

Comparison of competitive enzyme-linked immunosorbent assay and serum neutralization test for the detection of antibodies to vesicular stomatitis virus (New Jersey and Indiana serotypes) in equines from Costa Rica

Comparación de la técnica seroneutralización con la inmunoenzimática competitiva en la detección de anticuerpos contra el virus de la estomatitis vesicular (serotipos New Jersey e Indiana) en equinos de Costa Rica

Maren von Koeckritz-Blickwede¹, Gaby Dolz Wiedner²✉, Marco Vinicio Herrero²

- 1 Institut fuer Physiologische Chemie, Tierärztliche Hochschule Hannover, Buenteweg 17, 30559 Hannover, Germany. E-mail: maren.von.koeckritz-blickwede@tiho-hannover.de
- 2 Laboratorio de Entomología, Escuela de Medicina Veterinaria, Universidad Nacional. E-mail: gaby.dolz.wiedner@una.cr / herrero1958@hotmail.com

Received: 1st December 2014. **Corrected:** 15 June 2015. **Accepted:** 22 June 2015.

Abstract: A competitive enzyme-linked immunosorbent assay (c-ELISA) was compared to the serum neutralization test (SNT) for the detection of antibodies against vesicular stomatitis virus, New Jersey (VSV-NJ) and Indiana (VSV-IN) serotypes, using 214 equine serum samples from Costa Rica. In the c-ELISA for VSV-NJ 109 (50.93%) equine sera reacted positive, whereas in the SNT 138 (64.48%) serum samples reacted positive to VSV-NJ. In the c-ELISA for VSV-IN 26 (12.15%) sera reacted positive, whereas in the SNT 36 (16.83%) equine sera reacted positive to VSV-IN. The c-ELISA showed a moderate sensitivity compared to the SNT. The rapidity and ease of the c-ELISA system makes it suitable as a rapid diagnostic screening assay. However, because of the lower sensitivity of the c-ELISA, the SNT is considered the method of choice to detect equine VSV infections in Costa Rica.

Keywords: vesicular stomatitis virus, serological diagnosis, neutralizing antibodies, competitive ELISA, horses, Costa Rica.

Resumen: En este trabajo se comparó la técnica de seroneutralización (SNT) con un ensayo inmunoenzimático competitivo (c-ELISA) para la detección de anticuerpos contra el virus de la estomatitis vesicular, serotipos New Jersey (VSV-NJ) e Indiana (VSV-IN), utilizando 214 sueros equinos de Costa Rica. El c-ELISA detectó 109 (50.93%) sueros positivos; mientras que en SNT se determinó un total de 138 (64,48%) animales positivos a VSV-NJ. Un total de 26 (12.15%) sueros resultó positivos en c-ELISA; mientras que 36 (16,83%) sueros se determinó como positivos en SNT para VSV-IN. La técnica inmunoenzimática mostró una moderada sensibilidad, en comparación con la técnica de seroneutralización. Por su rapidez y fácil ejecución, el ELISA competitivo resulta una técnica importante de tamizaje; sin embargo, debido a la menor sensibilidad del c-ELISA, se considera la SNT como el método de elección para detectar infecciones equinas con VSV en Costa Rica.

Palabras clave: virus de la estomatitis vesicular, diagnóstico serológico, anticuerpos neutralizantes, ELISA competitivo, equinos, Costa Rica.

✉ Corresponding Author. Po Box: 86-3000 Heredia, Costa Rica. Email: gaby.dolz.wiedner@una.cr



INTRODUCTION

Vesicular stomatitis (VS) is a viral disease that primarily affects horses, cattle and swine and occurs in endemic and epidemic forms in the tropical and subtropical areas of the Americas (Afshar et al. 1993). From southern Mexico and throughout Central and South America the disease is considered endemic (Rodríguez et al. 1990). In addition, it is transmissible to man causing clinical symptoms ranging from flu-like illness to severe encephalitis (Rodríguez et al. 1993).

Vesicular stomatitis is mainly caused by two major serotypes, vesicular stomatitis virus-New Jersey (VSV-NJ) and vesicular stomatitis virus-Indiana (VSV-IN) of the genus *Vesiculovirus* of the *Rhabdoviridae* family and *Mononegavirales* order (Afshar et al. 1993).

Most clinical cases in endemic areas are caused by VSV-NJ (Atwill et al. 1993; McCluskey et al. 2003; Reis et al. 2011; Velasquez-Salinas et al. 2014). However, both serotypes cause vesicles and ulcerations on the epithelia of the horses' mouth, tongue and coronary bands of hooves (Sherril 1993). Numerous serological assays have been developed to detect antibodies to VSV: a serum neutralization test (SNT), a complement fixation test (CFT), and more recently competitive ELISA (Alonso et al. 1991; Afshar et al. 1993; Ahmad et al. 1993; Eernisse et al. 1995; Katz et al. 1997; Kweon et al. 2005; Lee et al. 2009; Heo et al. 2010).

The purpose of this paper was to compare the relative sensitivity and specificity of a competitive ELISA (c-ELISA) developed in Ames, Iowa, USA (Afshar et al. 1993; Katz et al. 1995), and prescribed by the World Organisation for Animal Health to the SNT as reference test, to detect antibodies in naturally infected equines from Costa Rica.

MATERIAL AND METHODS

Equine serum samples

Serum samples from 214 horses were collected between October 1998 and May 1999 from 19 different farms of Costa Rica (Blickwede et al. 2002).

Serum neutralization test (SNT)

The serum neutralization test was performed as described by Rodríguez et al. (1990). For the detection of neutralizing antibodies to VSV-NJ and VSV-IN test serum samples were heat inactivated (56°C, 30 min) and diluted from 1:8 to 1:512 (4-fold dilutions) with Dulbecco minimum essential medium in duplicate wells of tissue culture plates, and a 100-200 TCID₅₀ of VSV-NJ (Greentree strain) and VSV-IN (Chimayo strain) were added to each well. After incubation of the plates at 37°C in 5% CO₂ for 1 hour, Vero E6 cells (kindly supplied by Dr. Rodríguez, Plum Island, NY) were added as indicator cells (10,000 cells/well). Cell control, virus control, positive control, and negative control serum samples were included in each neutralization assay. All plates were read after 48 hours, and samples were considered positive if either well (New Jersey or Indiana) had a confluent monolayer of Vero cells in titers starting from 1:32.

Competitive enzyme-linked immunosorbent assay (c-ELISA)

The protocol of the c-ELISA developed by the National Veterinary Services Laboratories (NVSL) in Ames, Iowa, U.S.A. (Eernisse et al. 1995) was used in all the experiments, with some modifications: Microtiter plates (Polysorp, Fa. Nunc, Roskilde) were coated with 75 μ l recombinant antigen (protein N) of VSV-NJ and VSV-IN, diluted 1:2500 in 0.01 M PBS, pH 7.4, (exception: wells G11 and G12, that were used as blanks) and incubated 16 h at 4°C. The coating solutions were decanted; 100 μ l/well of blocking solution (0.01 M PBS, 5% nonfat dry milk, NFD) was added to the plates and incubated for 1 h at 37°C with constant agitation. The plates were then washed three times with washing buffer (0.002 M PBS, 0.05% Tween-20), and control and test serum samples diluted 1:8 in PBS (containing 1 M NaCl, 1% NFD) were added in duplicate wells (50 μ l/well) and incubated 30 min at 37°C with constant agitation. In the wells A1/A2 (diluent control) and G11/G12 (blank) no serum samples were added. Positive control serum samples were added to two wells. Polyclonal antibodies to VSV-NJ and to VSV-IN were diluted 1:2500 in PBS (containing 1 M NaCl, 1% NFD), 50 μ l were added to each well, and the plates were incubated 30 min at 37°C with constant agitation. After washing the plates, the conjugate goat-anti mouse-IgG (H+L)-peroxidase (Fa. Zymed) was diluted in 0.01 M PBS (containing 1% NFD, 12.5% goat serum), added to each well (50 μ l/well), and incubated for 1 h at 37°C with constant agitation. After washing, 50 μ l/well of peroxidase substrate (1 mg of tetramethylbenzidine 2 HCl, diluted in 10 ml of 0.05 M phosphate-citrate buffer, pH 5, containing 20 μ l of 3% H₂O₂) was added. The reaction was stopped with 2 M H₂SO₄ and the optical density (OD) recorded at 450 nm in a Labsystems Multiskan MS (Flow Laboratories). Results were interpreted as a reduction percentage (RP) of the OD. The mean of the optical density of the diluent control was considered as 0 RP. The following formula was used to determine the RP of the test serum samples: $RP = 100 - [(mean\ OD\ test\ serum / mean\ OD\ diluent\ control) \times 100]$. The cutoff points of the c-ELISA for VSV-NJ and VSV-IN were determined using 45 negative equine sera in SNT (titers of 1:8) to VSV-NJ and VSV-IN. Cutoff was defined as the mean of the RP of the negative serum samples plus three standard deviations. The cutoff point for VSV-NJ c-ELISA was 12.58 RP and for VSV-IN c-ELISA was 17.78 RP. Relative sensitivity, relative specificity and Kappa statistic of the c-ELISA compared to the SNT was determined using the EPIINFO program.

RESULTS

From the total of 214 sera samples analyzed by the SNT for antibodies to VSV-NJ, 138 (64.48%) sera samples were positive and 76 (35.52%) sera samples were negative, whereas in C-ELISA 109 (50.93%) sera samples reacted positively and 105 (49.07%) sera samples reacted negatively (Table 1). Compared to SNT, c-ELISA for VSV-NJ showed a relative sensitivity of 79%, a relative specificity of 100% and a Kappa statistic of 0.61.



Table 1: Comparison of results of SNT and c-ELISA detecting antibodies against VSV-NJ and VSV-IN in 214 equine sera.

	VSV-NJ			VSV-IN		
	SNT +	SNT -	Σ	SNT +	SNT -	Σ
C-ELISA +	109 (50.93%)	0 (0%)	109 (50.93%)	24 (11.2%)	2 (0.93%)	26 (12.15%)
C-ELISA -	29 (13.55%)	76 (35.52%)	105 (49.07%)	12 (5.61%)	176 (82.24%)	188 (87.85%)
Σ	138 (64.48%)	76 (35.52%)	214 (100%)	36 (16.83%)	178 (83.17%)	214 (100%)

From the total of 214 sera samples analyzed by the SNT for antibodies to VSV-IN, 36 (16.83%) sera samples were positive and 178 (83.17%) sera samples were negative, while in c-ELISA 26 (12.15%) sera samples reacted positive and 188 (87.55%) sera samples reacted negative to VSV-IN (Table 1). Compared to the SNT the c-ELISA for VSV-IN showed a relative sensitivity of 66.7%, a relative specificity of 98.9% and a kappa statistic of 0.74, respectively.

DISCUSSION

In the present study, the c-ELISA used for detecting antibodies to VSV-NJ in horses showed lower sensitivity than that reported by Eernisse et al. (1995). Although the concordance of both tests (c-ELISA and SNT for VSV-NJ) measured by the kappa coefficient (0.61) was good, Eernisse et al. (1995) and Alvarado et al. (2002) reported better concordance (0.88). One reason could be that the results of the previously mentioned authors were based on the detection of VSV-NJ antibodies in cattle and not in horses. Another reason could be the modification of the c-ELISA protocol (addition of 1 M NaCl to the diluent buffer of the serum samples and polyclonal antibodies), which is used to increase the specificity of immunoassays detecting specific bovine antibodies (Dolz & Moreno 1999; Alvarado et al. 2002), however, this may have affected the binding of equine antibodies, and consequently diminished the sensitivity. Nevertheless, the determined specificity of 100% in the present study was in accordance with Eernisse et al. (1995), who reported a specificity of 99% of the c-ELISA when compared to SNT. The analysis of the 29 serum samples with discrepant results in both assays to VSV-NJ showed that all samples that were positive in SNT were considered negative in c-ELISA. Similar results were obtained by Alvarado et al. (2002), when comparing sera of naturally infected bovines in Costa Rica with both techniques (SNT and c-ELISA). Results between c-ELISA and SNT may be discrepant due to the detection of different antigens in both assays. SNT detects antibodies directed against glycoprotein G, whereas c-ELISA detects antibodies directed against protein N (Alvarado et al. 2002). When discrepant sera was analyzed by immunoblotting, antibodies against glycoprotein G and protein N were visualized as thick and thin bands, respectively, leading to the assumption that the levels of antibodies against glycoprotein G and protein N were of high and low titer, respectively (results not shown). Therefore, probably the titer of antibodies against the

protein N was under the detection limit of the c-ELISA. This could explain why the c-ELISA failed to detect 29 sera as positive.

The results for c-ELISA for VSV-IN in 214 serum samples of equines also showed lower sensitivity (66.7%), as reported by Eernisse et al. (1995), but concordance, as measured by the Kappa statistic, was 0.74, and indicated a good agreement between SNT and c-ELISA. In this respect, Alvarado et al. (2002) also determined a good agreement between both tests (0.69) when used to detect bovine antibodies. From the total of 214 serum samples 200 (93.46%) showed identical results in both serologic tests and 14 (6.54%) showed different results in SNT and c-ELISA. The specificity determined at 100% in the present study was in accordance with Eernisse et al. (1995). The analysis of 14 serum samples with discrepant results in c-ELISA and SNT to VSV-IN showed that 8 serum samples that were considered positive in SNT and negative in c-ELISA were confirmed positive by WB (results not shown). Antibodies directed against the glycoprotein G of VSV-IN were detected more frequently and appeared as conspicuous bands, whereas protein N appeared less often and as weak bands, leading to the assumption that the levels of antibodies against protein N were of low titer, whereas antibodies against glycoprotein G were of high titer. This could explain why c-ELISA failed to detect sera as positive.

Although the c-ELISA described here showed lower sensitivity, it is recommended to use it as screening and diagnostic test in vesicular disease surveillance programs, since it is a quick, simple and inexpensive test compared to SNT. A further optimization of the sensitivity of c-ELISA for detection of equine VSV antibodies is recommended. SNT is the method of choice for detecting VSV infections, despite the disadvantages.

ACKNOWLEDGEMENTS

This investigation was supported by DAAD, Germany; Tierärztliche Hochschule Hannover, Germany and by grant No. FG-CR-106 (CSC-AES-5) "Natural cycle of vesicular stomatitis virus in endemic areas of Central America", RSED/FAS/USDA. The authors wish to thank DVM Luis Eduardo Forera, Rocío Cortes, Flor Vargas, Rodolfo Pereira and Jorge Prendas, Escuela de Medicina Veterinaria, and Universidad Nacional, for their technical assistance, and DVM Jaime Villalobos Vargas for the donated sera.

REFERENCES

- Afshar, A., Shafarchi, N.H. & Dulac, G.C. 1993. Development of a competitive enzyme-linked immunosorbent assay for detection of bovine, ovine, porcine and equine antibodies to vesicular stomatitis virus. *J. Clin. Microbiol.* 31: 1860-1865.
- Ahmad, S., Bassiri, M., Banerjee, A.K. & Yilma, T. 1993. Immunological characterization of the vesicular stomatitis virus nucleocapsid (N) protein expressed by recombinant baculovirus in *Spodoptera exigua* larva: use in differential diagnosis between vaccinated and infected animals. *Virology* 192: 207-216.



- Alonso, A., Martins, M.A., Gomes, M., Allende, R. & Sondahl M.S. 1991. Development and evaluation of an enzyme-linked immunosorbent assay for detection, typing and subtyping of vesicular stomatitis virus. *J. Vet. Diagn. Invest.* 3: 287-292.
- Alvarado, J.F., Dolz, G., Herrero, M.V., Salman, M. & McCluskey, B. 2002. Comparison of serum neutralization test and a competitive ELISA for the detection of antibodies to vesicular stomatitis virus-New Jersey and vesicular stomatitis virus-Indiana. *Journal Veterinary Diagnostic Investigation* 14: 240-242.
- Atwill, E.R., Rodríguez, L.L., Hird, D.W. & Rojas, O. 1993. Environmental and host factors associated with seropositivity to New Jersey and Indiana vesicular stomatitis viruses in Costa Rican cattle. *Prev. Vet. Med.* 15: 304-314.
- Blickwede, M., Dolz, G., Herrero, M.V., Tomlinson, S.M. & Salman, M. 2002. Neutralizing antibodies against vesicular stomatitis viruses (serotypes New Jersey and Indiana) in horses in Costa Rica. *J. Vet. Diagn. Invest.* 14: 438-441.
- Burkhard, C., Freer, G., Steinhoff, U., Li Y., Bishop, D.H., Zinkernagel, R.M. & Hengartner, H. 1994. Localization of t helper cell epitopes in the vesicular stomatitis virus: the nucleoprotein is responsible for serotype cross reactive T help. *Virol. Immunol.* 7: 103-111.
- Dolz, G. & Moreno, E. 1999. Comparison of agar gel immunodiffusion test, enzyme-linked immunosorbent assay and western blotting for the detection of BLV antibodies. *J. Vet. Med. B* 46: 551-558.
- Eernisse, K., Katz, J. & Landgraff, J. 1995. Evaluation of a competitive ELISA in an epizootic of vesicular stomatitis (abstr.). In: *Proceedings 38th Am. Assoc. Vet. Lab. Diag.* 124.
- Heo, J.S., Lee, H.S., Jeoung, H.Y., Ko, H.R., Kweon, C.H. & Ko, Y.J. 2010. Development of a blocking ELISA using a recombinant glycoprotein for the detection of antibodies to vesicular stomatitis New Jersey virus. *J. Virol. Methods* 164: 96-100.
- Katz, J.B., Shafer, A.L., Eernisse & K.A. 1995. Construction and insect larval expression of recombinant vesicular stomatitis nucleocapsid protein and its use in competitive ELISA. *J. Virol. Methods* 54: 145-157.
- Katz, J.B., Eernisse, K.A., Langraf, J.G. & Schmitt, B.J. 1997. Comparative performance of four serodiagnostic procedures for detecting bovine and equine vesicular stomatitis virus antibodies. *J. Vet. Diagn. Invest.* 9: 329-331.
- Kweon, C.H., Kwon, B.J., Kim, I.J., Lee, S.Y. & Ko, Y.J. 2005. Development of monoclonal antibody-linked ELISA for sero-diagnosis of vesicular stomatitis virus (VSV-IN) using baculovirus expressed glycoprotein. *J. Virol. Methods* 130:7-14.
- Lee, H.S., Heo, E.J., Jeoung, H.Y., Ko, H.R., Kweon, C.H., Youn, H.J. & Ko, Y.J. 2009. Enzyme-linked immunosorbent assay using glycoprotein and monoclonal antibody for detecting antibodies to vesicular stomatitis virus serotype New Jersey. *Clin. Vaccine Immunol.* 16: 667-671.

- McCluskey, B.J., Salman, M.D., Martínez, G.E., Serrano, A.O., Shaw, J.L. & Herrero, M.V. 2003. 3-year pilot study of sentinel dairy herds for vesicular stomatitis in El Salvador. *Prev. Vet. Med.* 58:199-210.
- Reis, J.L. Jr., Rodríguez, L.L., Mead, D.G., Smoliga, G. & Brown, C.C. 2011. Lesion development and replication kinetics during early infection in cattle inoculated with Vesicular stomatitis New Jersey virus via scarification and black fly (*Simulium vittatum*) bite. *Vet. Pathol.* 48:5.
- Rodríguez, L., Vernon, S., Morales, A.I. & Letchworth, J. 1990. Serological monitoring of vesicular stomatitis New Jersey virus in enzootic regions of Costa Rica. *Am. J. Trop. Med. Hyg.* 42: 272-281.
- Rodríguez, L., Letchworth, G.J., Spiropoulou, C.F. & Nichol, S.T. 1993. Rapid detection of vesicular stomatitis virus New Jersey serotype in clinical samples by using polymerase chain reaction. *J. Clin. Microbiol.* 31: 2016-2020.
- Sherril, L. 1993. Vesicular stomatitis in the horse. *Vet. Clin. North Am. Equine Pract.* 9: 349-53.
- Velázquez-Salinas, L., Pauszek, S.J., Zarate, S., Basurto-Alcantara, F.J., Verdugo-Rodríguez, A., Pérez, A.M. & Rodríguez, L.L. 2014. Phylogeographic characteristics of vesicular stomatitis New Jersey viruses circulating in Mexico from 2005 to 2011 and their relationship to epidemics in the United States. *Virology* 449: 17-24.

