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1 **Short communication**

2 **Development of a peptide ELISA for the diagnosis of Equine arteritis virus**

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19 **ABSTRACT**

20 A peptide-based indirect ELISA was developed to detect antibodies against Equine  
21 arteritis virus (EAV). Two peptides for epitope C of protein GP5 and fragment E of protein  
22 M were designed, synthesized, purified and used as antigens either alone or combined.  
23 Ninety-two serum samples obtained from the 2010 equine viral arteritis outbreak, analyzed  
24 previously by virus neutralization, were evaluated by the ELISA here developed. The best  
25 resolution was obtained using peptide GP5. The analysis of the inter- and intraplate  
26 variability showed that the assay was robust. The results allow concluding that this

1 peptide-based ELISA is a good alternative to the OIE-prescribed virus neutralization test  
2 because it can be standardized between laboratories, can serve as rapid screening, can  
3 improve the speed of diagnosis of EAV-negative horses and can be particularly useful for  
4 routine surveillance in large populations.

5 KEY WORDS synthetic peptides- ELISA- Equine arteritis virus

6

7 Equine arteritis virus (EAV) belongs to the order Nidovirales, family Arteriviridae,  
8 genus Arterivirus (Snijder and Meulenbergh, 1998). The most relevant feature of EAV  
9 infection is that it produces subclinical infection. However, the most important clinical signs  
10 of the disease are abortions, respiratory disease in adult animals and pneumonia in foals.  
11 In Argentina, although serological evidence was first documented in 1984 (Nosetto et al.,  
12 1984), EAV was first isolated in 2001 (Echeverría et al., 2003). Following the EAV  
13 outbreak in 2010, the number of samples sent to the laboratory for EAV analysis was  
14 significantly higher than the annual average of the previous years, reaching almost 5000  
15 samples analyzed over a period of seven months. This increase highlighted the need for  
16 an alternative technique to replace the virus neutralization test, which is, to date, the test  
17 for international trade prescribed by the OIE (OIE, 2012). The virus neutralization test  
18 detects antibodies against to EAV GP5 protein but is complex and high cost and requires  
19 72 h to yield a result. Other difficulties include the considerable interlaboratory variation  
20 and the contamination or nonspecific cellular cytotoxicity in sera from vaccinated horses  
21 (Newton et al., 2004). Although several ELISAs have been developed, none have been  
22 validated as extensively as the virus neutralization test. Some, however, offer comparable  
23 specificity and almost equivalent sensitivity. The aim of this work was to design an ELISA  
24 as a screening assay for EAV, using synthetic peptides. Indirect ELISA using peptides  
25 containing GP5 neutralization epitopes may provide a simpler and more cost-effective  
26 method to quantify EAV antibodies than the virus neutralization test. Another benefit of an

1 EAV ELISA is that it can provide a same-day test result compared with the 72 h needed for  
2 the virus neutralization test.

3

4 The two peptides used were a fragment of the V1 region of the GP5 protein –  
5 epitope C- (amino acids 67-90) – VFLDDQIITFGTGCNDTHSVPVST, and a fragment  
6 corresponding to the C terminal region of the M protein-Cterm -fragment E- (amino acids  
7 130-162) AVGNKLVDGVKTITSAGRLFSKRAAATAYKLQ. These peptides were designed  
8 according to the analysis of the Argentine LP02/C EAV strain (FIG. 1). The peptides were  
9 manually synthesized by solid phase peptide synthesis using the standard Fmoc (9-  
10 fluorenylmethyloxycarbonyl) protocols on a RinkMBHA resin of 0.6 mmol/g. The crude  
11 peptides were purified by semi-preparative HPLC on a Beckman System Gold with a  
12 reverse-phase C18 column, resulting in purity greater than 95%, checked by analytical  
13 HPLC on a Shimadzu system. The identity of the peptide was confirmed by mass  
14 spectrometry in positive ion mode ESI on a Bruker model apparatus. The two peptides  
15 were used either separately or together as antigens in the development of the ELISA.

16

17 Ninety-two horse serum samples from the 2010 EAV outbreak characterized  
18 previously by the virus neutralization test (46 positive and 46 negative) were obtained from  
19 the Laboratory of Virology of the School of Veterinarian Sciences of the University of La  
20 Plata (Buenos Aires, Argentina).

21

22 The optimal dilutions of each coating peptide, serum sample and secondary  
23 antibody were determined by checkboard titration in microtiter plates (Maxisorp Nunc,  
24 Roskilde, Denmark). Peptides were diluted from 100 µg/ml (stock solution of 2000 µg/ml)  
25 to 0.0488 µg/ml. Positive and negative sera were tested at dilutions from 1:2 to 1:256.

1 Horseradish peroxidase-conjugated rabbit anti-horse antibody (Sigma Chemical, St. Louis,  
2 MO, USA) was used at a dilution of 1:2000.

3

4 In a preliminary step, both peptides were evaluated either alone or in combination.  
5 Peptide GP5 showed better discrimination between positive and negative sera than  
6 peptide M or both (FIG. 2). Briefly, wells were coated with 100  $\mu$ l of peptides dissolved in  
7 50 mM carbonate/bicarbonate buffer, pH 9.6 ( $\text{Na}_2\text{CO}_3$  1.59 g,  $\text{NaHCO}_3$  2.93 g up to 1000  
8 ml  $\text{H}_2\text{O}$ ) at a concentration of 12  $\mu$ g/well and incubated at 37°C for 3 h and then at 4°C  
9 overnight. After removing the excess of unbound antigen, 100  $\mu$ l of blocking solution (PBS,  
10 0.05% bovine seroalbumin) was added to each well and the wells further incubated at  
11 37°C for 30 min and then rinsed with PBS containing 0.05 % Tween 20 (PBS-T). A 50- $\mu$ l  
12 volume of 1:8 dilutions of horse sera in blocking solution was added in duplicate and  
13 incubated at 37°C for 1 h. After rinsing with PBS-T, the wells were incubated with 50  $\mu$ l of  
14 horseradish peroxidase-conjugated rabbit anti-horse IgG (diluted 1:2000 in blocking  
15 solution) at 37°C for 1 h. Finally, after three washings, a 100- $\mu$ l volume of 1 mM 2,2'-azino-  
16 bis (3-ethylbenzothiazoline-6-sulfonic acid) ABTS (Sigma Chemical) substrate solution  
17 was added to each well and the wells then incubated at room temperature for 20, 30 and  
18 60 min. The optical density (OD) was read at 405 nm using an automatic ELISA reader.

19 The OD raw values were corrected according to the following formula: OD (sample)-  
20 (background OD of sample)/ OD (positive control)-(background OD of positive control). To  
21 determine the cut-off value of the ELISA, the OD values were analyzed with Stata\_SE 9.2  
22 software (Stata Corporation, TX, USA). In the first step, the values obtained were analyzed  
23 by Receiver Operating Characteristic (ROC) curves, where the true positive rate  
24 (sensitivity) is plotted as a function of the false positive rate (100-specificity) for different  
25 cut-off points. Each point on the ROC plot represents a sensitivity/specificity pair  
26 corresponding to a particular decision threshold. To evaluate intra-and inter-plate

1 repeatability, each serum sample was seeded in two wells of the same plate and then  
2 repeated on a new plate. The coefficients of variation (CVs) were calculated based on the  
3 raw values of OD between the wells of the same plate and between the wells of both  
4 plates.

5  
6 The optimal concentration of each peptide used was 12  $\mu\text{g}$  / ml and the optimal  
7 dilution of equine sera (1:8) in this indirect ELISA was determined by checkboard titration  
8 to give the maximum discrimination between the reference positive and negative sera  
9 selected. The optimal reading was set to 30 min. The best results with minimum  
10 background were obtained using peptide GP5 alone (FIG. 2). A total of 92 equine serum  
11 samples from the outbreak of equine viral arteritis of 2010, characterized previously as  
12 EAV-positive or EAV-negative by virus neutralization, were assayed using this ELISA. The  
13 analysis of the graphic of ROC showed that the area under the curve was 0.94, with a high  
14 confidence interval (95% CI) of 90–99%. The cut-off value selected was 0.5, with a  
15 sensitivity of 95.65% and a specificity of 80.43%. This cut-off value allowed correctly  
16 classifying 88.04% of the serum samples as true positive or true negative (Table 1 and  
17 FIG. 3).

18 To determine intra- and inter-plate repeatability, none of the coefficients of variation  
19 calculated exceeded the value reported as correct (Jacobson, 1998). The summary is  
20 shown in Table 2.

21  
22 The objective of this study was to design an ELISA as screening of EAV by using  
23 synthetic peptides as antigens. Other peptide-based ELISAs have been shown to be  
24 sensitive and specific indirect diagnostic tools in virology, such as to discriminate between  
25 serological responses to equine herpesvirus 1 and 4 (Lang et al., 2013), foot and mouth  
26 disease (Gao et al., 2012; Oem et al., 2005), classical swine fever (Lin et al., 2010),

1 equine infectious anemia (Soutullo et al., 2001) and porcine reproductive and respiratory  
2 syndrome virus (Plagemann, 2006). To define the cut-off value of the ELISA, the  
3 prevalence of the 2010 equine viral arteritis outbreak (2%) and the correlation of the ELISA  
4 test results with the virus neutralization test as gold standard were considered. A cut-off  
5 value of 0.5 allowed reaching a high percentage of sensitivity and clearly distinguishing the  
6 negative sera. By definition, a screening test must be easy to use and inexpensive and  
7 should be highly sensitive, so that it fails only in a small number of infected animals  
8 (Pfeiffer, 2002). Any positive result should undergo confirmatory testing and thus reduced  
9 specificity should be tolerated. The ELISA developed in the present work allowed  
10 separating the negative samples in a shorter time than virus neutralization.

11 The peptides designed in the present study represent the main neutralization site of  
12 GP5 and were strategically designed on the basis of the Argentine EAV sequences  
13 (Echeverría et al., 2010) to use this ELISA in infected horses of Argentina. Other authors  
14 have found that G16, located between amino acids 79 to 94, is a high antigenic peptide.  
15 (Kondo et al., 1998). The peptides designed overlap in 12 out of 16 amino acids. Other  
16 ovalbumin-conjugated synthetic peptide-based ELISAs designed with amino acids 81 to  
17 106 of GP5 of the EAV Bucyrus strain were used as diagnostic antigen. The sensitivity and  
18 specificity were 96.75% and 95.6% respectively (Nugent et al., 2000). In the ELISA  
19 developed in this work the sensitivity is almost the same. The strain variation and the  
20 region selected could be responsible of the difference of specificity. As horses in Argentina  
21 are infected with strains belonging to the European cluster (Metz et al., 2011) and  
22 vaccination is made with the American Bucyrus strain, it will be of interest to test whether  
23 this ELISA can distinguish EAV naturally infected horses from vaccinated ones. In this  
24 work, no positive sera from horses infected with the American strain were used. As  
25 suggested by Kondo et al. (1998), the reactivity to the peptide is highly specific to the  
26 homologous strain. These authors showed that a horse experimentally infected with a

1 heterologous strain (not American) does not react with the peptide by an ELISA designed  
2 over the Bucyrus strain. Other authors have been able to discriminate between serological  
3 responses to European-genotype vaccines and European-genotype field strains of porcine  
4 reproductive and respiratory syndrome virus, by using an ORF 4 peptide-based ELISA  
5 (Oleksiewicz et al., 2005).

6 To determine the significance of amino acid composition in the anti-EAV response,  
7 it would be of interest to include a larger ectodomain of EAV strains with larger variation in  
8 amino acid sequence than the LP02/C strain. Although the linear antigenic region of GP5  
9 was identified and comprises amino acids 75 to 98, it is uncertain whether the antibody  
10 response against an attenuated or inactivated EAV vaccine could be determined using a  
11 peptide ELISA.

12 ELISA procedures can be standardized between laboratories and could serve as  
13 rapid screening, thus improving the speed of diagnosis of EAV-negative horses and  
14 becoming useful for routine surveillance in large populations. Results of the present work  
15 show that the ELISA developed is a suitable alternative to the virus neutralization test for  
16 serodiagnosis of EAV in Argentina.



1

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3 are highly acknowledged.

4

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2 Figure legends

3

4 Figure 1: Alignment of the amino acid sequences of the GP5 and M proteins of laboratory  
5 and field strains of Equine arteritis virus (EAV). Neutralization sites B, C and D (variable  
6 region V1) are indicated in the boxes. The amino acid sequences of the synthetic peptides  
7 synthesized were based on the LP02/C strain of EAV (boxes in bold).

8

9 Figure 2: Reactivity of positive and negative EAV horse sera to synthetic peptides.

10

11 Figure 3: Statistical analysis of peptide-ELISA results. (A) Receiver Operating  
12 Characteristic (ROC) analysis using STATA SE 9.2 statistical analysis software (CI 95%  
13 0.90–0.99). (B) Report of sensitivity and specificity by STATA software. A cut-off value of  
14 0.5 classified serum samples correctly in the maximum value (88.04%), with highest  
15 sensitivity (95.65%) and good specificity (80.43%).

16

17

1

2 **Table 1:** Results of antibody detection over 92 analyses using the virus neutralization test  
3 and peptide-ELISA developed in the present work (cut-off 0.5).

4

5

	virus neutralization positive	virus neutralization negative	Total
<b>ELISA positive</b>	44	9	53
<b>ELISA negative</b>	2	37	39
<b>Total</b>	46	46	92

6

7

Sensitivity 95.65%

8

Specificity 80.43%

9

Positive predictive value 83.02%

10

Negative predictive value 94.87%

11

Kappa (95% CI)= 0.760 (0.696-0.825)

12

13

14

1

2 Table 2: Intra- and inter-plate precision of the peptide-ELISA

3

Precision test	Plate	maximum CV (%)	minimum CV (%)
<b>Intraplate repeatability</b>	Plate 1	10.70	0.21
	Plate 2	13.72	0.00
	Plate 3	15.59	0.00
	Plate 4	19.85	0.00
<b>Interplate repeatability</b>	Plate 1 vs. plate 2	14.89	1.44
	Plate 3 vs. plate 4	13.20	0.87

4

5

CV= coefficient of variation

Figure 1

gP5 protein	Neut. Site B	Neut. Site C	Neut. Site D		
LP02/R	HTALYNWSASKTCWY	CEFLDDQIIITFGTGCNDTYSVPVST	VLEQAHGPYSVLFDDMPPFI	110	
LT-LP-ARG	HTALYNCSASKTCWY	CEFLDDQIIITFGTGCNDTYSVPVST	VLEQAHGPYSVLFDDMPPFI	110	
LP02/C	HTALYNCSASKTCWY	CEFLDDQIIITFGTGCNDTHSVPVST	VLEQAHGPYSVLFDDMPPFI	110	
LP02/P	HTALYNCSASKTCWY	CEFLDDQIIITFGTGCNNTHSVPVST	VLEQAHGPYSVLFDDMPPFI	110	
LP01	HTALYNCSASETCWY	CVFLDEQVITIFGTGCNNTYSVPVST	VLEQAHGPYSVLFDDMPPFI	110	
EAV-UCD	H--MYNCSASKTCWY	CTFLDEQVITIGTDCNNAHAVSVAE	VLEQAHGPYSVLFDDMPPFI	108	
LP02/R	YYGREFGIFVMDVFMFY	PVVLVLFLLSVLPYATLILEMCVSI	LFVVYGLYSGAYLAMGIFA	170	
LT-LP-ARG	YYGREFGIFVMDVFMFY	PVVLVLFLLSVLPYATLILEMCVSI	LFVVYGLYSGAYLAMGIFA	170	
LP02/C	YYGREFGIFVMDVFMFY	PVVLVLFLLSVLPYATLILEMCVSI	LFVVYGLYSGAYLAMGIFA	170	
LP02/P	YYGREFGIFVMDVFMFY	PVVLVLFLLSVLPYATLILEMCVSI	LFVVYGLYSGAYLAMGIFA	170	
LP01	YYGREFGIFVMDVFMFY	PVVLVLFLLSVLPYVTLILEMCVSI	LFVVYGLYSGAYLAMGIFA	170	
EAV-UCD	YYGREFGIVLDVFMFY	PVVLVLFLLSVLPYATLILEMCVSI	LFIIYGIYSGAYLAMGIFA	168	
LP02/R	TTLVHHSVVLRLQLLWL	CLAWRYRCTLHASFISAEGKIYPVDP	GLPIAAAGN	222	
LT-LP-ARG	TTLVHHSVVLRLQLLWL	CLAWRYRCTLHASFISAEGKIYPVDP	GLPIAAAGN	222	
LP02/C	TTLVHHSVVLRLQLLWL	CLAWRYRCTLHASFISAEGKIYPVDP	GLPIAAAGN	222	
LP02/P	TTLVHHSVVLRLQLLWL	CLAWRYRCTLHASFISAEGKIYPVDP	GLPIAAAGN	222	
LP01	TTLVHHSVVLRLQLLWL	CLAWRYRCTLHASFISAEGKIYPVDP	GLPIAAAGN	222	
EAV-UCD	ATLAIHSIVVLRLQLLWL	CLAWRYRCTLHASFISAEGKVYPVDP	GLPVAAGN	220	
<b>M protein</b>					
LP02/C	MGAI DSFCGDGI LGEYLDYFI	LSVPLLLLLITRYVASGLVYVMTALFY	SFVLAAYIWFVIV	60	
LT-LP-ARG	MGAI DSFCGDGI LGEYLDYFI	LSVPLLLLLITRYVASGLVYVMTALFY	SFVLAAYIWFVIV	60	
LP02/P	MGAI DSFCGDGI LGEYLDYFI	LSVPLLLLLITRYVASGLVYVMTALFY	SFVLAAYIWFVIV	60	
LP02/R	MGAI DSFCGDGI LGEYLDYFI	LSVPLLLLLITRYVASGLVYVMTALFY	SFVLAAYIWFVIV	60	
LP01	MGAI DSFCGDGI LGEYLDYFI	LSVPLLLLLITRYVASGLVYVMTALFY	SFVLAAYIWFVIV	60	
EAV-UCD	MGAI DSFCGDGI LGEYLDYFI	LSVPLLLLLITRYVASGLVYVLAALFY	SFVLAAYIWFVIV	60	
LP02/C	GRAFSTAYAFVLLAAFL	LLLLIRMI VGVLPRLRS ICNHRQLV	VADFVDTPSGPVSI PRSTT	120	
LT-LP-ARG	GRAFSTAYAFVLLAAFL	LLLLIRMI VGVLPRLRS ICNHRQLV	VADFVDTPSGPVSI PRSTT	120	
LP02/P	GRAFSTAYAFVLLAAFL	LLLLIRMI VGVLPRLRS ICNHRQLV	VADFVDTPSGPVSI PRSTT	120	
LP02/R	GRAFSTAYAFVLLAAFL	LLLLIRMI VGVLPRLRS ICNHRQLV	VADFVDTPSGPVSI PRSTT	120	
LP01	GRAFSTAYAFVLLAAFL	LLLLIRMI VGVLPRLRS ICNHRQLV	VADFVDTPSGPVPI PRSTT	120	
EAV-UCD	GRAFSTAYAFVLLAAFL	LLVMRMI VGMMPRLRS IFNHRQLV	VADFVDTPSGPVPI PRSTT	120	
LP02/C	QVVVRNGY	TAVGNKLV	DGVKTI TSAGRLF	SKRAAATAYKLQ	162
LT-LP-ARG	QVVVRNGY	TAVGNKLV	DGVKTI TSAGRLF	SKRAAATAYKLQ	162
LP02/P	QVVVRNGY	TAVGNKLV	DGVKTI TSAGRLF	SKRTAATAYKLQ	162
LP02/R	QVVVRNGY	TAVGNKLV	DGVKTI TSAGRLF	SKRTAATAYKLQ	162
LP01	QVVVRNGY	TAVGNKLV	DGVKTI TSAGRLF	SKRTAATAYKLQ	162
EAV-UCD	QIVVRNGY	TAVGNKLV	DGVKTI TSAGRLF	SKRAAATAYKLQ	162

Figure 2

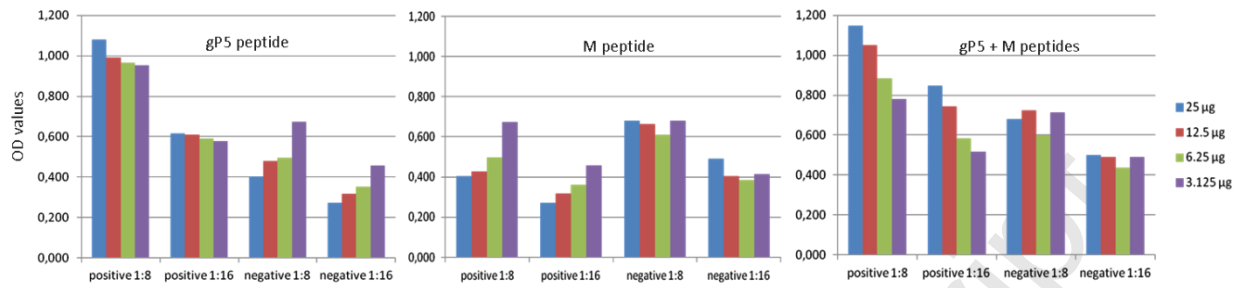
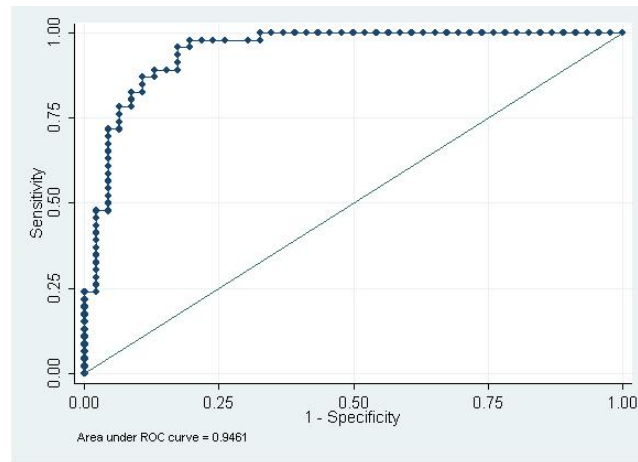




Figure 3



## Detailed report of Sensitivity and specificity

Cutpoint	Sensitivity	Specificity	Correctly Classified	LR+	LR-
( $\geq$ .3436773 )	97.83%	73.91%	85.87%	3.7500	0.0294
( $\geq$ .3493715 )	97.83%	76.09%	86.96%	4.0909	0.0286
( $\geq$ .3541396 )	97.83%	78.26%	88.04%	4.5000	0.0278
( $\geq$ .4003103 )	97.83%	80.43%	89.13%	5.0000	0.0270
( $\geq$ .5073701 )	95.65%	80.43%	88.04%	4.8889	0.0541
( $\geq$ .5333592 )	95.65%	82.61%	89.13%	5.5000	0.0526
( $\geq$ .5356866 )	93.48%	82.61%	88.04%	5.3750	0.0789
( $\geq$ .5465973 )	91.30%	82.61%	86.96%	5.2500	0.1053
( $\geq$ .562202 )	89.13%	82.61%	85.87%	5.1250	0.1316