Development and Preliminary Application of a Colloidal-Gold Dual Immunochromatography Strip for Detecting African Swine Fever Virus Antibodies

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April 16, 2024

Abstract

African swine fever (ASF) is an acute and highly contagious infectious disease caused by the African swine fever virus (ASFV). Currently, there is no vaccine against ASF worldwide, and no effective treatment measures are available. For this reason, developing a simple, rapid, specific, and sensitive serological detection method for ASFV antibodies is crucial for the prevention and control of ASF. In this study, a 1:1 mixture of gold-labeled p30 and p72 probes was used as the gold-labeled antigen. The p30 and p72 proteins and their monoclonal antibodies were coated on a nitrocellulose membrane (NC) as a test (T) line and control (C) line, respectively. A colloidal-gold dual immunochromatography strip (ICS) for ASFV p30 and p72 protein antibodies was established. The results showed that the colloidal-gold dual ICS could specifically detect ASFV antibodies within 5–10 min. There was no cross-reaction after testing healthy pig serum, porcine reproductive and respiratory syndrome virus (PRRSV), foot-and-mouth disease type A virus (FMDV-A), foot-and-mouth disease type O virus (FMDV-O), porcine circovirus type 2 (PCV-2), and swine fever (CSFV) positive sera. A positive result was obtained only for the positive control, P1. The sensitivity of the test strips was 1:256, which was equivalent to that of commercially available ELISA kits. Their coincidence rate with the two commercial ASFV ELISA antibody detection kits was higher than 98%. The test strips were stably stored at 18–25 °C and 4 °C for 4 and 6 months, respectively. The dual test strips prepared in this study had high sensitivity and specificity and were characterized by rapid detection, simple operation, and easy interpretation of results. Therefore, they are of great significance to diagnose, prevent, and control African swine fever.

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Running Title:

A new Colloidal-Gold Assay for Detecting Antibodies to African Swine Fever Virus

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SUMMARY

African swine fever (ASF) is an acute and highly contagious infectious disease caused by the African swine fever virus (ASFV). Currently, there is no vaccine against ASF worldwide, and no effective treatment measures are available. For this reason, developing a simple, rapid, specific, and sensitive serological detection method for ASFV antibodies is crucial for the prevention and control of ASF. In this study, a 1:1 mixture of gold-labeled p30 and p72 probes was used as the gold-labeled antigen. The p30 and p72 proteins and their monoclonal antibodies were coated on a nitrocellulose membrane (NC) as a test (T) line and control (C) line, respectively. A colloidal-gold dual immunochromatography strip (ICS) for ASFV p30 and p72 protein antibodies was established. The results showed that the colloidal-gold dual ICS could specifically detect ASFV antibodies within 5–10 min. There was no cross-reaction after testing healthy pig serum, porcine reproductive and respiratory syndrome virus (PRRSV), foot-and-mouth disease type A virus (FMDV-A), foot-and-mouth disease type O virus (FMDV-O), porcine circovirus type 2 (PCV-2), and swine fever (CSFV) positive sera. A positive result was obtained only for the positive control, P1. The sensitivity of the test strips was 1:256, which was equivalent to that of commercially available ELISA kits. Their coincidence rate with the two commercial ASFV ELISA antibody detection kits was higher than 98%. The test strips were stably stored at 18–25 °C and 4 °C for 4 and 6 months, respectively. The dual test strips prepared in this study had high sensitivity and specificity and were characterized by rapid detection, simple operation, and easy interpretation of results. Therefore, they are of great significance to diagnose, prevent, and control African swine fever.

Keywords: African swine fever virus; rapid antibody detection; colloidal-gold dual immunochromatography strip

INTRODUCTION

African swine fever (ASF) is an acute, severe, and highly contagious infectious disease. It is caused by African swine fever virus (ASFV) (Hakizimana et al., 2020). ASF was first described in the 1920s in Kenya (Lopez et al., 2020; Miao et al., 2019; Ros-Lucas et al., 2020). In August 2018, the first ASF outbreak in China was recorded (Zhu et al., 2020), and the disease has rapidly spread to all parts of China since then (Teklue et al., 2020). ASFV is a large, enveloped virus from the Asfarviridae family, with icosahedral morphology and an average diameter of 200 nm (Alejo et al., 2018; Alonso et al., 2018; Barrado-Gil et al., 2020; Wang et al., 2019). The only member of Asfarviridae is the genus *Asfivirus* , the only known arbovirus (Franzoni et al., 2020; Zhang et al., 2020; Zhu et al., 2020b). The ASFV genome ranges in length from approximately 170 to 193 kb, depending on the isolate, and contains approximately 160 open reading frames (ORFs) (de Villiers et al., 2010). Studies have shown that more than 50 proteins, including pp220, pp62, p72, p54, p30, and CD2v, are packaged into virus particles and play a role in viral infections. The p30 protein is among the viral proteins expressed early and is encoded by the *CP204L* gene (Hübner et al., 2018). It has a relative molecular weight of 30 kDa; it is among the most antigenic structural proteins involved in ASFV entry and infection and has the strongest immunogenicity (Jia et al., 2017; Petrovan et al., 2019). The p72 protein with a relative molecular weight of 73.2 kDa is the major ASFV structural protein, and it is the crucial antigenic protein encoded by the *B646L* (VP72) gene (Kazakova et al., 2017; Portugal et al., 2012). In addition, it is the main component of the viral icosahedral symmetrica; therefore, it has a high degree of antigen activity and immunogenicity. Both domestic pigs and wild boars are susceptible to ASFV infection. Depending on the virulence of the strain, ASF manifests as acute, subacute, and chronic infections in clinical practice. Acute infections typically cause high fever, skin cyanosis, and severe lymph node bleeding (Chen et al., 2016) with a mortality rate as high as 100% (Jackman et al., 2020; Mee et al., 2020). Compared with the former, subacute infections are not fatal. Although chronic infection is not fatal, it develops into a recessive or persistent infection. ASFV spreads rapidly, there are no effective treatment drugs and vaccines for immunization (Jackman et al., 2020), and culling is the most effective means of controlling the disease

(Gaudreault et al., 2020; Le et al., 2019). Therefore, ASF causes significant losses to the global pig industry (Cappai et al., 2020). The World Organization for Animal Health (OIE) lists ASFV as a statutory animal disease (Lopez et al., 2020) and a critical animal disease to be prevented in China (Fan et al., 2020).

In the absence of ASFV vaccination, a positive serological antibody test can confirm an ongoing or past infection. In epidemic areas, the antibodies in surviving animals last for months or years after subacute infection. Therefore, antibody testing can be applied for large-scale screening of chronic or non-infected animals. At present, the commonly used ASF serological antibody detection methods in laboratories include enzyme-linked immunosorbent assay (ELISA; Cubillos et al., 2013), fluorescent antibody test (FAT; Gallardo et al., 2015), and colloidal gold immunochromatography strips (ICS; Sastre et al., 2016), among others. Although ELISA can effectively detect ASFV-specific antibodies in pigs that survive ASFV infection (Bergeron et al., 2017), this method is time-consuming and requires professional technicians and specific equipment. Therefore, it is challenging to meet the requirements of a rapid, cheap, and efficient monitoring system for clinical antibody levels.

ICS is a new type of diagnostic technology that uses colloidal gold as a tracer and is combined with an antigen-antibody immune response. It is simple, fast, easy to interpret, does not require any equipment, and has a low cost (Yang et al., 2020). Therefore, it is suitable for on-site testing. Since the p30 and p72 proteins play essential roles in the early and late stages of infection, respectively, they have important application value in immunological diagnosis and development of new vaccines. This study aimed to establish an colloidal gold dual immunochromatographic test strip that can rapidly detect the level of antibodies after ASFV infection to efficiently monitor ASF. This would provide technical support for controlling the spread and prevalence of ASF.

MATERIALS AND METHODS

Recombinant Plasmids and Serum

African swine fever p30 and p72 proteins and p30 and p72 monoclonal antibodies were prepared in our laboratory. Forty attenuated ASFVΔ181/UK strain immune sera (immune serum with the highest blocking rate as determined by a Spanish ELISA kit was selected as the positive control P1), 14 ASFV infection inactivated sera, and 30 ASFV negative sera were provided by the African Swine Fever Regional Reference Laboratory (Lanzhou). CSFV, FMDV-A, FMDV-O, PRRSV, PCV-2 positive serum, and healthy swine serum were provided by the State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Reagents, Equipment, and Kits

Chloroauric acid was purchased from Sigma-Aldrich (St Louis, MO, USA); tri-sodium citrate was purchased from the Sinopharm Group (Shanghai, China); sample pads, absorbent pads, conjugate pads, nitrocellulose (NC) membranes, and PVC sheets were purchased from Shanghai Jiening Biotech Co., Ltd. (Shanghai, China); potassium carbonate was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); triple distilled water was used for preparing colloidal gold experiments. The ultrapure water system was purchased from MilliporeSigma (Burlington, MA, USA); an electronic balance model JY2001 was purchased from Shanghai Precision Science Instrument Co., Ltd. (Shanghai, China); the Biodot AD3200 gold standard diagnostic spotting system and Biodot CM4000 flat top fully automated stripper were obtained from BioDot Inc., (Irvine, CA, USA); an African swine fever (p30) ELISA antibody detection kit (lot number: B22013) was purchased from Svanova (Uppsala, Sweden); an African swine fever (p72) ELISA antibody assay kit (lot number: 171218) was purchased from Ingenasa (Madrid, Spain).

Purification of p30 mAb and p72 mAb

The ascitic fluid of both mAbs was centrifuged at 12,000 rpm for 20 min at 4 °C to remove the precipitate, and the supernatant was collected. An equal volume of saturated ammonium sulfate solution (50% saturation) was added, mixed, and incubated for 4–6 h at 4 °C, and the supernatant was discarded following centrifugation. The precipitate was dissolved in PBS (0.01 M, pH 7.4). The ammonium sulfate was removed by dialysis, and

the sample was subjected to affinity chromatography using a HiTrap Protein G affinity column first eluted with 10× the column volume of 0.02 M Na_3PO_3 buffer (pH 7) to wash the column. Protein samples were added at a speed of 1 mL/min using a syringe, followed by 5–10-fold column volume of Na_3PO_3 buffer wash and final elution with 2–5-fold column volumes of 0.1 M glycine-HCl (pH 2.7). The eluate was collected and concentrated by dialysis at 4 °C.

Titer detection of p30 mAb and p72 mAb

The p30 protein or p72 protein was coated on the ELISA plate at 1,000 ng/well, and the p30 mAb (1 mg/mL) and p72 mAb (1 mg/mL) were diluted from 10^2 to 10^7 for indirect ELISA detection. The antibodies were determined using OD_{450nm}.

Preparation of Colloidal Gold Solution

The principle of using 1% tri-sodium citrate to reduce hydrogen tetrachloroaurate hydrate ($HAuCl_4$) to prepare colloidal gold particles of different sizes was used (Liu et al., 2012;). One milliliter of 1% chloroauric acid was added to 100 mL of ultrapure water, mixed well, and heated until bubbles appeared uniformly and started rising upward, after which 2.0 mL of 1% trisodium citrate solution was added immediately, and heating continued for 8–10 min. The Erlenmeyer flask was carefully removed and allowed to cool to room temperature. Ultrapure water was added to a volume of 100 mL.

Determination of Optimal Labeling pH for p30 and p72 Protein

The pH of the gold solution was adjusted to 4–9 using 0.1 mg/mL K_2CO_3 solution; an appropriate amount of protein (60 μg/mL of p30 and 9.6 μg/mL of p72) was then added to the gold solution. After standing for 2h at room temperature, the solution was centrifuged at 12,000 rpm for 30 min at 4 °C; the supernatant was collected and coated on an ELISA plate; reactivity to ASFV-positive serum was measured by indirect ELISA. Finally, the pH was taken as the abscissa, and the $OD_{450\ nm}$ value was drawn as a scatter diagram to determine the optimum pH.

Determination of the Optimal Labeling Amount of p30 and p72 Protein

The p30 protein was diluted 2-fold from 0.1 to 0.00625 mg/mL using 0.1 M PBS, while the p72 protein was diluted 2-fold from 0.25 to 0.001952125 mg/mL using 0.1 M PBS. Afterward, 0.1 mL of the diluted p30 or p72 protein was added to the gold solution with adjusted pH, mixed thoroughly, and left to stand at room temperature for 8–10 min. Subsequently, 0.1 mL of 10% NaCl solution was added and mixed thoroughly, and the color change was observed after 2 h. The minimum protein label amount was recorded, and 20% was added to the minimum label amount to obtain the best protein label amount (Hampl et al., 2001).

Preparation and Purification of Gold Standard Probe

An appropriate volume of 0.1 mg/mL $K_2CO_3$was added to a 50.0 mL gold solution to adjust its pH, reacted for 30 min, and then reacted with an appropriate volume of 0.5 mg/mL p72 protein solution (or 0.1 mg/mL p30) for 45 min. Next, 10% BSA was added to a final concentration of 1%, then the reaction was continued for 45 min and the solution allowed to stand for 2 h at 4 °C. The solution was then centrifuged at 1,500 rpm for 15 min at 4 °C; the supernatant was carefully aspirated, and the precipitate was discarded. The supernatant was centrifuged at 12,000 rpm for 45 min at 4 °C, the resulting supernatant was discarded, and added the gold-labeled protein protectant to the precipitate to reach the original volume of the solution before centrifugation. The mixture was centrifuged again at 12,000 rpm for 45 min at 4 °C and the supernatant was discarded. The precipitate was resuspended in 1/20 of the original volume of the gold-labeled probe protector. Finally, 2.5 mL of p72 gold-labeled probe or 2.5 mL of p30 gold-labeled probe solution was obtained. The two gold-labeled probe solutions were mixed in a 1:1 ratio and placed in a refrigerator at 4 °C as the gold-labeled antigen.

Assembly of the Test Strip

An absorbent pad was sealed using a sample pad blocking solution (PBS solution containing 0.5% PVP,

0.5% Tween-20, 2% PEG20000, 1% BSA) for 0.5–1 h and preserved in a desiccant-containing paper bag. A conjugate pad was placed in a conjugate pad blocking solution (0.75% trehalose, 0.5% sucrose, 2% BSA, 0.5% Tween-20, 0.5% TritonX-100 in PBS); then, the conjugate pad was sprayed with a colloidal gold solution and placed in an oven at 37 °C for 3 h. After complete drying, tweezers were used to gently place the coated conjugate pad in a desiccant-containing paper bag and stored at room temperature. Subsequently, the conjugate pad was sprayed with a 1:1 mixture of p30 and p72 protein monoclonal antibodies (1 mg/mL) on the control line of the nitrocellulose membrane. Additionally, p72 (0.5 mg/mL) and p30 proteins (0.1 mg/mL) were sprayed on test lines 1 (T1) and 2 (T2) of the nitrocellulose membrane, respectively.

ICS Result and Reaction Principle

The serum was diluted with the sample diluent at a ratio of 1:10, and then 100 μL of the diluted sample solution was dropped onto the sample pad to observe the results within 5–10 min at room temperature. If the test and control lines appeared red, the test was positive; if the test line did not appear red, and the control line appeared red, the test was negative. If the control line was not colored, the test was deemed invalid (**Figure 7B**).

When the serum to be tested is added to the sample pad, it migrates forward by capillary action. If the serum reaching the conjugate pad contains p30 or p72 antibodies, the protein on the conjugate pad reacts with its corresponding antibody, thereby forming the corresponding antigen-antibody complex. It continues to migrate to T2 (p30) or T1 (p72) to form a red band visible to the naked eye. Regardless of whether the serum to be tested contains p30 and p72 antibodies, the gold-labeled protein will continue to migrate to the control line and react with the anti-p30 and -p72 monoclonal antibody complexes at the control line to produce a red band visible to the naked eye. Therefore, if the red bands are not displayed during the quality control assessment, the test strip is rendered invalid.

Evaluation of the ICS Performance

In the present study, to objectively evaluate the performance of the test strip, we verified its specificity, sensitivity, and stability. The test strips prepared in this study were used to test healthy pig serum; PRRSV, FMDV-A, FMDV-O, CSFV, and PCV-2 positive sera; and a positive control, P1 to test their specificity.

The positive control P1 was diluted with a sample diluent at ratios ranging from 1:2 to 1:512, and each solution was used as an independent sample for testing according to the steps outline in the section "ICS Result and Reaction Principle" below. The results were observed within 5–10 min and the sensitivity of the test strip was determined.

The test strips were stored at room temperature (18–25) and at 4 to determine their stability. Test strip stability was determined at 0, 1, 2, 3, 4, 5, 6, 7, and 8 months (Liu et al., 2017).

The Growth and Decline of p30 and p72 Antibody

To effectively apply the test strips prepared in this study to clinical practice tests, we first used the attenuated ASFVΔ181/UK strain to immunize pigs No. 1–5 and then obtained antibody tests for each at 0, 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, and 29 days. The test strips prepared in this study were used to detect antibody growth and decline. The strips were compared against the Svanova p30 indirect ELISA kit and Ingenasa p72 blocking ELISA kit.

Clinical Sample Application

The ASFV antibody colloidal gold dual immunochromatographic test strips prepared in this study, the Svanova ASFV p30 antibody detection kit, and the Ingenasa ASFV p72 ELISA kit were used to test 84 clinical serum samples (40 attenuated ASFVΔ181/UK strain immune sera, 14 infected inactivated sera, and 30 ASFV negative sera). The coincidence rate of the ASFV antibody colloidal gold test strip was compared with that of the two commercial ELISA kits (Svanova and Ingenasa).

RESULTS

Identification and Purification of p72, p30 Recombinant Protein

The expressed p30 recombinant protein and p72 truncated protein were purified by nickel column chromatography and subjected to SDS-PAGE, resulting in bands of approximately 45 and 38 kDa, respectively. This indicated that the purified protein of interest had a single band that was consistent with the predicted band size, and the target protein was purified successfully (**Figure 1**).

Figure 1— Purification of p30 recombinant protein and p72 truncated protein. 1: SDS-PAGE analysis of p30 recombinant protein, 2: SDS-PAGE analysis of p72 truncated protein, M: Marker.

Purification of p30, p72 mAb

The collected eluates were subjected to SDS-PAGE. The purified heavy and light chains of the p30 and p72 antibodies had relative molecular weights of approximately 55 and 25 kDa, respectively (**Figure 2**), indicating that the purified antibody band was correct and the purity was high.

Figure 2— Purification of ascites with p30 and p72 mAb. 1: the size of heavy and light chain bands after purification of p30 mAb, 2: the size of heavy and light chain bands after purification of p72 mAb, M: Marker.

Titer detection of p30 mAb and p72 mAb

When the OD_{450nm} value is greater than 1, the results of indirect ELISA are judged to be positive. As shown in **Figure 3** , the antibody titers of the p30 and p72 monoclonal antibodies were both 2^6, indicating that the antibody level was high and the antigen-binding was strong.

Figure 3— Titer detection of p30 mAb and p72 mAb

Preparation of Colloidal Gold Solution

As shown in Figures 4A and 4B, the prepared gold solution was clear and bright wine-red, with a maximum absorption peak wavelength of 520 nm, according to the regression equation Y = 0.786X + 505.53 (Y is the maximum absorption wavelength and X is the particle size). It can be seen that the average particle size of the colloidal gold is approximately 18.4 nm, and all the three parameters meet the requirements of obtaining a good quality colloidal gold solution (Safenkova et al., 2010).

Figure 4— Nanogold solution ocular view (A), UV scan figure (B)

Determination of Optimum pH of p30 and p72 Protein

An indirect ELISA was used to test the supernatant of the gold-labeled solution at pH 4–9 after centrifugation. When the OD_{450nm} value of the supernatant was at its lowest, the unlabeled protein content in the supernatant was also at its lowest, implying that the protein bound to the surface of gold particles was at its highest at this pH. As shown in **Figures 5A** and **5B** , the OD_{450nm} of the supernatant was lowest when the pH of the colloidal gold solution was 8 and 7. Therefore, the optimal pH values for the p30 and p72 proteins were 8 and 7, respectively.

Figure 5— Determination of the optimal pH value for p30 protein (A) and p72 protein (B). Error bars correspond to the standard deviation of the data ($n = 3$).

Determination of the Optimal Protein Amount of p30 and p72

The p30 and p72 proteins were diluted to different concentrations and verified using a 10% NaCl destructive test. As shown in **Figures 6A** and **6B** , the optimal labeling concentrations for p30 and p72 were 60 and 9.6 μg/mL, respectively.

Figure 6— Determination of the optimal labeling of p30 protein (A) and p72 protein (B). A1–A6: p30 protein was diluted from 0.1 to 0.00625 mg/mL; B1–B8: The p72 protein was diluted from 0.25 to 0.001953125 mg/mL.

The structure of the colloidal gold test strip and the result from ICS

As shown in **Figure 7A** , the immunochromatographic strip was mainly constructed from a sample pad, absorbent pad, conjugate pad, nitrocellulose (NC) membrane, and a PVC sheet.

Figure 7 — The structure of the test strip (A) and ICS results (B).

Specificity

The test strip was used to test the positive control serum P1; PRRSV, FMDV-A, FMDV-O, PCV2, and CSFV positive sera; and healthy pig serum. The test results showed that only P1 was positive, and there was no cross-reactivity with other sera (**Figure 8**).

Figure 8 —Specificity of the test strip. 1: positive control serum P1, 2: PRRSV positive serum, 3: FMDV-A positive serum, 4: FMDV-O positive serum, 5: PCV-2 positive serum, 6: CSFV positive serum, 7: healthy pig serum.

Sensitivity

The test strip was used to test the positive control serum P1, which tested positive when diluted to 1:256, indicating that the minimum detection limit was 1:256 (**Figure 9**). The Svanova and Ingenasa ELISA kits yielded positive results when P1 was diluted to 1:512 and 1:256, respectively (**Table 1**). Therefore the sensitivity of the strips prepared in this study was comparable to those of two commercial ELISA kits.

FIGURE 9— Sensitivity of the test strips. 1–9: the positive control serum P1 was diluted from 1:2 to 1:512.

Table 1 — Svanova and Ingenasa ELISA kit sensitivity

positive control serum P1 dilution factor	Svanova ELISA kit (PP value)	Ingenasa ELISA kit (blocking rate*100%)
1:2	125.6	96
1:4	117.3	93.1
1:8	98.2	90.6
1:16	85.4	86.4
1:32	77.6	72.8
1:64	55.7	62.8
1:128	39.4	51.3
1:256	26.5	42.1
1:512	14.2	28.3
1:1024	8.6	11.2

Stability

Stability is an important factor in field applications. The test strips were stored at room temperature (18–25 °C) and at 4 °C. The stability test was carried out for both groups of strips at 0, 1, 2, 3, 4, 5, 6, 7, and 8 months. **Table 2** shows that the strip can be stored for up to 4 months at room temperature and for up to 6 months at 4 °C.

Table 2 —Test strip stability

Preservation temperature	Preservation time 0	Preservation time 1	Preservation time 2	Preservation time 3	Preservation 4
Room temperature(18–25)	+++	+++	+++	++	++
4	+++	+++	+++	+++	+++

+ + +, the effective rate was 100%; + +, the effective rate was 66%; +, the effective rate was 33%; -, the effective rate was 0%.

Growth and Decline of p30 and p72 Antibody

Pig NO.1 p30 and p72 antibodies were detected gradually from the 7th and 9th day, respectively, and then at a steady upward trend (**Figure 10A**); Pig NO.2 p30 and p72 antibodies were detected gradually from the 7th and 9th day, respectively, and then at a steady upward trend (**Figure 10B**); Pig NO.3 p30 and p72 antibodies were gradually detected from the 9th and 11th day, respectively, and then at a steady upward trend (**Figure 10C**); Both p30 and p72 antibodies of pig NO.4 were detected gradually from day 11, and then at a steady upward trend (**Figure 10D**); Pig NO.5 p30 and p72 antibodies were gradually detected from the 11th and 15th day, respectively; afterward, a steady upward trend was observed (**Figure 10E**). As shown in**Figure 10F** and **Figure 10G** , the above results are consistent with the test results of the commercial ELISA test kits (the Svanova p30 indirect ELISA result was positive when the PP value was greater than 10, and the Ingenasa p72 blocking ELISA was positive when the blocking rate was greater than 40%).

FIGURE 10— Five pigs were immunized with the attenuated Δ181/UK strain; the test strips prepared in this study, the Svanova p30 and Ingenasa p72 ELISA kits, were used to detect antibody growth and decline. A–E: Pigs No. 1–5 test strip antibody growth and decline results; F: Pigs No. 1–5 Svanova p30 ELISA results; G: Pigs No. 1–5 Ingenasa p72 ELISA results.

Practicability

Two commercial ELISA kits (Svanova and Ingenasa) and the test strips prepared in the present study were used to simultaneously test 84 clinical serum samples (attenuated 40 ASFVΔ181/UK strain immune sera, 14 infection-inactivated sera, and 30 ASFV-negative sera). The results showed that the coincidence rates of the test strip, Svanova, and Ingenasa ELISA kits were 98.8% [(39+14+30)/(54+30)], 100% [(40+14+30)/(54+30)], and 100% [(40+30)/(40+30)], respectively. The coincidence rate between the test strip and the Svanova ELISA kit was 98.8% [(39+14+30)/(40+14+30)], and the coincidence rate between the test strip and the Ingenasa ELISA kit was 98.6% [(39+30)/(40+30)] (**Table 3**).

Table 3 —The results of the compliance test

Sample	Number of samples	Test strip	Svanova ELISA	Ingenasa ELIS
40 ASFVΔ181/UK attenuated strain immune serums	54	39	40	40
14 infection inactivated serums		14	14	0
30 ASFV negative serums	30	30	30	30

DISCUSSION

Since the first case of African swine fever occurred in August 2018, the Chinese pig industry has been devastated. In the absence of commercial vaccines and therapeutic drugs, early, effective, rapid, and convenient detection is essential for the prevention and control of ASF. Therefore, it is urgent to establish a rapid and efficient ASF antibody detection method.

At present, the commonly used methods of serological detection of antibodies include ELISA, indirect immunofluorescence test (IFA), western blotting (WB), and colloidal gold immunochromatographic detection technology. Compared with other methods, colloidal gold immunochromatographic detection technology is simple, portable, and has a rapid response; results can be observed with the naked eye within 5–10 min. Therefore, it is suitable for rapid high-throughput detection at the grassroots level or on-site (Lei et al., 2020; Sun et al., 2020; Yang et al., 2020a). Colloidal gold immunochromatographic detection technology has diagnostic significance for serological diagnosis. The ASFV structural proteins primarily include p30, p72, and p54 (Costard et al., 2009; Neilan et al., 2004). The p30 protein is generally expressed and secreted in the early stages of viral infection and has good antigenicity; thus, it can be used to detect infection early. The p72 protein is mainly expressed in the late stages of viral infection; it is highly conserved, and its secretion and expression show good antigenicity (Bao et al., 2019; Fernández-Pinero et al., 2013; Olasz et al.,

2019). Considering the advantages of colloidal gold immunochromatographic test strips and the combined characteristics of p30 and p72 as diagnostic antigens, we established an ASFV antibody dual colloidal gold immunochromatographic detection method that can quickly and conveniently monitor the occurrence and development of ASF infection in real time.

The stability of a test strip plays a vital role in field applications. Accordingly, the quality of the gold solution and the purity and concentration of the raw materials (protein or antibody) all affected and restricted the stability of the test strip.

The quality of the gold solution is one of the decisive factors determining whether colloidal gold immunochromatographic detection technology can be successfully established. If the diameter variation range of the gold particles is too large, the stability of the test strip will be affected. At the same time, if the size of the gold particles is not uniform, the gold-labeled probes will easily precipitate, resulting in false positives. Therefore, preparing a colloidal gold solution with uniform particles and good dispersion is especially important for subsequent experiments. According to Wang et al. (2004), if the maximum absorption peak and its corresponding OD_{450nm} value are between 0.8 and 1.2, it can be preliminarily considered that the gold solution meets the conditions. Therefore, to obtain a good-quality gold solution, we systematically explored the amount of reducing agent required and obtained a clear and bright wine-red gold solution. The maximum absorption peak λmax (520 nm) of the gold solution and its corresponding OD_{450nm} value (0.925) were obtained using an ultraviolet scanner. These values indicated that the conditions were met. Many tests have proven that the purity and concentration of the raw materials directly affect the quality of the gold-labeled probe. The recombinant p30 protein prepared in this study had a His-tag and contained more imidazole after purification through a nickel column. At the same time, p30 protein is an inclusion body protein, and a high concentration of urea is used when it is dissolved. If the colloidal gold label contains imidazole and urea, a large amount of colloidal gold can accumulate, precipitate, or even cease activity. Therefore, the raw materials must be fully dialyzed to remove urea and imidazole before labeling the gold particles. The main component of the dialysate was 20 mM Tris-HCl. A small amount of salt can also be added to ensure a balance within the entire system.

To evaluate the practicability of the test strips prepared in this study, we tested 84 serum samples simultaneously using two commercial ELISA kits. The results indicated that the test strips and the two commercial ELISA kits had coincidence rates higher than 98%. Test strip specificity was evaluated by testing for cross-reactivity with pig-derived CSFV, PRRSV, FMDV-A, FMDV-O, and PCV-2 positive sera, which are clinically similar to ASF and easily confused for it. The results showed that the test strip had good specificity. The test strip sensitivity was assessed using different test dilutions of attenuated Δ181/UK strain immune serum, and the results revealed a high sensitivity of the test strips (p30 ELISA titer: 1:512, p72 ELISA titer: 1:256). Notably, the test strips were able to detect the growth and decline of antibodies from days 0 to 29 after challenge with an attenuated strain of ASFVΔ181/UK, enabling us to determine the period during which the antibodies corresponding to p30 and p72 appeared. The earliest appearance of p30 occurred on the 7th day, and the latest was on the 11th day. The earliest appearance time of p72 was generally the 9th day, and the latest was on the 15th day. Therefore, the present study demonstrated the dynamic monitoring of ASF by observing the growth and decline of the two antibodies.

Currently, the development of African swine fever primarily shows three trends. 1) Infection of a pig herd with a virulent ASFV strain will cause high mortality over a short period, with a fatality rate as high as 100%. Since disease duration is short and mortality is close to 100%, antibody testing is not applicable. 2) By monitoring the prevalence of ASF in seven Chinese provinces from June to December 2020, it was shown that there were natural mutations or deletions in the ASFV strains, resulting in a non-blood cell adsorption phenotype. Following virulence tests in pigs, it was observed that a toxic dose of 10^6 $TCID_{50}$ could cause fatal acute or subacute disease in pigs. The remaining non-fatally diseased pigs will naturally recover after tolerance but become carriers for a long time, and the virus is easily transmitted since a 10^3TCID_{50} dose could cause non-lethal chronic or persistent infections. Compared with the former, the latter trend has a higher survival rate, but more pigs are infected, and transmission is greater. Therefore, it is easy to cause

a second ASF pandemic, which will bring a new level of destruction to the world's breeding industry. Since surviving animals can maintain high antibody levels for a long time, the detection of ASFV-specific antibodies is of great significance for determining the infection status of the host. Therefore, the test strips prepared in this study could be effectively applied to detect the presence of mutant strains. Dynamic monitoring of antibodies in pigs provides a powerful technical means for the prevention and control of ASF, ensuring that economic losses are reduced by minimizing the risk of transmission. 3) Through the continuous efforts of researchers, an effective ASF vaccine should be successfully developed in the near future, and antibodies that can inhibit or kill ASFV can be obtained in pig populations after comprehensive immunization. The test strips prepared in this study can be effectively used to evaluate the immune effects of vaccines. In addition, this study uses a test strip to detect two antibodies simultaneously, which not only achieves the effect of diversified detection but also lowers costs.

Conclusion

Compared with a single p30 or p72 protein test strip, the p30 and p72 protein test strips can be more accurate in determining the organism's infection period; at the same time, it has a good specificity, high sensitivity, good repeatability, and is fast and simple. It only takes 5-10 min to observe the results with the naked eye; this technology is expected to enable on-site or laboratory testing of African swine fever antibodies and provides a new solution for the rapid diagnosis of African swine fever.

Data Availability Statement

The data that supports our fingings are available in the manuscript .

Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. All animal experimental procedures have been reviewed and approved by the Animal Care and Use Committee of Lanzhou Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (approval ID: SYXK(Gan) 2015-0003).

ACKNOWLEDGMENTS

This work was supported by the National Key R&D Program of the Ministry of Science and Technology (2018YFC0840400).

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

Author contributions

Ying Wan: Conceptualization, Methodology, Formal analysis, Writing - original

draft. Zhengwang Shi : Formal analysis, Investigation. Gaochaung Peng: Investigation, Validation. Lijuan Wang: Methodology, Validation. Juncong Luo: Formal analysis. Yi Ru: Formal analysis. Gaijing Zhou: Formal analysis. Yuan Ma: Investigation. Rui Song: Investigation. Bo Yang: Investigation. Liyan Cao: Investigation. Hong Tian: Writing – review and editing, Supervision. Haixue Zheng: Project administration, Funding acquisition.

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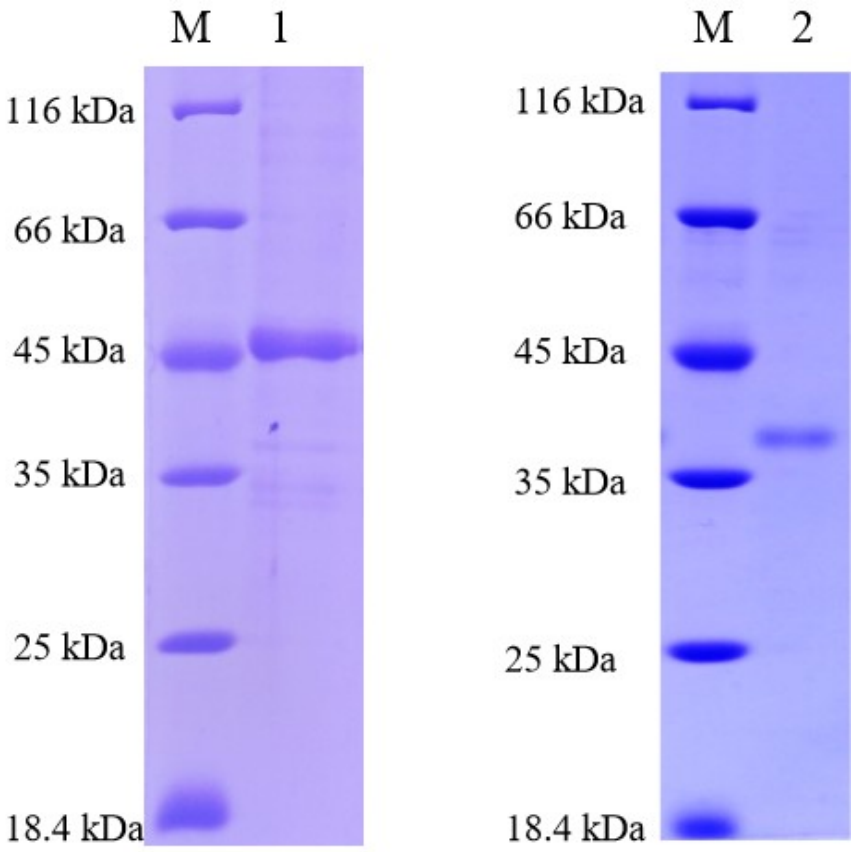

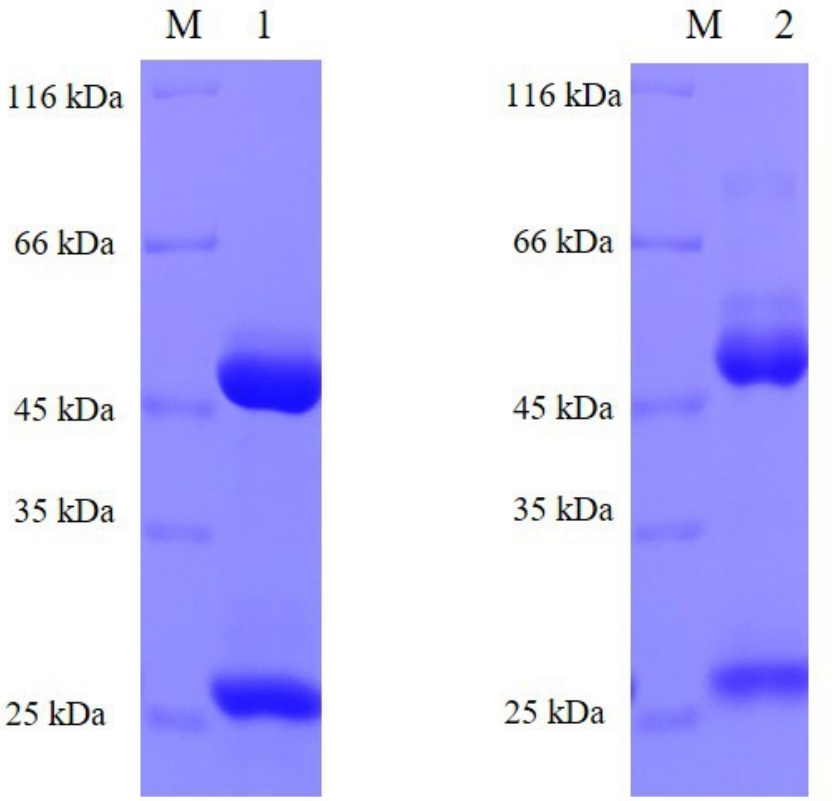

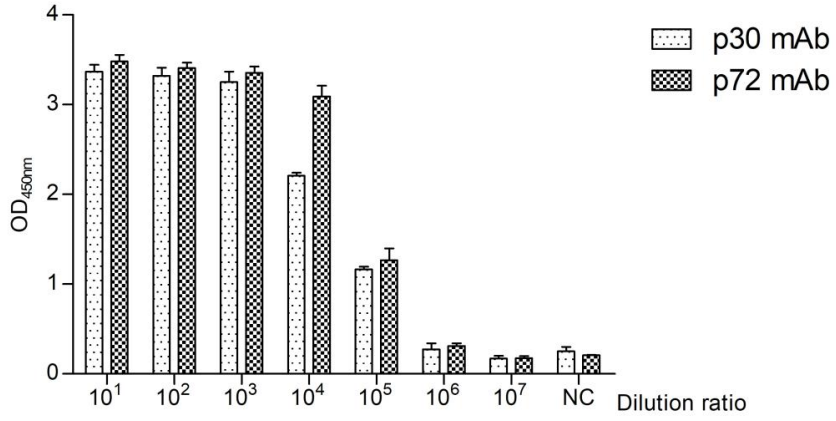

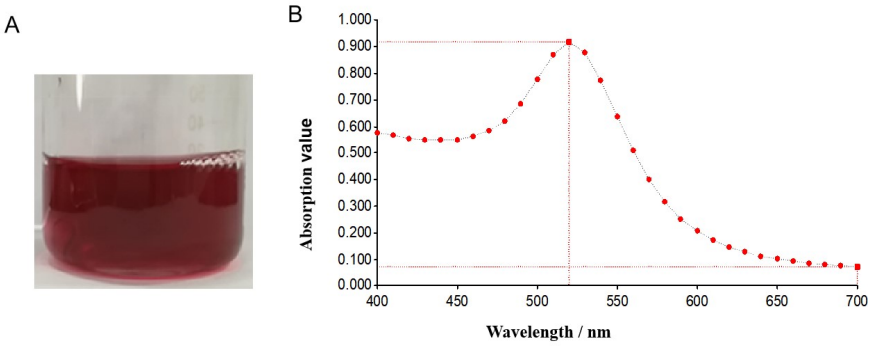

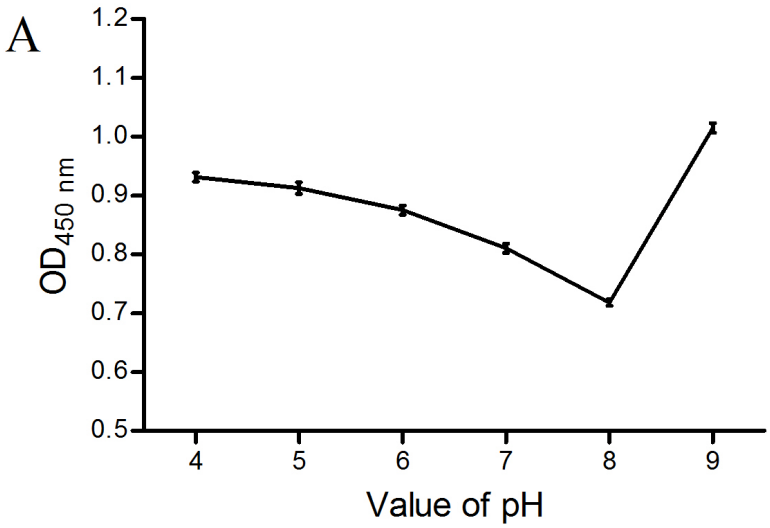

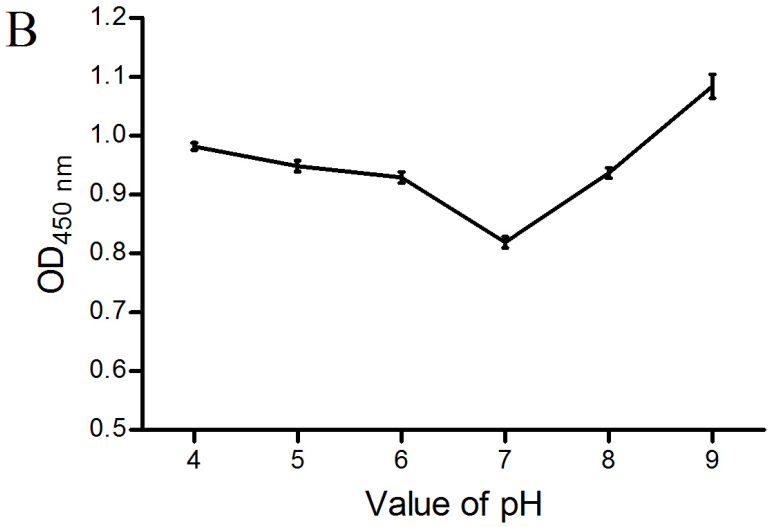

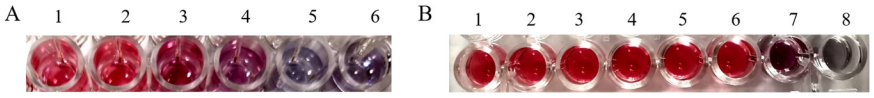

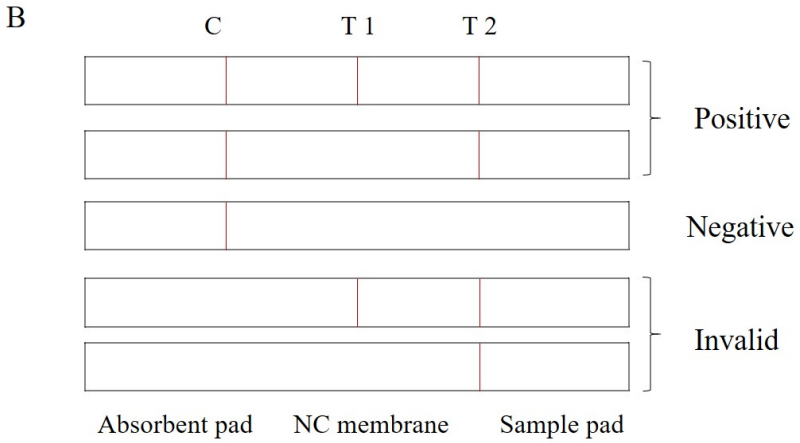

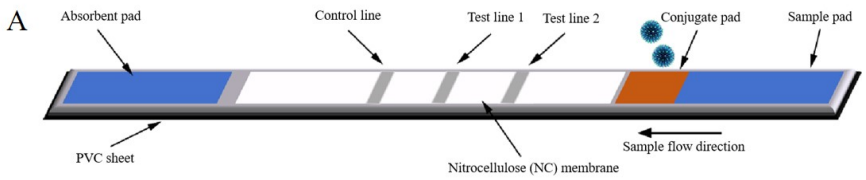

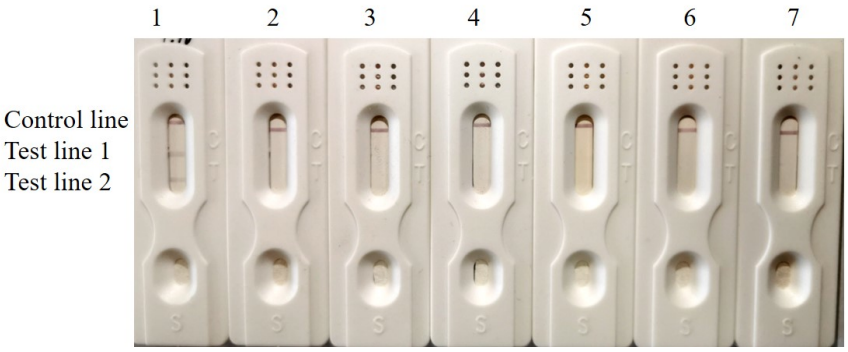

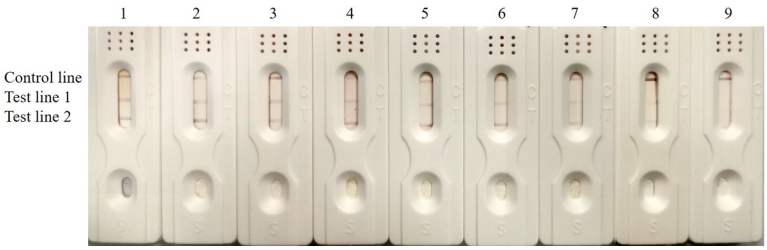

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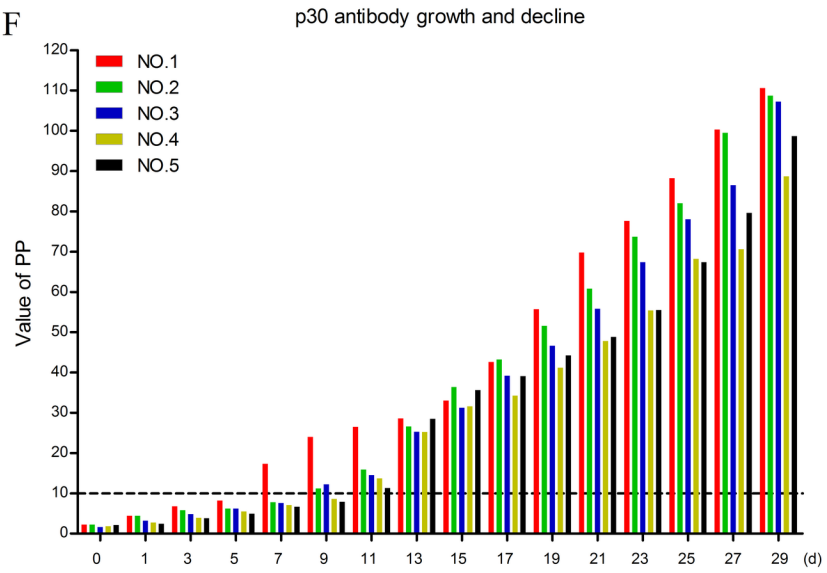

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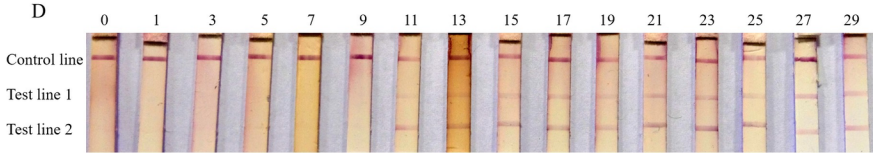

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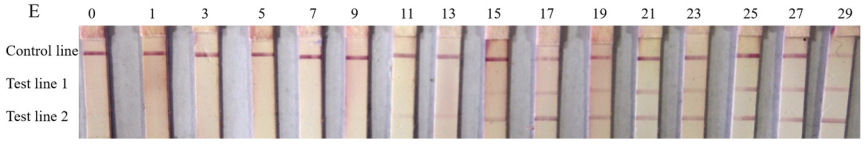

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