RESEARCH REPORT
SWINE HEALTH

Title: Improving African swine fever diagnostics through the use of Multi Antigen Print Immunoassay (MAPIA) technology – NPB-CEEZAD #16-021

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Industry Summary:

OIE-approved assays to identify the presence of African Swine Fever Virus (ASFV)-specific antibodies include an initial screening by ELISA-based tests followed by a confirmatory assay. False positive reactions in ELISA are not uncommon, especially when serum samples are in poor condition. The use of an ELISA test subsequently followed by confirmatory assays increases the specificity of the final results, and is recommended by the OIE. The Immunoblotting (IB) or Western blot (WB) test is the most widely used confirmatory method, allowing the visualization of antibody responses to ASFV proteins of various sizes. However the production, performance and interpretation of this test present certain limitations. It is a time consuming process, and needs to be performed in high containment BSL-3 facilities. In addition, there are significant difficulties in standardization and scalability of the WB assay, and it is often difficult to interpret the results of the assay due to a high background signal.

In this project, we utilized Multi Antigen Print Immunoassay (MAPIA) technology to develop a test for the detection of ASFV-specific antibodies as alternative to the current method based on WB analysis with cell-propagated ASFV antigens. The ASF MAPIA assay developed in this project uses recombinant ASFV proteins printed on nitrocellulose membranes for ASFV-specific antibody detection. Strips containing recombinant ASFV proteins can be produced in a BSL-2 laboratory and are easy to standardize and to scale up. Test evaluation data generated in this project demonstrated good specificity and ease of interpretation of the ASF MAPIA test results.

Keywords: African Swine Fever, MAPIA, recombinant proteins, p22, p30, p54, p72

Scientific Abstract:

Serological diagnosis of complex viruses that generate a highly heterogeneous antibody repertoire, such as African swine fever (ASF), requires tests based on cocktails of antigens. The general procedure for antibody detection of ASF in affected countries consists of screening of sera by ELISA, followed by confirmation by WB using nitrocellulose strips containing ASFV proteins. Poorly preserved sera can produce false-positive reactions by ELISA or false-negative results due to a loss of antibodies against viral antigens present in the ELISA. In both cases, the WB is a valuable assay

to confirm ELISA results. In this project, we tried to improve the current OIE confirmatory WB test by developing a Multi-Antigen Print Immunoassay (MAPIA) test for ASFV serological diagnosis. The ASF MAPIA is based on the application of recombinant ASFV antigens to nitrocellulose membranes by micro-aerosolization (printing), followed by antibody detection using anti-swine peroxidase antibodies and standard chromogenic substrate. The use of well-defined recombinant ASFV proteins printed on a nitrocellulose membrane allows simple interpretation of the test results when compared to the conventional WB test. We concluded that the ASF MAPIA is a sensitive, specific, safe, and rapid test format. Production of MAPIA strips can be done in BSL-2 laboratories and the system can be easily standardized and scaled up. The ASF MAPIA might be an economically viable alternative to the existing confirmatory WB test for ASF serological diagnostic.

Introduction:

African swine fever (ASF) is one of the most important diseases of swine, whose presence in affected countries causes serious socio-economic consequences (Mur L et al., 2012; Sánchez-Vizcaíno, J. and L. Mur, 2013; Sánchez-Vizcaíno et al, 2013). Its recent introduction into Eastern European countries, Belgium and Southeast Asia has increased the concerns about the potential of further spread, especially within western EU and eventually to North America. Therefore it is important to develop robust serological tests as effective countermeasures. ASF is routinely diagnosed with both molecular (normally PCR) and serological tests. The long persistence of anti-ASFV antibodies in chronically-infected animals makes serological assays an essential part of ASF diagnosis.
OIE-approved assays to identify the presence of ASFV-specific antibodies include an initial screening by ELISA, followed by a confirmatory assay. False positive reactions in ELISA tests are not uncommon, especially when serum samples are in poor condition. The use of an ELISA test subsequently followed by confirmatory techniques increases the specificity of the final results and is recommended by the OIE (OIE, 2013). The Immunoblotting (IB) or Western blot (WB) test based on ASFV antigens produced in cells is the most widely used confirmatory method, allowing the visualization of antibody responses to ASFV proteins (OIE, 2013; Pastor et al., 1989). However the production, performance and interpretation of the WB test presents certain limitations. It is tedious, time consuming and needs to be performed in high containment facilities (BSL3Ag). In addition, there are significant difficulties in standardization and scalability of the WB, and it is often difficult to interpret the results of the assay due to a high background signal.
The Multi Antigen Print Immunoassay (MAPIA) has been employed for the serological diagnosis of several infectious diseases demonstrating many benefits compared to traditional serological methods (Lyashchenko et al., 2000). Specifically, the ability of this technique to include multiple antigens makes it highly specific. The MAPIA is an antibody detection method that directly sprays (prints) purified proteins in lines onto nitrocellulose membranes upon which classical antibody detection methods can be performed. The ASF MAPIA can be used as an alternative to the immunofluorescence antibody test (IFA) or the WB test to confirm positive or negative results obtained with individual sera tested by ELISA. It is highly specific, rapid, enables easy and objective interpretation of the results and a good recognition of weak-positive samples. The ASFV MAPIA contains selected recombinant ASFV proteins with which ASFV-specific antibodies in acute, chronic, and convalescent pig sera will react. Importantly, the test can be performed in 2.5 - 3 hours and, if needed, outside of a conventional diagnostic laboratory (i.e. the field). The successful development and implementation of the ASF MAPIA test will significantly improve the current diagnostic methods available for ASF. Early confirmation of ASF results is critical to avoid unnecessary loss of livestock or delays in the implementation of control measures.

Objectives:

The overall objective of this project was to develop a MAPIA-based serological assay for the confirmation of ASF infection. The specific objectives of this work include:

1. Production and evaluation of candidate ASFV recombinant proteins.

2. Production of hyperimmune sera in pigs against selected recombinant proteins as reference controls.
3. Development and optimization of a prototype MAPIA for the serological diagnosis of ASF.
4. Validation of the prototypes with ASF serum samples from experimentally infected pigs and well characterized field samples.

Material & Methods:

Production of recombinant proteins of interest for ASF sero-diagnostics and MAPIA strip preparation

ASF recombinant proteins, including p22, p30, p54, pp62 and p72 were produced in the baculovirus expression system in Sf9 cells. Sequence of the ASFV strain Georgia 2007/1 (GenBank accession # FR682468) was used as a reference for the cloned ASFV genes. The correct expression and reactivity of ASFV recombinant proteins were evaluated by western blot using anti-HisTag antibodies and polyclonal serum against ASFV.
The ASFV proteins plus two internal controls were immobilized by printing on nitrocellulose membrane (GE Healthcare #10600018) in parallel bands using the BioDot ZX 1010 dispensing system. Porcine serum at a 1:100 dilution was used as internal swine immunoglobulin positive control, and recombinant EHDV protein, expressed in baculovirus, was used as internal negative control.
Each recombinant ASFV protein was used at 150 µg/ml concentration and printed at 50mm/second speed with a volume of 0.5 µl/mm.

Production of hyperimmune sera in swine

Porcine hyperimmune sera against ASFV proteins (ASFV p22, p30, p54, p72) were produced at the KSU Large Animal Research Center (LARC). Three-week old piglets were immunized with the selected ASFV recombinant proteins. The pigs were immunized intramuscularly with recombinant proteins (50µg in 0.5 ml of PBS, pH 7.4) emulsified with Emulsigen-D adjuvant (MVP Technologies). Two subsequent boosts were administered three weeks apart. In order to assess the immune response to each antigen before final serum collection, blood samples were collected one week after each boost and titers of serially diluted sera were determined using an indirect ELISA using the respective recombinant proteins as coating antigen. Negative swine serum (collected prior inoculation) was used as negative control to determine the background performance of the assay.

Optimization of the ASF MAPIA assay

The workflow of the ASF MAPIA is presented in Figure 1. Several materials and conditions were assayed during the optimization of the MAPIA prototype to obtain the best possible results. Specifically, the following conditions were assayed for optimization:

- Membranes: three different types of membranes were tested (0.45 µm pore nitrocellulose, conventional and supported, and 0.45 µm pore PVDF membrane).
- Printing conditions: rate, protein volume dispenser, speed.
- Protein concentration: 100 - 400 µg/ml were assessed.
- Drying membrane conditions: from 15 min to one hour at room temperature and in a warm chamber (37^{o}C).
- Conjugates: three different peroxidase conjugated antibodies (Anti-swine IgG) from different companies were assayed at different concentrations.
- Incubation conditions of antiserum: room temperature vs warm chamber (37°C)
- Blocking buffer: 1% to 10% milk and 5% BSA were assayed.

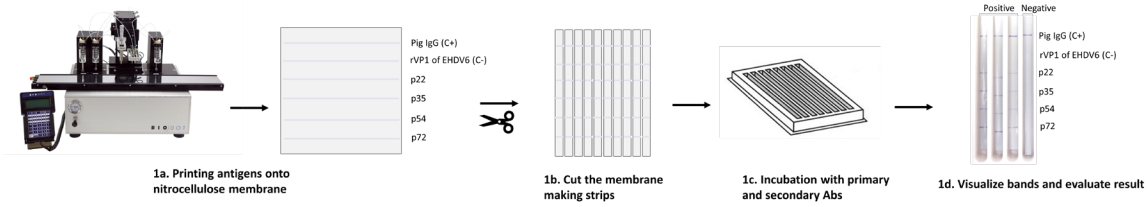

Figure 1. ASF Multi-Antigen Print Immunoassay workflow.

Results:

Production of ASFV proteins

A total of 17 ASFV proteins with histidine tags were produced by cloning and expression in two different systems (i.e. baculovirus Sf9 cell and *E.coli*) based on sequences of the ASFV Georgia 2007-1 strain (Gallardo et al., 2006, Gallardo et al., 2009). The proteins were purified using nickel columns.
Purified ASFV proteins were sent to the Universidad Complutense de Madrid (UCM) in Madrid to analyze the reactivity of those proteins using well characterized ASFV sera from experimentally infected pigs (early and late after infection) and field sera as well as ASF-specific pig hyperimmune sera. This approach allowed us to select ASFV proteins that are detected at early and late stages of ASFV infection.

MAPIA prototype

Based on the results of our indirect ELISA and preliminary MAPIA tests, we decided to include the following proteins in the MAPIA prototype:

- P22 expressed in Baculovirus system
- P30 expressed in *E.coli*
- P54 expressed in Baculovirus system
- P72 expressed in Baculovirus system

The optimization of the ASF MAPIA assay was performed at KSU and the National Centre for Foreign Animal Disease (NCFAD) – Winnipeg, Canada, using reference serum obtained from the EU Reference Laboratory for ASF in Spain, and well characterized serum samples collected from ASFV-infected pigs at the NCFAD and KSU. The well characterized serum samples also included ASFV protein-specific hyperimmune serum prepared at KSU. The reference samples included strong ASFV positive, weak ASFV positive and ASFV negative porcine sera. In addition, commercial pig serum (Gibco lot#1671328) and five field serum samples from the Canadian Annual Porcine Sero-surveillance were used. The ASFV reactivity of the above samples was confirmed by WB and the commercially available blocking ELISA INgezim PPA Compac kit. MAPIA results were evaluated visually, by the presence of colored bands with defined intensity relative to a defined band included in the test as internal control.

The final ASF MAPIA test procedure was determined as follows:

- Put MAPIA strip in well of a mini-tray
- Add 1 ml of blocking buffer (1xCasein blocking buffer) into wells
- Incubate 1 hour at room temperature on shaker
- Remove blocking buffer from wells of mini-tray
- Add the test serum at a 1:1000 dilution in blocking buffer
- Incubate 1 hour at room temperature on shaker
- Wash the strips with wash buffer (PBS/T20) 3 times, each for 10 minutes
- Add anti-swine peroxidase antibody (Peroxidase conjugated-anti-swine IgG [H+L]) (Jackson ImmunoResearch, Inc.), diluted 1:15,000 in blocking buffer
- Incubate 1 hour at room temperature on shaker
- Wash the strips with wash buffer 3 times as described above
- Add 500µl TMB substrate (SigmaAldrich # T0565-100ML)
- Incubate 10 minutes at room temperature on shaker
- Rinse the strip with water
- Interpret test result based on internal positive and negative control reactivity

Evaluation of ASF MAPIA test

Several factors were evaluated during the production of the MAPIA strips: buffers, nitrocellulose membranes, protein concentrations, incubation time, etc. Based on the results of the optimization procedure, preliminary batch strips were produced for testing with sera from experimentally infected animals. The printed nitrocellulose membranes were dried at 37°C for 30 min, then incubated at 22^{0}C for 24 hours before cutting into 3 mm strips. The strips were kept at 4^{0}C until use.

Evaluation of the ASF MAPIA with well characterized ASFV positive sera.

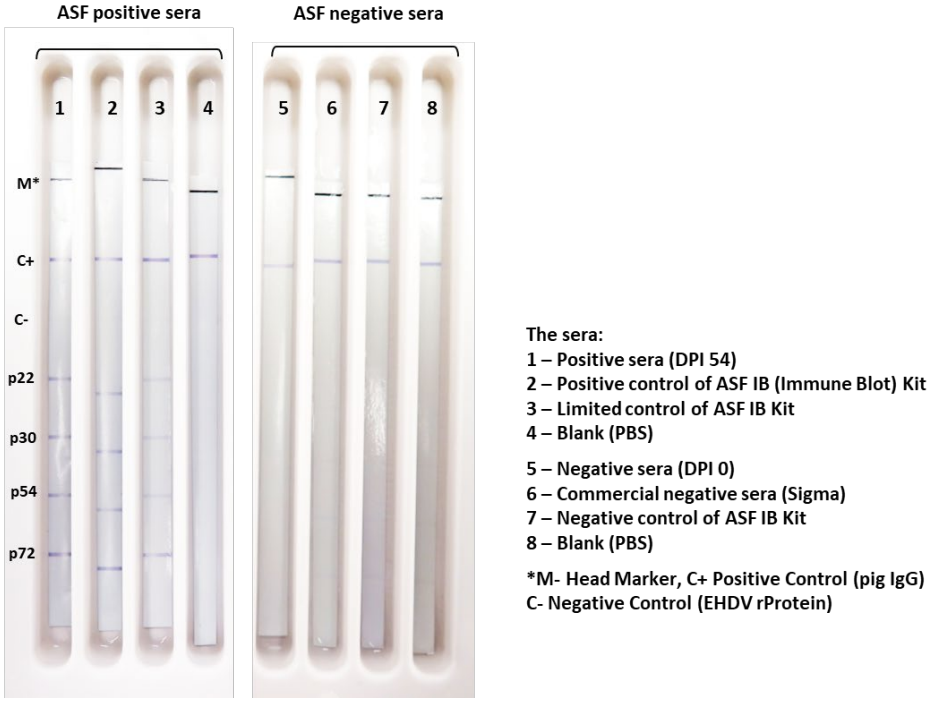

Figure 2. ASF MAPIA validation with ASFV positive and negative serum.

Well characterized samples of ASF positive and negative serum were tested using the ASF MAPIA strips (Figure 2). The positive pig serum (pig #2, DPI 54) and two positive serum control samples which are provided as part of the confirmatory WB kit (CISA/ASF/IB/Spain) obtained from CISA-Spain, were confirmed as positive by the ASF MAPIA test. These 3 sera were also positive with the INgenesa ASF Compac ELISA.

The ASF MAPIA results were evaluated visually, by the presence of a colored band with defined intensity. All three ELISA positive sera used in this experiment developed positive staining for all four recombinant ASFV proteins printed on the test strip, with a low background.

Evaluation of the ASF MAPIA with serum from experimentally ASFV infected pigs

The evaluation of the MAPIA was performed with experimental serum samples from pigs infected with attenuated ASFV. Each sample, used in MAPIA test, was also tested by an ASF Ab Indirect ELISA and the commercially available INgezim PPA Compac ELISA.

The evaluation was done with serum samples collected at different days post-infection from pigs experimentally infected with low-virulent ASFV strain OURT88/3 and also challenged with moderately virulent ASFV Malta'78 strain at the NCFAD. Serum samples collected from 2 pigs on days 0, 10, 12, 14, 18, 28 and 54 post infection were used for evaluation. ASF MAPIA strips showed positive results starting as early as 10 -12 days post inoculation, and highest intensity of staining was detected with serum from 54 or 56 days post infection (Figure 3). The sensitivity of the ASF MAPIA assay was comparable to that of the INgenesa ASF Compac ELISA.

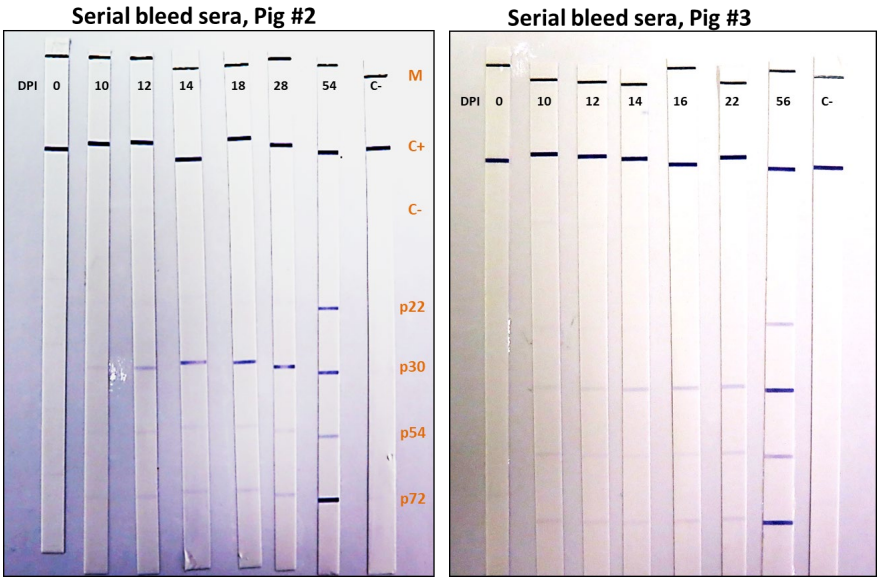

Figure 3. ASF MAPIA validation with 2 sets of serum samples collected from experimentally infected pigs.

Evaluation of the ASF MAPIA with sera from vaccination and challenge studies at KSU.

Final optimization of the ASF MAPIA was performed using serum samples which were collected from pigs after ASFV vaccination and challenge at the Biosecurity Research Institute at KSU. In these studies, pigs were inoculated with modified live attenuated ASFV and challenged with a lethal dose of ASFV Arm07. ASF MAPIA strips were prepared as described above with p22, p30, p54, pp62 and p72 ASFV recombinant proteins. Normal pig serum was printed on the strips as internal positive control antigen and recombinant protein VP2 of EHDV1 was printed on the strips as

internal negative control antigen. The optimized ASF MAPIA procedure used for the testing was very similar to the final test procedure provided above, with slight modifications as follows:

- Put MAPIA strips in channels of mini tray
- Add 1 ml of 1 x Casein blocking buffer into wells
- Incubate 1 hour at room temperature on a shaker
- Remove blocking buffer from wells of mini-tray after the incubation
- Add 800 ul of test serum at a 1:1000 dilution in blocking buffer
- Incubate 1 hour at room temperature on shaker
- Wash the strips with 800 ul of wash buffer (PBS/T20) 3 times, each for 10 minutes each
- Add 800 ul of anti-swine peroxidase antibody (Peroxidase conjugated-anti-swine IgG [H+L]; (Abcam)), diluted 1:20,000 in blocking buffer
- Incubate 40 minutes at room temperature on shaker
- Wash the strips with wash buffer 3 times as described above
- Add 400µl TMB substrate (Sigma Aldrich # T0565-100ML)
- Incubate 10 minutes at room temperature on shaker
- Rinse the strip with water
- Interpret test result based on reaction with pos. and neg. internal control reactivity

The results of this modified ASF MAPIA procedure are shown in Figure 4. Sera samples, collected from pigs prior to vaccination (lines 1 and 2) tested negative (no staining), while sera samples, collected on day 13 post challenge from vaccine protected animals (lines 3 through 8) tested positive; i.e. there is a presence of colored bands corresponding to the respective ASFV proteins.

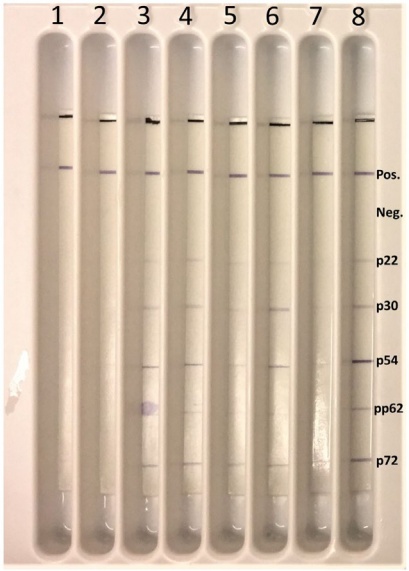

Figure 4. ASF MAPIA validation with serum from vaccinated and ASFV challenged pigs: lines 1 and 2 – negative serum, collected prior to ASFV vaccination; lines 3 through 8 – serum collected from pigs on day 13 post ASFV challenge. Pos. – normal pig serum, printed as positive control for the anti-swine IgG antibody; Neg. – recombinant VP2 protein from EHDV1, printed as negative protein control; p22, p30, p54, pp62 and p72 - ASFV recombinant proteins.

The results of these tests further confirmed utility of the ASF MAPIA for the detection of antibodies against ASFV proteins.

Evaluation of the ASF MAPIA with false positive sera.

ASF MAPIA strips were also tested with five false positive pig serum samples. These samples were collected during annual disease surveillance in Manitoba, Canada, and were tested false positive in an ASF Ab Indirect ELISA with p72 antigen. The results of the testing are shown in Figure 5. ELISA false positive serum was tested side by side by the ASF MAPIA test and the confirmatory WB test commercially available from CISA, Spain. Sera 37, 66,179 and 180 tested negative in the ASF MAPIA; only serum 49 showed weak reaction with one protein (p72) on the ASF MAPIA strip. In contrast, multiple bands were observed when these sera were tested in the confirmatory WB test, but the patterns of the reactive bands were not exactly the same as in ASFV positive antibody controls. This demonstrated and confirmed the specificity of the ASF MAPIA test even with false negative sera which showed cross-reactivity with other antigens in the confirmatory WB test (CISA/ASF/IB/Spain).

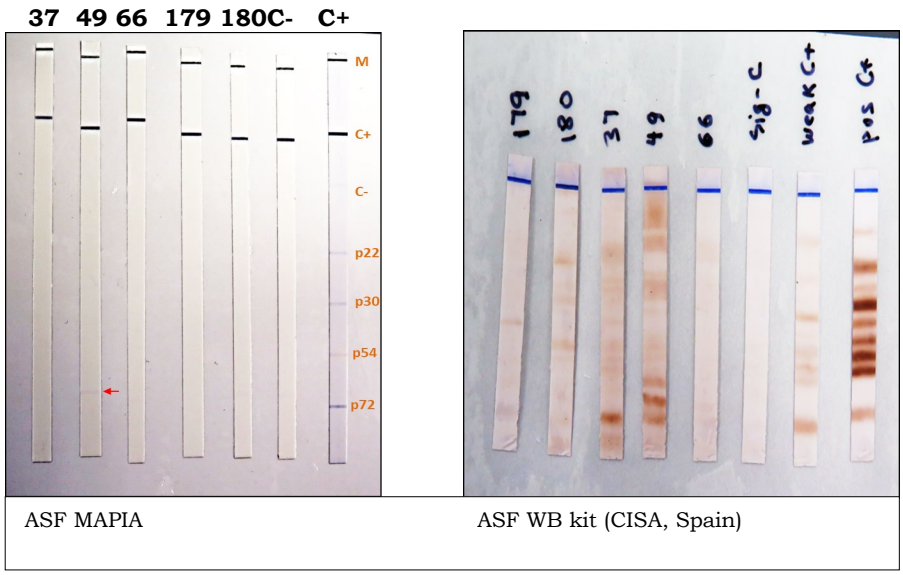

Figure 5. ASF MAPIA validation with false positive serum (number 37, 49, 66, 179, 180) and respective controls (C-negative pig serum, C+ positive control sera, provided in the WB kit)

Discussion

Serological diagnosis of complex viruses that generate a highly heterogeneous antibody repertoire, such as African swine fever (ASF), requires tests based on cocktails of antigens. The general procedure for antibody detection of ASF in affected countries consists of screening of sera by ELISA, followed by confirmation by Western blot using nitrocellulose strips containing ASFV proteins produced in infected cells. Poorly preserved sera can produce false-positive reactions by ELISA or false-negative results due to a loss of antibodies against the viral proteins presented in the ELISA. In both cases, the confirmatory Western blot is a valuable assay to confirm ELISA results. At the same time, the production of the confirmatory WB test requires work with live ASFV under BSL3 conditions, the test is difficult to standardize and interpretation of the test results is often difficult due to the high background and variability between different WB lots. In order to overcome these challenges, we developed an ASF MAPIA system for ASFV serological diagnosis. The ASF MAPIA is based on the application of recombinant ASFV antigen to nitrocellulose membranes by micro-aerosolization (printing), followed by antibody detection using an anti-swine peroxidase antibody and a standard chromogenic substrate. The use of well-defined recombinant ASFV proteins printed on a nitrocellulose membrane improves and simplifies the interpretation of the test results when compared to the conventional confirmatory Western blot. Multiple proteins were tested and a final combination of highly immunoreactive recombinant ASFV proteins were selected for printing on the membrane. Conditions of the ASF MAPIA test were optimized using ASFV-specific hyperimmune sera and well characterized ASFV – positive serum samples from several

experimental ASFV infection and field studies. Comparison with the confirmatory WB test (CISA/ASF/IB/Spain) showed significantly lower background and easy interpretation of the test results. Evaluation with sets of sera collected from animals after experimental inoculation with attenuated ASFV (from 0 to 54 days post inoculation) and after vaccination and challenge experiments, demonstrated utility of the developed test for antibody detection. We concluded that the ASF MAPIA is a sensitive, specific, safe, and rapid test system. The production of ASF MAPIA strips can be performed in BSL-2 laboratories and the system can be easily standardized and scaled up. Therefore, the ASF MAPIA might be an economically viable alternative to the existing confirmatory Western blot test for ASFV serology confirmation.

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