

DEVELOPMENT OF AN ELISA TEST TO IDENTIFY ANTIBODIES TO VESICULAR STOMATITIS (INDIANA-3) VIRUS

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Summary. The competitive ELISA in liquid phase was adapted to identify antibodies to vesicular stomatitis Indiana-3 virus using the viral glycoprotein as antigen. Test validity and reproducibility were determined by testing 553 sera from negative animals and 305 sera from positive animals, obtaining values of 99% sensitivity and 100% specificity. No significant differences were observed among repeated tests. Predictive values for positive and negative results, and the overall value of the technique studied indicate that it is a satisfactory method for use in the identification of antibodies to vesicular stomatitis virus.

The vesicular stomatitis (VS) virus (VSV) belongs to the *Rhabdoviridae* family, genus *Vesiculovirus*, which includes two serotypes: New Jersey and Indiana (17). The Indiana serotype is subdivided into three subtypes (9): VSV-Indiana-1, identified together with VSV-New Jersey in endemic regions of the United States' southeast, Mexico, Central America, Panama, Venezuela, Colombia, Ecuador, and Peru (18,20); VSV-Indiana-2, identified in Trinidad (13), Argentina (11), and Brazil (21); and VSV-Indiana-3, isolated from domestic animals in Brazil only (4).

VS affects mainly horses, cattle, and swine causing vesicular lesions in the tongue, feet, and udder. It is included in list "A" of the International Office of Epizootics, together with other vesicular diseases. These diseases are characterized for having a great ability to spread and may extend beyond national boundaries, with serious socio-economic

and health consequences. Its incidence in the foreign trade of animals and livestock products is therefore important. The presence of VS in certain regions is a limiting factor for the commercial exchange of livestock products.

Today, the movement of animals among different regions, as a result of the import/export activities, includes the previous analysis of sera to detect the presence of antibodies to VSV. The most used serological tests for this purpose are the virus neutralization (1,6,8,10) and ELISA techniques (3,22,25). Virus neutralization is a sensitive test that requires the use of infectious virus, a laboratory infrastructure for cell cultures, and a 2 to 3-day period to obtain the results. In some cases, when analyzing sera from uninfected healthy animals, nonspecific reactions were observed. These sera came from VS-free regions, without suspected epidemiologic clinical disease (14,24). Using an indirect ELISA developed to identify antibodies to VSV-New Jersey (25), cross-reactions were found between both VSV serotypes. Subsequently, the development of a competitive ELISA in liquid phase (3), using viral glycoproteins as antigen,

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provided more specific results, allowing the differentiation between VSV-New Jersey and VSV-Indiana-1 antibodies.

The purpose of the present study was an assessment of the validity and reproducibility of the competitive ELISA in liquid phase, using viral glycoprotein (non-infectious) as antigen, to identify antibodies against VSV-Indiana-3.

MATERIALS AND METHODS

Antigens

Virus: The VSV-Indiana-3 Br/86 strain isolated in 1986, from a bovine tongue epithelium of Resende county (RJ, Brazil), was replicated in BHK21 cells. When approximately 80% cytopathic effect was observed, the viral suspensions were submitted to freezing and thawing processes. Then, they were clarified by centrifugation at 1000g for 10 minutes. The supernatant obtained was placed in 1 ml volume and stored in liquid nitrogen at -170°C. The antigen obtained (VNAg) was used to obtain the glycoproteins.

Glycoprotein (GLAg): The glycoprotein was prepared according to the methods previously described (3) from VNAg suspensions. The GLAg was used in the ELISA test.

Antigen for production of capture sera (IBRS-2Ag): The VSV-Indiana-3 Br/86 strain was replicated in IBRS-2 cells. The method used to obtain the virulent suspensions was the same as the VNAg. Once the IBRS-2Ag was obtained it was concentrated by ultracentrifugation at 75000 g for 60 minutes (Sorvall 75). The sediment was resuspended to 5% of the original volume in phosphate buffer (PBS). This antigen was used in the production of hyperimmune rabbit serum.

Hyperimmune sera

The hyperimmune rabbit serum (trapping antibodies) and the hyperimmune guinea pig serum (detector antibodies) for the VSV-Indiana-3 Br/86 were produced according to the methods previously described (3).

Reference sera

The serum obtained from a convalescent horse naturally infected with VSV-Indiana-3 Br/86 was used as positive control serum for all tests. Fetal bovine serum was used as negative control. As heterologous control, two sera from cattle inoculated experimentally with VSV-New Jersey or with VSV-Indiana-1 (kindly supplied by ICA-Colombia) were used.

Twenty adult guinea pigs were inoculated by the intradermoplantar route with VSV-Indiana-3 Br/86, previously adapted to this species. Serum samples were collected prior to inoculation and at 3, 5, 7, 14, 21, 28, and 90 days post-infection (dpi). Once inactivated at 56°C for 30 minutes, sera were maintained at -20°C.

Sera for the execution of serological tests

For reasons of biological safety, inoculation of bovines and equines with VSV was impossible. Epidemiological criteria were adopted to classify the sera into positive and negative groups. A collection of negative sera were formed with animals from VSV free areas (18,20), and another collection of positive sera was obtained from animals from VSV-Indiana 3 Br/86 outbreak areas (18,20). All sera were inactivated and stored at -20°C.

Sera from VSV-Indiana-3 free regions: This group was formed by 533 serum samples, which consisted of: 294 bovine sera and 56 swine sera from Chile (Livestock Agricultural Service [SAG], Chile); 102 bovine sera from Uruguay (Directorate of Foot-and-Mouth Disease Control [DILFA], Uruguay); 25 bovine sera from Italy, 32 horse sera from USA; and 24 horse sera from Argentina (PANAFTOSA).

Sera from regions with VSV-Indiana-3 activity: This group was formed by 305 sera belonging to the serum collection at PANAFTOSA, and they were collected in different states of Brazil: 65 horse sera with vesicular lesions by VSV-Indiana-3; 19 bovine sera with vesicular lesions by VSV-Indiana-3; 221 sera from swine clinically healthy from slaughterhouses of the state of Pará and previously diagnosed as positive by virus neutralization test (5).

ELISA

The reagents used in the competitive ELISA in liquid phase were previously titrated, to determine the optimal dilution, using the indirect sandwich ELISA (23). As solid support, flat-bottomed polystyrene plates (Nunc Maxisorp*) were used in all the ELISA tests. The trapping antibodies were diluted in 0.05M carbonate/bicarbonate buffer, pH 9.6, the peroxidase substrate in acid buffer, and the remaining test reagents in dilution buffer (PBS pH 7.4 with 0.05% Tween 20, 1% ovalbumin, 2% normal rabbit serum, and 2% normal serum from each animal species in study were added). All incubation stages were then washed three times (200 µl/well/wash) with physiologic saline solution containing 0.05% Tween 20.

Capture: The trapping rabbit antibodies were placed at the optimal dilution in a plate, in a volume of 100 µl/well, and incubated for 18 h at 4°C. The reagent not adsorbed was eliminated by inversion of plates and the sensitized plates were then blocked with 100 µl/well ovalbumin (GV SIGMA) 1% in PBS for 60 minutes at 25°C. After this stage, the excess reagent was removed by simple inversion of plates. Next, they were sealed with plastic, and stored at -20°C until ready to use.

Liquid phase: The problem sera and the negative and positive controls were diluted in an auxiliary plate, from neat serum in base 5 through 1:3125. The glycoprotein previously titrated was added to these dilutions in equal volume. Controls of antigen and background readings were included (Figure 1). The serum antigen mixture was incubated for 60 minutes at 37°C.

Transference: The pre-sensitized plates, were defrosted and stored for 20 minutes at 4°C, 20 minutes at 25°C, and 20 minutes at 37°C. Afterwards, the serum-virus mixture was transferred to this plate at 50 µl/well starting at line H and ending at line A. The precaution was taken of proper mixing with the pipette before removing the sample

to be transferred in each line. At this stage, the plate was incubated for 30 minutes on a plate shaker at 37°C on a slow orbital shaker.

Detector: Serum detectors were diluted in a working dilution and incubated for 30 minutes at 37°C, prior to being placed in the plate. When antibodies in guinea pig and swine sera were studied, a detector prepared in mice was used to avoid guinea pig/swine cross reactions. For other species, a guinea pig detector was always used in a volume of 50 µl/well, and incubated for 30 minutes at 37°C on a slow orbital shaker.

Conjugate: The anti-species-peroxidase conjugate was added at a volume of 50 µl/well and incubated as in the previous stage. Afterwards, OPD substrate (orthophenylenediamine) diluted in acid buffer was added, and incubated for 15 minutes at 25°C, protected from the sunlight. Then, the reaction was stopped adding 3N H₂SO₄ (50 µl/well) to the substrate that was on plate.

Reading and interpretation of the ELISA test: The test was read with multiskan at 492nm. The value 100% of absorbance was considered the average of the reading obtained in 12 wells corresponding to the antigen control (line G). From this value, serum titrations were calculated, corresponding to the dilution expressed in log₁₀, giving 50% of the value obtained in the antigen control.

Statistical analysis

From the data obtained in the different stages of the experiment, the sensitivity and specificity of the ELISA were calculated as well as the predictive values of positive (VPR+) and negative (VPR-) results. In addition, the overall value of the test was determined (12).

For the analysis of reproducibility of the ELISA test an experimental chart of randomized blocks, with four reproductions in five blocks, was used. The significance level was calculated by F test, establishing as working hypothesis that there was no significant difference among repeated tests

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Sera:	N° 1		N° 2		N° 3		N° 4		N° 5		Pos.	Neg.
	1	2	3	4	5	6	7	8	9	10	11	12
A	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:20	1:2
B	1:10										1:100	1:10
C	1:50										1:500	1:50
D	1:250										1:2500	1:250
E	1:1250										1:12500	1:1250
F	1:6250										1:62500	1:6250
G	ANTIGEN CONTROL											
H	BACK GROUND READINGS											

FIGURE 1. Plan of the ELISA plates showing positions problem of sera, positive and negative serum controls and antigen and blank controls.

of the same serum. The level of rejection of the null hypothesis was established at 5%, corresponding to $F_t = 3,06$ (16).

RESULTS

Titration of reference sera

Figures 2 and 3 indicate the charts of reference sera titrated by ELISA using VSV-Indiana-3 Br/86 antigen. The distribution of the results of titrations of animal sera from VSV free and endemic areas is indicated in Figure 4.

Validity of the ELISA test

Sensitivity and specificity: From the observations made of the results presented in Figure 4, the 1.0 log 10 value was elected as discriminating positive/negative value of the ELISA. Table 1 was prepared, from which the specificity (Sp) value of 100% and sensitivity (S) of 99% were calculated as well as the VPR+, VPR-, and the global value of the test. These are shown in Table 2, together with the proportions of the validity parameters of the ELISA test and are compared to the epidemiological criteria for the confidence limits equal to 95%.

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Repeatability: The results of the reproductions of titrations of 3 sera and their analysis of variance are presented in Tables 3 and 4. The null hypothesis was accepted in all cases ($F_c < F_t$).

DISCUSSION

As judged by the VPR+ and VPR- as well as the global value determined in this study, the competitive ELISA in liquid phase is a satisfactory

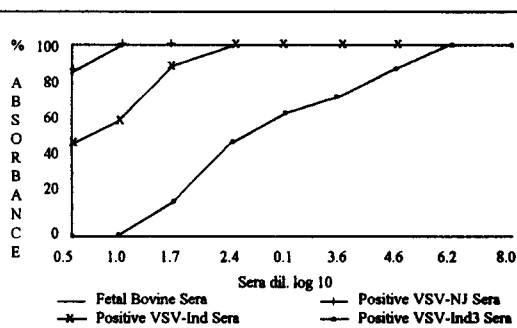


FIGURE 2. ELISA titers of reference sera tested with the VSV Indiana-3 Br/86 glycoprotein antigen.

procedure to identify antibodies to VSV-Indiana-3. Also, the values of sensitivity and specificity obtained were similar with those previously reported for New Jersey and Indiana-1 serotypes (3).

The results of reference sera (Figure 2) showed that the ELISA is specific for the identification of antibodies against the homologous antigen. Minimal cross-reactions were seen between New Jersey and Indiana serotypes. This cross-reactivity is based on the common antigenic determinants that both serotypes share in the VSV glycoprotein (15). The cross-reactions between VSV-Indiana-1 and Indiana-3 may be explained by the close serologic relation between these viruses, as previously described (2,7,9,19).

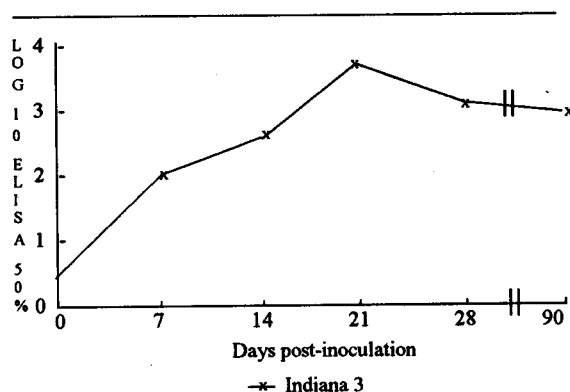


FIGURE 3. ELISA titers to reference sera from guinea pigs inoculated with VSV Indiana-3 Br/86 using VSV Indiana-3 Br/86 glycoprotein antigen.

When sera from guinea pigs inoculated with VSV-Indiana-3 were studied, the ELISA was able to detect an increase in the antibody titre at 3 dpi, reaching its peak at 21 dpi (Figure 3).

The distribution of results in Figure 4 shows that there is no overlapping between the values of negative and positive sera. This could be due to the fact that the positive sera belonged to animals with clinical symptoms, or recovered from the disease and consequently, had high antibody

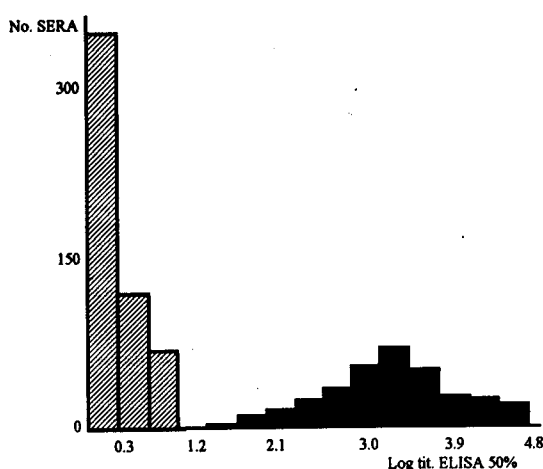


FIGURE 4. Results of titrations by ELISA of sera from regions with and without VSV Indiana-3 viral activity.

titres. It was not possible to have sera taken during the initial stages of the disease, which would probably have shown some overlap both curves.

The ELISA threshold value chosen was 1.0 (log 10). However, if the animal were at the initial stages of disease, it could have antibody titres below 1.0. This potential limitation can be overcome by analyzing at least two serum samples obtained 2-3 weeks apart.

To minimize the unspecific reactions in the test, rabbit antisera produced with antigen replicated in IBRS-2 cells was used as trapping antibody. The detector guinea pig antisera was

TABLE 1. Sera from regions with and without VSV Indiana-3 activity, classified by the ELISA test on the basis of its discriminant value

Region	Value		Total
	$\geq 1,0$	$< 1,0$	
Activity	302	3	305
No activity	0	533	533
Total	302	536	838

TABLE 2. Values obtained to determine the validity of the ELISA test in comparison with the epidemiologic method for 95% confidence limit.

	%	LC
Sensitivity	99,0	0,674
Specificity	100,0	-
VPR +	100,0	
VPR -	99,4	0,523
Global Value	99,6	0,400

VPR = Productive value.

produced by hyperimmunization of guinea pigs with infectious VSV Indiana-3 previously adapted to this species. By working with glycoprotein as antigen, unspecific reactions given by internal proteins M,N,NS and L (25) were avoided.

Another important aspect of this study was the adsorption of all test reagents prior to use. For that, we prepared a diluent buffer containing 2% normal rabbit serum and 2% normal serum of each

TABLE 3. Values expressed as \log_{10} of repeat titrations of three sera by ELISA.

Sera		Treatments				
		A	B	C	D	E
No. 1		3,63	3,26	3,26	3,55	3,38
	R	3,57	3,26	3,26	3,58	3,43
	E	3,20	3,40	3,40	3,52	3,52
	P.	3,34	3,43	3,42	3,41	3,43
	Totals	13,74	13,35	13,34	14,06	13,76
No. 2		2,60	2,09	2,19	2,32	2,15
	R	2,56	2,05	2,20	2,32	2,20
	E	2,40	2,38	2,33	2,15	2,36
	P.	2,31	2,37	2,25	2,37	2,30
	Totals	9,87	8,89	8,97	9,16	9,01
No. 3		2,92	2,60	2,56	2,90	2,73
	R	2,83	2,55	2,60	2,91	2,71
	E	2,66	2,80	2,78	2,71	2,75
	P.	2,74	2,76	2,74	2,57	2,74
	Totals	11,75	10,71	10,68	11,09	10,93

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species under test. Once diluted, all test reagents were incubated at 37°C for 30 minutes, avoiding gradients temperature in the plate. Thus, it was possible to obtain a minimal background when reading the plates. The practice to block the sensitized plates with ovalbumin (GV), allowed us to conserve trapping antibody stability for over a 12-month period at -20°C. When the plates were used immediately after sensitization with the trapping antibodies, it was not necessary to block them with ovalbumin.

TABLE 4. Analysis of variance of titrations of three sera repeated 20 times, by ELISA.

Causes of variation		GL	SQ	QM	Fc	Ft*
Sera No. 1	Treatment	4	0,091	0,023	1,77	< 3,06
	Exper.error	15	0,197	0,013		
	Total	19	0,288			
Sera No. 2	Treatment	4	0,159	0,040	2,85	< 3,06
	Exp. error	15	0,216	0,014		
	Total	19	0,375			
Sera No. 3	Treatment	4	0,045	0,011	0,846	< 3,06
	Exp. error	15	0,197	0,013		
	Total	19	0,242			

* alfa = 5%

The sensitivity, specificity and repeatability of the ELISA as standardized under the conditions of the present study, as well as the speed when reading and easy performance of the test process itself, are the main advantages of the test herein reported. These conditions would make the ELISA to be first choice over the other serological tests, such as the virusneutralization, for antibody detection in animal populations. Also, the ELISA has the

advantage of using a non-infectious antigen, which enables its use in disease-free regions, for providing support to the epidemiological surveillance programs of vesicular diseases.

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Announcements

Meetings of the South American Commission for the Control of Foot-and-Mouth Disease (COSALFA)

Meetings to discuss matters related to the prevention and control of foot-and-mouth disease are held annually by the member countries of COSALFA. Prior to each meeting, a Seminar on a topic selected during the preceding Seminar is also held.

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International Seminar on Local and Zonal Plans with Mobilization of Resources and Community Participation of Eradication of Foot-and-Mouth Disease. March 30 to April 1, 1992.

1993 - XX Regular Meeting of COSALFA. March 25 and 26, 1993, Montevideo, Uruguay

International Seminar on Foot-and-Mouth Disease Eradication, its Technical and Administrative Bases and its Consequences on the Commercialization of Animals and their Products and by-products. March 22 to 24, 1993.

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- . Development of programs of animal health services.