower variances of NE. in order to improve the predictive ability of ELISA.

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ISPITIVANJE MOGUĆNOSTI UTVRĐIVANJA FUNKCIONALNOG MODELA UPOREDNIM ISPITIVANJIMA TITRA ANTITELA ZA BOVINI HERPESVIRUS-1 IMUNOENZIMSKOM I METODOM MIKRO NEUTRALIZACIJE

S. LAZIĆ, G. PEROVIĆ, RUŽICA AŠANIN, L. DRAŽIĆ, DUBRAVKA MILANOV i BRANKA VIDIĆ

SADRŽAJ

Uporednim ispitivanjima uzoraka krvnih seruma krava i teladi ELISA metodom i metodom mikro virusneutralizacije, vršeno je utvrđivanje antitela za goveđi hrpesvirus-1 (BHV-1). Statističkom analizom dobijenih rezultata utvrđen je adekvatan model regresionih zavisnosti neto ekstinkcija od vrednosti titra neutralizujućih antitela.

Međutim, ispitivanje mogućnosti prognoziranja vrednosti titra antitela na osnovu neto ekstinkcija, prema rezultatima izvedenih ispitivanja, nije se moglo u potpunosti ostvariti. Utvrđeno je, da bi 30% prognoza bilo ispravno, dok je kod određenih vrednosti titra neutralizujućih antitela taj procenat mnogo veći i iznosi više od 80%.