DR. LUCILA OF GARCIA (Orcid ID : 0000-0002-1625-422X)

DR. ADRIANA ROSA SOUTULLO (Orcid ID : 0000-0001-5343-2067)

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Validation of an indirect *in-house* ELISA using synthetic peptides to detect antibodies anti-gp90 and gp45 of the Equine Infectious Anemia Virus (EIAV)

Romina C. Rusi[1,2], L. Garcia[1], María S. Cámara[3] and Adriana R. Soutullo*[1,2]

Romina Cecilia Rusi (ORCID 0000-0001-7888-7623)
Lucila Garcia (ORCID 0000-0001-1625-422x)
María Silvia Cámara (ORCID 0000-0002-0811-1026)
Adriana Rosa Soutullo (ORCID 0000-0001-5343-2067)

[1]Laboratorio de Diagnóstico e Investigaciones Agropecuarias, Sub- Dirección de Ganadería y Sanidad Animal, Ministerio de la Producción, Ciencia y Tecnología de la Provincia de Santa Fe, Boulevard Pellegrini 3100, S3000ZAA Santa Fe, Argentina; [2]Laboratorio de Inmunología Experimental. Cátedra de Inmunología Básica. Facultad de Bioquímica y Ciencias Biológicas. Universidad Nacional del Litoral. S3000ZAA Santa Fe, Argentina and [3]Laboratorio de Control de Calidad de Medicamentos. Cátedra de Control de Calidad. Facultad de Bioquímica y Ciencias Biológicas. Universidad Nacional del Litoral. S3000ZAA Santa Fe, Argentina.

*Corresponding author email: soutullo@fbcb.unl.edu.ar

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Current address for Dr Garcia: Instituto de Biología Molecular y Celular de Rosario (IBR)-Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Ocampo y Esmeralda S/N, S2002LRK, Rosario, Argentina.

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Running title: $ELISA_{gp90/45}$ is a robust diagnostic test and outperforms the AGID test

Summary

Background: Equine infectious anaemia (EIA) is controlled by identification of seropositive animals. The official diagnostic method is agar gel immunodiffusion (AGID) test, which detects antibodies against a viral core protein (p26). Although AGID is inexpensive and specific, report of results takes considerable time and the test has low analytical sensitivity.

Objective: To validate our *in-house* indirect $ELISA_{gp90/45}$, following the World Organization of Animal Health (OIE) criteria.

Study design: Test validation.

Methods: Synthetic peptides gp90 and gp45 were used as antigens in $ELISA_{gp90/45}$. Tests used for validation, calibration and linear working operating range, analytical and diagnostic sensitivity and specificity, repeatability and reproducibility were assessed by comparing them with the AGID test and using 1844 equine sera grouped into five different panels.

Results: We were able to replace the National References Sera with our Internal Reference Sera. $ELISA_{gp90/45}$ had acceptable repeatability and reproducibility. Analytical sensitivity of the $ELISA_{gp90/45}$ was 800 times greater than that of AGID test for positive sera and 400 times greater for weak positive sera. $ELISA_{gp90/45}$ also showed optimal analytical specificity, since no cross-reactivity was detected with antibodies against other equine viruses. One sample was positive by AGID test and negative by ELISAgp90/45. $ELISA_{gp90/45}$ was performed using 243 EIA positive and 878 negative equid sera, and showed a diagnostic sensitivity of 99.59% [CI 97.73 - 99.99 %] and a diagnostic specificity of 90.32% [CI 88.17 - 92.19 %], compared to AGID test; thus, it was demonstrated to be a robust test.

Main limitations: Samples were derived from naturally infected equid populations showing heterogeneous clinical states: therefore, their status was uncertain and some horses were sampled more than once. The AGID test may not be the most useful gold standard.

Conclusion: ELISA$_{gp90/45}$ is a useful tool for the diagnosis of EIAV infection and meets validation requirements established by the OIE.

1. Introduction

Equine Infectious Anaemia (EIA) is one of the 11 notifiable equine diseases declared by the World Organization for Animal Health (OIE). The best strategy to prevent EIAV transmission is to detect infected horses during the first month of disease, when the viral load is still high. The only reliable indicator of EIAV infection has been the presence of specific antibodies against the envelope glycoproteins (gp90 and gp45) detected approximately between 10 and 30 days after exposure. However, the official diagnostic test, the agar gel immunodiffusion (AGID) test, only detects antibodies against the major core protein (p26) of EIAV usually between 14 and 45 days after infection, with some EIAV-infected horses being detected 180 days after infection.[1,2] Equids recently infected with EIAV develop low levels of antibodies against p26 antigen; therefore, they may be falsely reported as negative by the AGID test. By contrast, the high level of antibodies against glycoproteins, which are present before the appearance of antibodies anti core protein (p26), can be detected by Enzyme-linked immunosorbent assay (ELISA) and/or Immunoblot (IB).[2–4] For this reason, the OIE currently recommends both techniques (ELISA and AGID test) to evaluate EIAV infection and the efficiency of eradication policies.[5]

Several reports have described ELISA tests using purified viral proteins, recombinant proteins and/or synthetic peptides as antigen. All of them have shown correlation with the AGID test, and some of them, a higher sensitivity and analytical capacity to report results in a shorter period. [4,6–10] However, most ELISAs detect antibodies that recognise only one EIAV protein, mainly the nucleoprotein p26, as the AGID test does, or only one of the envelope glycoproteins. Scicluna et al.[4] proposed that horses should be defined as serologically positive for EIAV when their sera react with two or more EIAV structural proteins (p26 and gp45 or gp90) in IB test.[4] In a previous report, we described the design of an ELISA that can detect antibodies using synthetic peptides from gp45 and gp90 as antigens.[11] Here, following the validation guidelines defined by the OIE, as requested by the national authorities of animal health (SENASA) in Argentina,[12,13] we show that our *in-house* indirect ELISA$_{gp90/45}$ can be successfully applied not only in other laboratories but also in national and/or international control or surveillance programs.

2. Materials and Methods

A four-step procedure based on OIE criteria[5] was followed to validate our *in-house* ELISA$_{gp90/45}$ (Fig. S1).[5]

2.1. Preliminary consideration

2.1.1. Fitness for intended purpose

$ELISA_{gp90/45}$, is a serological method either to detect EIA-infected equids or to certify EIA-free herd population; thus, it might be a useful tool in national control or surveillance programs.

2.1.2. Study design

The *in-house* indirect ELISA validated in this work was developed by Soutullo et al. [11] As antigens, $ELISA_{gp90/45}$ uses gp90 and gp45, two different synthetic peptides derived from the EIAV surface glycoproteins. The peptides synthesised from gp90 represent highly conserved and immunodominant regions located close to the C-terminal domain of this glycoprotein. Peptide gp45 overlaps the immunodominant epitope CIERTHVFC, between the cysteine residues 536 and 544 of the transmembrane glycoprotein gp45, and includes a hydrophilic chain close to the N-terminal end of this nonapeptide loop. This peptide was used in the cyclic form and the peptide gp90 in the linear form. Sequences of both peptides used in this study are shown in Table S1.

Sample sera were collected from the blood of naturally infected and uninfected horses from rural areas in the province of Santa Fe, Argentina. Each sample was evaluated using the AGID test, as a comparative method, following OIE recomendations[5]. Serum samples were grouped into five panels for the validation procedure (Table 1). Panel 1 (Reference sera) was used during the optimisation and standardisation stage, Panels 2 and 3 consisted of reference samples analysed by Regional Laboratory of SENASA, negative and positive by AGID test, respectively, and were used to determine analytical specificity and sensitivity and to calculate the cut-off value. The minimum number of samples to be used as reference samples (panels 2 and 3, Table 1) was obtained using the formula proposed by the OIE[5,14] :

$$n = \frac{(DSn)(1 - DSn)(c)^2}{e^2}$$

where n is the minimum number of samples -animals of known health status- required to establish the cut-off value; DSn is diagnostic sensitivity/specificity, i.e. the expected proportion of infected animals in a given population that will give positive results; c is the estimated confidence interval; and e is the percentage of error, expressed as a decimal number.

We calculated the minimum number of sera considering a diagnostic sensitivity or specificity of 98%,[11] with an error of 2% and a confidence level of 99%. The minimum number calculated was 325 sera per panel,

but the real number of examined samples is that reported for panel 2 *(n=374)* and 3 *(n=333)* of Table 1, which is higher than the number necessary to calculate the cut-off value.

Repeatability and reproducibility were determined using panel 4 and the diagnostic sensitivity and specificity were determined using sera from panel 5 of Table 1.

2.1.3. Assay procedure

$ELISA_{gp90/45}$ procedure was set up following the recommendation described by Soutullo et al. [11] Briefly, peptides were dissolved in 0.05 M sodium carbonate, pH 9.6, at 0.5 ⌈g/100 µL. Each peptide dilution was adsorbed individually in a Microtiter plate (Costar No. 2580). The plates were sealed and incubated at 37°C for 3 hours, washed with distilled water and kept at 4°C. The antigen-coated wells were filled with 200 µL of 4% (w/v) of no-fat milk and 1% gelatin type B (Merck-Sigma) in phosphate-buffered saline (PBS) and incubated at 37°C for 1 hour. The wells were washed five times with PBS-T [PBS, pH 7.5 containing 0.01% (v/v) Tween 20], and 100 µL of serum samples, diluted 1:200 in PBS-T or at the dilutions corresponding to each assay, were added to each well. The plates were sealed and incubated at 37°C in wet chamber for 1 h. Then, they were washed with PBS-T, and incubated with 50 µL of peroxidase conjugated rabbit serum specific for equine immunoglobulin IgG (Merck-Sigma) diluted 1:20,000 in PBS-T at 37°C for 30 min. After five washings, colorimetric reaction was developed by adding 50 µL/well of TMB Substrate solution (Thermo Fisher Scientific). The optical density (OD) of the samples was read at 450 nm using a Multiskan Automatic ELISA Plate Reader (Thermo Fisher Scientific/Labsystem). $ELISA_{gp90/45}$ includes three Internal Reference Sera: Internal Reference Positive Control (IRPC) in triplicate; Internal Reference Strong Positive Serum (IRSPS) in duplicate and Internal Reference Negative Control (IRNC) in triplicate, described in panel 1b (Table 1). All of them were used as internal controls for every run of $ELISA_{gp90/45}$. The results are expressed as Percentage of Positivity (PP), which was calculated using the following formula[15]:

$$PP[\%] = 100 \times \frac{[OD\ (sample) - OD\ (IRNC)]}{[OD\ (IRPC) - OD\ (IRNC)]}$$

2.1.4. Optimisation and standardisation

To meet the OIE criteria, first it was necessary to determine if the dilutions of the Internal Reference sera used were within the linear working range of sigmoidal dose-response curve. The Internal Reference Sera (panel 1a, Table 1) and National Reference Sera (panel 1b, Table 1) were used in two-fold serial dilutions (1:25 to 1:800)

in PBS-T in triplicate and their ODs were plotted as a dose-response curve using GraphPad version 6.0. The linear ranges were established considering the linear part of the sigmoidal dose-response curve, between 20% and 80% of the obtained OD for each peptide. For this, 100% was considered the maximum absorbance value obtained in each test.

To improve calibration of the Internal Reference Positive Control, we built a dose-response curve by plotting the PPs and the logarithms of dilution of IRSPS (1:25 to 1:800), on the y and x-axes, respectively. PPs were calculated considering the OD of IRPC diluted 1:50 or 1:200, minus OD of IRNS diluted 1:200, as 100%.

To study whether the Internal Reference Sera could replace the National Reference Sera, Slope and logEC50 (log of the dilution whose OD corresponds to 50% absorbance) of these dose-response curves were statistically compared using an F test provided by the software. This test is the extra-sum-of-squares F test, an adaptation of analysis of variance (ANOVA), based on tests of traditional statistical hypothesis. A p-value greater than 0.05 indicates a lack of statistically significant differences between the curves and, therefore, that replacement is possible.

2.2. Cut-off values

The cut-off values were determined using Receptor-Operator Curve (ROC) analysis. PP of sera analysed by AGID test (panels 2 and 3, Table 1) were used with MedCalc software version 12.7.0 (www.medcalc.org, Belgium). The AGID test was considered the gold standard for ROC analysis, since it is the official confirmatory test. The reproducibility and analytical and diagnostic performances were calculated using both cut-off values defined in each ELISA ($ELISA_{gp90}$ or $ELISA_{gp45}$) by ROC. The purpose of this assay is to detect positive sera as early as possible; therefore, the cut-off values were selected in order to maximise the sensitivity of the test. We considered a positive result by $ELISA_{gp90/45}$ when the PP of at least one of the ELISAs (gp90 or gp45) exceeded the established cut-off value.

2.3. Diagnostic performance characteristics

2.3.1. Diagnostic sensitivity (DSe) and specificity (DSp)

The diagnostic sensitivity and specificity were determined using sera from panel 5 of Table 1. Sera evaluated by $ELISA_{gp90/45}$ were analysed by the AGID test. The sera were classified as True Positive (TP) (Positive Coggins Test, Positive $ELISA_{gp90/45}$) and True Negative (TN) (Negative Coggins Test and negative $ELISA_{gp90/45}$). Alternatively, they were classified as false positives (FP) or false negatives (FN) if the $ELISA_{gp90/45}$ did not agree with the AGID test. The diagnostic sensitivity was calculated as TP/(TP+FN) and the diagnostic specificity was calculated as TN/(TN+FP). Both results are expressed as percentages. Confidence intervals (CI) were obtained using Epitools version 0.5-10.1 (Cloper-Pearson).

2. 4. Analytical performance characteristics.

2.4.1. Repeatability.

Intra and inter-plate variability was evaluated to determine if the variation of the assay affects its precision. Intra-plate variability was evaluated using each peptide, gp90 or gp45, absorbed onto a 44-well plate. Three strong positive (SP) sera, three weak positive (WP) sera, and three negative (N) sera were selected from panel 4 of Table 1 and assayed in quadruplicate by a single analyst. This test also evaluated if the location of the sera in the plate interfered with the reading. Inter-plate variability was assessed on three days distributed in consecutive weeks. $ELISA_{gp90/45}$ was run by three analysts and it was performed in a 62-well plate for each peptide. The three SP, the two WP, and the three N sera were assayed in sextuplicate.

2.4.2. Analytical specificity and sensitivity.

Analytical specificity (ASp) defines how the test distinguishes between the target analyte and other components present in the matrix considering three aspects: selectivity, exclusivity, and inclusivity. Selectivity is the capability to detect the analyte, even in the presence of interfering substances, such as matrix components and degraded products, such as haemolytic sera. Exclusivity is the ability of the assay to detect the target analyte, excluding all other cross-reactive biological substances present in the sample. Forty sera that have antibodies against Influenza virus, Equine Arteritis virus, and Equine Herpes virus types 1 and 3, which were negative to the AGID test, were tested by $ELISA_{gp90/45}$ (panel 2c, Table 1). Inclusivity, the ability of the assay to recognise various serotypes of the same biological entity, was assessed by analysing panel 3e of Table 1, which includes AGID test-confirmed positive sera collected from different regions of Argentina between 1998 and 2018.

For analytical sensitivity (ASe), $ELISA_{gp90/45}$ and AGID test were compared using endpoint dilutions to calculate the limit of detection (LOD). One positive and one WP sera from panel 3b and 3c of Table 1, respectively, were diluted in the IRNS (panel 1b, Table 1). Two-fold serial dilutions (1:2 to 1:256) were tested by AGID test and each of them was also tested by the $ELISA_{gp90/45}$ and in turn diluted 1:200 (1:200- 1:51,200). Final titration points were established in each of the mentioned methods and the LOD was calculated. The results were expressed as the log10 of the inverse of the last dilution detected as positive by two assays.

2.5. Reproducibility

To determine the reproducibility of $ELISA_{gp90/45}$, the assay was carried out in three laboratories by three operators using the same batch of samples and reagents. The laboratories had experience in handling the ELISA method (Immunological Technology Laboratory of the Faculty of Biochemistry and Biological

Sciences of the Universidad Nacional del Litoral, Animal Health Laboratory of the Instituto Nacional de Tecnologia Agropecuaria (INTA) Rafaela, and Laboratory of the Center of Comparative Medicine of the Instituto de Ciencias Veterinarias del Litoral; ICIVET). Twenty sera: 8 N (panel 2d, Table 1), 8 positives (panel 3b, Table 1) and 4 WP by AGID test (panel 3c, Table 1) with different levels of reactivity, and the internal controls were blindly distributed in identical aliquots. Median values and the interquartile range (IQR) were calculated for each laboratory. These results were compared using the Kruskal-Wallis test to evaluate concordance ($p>0.05$). Data distribution was graphically represented using Whisker-Box diagrams.

3. Results

3.1. Optimisation and standardisation: Calibration and determination of the working operating range

To determine the linear operating range of the assay, a dose-response curve was plotted for each antigen employed in the ELISA, gp90 (Fig. 1A-D) and gp45 (Fig. 1E-H), using internal controls. A curve for each internal control was compared with the corresponding national reference sera. Thus, IRPC and IRSP were compared with NRSPS (Fig. 1A and 1E; Fig. 1B and 1F); NRWPS with IRWPS (Fig 1C and 1G); and IRNS with NRNS (Fig 1D and 1H). All calibration curves showed the typical sigmoidal shape; therefore, we were able to determine the linear operating range of the assay (Fig. 1A-H). The calculated linear ranges were [1:80 to 1:300] and [1:75 to 1:300] for IRSP in the $ELISA_{gp90}$ and $ELISA_{gp45}$, respectively, and [1:50 to 1:250] and [1:40 to 1:200] for IRNS in the $ELISA_{gp90}$ and $ELISA_{gp45}$, respectively. Those linear ranges are concordant with the optimal dilution established in our previous work[11]. However, IRPC behaved differently, since 1:50 was not within the calculated linear range ($ELISA_{gp90}$ [1:125 to 1:500] and $ELISA_{gp45}$ [1:65 to 1:300]). Therefore, to improve calibration and determine the optimal dilution of the IRPC, the PPs were calculated considering the OD of IRPC, diluted 1:50 (blue) or 1:200 (black), minus OD of IRNS diluted 1:200, as 100% of PP (Fig. 2). PPs calculated for $ELISA_{gp90}$ (60.2 [95% CI: 34.85-85.55]; 61.70 [95% CI: 35.77-87.63]) or $ELISA_{gp45}$ (80 [95% CI: 56.12-103.9]; 68 [95% CI: 50.82-85.18]) were similar for both dilutions used for the IRPC (Fig. 2A) and were within the same linear range [1:50 to 1:800]. Interestingly, PPs from $ELISA_{gp45}$ using IRPC diluted 1:50 were higher than when 1:200 was used (Fig. 2B). Hence, we decided to use IRPC diluted 1:50.

Moreover, results from the F test ($p>0.05$) indicated that it is appropriate to replace the National Reference Sera with our Internal Reference Sera in the $ELISA_{gp90/45}$ ($logEC50_{gp90}$ 1:50 0.9501 [95% CI 0.5622-1.338] vs. 0.7569 [95% CI 0.3206-1.193] and $logEC50_{gp45}$ 1:50 0.8937 [95% CI 0.6311- 1.156] vs. 0.6664 [95% CI 0.3360- 0.9968]).

3.2. Cut-off values

ROC curves were plotted for each peptide (Fig. 3). The ROC curve analysis for $ELISA_{gp90}$, had an area under the curve (AUC) of 0.991, with standard error (SE) of 0.00356 and confidence interval (CI) (95%) between 0.981 and 0.997. The cut-off value of PP established for the $ELISA_{gp90,}$ corresponding to the highest sensitivity (96.10%) and specificity (98.13%) values, was 18.5 PP (Fig 3A). On the other hand, the ROC curve analysis for $ELISA_{gp45}$ as an antigen had an AUC of 0.983, with SE of 0.00519 and CI (95%) between 0.971 and 0.992. The cut-off value for $ELISA_{gp45}$ was 14 PP, considering a diagnostic sensitivity and specificity of 99.59% [CI 97.73 - 99.99 %] and 90.32% [CI 88.17 - 92.19 %], respectively (Fig 3B).

The purpose of this assay is to detect positive sera as early as possible; therefore, we decided to increase the sensitivity of the test, considering a positive sample if, at least, one of PP of any of the ELISAs, gp90 or gp45, was higher than the calculated cut-off value.

3.3. Analytical performance characteristics.

3.3.1. Repeatability

The OD mean values, standard deviation (SD) and coefficients of variation (CV) were calculated for the three N, three WP and three SP sera (Table S2). For the intra-plate variability study, most of the CV percentages were below 20%, except for one SP serum and one WP serum against antigen gp45, which were 20.9% and 29.3%, respectively (Table S2). For the inter-plate variability study, all the CV percentages were below 20%, except for one SP serum against antigen gp45, which had a CV of 23.62% (Table S3). The SD of the intra-plate and inter-plate replicates indicates that $ELISA_{gp90/45}$ is a reproducible assay.

3.3.2. Analytical specificity (ASp) and sensitivity (ASe).

Before calculating the ASp and ASe for our $ELISA_{gp90/45}$, we decided to evaluate the possible interference of hemolysis with our assay. Hemolytic and non-haemolytic sera were added to sera from panels 2 and 3 (Table 1) and analysed by AGID test and $ELISA_{gp90/45}$. The concordance of results obtained between AGID test and $ELISA_{gp90/45}$ indicated that haemolysis did not interfere with the results of our test (data not shown).

None of the panel 2c sera were positive by $ELISA_{gp90/45}$, suggesting that EIAV does not present cross-reactivity with any of the evaluated equine viruses (Equine Herpes Virus-1, Equine herpes Virus-3, Equine Viral Arteritis and Equine Influenza Virus). In contrast, $ELISA_{gp90/45}$ detected as positive all sera included in panel 3e. These results demonstrate the high level of ASp of our $ELISA_{gp90/45.}$

Reactivity was obtained up to a 1:1600 dilution (1:8 diluted 200 times) for $ELISA_{gp90/45}$ and 1:2 for AGID test when positive serum was used. Antibodies in the WP were detected at 1:400 (1:2 diluted 200 times)

dilution by $ELISA_{gp90/45}$ but only weakly detected by AGID test when that serum was undiluted (Table S4). The log10 of LOD of $ELISA_{gp90/45}$ was 3.2 and 2.6, and AGID test was 0.6 and 0, for positive and WP sera, respectively (Table S4). Our results show that $ELISAgp_{90/45}$ outperformed the AGID test in terms of ASe.

3.4. Diagnostic performance characteristics

3.4.1. Diagnostic sensitivity (DSe) and specificity (DSp)

The DSe and DSp were determined using sera from panel 5 of Table 1. The results of the $ELISA_{gp90/45}$ were compared with those of the AGID test (Table 2). The DSe was 99.59% [CI 97.73 - 99.99%] and DSp was 90.32% [88.17 - 92.19%]. Of 86 samples with discordant results, 85 were $ELISA_{gp90/45}$-positive, AGID-negative, with 27 of them being positive for both peptides used as antigen (gp90 and gp45). Only one serum was AGID-positive but $ELISA_{gp90/45}$-negative.

3.5. Reproducibility

The PP values reported by the three laboratories for $ELISA_{gp90}$ were: A [median=38.75, IQR=74.38], B [median=33.65, IQR=74.32], C [median=49.66, IQR=81.06], whereas for $ELISA_{gp45}$, PP values were: A [median=50.26, IQR=74.67], B [median=83.06, IQR=101.59], C [median=81.17, IQR=91.74] (Fig. 4 A and B). Moreover, the results obtained for 20 sera by each laboratory were concordant ($p>0.05$, Kruskal-Wallis) and were correctly classified as positive or negative based on previously obtained cut-off values ($ELISA_{gp90}$: 18.5 PP and $ELISA_{gp45}$: 14 PP) to determine diagnosis (Table S5).

4. Discussion

Equine infectious anaemia is currently controlled exclusively by identification of seropositive animals. In most countries, including Argentina, the official diagnostic assay is the AGID test, which detects only antibodies specific to the major core protein (p26).[16,17] Nowadays, for surveillance purposes, the OIE considers ELISA as a secondary method to the AGID test, and recommends that any ELISA-positive sample must be confirmed using either the AGID test or the IB test.[5,18] However, numerous authors reported that several factors limit the use of the AGID test.[17–20] The exclusive use of the AGID test as a reference can make it difficult to interpret EIA diagnostics in animals with low virus load and specific antibody levels against p26 protein; for this reason, many infected horses were not detected. [17]

Accordingly, our purpose was to validate our $ELISA_{gp90/45}$, taking into account the OIE criteria,[5,12,13] using AGID test as the gold standard test, even though it is an imperfect reference test. [4,9,21]

The indirect ELISA$_{gp90/45}$, developed previously and validated here, is able to detect antibodies against conserved regions of gp90 and gp45 proteins. These specific antibodies against envelope proteins are typically detected during the first 10 or 15 days post-infection, earlier than detection of antibodies anti-p26 by AGID test. [22,23] Antibodies against gp90 and gp45 are 10-100 times more abundant than those directed towards the p26 viral capsid during the entire course of infection.[17,23,24] Most previous ELISA tests identify anti-p26 antibodies, as the AGID test. [25,26] Synthetic peptides or recombinant proteins have been used, to a lesser extent, as antigens to verify the presence of antibodies anti gp45 or gp90, and very few previous assays have combined core and one of the *env* proteins as antigen. [17,27] In this work we validated our previously reported ELISA that uses two synthetic peptides from both envelope proteins, .[11]

In agreement with the OIE requirement during the process of optimisation, calibration of standards and setting of the operating range of this assay, we confirmed that the dilutions of the Internal Reference Sera used in our previous report were within the linear operating range and verified that it is possible to replace the National References Sera with our Internal Reference Sera in the ELISA$_{gp90/45}$.[11,28] This step allowed us to demonstrate the use of these Internal Reference Sera as quality control of ELISA$_{gp90/45}$. Indeed, the results, expressed as PP, should be more reliable if the IRPC had higher reactivity than the NRSPS.

Results obtained in this validation study showed that the ELISA$_{gp90/45}$ has high repeatability. Most of the obtained coefficients of variation were less than 20%, except for two sera, one of them a WP serum by AGID test.[5] Similar results were obtained previously with tests detecting antibodies directed against EIAV using recombinant gp90 or gp45 as antigen in their indirect ELISA.[17,20,27]. By contrast, the competitive ELISA that detected antibodies directed against the viral core protein p26 had higher CV, up to 30%, particularly in negative sera.[25] Moreover, the ELISA$_{gp90/45}$ had high reproducibility. Our inter-laboratory tests showed a SD lower than 20% in most cases. Only in four cases was the SD between 20-23%. Regarding the diagnosis, the results obtained by each of the three laboratories coincided with the diagnosis of the AGID test. On the other hand, the statistical analysis confirmed the lack of significant differences among laboratories; this result determines the accuracy and precision of ELISA$_{gp90/45}$, demonstrating its robustness.

Our validation data showed that ELISA$_{gp90/45}$ has satisfactory analytical performance characteristics. The LOD of ELISA$_{gp90/45}$ is higher than that of the AGID test. The analytical sensitivity of the ELISA$_{gp90/45}$ was 400 times higher than that of the AGID test for positive sera and 200 times higher for WP sera. Based on our results, we consider that our *in-house* assay is able to detect antibodies earlier than AGID test, even when the quantity of antibodies would not be sufficient to precipitate with antigen in AGID test. Our data also confirm the lower analytical sensitivity of the AGID test and provide further consistent evidence of the need to replace the AGID test with more sensitive methods for the serological diagnosis of EIA; however, regulatory

organisms in some countries have put up resistance to the adoption of other methods to date[4,9,17,19,20,22,25]. $ELISA_{gp90/45}$ also showed optimal analytical specificity; since no cross-reactivity was detected with antibodies against the most frequently reported equine viruses. Indeed, the $ELISA_{gp90/45}$ has the capacity to detect all serum samples from different areas of our country, AGID test positive, and confirmed by other technical approaches. A similar characteristic regarding analytical specificity was reported elsewhere.[20,25]

The purpose of this assay is to detect infected animals as early as possible; therefore, we selected both cut-off values considering the highest sensitivity and specificity. We defined a positive result of the $ELISA_{gp90/45}$ when at least one of the PP values calculated for each antigen, gp90 and gp45, was higher than the cut-off value calculated for each of them. Moreover, when the $ELISA_{gp90/45}$ detects antibodies against both *env* peptides, the equine should be considered infected, an approach resembling the interpretation of IB test, adopted for HIV[29]: an equine is serologically positive for EIAV when the presence of antibodies is detected against at least two of the three principal structural proteins, p26, gp45 and gp90.[4,20] Our results demonstrated that detecting antibodies against more than one antigen guarantees a high specificity for the diagnosis of EIAV, because 218 of 242 true positive sera were positive simultaneously by $ELISA_{gp90}$ and $ELISA_{gp45}$. Indeed, 6 of 11 weak positive sera by AGID test had the same results with both peptides, and the remaining 5 had antibodies only against peptide gp45 (data not shown). Similar sensitivity was reported using recombinant gp45 as antigen in an indirect rgp45 ELISA during the validation procedure.[22]

Our specificity results demonstrated that 85 serum samples were positive by $ELISA_{gp90/45}$ but negative by AGID test; therefore, these horses would be considered "non-infected" by the latter test. Nevertheless, we consider that 27 of them were infected because they had antibodies against both antigens. The remaining 58 serum samples were positive by one of the $ELISA_{gp90/45}$, 42 only by $ELISA_{gp45}$ and 16 by $ELISA_{gp90}$ (data not shown). This result means that we detected about 25% more EIAV-infected horses than the AGID test. Another indirect ELISA, which uses p26 as antigen, is able to detect about 20% more positive sera than AGID test.[9]

Previous reports indicated that the AGID test can report false negatives, thus allowing the free movement of EIAV-infected equids, with an increase in the risk for transmission of the disease.[4,9] There are many reasons to explain the false negative results of the AGID test including misreading, especially with weak reactions, and some horses that simply fail to produce enough antibodies for detection by the AGID test.[4,9] In such circumstances, weak serological reactions are associated with new cases of animals that are at the initial phase of antibody production.[20] Hence, the diagnostic sensitivity (99.59%) of $ELISA_{gp90/45}$ is an essential characteristic; therefore, this test might be included in official programs for the control of EIA disease.

Only one sample had discordant results, being negative by $ELISA_{gp90/45}$ but positive by AGID test. Similar discrepant results, reported by Naves et al., were observed between the AGID test, IB and the indirect

ELISA, which uses a synthetic peptide gp45 as antigen with similar sequence to that of our gp45 peptide. [17] They found one donkey sample serum AGID test positive and negative by their ELISA and antibodies against gp90; gp45 and p26 protein in IB.[17] Discordant results could be explained in cases of equids that had been exposed to the same related Retrovirus (e.g. Bovine Immunodeficiency Virus). The gag gene protein determinants are conserved among different Retroviruses, like HIV, EIAV and Bovine Immunodeficiency Virus.[30] Serological cross-reactions between these lentiviruses are probably indicative of immune recognition of highly conserved conformational epitopes among these phylogenetically related retroviruses.[31–34] Another cause of cross-reactivity may be related to the impurity of the recombinant p26 antigen used in AGID test kits when it was produced using *Escherichia coli*. [24] This problem does not occur with synthetic peptides, used with more than 95% of purity.[4,24,35] This background of reactivity against the p26 antigen in equine populations can be best demonstrated using IB tests, and these samples should be considered false-positive when they only have antibodies that recognise the p26 antigen. Nevertheless, the anti-p26 cross-reaction rarely produces a response strong enough to result in an AGID test reaction that could be interpreted as positive.[18]

We recommend that, in future trials, samples with discordant results should be tested with the IB test, which detects antibodies against p26, gp90, and gp45 viral proteins. IB has higher sensitivity than the AGID test and it allows an earlier detection of infection, reducing the risk of transmission.[20] Moreover, negative results by different serological methods do not ensure that the horses are uninfected; indeed, we have demonstrated that some infected animals remained serologically negative for at least two years, although their sera were analysed by multiple assays, including the sensitive IB test.[36]

Taking into account the increasing evidence of the limitations of AGID test in diagnosing all EIAV-infected animals, it is essential to reconsider the OIE diagnostic recommendations of using only this test for international trade. To obtain higher diagnostic accuracy, both techniques should be considered in our country to combine the specificity of the AGID test with the higher sensitivity of the $ELISA_{gp90/45}$; thus, both anti p26 antibodies and both glycoproteins could be detected simultaneously, similarly to IB test.

One of the limitations of this study was that samples were derived from naturally infected equid populations showing heterogeneous clinical states. Thus, most of the sampled horses were asymptomatic during the inapparent stage and some of them had febrile episodes. Moreover, some horses were sampled on multiple occasions. In addition, the AGID test may not be the most useful gold standard. However, in Argentina as OIE recommendations, it is the reference method. For this reason, the AGID test was selected for this validation.

The current study is the first validation trial of indirect $ELISA_{gp90/45}$ to detect antibodies against two *env* EIAV antigens, following OIE criteria in Argentina. Since this validation is in compliance with

international standards, we will proceed with its submission for approval by Argentine veterinary health authorities, so that the *in-house* ELISA$_{gp90/45}$ can be employed to certify freedom from infection during the control or surveillance programs and to detect the presence of the agent in individual animals by private laboratories.

Authors' declarations of interest

No competing interests have been declared.

Ethical animal research

Research ethics committee oversight not currently required by this journal: the study was performed on material from research laboratory archives and excess sera collected previously during clinical procedures and health screening.

Informed consent

Explicit owner informed consent for inclusion of samples from client-owned animals in this study was not sought but owners were given the option to opt out of research.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Source of funding

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Authorship

A.R. Soutullo conceived and designed the study. R.C. Russi and L. Garcia contributed with the study execution and data interpretation. M.S. Cámara contributed with data analysis. A.R. Soutullo, L. Garcia and R.C. Russi wrote the manuscript. All co-authors approved the final version of the manuscript.

Table 1: Panels. Horse sera employed for the validation of indirect ELISA$_{gp90/45}$ for Equine Infectious Anaemia Virus.

Panel	Description
Panel 1	Reference sera, 7 sera 1a) Official SENASA National Reference Sera: 1 National Reference Strong Positive Serum (NRSPS). 1 National Reference Weak Positive Serum (NRWPS). 1 National Reference Negative Serum (NRNS). 1b) Internal Reference Sera: 1 Internal Reference Positive Control (IRPC): purified equine gamma globulin obtained from a naturally infected horse¶. 1 Internal Reference Strong Positive Serum (IRSPS) from pooled sera (30 positive sera). 1 Internal Reference Weak Positive Serum (IRWPS) from pooled sera (15 strong positive and 15 weak positive). 1 Internal Reference Negative Serum (IRNS) from pooled sera (30 negative). All sera included in this panel were analysed by AGID test.
Panel 2	Negative AGID test: reference samples analysed by Regional Laboratory of SENASA, 374 sera†, grouped as: 2.a) 201 sera from clinically healthy mixed-breed horses from different rural areas of Santa Fe, Entre Rios and Cordoba provinces without any cases of EIAV reported for the last10 years later. 2.b) 99 sera from 33 Thoroughbred horses for sport competition purpose (Polo), checked three times, every four months during a year, but exposed to an outbreak of EIA. 2.c) 40 sera from 40 Thoroughbred horses for sport competition purpose, without any reported outbreak of EIAV, but infected with other viral infectious diseases, Equine Herpes Virus-1 (20); Equine Herpes Virus-3 (20); Equine Viral Arteritis (20); Equine Influenza Virus (20) §. 2.d) 34 sera from healthy mixed-breed horses from rural areas of Patagonia, a region officially determined as free of EIAV infection, by SENASA¥.
Panel 3	Positive AGID test: Reference samples analysed by Regional Laboratory of SENASA, 333 sera†, grouped as: 3.a) 16 sera from 16 Thoroughbred horses for sport competition purpose, with EIAV acute disease, during the same outbreak of EIA, mentioned in panel 2.b. 3.b) 25 sera from Laboratory of References of Equine Infectious Anemia disease of SENASA, provided annually for validation of performance of official AGID test. 3.c) 42 sera weak positive by AGID test, from mixed-bred horses. 3.d) 189 sera from inapparent infected mixed-breed horses, localised in different stables near coastal areas, with prevalence higher than 20%. 3.e) 61 sera from EIAV-infected horses from different provinces of Argentina, confirmed by Immunoblot and/or PCR and/or Lymphoproliferation assays.

Panel 4	Precision panel from Laboratory of References of Equine Infectious Anemia disease of SENASA, provided for validation of performance of official AGID test. 3 strong positive (SP) sera 3 weak positive (WP) sera 3 negative (N) sera
Panel 5	1121 Field sera analysed by AGID test (243 positives, 878 negatives).

¶Kindly provided by Vet Eduardo Lucca, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral

†Calculated according to Jacobson et al. [14].

§Kindly provided by Dr Maria Edith Barrandeguy, Instituto Nacional de Tecnología Agropecuaria (INTA), Castelar, Buenos Aires, Argentina.

¥Kindly provided by Vet Carlos Vaghi. Servicio Nacional de Sanidad Animal (SENASA), Regional Patagonia Sur, Argentina.

Table 2: Diagnostic Sensitivity and Specificity. Results of $ELISA_{gp90/45}$ compared with those of AGID test for the evaluation of diagnostic sensitivity and specificity. Sera were collected from different regions of Argentina.

		AGID Test		
		NEGATIVES	POSITIVES	Total
$ELISA_{gp90/45}$	NEGATIVES	793	1	794
	POSITIVES	85	242	327
	Total	878	243	**1121**

Figure legends

Figure 1: Calibration and determination of working operation range. Linear range for Internal Reference Materials and National Reference Sera represented as OD (optical densities) vs. Log dilution. A-D: Values obtained from $ELISA_{gp90}$. E-H: Values obtained from $ELISA_{gp45}$. Fig.1A and 1E: IRPC vs. NRSPS; Fig. 1B and 1F: IRSPS vs. NRSPS; Fig. 1C and 1G: IRWPS vs. NRWPS; Fig. 1D and 1H: IRNS vs. NRNS. IRPC, Internal Reference Positive Control; NRSPS, National Reference Strong Positive Serum; IRSPS, Internal Reference Strong Positive Serum; IRWPS, Internal Reference Weak Positive Serum; IRNS, Internal Reference Negative Serum; NRNS, National Reference Negative Serum.

Figure 2: Optimization of the IRPC. Dose-response relationship between PPs Log dilution of the IRSPS (1:25 to 1:800). The PPs were calculated considering the OD of IRPC as 100% of PP, diluted 1:50 (blue) or 1:200 (black). Values obtained using synthetic peptide gp90 (Fig 2.a) or gp45 (Fig.2b) as antigen in ELISA. IRPC, Internal Reference Positive Control; IRSPS, Internal Reference Strong Positive Sera; PP, Percentage of Positivity.

Figure 3: Determination of diagnostic performance. A) ROC curve of ELISA using gp90 as antigen. B) ROC curve using gp45 as antigen. Sensitivity is represented on the Y axis and 100-specificity on the X axis for each PP value obtained in the $ELISA_{gp90/45}$. ROC, Receptor-Operator Curve.

Figure 4: Evaluation of Reproducibility. A) PP obtained for the 20 samples (8 replicates/sample) by each laboratory (A, B or C) using gp90 as antigen. B) PP obtained for the 20 samples (8 replicates/sample) from each laboratory (A, B or C) using gp45 as antigen. There is no significant difference in results among laboratories ($p>0.05$; Kruskal-Wallis test).

Supporting Information

Figure S1: Representation of validation process. The flow chart depicts the steps of the validation process of the $ELISA_{gp90/45}$.

Table S1: Synthetic peptides. Sequences of synthetic peptides used as antigens in $ELISA_{gp90/45.}$ Peptide gp90 represents C-terminal region in the glycoprotein gp90. Peptide gp45 represents a loop region in the N-terminal region of the glycoprotein gp45. Peptides gp90 and gp45 were used as linear and cyclic conformation, respectively.

Table S2: Intra-plate variability data. Mean optical density (OD) of 3 strongly positive sera (SP1, SP2, SP3), 3 weakly positive sera (WP1, WP2, WP3) and 3 negative sera (N1, N2, N3) evaluated in quadruplicate. The standard deviation (SD) of the mean OD and the coefficients of variation (CV) obtained for each gp90 and gp45 peptide are expressed.

Table S3: Inter-plate variability data. Percentage of Positivity (PP) of 3 strongly positive sera (SP1, SP2, SP3), 2 weakly positive sera (WP1, WP2) and 3 negative sera (N1, N2, N3) evaluated by three different analysts. The mean of the PP and the coefficients of variation (CV) obtained for each gp90 and gp45 peptide are shown.

Table S4: Analytical Sensitivity. Maximum reactive dilution of the positive and weak positive sera by AGID test and $ELISA_{gp90/45}$. Results: strong positive (++), weak positive (+), negative (-); W: weak; E: equivocal.

Table S5: Diagnostic reproducibility. Mean Percentage of Positivity (PP) of 8 negative sera (#1- #8), 8 strongly positive sera (#9-#16) and 4 weakly positive sera (#17-#20) evaluated in quadruplicate in three laboratories on two different days.

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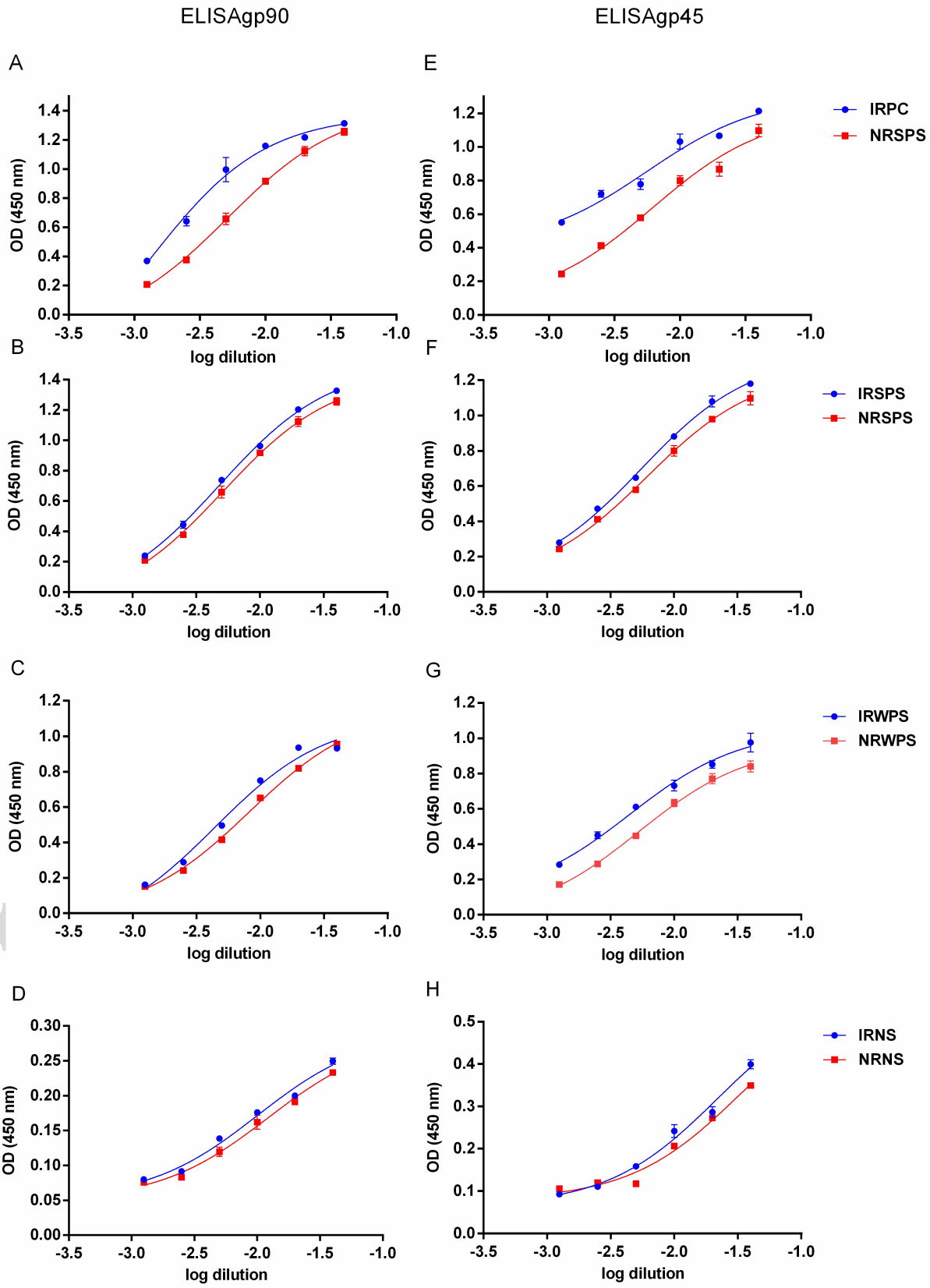

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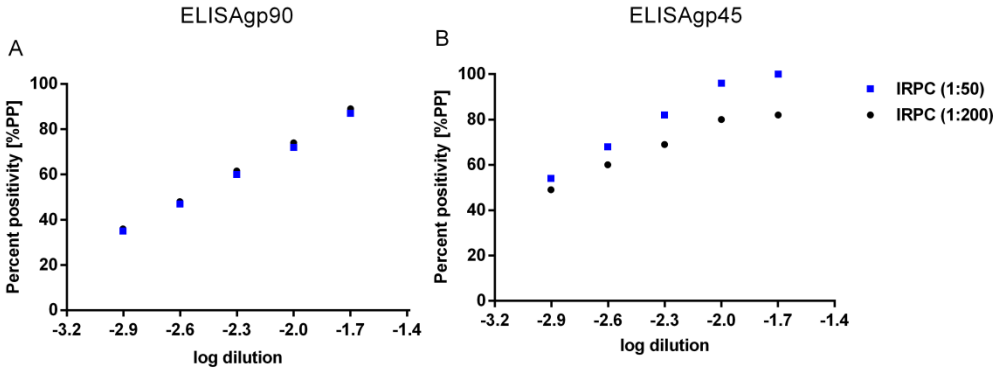

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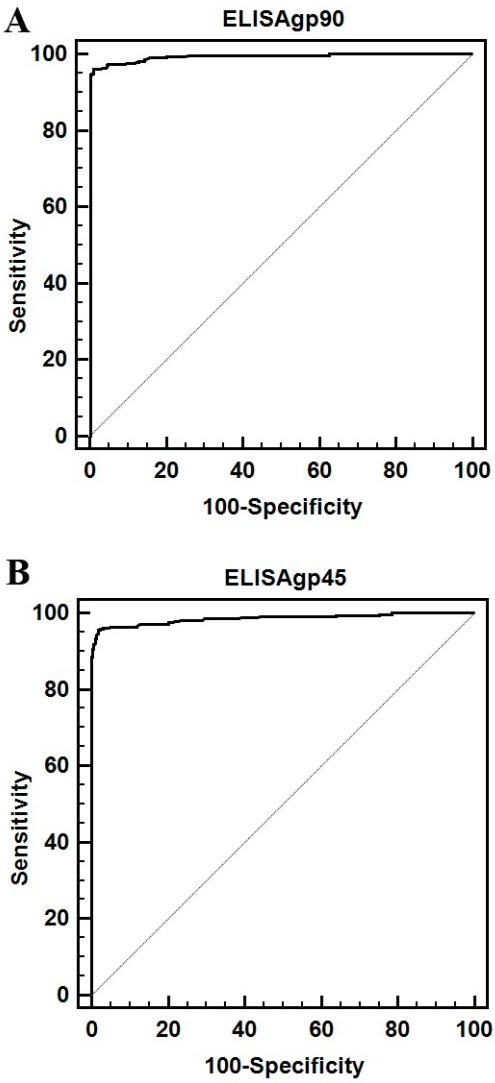

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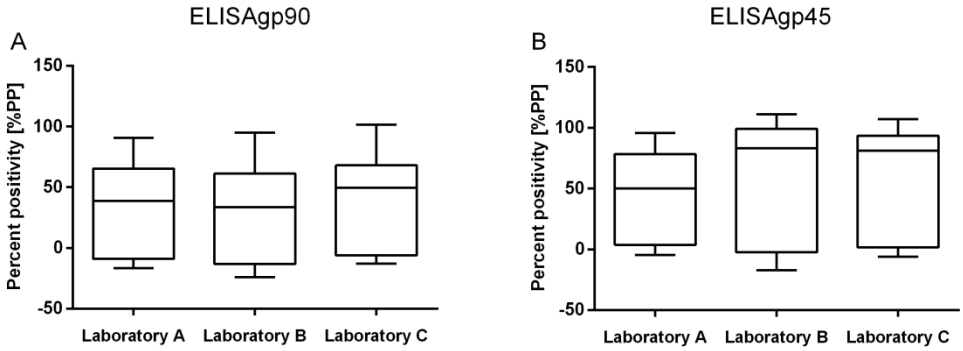

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