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Original Research

Evaluation of Equine Infectious Anemia Virus by the Indirect Enzyme-linked Immunosorbent Assay EIA-LAB as Screening Tools in Mexico

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ABSTRACT

Equine infectious anemia is a worldwide distributed disease that affects the *Equide* family. Commercial effective vaccine is not available, for that reason control of the disease depends on diagnostic tools. To improve the efficiency of the diagnostic program in Cuba, LABIOFAM Group, developed an indirect enzyme-linked immunosorbent assay (ELISA), ELISA kit, to complement the diagnostic system that currently uses the agar gel immunodiffusion (AGID) kit. The ELISA AIE-LAB Kit was evaluated in a Mexican context, compared with the gold standard test Agar gel immunodiffusion, AGID AIE-LABIOFAM, and commercial AGID kit. The analytical sensitivity was determined using serial dilutions twofold of the positive control serum to establish the range of detected antibodies in relation to the cutoff value of the plate (OD 0.300). A precision study was carried out to evaluate repeatability, intermediate precision, and reproducibility by estimating the standard deviation and coefficient of variation. The precision results were satisfactory and the values of the coefficient of variation were considered adequate to guarantee an excellent consistency of the ELISA AIE-LAB. The diagnostic performance of the ELISA AIE-LAB involved the evaluation of specificity, sensitivity, and concordance in comparison with both AGID tests. The diagnostic sensitivity was 100% and the specificity 97.6%, with a very good degree of concordance (Kappa = 0.9). The results suggest that the ELISA AIE-LAB test could be used in Mexico as a diagnostic system for the detection of specific antibodies against the equine infectious anemia virus, as per current international norms.

1. Introduction

Equine infectious anemia virus (EIAV) is a member of *Retroviridae* family, genus *Lentivirus,* recognized as a pathogen with significant economic importance for horse industry. Currently, there is no vaccine or treatment for EIA, and the control of the disease depends on diagnostic tools' efficacy [1].

Agar gel immunodiffusion (AGID) also known as Coggins test [2] is an effective tool for detection of specific EIAV antibodies based on the immunoreaction of antigen and samples' antibodies. As per the World Organization for Animal Health (OIE), AGID is considered as the confirmatory standard test for the diagnosis of the disease [3]. Results' interpretation is conducted by visual reading of a precipitation line that can be highly subjective generating many samples misinterpreted by inexperienced laboratory personnel [4].

During the last few years, the detection of EIA antibodies by enzyme-linked immunosorbent assay (ELISA) has been applied in some countries using different designs and antigens [5–7].

Animal welfare/ethical statement: The research was performed in accordance with the ethical standard laid down in the 1996 declaration of Helsinki and its later amendments.

Conflict of interest statement: The authors declare that they have no conflict of interest.

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Validation assays have pointed excellent agreement between these ELISAs and the gold standard test [6,7]. In addition, scientific reports have indicated that ELISAs are more sensitive technique than AGID, with a higher sample processing capacity [8–10].

Most AGID and ELISA diagnostic kits allow the detection of p26 antibodies, but in recent years, other EIAV proteins have been used as antigens for enhancing the sensitive of tests. Glycoprotein gp45 is an immunogenic viral envelope protein that mediates virus-host membrane fusion and has been used as antigen in different ELISA tests [11–13].

Routine diagnosis process established by Cuban sero-surveillance program for EIA use the antigen-antibody AGID-AIE Kit produced by LABIOFAM. Recently, this laboratory also has developed an indirect ELISA AIE-LAB test using gp45 synthetic peptide which has the advantage of allowing efficient and accurate processing of a lot of serum samples' number within a relatively short period and of providing an objective interpretation by the use of a spectrophotometer. Previews validation studies using equine serum from different regions of Cuba have indicated a good agreement between these ELISA and AGID-AIE LABIOFAM diagnostic kit. However, ELISA AIE-LAB has not been validated in other conditions outside Cuba. One of the evidences about value and efficacy of a diagnostic test is that capacity of giving successful results in other laboratories under different conditions and countries [14].

Mexico has a worldwide consolidated equine industry, with a population that was estimated on 6 385 102 animals, in accordance with data provided by FAOSTAT for 2018 [15]. Equine are valuable for agriculture, sport, and meat production [16,17] and for all these activities is important count with a certainly diagnostic system that establish that animals are free of EIAV. Information of the virus in Mexico is limited [18]. The commercially available cELISA (IDEXX) designed to detect antibodies to p26 that has been approved for EIA diagnosis in several countries. Recently a study of serological diagnosis using a commercial cELISA (IDEXX, USA) indicated the presence of EIAV in 37.7% of a 56 population of work equines at the municipality of Veracruz, Mexico [19].

For all that reason, the aim of this study was to evaluate the performance of ELISA AIE-LAB in a Mexican context, using AGID-AIE Kit (LABIOFAM, Cuba) and DyaSystems EIA-AGID test kit (IDEXX, USA) as quality control diagnostic kits. For the first time, we presented ELISA AIE-LAB results, a designed and produced kit by Cuba industry, used for the diagnostic of EIAV with Mexican samples.

2. Material and Methods

2.1. Serum Samples

A panel of 96 individual horse serum samples (86 negative and 10 positive) collected from State of Mexico, donned by the Research and Advanced Studies in Animal Health Center (CIESA) and National Center of Animal Health Diagnostic Services (CENASA) was used in the study for the evaluation of ELISA AIE-LAB.

2.2. Agar Gel Immunodiffusion, AGID Test

AGID-AIE Kit (LABIOFAM, Cuba) and DyaSystems EIA-AGID test kit (IDEXX, USA) were used following the manufacturer's recommendations and also OIE manual indications for EIA diagnosis [3]. AGID tests were performed, in a 15 × 90 mm diameter petri dish with 15 mL of 1% Noble agar. After hardened, agar was perforated with a mold that originates a central and six peripheral wells. The measure of these was 5.3 mm in diameter and 2.4 mm of distance between wells. Antigen p26 protein (24 μL) was placed in the central well and positive control sera interleaved with diagnostic target serum samples (24 μL) were placed in peripheral wells. The AGID test results were interpreted either as positive by visual reading of precipitation line curvature or negative by the absence of the line, after 48 hours–72 hours of incubation at room temperature (20°C–25°C). The AGID tests were considered valid only if the negative and positive controls included on each test plate yielded the expected results. Samples were retested when interpretation of the results was doubtful.

2.3. Enzyme-linked Immunosorbent Assay

ELISA test was carried out following the manufacturer instructions and OIE manual recommendations [3]. Serum samples (5 μL) and buffer dilution (95 μL) were added in microtitration plates provided by the manufacturer and incubated at 37°C for 1 hour. Then six steps of washed with buffer (PBS-Tween 20) were carried out and anti-equine IgG-conjugated peroxidase (100 μL/well) was added. An incubation step was performed at 37°C for 1 hour and later plates were washed four times. Orthophenylenediamine tablet (Sigma-Aldrich, USA) in phosphate-citrate substrate buffer (100 μL) was added per well and incubated at 37°C for 15 minutes. Finally, reactions were stopped with 100 μL of 2M H_2SO_4, and absorbance was read at 492 nm in a microplate SpectraMax M5 reader (Molecular Devices, USA).

2.4. Data Analysis

Some general aspects were analyzing such as content availability of the liquid components, manual of instruction, and organoleptic properties by visual examination. The analytical sensitivity was evaluated using twofold serial dilutions of the positive control serum from 1/5 to 1/1280, to determine the maximum dilution where antibodies were detected considering the cutoff value of the plate, optical density (OD) 0.300.

A precision study was performed to estimate the repeatability, intermediate precision, and reproducibility. The repeatability was determined using 12 replicates of positive and negative serum control from the ELISA and a positive field serum. For the intermediate precision estimation, the design was the same as the repeatability assay but evaluated in three different days. The reproducibility was assessed in two laboratories with different equipment and personnel. The standard deviation and the intra-assay and interassay variation coefficients (CV) were also calculated [20]. The diagnostic performance of ELISA AIE-LAB included the evaluation of specificity, sensitivity, and concordance against both AGID tests [21,22]. In addition, robustness of ELISA was demonstrated by the evaluation of absorbance value using conjugate reagent after 24 hours at room temperature.

3. Results

ELISA AIE-LAB kit contains 10 components that were supplied in enough quantity for the evaluation of 192 samples. Liquid components showed good transparency without sediments in none of the flasks. In addition, the color of these components did not change in the flasks or in the microplate during the assays. The evaluation of ELISA AIE-LAB robustness challenging the anti-IgG equine peroxidase conjugate at room temperature for 24 hours did not affect its functionality. Furthermore, changes of the physical appearance of the sera samples (lipemic and hemolytic sera) did not influence in the expected results.

ELISA AIE-LAB allowed the detection of antibodies from a 1/5 dilution of the positive control serum to a 1/160, which represents the detection limit of the assay, maximum and minimum range of

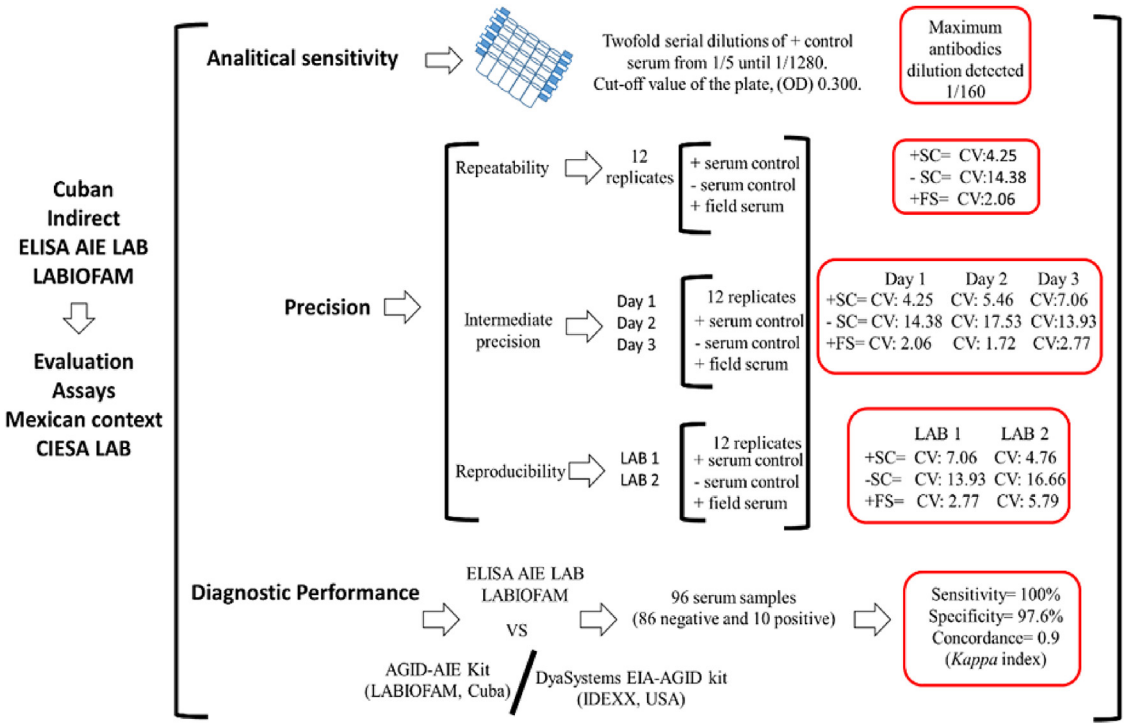

Fig. 1. ELISA AIE-LAB evaluation assays for performance of diagnostic kit in Mexico.

the OD values detectable with the kit. The controls' sera results were OD of positive control serum > 1.0 and OD of negative control serum between 0.025−0.05 and (P/N) ≥ 6.0 (P: OD media of positive control and N: OD media of negative control).

The validation parameters evaluated were repeatability and reproducibility based on standard deviation (SD) and CV estimation. In addition, accuracy was estimate from concordance *(Kappa* index), diagnostic sensitivity, and specificity with respect to those of the AGID (Fig. 1). For the repeatability trials (intraplate variability), all CV values were less than 20% as expected for both controls' serums and a positive serum from CENASA, Mexico (Table 1). The CV values confirmed the satisfactory compliment of valid parameters of ELISA AIE-LAB in the repeatability assay. The results of repeatability trials to determine the intermediate precision of the system are summarized (Table 2). The reproducibility results of trials showed CV values of 0.24 for positive control serum, 11.72 for negative control serum, and 2.26 for positive serum from SENASA, Mexico (Table 3). All CV values obtained were lower than 20%. The precision results (repeatability and reproducibility) were satisfactory, and CV values were considered adequate to guarantee excellent precision and consistency of the ELISA AIE-LAB (Fig. 1).

Correlation between ELISA AIE-LAB and AGID test results was similar for both AGID techniques used as confirmatory control test. Only two samples (sample 2 and 3) were identified as reactive serums by ELISA AIE-LAB and were detected as negative by both AGID test (Table 4) (Fig. 2).

The parameters of performing diagnosis for the ELISA in comparison with the AGID technique are showing in Table 5. The ELISA AIE-LAB showed a 100% sensitivity and 97.6% specificity, with a concordance 0.9, values considered adequate for the test.

4. Discussion

Equine infectious anemia virus causes a persistent infection where animals remain viraemic carriers for life. Antibody response usually persists and antibody-positive animals older than 6 months are identified as infectious with the potential to transmit the virus to other horses. Currently, there is no vaccine or effect treatment for EIA, and that is why the control strategy consists in the detection and segregation of infected animals [1,3]. The AGID or Coggins test have been used since 1973 [23] as the official diagnosis test established by OIE, also prescribed as mandatory test for international movement of equine [14]. However, this test has a low sensitivity which can originate false results making more difficult of the control and eradication of EIA. Even though the specificity of the AGID test is very high, its sensitivity is lower, and horses can transmit EIAV even though their AGID tests were negative. In addition, to develop a more rapid and sensitive diagnostic tools, several laboratories have developed ELISA [7,10].

The ELISA AIE-LAB is an indirect heterogenic system designed and produced in Cuba with the purpose of enhancing the efficiency of the National Diagnostic Program of EIA. The ELISA EIA-LAB uses a synthetic peptide from gp45 protein as antigen for detection of EIAV antibodies presenting advantages over the use of natural antigens because they increase the sensitivity and eliminates crossed reactions [24]. The primary goal of this study was to evaluate the

Table 1
Repeatability study of the ELISA AIE-LAB (intraplate variability).

Positive Control Serum			Negative Control Serum			Positive Serum		
X	SD	CV	X	SD	CV	X	SD	CV
1.92	0.08	4.25	0.09	0.01	14.38	1.83	0.04	2.06

Abbreviations: X, mean value; SD, standard deviation; CV, coefficient of variation.

Table 2
Intermediate precision of ELISA AIE-LAB.

Days	Positive Control Serum			Negative Control Serum			Positive Serum		
	X	SD	CV	X	SD	CV	X	SD	CV
1	1.92	0.08	4.25	0.09	0.01	14.38	1.83	0.04	2.06
2	2.15	0.12	5.46	0.09	0.02	17.53	1.93	0.03	1.72
3	2.24	0.16	7.06	0.12	0.02	13.93	1.96	0.05	2.77

Abbreviations: X, mean value; SD, standard deviation; CV, coefficient of variation.

ELISA AIE-LAB performance in CIESA laboratory of UAEM University. The components provided in the kit presented adequate organoleptic characteristics (color and texture) in accordance with establish by the manufacturer. The color present in positive and negative serum controls facilitates identification of the reagents. The design of color change of control serum at contact with the reagent sample diluent allows easy visualization in the support of the reaction. The chemical properties and functional characteristic of the components were satisfactory to achieve established limit of OD. The detection limit established by the manufacturer provided an extensive range of dilutions for the positive serum that increase the sensitivity of the technic. The anti-IgG equine-peroxidase conjugate activity was not affected by change in temperature. All these results were a sample of the robustness of the ELISA AIE-LAB, which indicates the ability to maintain the same results in the face of small changes that may occur during the test.

The repeatability is an indicator of the coincident results between the replica of the same sample in one or different trials. At least a minimum of three samples representing analytic activity within the operating range of the assay have to be used for the repeatability evaluation. Reproducibility is the ability of a diagnostic test to provide consistent results, as a parameter of precision, when the evaluation of aliquots of the same sample tested with a kit in different laboratories, located in distinct regions or countries, achieves the same results [14].

ELISA EIA-LAB showed a satisfactory result in repeatability trials (intraplate variability) and reproducibility because all CV values were less than 20%. The CV values of positive serum were less than 15%, however, CV of the negative serum had a different behavior associated with low-OD values (<1.0). Small numerical variations in OD of negative serums can produce higher CV values because the calculation of CV (CV = standard deviation/mean of OD) using a low value of denominator (low mean OD) mathematically produce a higher numerical result. These results are in agreement with another ELISA kits in repeatability assay when CV values obtain had been 2.8% [11] and 12.73% [25] for positive sera and less than 20% for the negative serum [26–28]. Previously reported CV values of reproducibility for positive samples have been between the range of 3 and 15% [11,13] and CV values for negative samples even more than 20% [11,25].

The comparison of ELISA EIA-LAB and AGID test originated two discordant results, which have been expected because OD values for these two serums were around the gray zone established by the manufacturer, which indicates that these are weak serums and are not visible in the precipitation line by AGID. Lower antibody concentration in a serum sample may originate negative AGID interpretation, while are positive by ELISA [5,10,11]. In addition, positive ELISA results may be due to the presence of gp45 antibodies, produced in the early stages of viral infection, which are not possible to detect by AGID test because it only uses the p26 protein as an antigen [27]. Immune response against gp45 and gp90 envelope proteins, encoded by *env* gen [29], is developed in first stage of infection. Previous studies have reported that antibodies against capsid p26 protein are produced after glycoproteins antibodies because gp45 is a transmembrane protein and gp90 an integral membrane protein, both more exposed from immune system recognition [30,31]. Despite the rapid and high rate of *env* gene mutations, as a viral mechanism for evasion of the immune system response, conserved regions in the *env* sequence have been identified for the diagnostic and production of immunogenic peptides [24,32,33].

Table 3
Reproducibility results of ELISA AIE-LAB.

Laboratory	Positive Control Serum			Negative Control Serum			Positive Serum		
	X	SD	CV	X	SD	CV	X	SD	CV
Lab 1	2.24	0.16	7.06	0.12	0.02	13.93	1.96	0.05	2.77
Lab 2	2.25	0.11	4.76	0.11	0.02	16.66	1.89	0.11	5.79

Abbreviations: X, mean value; SD, standard deviation; CV, coefficient of variation.

Table 4
Diagnosis Comparison of AGID EIA IDEXX Commercial System: AGID AIE-LABIOFAM and ELISA AIE-LAB.

ELISA AIE-LAB	AGID IDEXX and AGID AIE-LABIOFAM		
	Positive	Negative	Total
Positive	10	2	12
Negative	0	84	84
Total	10	86	96

Enzyme-linked immunosorbent assay system reached an appropriate performance in comparison with AGID, which is the method accepted for EIA diagnosis by the OIE. The analysis of the results demonstrates high levels of sensitivity (100%) and specificity (97.6%), despite the low number of samples evaluated. The strong points of the ELISA AIE-LAB arising from this assay were diagnostic sensibility and precocity, which could be due to the antigen used. False negative results can be the product of low specificity or sensitivity of diagnostic test, making difficult to establish a good control program for disease eradication [4]. Different classical methods are been available for antigens preparation, usually using infected spleen or primary cell culture equine. These techniques have disadvantages because are expensive, laborious, and the antigen can be contaminated by another proteins, generating nonspecific results in diagnostic tests [14]. Alternative antigen production for serologic diagnosis confers enhancements in the techniques and consequent improvement of the programs for EIA eradication [22]. Many authors have reported the use of envelop synthetic peptides (gp45) as antigen for ELISA with excellent agreement in comparison with AGID [12,13]. The calculated ELISA AIE-LAB effectiveness was bigger than 97%, and it represents a general capacity to detect all true positive and negative serums correctly. The ELISA AIE-LAB was designed for the screening of the equine infectious anemia, aiming to bring high sensitivity and specificity. The concordance degree between both methods tested was very good (Kappa = 0.9) and demonstrated that this system is appropriated for the purpose it was designed [14,34]. Control's serum OD values were correct, and the assay was not invalidated. No change of coloration, or sedimentation was observed in the flask of the conjugate. The distilled water used to dilute the washing solution did not modify the functionality of ELISA. The behavior of the general stability of the components and their functionality after the proper transfer was demonstrated by the OD values of the controls and the result of the precision trial.

In addition, our results agree with those presented recently where an ELISA using peptides of the gp45 protein as antigen was evaluated for the diagnosis of serum samples from horse, donkey,

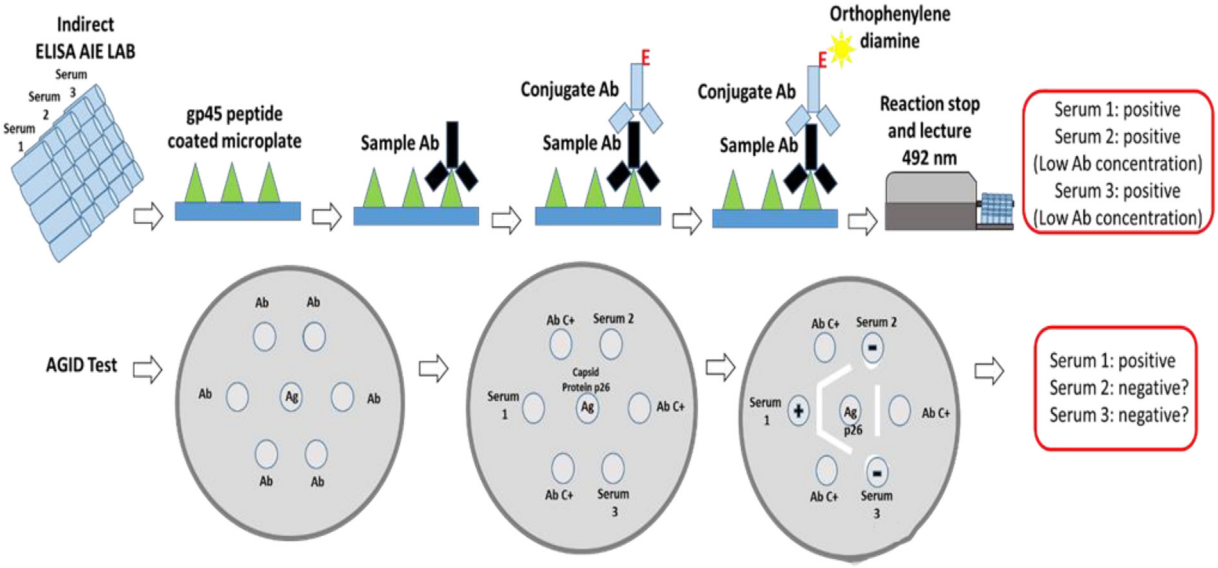

Fig. 2. Principle and method of ELISA AIE-LAB and AGID test.

and mule, with 96.1% concordance, 98.6% sensitivity, and 95.6% specificity compared with AGID. The sensitivity and specificity of this ELISA was also > 90% when tested in individual equid species, indicating the potential of the gp45 antigen–based diagnostic design, especially in donkeys and mules, which have a demonstrated tendency of equivocal results in AGID [35].

The ELISA is a technique that offers greater sensitivity and diagnostic specificity, capable of detecting EIAV-specific antibodies between 10 and 14 days after infection [35], whereas the AGID test does not have the sensitivity to detect antibodies during the first day of infection. Unfortunately, animals with low titers of antibodies can originate false negative results, if only AGID test is used in diagnostic schedules. This situation is very important due to enhance risk of widespread of the disease because positive animals escape the diagnostic filter, allowing the mobilization of infected animals [4]. Despite AGID test limited capacity due to low-diagnostic sensitivity and difficult line interpretation by visual recognition, this technique continues as the established official test for diagnosis of EIA [3]. Consequently, some countries have been established the ELISA as the sero-screening official diagnostic tool, with the confirmation of positive samples by AGID test, before taking any action with the infected animal [3,5,34]. Surveillance programs in Mexico establish AGID kit as diagnostic test after OIE indications. Contrasting with the official stablished immunodiffusion test, ELISA is an immunoenzymatic technique that allows obtaining results in short periods of time, being more effective to issue criteria and authorization for the transfer of animals, adding efficiency to equine production and activities. Furthermore, recent studies have shown that the scientific production dedicated to the investigation of this virus in Mexico is deficient, despite being one of the main producing countries of equines [18]. The implementation of the diagnosis using ELISA systems in Mexico could be an alternative to expand EIA screening studies, as well as carry out epidemiologic and prevalence analyzes in higher risk areas because it is an economical technique with high capacity for processing a large number of samples in a single round test. Taking to account the results obtained in the present work, we propose the use of ELISA EIA-LAB as screening tool for routine diagnosis and confirmation of positive samples by AGID the gold standard test.

Table 5
Evaluated parameters of ELISA AIE-LAB relative to commercial AGID IDEXX and AGID AIE-LABIOFAM.

Parameter	Percent (%)
Sensitivity	100
Specificity	97.6
PPV	83.3
PNV	97.67
Efficacy	97.91
Concordance (kappa)	0.9 (very good)

Abbreviations: PPV, predictive positive value; PNV, predictive negative value.

5. Conclusions

The ELISA AIE-LAB shows good results as a diagnostic system and could be used in Mexico for the detection of specific antibodies against the EIAV. Considering the limitations of the AGID test, many authors share the idea that the OIE diagnostic recommendations for international trade in equines need to be modified by adding the ELISA in the routine diagnosis. In addition, the combination of the ELISA and AGID test as a diagnostic algorithm confers greater sensitivity and specificity, which could improve the accuracy of EIA surveillance programs in Mexico.

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