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Short communication

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

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ABSTRACT

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

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

KEY WORDS synthetic peptides- ELISA- Equine arteritis virus

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

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

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

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

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

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

In a preliminary step, both peptides were evaluated either alone or in combination. Peptide GP5 showed better discrimination between positive and negative sera than peptide M or both (FIG. 2). Briefly, wells were coated with 100 µl of peptides dissolved in 50 mM carbonate/bicarbonate buffer, pH 9.6 (Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g up to 1000 ml H_2O) at a concentration of 12 µg/well and incubated at 37ºC for 3 h and then at 4ºC overnight. After removing the excess of unbound antigen, 100 µl of blocking solution (PBS, 0.05% bovine seroalbumin) was added to each well and the wells further incubated at 37ºC for 30 min and then rinsed with PBS containing 0.05 % Tween 20 (PBS-T). A 50-µl volume of 1:8 dilutions of horse sera in blocking solution was added in duplicate and incubated at 37ºC for 1 h. After rinsing with PBS-T, the wells were incubated with 50 µl of horseradish peroxidase-conjugated rabbit anti-horse IgG (diluted 1:2000 in blocking solution) at 37ºC for 1 h. Finally, after three washings, a 100-µl volume of 1 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ABTS (Sigma Chemical) substrate solution was added to each well and the wells then incubated at room temperature for 20, 30 and 60 min. The optical density (OD) was read at 405 nm using an automatic ELISA reader.

The OD raw values were corrected according to the following formula: OD (sample)-(background OD of sample)/ OD (positive control)-(background OD of positive control). To determine the cut-off value of the ELISA, the OD values were analyzed with Stata_SE 9.2 software (Stata Corporation, TX, USA). In the first step, the values obtained were analyzed by Receiver Operating Characteristic (ROC) curves, where the true positive rate (sensitivity) is plotted as a function of the false positive rate (100-specificity) for different cut-off points. Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. To evaluate intra-and inter-plate repeatability, each serum sample was seeded in two wells of the same plate and then repeated on a new plate. The coefficients of variation (CVs) were calculated based on the raw values of OD between the wells of the same plate and between the wells of both plates.

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

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

The objective of this study was to design an ELISA as screening of EAV by using synthetic peptides as antigens. Other peptide-based ELISAs have been shown to be sensitive and specific indirect diagnostic tools in virology, such as to discriminate between serological responses to equine herpesvirus 1 and 4 (Lang et al., 2013), foot and mouth disease (Gao et al., 2012; Oem et al., 2005), classical swine fever (Lin et al., 2010), equine infectious anemia (Soutullo et al., 2001) and porcine reproductive and respiratory syndrome virus (Plagemann, 2006). To define the cut-off value of the ELISA, the prevalence of the 2010 equine viral arteritis outbreak (2%) and the correlation of the ELISA test results with the virus neutralization test as gold standard were considered. A cut-off value of 0.5 allowed reaching a high percentage of sensitivity and clearly distinguishing the negative sera. By definition, a screening test must be easy to use and inexpensive and should be highly sensitive, so that it fails only in a small number of infected animals (Pfeiffer, 2002). Any positive result should undergo confirmatory testing and thus reduced specificity should be tolerated. The ELISA developed in the present work allowed separating the negative samples in a shorter time than virus neutralization.

The peptides designed in the present study represent the main neutralization site of GP5 and were strategically designed on the basis of the Argentine EAV sequences (Echeverría et al., 2010) to use this ELISA in infected horses of Argentina. Other authors have found that G16, located between amino acids 79 to 94, is a high antigenic peptide. (Kondo et al., 1998). The peptides designed overlap in 12 out of 16 amino acids. Other ovoalbumin-conjugated synthetic peptide-based ELISAs designed with amino acids 81 to 106 of GP5 of the EAV Bucyrus strain were used as diagnostic antigen. The sensitivity and specificity were 96.75% and 95.6% respectively (Nugent et al., 2000). In the ELISA developed in this work the sensitivity is almost the same. The strain variation and the region selected could be responsible of the difference of specificity. As horses in Argentina are infected with strains belonging to the European cluster (Metz et al., 2011) and vaccination is made with the American Bucyrus strain, it will be of interest to test whether this ELISA can distinguish EAV naturally infected horses from vaccinated ones. In this work, no positive sera from horses infected with the American strain were used. As suggested by Kondo et al. (1998), the reactivity to the peptide is highly specific to the homologous strain. These authors showed that a horse experimentally infected with a heterologous strain (not American) does not react with the peptide by an ELISA designed over the Bucyrus strain. Other authors have been able to discriminate between serological responses to European-genotype vaccines and European-genotype field strains of porcine reproductive and respiratory syndrome virus, by using an ORF 4 peptide-based ELISA (Oleksiewicz et al., 2005).

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

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

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Figure legends

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

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

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

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

	virus neutralization positive	**virus neutralization negative**	**Total**
ELISA positive	44	9	53
ELISA negative	2	37	39
Total	46	46	92

Sensitivity 95.65%
Specificity 80.43%
Positive predictive value 83.02%
Negative predictive value 94.87%
Kappa (95% CI)= 0.760 (0.696-0.825)

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

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

CV= coefficient of variation

Figure 1

gP5 protein — Neut. Site B, Neut. Site C, Neut. Site D

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LP02/R       HTALYNWSASKTCWYCEFLDDQIITFGTGCNDTYSVPVSTVLEQAHGPYSVLFDDMPPFI 110
LT-LP-ARG    HTALYNCSASKTCWYCEFLDDQIITFGTGCNDTYSVPVSTVLEQAHGPYSVLFDDMPPFI 110
LP02/C       HTALYNCSASKTCWYCVFLDDQIITFGTGCNDTHSVPVSTVLEQAHGPYSVLFDDMPPFI 110
LP02/P       HTALYNCSASKTCWYCEFLDDQIITFGTGCNNTHSVPVSTVLEQAHGPYSVLFDDMPPFI 110
LP01         HTALYNCSASETCWYCVFLDEQVITFGTGCNNTYSVPVSTVLEQAHGPYSVLFDDMPPFI 110
EAV-UCD      H--MYNCSASKTCWYCTFLDEQVITIGTDCNNAHAVSVAEVLEQAHGPYSVLFDDMPPFI 108

LP02/R       YYGREFGIFVMDVFMFYPVLVLFFLSVLPYATLILEMCVSILFVVYGLYSGAYLAMGIFA 170
LT-LP-ARG    YYGREFGIFVMDVFMFYPVLVLFFLSVLPYATLILEMCVSILFVVYGLYSGAYLAMGIFA 170
LP02/C       YYGREFGIFVMDVFMFYPVLVLFFLSVLPYATLILEMCVSILFVVYGLYSGAYLAMGIFA 170
LP02/P       YYGREFGIFVMDVFMFYPVLVLFFLSVLPYATLILEMCVSILFVVYGLYSGAYLAMGIFA 170
LP01         YYGREFGIFVMDVFMFYPVLVLFFLSVLPYVTLILEMCVSILFVVYGLYSGAYLAMGIFA 170
EAV-UCD      YYGREFGIVVLDVFMFYPVLVLFFLSVLPYATLILEMCVSILFIIYGIYSGAYLAMGIFA 168

LP02/R       TTLVVHSVVVLRQLLWLCLAWRYRCTLHASFISAEGKIYPVDPGLPIAAAGN 222
LT-LP-ARG    TTLVVHSVVVLRQLLWLCLAWRYRCTLHASFISAEGKIYPVDPGLPIAAAGN 222
LP02/C       TTLVVHSVVVLRQLLWLCLAWRYRCTLHASFISAEGKIYPVDPGLPIAAAGN 222
LP02/P       TTLVVHSVVVLRQLLWLCLAWRYRCTLHASFISAEGKIYPVDPGLPIAAAGN 222
LP01         TTLVVHSVVVLRQLLWLCLAWRYRCTLHASFISAEGKIYPVDPGLPIAAAGN 222
EAV-UCD      ATLAIHSIVVLRQLLWLCLAWRYRCTLHASFISAEGKVYPVDPGLPVAAAGN 220
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M protein

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LP02/C       MGAIDSFCGDGILGEYLDYFILSVPLLLLITRYVASGLVYVMTALFYSFVLAAYIWFVIV 60
LT-LP-ARG    MGAIDSFCGDGILGEYLDYFILSVPLLLLITRYVASGLVYVMTALFYSFVLAAYIWFVIV 60
LP02/P       MGAIDSFCGDGILGEYLDYFILSVPLLLLITRYVASGLVYVMTALFYSFVLAAYIWFVIV 60
LP02/R       MGAIDSFCGDGILGEYLDYFILSVPLLLLITRYVASGLVYVMTALFYSFVLAAYIWFVIV 60
LP01         MGAIDSFCGDGILGEYLDYFILSVPLLLLITRYVASGLVYVMTALFYSFVLAAYIWFVIV 60
EAV-UCD      MGAIDSFCGDGILGEYLDYFILSVPLLLLLTRYVASGLVYVLAALFYSFVLAAYIWFVIV 60

LP02/C       GRAFSTAYAFVLLAAFLLLLIRMIVGVLPRLRSICNHRQLVVADFVDTPSGPVSIPRSTT 120
LT-LP-ARG    GRAFSTAYAFVLLAAFLLLLIRMIVGVLPRLRSICNHRQLVVADFVDTPSGPVSIPRSTT 120
LP02/P       GRAFSTAYAFVLLAAFLLLLIRMIVGVLPRLRSICNHRQLVVADFVDTPSGPVSIPRSTT 120
LP02/R       GRAFSTAYAFVLLAAFLLLLIRMIVGVLPRLRSICNHRQLVVADFVDTPSGPVSIPRSTT 120
LP01         GRAFSTAYAFVLLAAFLLLLIRMIVGVLPRLRSICNHRQLVVADFVDTPSGPVPIPRSTT 120
EAV-UCD      GRAFSTAYAFVLLAAFLLLVMRMIVGMMPRLRSIFNHRQLVVADFVDTPSGPVPIPRSTT 120

LP02/C       QVVVRGNGYTAVGNKLVDGVKTITSAGRLFSKRAAATAYKLQ 162
LT-LP-ARG    QVVVRGNGYTAVGNKLVDGVKTITSAGRLFSKRAAATAYKLQ 162
LP02/P       QVVVRGNGYTAVGNKLVDGVKTITSAGRLFSKRTAATAYKLQ 162
LP02/R       QVVVRGNGYTAVGNKLVDGVKTITSAGRLFSKRTAATAYKLQ 162
LP01         QVVVRGNGYTAVGNKLVDGVKTITSAGRLFSKRTAATAYKLQ 162
EAV-UCD      QIVVRGNGYTAVGNKLVDGVKTITSAGRLFSKRAAATAYKLQ 162
```

Figure 2

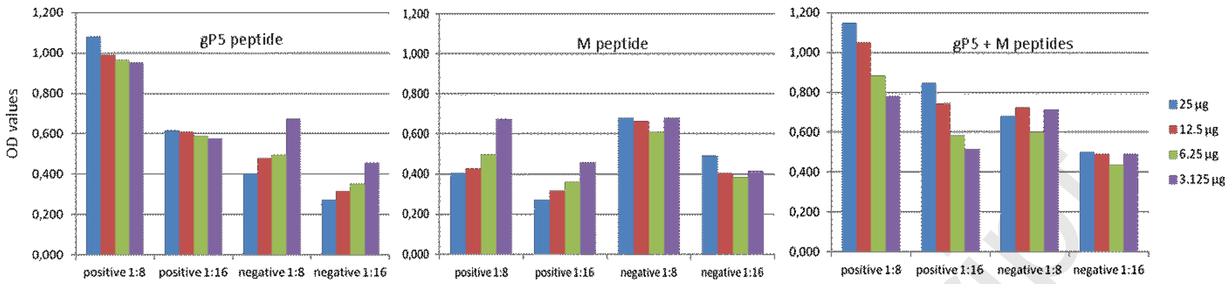
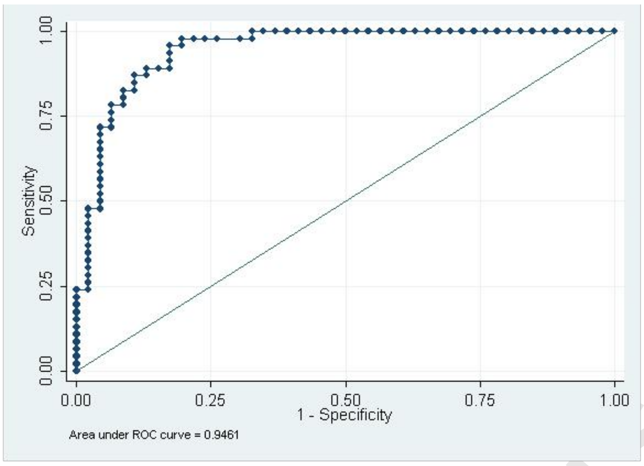

gP5 peptide
M peptide
gP5 + M peptides
OD values
1,200
1,000
0,800
0,600
0,400
0,200
0,000
positive 1:8
positive 1:16
negative 1:8
negative 1:16
25 µg
12.5 µg
6.25 µg
3.125 µg

Figure 3



Detailed report of Sensitivity and Specificity

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