RESEARCH REPORT
SWINE HEALTH

Title: Development of ASFV-specific monoclonal antibodies and mAb-based blocking ELISA – **NPB #19-117:**

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Date Submitted: January 1, 2021

Industry Summary: Historically, the greatest threats to the US swine production are from foreign animal diseases, such as African swine fever (ASF). ASF virus (ASFV) infection can have a deleterious effect on swine production, causing excessive morbidity and mortality in domestic pigs. Currently, no vaccine and treatment are available for ASFV. The only way to control the disease is to quarantine, isolate and eliminate the infected animals in order to stop the spreading of the disease. High levels of biosecurity measures, including surveillance along with rapid diagnostics are critical to identify infected animals. Monoclonal antibody (mAb) is a key reagent for detecting of viral infection. This study generated a panel of specific mAbs against selected immunogenic ASFV proteins. Their application in the detection of ASFV infection was tested in various diagnostic assays, including the immunofluorescent assay (IFA) and immunohistochemistry (IHC). One of the mAbs was further used to develop a mAb-based bELISA for detecting host antibody response against ASFV infection. The bELISA showed good diagnostic sensitivity and specificity, and it is highly repeatable. The availability of ASFV-specific mAbs and bELISA provides valuable tools in ASFV epidemiological survey and outbreak investigation. For more information, please contact Dr. Ying Fang, University of Illinois at Urbana-Champaign, Phone: 217-300-5483, E-mail: yingf@illinois.edu.

Key Findings:

- A panel of ASFV-specific mAbs has been generated;
- An ASFV anti-p30 mAb-based bELISA has been developed;
- This panel of mAbs and bELISA provides valuable tools in ASFV epidemiological surveys and outbreak investigations;

Keywords: African swine fever virus, monoclonal antibody, blocking ELISA.

Scientific Abstract:
Recent outbreaks of African Swine Fever virus (ASFV) in China and some European countries pose the potential pandemic threat to global swine industry. Highly sensitive and specific diagnostic reagents and assays are urgently needed for rapid detection and implementation in quarantine and elimination of infected animals. In this study, we generated a panel of specific mAbs against selected immunogenic ASFV proteins, including p10, p14.5, p22, p30, p49, p54, p72, and CD2v. These mAbs were initially screened by immunofluorescent assay using *in vitro*

expression system. The antibody reactivity was confirmed in virus-infected cells. Their application in the detection of ASFV infection was further tested using the methods of IFA, IHC, Western blotting, immunoprecipitation and ELISA. One of the anti-p30 mAbs showed specific blocking activity against ASFV specific serum antibodies in a blocking ELISA (bELISA) format. Subsequently, an anti-p30 mAb-based bELISA was developed. Serum standards were established, which includes high positive, low positive and negative standard. Analytical sensitivity analysis showed that the 1:64 dilution of high positive standard serum is the limit of detection. Receiver Operating Characteristic (ROC) analysis calculated a diagnostic sensitivity of 98.11% and a diagnostic specificity of 99.42%. A cut off value of the assay was determined as percentage of inhibition (PI) at 45.92%. The coefficient of variation of an internal quality control serum was 6.81% for between-runs, 6.71% for within-run, and 6.14% for within-plate. These results indicate that the bELISA can be used as a serological test for ASFV with high levels of sensitivity, specificity and repeatability. The availability of the panel of mAbs and bELISA provides important tools in aid of ASFV diagnostics and research.

Introduction:

African swine fever virus (ASFV) is a large double-stranded DNA virus that belongs to the family *Asfarviridae*, genus *Asfivirus* (Dixon et al., 2013). The virus is enveloped with two membranes at its inner and outer sides, wrapped around an icosahedral capsid. The viral genome varies in size between 170 and 190 kb, which encodes over 170 proteins. ASFV can cause lethal hemorrhagic fever in domestic pigs, characterized by high morbidity and mortality. The virus is directly transmitted by contacting with other infected animals and contaminated feed or fomites, and is also transmitted indirectly through the soft ticks of genus Ornithodoros (Sánchez-Vizcaíno and Neira, 2012). ASFV was originated in Africa, where warthogs and bushpigs are reservoirs (Montgomery, 1921; Oura et al., 1998). The virus initially spread from West Africa to Europe in the middle of the last century. It was finally eradicated from most areas of Europe during the mid-1990s, but ASFV remains endemic in the island of Sardinia, Italy (Martinez-Lopez et al., 2015). Since 2007, ASFV again spread out of Africa to Caucasus and then Eastern Europe, causing outbreaks in the Russian Federation and several neighboring countries, including Belarus, Ukraine, Lithuania, Estonia, Poland, Latvia, Czech Republic, Romania and hungary. In Europe, by July 2018, 334 outbreaks were detected, mostly in domestic farms. From then on, ASF spread widely in Romania with outbreaks in more than 1,000 domestic pig farms in 2018 and about 2,500 in 2019 according to Animal Disease Notification System of the European Commission (ADNS) (Boklund, et al., 2020). In 2020, as of June 14, the EC-ADNS had recorded a total of 311 ASF outbreaks in domestic pigs in Europe (*https://www.feedstrategy.com/african-swine-fever/new-asf-outbreaks-among-polish-romanian-pigs/*). German also reported their first case on September 10, 2020 (OIE). As of July 24, 2020, China has reported 178 ASF outbreaks (including four outbreaks in wild boar) in 31 provinces (Wu, et al., 2020). By October 26, 2020, ASFV has been spreading in more than 50 countries in Africa, Asia and Europe (*https://www.nationalhogfarmer.com/animal-health/fao-oie-kick-initiative-stop-spread-african-swine-fever*). These new outbreaks pose the potential threat to US swine industry.

Currently, there is no effective vaccine and treatment available against ASFV infection. Protective immunity against ASFV is poorly understood. Humoral antibody responses have been demonstrated as important components contributed to protection (Onisk et al., 1994). Pigs infected with ASFV develop antibody responses, which can be detected since 7 days post-infection (Cowan, 1961; Malmquist, 1963). Neutralizing antibodies directly against the three proteins (p30, p54, and p72) on the virion have been described (Gomez-Puertas et al., 1996; Onisk et al., 1994; Ruiz et al., 1986; Zsak et al., 1993). Among all the ASFV proteins that have been analyzed as far, p30 protein is determined to be a highly immunogenic protein, which is capable to stimulate the highest level of antibody response during the virus infection (Giménez-Lirola et al., 2016). Recombinant p30 fused with p54 induced neutralizing antibodies correlated with a reduction in viremia levels in vaccinated pigs (Barderas et al., 2001). It was reported that antibodies to p72 and p54 inhibited viral attachment to cells, and antibodies to p30 inhibited virus internalization (Gomez-Puertas et al., 1996). In a recent study (Jankovich et al., 2018), a total of 47 ASFV proteins were screened in order to identify the immunogenic and protective antigens for vaccine development. Among these proteins, several of antigens including p30, p54, and p72, were tested to be able to induce certain levels of antibody responses in immunized

pigs. These antigens were selected based on their (known or predicted) properties of being present on the surface of the intracellular mature virion or extracellular viral particles. The results demonstrated that these antigens are potentially important for induction of protective antibody responses, which could be implicated in vaccine development as well as in serological assays for detection of ASFV infection.

Although much of the progress has been made in identification of protective antigens as described above, the exact immune mechanisms and range of critical viral immune determinants have yet to be defined for developing effect vaccines and antiviral measures. Currently, the only strategy to control the ASF is to quarantine and eliminate the infected animals; therefore, highly sensitive and specific diagnostic reagents and assays are urgently needed for rapid detection and isolating the ASFV-infected pigs. Monoclonal antibody is a key reagent for the detection of viral infection, while mAb-based bELISA provides the similar level of sensitivity but higher level of specificity than traditional ELISA. Based on the immunogenic nature of ASFV proteins described above, in this study, we developed and characterized a panel of monoclonal antibodies for use in ASFV diagnostics and research. In addition, an anti-p30 mAb-based block ELISA (bELISA) has been developed to improve the specificity of the current ELISA tests for accurately identification of ASFV infection.

Objectives:
Objective 1). To generate mAbs against a panel of immunogenic proteins of ASFV.
Objective 2). To develop mAb-based bELISA for detecting host antibody responses against ASFV infection.

Materials & Methods:
Objective 1). To generate mAbs against a panel of immunogenic proteins of ASFV.
ASFV proteins used for mAb production were selected based on their known or predicted properties of being present on the surface of intracellular mature virion or extracellular viral particles. Antigenic regions of the viral proteins were expressed for mice immunization, and spleenocytes from immunized mouse were fused with mouse myeloma cells to generate hybridomas. ASFV specific mAbs from hybridoma culture were initially screened using *in vitro* expression system and then confirm the reactivity in ASFV-infected cells.

Antigen production: Recombinant protein expression and purification were conducted as described in our previous publications (Brown et al., 2009; Langenhorst et al., 2012). Briefly, the genomic region encoding antigenic domain of each viral protein was amplified by PCR and then cloned into the protein expression vector pET 28a (Novagen) as fusion proteins containing an amino-terminal 6-His tag for facilitating protein purification. Recombinant proteins were expressed in *E. coli* BL21 (DE3) cells (Stratagene). Proteins were purified by nickel-affinity chromatography, analyzed by SDS-PAGE and western blot using the mAb against His-tag. For non-soluble proteins, they were purified as denatured form first and then refolded in refolding buffer overnight. The refolded proteins were concentrated by tangential flow filtration (Pellicon XL Ultracel PLC 5 kd, Millipore) and dialyzed against 20mM Tris-HCl, PH 8.0. Protein concentration was measured by Lowry protein assay following the standard protocol.

Generation and charaterization of mAbs: BALB/c mice were immunized 3 times with 50-100 μg purified ASFV antigen mixed with Freund's incomplete adjuvant at 2-3 weeks intervals. Mouse splenocytes were fused with NS-1 myeloma cells. Specific mAbs were initially screened by immunofluorescence assay (IFA) using the Vero cells transfected with a plasmid expressing the corresponding ASFV protein. Reactivity of the mAbs was confirmed by IFA using Vero cells and porcine macrophages infected with ASFV strain Georgia/2007. The specificity of the anti-ASFV mAbs was further determined by Western blotting and/or immunoprecipitation using Vero cells transfected with a plasmid expressing the corresponding ASFV protein. Hybridomas producing ASFV-specific mAbs were subcloned and expanded.

Objective 2). To develop mAb-based bELISA for detecting host antibody responses against ASFV infection.

The ASFV specific bELISA was developed using recombinant p30 as the antigen and a biotinylated mAb against p30 as the secondary antibody for detection. Test conditions were optimized using standard serum samples generated from pigs immunized with Alphavirus replicon particles expressing p30 antigen. Validation of the bELISA was performed using serum samples from pigs immunized with experimental vector vaccines as well as serum samples from ASFV-infected and non-infected pigs. Diagnostic sensitivity and specificity were determined by receiver operating characteristic (ROC) analysis.

Serum samples: Four sets of serum samples are used for bELISA development and validation. The first set of samples (74) was collected from piglets immunized with recombinant Alphavirus replicon particles expressing ASFV p30 protein. This set of samples is from BSL2 laboratories at K-State (72 samples) and Iowa State University (2 samples). The second set of samples (8) was collected from piglets immunized with recombinant adenovirus expressing p30 protein. The third set of samples (24) was collected from piglets infected with ASFV OURT 88/3 strain (23 samples) and Georgia/07 strain (1 sample), which are currently stored in BSL3 laboratory at Kansas Biosecurity Research Institute. These three sets of samples are used as positive validation-testing population. The fourth set contains 810 known ASFV negative serum samples, which collected from previous pig experiments in PI's lab (510 samples) and Iowa State Veterinary Diagnostic Laboratory in the US (300 samples) (Chen et al., 2016; Li et al., 2016; Li, Shang, et al., 2018). These samples are used as negative-testing validation population.

Establishment of internal control standards for bELISA: Internal positive control standards were established using serum samples from pigs immunized with recombinant Alphavirus replicon particles expressing p30 protein (RP-p30), while internal negative control standard was established using serum samples from negative control pigs. Large quantity of the hyperimmune serum from the terminal bleed of a group of RP-p30 immunized pigs was pooled into a single lot of positive control serum. Similarly, large quantity of the negative serum from the group of negative control pigs was pooled into a single lot of negative serum standard.

Development of bELISA: The bELISA test is established based on ASFV p30 antigen and anti-p30 mAb. The antigen concentration of p30 protein was optimized so that the anti-p30 mAb generated an optical density (OD) of approximate 2 in the absence of a competitor. Using the standard serum samples, the test conditions of other components were also optimized by checkerboard titrations. The result (OD value) was be quantified by reading at 405 nm with an Epoch 2 Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT). The raw plate data were copied to an Excel spreadsheet to calculate the percent inhibition (PI) using the formula: PI = [1- (OD of sample – OD of buffer) / (OD of negative control – OD of buffer)] x 100.

IFA confirmation test: IFA was performed as a confirmation test for bELISA. The assay was performed using Vero cells transfected with a plasmid expressing ASFV p30 protein. Transfected cells were fixed with 80% acetone, and then blocked with 2% BSA. Swine serum samples were added and incubated at 37°C for 1h, followed by washes, and incubation with FITC-conjugated goat anti-swine secondary antibody at 37°C for 1h.

Test validation: Test validation used the samples from experimentally immunized/infected pigs. To assess the cutoff value, diagnostic sensitivity, and diagnostic specificity, serum samples from individual pigs with known ASFV infection/immunization status were analyzed using the bELISA and the IFA. ROC analysis was performed to analyze the bELISA results obtained with the positive- and negative-testing sample populations in order to determine an optimized cutoff that maximizes both the diagnostic specificity and diagnostic sensitivity of the assays. The analysis was conducted using GRAPH ROC software (*http://members.tripod.com/refstat/GraphROC.htm*). Subsequently, the repeatability of the bELISA was assessed by running the same lot of internal control sera. The within-plate precision was calculated from 40 replicates on one plate, while within-run precision was calculated using a standard serum on 10 plates in one run, and between-run precision was calculated from a standard serum in 10 different runs. Means, standard deviations, percent coefficient of variation (% CV), and Levey-Jennings control charts was calculated using Control Chart Pro Plus software.

Results:

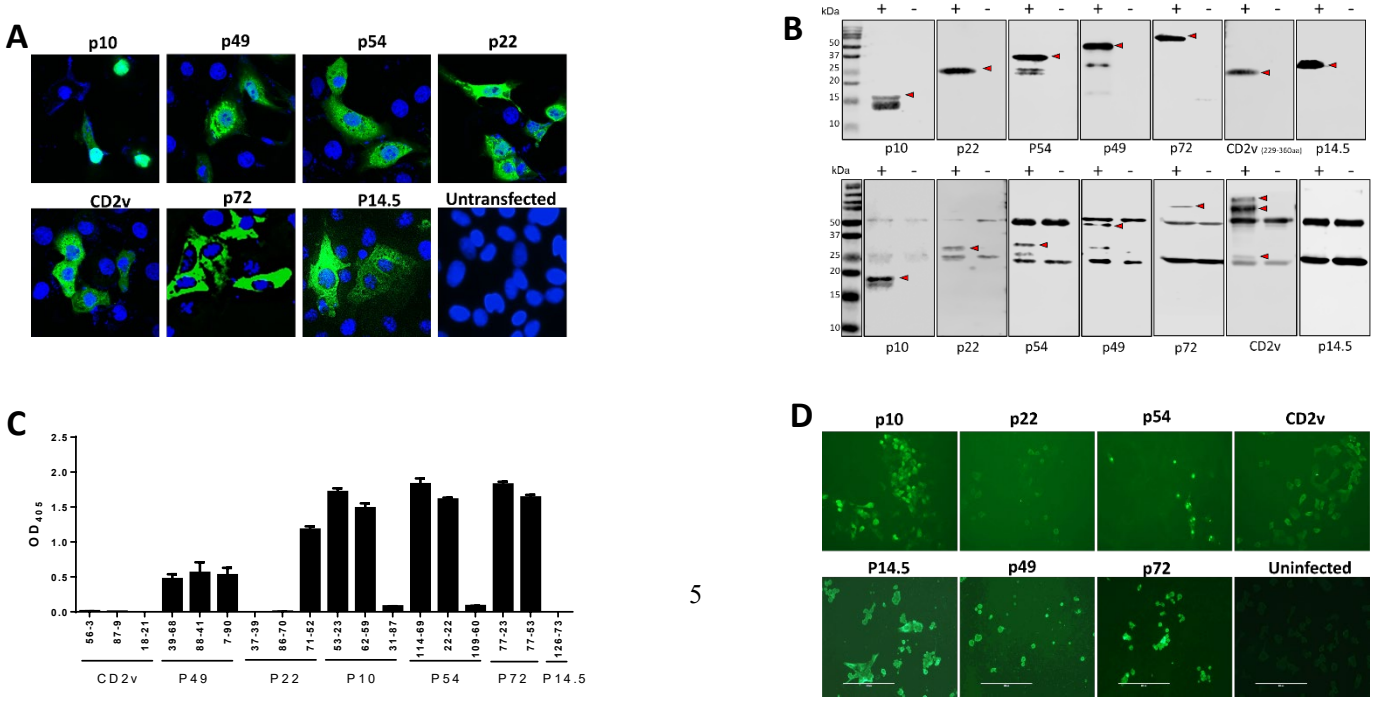
Objective 1). To generate mAbs against a panel of immunogenic proteins of ASFV

Initial hybridoma screening by IFA using MARC-145 cells that express each individual ASFV protein resulted in a total of 74 mAbs against ASFV p10, p14.5, p22, p30, CD2v, p49, p54, and p72 proteins (Figure 1A). Two to three hybridoma clones from each antigen were expanded for further characterization. A total of 20 mAbs against 8 ASFV antigens were generated (Table 1).

Western blot (WB) and immunoprecipitation (IP) were performed using the lysate of transfected 293T cells expressing a specific viral protein. In western blot analysis (Fig. 1B, top panel), all mAbs specifically detected protein bands for p10, p49, p54, CD2v, p14.5, p72, and p22. As expected, these bands were not detected in mock-transfected cells. MAbs against p54, p22, p10, p49, CD2v, and p72 also detected corresponding proteins in immunoprecipitated proteins from transfected cells (Fig. 1B, bottom panel). This panel of mAbs were further tested on indirect ELISA using recombinant proteins as the coating antigens. As is shown in Figure 1C, mAbs against p72, p54 (#114-69, #22-22), p10 (#53-23, #62-59), and p22 (#71-52) showed high reactivity with OD_{405} value above 1.0, while low reactivity was observed for mAbs against p49 (#39-68, #88-41, and #7-90). No reactivity was detected for mAbs against CD2v and p14.5. Specificity of these mAbs were further confirmed by IFA test using ASFV-infected Vero cells. As shown in Figure 1D, anti-p10, anti-p14.5, anti-p49, anti-p54, and anti-p72 mAbs showed strongly reactivity with the viral proteins, while anti-p22 and anti-CD2v showed weak reactivity.

Antigen	Protein Function	mAb	Assays			
			IFA	WB	IP	ELISA
CD2v	Glycoprotein inserted into virus' external envelope; host cell CD2-like protein, function as viral hemagglutinin to mediate hemadsorption by ASFV-infected cells	#87-9, #56-3, #18-21	+	+	+	-
p54	Structural protein binds to LC8 chain of dynein, involved in virus entry; required for recruitment of envelope precursors to the factory for generating viral particles	#114-69, #22-22, #109-60	+	+	+	+
p10	Structural protein involved in virus adsorption and has DNA binding capacity	#31-87, #53-23, #62-59	+	+	+	+
p49	Structural protein required to form the vertices of the icosahedral capsid	#7-90, #88-41, #39-68	+	+	+	+
p22	Envelope protein	#37-39, #71-52, #86-70	+	+	+	+
p14.5	DNA-binding protein on the surface of intracellular virions; required for movement of virions to plasma membrane	#126-73	+	+	-	-
p72	Major capsid protein, involved in virus entry	#77-23, #77-53	+	+	+	+
p30	Phosphorylated protein; involved in virus entry	#25-18, #144-12	+	+	+	+

Table 1. Characterization of ASFV specific monoclonal antibodies



Objective 2). To develop mAb-based bELISA for detecting host antibody responses against ASFV infection
Initially, p30 antigen was prepared for the assay development. Recombinant p30 protein was expressed and purified from *E. coli*. The purity of the antigen was verified through SDS-PAGE (Fig. 2A) and the specificity of the protein was confirmed by Western-blot analysis (Fig. 2A). Next, we tested the p30 antigen coating gradient. The result showed that the coating amount of 300ng generated the highest OD value (Fig. 2B). Subsequently, serum standards were established, including high positive (OD of 1.5-2.0), medium positive (OD of 1.0-1.5), low positive (OD of 0.8-1.0), and negative controls (OD of 0-0.3) (Fig. 2B). Analytical sensitivity analysis showed that the 1:64 dilution of high positive standard serum is the limit of detection (Fig. 2D).

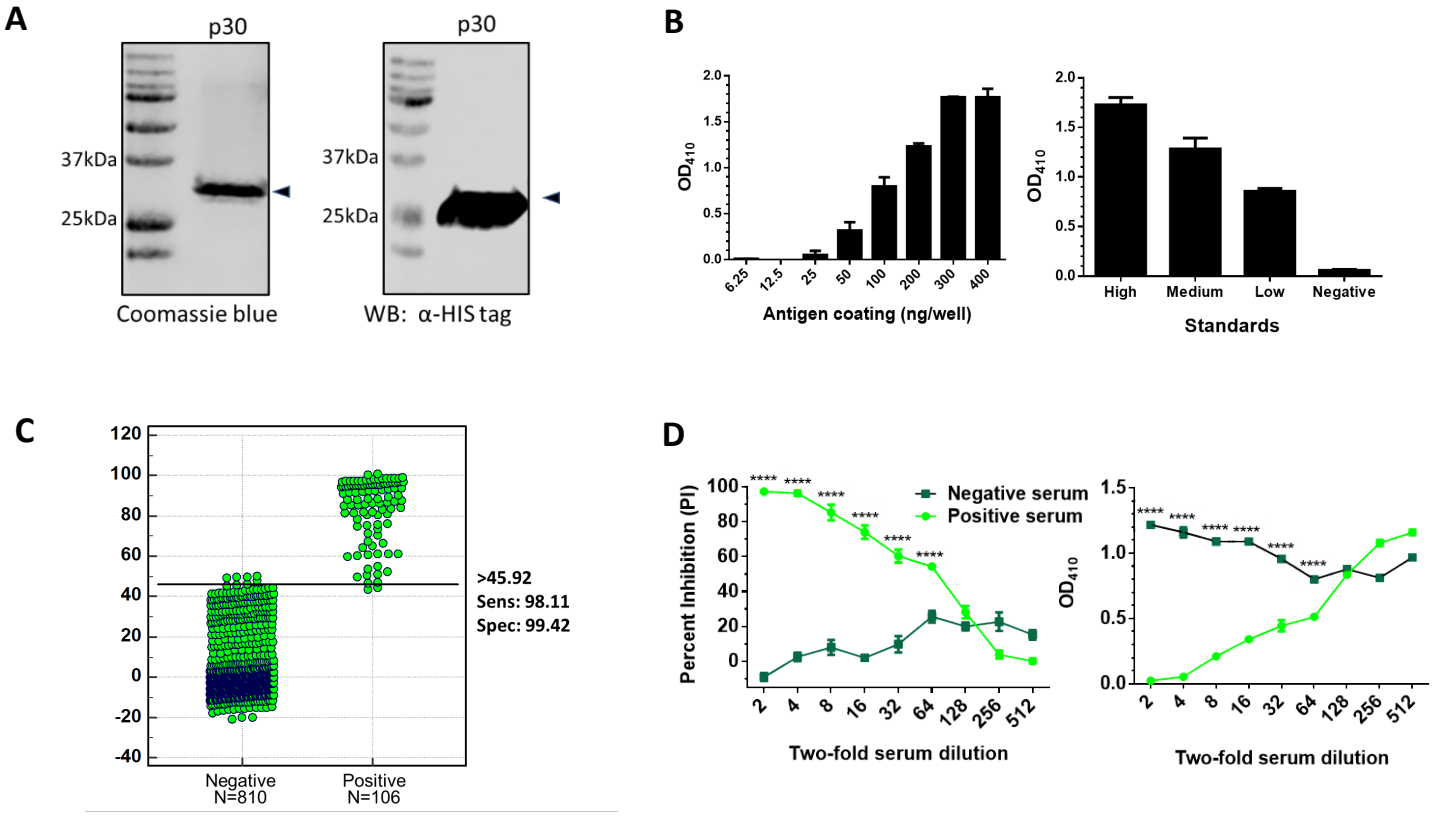


Figure 2. ASFV anti-p30 mAb-based bELISA development. (**A**) SDS gel electrophoresis and western blot analysis of ASFV p30 protein preparation. The left picture is SDS-PAGE of p30 protein, followed by Coomassie blue staining. Western blotting was performed by probing membrane with IRDye 800CW-conjugated anti-HIS antibody. Blot imaging was performed using an odyssey infrared imaging system (Li-Cor Bioscience). (**B**) Antigen-coating gradient and establishment of standards for bELISA validation. Best antigen coating amount was determined by a serial dilution of p30 protein. Control standards representing high-positive, medium-positive, low-positive, and negative were shown in right graph. (**C**) Cutoff determination, diagnostic sensitivity, and diagnostic specificity. A total of 810 known-status negative serum samples and 106 positive serum samples were utilized to determined cut-off of the bELISA assay. Receiver operating characteristic (ROC) analysis was performed using MedCalc version 11.1.1.0 (MedCalc software, Mariakerke, Belgium). **(D)** Analytical sensitivity of mAb-based bELISA. Nine 2-fold serial dilutions of a high-positive internal control serum and a negative internal control serum were tested. The analytical sensitivity is the largest dilution of a high-positive serum in which antibody is no longer detected. Left graph represents percent inhibition value and right graph shows the OD value. Analysis of variance (ANOVA) was performed using GraphPad InStat Prism software (version 5.0) to evaluate differences between the dilutions of the two control sera.

To determine the cutoff value and diagnostic performance, 106 serum samples from the positive-testing population and 810 serum samples from the negative-testing population were analyzed with the bELISA. Serum antibody status was confirmed by IFA using ASFV-infected cells or transfected cells that express p30. ROC analysis was performed to determine an optimized cutoff that maximizes both the diagnostic specificity and diagnostic sensitivity of the bELISA. As shown in Fig. 2C, an optimized cutoff at 45.92% of inhibition (PI) maximizes the

efficiency of the bELISA, which calculated a diagnostic sensitivity of 98.11% with a 95% confidence interval of 93.4 to 99.8% and a diagnostic specificity of 99.42% with a 95% confidence interval of 98.7 to 99.8%. To determine the level of precision for the bELISA, the internal negative control serum standard was used to run the repeatability. The coefficient of variation was 6.81% for between-runs, 6.71% for within a run, and 6.14% for within-plate. These data indicate the assay is highly repeatable.

Discussion:

Although the US is currently free from ASFV, outbreaks in other countries pose an increased threat to the US. The introduction of ASFV to the US could cause billions in economic losses to swine industry, which could also devastate international trade and markets. We have already learned a lesson from the introduction of porcine epidemic diarrhea (PED) disease in the US swine farms, which caused substantial economic losses to swine producers. Currently, no vaccine and treatment are available for ASFV. The only way to control the disease is to quarantine, isolate and eliminate the infected animals in order to stop the spreading of the disease. High levels of biosecurity measures, including surveillance along with rapid diagnostics are critical to identify infected animals. Serology tests are commonly used diagnostic tools for detecting viral infection. The current serological assays for ASF approved by the World Organization for Animal Health (OIE) are using live virus as antigen, which involves high containment facilities and select agent permits. To overcome this problem, several serological assays were developed using recombinant ASFV proteins expressed in *E. coli* or by baculovirus. These assays showed good sensitivity and specificity under the laboratory controlled settings; however, one of the disadvantages continues to be the number of false positive results obtained with field samples. Therefore, a second confirmatory test is required. The mAb-based bELISA can provide the similar level of sensitivity but higher level of specificity when compared with traditional ELISA. We believe the mAbs and bELISA developed in this study will be valuable tools for ASFV surveillance, which will help achieving the ultimate goal to stop spreading the virus and prevent it from reaching the U.S.

The lack of effective vaccine and treatment for ASFV infection also reflects our poor knowledge on viral pathogenic mechanisms and host immune responses. One of the key reagents to study the viral protein structure-function and design antiviral intervention strategies is the mAb. In this study, we generated a total of 74 mAbs against a panel of ASFV immunogenic proteins, including p10, p14.5, p22, p30, CD2v, p49, p54, and p72 proteins. They were selected based on their (known or predicted) properties of being present on the surface of the intracellular mature virion or extracellular viral particles. Previous studies demonstrated that these antigens are potentially important for induction of protective antibody responses; thus, the antigens and mAbs generated in this project could be important tools for basic mechanism studies toward developing vaccines and other antiviral strategies.