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Development and Application of an Immunochromatographic Strip to Monitor the Vaccination Efficacy of Vaccinated Pigs Against CSFV with E2 Protein

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Pig vaccination is a key strategy for the prevention and control of Classical Swine Fever in developing countries

SUMMARY

Pig vaccination is a key strategy for the prevention and control of Classical Swine Fever in developing countries. To validate the efficacy of the vaccine, a simple and rapid routine test is needed to monitor the antibody response against Classical Swine Fever Virus (CSFV) induced after vaccination. A rapid immunochromatographic strip reactive when antibody titers against E2 protein are greater than 1:50 according to neutralization peroxidase-linked assay (NPLA) as a gold standard technique was developed. This titer is considered protective against classical swine fever virus after vaccination with Porvac® subunit vaccine based on the E2 protein. The E2 glycoprotein is a fundamental component of the envelope of CSFV. This glycoprotein was labeled with colloidal gold. The conjugate was dispensed onto a conjugate pad as assay detector. The unconjugated E2 protein and a mouse monoclonal antibody against to E2 protein were printed into a nitrocellulose membrane for the test and control lines, respectively. The serum dilution that differentiates protected and unprotected animals was established using sera with titers of 1/40 determined by NPLA. A 1/32 dilution of swine sera makes possible to set pigs protection against CSFV after the application of Porvac®. The diagnostic sensitivity and diagnostic specificity of this strip were above 94% and 96% respectively, the results were correlated with the NPLA. This diagnostic test allowed determines that Porvac has an efficacy of 93.1% in two pig farm. Also, this strip could be used for rapid detection of antibodies against CSFV in the field conditions, under non-vaccination areas.

INTRODUCTION

Classical swine fever (CSF) is a highly contagious viral disease of domestic pigs and wild boars (Moennig et al., 2003) which causes great economic losses, due to high mortality rates, mandatory stamping out policies and the prohibition of trade in live pigs and pork products (Terpstra and de Smit, 2000). The causal agent is the classical swine fever virus (CSFV) of the genus Pestivirus, of the Flaviviridae family (Simmonds et al., 2012). The E2 glycoprotein exposed on the external surface of the virus (Weiland et al., 1999) is the most immunogenic, responsible for the induction of neutralizing antibodies and protection against

lethal challenge (Hulst et al., 1993). It has been the main component in the design of CSFV marker vaccines (Beer et al., 2007; Dong et al., 2006; Ganges et al., 2008).

In Cuba CSF is the first zoosanitary problem of pig farming. The live attenuated lapinized vaccine (Chinese strain produced by Labiofam, SA) has kept Cuba with the disease under control since the mid-1970s. However, since 1993 CSF became an endemic disease with outbreaks every year (Díaz de Arce et al, 1999; Díaz de Arce et al., 2005). Despite the vaccination program implemented the effectiveness of these vaccines fails due to factors associated with the loss of the cold chain, neutralization by maternal antibodies, lack of vaccination coverage mainly in inaccessible rural areas and backyard farms, which leads to the possible appearance of carrier animals (Ganges et al., 2008; Coronado et al., 2017; Muñoz-González et al., 2015a)

Considering the complex epidemiological situation in endemic areas, even under regular vaccination with live attenuated vaccines (de Arce et al., 2005; Ji et al., 2014; Moennig, 2000; Pérez et al., 2012; Shen et al., 2011), the development of a vaccine capable of inducing strong viral protection, protecting against transplacental transmission of CSFV, and easy to implement in current endemic countries became a priority.

Muñoz-González, S et al., 2017, demonstrated that Porvac®, a new subunit vaccine against CSFV based on the E2 glycoprotein, fused to the stimulating molecule of the immune system CD154 (Prieto et al., 2014 and Suarez et at 2017) confers protection against vertical transmission, as well as being a promising alternative to live attenuated vaccine for developing countries. Vaccination is a key strategy for the prevention and control of classical swine fever in developing countries. To validate the efficacy of a vaccine, a rapid and simple routine test is needed to monitor the antibody titers against Swine Fever Virus induced after vaccination. The most commonly used serological tests are the virus neutralization test and the enzyme-linked immunosorbent assay (ELISA). Although these assays provide accurate and sensitive antibody detection, they require specialized equipment and technical expertise.

The immunochromatographic strip has several advantages over traditional immunoassays, such as simplicity of the procedure, quick operation and immediate results, low cost, no requirement for trained technicians or expensive equipment. In the last decade it has been applied in various fields such as allergies, infectious diseases, environmental pollutants, drugs, fertility and in veterinary medicine.

An immunochromatographic strip for a rapid, visual and qualitative determination of specific antibodies against the E2 protein of CSFV in serum, that correlate with the neutralization peroxidase-linked assay (NPLA), was developed. This system could be useful for determining the presence of antibodies as infection markers, especially in cases of subacute or chronic forms of the diseases. In this work, this methodology was adapted to monitor the vaccination efficacy, creating the test conditions that allow differentiating between protected and non-protected animals against CSFV, after the application of Porvac®.

EXPERIMENTAL SECTION

Serum Samples

To determine the dilution of the samples to determine the efficacy of vaccination and to evaluate the diagnostic accuracy, the group of clinical trials of the Center for Genetic Engineering and Biotechnology of Havana, Cuba provided sera from pigs vaccinated against classical swine fever with Porvac® vaccine. These serum samples were provided with information on neutralizing antibody values, determined by the NPLA technique (standard reference method). Serum samples were stored at -20 °C. Each sample was evaluated for anti-E2 antibodies with three independent lots of the anti E2-CSF immunochromatographic strip.

To evaluate the applicability of the strip, 110 sera from vaccinated pigs with Porvac®, proceeding of Sancti Spíritus farms, were used.

E2 Protein and Its Conjugation to Colloidal Gold

The active pharmaceutical ingredient in Porvac® vaccine was supplied by its manufacturer, the Center for Genetic Engineering and Biotechnology of Camagüey, Cuba. It is a chimeric protein formed by the fusion of the extracellular region of the E2 glycoprotein of the Margarita strain of CSFV and the extracellular segment of the CD154 pig molecule.

The conjugation of the E2 protein, by the adsorption method, was carried out according to Bailes et al., 2012, using 40 nm

colloidal gold particles, supplied by British BioCell International (BBI). The protein was solubilized in 5 mM Tris-HCl buffer, pH 8.0. This pH value corresponds to 0.5 units above the isoelectric point (PI) of the protein. The colloidal gold was also adjusted to pH 8 with 0.1 M K2CO3.

The reaction mixture between the protein and colloidal gold was done in a ratio 1:5 v/v. The amount of protein in relation to a fixed volume of gold was determined by the following flocculation test: 500 µL of 40nm colloidal gold was mixed at pH 8 (adjusted with 0.2 M potassium carbonate) with 100 µL of the protein solution in a range between 4 and 20 µg. The mixture was homogenized and incubated for 10 min at room temperature and 500 µL of 10% sodium chloride solution was added to each reaction mixture. Finally, the content of the conjugate in the mixture was quantified by measuring the absorbance at 530 nm in a spectrophotometer. (Thermo Scientific GENESYS 10S UV-Vis, Madison, USA)

Once the optimal amount of protein had been selected, the scale up of reaction mixture of the conjugate was homogenized for 30 min at a temperature between 20 °C and 25 °C, with stirring at 300 rpm. The free sites on the colloidal gold particles were blocked with an adequate volume of 10% BSA, for a final concentration of 1%. The final mixture was centrifuged at 12000xg for 1 hour at 4 ?C. The conjugate obtained (pellet) was resuspended in a proportion of 300 µL of 1% sucrose solution and 0.05% sodium azide, for each 100 mL of colloidal gold used. The conjugate obtained was adjusted by dilution to an absorbance of 20, at a wavelength of 530 nm.

Preparation of the Immunoreactive Strip

The strip components were nitrocellulose membrane of 15 µm (MDI,type CNPC-SS12-L2-H50, dimensions 7,5 x 26 cm, 1,5 cm of nitrocelullose), pad for the antigen-gold conjugate (MDI,type PTR-5, dimensions 2,7 x 26 cm) and absorbent pad (MDI,type AP080, dimensions 3,5 x 26 cm) (Figure 1). For coating the macroporous material with the conjugate, these were previously immersed for 10 minutes at room temperature in a conjugate release solution (0.1% Tween 20 and 0.02% sodium azide) and dried for 2 h at 37 ?C. The conjugate was diluted to an absorbance of 10, at a wavelength of 530 nm and dispensed onto the antigen-gold conjugate pad.

To establish the most favorable condition for adsorption of the E2 protein to the membrane in the test line, six conditions were tested in which the most important factors that could modify this process were combined: ionic strength, alcohol addition, detergents addition and protein concentration in the coating of membrane.

Condition 1: 2 mg mL of E2 protein dissolved in 150 mM PBS

Condition 2: 1 mg/mL of E2 protein dissolved in 85 mM PBS and 3% (v/v) of methanol

Condition 3: 1 mg/mL E2 protein dissolved in 85 mM PBS

Condition 4: 4 mg/mL of E2 protein dissolved in 20 mM PBS and 0.05% (w/v) of SDS

Condition 5: 2 mg/mL of E2 protein dissolved in 20 mM PBS and 0.025% (w/v) of SDS

Condition 6: 2 mg/mL of E2 protein dissolved in 10 mM PBS and 0.025% (w/v) of SDS

The mixture of components of the control line, for coating the nitrocellulose membrane, consisted of a monoclonal antibody against E2 protein provided by Center for Genetic Engineering and Biotechnology of Sancti Spítitus, Cuba at 4 mg/mL and 0.2 mg/mL of poly L-lysine, dissolved in 150 mM of PBS.

Both the macroporous material and the nitrocellulose membrane were sprayed with the BioDotQuanti 2000. BioJet, England and then dried in an oven at 37 ° C, for 2 h and 1 h respectively.

The nitrocellulose membrane cards were blocked for 1 h at room temperature in blocking solution (0.02% (v/v) Tween 20, 0.02% (w/v) sodium azide and 5% (w/v) sucrose, dissolved in 15 mM PBS). Later they were washed and dried in the oven at 37 °C for 2 h.

The best condition for adsorption of the E2 protein to the membrane was chosen taking into account the signal of antibody detection on the strip, generated by a pool of sera of Porvac® vaccinated pigs with a titer of 1/100, according to the NPLA.

Evaluation Of Conditions That Contribute To The Storage Life Of The Strip

To increase the stability of the immunochromatographic strip, sucrose was added in a washing solution and trehalose was added in the coating solutions of both control line and test line of the strip. Effect of thermopreservatives on the stability of immunochromatographic strips placed at 60 ?C for 5 days was evaluated. Three treatments were assayed:

T1: Treatment where the control line is coated with mAb CBSS E2.1 at 4 mg/mL; 0.2 mg/mL poly L-lysine; 0.1% (w/v) trehalose and 20 mM PBS. The test line was coated with the E2 protein at 4 mg/ mL dissolved in 0.1% (w / v) of trehalose; 0.05% (w / v) SDS and 20 mM PBS. A 15 min washing step was carried out after blocking with the 5 mM Na2HPO4 solution at pH 7.5; 0.01% (w / v) of SDS and 5% (w / v) of sucrose.

T2: Treatment where it is coated in the same conditions as T1, but without the washing step after blocking.

T3: Treatment where the control line is coated with the monoclonal antibody anti E2 (CBSS E2.1) at 4 mg/mL and 0.2 mg/mL of poly L-lysine, dissolved in 150mM of PBS. The specific line was coated with the E2CD protein at 4 mg/mL, dissolved in 0.05% (w/v) of SDS and 20 mM of PBS. A wash step was performed as in T1 treatment.

The signal on the strip is generated by a pool of anti E2 antibody-positive serum from pigs vaccinated with Porvac®, with a titer of 1/100, according to the NPLA technique. The intensity of the signals of the control line and the specific line was visually evaluated on the strip. In addition, the stability of the strip was studied in real time for 1, 12, 18, 24 and 30 months. The strips were placed in aluminum bags with desiccant (silica gel) and stored at 2-8 ?C, 20-25 ?C and 28-32 ?C. The storage life of the strips was determined by testing six strips stored at each temperature with an anti E2 antibody-positive serum and a negative serum.

Test Strip Assembly

The NC membrane is coupled in the center of a polyvinyl chloride support. The absorbent pad and the conjugate pad were glued by overlapping 1 mm at the top and bottom of the NC Membrane, respectively. The sample pad was then glued by overlapping 2 mm on the bottom of the conjugate pad (Figure 1). The master assembled card was cut into 4 mm wide strips using a BioDot CM5000-guillotine-cutter, USA.

Procedure and Principle of the Test

The swine serum moves by capillary action through the macroporous material once the corresponding end of the test strip is introduced into it, binding the E2 protein labeled with colloidal gold, forming an antigen-antibody immune complex. Both the immune complex formed, and the free conjugate moves through the nitrocellulose membrane towards the results window of the strip, where two independent reactions occur. First, the immune complex binds the E2 protein attached to the NC membrane, making a colored line (test line). The free conjugate and excess of immune complex are captured by a mouse monoclonal antibody against E2 antigen, mixed with poly L-lysine, fixed to the solid phase in a second line (control line). This line indicates that the test has worked correctly. Once these lines are formed, they do not disappear. The qualitative determination of anti E2 antibodies by this immunoassay was performed by introducing the strip into the diluted serum and the result was evaluated after 15 minutes.

Establishment of Dilution Conditions for Samples to Monitor Vaccination Efficacy

To provide a criterion of protected or unprotected animal when the sera are evaluated with the immunoreactive strip, it was considered that a cut-off value (criterion of protected animal) for the strip was established, a titer of 1/50 by NPLA (Bouma A et al, 1999). In view of this, a group of 12 sera from vaccinated animals with non-protective titers (1/40 by NPLA) was formed by diluting the sera with a pool of negative pig sera, to find the dilution in which no sign on the strip. With each one of them, 1/4, 1/8, 1/16, 1/32 and 1/64 dilutions were made serially with a mixture of rabbit serum.

Assessment of Diagnostic Accuracy

Diagnostic accuracy was assessed using the following fundamental parameters: sensitivity, specificity, and predictive values. The concordance between the strip and the NPLA results, taken as the gold standard, was estimated by the Cohen´s kappa coefficient. The following serum samples were incorporated in this study. Vaccinated animal samples and diluted with a pool of negative sera to titer ?1/50 determined by NPLA (n=39).Vaccinated animals samples with titer ?1/50 determined by NPLA (n=88) and non-vaccinated animals samples and negative by NPLA (n=38).

Application Of The Immunochromatographic Strip In Field Conditions

The immunochromatographic strip was used to detect pig antibodies anti E2 in 110 sera collected from some farms in Sancti Spiritus province. These serum samples were also evaluated for anti-Erns antibody using a homemade indirect ELISA to differentiate infected from vaccinated animals.

RESULTS AND DISCUSSION

The evaluation of the effectiveness of immunization depends mainly on serological tests. The immunochromatographic strip has several advantages over traditional immunoassays, such as the simplicity of the procedure, fast operation, immediate results, low cost, and no requirement for technical experts or expensive equipment. Due to these characteristics, the immunochromatographic strip test is suitable for in situ detection of antibodies. It has the potential for use in the field conditions to control vaccine-induced antibodies. Detection of the anti E2 antibody is the basis for establishing an adequate immune program and evaluation of the effect of Porvac® vaccination in pigs.

Conjugation of the E2 Protein to Colloidal Gold

It is necessary to perform a saturation isotherm to determine the protein: gold ratio for each protein and each size colloidal gold (Oliver C., 1994). In the conjugation process was kept the volume ratio between protein and colloidal gold 1:5. To guarantee the reproducibility of the system, it is important to maintain a constant ratio, because the kinetics of gold-protein conjugation depends on it. Bailes et al., 2012 conjugate proteins to 40 nm colloidal gold from the BBI using the same ratio 1:5, likewise Zhang et al., 2009 conjugate both antigens and IgG using 40 nm manufactured gold nanoparticles in the same proportion.

The flocculation test showed that from 10 µg of E2, the entire surface of the colloidal gold particles is saturated (Figure 2). For safety in the conjugation process, 11 µg of E2 protein were used for every 500 µL of colloidal gold.

Preparation of the Immunoreactive Strip

To evaluate the E2 protein adsorption to the nitrocellulose membrane, six conditions of dilutions were tested. The most intense signal was obtained with the condition 4 : 4 mg / mL of E2 dissolved in 20 mM of PBS and 0.05% (p / v) of SDS. The high ionic strength in the protein dilution buffer interferes with electrostatic interactions that enhance the binding of the protein to nitrocellulose (Jasdeep K et al., 2007).

Besides the low ionic strength, another factor that could facilitate the adsorption of the protein to the nitrocellulose membrane is the addition of alcohols in the buffer (Jasdeep K et al., 2007). In this experiment, the inclusion of methanol at 3% (v/v) (condition 2) did not show an appreciable effect in relation to the condition containing the buffer with the same molarity and the same concentration of E2 (condition 3).

Evaluation of Conditions that Contribute to the Storage Life of the Strip

In order to increase the stability of the immunoreactive strips, the influence of sucrose and trehalose as stabilizers was evaluated through an experiment that included three treatments (T1, T2 and T3). Sucrose was incorporated in a washing step (5 mM Na2HPO4 solution at pH 7.5; 0.01% (w / v) of SDS and 5% (w/v) of sucrose) after the blocking step of the nitrocellulose membrane. Trehalose was added directly into the membrane coating mixes.

Regarding the intensity and stability of the specific line, the best of the treatments tested after an accelerated stability study was T1 (4 mg/mL of E2 dissolved in 20 mM PBS: 0.05% (w/v) SDS and 0.1% (w/v) trehalose). With this result it was evidenced that sugar enhanced positively the stability of coated protein (Figure 3).The worst treatment was T2, which only differs from T1 in the membrane washing step during 15 min, after the blocking step. In this way, the observed thermopreservative effect of sucrose and the favorable effect of washing were probably because of the removing excess of the blocking protein (BSA) from the nitrocellulose membrane.

Has been reported that with protein dehydration during manufacture drying steps of the strips, the water molecules that were part of the structure of the protein are lost and this fact could induce an instability in its structure. This instability can be avoided by mixing proteins with sugar molecules, such as trehalose and sucrose, to replace the sites occupied by water (Se-

Hwan Paek et al., 2000)

Regarding the stability of the control line, the best treatment was T3 (AcM CBSS E2.1 at 4 mg / mL and 0.2 mg / mL of poly L-lysine, dissolved in 150 mM of PBS). The most important factor was the relatively higher ionic strength in the T3 buffer. The low ionic strength favors the adsorption of the antibodies to the nitrocellulose membranes; however, it does not appear to favor the adsorption or stability of the poly L-lysine. The immunochromatographic strips were stable for five days at 60 ?C, when trehalose was added to the specific line and sucrose in a washing step after blocking.

The real-time stability was performed, and the anti E2-CSF immunochromatographic strip correctly identified all positive and negative samples after 30 months of storage between 2-8 ?C and after six months of storage between 28-32 ?C. The results showed that the strips sealed with desiccant in an aluminum bag could be stored for at least 30 months at 4?C with no signi?cant loss of sensitivity. Several CSFV endemic countries are located in tropical areas (Spickler and Roth, 2003), taken into account, the thermal stability of the diagnostician, accompanying the subunit vaccine is a practical added value for those regions where the cold chain could fail and also exist warm temperature in field conditions.

Establishment of the Dilution Conditions of Serum Samples to Monitor the Efficacy of Vaccination with E2

Several researchers have demonstrated a correlation between vaccine induced antibody levels at the time of challenge and protection against lethal infection. Terpstra and Wensvoort (1988) found that if serum neutralizing antibody titers against C-strain virus, as measured by NPLA, were greater than or equal to 12.5 and less than 25 then these pigs were able to survive a lethal challenge whilst remaining viraemic and able to transmit virus. Pigs with antibody titers greater than or equal to 25 and less than 50 displayed some clinical signs without evidence of transmission. Antibody titers greater than or equal to 50 resulted in complete protection with no virus transmission or clinical signs (Terpstra and Wensvoort, 1988). (Terpstra and Wensvoort, 1988). Vaccination with 32 ?g of baculovirus expressed E2 subunit vaccine on the other hand conferred protection against lethal challenge if the serum antibody titer measured by NPLA was greater than or equal to 50 (Bouma et al. 1999). Based on those reports, a titer measured by NPLA greater than 1:50 was taken as a protective value.

The dilution of the sera was established, which allows the differentiation between protected and unprotected animals. For this, 12 sera of animals vaccinated by Porvac® were diluted to NPLA titers of 1/40 to find the minimum dilution in which no signal appears on the strip. In this way, when evaluating sera from unprotected animals, test will be negative; minimizing false positives, so that the diagnostic specificity of the strip is not affected. The working dilution established for the sera from animals vaccinated with E2 was 1/32, which was the minimum dilution in which no protection signal appears in any of the evaluated samples. (Table 1)

Sastre et al, 2016 developed a lateral duplex flow assay for simultaneous detection of antibodies against African swine fever virus (ASFV) and CSFV (anti E2), whose main use is to differentiate both diseases. On the other hand, Li et al. 2012 developed an immunochromatographic strip that detects anti E2 and anti Erns antibodies with a chimeric protein, in order to detect antibodies against CSFV, they recommended a possible use to verify vaccination efficacy. However, to our knowledge, no author has established conditions to associate a positive signal on the strip with a protective antibody titer. The use of the test strip with 1/32 diluted porcine sera allows evaluating the immunogenic capacity of a vaccine by detecting antibody titers higher than 1/50 by NPLA, capable of protecting the herd.

Evaluation of Diagnostic Accuracy and Strip Agreement Using NPLA as Gold Standard

For the evaluation of the diagnostic accuracy parameters and the concordance of the strip with the NPLA, pig sera were used diluted 1/32 with a pool of rabbit serum. A total of 165 serum samples were tested with three lots of the immunochromatographic strip, using one strip by serum sample (Table 2).The NPLA was used as the gold standard for comparison. The strip showed high values of diagnostic accuracy. The corresponding values of sensitivity and specificity were above 94% and 96%, respectively in the three evaluated lots. The positive predictive values were higher than 96% and the negative predictive values, over 93% (Table 3).

Taking into account that this strip will mainly be used to determine the immune status in vaccinated animals, the diagnostic specificity should be an essential parameter. This would imply a low fraction of false positives during post-vaccination immunity evaluation. The 1/32 dilution of the sera determines that NPLA values less than 1/50 are not detected by the strip, which reduces false positives to less than 4%.

Kappa index is the fraction of coincident results between two tests performed on the same sample beyond that expected by

random. A kappa index of 0.9 was obtained, which qualifies the concordance between the strip and the NPLA as very good, according to Landis and Koch, 1977.

Application of the Immunochromatographic Strip in the Field

The OIE recommends that the efficacy of vaccination, both for live attenuated vaccines and for subunit vaccines, be estimated in vaccinated animals directly, by evaluating their resistance to live virus challenge (OIE Terrestrial Manual, 2019). This procedure is restricted to areas where all security conditions prevent the spread of the virus. The evaluation of neutralizing antibodies in vaccinated animals has been proposed as alternative to measure efficacy once the vaccine has been applied under uncontrolled conditions (Freitas et al.,2009). NPLA titers have been reported that confer protection to vaccinated animals against classical swine fever virus (?1/50 for subunit vaccines. Bouma et al, 1999).This technique has the disadvantage that it requires specialized equipment, culture of viruses in PK15 cells, intensive labor, high cost, technical expertise, and is suitable for laboratory use only.

The use of the lateral flow immunochromatographic strip could substitute the NPLA when the test result is correlated with protective titer quantified by NPLA. Besides, the strip has advantages as procedural simplicity, immediate results, low cost, and no requirements for technical expertise or specialized equipment.

The strip was applied to measure the efficacy of Porvac® by determining anti-E2 antibodies in sera from 101 vaccinated pigs from two pig farms. This test does not allow differentiation of Porvac vaccinated animals from infected animals. For this reason it was previously tested that these serum samples did not have detectable antibodies against CSFV, consistent with a home-made anti-Erns ELISA (Farm1, 38 pigs and Farm2, 63 pigs). According to the immunochromatographic strip, 94 out of 101 samples were positive, which means that 93.1% of the animals had protective antibodies against CSVF, which corresponds to the effectiveness of the vaccine in this sample.

In Porvac phase III clinical trials, neutralizing antibody titers greater than 1: 100 by NPLA were obtained in 100% of animals and titers greater than 1: 200 by NPLA in 98% of animals (Unpublished data). The difference with our results (?7%) could be attributed to the fact that this study was conducted under uncontrolled conditions. Factors associated with poor management and poor practice in vaccination have been reported to explain many of the failures of vaccination in endemic areas (Coronado et al., 2019)

The strip demonstrated its applicability to monitor E2 vaccine-induced antibodies. Monitoring the general rise and fall of antibody levels in pig herds can help to some extent in assessing the response to the vaccine. Besides test before and after vaccination can be useful to identify the induction of vaccine-related antibodies. The strip could also be used as a serological diagnostic tool for classical swine fever virus in unvaccinated herds.

CONCLUSION

The developed immunochromatographic strip can qualitatively detect anti-CSFV E2 antibodies and predict their protective values. Although it cannot quantitatively specify the level of antibodies, the quantitative data of the strip was acquired taken NPLA as a gold standard. When the NPLA titer was ? 1/50, the strip gave a positive result, when the NPLA titer was ?1/50, the strip gave a negative result. A positive test result means that the antibodies are enough to protect against viral infection. The diagnostic sensitivity was 94% and diagnostic specificity was 96% according to the neutralizing antibody titer given by the NPLA.

The anti E2-CSF immunochromatographic strip is suitable for monitoring vaccination efficacy when E2 protein is used as an immunogen. Their characteristics make these devices very useful especially in countries with poor laboratory or even absent. Since this method cannot differentiate vaccine induced antibodies from a response to natural virus infection, the situation of each country and the vaccination programs used must be taken into account in order to correctly interpret serological results.

CONFLICT OF INTEREST

Authors declare no conflict of interest to publishing this information that has not been publishing in any other scientific journal.

REFERENCES

1. Altman DG, Machin D, Bryant TN, Gardner MJ (Eds) (2000) Statistics with confidence, 2nd ed. BMJ Books.
2. Beer, M., Reimann, I., Hoffmann, B., Depner, K., 2007. Novel marker vaccines against classical swine fever. Vaccine

25, 5665–5670. http://dx.doi.org/10.1016/j.vaccine.2006.12.036.

3. Bailes,J., Mayoss,S.,Teale,P.,Soloviev, M., 2012.Chapter 4: Gold Nanoparticle Antibody Conjugates for use in competitive lateral flow assays. Mikhail Soloviev (ed), Nanoparticles in Biology and Medicine Methods and Protocols Methods in Molecular Biology, vol 905. DOI 10 1007/978-1-61779-953-2-4. Springer Science +Business Media, LLC
4. Bouma, A., de Smit, A., de Kluijver, E., Tersptra, C., Moormann, R., 1999. Efficacy and stability of a subunit vaccine based on glycoprotein E2 of classical swine fever virus. Veterinary Microbiology 66 (2): 101-114.
5. Clavijo,A.,Lin,M.,Riva,J,Zhou,EM.2001. Application of competitive enzyme-linked immunosorbent assay for the serologic diagnosis of classical swine fever virus infection. J Vet Diagn Invest. 3,357–360
6. Coronado, L., Liniger, M., Muñoz-Gonzalez, S., Postel, A., Perez, L.J., Perez-Simó, M., Perea, C.L., Frias, M.T., Rosell, R., Grundhoff, A., Indenbirken, D., Alawi, M., Fischer, N., Becher, P., Ruggli, N., Ganges, L., 2017. Novel poly-uridine insertion in the 3'UTR and E2 amino acid substitutions in a low virulent classical swine fever virus. Vet.Microbiol. 201, 103–112. http://dx.doi.org/10.1016/j.vetmic.01.013
7. Coronado,L., Bohórque,J., Muñoz-González,S., Perez,L., Rosell,R., Fonseca,O.,Delgado,L.,Perera, C.,Maria Teresa Frías,MT., Ganges,L.,2019. Investigation of chronic and persistent classical swine fever infections under field conditions and their impact on vaccine efficacy. BMC Veterinary Research 15:247 https://doi.org/10.1186/s12917-019-1982-x
8. Díaz de Arce, H., Ganges, L., Barrera, M., Naranjo, D., Sobrino, F., Frías, M.T., Núñez, J.I., 2005. Origin and evolution of viruses causing classical swine fever in Cuba. Virus Res. 112, 123–131.
9. Díaz de Arce, H., Núñez, J.I., Ganges, L., Barrera, M., Frías, M.T., Sobrino, F., 1999. Molecular epidemiology of classical swine fever in Cuba. Virus Res. 64, 61–67
10. Dong, X.N., Qi, Y., Ying, J., Chen, X., Chen, Y.H., 2006. Candidate peptide-vaccine induced potent protection against CSFV and identified a principal sequential neutralizing determinant on E2. Vaccine 24, 426–434. http://dx.doi.org/10.1016/j.vaccine.2005.08.002
11. Freitas,T., Caldas,L.,Esteve,E.,Duarte,A.,Rebello,M.,2009.Classical Swine Fever: humoral neutralizing antibody induced by a live attenuated vaccine. Revue Méd. Vét. 160, 6, 314-318
12. Ganges,L., Nuñez,J.I., Sobrino,F., Borrego, B., Fernández-Borges,N., Frías-Lepoureau, M.T., Rodríguez, F., 2008. Recent advances in the development of recombinant vaccines against classical swine fever virus: cellular responses also play a role in protection. Vet. J. 177, 169–177. http://dx.doi.org/10.1016/j.tvjl.2007.01.030.
13. Hulst, M.M., Westra, D.F., Wensvoort, G., Moormann, R.J., 1993. Glycoprotein E1 of hog cholera virus expressed in insect cells protects swine from hog cholera. J.Virol. 67, 5435–5442.
14. Ji, W., Niu, D.-D.D., Si, H.-L.L., Ding, N.-Z.Z., He, C.-Q.Q., 2014. Vaccination influences the evolution of classical swine fever virus. Infect. Genet. Evol. 25, 69–77. http://dx. doi.org/10.1016/j.meegid.2014.04.008.
15. König, M., Lengsfeld,T., Pauly, T., Stark,R., Thiel, H.J., 1995. Classical swine fever virus:independent induction of protective immunity by two structural glycoproteins. J.Virol. 69, 6479–6486.
16. Li,Y.,Shen,L., Sun,Y.,Yuan,J., Huang,J., Li,C.,Li,S., Luo, Y.,Ji.H.,2013,Simplified serum neutralization test based on enhanced green fluorescent protein-tagged classical swine fever virus. J Clin Microbiol.51,2710–2712.
17. Mercaldo N., Lau ,K., Zhou, X.,2007. Confidence intervals for predictive values with an emphasis to case-control studies. Statistic in Medicine 26(10):2170-83. doi: 10.1002/sim.2677.
18. Meyers, G., Thiel, H.J., 1996. Molecular characterization of pestiviruses. Adv. Virus. Res. 47, 53–118.
19. Moennig, V., 2000. Introduction to classical swine fever: virus, disease and control policy. Vet. Microbiol. 73, 93–102.
20. Moennig, V., Floegel-Niesmann, G., Greiser-Wilke, I., 2003. Clinical signs and epidemiology of classical swine fever: a review of new knowledge. Vet. J. 165, 11–20.
21. Muñoz-González, S., Perez-Simó, M., Muñoz, M., Bohorquez, J.A., Rosell, R., Summerfield, A., Domingo, M., Ruggli, N., Ganges, L., 2015a. Efficacy of a live attenuated vaccine in classical swine fever virus postnatally persistently infected pigs. Vet. Res. 46, 78. http://dx.doi.org/10.1186/s13567-015-0209-9.
22. Muñoz-González,S.,Sordo,Y., Pérez-Simó,M., Suarez,M., Canturric,A., Rodriguez,MP., Frías-Lepoureaud,MT., Domingo,M., Estradab,MP., Ganges, L., 2017.Corrigendum to "Efficacy of E2 glycoprotein fused to porcine CD154 as a novel chimeric subunit vaccine to prevent classical swine fever virus vertical transmission in pregnant sows". Veterinary Microbiology ,http://dx.doi.org/10.1016/j.vetmic.2017.10.014
23. Oliver C.1994, Conjugation of colloidal gold to proteins. Methods Mol Biol.34,303-307.
24. Pérez, L.J., Díaz de Arce, H., Perera, C.L., Rosell, R., Frías, M.T., Percedo, M.I., Tarradas, J., Dominguez, P., Núñez, J.I., Ganges, L., 2012. Positive selection pressure on the B/C domains of the E2-gene of classical swine fever virus in endemic areas under Cstrain vaccination. Infect. Genet. Evol. 12, 1405–1412. http://dx.doi.org/10.1016/j. meegid.2012.04.030

25. Prieto, Y., Méndez, L., Rodríguez, E., Rodríguez, M P., Lorenzo, E., Suarez, M., Rodríguez, A., Estrada, MP., Pimentel, E. 2014. Transferencia a la etapa de desarrollo: producción y purificación de proteína e2cd como antígeno vacunal contra la peste porcina clásica. Centro de Ingeniería Genética y Biotecnología.
26. Rümenapf, T., Meyers, G., Stark, R., Thiel, H.J., 1991. Molecular characterization of hog cholera virus. Arch. Virol. 7–18.
27. Saegerman, C., De Waele, L., Gilson, D., Godfroid, J., Thiange, P., Michel, P., Limbourg, B., Vo, T.K., Limet, J., Letesson, J.J. and Berkvens, D. 2004. Evaluation of three serum ELISAs using monoclonal antibodies and protein G as peroxidase conjugate for the diagnosis of bovine brucellosis. Vet. Microbiol. 100, 91-105
28. Sastre,P., Pérez,T., Costa,S., Yang,X., Räber,A., Blome,S.,Goller,KV., Gallardo, C., Tapia,I., García,J., Sanz,A., Rueda,P.,2016. Development of a duplex lateral flow assay for simultaneous detection of antibodies against African and Classical swine fever viruses. Journal of Veterinary Diagnostic Investigation. 28, 543 –549. DOI: 10.1177/1040638716654942
29. Shen, H., Pei, J., Bai, J., Zhao, M., Ju, C., Yi, L., Kang, Y., Zhang, X., Chen, L., Li, Y., Wang, J., Chen, J., 2011. Genetic diversity and positive selection analysis of classical swine fever virus isolates in south China. Virus Genes 43, 234–242. http://dx.doi.org/10.1007/s11262-011-0625-5.
30. Silva, E., Izquierdo, M., Nibot, C. and Martín, R.Z. 2006. Evaluation of the DAVIH BRU 3 ELISA system in the diagnosis of Brucellosis in different animal species. Rev. Salud. Anim. 28(1), 31-35
31. Simmonds, P., Becher, P., Collett, M., Gould, E.A., Heinz, F.X., Meyers, G., Monath, T., Pletney, A., Rice, C.M., Stiasny, K., Thiel, H.-J., Weiner, A., Bukh, J., 2012. Family Flaviviridae. In: King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.), Ninth Report of the International Commitee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, C.A, pp. 1004–1020.
32. Spickler, A.R., Roth, J.A., 2003. Adjuvants in veterinary vaccines: modes of action and adverse effects. J. Vet. Intern. Med. 17, 273–281
33. Suárez, M., Sordo, Y., Prieto, Y., Rodríguez, M.P., Méndez, L., Rodríguez, E.M., Rodríguez-Mallon, A., Lorenzo, E., Santana, E., González, N., Naranjo, P., Frías, M.T., Carpio, Y., Estrada, M.P., 2017. A single dose of the novel chimeric subunit vaccine E2-CD154 confers early full protection against classical swine fever virus. Vaccine 35,4437–4443. http://dx.doi.org/10.1016/j.vaccine.2017.05.028. 28688785
34. Terpstra C., Wensvoort G.,1987. Influence of the vaccination regimen on the herd immune response for swine fever. Vet. Microbiol. 13, 143-151.
35. Terpstra C., Wensvoort G., 1988.The protective value of vaccine induced neutralizing antibody titre in swine fever. Vet. Microbiol. 16, 123 - 128.
36. Terpstra, C., Smit, A.J., 2000. The 1997/1998 epizootic of swine fever in the Netherlands: control strategies under a non-vaccination regimen. Vet. Microbiol. 77, 3–14.
37. Thiel, H.J., Collett, M.S., Gould, E.A., Heinz, F.X., Houghton, M., Meyers, G., Purcell, R.H., Rice, C.M., 2005. Family Flaviviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, pp. 979–996.
38. Weiland, F., Weiland, E., Unger, G., Saalmüller, A., Thiel, H.-J., 1999. Localization of pestiviral envelope proteins Erns and E2 at the cell surface and on isolated particles. J. Gen. Virol. 80, 1157–1165.
39. Li,X., Wang,L., Shi,X., Zhao,D., Yang,J., Yang,S., Zhang,G.,2012. Development of an immunochromatographic strip for rapid detection of antibodies against classical swine fever virus. Journal of Virological Methods. 180 , 32–37
40. Zhang G., Guo J., Wang X.,2009. Immunochromatographic Lateral Flow Strip Tests. In: Rasooly A., Herold K.E. (eds) Biosensors and Biodetection. Methods in Molecular Biology™, vol 504. Humana Press

FIGURES and TABLES

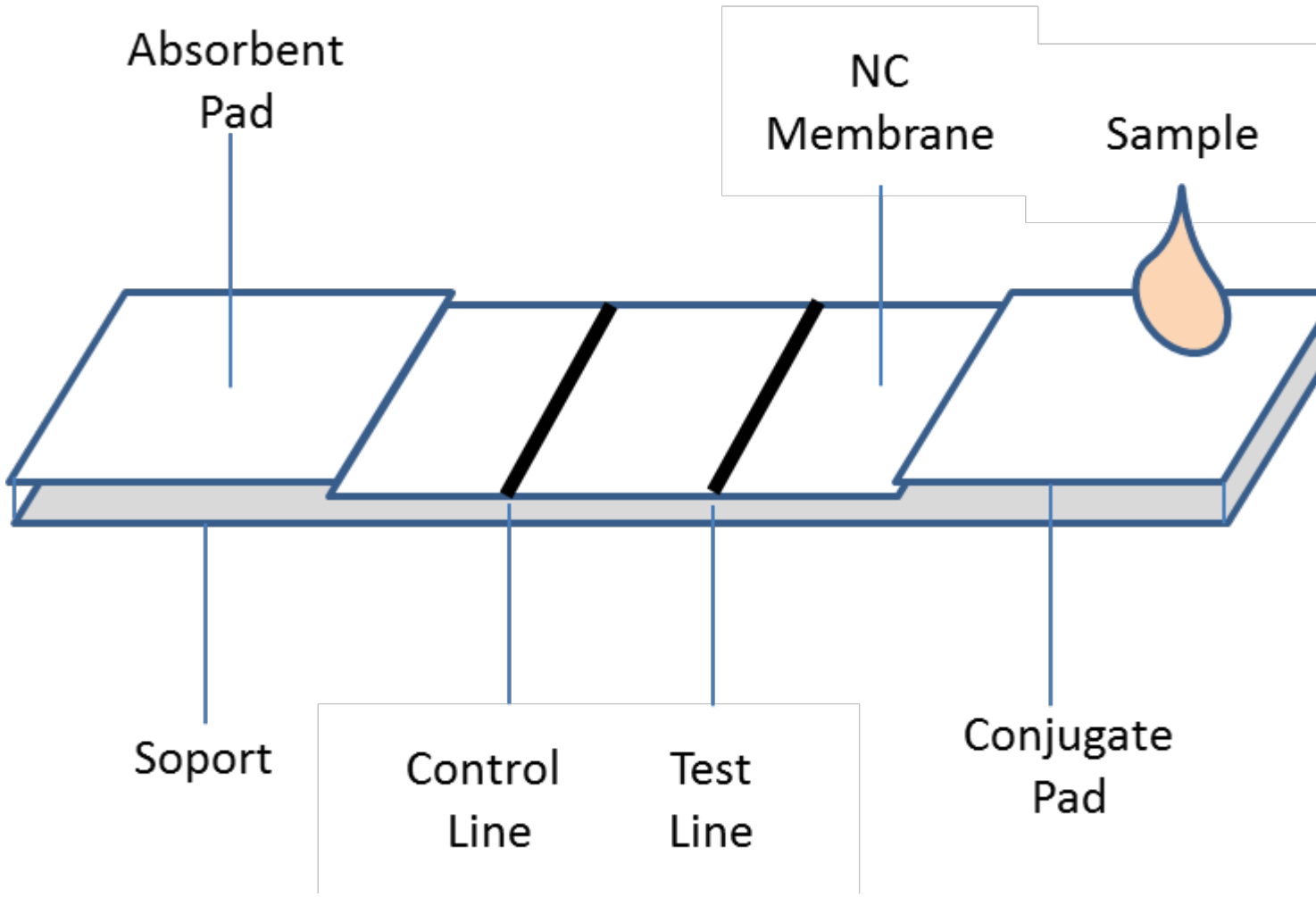

Figure 1. Structure Diagram Of The Test Strip For Anti E2 Antibody Detection.

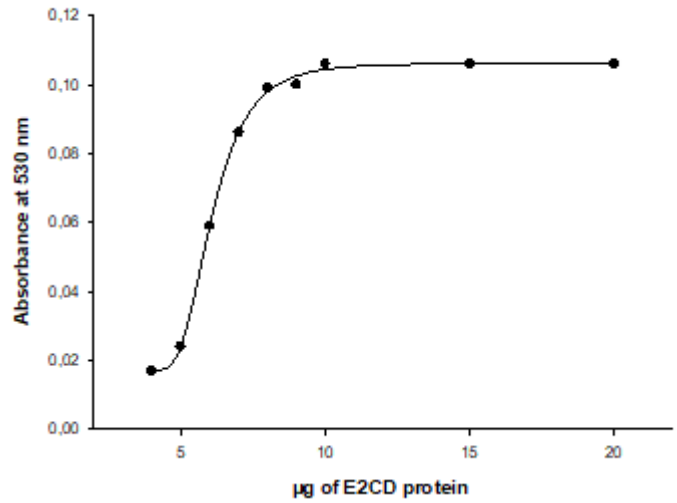

Figure 2. Influence of E2 protein concentration in the stability of colloidal gold conjugate, evaluated through flocculation test.

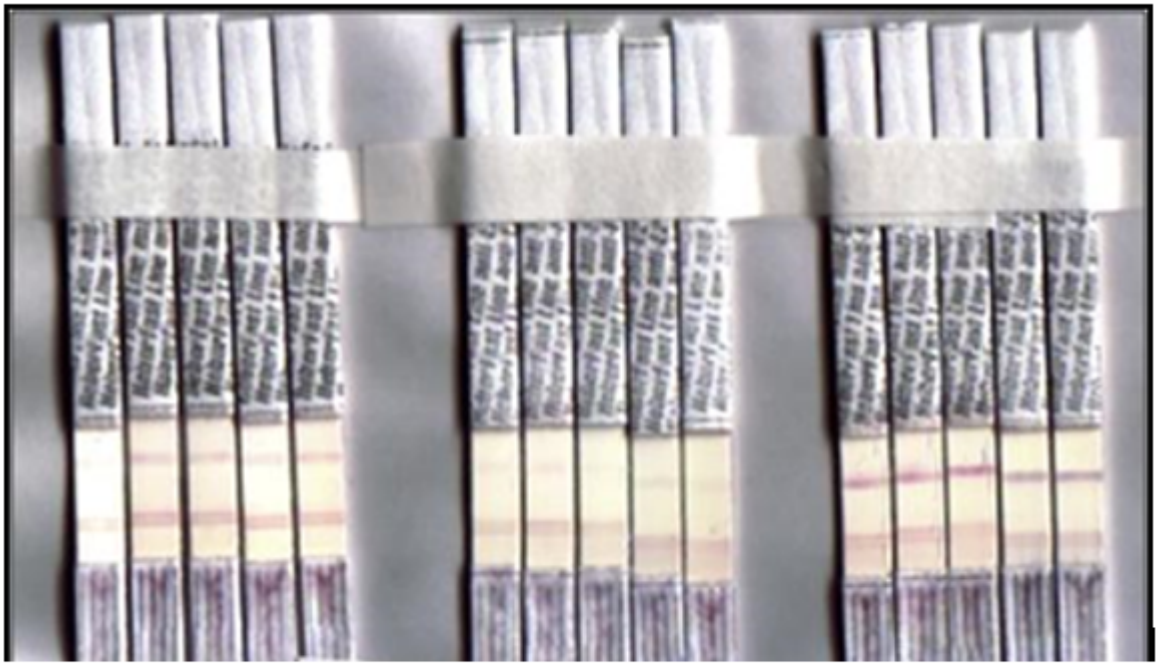

Figure 3. Effect of thermopreservatives on the stability of immunochromatographic strips placed at 60 ?C for 5 days. The influence of sucrose and trehalose as preservatives was evaluated through an experiment that included three treatments (T1, T2 and T3).The signal on the strip is produced by a pool of sera from pigs vaccinated with Porvac®, with a titer of 1/100, according to the NPLA technique. Evaluating the stability and intensity of the lines, the best treatments were T1for the specific line and T3for control line.

TABLE I

Dilution Factor	Samples						
	5	6	8	10	11	12	13
4	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+
16	+	+	+	+	-	-	-
32	-	-	-	-	-	-	-
64	-	-	-	-	-	-	-

Table 1 Dilution of the samples of vaccinated animals that allows differentiating between protected and unprotected animals.

TABLE II

Lot			NPLA	
			Negatives	Po
PT1502	Strip	Negatives	75	
		Positives	2	
		Total	77	
PT1503	Strip	Negatives	74	
		Positives	3	
		Total	77	
PT1601	Strip	Negatives	74	
		Positives	3	
		Total	77	

Table 2. Comparison of the newly developed immunochromatographic strip test with the NPLA assay

TABLE III

Strip lot	Sensibility (%) (CI 95 %)	Specificity (%) (CI 95 %)	Positive Predictiv Value (%) (CI 95 %)
PT1502	95.45 (88.77% to 98.75%)	97.4 (90.93% to 99.68%)	97.67 (91.44% to 99.40
PT1503	94.3 (87.24% to 98.13%)	96.1 (89.03% to 99.19%)	96.51 (90.11% to 98.82
PT1601	97.7 (92.03% to 99.72%)	96.1 (89.03% to 99.19%)	96.63 (90.43% to 98.86

*Sensitivity and specificity of the test were calculated as 100*TP/(TP+FN) and 100*TN/(TN+FP), respectively, where TP means true positive values, FN means false negative values, TN menas True negative values and FP means False positive values . CI represents confidence intervals at 95%. Confidence intervals for sensitivity and specificity are "exact" Clopper-Pearson confidence intervals. Confidence intervals for the predictive values are the standard logit confidence intervals given by Mercaldo et al. 2007.*

Table 3. Evaluation of diagnostic accuracy