Original Article

Rabbit polyclonal antibody against Erns protein for the detection of Classical Swine Fever Virus

Anticuerpo policlonal de conejo contra la proteína Erns para la detección del virus de la peste porcina clásica

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ABSTRACT: Classical swine fever (CSF) is an endemic disease in many countries. A key tool for laboratory confirmation of CSF is a differential diagnosis between vaccinated and actually infected pigs. Many enzyme-linked immunosorbent assays (ELISA) are based on the capture of antibodies in animal sera, but these tests cannot be used in animals that are persistently infected. In these cases, an antigen-capture ELISA may be a good choice to differentiate vaccinated animals from those that are infected. In order to produce a polyclonal antibody that allows this technique, two rabbits were immunized with recombinant Erns protein. The antibody was conjugated to horseradish peroxidase (HRP) and the optimal working dilution was 1:64 000 in a direct ELISA. Different coating conditions were evaluated to trap recombinant Erns in a sandwich ELISA and it was concluded that coating at 10 µg/mL would ensure greater capture. The ELISA was able to detect Erns from a mixture of sera from pigs vaccinated with the Chinese strain of classical swine fever virus (CSFV). In addition, no reaction was observed in sera from healthy animals or in sera from animals vaccinated with Porvac®, an E2 protein subunit vaccine. It was concluded that the generated polyclonal antibody can recognize the viral Erns protein in pig sera, so it could be used in a sandwich ELISA to differentiate healthy animals from those infected with CSFV.

Key words: anti-Erns polyclonal antibody, CSFV, ELISA.

RESUMEN: La peste porcina clásica (PPC) es una enfermedad endémica en numerosos países. Una herramienta clave para la confirmación en laboratorio de la PPC es el diagnóstico diferencial entre cerdos vacunados y los que están realmente infectados. Muchos ensayos inmunoadsorbentes ligados a enzima (ELISA) están basados en la captura de anticuerpos en los sueros, pero estos ensayos no pueden utilizarse en aquellos animales que están persistentemente infectados. En tales casos, un ELISA de captura de antígeno podría ser una buena elección para diferenciar los animales vacunados de aquellos que están infectados. Con el objetivo de producir un anticuerpo policlonal que permita esta técnica, se inmunizaron dos conejos con proteína Erns recombinante. El anticuerpo fue conjugado a peroxidasa de rábano picante (HRP) y la dilución óptima de trabajo fue de 1:64 000 en un ELISA directo. Se evaluaron diferentes condiciones de recubrimiento para atrapar Erns recombinante en un ELISA y se concluyó que el recubrimiento a 10 µg/mL aseguraría mayor captura. En el ELISA se pudo detectar la Erns de una mezcla de sueros de cerdos vacunados con la cepa china del virus de la peste porcina clásica (VPPC). Además, no se observó reacción en sueros de animales sanos ni con sueros de animales vacunados con Porvac®, vacuna de subunidad basada en la glicoproteína E2. Se concluyó que el anticuerpo policlonal generado puede reconocer la proteína Erns viral en sueros porcinos y que podría ser utilizado en un ELISA sándwich para el diagnóstico de la PPC, en aquellos animales persistentemente infectados o inmunodeprimidos.

Palabras clave: anticuerpo policlonal anti-Erns, VPPC, ELISA.

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INTRODUCTION

CSF is highly contagious viral disease affecting domestic pigs and wild boar. It is endemic in several South and Central American countries, in the Caribbean region, as well as in Asia, and in some Eastern European countries (1). The economic and social consequences of its impact place CSF on the list of notifiable diseases for the World Organization for Animal Health (2). Its etiologic agent is CSFV, an enveloped virus with a single plus strand RNA genome, included on the genus *Pestivirus* in the Flaviviridae family (3).

The serological analysis is a very used tool for the diagnosis of CSF (4). However, pigs born from vaccinated mothers may present anti-CSF antibodies by passive immunity. This, although giving them clinical protection, does not imply that animals are free from infection (5). In addition, persistently infected pigs with low or moderate virulent CSF isolate do not develop immune response, even when they shed high viral load in their excretion (6). Reverse transcription-polymerase chain reaction (RT-PCR) assay is the best tool available for the diagnosis of CSF (4), but this technique could be costly in many countries. Hence, ELISA antigen tests could be a useful and easily performed tool for this purpose.

Currently there are numerous ELISAs to detect viruses and antibodies to evaluate the effectiveness of developed vaccines. Glycoproteins E2 and Erns are among the most immunogenic proteins of CSFV, both of them are candidates for the development of a DIVA vaccination system (7). The glycoprotein Erns is the second most immunogenic within the viral envelope, and it is able to induce a neutralizing antibody response, which explains its use as an attractive protein for the immunological diagnosis of infection by CSFV (1). The available ELISA tests in the market are expensive to monitor CSFV in developing countries (8). An anti-Erns antibody that allows the development of an ELISA to capture Erns in samples from infected animals is advantageous for the differential diagnosis of CSF as accompanying test to E2 marker vaccine. This would ensure in the diagnosis of animals to differentiate among the immunized, the infected and the healthy. Therefore, the aim of this work is to produce a polyclonal antibody capable of recognizing viral Erns in pig sera.

MATERIALS AND METHODS

Purification of Erns antigen

The protein was extracted from *Escherichia coli* BL21 DE3 cells transformed with pET22b-Erns, which expressed a truncated form of Erns fused to a 6-histidine tag at the C-terminal end and to pelb leader sequence by the N-terminal end. Chromatography was performed by metal chelate affinity chromatography (IMAC) under denaturing conditions, using 6.5 mL of Chelating Sepharose ™ Fast Flow matrix charged with Ni^{2+} and packed in a C10/10 column (GE Healthcare). The column was equilibrated with binding buffer (20 mM Tris-HCl, 200 mM NaCl, 2 mM Imidazole, 8 M Urea, pH 6) at a volumetric flow rate of 0.6 mL/min. Purification processes included a washing with 50 mM Imidazole, and protein elution was performed with 200 mM Imidazole.

SDS-PAGE

Protein electrophoresis was performed on a 12 % polyacrylamide denaturing gel with 2 % Sodium Dodecyl Sulfate (SDS). Samples were diluted in loading buffer (125 mM Tris-HCl, pH 6.8, 1 % (w/v) SDS, 5 % (v/v) glycerol, 10 mM Dithiothreitol, 0,005 % (w/v) Blue Bromophenol) and boiled for 10 minutes. The samples were run for about 45 minutes at a constant voltage of 200 V in running buffer (25 mM Tris-HCl, pH 8.8; 192 mM Glycine; 35 mM SDS). The gel was stained with a 0.1 % (w/v) Coomassie R-250 solution, 40 % (v/v) methanol, 10 % (v/v) acetic acid for 20 minutes and the bands were visualized after gel destaining with a 10 % (v/v) methanol and 10 % (v/v) acetic acid.

Detection of proteins by western blotting

For western blotting assay, proteins were transferred (100 V, for 1 h) from the polyacrylamide gel to a PVDF membrane (Roche Diagnostics, Germany). As the primary antibody,

the purified anti-Erns polyclonal antibody was used (5 µg/mL) in skimmed milk powder (1 %) - PBS-1X-Tween 20 (0.05 %). As the secondary antibody, an anti- rabbit IgG conjugated to alkaline phosphatase diluted 1: 20000 (Sigma Aldrich, USA) was used. Colorimetric detection was performed with 5-bromo-4-dichloro-indolyl phosphate and nitroblue tetrazolium (Promega, USA).

Immunization of rabbits with Erns antigen

Two white New Zealand male rabbits weighing approximately 1.5 kg, supplied by the National Laboratory Animal Production Center (CENPALAB), were immunized subcutaneously with 217 µg of purified Erns each one. The protein was previously diluted until the urea decreased from 8 M to 2 M, to avoid toxic effects for immunized animals. For the first dose, the antigen was emulsified with a 1:1 ratio in complete Freund's adjuvant (CFA). Six subsequent immunizations were performed every 28 days; the first two with 100 µg and the rest with 50 µg of antigen emulsified with a 1:1 ratio in incomplete Freund's adjuvant (IFA). Blood samples were taken by puncture of the ear vein to sera titration. At the end of the immunization scheme, exsanguination was performed by cardiac puncture. Collected blood was incubated for 30 min at 37°C and then for 30 min at 4°C. The serum was extracted by centrifugation at 600 x g during 20 min. Animal experiments were carried out in accordance with the legal requirements of the national authorities.

Purification of polyclonal anti-Erns antibody

A volume of 10 mL of immunized rabbit serum was filtered through glass wool and precipitated by 50 % (w/v) of $(NH_4)_2SO_4$. The resulting pellet was resuspended into 3 volumes of binding buffer (1.5 M Glycine, 3 M NaCl, pH 8.9) and charged to an nProtein A Sepharose Fast Flow (GE Healthcare) matrix packaged in a C10/10 column (GE Healthcare) with a height of 8.5 cm and equilibrated with the same buffer. Elution was performed at acidic pH with 0.1 M citrate buffer pH 3.0. The flow was kept constant at 0.6 mL/min throughout the process. The chromatographic profile was monitored by measuring absorbance at 280 nm. The eluate was neutralized with 2 M Tris-HCl and dialyzed on 20 mM Tris-HCl, 150 mM NaCl pH 7 for 16 h, and then it was filtered by 0.2 µm membranes. Thimerosal was added as a preservative at a final concentration of 0.02 % (v/v). The purity was evaluated by SDS-PAGE under reducing conditions, and the protein concentration was determined by Lowry´s method using bovine serum albumin as standard (9).

ELISA

Immunoenzymatic assays were performed on a 96-well polystyrene microtiter plate (Greiner, Microlon, and High Binding Germany). The coating was performed in 0.1 M carbonate buffer pH 9.5 during 1h at 37°C with purified Erns (10 µg/mL) in titration ELISAs and anti-Erns polyclonal antibody in all antigen capture ELISAs. In blocking step, skimmed milk powder 3 % (Oxoid, UK) (w/v) in PBS was used for 1 h at 37°C. In the case of the conjugate in indirect ELISA, an anti-rabbit IgG antibody coupled to peroxidase produced in sheep was used, according to the supplier (CIGBSS, Cuba). In the direct ELISAs, the obtained polyclonal antibody, conjugated to peroxidase, was used. All washing steps were performed with wash solution (50 mM Tris, 140 mM NaCl, 0.05 % Tween 20). Dilutions of the samples and antibodies were performed in a 1 % (w/v) skimmed milk powder solution in PBS buffer, and incubations were performed at 37°C in a wet chamber. In all assays 100 µL were added per well except the blocking step which was carried out with 300 µL. The revealed reaction was developed with a solution of Ortho-phenylenediamine (0.5 mg/mL, dissolved in 0.0243 M citric acid with 0.0514 M disodium hydrogen phosphate and 0.015 % hydrogen peroxide). The development time was of 15 minutes in all cases and reaction was stopped with 2 M H_2SO_4. The reading was carried out at 492 nm in a plate reader (Multiskan Titertek).

Conjugation of anti-Erns polyclonal antibody to horseradish peroxidase

Conjugation was performed by the periodate method (10). Briefly, 1 mL of purified antibody

(8 mg/mL) in carbonate buffer 10 mM (pH 9.5) was added to the activated enzyme and it was put on the stirrer for 2h at 25ºC. Then, 0.4 mg of sodium borohydride was added to the conjugated solution. Precipitation by ammonium sulfate was carried out and pellet was resuspended in 1.5 volumes of PBS. The conjugated solution was dialyzed in PBS buffer, and bovine serum albumin (BSA) was added at a concentration of 10 mg/mL. After BSA was completely hydrated, an equal volume of 99 % glycerol was added along with thimerosal solution to a final concentration of 0.01 %.

RESULTS AND DISCUSSION

Antibodies against CSF markers, like Erns protein, are vital for tests based on antigen capture such as antigen capture ELISAs. An important step for this type of ELISA is to ensure biological reagents (antibodies) with high purity to avoid unexpected results in the test.

Purification of the polyclonal anti-Erns antibody from the sera of the immunized rabbits

In order to clarify the serum, it was filtered by glass wool to contribute to delipidation and avoid unwanted interactions with the matrix. The purification of anti-Erns antibodies was carried out by Protein A affinity chromatography. An eluate with 32 mg of total IgG (4 mg of IgG/mL of Protein A matrix) was collected with purity higher than 90 %. One of the most frequent contaminants in antibody purification is albumin, due to its high concentration in animal sera. In order to eliminate part of the total albumin into the clarified serum, it was precipitated with ammonium sulfate. Regarding the chromatogram of the process (Fig. 1A), it could be seen that the peak of the elution corresponded to a high purity fraction of the polyclonal antibody (Fig. 1B). The absorbance corresponding to the non-absorbed fraction indicated that the clarification of the serum with glass wool and the ammonium sulfate precipitation contributed to the purity of eluted antibody as they prevented the overloading of the matrix. However, a band of approximately 70 kDa was observed in the not-bound fraction corresponding to albumin, which gave an idea of its high amounts in serum. (Figure 1B).

Specificity and cross-reactivity of the anti-Erns polyclonal antibody

To determine the reactivity of the purified polyclonal antibody with the corresponding protein, a western blot assay was performed using the previously purified protein. The

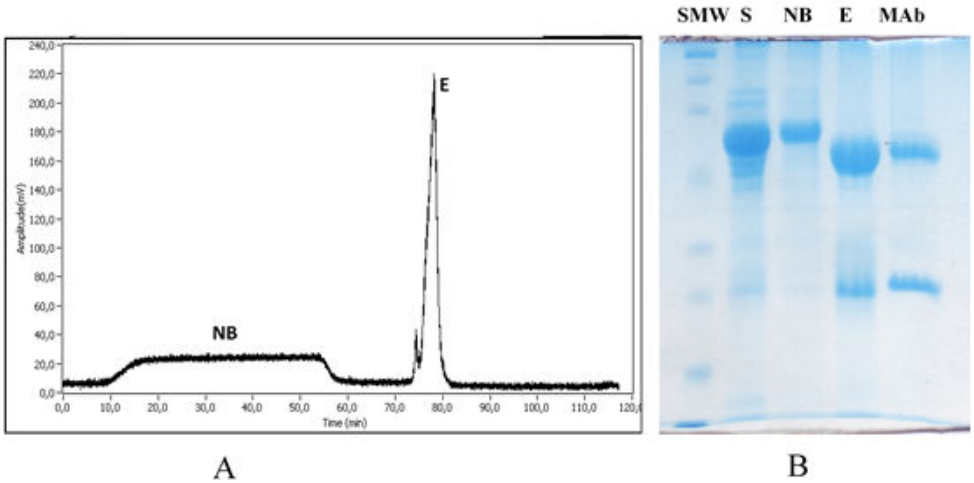

Figure 1. Chromatogram (A) and 12 % polyacrylamide denaturing gel electrophoresis (B) of Protein A affinity purification of rabbit anti-Erns polyclonal antibody. NB: proteins that not stick to the matrix. E: antibody elution at pH 3. SMW: standard molecular weight Pre-stained SDS PAGE Standard Broad range- Biorad. **S:** Rabbit serum immunized with Erns. **E:** elution at pH3 of Protein A sepharose matrix (polyclonal anti-Erns antibody). MAb: anti-IL2h monoclonal antibody used as control / *Cromatograma (A) y electroforesis en gel desnaturalizado de poliacrilamida al 12% (B) de la proteína A, purificación de la afinidad del anticuerpo policlonal anti-Erns de conejo. NB: proteínas que no se adhieren a la matriz. E: elución de anticuerpos a pH 3. SMW: peso molecular estándar Pre-teñido SDS PAGE Estándar de amplio rango- Biorad. S: suero de conejo inmunizado con Erns. E: elución a pH 3 de la matriz de la proteína A sefarosa (anticuerpo policlonal anti-Erns). MAb: anticuerpo monoclonal anti-LI2h usado como control.*

antibody could recognize a band of approximately 19 kDa, corresponding to the truncated Erns (Fig. 2B). This implied that the immunization procedure was efficient.

The reactivity of the antibody against other unrelated proteins was assessed by an indirect ELISA (Fig. 2A). The antibody showed no reactivity against any of the adjuvants used in immunization (complete and incomplete Freund's adjuvants). As unrelated protein, the recombinant p24 (belonging to the human immunodeficiency virus) was used. This protein had a 6 histidine tag as well as the truncated Erns used in immunization. No reaction indicated that there were not antibodies in the serum capable of recognizing this fragment used in the production of chimeric proteins. Another negative control used was the extract of *Escherichia coli* BL21-DE3 transformed with the PET-22 plasmid, but without the insert containing the sequence encoding the truncated Erns.

The reactivity of the antibody was observed against proteins of the strain used for the production of the antigen. However, the antibody (10 µg/mL) had a 5-fold higher affinity for the truncated Erns than for *E. coli* contaminants and the monoclonal antibody evaluated as unrelated protein. Besides, upon dilution of the antibody up to 1250 ng/mL, the corresponding absorbance was greater than 1 and no cross-reactivity was observed at this concentration. This cross-reactivity was attributed to small concentrations of contaminants that may have been more immunogenic than the truncated Erns, but also other interactions (not antigen-antibody) could have influenced the assay.

Conjugation of the anti-Erns polyclonal antibody to horseradish peroxidase

In order to reduce the incubation steps in the immuno-enzymatic assays for Erns detection, the antibody was conjugated to peroxidase. Direct ELISA was developed with coated Erns (10 µg/mL) to evaluate working dilution. The antibody was added at an initial dilution of 1:500 and serial 1:2 dilutions were performed. The optimal dilution work was taken when the observed absorbance was approximately 1 because with it, the signal over-saturation would

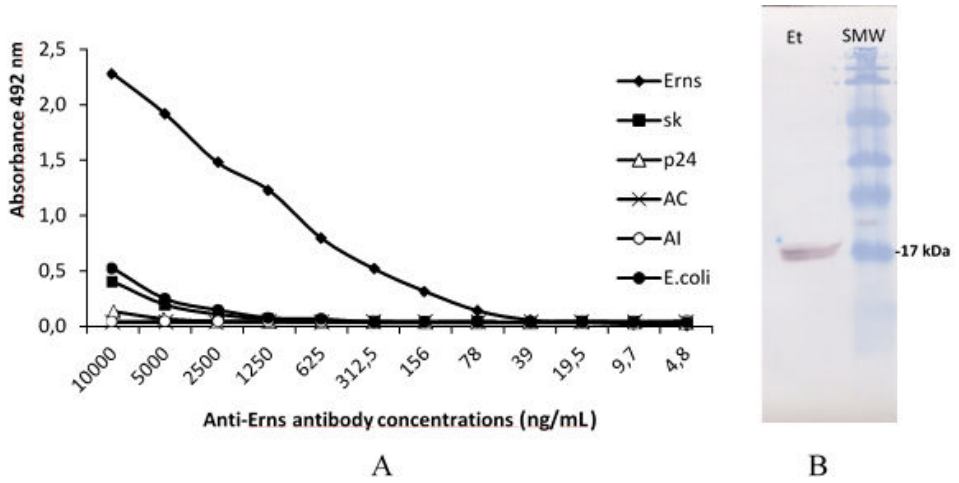

Figure 2. A: ELISA to evaluate the titer of anti-Erns polyclonal antibody purified by protein A. Erns: Coating with truncated Erns (10 µg/mL). Sk: Coating with anti-streptokinase monoclonal antibody (10 µg/mL). P24: Coating with recombinant p24-His protein from Human Immunodeficiency Virus. CFA: Coating with complete Freund's adjuvant 1: 100 (v/v). IFA: Coating with incomplete Freund's adjuvant 1: 100 (v/v). E. coli: Coating with *E. coli* BL21-DE3 (transformed with plasmid PET22b) protein extract, approximately 10 µg/mL calculated from the estimation that 16 % of the *E. coli* wet weight biomass is protein. B: Western blot performed to assess specificity of the polyclonal antibody with the truncated Erns. Et: 5µg of Erns truncated. SMW: Pre-stained SDS PAGE -Biorad standard molecular weight. */ ELISA para evaluar el título del anticuerpo policlonal anti-Erns purificado por la proteína A. Erns: recubrimiento con Erns truncado (10 µg/mL). Sk: recubrimiento con anticuerpo monoclonal anti-estreptoquinasa (10 µg/mL). P24: recubrimiento con la proteína recombinante p24-H de su virus de inmunodeficiencia humana. CFA: recubrimiento con el adyuvante completo de Freund 1: 100 (v/v). IFA: recubrimiento con el adyuvante incompleto de Freund 1: 100 (v/v). E. coli: recubrimiento con extracto de proteína E. coli BL21-DE3 (transformada con el plásmido PET22b), aproximadamente 10 µg/mL calculado a partir de la estimación de que el 16% de la biomasa de peso húmedo de E. coli era proteína. B: Western blot realizado para evaluar la especificidad del anticuerpo policlonal con la Erns truncada. Et: 5µg de Erns truncado. SMW: Pre-teñido SDS PAGE -Biorad peso molecular estándar.*

be avoided in the plate reader. As shown in Figure 3, absorbance was about 1 with a dilution of 1: 64000, so this could be taken as working dilution. This conjugated antibody could also be used in other assays such as western blot and dot-blot, but for these kinds of assays, the optimal dilution and substrate conditions must have been established to ensure the best performance for each technique.

Coating antibody concentration

Four coating concentrations were evaluated with the antibody to determine the optimal capture conditions. Eight dilutions of the used samples were evaluated: purified recombinant Erns as positive control, P24 as a negative control and a mixture of sera from healthy pigs to evaluate the possible recognition of nonspecific proteins in sera (Fig. 4). The assay demonstrated that coating at 10 µg/ mL was the condition at which higher Erns was captured. It was also observed that the antibody did not capture proteins from healthy pig sera.

Viral Erns capture in pig sera

The best coating concentration (10 µg/mL) was used in a Sandwich ELISA (Fig. 5). The polyclonal antibody could recognize Erns from the sera of individual animals positive to CSFV and from a pool of sera which belonged to the animals vaccinated with the Chinese strain of CSFV. Other authors have also detected viral Erns in sandwich ELISA for the diagnosis of CSF and they have obtained successful results when compared with more specific techniques such as RT-PCR (5). Nevertheless, in some cases, good results have not been obtained with the antigen-capture ELISAs for the diagnosis of CSF (4).

Negative samples (serum from animals vaccinated with Porvac®) were evaluated in the same ELISA. The fact that it did not recognize negative sera, including healthy animals immunized with E2, supported the hypothesis that the antibody could be a useful tool to distinguish vaccinated animals from those infected with CSFV. Besides, the development of an antigen capture technique by ELISA could work like a complement in the diagnosis of CSF in passively immunized animals and in persistently infected pigs a few days after birth with moderately virulent strains of CSFV that did not rise up an immune response. Unlike the direct ELISA, where the antibody detected *E. coli* BL21DE3-Pet22b proteins coated at 10 µg/mL (Fig. 2A), this non-specific reactivity was not observed in the sandwich ELISA. In addition, there was no response against the sera from animals immunized with E2.

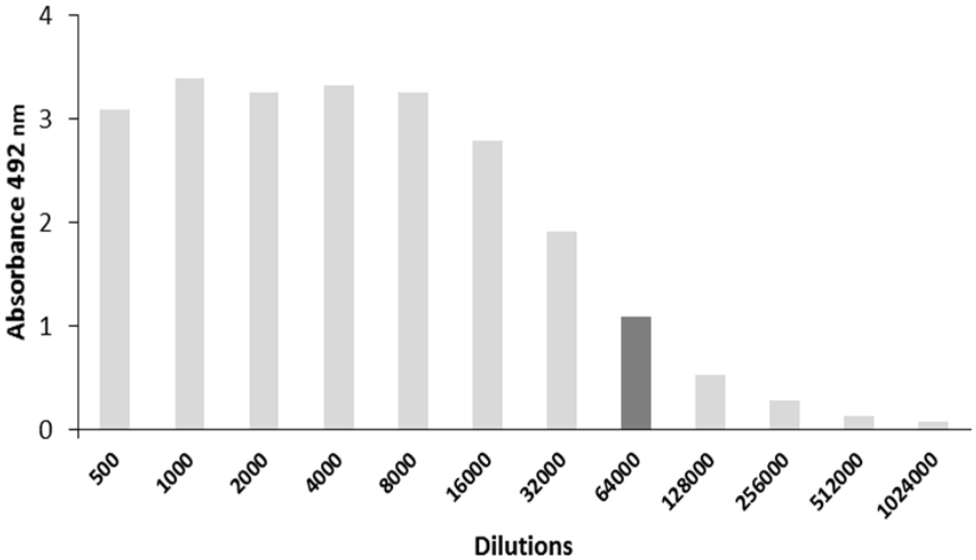

Figure 3. Direct ELISA to evaluate the titer of the antibody conjugated to horseradish peroxidase. In dark, the optimal work dilution of antibody for this assay. / *ELISA directo para evaluar el título del anticuerpo conjugado con peroxidasa de rábano picante. En la oscuridad, la dilución óptima del anticuerpo en el trabajo para este ensayo*

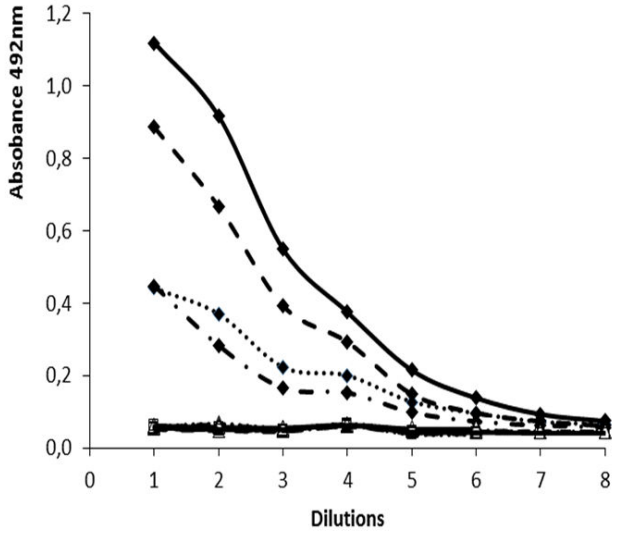

Figure 4. Sandwich ELISA with anti-Erns polyclonal antibody as capture and the same conjugated antibody for developing. Coating concentrations with antibody 1 (▪▪▪), 2 (— ▪ —) 5 (— —) and 10 (——) µg/mL. Samples of Erns (♦), p24 (Δ) and negative sera (□) were used. Eight serial dilutions of 1: 2 were made to all samples. In the case of Erns and p24, 1 µg/mL was started. / *ELISA sándwich con anticuerpo policlonal anti-Erns como captura y el mismo anticuerpo conjugado para el desarrollo. Concentraciones de recubrimiento con el anticuerpo 1 (▪▪▪), 2 (— ▪ —) 5 (— —), y 10 (——) µg/mL. Se utilizaron muestras de Erns (♦), p24 (Δ) y sueros negativos (□). Se hicieron ocho diluciones en serie de 1: 2 a todas las muestras. En el caso de la Erns y la p24, se inició 1 µg/mL.*

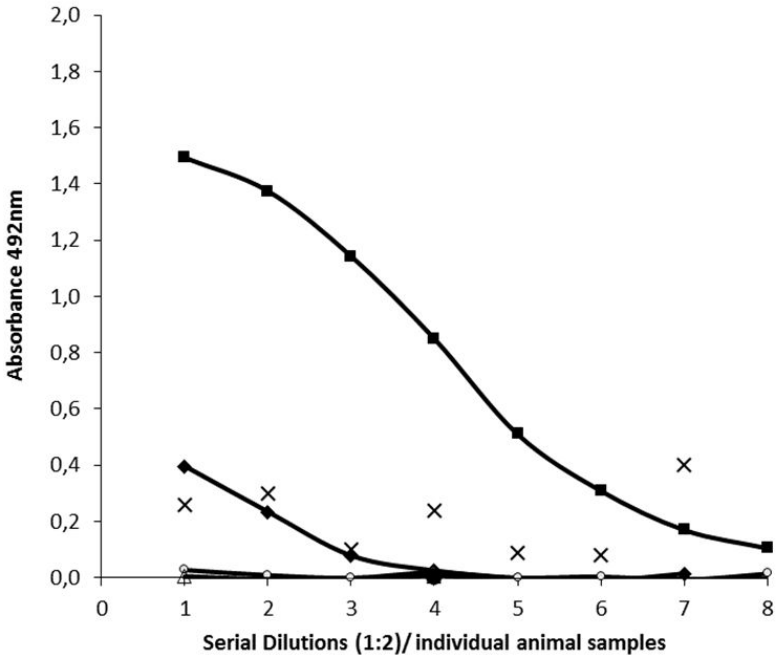

Figure 5. Sandwich ELISA developed to evaluate the recognition of Erns in a pool of positive pig sera and individual positive sera. In the case of serum samples, dilutions of 1: 2 (v/v) were started. Eight serial dilutions of 1: 2 were made to all samples. In the case of Erns and the *E. coli* BL21-pET22b protein extract, 1 µg / mL was started. Erns curve (■), Pool of positive sera (♦), Individual sera of vaccinated animals with the Chinese strain (×), Negative serum (○), *E. coli*-pET22b protein extract (Δ), Serum of animals immunized with a DIVA vaccine based on E2 glycoprotein (●). / *ELISA sándwich desarrollado para evaluar el reconocimiento de Erns en un conjunto de sueros de cerdo positivos y sueros positivos individuales. En el caso de las muestras de suero, se iniciaron diluciones de 1: 2 (v/v). Se hicieron ocho diluciones seriadas de 1: 2 en todas las muestras. En el caso de Erns y el extracto de proteína E. coli BL21-pET22b, se inició 1 µg / mL. Curva de Erns (■), Pool de sueros positivos (♦), Sueros individuales de animales vacunados con la cepa china (×), Suero negativo (○), Extracto de proteína E. coli-pET22b (Δ), Suero de animales inmunizados con una vacuna DIVA basada en la glicoproteína E2 (●).*

CONCLUSIONS

In this work, a rabbit anti-Erns polyclonal antibody, which recognized specifically the recombinant protein Erns used in the immunization scheme, was obtained. It was also able to conjugate the antibody to horseradish peroxidase, and the working dilution of the conjugate in a direct ELISA was 1:64000. A sandwich ELISA was performed with the polyclonal antibody to capture native Erns from vaccinated pig sera with the Chinese strain, and it was able to detect signals in those animals immunized with this vaccine, showing that the antibody could be used as a tool for CSF diagnosis.

REFERENCES

1. Méndez LP. Obtention of a differential diagnostic system for Classical Swine Fever, M. S thesis, Center for Genetic Engineering and Biotechnology, Havana city, Cuba. 2010.
2. Blome S, Staubach Ch, Henke J, Carlson J, Beer M. Classical Swine Fever-An Updated Review. Viruses. 2017;9:86.
3. Moennig V. Introduction to classical swine fever: virus, disease and control policy. Vet Microbiol. 2000;73:93102.
4. Dewulf J, Koenen F, Mintiens K, Denis P, Ribbens S, de Kruif A. Analytical performance of several classical swine fever laboratory diagnostic techniques on live animals for detection of infection. J Virol Methods. 2004;119:137-143.
5. Prodanov J, Došen R, Valčić M, Petrović T, Pušić I, Maljković M, *et al*. Detection of Classical Swine Fever Virus in blood samples in experimentally infected piglets of different immunological status. Lucrări Stiinłifice Medicină Veterinară. 2009;42 (1):151-159.
6. Muñoz SG, Perez MS, Bohorquez M, Rosell JA, Summerfield R, Domingo AM, *et al*. Efficacy of a live attenuated vaccine in Classical Swine Fever Virus postnatally persistently infected pigs. Vet Res. 2015;46(1):78.
7. Oirschot JT. Vaccinology of classical swine fever: from lab to field. Vetmic. 2003;96:367-384.
8. Ferrer E, Fonseca O, Irian MP, Abeledo MA. La Peste Porcina Clásica en las Américas y el Caribe. Actualidad y Perspectivas de Control y Erradicación. Rev Salud Anim. 2010;32(1):11-21.
9. Lowry OH, Rosebrough J, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193-275.
10. Nakane PK, Kawaoi. Peroxidase-labeled Antibody: A New Method of Conjugation. J Histochem Cytochem. 1974;22(12):1084-1091.

Los autores de este trabajo declaran no presentar conflicto de intereses.
Los autores de este trabajo declaran presentar una participación igualitaria en la concepción, ejecución y escritura de la investigación.
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