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Protocols

Serological diagnosis of equine infectious anemia in horses, donkeys and mules using an ELISA with a gp45 synthetic peptide as antigen

João Helder F.F. Naves[a], Fernanda G. Oliveira[a], Juliana M. Bicalho[a], Paula S. Santos[b], Ricardo A. Machado-de-Ávila[c], Carlos Chavez-Olortegui[d], Rômulo C. Leite[a], Jenner K.P. Reis[a,*]

[a] Laboratório de Retroviroses da Escola de Veterinária da Universidade Federal de Minas Gerais, Brazil
[b] Laboratório de Nanobiotecnologia, Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, Uberlândia, Brazil
[c] Laboratório de Fisiologia Experimental, Programa de Pós-graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, Brazil
[d] Laboratório de Imunoquímica de Toxinas Naturais do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, Brazil

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ABSTRACT

Equine infectious anemia (EIA) is a disease caused by a *Lentivirus* that is currently controlled exclusively by identification of seropositive animals. In most countries, including Brazil, the official diagnostic test for EIA is the agar gel immunodiffusion test (AGID). Although this assay has a high specificity it can produce false negative reactions or equivocal results due to weak precipitation lines, especially in samples from donkeys, mules or newly infected equids. In this pioneering study, it was used overlapping synthetic peptide pools to map and identify a consensus, widely recognised antibody epitope within *env* encoding the EIAV envelope proteins. A 20-mer soluble peptide encompassing this epitope (pgp45) was then synthesized and tested in an indirect ELISA test. Using a panel of 859 EIA positive and negative equid serum samples, the pgp45 ELISA had 96.1% concordance, 98.6% sensitivity and 95.6% specificity respectively, when compared to AGID. The sensitivity and specificity of the pgp45 ELISA was also > 90% when tested in individual equid species including horses (*Equus caballus*), donkeys (*Equus asinus*) and mules (*Equus caballus* x *Equus asinus*). Moreover, in a horse experimentally infected with the pathogenic Wyoming EIAV strain viral-specific antibodies were detected at 10 days post-infection (dpi) whereas in AGID no specific antibody was detected until 18 days of experimental infection. This peptide can now be used as an antigen in serological tests, especially for rapid screening of large numbers of equids, where it may contribute significantly in the control of EIA, especially at sites with high populations of donkeys and mules.

1. Introduction

Equine infectious anemia (EIA) is caused by *Equine infectious anemia virus* (EIAV) a virus that is a member of the *Retroviridae* family, genus *Lentivirus*. EIAV infects horses (*Equus caballus*), donkeys (*Equus asinus*) and mules (*Equus caballus* x *Equus asinus*) and causes a persistent infection (Leroux et al., 2004). EIAV is a notifiable disease and one of the most important viral diseases affecting Equidae worldwide, causing substantial losses for horse owners (Almeida et al., 2006; Leroux et al., 2004). To date, there is no vaccine or effective treatment for the virus, and infected animals can become lifelong asymptomatic carriers and potential transmitters of EIAV (Almeida et al., 2006; Leroux et al., 2004).

The control of EIA is based primarily on identifying animals infected with EIAV by serological diagnosis and the agar gel immunodiffusion (AGID) test, which uses the viral core p26 protein as an antigen is recommended by the World Organisation for Animal Health (OIE) (OIE, 2013) and Ministry of Agriculture, Livestock and Supply (Ministério da Agricultura, Pecuária e Abastecimento - MAPA) in Brazil (Brasil, 2004). In many countries the only officially recognized test for diagnosis of EIAV is the AGID (Issel et al., 2014). Although the AGID test is simple to perform, it is not a rapid test, given that the readings can only be carried out after 24 h and cannot be automated (Scicluna et al., 2013). In addition, previous publications have demonstrated, the AGID has

Abbreviations: AGID, agar gel immunodiffusion; EIA, Equne infectious anemia; EIAV, *Equine infectious anemia virus*; ELISA, enzyme-linked immunosorbent assay; OIE, World Organisation for Animal Health; MAPA, Ministry of Agriculture, Livestock and Supply – Brazil

* Corresponding author.
E-mail addresses: jhelder83@yahoo.com.br (J.H.F.F. Naves), fe_goliveira@yahoo.com.br (F.G. Oliveira), julianambicalho@gmail.com (J.M. Bicalho), paulasantos.bio@gmail.com (P.S. Santos), r_andrez@yahoo.com (R.A. Machado-de-Ávila), olortegi@icb.ufmg.br (C. Chavez-Olortegui), romulocnpq@gmail.com (R.C. Leite), jenner@ufmg.br (J.K.P. Reis).

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relatively low sensitivity and that readings can be subjective leading to false negative or equivocal results, especially in cases of recent exposure (Issel et al., 2013; Nardini et al., 2017; Oliveira et al., 2017; Scicluna et al., 2013). Among the primary immunogens encountered after primary infection are the EIAV envelope glycoproteins encoded by the viral gene *env* that comprise the surface unit (gp90) and transmembrane (gp45) proteins (Chong et al., 1991; Craigo et al., 2013). Therefore one of the objectives of this study was to identify conserved, broadly reactive epitopes within the viral envelope glycoproteins and to determine if peptides encompassing these epitopes can be used as antigens in new diagnostic tests, that possess greater sensitivity than AGID (Issel et al., 2013; Paré and Simard, 2004; Reis et al., 2012).

Accurate tests are even more important for donkeys and mules as studies have demonstrated there is a higher probability for obtaining false-negative or equivocal results in AGID with serum samples from these species than with horses or ponies (Issel et al., 2013; Scicluna et al., 2013). Moreover in comparative infection studies using the same EIAV strains donkeys had significantly lower plasma-associated viral RNA levels and took longer to seroconvert than horses (Cook et al., 2001). Similar findings consistent with low viremia levels and discrepant results between AGID and other serological tests have also been reported in the case of donkeys and mules (Oliveira et al., 2017; Spyrou et al., 2003). Therefore, areas of the globe where donkeys and mules are abundant may complicate EIA control efforts especially if AGID is the only available serological test (Cook et al., 2001; Issel et al., 2013; Scicluna et al., 2013; Spyrou et al., 2003).

Consequently, new EIA diagnostic tests are needed that are faster, more sensitive and that use antigens with improved EIAV detection in donkeys, mules and in equids that have been recently exposed to the virus (Cook et al., 2013; Coutinho et al., 2013; Scicluna et al., 2013). In this study, SPOT synthesis (Frank, 1992; Molina et al., 1996) was used to produce a panel of overlapping uniformly sized peptides (15-mers) that represented both the gp90 and gp45 EIAV envelope glycoproteins in their entirety. These peptides were used in epitope mapping experiments to identify an immunodominant epitope within gp45 that was widely recognized by antibodies isolated from EIAV infected horses, mules and donkeys. Moreover, it was demonstrate this peptide can be used effectively as an antigen in indirect ELISA tests for EIA.

2. Materials and methods

2.1. Epitope mapping

Two hundred eighty-three overlapping pentadecapeptides (15-mers) frameshifted by 3 amino acid residues representing the entire gp90 and gp45 protein sequences encoded by the *env* gene of a North American EIAV strain (Rushlow et al., 1986) (Genbank M16575) were produced by SPOT synthesis using a cellulose support membrane (Frank, 1992).

Following synthesis peptides that were immunoreactive were identified using separate pools of EIAV antibody positive and negative serum samples. Samples from EIAV infected or uninfected horses, mules and donkeys were analyzed separately with each pool consisting of material collected from 5 individual animals. The EIA status of each serum donor was determined previously by official laboratories in Brazil (MAPA). SPOT synthesis membranes were blocked by addition of 0.1% TBS-tween buffer, 5% sucrose and 5% casein and incubation at room temperature for 12 h. The membranes were then washed, with 0.1% TBS-Tween-20 buffer for 10 min. Next, they were incubated (2 h) with the serum sample pools using serial dilutions from 1/125 to 1/1000 in blocking solution.

After washing, membranes were incubated (1 h) with rabbit anti-horse IgG conjugated with alkaline phosphatase (Sigma-Aldrich) diluted (1:2000) in blocking solution. This was followed by two washes (10 min each at room temperature) in 0.1% TBS-Tween and two more washes (10 min at room temperature) in citrate buffered saline (CBS) (pH 7.0). Next, a substrate containing MTT (3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide), BCIP (5-bromo-4-chloro-3-indolyl phosphate) and $MgCl_2$ (Sigma-Aldrich) was added. This reaction was stopped with distilled water after twenty minutes of incubation. All incubation steps were carried out while stirring.

Membranes were regenerated after each immunochemical assay by three 10-minute washes in DMF, three 10-minute washes in reagent A (8 M urea, 1% SDS, and 0.1% 2-mercaptoethanol) and three 10-minute washes in reagent B (ethanol, water, and acetic acid at 50:40:10 v/v/v). Membranes that were not used immediately after regeneration were kept frozen at - 20 °C.

The synthesized peptides and the mean reactivity of positive spots with numerical (0–4) and color intensity (no reactivity – no color, more reactive – darker) were determined for the soluble synthesis.

2.2. Bioinformatic analysis and prediction of 3D protein structure

The amino acid sequences of individual immunoreactive SPOT synthesized peptides were analyzed using the Blast bioinformatic tool available at http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=110073 to determine if these were localized in known hypervariable regions encoded by the *env* gene (V1-V8) as established by Leroux et al. (1997, 2001) and Cook et al. (2013) along with the extent of predicted amino acid sequence identity with other EIAV field strain *env* sequences that have been molecularly characterized to date: Wyoming (Petropoulos, 1997); Liaoning (Tu et al., 2007); Ireland (Quinlivan et al., 2013) and Miyazaki2011-A (Dong et al., 2013) from GenBank (http://www.ncbi.nlm.nih.gov/genbank). All amino acid sequences were aligned and compared using the clustalW2 algorithm within the Biological sequence alignment editor for Windows (BioEdit v 7.0.9.1).

Tools from ExPASy (http://expasy.org/proteomics) were used to determine the molecular weight, hydropathicity and isoelectric point (pI) of each immunoreactive peptide. Molecular weight and pI were determined with the Compute pI/MW tool (http://www.expasy.org/) and hydropathicity was determined via the Hopp & Woods algorithm (http://www.expasy.org/). Structural modelling of the three-dimensional structure of predicted EIAV viral envelope protein sequences was accomplished using the I-Tasser platform (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Roy et al., 2010) and analyzed using with PyMOL (http://www.pymol.org).

2.3. Soluble peptide synthesis for use in ELISA

As a result of methods outlined above one relatively conserved immunoreactive SPOT peptide (pgp45) was identified. Larger amounts of this peptide for use as an ELISA antigen were synthesized using the Merrifield method (Merrifield, 1969) in conjunction with an automated synthesizer (ResPepSL / Spot Automatic Synthesizer Intavis GmbH, Koln, Germany) (Gausepohl et al., 1992). The methodology consisted of attaching the C-terminal amino acid to a solid insoluble support (a resin) and then lengthening the peptide chain by the successive separate addition of each amino acid residue. The amino acids were protected from undesirable side chain reactions and amine function of FMOC groups.

2.4. Indirect ELISA test (pgp45 ELISA)

The pgp45 peptide was used as an antigen in an indirect ELISA test to detect EIAV-specific antibodies in serum samples from horses, donkeys, and mules. The serum panel employed consisted of 859 samples (474 horse, 179 donkey and 206 mule) all collected from equids within Minas Gerais State, Brazil. Based on AGID testing (Coggins and Norcross, 1970) this panel comprised 143 EIAV antibody positive and 716 negative specimens. In addition, positive and negative reference sera (as tested by AGID were provided by official laboratories in Brazil

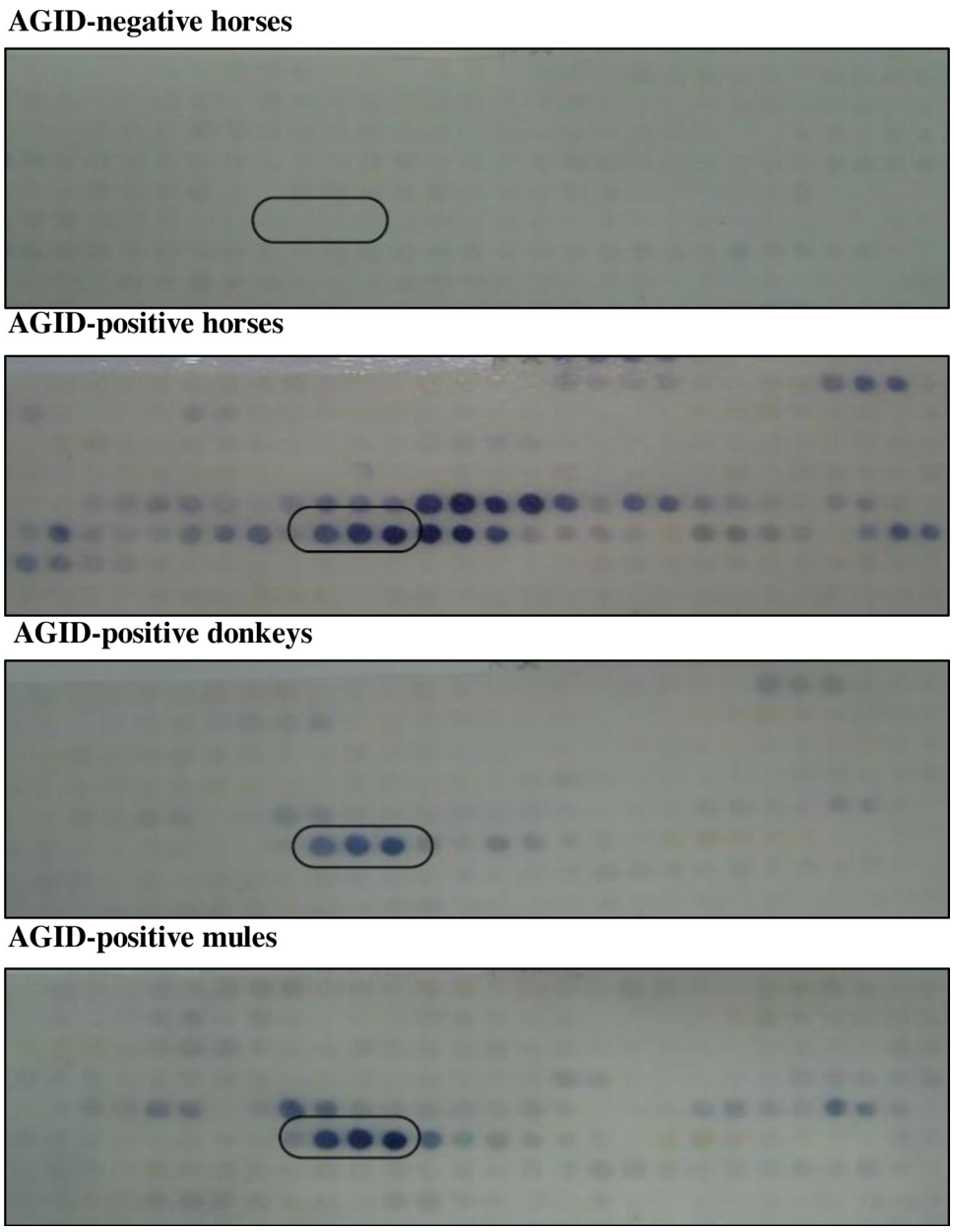

Fig. 1. Epitope mapping to characterize antibody reactivity to viral envelope glycoproteins in EIAV infected horses, mules and donkeys. SPOT synthesis was used to produce 283 overlapping 15 mer peptides bound to cellulose membranes. These membranes were used to identify antibody reactions to specific peptides using separate serum pools (each derived from five individual animals) from AGID test positive horses, donkeys or mules. A serum pool derived from 5 AGID-test non-reactive horses was employed as a negative control. Reactivity was visualized using a rabbit anti-horse IgG alkaline phosphatase conjugate in conjunction with a MTT, BCIP, $MgCl_2$ substrate. Reactivity against identical peptides in serum pools from all three EIAV-infected species resulting in selection of the pgp45 ELISA antigen is indicated by brackets.

(MAPA). These were used in a serial twofold dilution series to determine the optimum serum dilution for providing the highest discrimination between EIAV-antibody positive and negative samples. Briefly, the ELISA protocol consisted of coating each well of an ELISA 96-well plate (Nunc-Immuno Plate Maxisorp) with pgp45 at 0.5 ng/well in 50 mM carbonate / bicarbonate buffer (pH 9.6) and incubating for 18 h at 4 °C. The plates were washed 2 times with PBS (phosphate buffered saline, pH 7.0) containing 0.01% Tween 20 (PBS-Tween) and then incubated for 1 h with blocking solution consisting of nonfat dried milk (5%) in PBS-Tween. Following 3 washes in PBS-Tween 100 µl of each equine serum sample (diluted to 1/100) was added to duplicate wells and incubated for 1 h at room temperature. In addition the EIA reference positive and negative serum samples (2 wells each) were included in each plate as controls. At the end of the incubation period all plates were washed 3 times with PBS-Tween and incubated for 1 h at room temperature with rabbit anti-horse IgG peroxidase conjugate (Sigma-Aldrich), diluted to 1:5000 in PBS-Tween. After 3 additional washes with PBS-Tween, 100 µl of substrate o-phenylenediamine (OPD) at 0.5 mg/ml plus hydrogen peroxide (H_2O_2) in phosphate-citrate buffer (0.1 M citric acid, 0.2 M sodium phosphate) at pH 5) was added and incubated for 10 min at room temperature. The reaction was stopped with 1 N sulphuric acid and optical densities read with a spectrophotometer at a wavelength of 492 nm.

The minimum sample size required to generate an estimate of true prevalence was based on an equation described by (Thrusfield, 1995):

$$n = \frac{Z^2 . P . (P - 1)}{d^2}$$

At a confidence level of 95% (Z = 1.96), with an expected prevalence of 15% (P = 0.15) and precision of 5% (d = 0.05) the calculated minimum sample size (n) to obtain a statistically significant estimate of true prevalence was 196.

The cut-off point for positive and negative sera in pgp45 ELISAs was determined by Roc curve analysis in MedCalc software version 13.0.0.0 (MedCalc Software, Mariakerke, Belgium). The relative sensitivity and specificity of pgp45 ELISA was first separately determined for horses, donkeys and mules and then for all equids as a group. Afterwards, the Kappa test (Landis and Koch, 1977) was conducted (Bioestat 5.0 software) in the same way to assess concordance between the observed and

expected AGID and pgp45 ELISA tests.

To evaluate the performance of the pgp45 ELISA in diagnosis of newly infected animals, samples of blood were collected from a 5-year old stallion experimentally inoculated intravenously with 10^3 $TCID_{50}$ of the Wyoming strain of EIAV ($EIAV_{WYOMING}$) at 3 day intervals from 0 to 18 days post-infection. During this process this horse was kept isolated in a screened horse stall with the rectal temperature and overall condition evaluated daily as an indication of viral replication. Serum collected from this animal was previously tested in AGID and rgp90 ELISA (cut-off 0,225) (Reis et al., 2012).

2.5. Quality assurance and repeatability of ELISA

The repeatability (intra-plate variability) was assessed by the same analyst testing two samples (negative and positive control) four times on the same plate and on the same day. The mean and coefficients of variation (CVs) were computed using Excel 2007 (Microsoft Corp, USA). The reproducibility (inter-plate variability) of the test results was assessed by testing two samples (negative and positive control) 20 times. The results were obtained by use of distinct lots, on different days, by different analysts. The mean and coefficients of variation (CVs) were computed.

2.6. Analysis of discrepant samples between pgp45 ELISA and AGID

Blood serum samples showing discrepant results between pgp45 ELISA and AGID were evaluated by the immunoblot assay according to the establishment of OIE (OIE, 2013). The immunoblot membranes were provided by Dr. C.J. Issel (Gluck Center, University of Kentucky, KY, USA) and prepared as described previously (Issel and Cook, 1993). The protocol used for Immunoblot was also as previously described (Issel et al., 2013).

3. Results

3.1. Epitope mapping

More widespread activity against viral envelope derived peptides was observed with EIAV infected horse rather than mule or donkey pooled serum samples in epitope mapping experiments (Fig. 1). However, intense reactivity with SPOT-peptides 174–176 even at serum dilutions of 1/1000 was observed with all EIAV infected equid samples (Fig. 1). Therefore, sequences associated with these peptides were selected for the synthesis of a potential ELISA antigen. In contrast to serum samples from infected equids, pooled serum samples from horses (Fig. 1), donkeys or mules (data not shown) with no evidence of exposure to EIAV showed no reactivity to any of the 283 peptides evaluated in the immunochemical assay.

3.2. Bioinformatic analysis and prediction of the 3D protein

The peptides at SPOT positions 174–176 encompassed a 20 amino acid residue sequence (KERQQVEETFNLIGCIERTH) mapping to predicted amino acid positions 521–540 within *env*. Therefore, this sequence is within the extracellular domain of the gp45 transmembrane glycoprotein (Fig. 2). Comparison of this sequence with equivalent regions found within other previously characterized EIAV strains (Blast bioinformatic tool available at http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=110073) demonstrated 100%, 90%, 80% and 70% amino acid identity with $EIAV_{WYOMING}$, $EIAV_{IRELAND}$, $EIAV_{LIAONING}$ and $EIAV_{MIYAZAKI}$ strains respectively (Table 1). Only two changes in the amino acid classification were observed in the sequence of the selected peptide and other changes appear to be conservative and/or involve the preservation in the number of charged residues (Table 1).

Bioinformatic analysis showed the selected peptide had a molecular weight of 2430.72 daltons and an isoelectric point (pI) of 5.57. A pI below 7 indicates that the peptide is negatively charged. Negative hydropathicity averaged 1.005, indicating that the peptide is hydrophilic consistent with its location within the extracellular domain of gp45. Moreover three-dimensional modelling of gp45 based on the predictive sequences 2b4cG, 1z8lA, 3j5mA, 2cmrA, 4n4jA and 1g9mG from the Protein Data Bank (PDB) (Fig. 3) suggests the 20mer peptide (yellow spheres) is located on the outer surface of the gp45 protein (purple spheres) and therefore likely to be readily exposed to humoral immune responses.

3.3. Indirect ELISA

Field sample testing was carried out on 859 blood serum samples (comprising 474 horses, 206 mules and 179 donkeys). Animals showing optical densities above 0.251 in the pgp45 ELISA are classified as positive. This cut-off was selected based on the Roc curve using all three equid species (859 sera samples). The results of sensitivity and specificity parameters as well as the level of concordance between the pgp45 ELISA and AGID tests involving serum samples from either a single equid species (horses, donkeys or mules) or a combination of all species are demonstrated in Table 2.

The pgp45 ELISA along with another ELISA test (rgp90) based on a peptide derived from gp90 detected EIAV-specific antibodies at 10 days post-infection from the horse experimentally inoculated with $EIAV_{WYOMING}$ (Fig. 4). In contrast positive test results using serum from this animal were not produced until 18 days post-infection with AGID. The $EIAV_{WYOMING}$ infected horse showed characteristic acute signs of EIA, including a febrile response that is usually associated with high blood-associated viral burdens from 10 to 14 days post inoculation (Fig. 4).

3.4. ELISA quality assurance and reproducibility

Within plate variation, based on the mean optical density (OD) value of the negative control was 0.176 (range 0.122–0.230) (mean ± 2 standard deviations) and the CV was 7.44%. The mean OD value of the reference positive control was 0.662 (range 0.588–0.736) (mean ± 2 standard deviations) and the CV was 6.34%. Regarding the reproducibility, the mean OD value of the negative control was 0.144 (range 0.068–0.220) (mean ± 2 standard deviations) and the CV was 22.86%. The mean OD value of the positive control was 0.815 (range 0.531–1.199) (mean ± 2 standard deviations) and the CV was 21.32%. The standard deviation of the replicates indicates that pgp45 ELISA is a very reproducible test.

3.5. Analysis of discrepant samples between pgp45 ELISA and AGID

Of the 859 serum samples tested in the pgp45 ELISA only 33 (3.8%) had discrepant results (8 donkeys, 11 horses and 14 mules) compared to the AGID test. These samples were submitted to evaluation using immunoblot (Issel et al., 2013) as a confirmatory test. Thirty-one of the discrepant samples were classified as pgp45 ELISA positive/ AGID negative and in immunoblot 81.8% (26/31) were considered EIAV seropositive. The other two animal samples were diagnosed as pgp45 ELISA negative / AGID positive. Of these one was confirmed as positive in immunoblot (Table 3).

4. Discussion

Identification of viral protein components that are both highly reactive with specific antibodies and relatively conserved between isolates will enable the development of more accurate immunoassays for detection of equids that have been exposed to EIAV. Epitope mapping experiments using a series of overlapping peptides representing both the gp90 and gp45 viral envelope glycoproteins were conducted to identify antigens capable of detecting all equids (horses, donkeys and

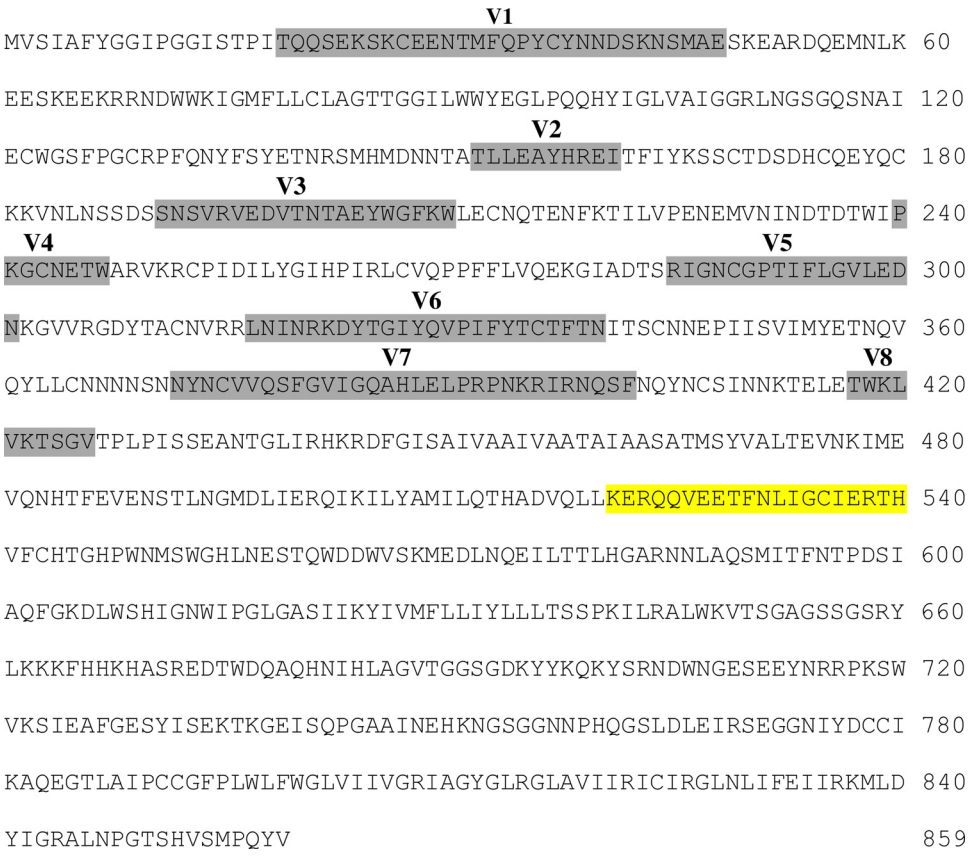

Fig. 2. Predicted amino acid sequence of the EIAV envelope glycoproteins gp90 (aa 1–432) and gp45 (aa 433–859) from the $EIAV_{WYOMING}$ strain (GenBank M16574) (Rushlow et al., 1986). The 8 hyper-variable regions (V1 – V8) in gp90 are highlighted in grey while the peptide selected as an antigen in the pgp45 ELISA is shown in yellow (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

mules) infected with EIAV. These proteins were chosen because the levels of specific antibodies produced by infected hosts against them are approximately 10–100 times more abundant than those directed towards the p26 viral capsid (Montelaro et al., 1984; Rwambo et al., 1990).

Conserved and hypervariable regions exist within env (Cook et al., 2013; Leroux et al., 1997; Montelaro et al., 1993; Quinlivan et al., 2013). A comparison of the aligned EIAV isolate amino acid sequences from the Wyoming, Liaoning, Ireland and Miyazaki2011-A strains and our synthesized peptide sequence showed that the peptide lies within a conserved region of the EIAV *env* gene and, more specifically, within the region that encodes gp45 and is not hypervariable (Chong et al., 1991). Conserved epitopes were identified in the gp45 protein (Belshan et al., 2001), which has a primary immunodominant domain and is highly conserved across retroviruses (Chong et al., 1991). Only two changes in the amino acid classification were observed in the sequence of the selected peptide. These changes were in the Liaoning and Miyazaki2011-A sequences. The first occurred in position three of the peptide sequence, where an arginine (R), which is classified as a positively charged polar amino acid, was exchanged for a glutamine (Q), which is classified as neutral polar. The second change occurred in position five of the peptide sequence, where a glutamine (neutral polar) was exchanged for a lysine (positively charged polar) The three 3D structures of the predicted proteins show that the epitope is exposed and is located in a hydrophilic region (Fig. 3). The position of the protein gives it an immunogenic character and facilitates its recognition by anti-EIAV antibodies.

One of the challenges of working with synthetic peptides is that they often do not adhere well to solid support matrices such as polystyrene or polypropylene ELISA microplates. This problem can be attributed to the size of the peptide and the electrostatic charges of the constituent amino acids (Ball et al., 1992; Geerlings et al., 1988). To achieve better peptide adsorption to the plate, a leucine (L) residue was removed from the leading N-terminus of the peptide, which exposed a lysine (K). Lysine at the N-terminus of the peptide allows for hydrogen bonding, which makes it positively charged and hydrophilic and favors its bonding with negatively charged regions of antibodies (Machado de Avila et al., 2011). The peptide developed in the current study seems to have achieved good adsorption, given that it was capable of efficiently distinguishing between positive and negative EIA sera.

In the analysis of 859 serum samples concordance between pgp45 and AGID was 96.1%. Of the 33 discordant results 31 (3.6%) were positive in the pgp45 ELISA but negative in AGID while the remainder were pgp45 negative and AGID positive. Confirmatory examination by

Table 1
Peptide similarity analysis of pgp45 using samples isolated from the EIAV Wyoming, Liaoning, Ireland and Miyazaki2011-A strains. Amino acids similar to the sequence of pgp45 are indicated by a point; those that displayed any changes are listed under the appropriate letter of its nomenclature, and those marked in bold are those that displayed changes in classification.

n° aa	Sequence																				GenBank	EIAV isolate
20	K	E	R	Q	Q	V	E	E	T	F	N	L	I	G	C	I	E	R	T	H	–	
859	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NP_056903	Wyoming
842	.	.	Q	.	K	I	.	.	.	.	.	.	.	.	.	.	.	.	S	.	AAK21107	Liaoning
867	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	AFW99167	Ireland
866	.	.	Q	.	K	I	.	.	.	.	.	M	.	.	.	A	.	.	S	.	AFV61762	Miyazaki2011-A

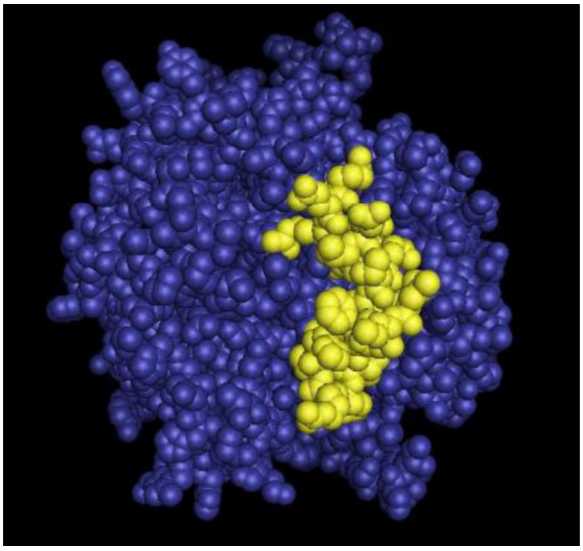

Fig. 3. Tertiary structure of the viral gp45 envelope glycoprotein from the $EIAV_{WYOMING}$ strain (GenBank M16574) (Rushlow et al., 1986) based on the I-Tasser predictive algorithm (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and as visualized using PyMOL (http://www.pymol.org). The majority of the amino acid sequence of the molecule is shown in purple with the pgp45 ELISA peptide antigen highlighted in yellow (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

immunoblot demonstrated that 26 of the 31 pgp45 positive/AGID negative samples possessed EIAV-reactive antibodies. In addition, immunoblot testing revealed that one of the pgp45 negative/AGID positive serum group also contained EIAV-specific antibodies. Therefore, based on the serum panel employed in this study pgp45 ELISA identified 3.03% more EIAV seropositive equids than AGID. These findings are completely consistent with results generated during the Italian national EIAV surveillance program where direct comparisons were performed between EIA-ELISA and AGID-based screening protocols (Issel et al., 2013; Scicluna et al., 2013). The higher sensitivity of the pgp45 ELISA strongly suggest that it will be superior to AGID for initial screening of equid populations for EIAV-specific antibodies. Moreover, on the basis of the samples tested the pgp45 ELISA had false-positive and false-negative rates of only 0.58% and 0.12% respectively.

Another potential advantage of the pgp45 ELISA is that during sequential analysis of serum samples from a horse experimentally inoculated with $EIAV_{WYOMING}$ it detected virus-specific antibodies at 10 days postinfection whereas in AGID no specific antibody was detected until 18 days of experimental infection. In cases of suspected recent EIAV exposure, detection at an early time-point in the infection cycle can be critical to minimizing the spread of this virus. These results reported here are in agreement with previous studies where EIAV-specific antibodies are detected between 14 and 28 days after infection when using ELISA and Immunoblot, but not when using AGID alone (Ball et al., 1992; Hammond et al., 1997; O'Rourke et al., 1988; Rwambo et al., 1990). Furthermore, in a study by Issel et al. (2013) ponies were inoculated intravenously with a genetically engineered strain of EIAV that had significantly decreased replication potential *in vivo*. In 25% of these animals EIAV-specific antibodies were not detectable by AGID for up to 180 days postinfection but were diagnosed as positive within 35 days by ELISA and immunoblot.

The use of synthetic peptides as serological diagnostic antigens provides several advantages over some conventional antigen production methods. For example, they circumvent the need to culture viruses, which is laborious, time consuming, expensive and can result in protein contamination from the cells in which the viruses were cultivated (Sugiura et al., 1995). Furthermore, while viral antigens can be produced using recombinant DNA technology this can also present considerable technical difficulties in certain cases. In some host microorganism systems, particularly *E. coli* high level expression of membrane-associated viral glycoproteins such as EIAVgp90 is often

Table 2
Evaluation of sensitivity, specificity and concordance between results of the pgp45 ELISA and AGID using the kappa index of field samples from all total equids (A) and in horses (B), donkeys (C) and mules (D).

A - Equids	AGID			Sens.%	Sp%	Conc.	Kappa
pgp45ELISA	Positive	Negative	Total				
Positive	141	31	172	98.6	95.6	96.1	0.87
Negative	2	685	687				
Total	143	716	859				
B - Horses	AGID			Sens.%	Sp%	Conc.	Kappa
pgp45ELISA	Positive	Negative	Total				
Positive	65	10	75	98	97.5	97.7	0.91
Negative	1	398	399				
Total	66	408	474				
C – Donkeys	AGID			Sens.%	Sp%	Conc.	Kappa
pgp45ELISA	Positive	Negative	Total				
Positive	41	7	48	97	95	95,5	0.88
Negative	1	130	131				
Total	42	137	179				
D – Mules	AGID			Sens.%	Sp%	Conc.	Kappa
pgp45ELISA	Positive	Negative	Total				
Positive	35	14	49	100	91	93.2	0.79
Negative	0	157	157				
Total	35	171	206				

Sens.- Sensitivity; Sp. - Specificity; Conc.- Concordance.

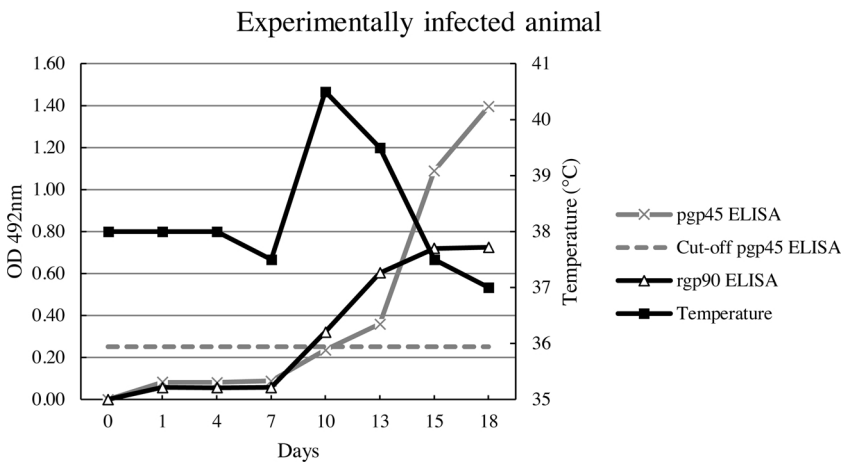

Fig. 4. Reactivity profile of sera from an animal experimentally inoculated with $EIAV_{WYOMING}$ showing the pgp45 ELISA and the pgp90 ELISA (Reis et. al. 2012) and the body temperature of the animal during 18 days of experimental infection.

Table 3
Discrepant results between pgp45 ELISA and AGID tested on Immunoblot.

Animals	Species	pgp45 ELISA	AGID	Immunoblot p26	gp45	gp90
1	Donkey	+	–	–	–	–
2	Donkey	+	–	+	+	+
3	Donkey	+	–	+	+	+
4	Donkey	+	–	+	+	+
5	Donkey	+	–	+	+	+
6	Donkey	+	–	+	+	+
7	Donkey	+	–	–	–	–
8	Donkey	–	+	–	–	–
9	Equine	+	–	+	+	+
10	Equine	+	–	+	+	+
11	Equine	+	–	+	+	+
12	Equine	+	–	+	+	+
13	Equine	+	–	+	+	+
14	Equine	+	–	–	–	–
15	Equine	+	–	+	+	+
16	Equine	+	–	+	+	+
17	Equine	+	–	+	+	+
18	Equine	–	+	+	+	+
19	Equine	+	–	+	+	+
20	Mule	+	–	+	+	+
21	Mule	+	–	+	+	+
22	Mule	+	–	+	–	–
23	Mule	+	–	+	+	+
24	Mule	+	–	+	+	+
25	Mule	+	–	+	+	+
26	Mule	+	–	+	+	+
27	Mule	+	–	–	–	–
28	Mule	+	–	+	+	+
29	Mule	+	–	+	+	+
30	Mule	+	–	+	+	+
31	Mule	+	–	–	–	–
32	Mule	+	–	+	+	+
33	Mule	+	–	+	+	+

+: Positive result -: Negative result.

highly detrimental resulting in intense selection pressure to either minimize or even eliminate synthesis of these molecules (Cunningham et al., 1993). In other cases recombinant proteins may be produced in an almost insoluble form or otherwise difficult to purify (Fahnert et al., 2004). These problems do not occur with synthetic peptides. Chong et al. (1991) used a linear synthetic peptide (IERTHVF) that was detected in only 45% of animals that were proven to be positive for EIA. Soutullo et al. (2001) used bioinformatics to develop a cyclic synthetic peptide with an amino acid sequence in the gp45 region (ERQQVEETFNLIGCIERTHVFCHTG). This peptide had excellent reactivity and identified 92% of positive animals. These results suggest that functionality depends significantly on conformation, given that very low reactivity was observed in linear forms. Nevertheless, the peptide in the present study detected 98.6% of positive animals despite being linear and very similar to the larger peptide used by Soutullo et al. (2001). The use of antigens obtained from the gp45 region in indirect ELISA has already been described in studies using equine positive and negative sera for EIA. Recently, Du et al. (2018) described the use of recombinant gp45 as an antigen of an indirect ELISA for the detection of antibodies to EIAV in horses infected or vaccinated in China. The rgp45 indirect ELISA showed sensitivity of 90.0% and specificity of 99.3% in relation to AGID (Du et al., 2018).

Available AGID and ELISA generally use the p26 protein as an antigen to detect anti-p26 antibodies resulting from EIAV infection being ELISA in general more sensitive than AGID (Alvarez et al., 2007; Coutinho et al., 2013; Issel and Cook, 1993; Soutullo et al., 2007) but, using only this protein as an antigen decreases the sensitivity of these tests (Issel et al., 2013; Scicluna et al., 2013). Exclusive use of the AGID test can make it difficult to interpret EIA diagnostics, especially in donkeys and mules. Sometimes, animals that have been inoculated with a virus and are known to be positive are not detected or produce extremely weak precipitation lines. This may be due to low viremia levels and consequently because of it, low quantities of specific antibodies are induced (Cook et al., 2001; Issel et al., 2013; Scicluna et al., 2013). Donkeys that were experimentally infected with two different EIAV strains had virus levels associated with plasma and nucleic acid levels that were significantly lower than those in ponies and horse that had been inoculated with the same strains (Cook et al., 2001). Mules may produce contradictory results in diagnostic tests. A study in Italy showed that mules tested positive in ELISA but negative in AGID. Therefore, they evaluated 5 positive mules and 5 negative mules that displayed weakly positive results in AGID and entirely positive results in ELISA and immunoblotting. To determine whether there was correlation between viral load and antibody response all animals were immunosuppressed with dexamethasone. None of the animals exhibited clinical characteristics of EIA during the test, and none of the animals that were negative in AGID showed seroconversion. However, all of the mules tested positive by nested PCR and were found to express similar proviral DNA sequences (Scicluna et al., 2013).

Issel et al. (1988) and Chong et al. (1991) proposed a more sensitive diagnostic test that uses EIAV envelope proteins as antigens. This test can detect the true serological "status" of the animal and decrease nonspecific or difficult-to-interpret AGID results. An indirect ELISA test using a recombinant antigen from the EIAV gp90 protein was very robust at diagnosing EIA in horses, however, the sensitivity and specificity were lower when the sera of donkeys were evaluated (Oliveira et al., 2017; Reis et al., 2012). Results from donkey and mule samples can be

difficult to interpret via AGID and can sometimes produce false negatives (Cook et al., 2001; Issel et al., 2013; Scicluna et al., 2013; Spyrou et al., 2003). pgp45 ELISA, conversely, has been shown to be highly efficient, sensitive and specific for horses, donkeys and mules, and it can be an effective EIA diagnostic alternative for these species. Therefore, this the first study to base antigen design on the EIAV-specific antibody reactivity in different equid species.

Author conflicts of interest

The authors declare that there are no conflicts of interest.

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