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Design and validation of an ELISA for equine infectious anemia (EIA) diagnosis using synthetic peptides

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Abstract

Three peptides derived from the equine infectious anemia virus (EIAV) surface proteins were synthesized to design and validate an ELISA for EIA diagnosis. Peptides identified as gp90-I and gp90-II correspond to the N- and C-terminal part of the surface glycoprotein gp90. Peptide gp45-1 overlaps the immunodominant epitope CIERTHVFC of the transmembrane glycoprotein gp45, and includes a hydrophilic chain close to the N-terminal end of this nonapeptide loop. Serum samples from 140 naturally infected horses with EIAV and a panel of 167 non-immune equine sera obtained from non-infected animals were used. Differences in reactivity between positive and negative serum samples were clearly distinguished. Samples considered weak positive to the agar gel immunodiffusion (AGID) test were “true” positive in the ELISA. These results are consistent with the improved sensitivity of the ELISA in comparison with the AGID test. The cyclic peptide that mimics the immunodominant sequence of gp45 showed excellent reactivity, thus suggesting that its functional activity depends significantly on its conformation, since very low reactivity was observed in the linear form of the peptide. The detectability indices of positive and negative sera reached 98% when gp90-II and gp45-I synthetic peptides were used in the same assay, illustrating the high specificity and sensitivity of the assay. Our study represents a first approach for the design of a diagnostic kit, which would allow the rapid analysis of a large numbers of serum samples

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from horses, and could be applied in endemic areas with different prevalence of infection.
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1. Introduction

Equine infectious anemia virus (EIAV), a member of the lentivirus subfamily of the *Retroviridae*, causes a chronic infection with world-wide spread in the *Equidae* family; it has been evidenced in horses, donkeys and mules (Montelaro et al., 1984; Hammond et al., 1997). Once an animal is infected, it will remain infected for the rest of its life (Montelaro et al., 1984; Clements and Zink, 1996; Issel et al., 1982).

The virus possesses the surface glycoproteins, gp90 and gp45 and four major non-glycosylated internal core proteins, known as p26, p15, p11 and p9 (Parekh et al., 1980; Montelaro et al., 1984; Hussain et al., 1988).

At the initial stage of acute equine infectious anemia (EIA), the infection is characterized by recurrent febrile episodes with high virus load and anemia. After resolution of the primary viremia, most animals develop chronic EIA, which is characterized by irregular and less frequent viremia, associated with weight loss and anemia. The disease then progresses to an asymptomatic but still infectious stage which remains for the rest of the animal's life (Hammond et al., 1997).

Most infected horses develop a vigorous humoral immune response, the envelope glycoproteins being the major immunogens. Each episode of viremia is associated with the appearance of a new and predominant antigenic variant of the virus, caused by point mutations in the *env* gene that codes the viral surface proteins (Montelaro et al., 1984).

Coggins et al. (1972) developed an agar gel immunodiffusion (AGID) test to detect precipitating antibodies to the core protein p26, which is known to be group-reactive and antigenically stable (Payne et al., 1984). The AGID test is the only test officially recognized by the Office International des Epizooties (OIE).

A positive AGID test result is reliable and confirms that the virus is present (Issel and Cook, 1993); infected horses produce antibody to EIAV proteins as early as 12 days after infection, but usually become AGID test-positive 15–25 days post-inoculation (Coggins et al., 1972).

Since the late 1980s, the enzyme-linked immunosorbent assay (ELISA) has been introduced for faster and more sensitive serodiagnosis. A competitive ELISA (CELISA) has been developed to detect antibodies against p26 protein, using purified antigens from cell culture-adapted EIAV strain Wyoming. Although the authors found a good correlation between AGID and CELISA, the latter could not detect the “weak positive” International Reference Serum (Bürki et al., 1992; Issel and Cook, 1993). Another ELISA was developed using the amino-terminal portion of a recombinant gp45 protein as antigen. Even though all sick horses ($n = 15$) were positive to ELISA, the authors could not be sure that the recombinant antigen contained conserved epitopes (Lew et al., 1993).

Epitope mapping of EIAV surface proteins (gp90 and gp45) revealed the presence of several conserved and immunodominant epitopes among the different antigenic variants

(Ball et al., 1992). These conserved and highly antigenic epitopes may become valuable diagnostic antigens, since both glycoproteins are recognized by the reference serum and are detected earlier than precipitating antibodies in the course of infection (Hussain et al., 1988; Carpenter et al., 1991; Chong et al., 1991; Ball et al., 1994). The aim of this work was to evaluate the performance of an EIAV ELISA designed with linear and cyclic synthetic peptides from gp90 and gp45. This assay could valuable prove for large throughput screening and for diagnosing EIA in earlier phases of the infection, when the results of the traditional Coggins test are still negative.

2. Materials and methods

2.1. Peptide synthesis

The synthesized peptides from gp90 represent highly conserved and immunodominant regions located in immediate proximity of the N-terminal (peptide gp90-I) and C-terminal (gp90-II) domains of this protein.

Peptide gp45-I overlaps the immunodominant epitope CIERTHVFC, between the cysteine residues 536 and 544, and includes a hydrophilic chain close to the N-terminal end of this nonapeptide loop. This peptide was used in the cyclic form, by means of a bridge between the cysteine residues, to introduce a conformational constraint and to improve its biological activity. This was accomplished by oxidation of the dithiol form of the peptide with iodine in alkaline medium. The reaction was monitored by the Ellman test (Ellman, 1959).

Peptides corresponding to the primary amino acid sequence of EIAV envelope glycoproteins were synthesized on solid phase, either manually or automatically, on an EPS 221 (ABIMED) automatic synthesizer using 9-fluorenylmethoxycarbonyl (Fmoc) chemical strategies and following standard protocols (Atherton and Sheppard, 1989; Fields and Noble, 1990).

For batch and automatic peptide synthesis, Rink amide resin (Novabiochem, San Diego, CA) and Tentagel S Ram resin (Peptide International, USA) were used, respectively.

The Fmoc protected amino acids were purchased from Novabiochem (San Diego, CA). The following side chain protected amino acids were used: Fmoc-Thr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Cys(Trt)-OH.

Coupling efficiencies were evaluated by the ninhydrin colorimetric assay (Kaiser et al., 1970), and the corresponding deprotections by quantitative measurement of the Fmoc-piperidine adduct.

Peptides were cleaved from the solid support by trifluoroacetic acid and scavengers were added to the cleavage mixture. The products were isolated by precipitation with cold ether, and finally lyophilized.

Peptides were purified by solid phase extraction methods, with C18 Sep-Pak cartridges (Waters), and then analyzed by reverse phase high pressure liquid chromatography

(modular Gilson System) using an Ultrasphere ODS 5 μm 4.6 mm \times 25 cm column (Beckman).

2.2. *Sample collection*

Equine serum samples were collected from blood of naturally infected and uninfected horses from ranches in the province of Santa Fe, Argentina. The samples were first checked with the AGID test and then with ELISA protocols.

Negative sera were selected by a sequence of three negative samplings carried out at 30-day intervals. The first sample was chosen for the experimental work.

A total of 307 serum samples were used in ELISA with gp90-II synthetic antigen; 167 were negative and 140 positive to the AGID test; 40 of the latter were weak positive.

A total of 286 serum samples were checked with ELISA using gp45-I synthetic antigen; 168 negative and 118 with positive reaction to the AGID test, 41 of the latter showed weak reactivity.

Most of the serum samples ($n = 278$) were simultaneously checked with gp45-I and gp90-II synthetic epitopes.

2.3. *Coating of microtiter assay plates with synthetic peptides: passive-coating method*

Each peptide was dissolved in 0.05 M sodium carbonate, pH 9.6, and adsorbed to the wells of a microtiter plate (Costar No. 2580). In order to determine the optimum concentration to be immobilized, we performed preliminary assays using 1–10 $\mu\text{g}/\text{ml}$ solutions. Best results were obtained with 5 $\mu\text{g}/\text{ml}$, and 100 μl of this solution was added to each well.

The plates were sealed and incubated at 37°C for 3 h, then kept at 4°C and washed five times with distilled water. The dried microtiter plates were stored at 4°C.

2.4. *ELISA protocol*

The antigen-coated plates were filled with a solution of 0.5% (w/v) bovine serum albumin (BSA) (Sigma) in PBS and incubated at 37°C for 1 h. After washing five times with PBS-T [PBS 2 \times pH 7.5 containing 0.01% (v/v) Tween 20], 100 μl of serum sample, diluted 1/200 in PBS-T, was added to each well. This serum dilution was selected because it showed the highest relation between OD positive and negative serum controls, tested at 1/25, 1/50, 1/100, 1/200, and 1/400. The plates were sealed and incubated for 1 h at 37°C in wet chamber. After that, they were washed with PBS-T, and then 50 μl of peroxidase-conjugated anti-horse immunoglobulin (Sigma) diluted 1/20,000 in PBS-T was added; the plates were incubated for 30 min at 37°C. The color reaction was developed by adding 50 $\mu\text{l}/\text{well}$ of a solution containing 3,3',5,5'-tetramethylbenzidine (TMB) (1 mg/ml) in 0.05 M citrate buffer (pH 4.0) with 0.05% (v/v) H₂O₂. The plates were incubated at 37°C in the dark for 5 min. Color development was halted by the addition of 50 $\mu\text{l}/\text{well}$ of 2.5 M H₂SO₄. Absorbance measurements were made at 450 nm using a Multiskan Automatic ELISA Plate Reader (Labsystem).

The following controls were included for each assay:

1. Negative control serum (NCS): pool of 30 negative sera to the AGID test obtained from a farm without previous reports of infection over the last 10 years. This panel of 30 negative sera was obtained from a racehorse breeding ranch with 10-year absence of clinical and serological disease checked twice annually (two annual controls to the total of the population).
2. Pool of 15 positive sera to the AGID test from farm areas with high prevalence of EIAV infection. The negative and positive controls were diluted 1/200 in PBS-T.
3. Reference standard positive control (RSPC): purified equine gammaglobulin, diluted 1/50 in PBS-T, obtained from a naturally infected horse.

2.5. Result expression

The optical density (OD) of each tested sample was expressed as a percentage of a high positive reference standard, calculated by Eq. (1). This relative measurement resulted in a uniform scale of 0–100 percent positivity (PP). This form of data expression was recommended during a joint FAO/IAEA Meeting of Consultants where the following aspects of ELISA were reviewed: data expression, primary reference standards, quality assurance, and diagnostic validation in infectious diseases (Wright et al., 1993).

$$PP (\%) = \frac{100 \times OD (\text{Sample}) - OD (\text{NCS})}{OD (\text{RSPC}) - OD (\text{NCS})} \quad (1)$$

where NCS is the negative control sera and RSPC is the reference standard positive control.

The results (expressed as PP) of the negative and positive sera to the AGID test were processed with SPSS, version 7.5 for Windows, to determine the descriptive measures as mean (\bar{X}) and standard deviation (S.D.).

To determine the cut-off, the frequency distribution of both positive and negative AGID tests were plotted. The corresponding adjustment to the normal distribution was carried out by means the Kolmogorov–Smirnov test.

The positive and negative detectability indices [DI(+), DI(-)] and predictive values [PV(+), PV(-)] were calculated by Bayes' theorem using the following equations (Tijssen, 1993):

$$DI(+) = \frac{\text{True positives}}{\text{True positives} + \text{False negatives}} \times 100 \quad (2)$$

$$DI(-) = \frac{\text{True negatives}}{\text{True negatives} + \text{False positives}} \times 100 \quad (3)$$

$$PV(+) = \frac{\text{True positives}}{\text{True positives} + \text{False positives}} \times 100 \quad (4)$$

$$PV(-) = \frac{\text{True negatives}}{\text{True negatives} + \text{False negatives}} \times 100 \quad (5)$$

3. Results

The sequences of the three peptides used in this study are shown in Table 1. Peptide homogeneity was more than 90%, as determined by analytical HPLC.

In a preliminary study, the reactivity of the three peptides was compared by means of ELISA using 32 immune horse sera; 12 samples showed weak positive reaction in the AGID test. Peptides gp90-II and gp45-I were the most immunoreactive, and were considered the best antigens for this study. The results are shown in Fig. 1.

The means and standard deviations (S.D.) of the PP were calculated for the gp45-I and gp90-II synthetic peptides. The results are shown in Table 2. Fig. 2 shows the frequency distributions of ELISA-PP of sera negative and positive for EIAV-antibodies to the gp45-I and gp90-II peptides. Cut-off values of 22 and 28%, respectively, for each peptide were determined at a confidence limit of 95%.

The detectability indices and predictive values are shown in Table 3. Best results were obtained when the serum samples ($n = 278$) were simultaneously evaluated with both antigens.

These results demonstrate that this diagnostic system would be especially useful in areas of low prevalence (<5%); indeed, false negative results could not be found in ranches with a maximum of 100 horses. In areas of high prevalence (>50%), at least one horse serum out of 100 would be considered as a false negative result.

Two sera positive in the AGID test were detected as non-reactive by ELISA, using gp45-I + gp90-II peptides, probably because the main antigen used in the AGID test is the p26 core protein, whereas this ELISA was designed only with surface protein antigens.

Three other sera negative in the AGID test did react in the ELISA. Table 4 shows the PP for these five horse sera.

4. Discussion

In the transmembrane glycoprotein gp45 of EIAV, two neighboring cysteine residues, conserved in most retroviruses, define a potential loop structure between residues 536–544. In the lentiviruses, this potential loop constitutes the principal immunodominant domain (PID), a B-cell epitope present in nearly all infected animal's sera (Pancino et al., 1995).

Chong et al. (1991) reported results obtained with different overlapping peptides from gp45. A peptide termed R33, with the sequence IERTHVF (region 537–543), was tested

Table 1
Synthetic peptide sequences

Identification	Sequence	No. of amino acid residues
gp45-I	ERQQVEETFNLIGCIERTHVFCHTG (523–547)	25
gp90-II	ETWKLVKTSQVTPPLPISSEANTGLIRHKR (409–436)	29
gp90-I	YGGIPGGISTPITQQSEKSK (1–20)	20

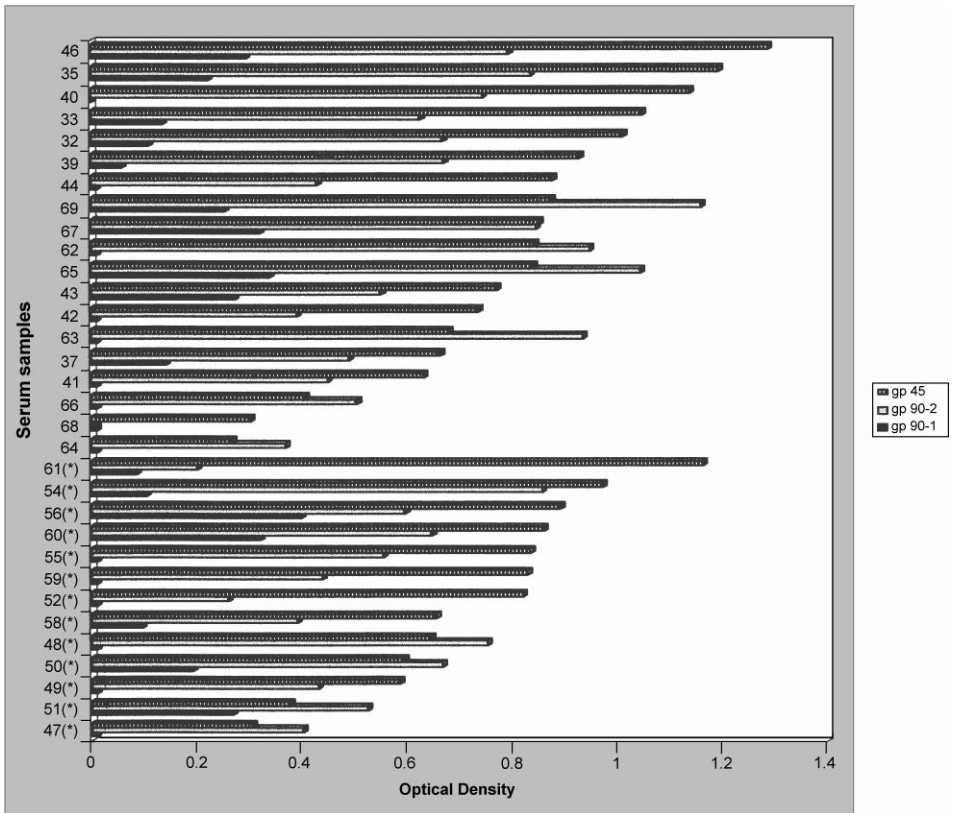


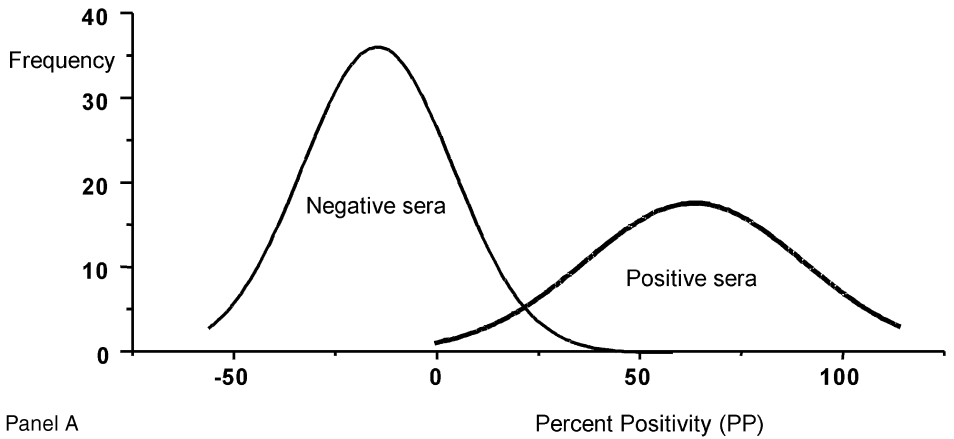
Fig. 1. Reactivity of 32 immune horse sera with EIAV gp45-I, gp90-I and gp90-II synthetic peptides in ELISA. (*) Serum samples with weak positive reaction in the AGID test.

with 20 sera and showed a low reactivity of 45%. Other, more relevant, antigenic sequences were identified by the authors close to the N- and C-terminal extreme of peptide R33.

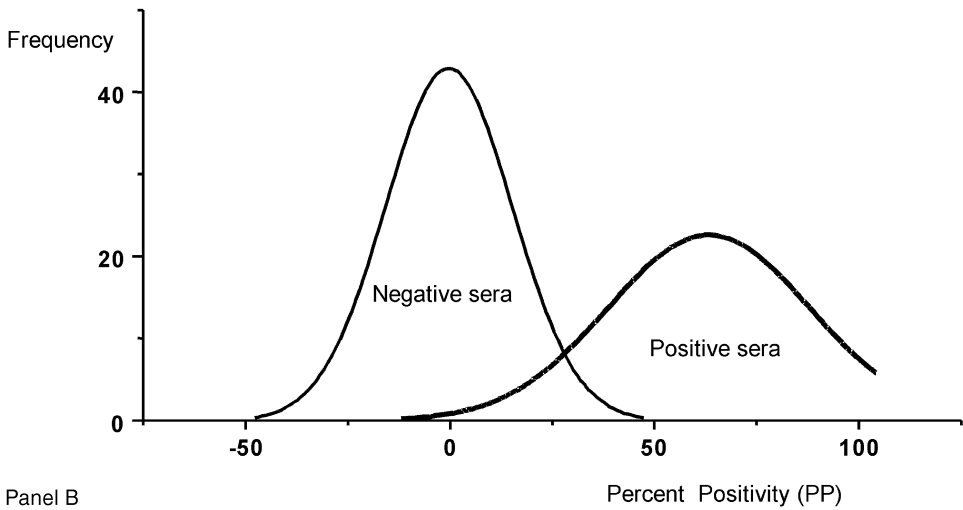
We selected a peptide of 25 amino acids (523–547) containing the complete loop structure and extending towards the N- and C-terminal parts of this transmembrane

Table 2
Mean of PP and S.D. values of ELISA gp45-I and ELISA gp90-II

ELISA	AGID test	Sample number (n)	PP (%)	
			Mean	S.D.
gp45-I	Positive	118	63.35	26.82
	Negative	168	–14	18.54
gp90-II	Positive	140	63.31	24.71
	Negative	167	–0.33	15.52



Panel A



Panel B

Fig. 2. Panel A: frequency distribution of PP in ELISA gp45-I. Panel B: frequency distribution in ELISA gp90-II.

Table 3

Detectability index and predictive values for ELISA gp45-I, ELISA gp90-II and ELISA gp45-I + gp90-II

	Detectability index (%)		Predictive value (%)	
	Positive	Negative	Positive	Negative
gp45-I ($n = 286$)	92	98	97	95
gp90-II ($n = 307$)	91	99	99	93
gp45-I + gp90-II ^a ($n = 278$)	98	98	97	99

^a The results were considered positive by ELISA test when at least one PP result (45 and/or 90) was greater than the cut-off established for each peptide.

Table 4
Percent positivity (ELISA) in five horse sera without a correlation to AGID test results

Serum No.	AGID test	PP (%)	
		ELISA gp90-II (cut-off: 28 PP)	ELISA gp45-I (cut-off: 22 PP)
1	Weak positive	15	0.6
2	Weak positive	25	11
3	Negative	47	59
4	Negative	20	26
5	Negative	22	24

glycoprotein. To predict the physicochemical and antigenic characteristics of the sequences, we applied several algorithms included in the Antheptot 2.9 package program and Peptide Companion version 1.24 (1994), CoshiSoft/Peptide Search, licensed to Peptides International (USA).

The N-terminal extreme of peptide gp45 was characterized by the presence of high hydrophilicity amino acid residues (Hoop and Woods, 1981), and was predicted to be the most antigenic part of the whole sequence (Welling et al., 1985; Parker et al., 1986). The immunoreactivity of peptide gp45-I was significant only when it was used in its cyclic form. Even though synthetic peptides mainly mimic sequential epitopes, preferential conformations on the solid phase may play an important role in epitope functional activity.

We had previously investigated the relevance of this conformational factor using model peptides of different lengths, containing the immunodominant epitope CSGKLIC of gp41 human immunodeficiency virus type 1 (HIV-1) transmembrane glycoprotein. We have noticed that only cyclic peptides were immunoreactive, and also that amino acid residues flanking the epitopes may influence molecular recognition (Aleanzi et al., 1996). An antigenicity study of linear and cyclic peptides that imitate the disulfide loops in gp125 of human immunodeficiency virus type 2 (HIV-2) has also been reported (Belhadj Jrad and Bahraoui, 1998).

Peptides gp90-I and gp90-II are characterized by the presence of more hydrophilic amino acid residues in proximity of their C-terminal extremes with high solvent accessibility. These peptides were predicted as the most antigenic sites. Of both antigens, gp90-II was significantly more immunoreactive than gp90-I. Both gp90-II and gp45-I showed a remarkable immunoreactivity, as they reacted with the positive control sera at dilutions up to 1/3200 (data not shown).

When serum samples positive in the AGID tests were checked with ELISA, high PP values with gp45-I and gp90-II peptides were revealed, and also weak AGID samples were “true” positives in the ELISA.

The detectability indices of positive and negative sera were above 90% in all cases and reached 98% when they were simultaneously evaluated with the gp45-I and gp90-II peptides. This illustrates the high specificity and sensitivity of the assay. The indices were calculated based on the AGID test results; however, this test is not considered as the “gold standard” because horses in the early stages of infection may not give a positive reaction in an AGID test.

The detection of proviral sequences by polymerase chain reaction (PCR) in peripheral blood cells of horses is not always possible because provirus levels in the peripheral monocytes decrease to undetectable levels (<1 copy in 5×10^6 cells) after each bout of viremia (O'Rourke et al., 1991). Furthermore, PCR still needs to be validated under multi-centric evaluation before being used as the "gold standard" (Desmetre, 1999).

The only way to ascertain that a negative AGID result is true is to test each animal again after 30 and 60 days. If the last sample does not react in AGID test, then the first serum was a true negative. In our hands, however, two weak positive sera to the AGID test were found negative in our ELISA using gp45-I and gp90-II. Bürki et al. (1992) had reported that weak AGID reactive samples recognized only p26 in a Western blot, which could account for these unexpected results. The results obtained with five horse sera that did not correlate with the AGID test (Table 4) will be evaluated using the Western blot technique.

We suggest that peptides gp45-I and gp90-II should be included in the ELISA, and we estimate that the addition of relevant epitopes from the p26 core protein may improve these results. Our study represents a first approach towards the design of a diagnostic kit, which would allow the rapid analysis of large numbers of serum samples from horses and could be applied not only in endemic areas with high prevalence of infection, but also in areas with low prevalence.

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