J Vet Sci. 2022 Jul;23(4):e55
https://doi.org/10.4142/jvs.21134
pISSN 1229-845X·eISSN 1976-555X

Journal of Veterinary Science

Original Article
Immunology

Identification of a conservative site in the African swine fever virus p54 protein and its preliminary application in a serological assay

Lingyu Xu [1,†], **Chenfu Cao** [2,†], **Zhiyi Yang** [1], **Weixin Jia** [1,*]

[1]National and Regional Joint Engineering Laboratory for Medicament of Zoonoses Prevention and Control, College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, China
[2]Shenzhen Customs District P.R. China, Shenzhen 518045, China

Received: Aug 16, 2021
Revised: Apr 25, 2022
Accepted: Apr 27, 2022
Published online: May 16, 2022

***Corresponding author:**
Weixin Jia
National and Regional Joint Engineering Laboratory for Medicament of Zoonoses Prevention and Control, College of Veterinary Medicine, South China Agricultural University, No. 483, Wushan Road, Tianhe District, Guangzhou 510642, China.
Email: jiaweixin@scau.edu.cn
https://orcid.org/0000-0002-6335-9067

[†]Lingyu Xu and Chenfu Cao equally contributed to this work.

© 2022 The Korean Society of Veterinary Science
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (https://creativecommons.org/licenses/by-nc/4.0) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

ABSTRACT

Background: ASF was first reported in Kenya in 1910 in 1921. In China, ASF spread to 31 provinces including Henan and Jiangsu within six months after it was first reported on August 3, 2018. The epidemic almost affected the whole China, causing direct economic losses of tens of billions of yuan. Cause great loss to our pig industry. As ELISA is cheap and easy to operate, OIE regards it as the preferred serological method for ASF detection. P54 protein has good antigenicity and is an ideal antigen for detection.
Objective: To identify a conservative site in the African swine fever virus (ASFV) p54 protein and perform a Cloth-enzyme-linked immunosorbent assay (ELISA) for detecting the ASFV antibody in order to reduce risks posed by using the live virus in diagnostic assays.
Method: We used bioinformatics methods to predict the antigen epitope of the ASFV p54 protein in combination with the antigenic index and artificially synthesized the predicted antigen epitope peptides. Using ASFV-positive serum and specific monoclonal antibodies (mAbs), we performed indirect ELISA and blocking ELISA to verify the immunological properties of the predicted epitope polypeptide.
Results: The results of our prediction revealed that the possible antigen epitope regions were A23–29, A36–45, A72–94, A114–120, A124–130, and A137–150. The indirect ELISA showed that the peptides A23–29, A36–45, A72–94, A114–120, and A137–150 have good antigenicity. Moreover, the A36–45 polypeptide can react specifically with the mAb secreted by hybridoma cells, and its binding site contains a minimum number of essential amino acids in the sequence 37DIQFINPY44.
Conclusions: Our study confirmed a conservative antigenic site in the ASFV p54 protein and its amino acid sequence. A competitive ELISA method for detecting ASFV antibodies was established based on recombinant p54 and matching mAb. Moreover, testing the protein sequence alignment verified that the method can theoretically detect antibodies produced by pigs affected by nearly all ASFVs worldwide.

Keywords: African swine fever virus; antigenic site; conservative; monoclonal antibody

Generated by xmlinkpress

ORCID iDs
Lingyu Xu
https://orcid.org/0000-0002-8301-0698
Chenfu Cao
https://orcid.org/0000-0001-9362-3845
Zhiyi Yang
https://orcid.org/0000-0003-4735-0625
Weixin Jia
https://orcid.org/0000-0002-6335-9067

Author Contribution
Conceptualization: Cao C, Xu L; Data curation: Lin Y; Formal analysis: Xu L; Funding acquisition: Jia W; Investigation: Cao C; Methodology: Xu L; Project administration: Xu L; Resources: Yang Z; Software: Xu L; Supervision: Jia W; Validation: Xu L; Visualization: Yang Z ; Writing - original draft: Cao C, Xu L; Writing - review & editing: Xu L.

Conflict of Interest
The authors declare no conflicts of interest.

Funding
This work was supported by Key-Area Research and Development Program of Guangdong Province (2019B020211003), Science and Technology Program of Guangdong Province (2021B1212030015), Science and Technology Project of General Administration of Quality Supervision, Inspection and Quarantine of China (2016IK233).

INTRODUCTION

African swine fever (ASF) is an infectious disease in domestic pigs and wild boars characterized by high fever, hemorrhaging of the reticuloendothelial system, and high mortality. Although ASF outbreaks can have severe socioeconomic consequences, there is currently no effective vaccine against ASF, which is now ubiquitous in Georgia [1], Ukraine, the Russian Federation, Iran, and numerous countries in sub-Saharan Africa [2]. In China, the first case of ASF was reported on August 3, 2018, and as the disease continues to spread, it seriously threatens China's population of domestic pigs, which accounts for more than 50% of all pigs worldwide [3,4].

The ASF virus (ASFV), the only DNA virus of the Asfarviridae family, has a positive icosahedral structure 170–190 kb in length and contains 14 open reading frames that can encode the viral antigen epitope [5,6]. Within them, the structural proteins are p30 (CP204L), p54 (E183L), p72 (B646L), A104 R, and p10 (K78R). Encoded by the E183L gene, the p54 protein is an important structural protein of ASFV with a molecular weight of approximately 25 kDu. Temporarily expressed in the endoplasmic reticulum membrane, the p54 protein can be secreted outside the virus and infect cells in the form of a disulfide bond-linked type I membrane binder [7,8]. Antibodies can be detected 7–10 days after the attenuated challenge. Equivalent to the level of OIE whole virus antigen enzyme-linked immunosorbent assay (ELISA), the ELISA antibody detection method established with the p54 protein has high sensitivity, specificity, and stability and is currently the most comprehensive method of evaluating the best ELISA diagnostic antigens.

In our study, we applied bioinformatics methods to predict the epitope of the p54 protein and used the ASF-positive serum and anti-p54 protein monoclonal antibody (mAb) to verify the predicted epitope with ELISA. After designing 19 polypeptides for the most likely antigenic sites, we determined the precise amino acid sequence of the epitope by changing the number of amino acids at both ends of the polypeptide in order to accurately locate the B cell epitope of the p54 protein. The results lay the foundation for developing ASF vaccines and detection methods with high sensitivity.

MATERIALS AND METHODS

Animals, plasmids, strains, polypeptides, and standard serum

To begin, 6–8-wk-old SPF-grade BALB/c female mice were purchased from Guangdong Experimental Animal Center. The pET-52b(+) vector, the pET-52b(+)3C/LIC Vector Kit, and *Escherichia coli* DH5α and *E. coli* BL21(DE3) were purchased from Novagen, whereas the DH10 Bac cells and the vector pFastBac/NT-TOPO were purchased from Invitrogen. The pMD18-T-p54 plasmid (including the p54 target gene with a fragment size of 462 bp), the plasmid pMD18T-vp54 (including the full-length ASFV p54 gene with a fragment size of 558 bp), and insect cell Sf9 and mouse myeloma cell SP2/0 were stored at the transplantation center of the Shenzhen Entry–Exit Inspection and Quarantine Bureau. The synthetic antigen epitope peptides were completed by Shanghai Qiangyao Biological Technology (China).

Standard ASF-positive and -negative sera were provided by the IAH Laboratory, while the positive and negative reference sera for other pig diseases were obtained from the Animal Inspection and Quarantine Laboratory of the Shenzhen Entry–Exit Inspection and

https://doi.org/10.4142/jvs.21134

Quarantine Bureau. Sheep anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) and rabbit anti-pig IgG-HRP were purchased from Thermo Fisher (USA).

Main reagents and kits

PrimeSTAR Max DNA Polymerase was purchased from Dalian Bao Biological Engineering; isopropyl thiode-β-D-galactoside (IPTG) and the Ni-NTA Fast Start Kit were purchased from Qiagen; Platinum Pfx DNA Polymerase, the PureLink HiPure Mini Plasmid Purification Kit, Cellfectin reagent, ampicillin, gentamicin, and others were purchased from Invitrogen; Furch's complete adjuvant, Furch's incomplete adjuvant, and mAb subclass identification kits were purchased from Sigma; and ProteOn GLM chips, the ProteOn Regeneration and Sensor Chip Conditioning Kit, and the ProteOn Immobilization Buffer Kit were purchased from Bio-Rad Laboratories (USA).

Prokaryotic expression of the ASFV p54 protein

Using the pMD18-T-p54 plasmid as a template for PCR amplification, the PCR product was recovered and purified. The target gene was ligated to the pET-52b(+)3C/LIC vector using the T4 DNA polymerase, the enzyme-linked product was transformed into *E. coli* competent cell DH5α, and, following extraction, the pET-52b(+)3C/LIC-p54 recombinant plasmid was obtained. After the recombinant plasmid was transformed into BL21(DE3) competent cells, it was cultured to an optical density $(OD)_{600}$ value of 0.6, and IPTG was added at a final concentration of 1 mM to induce protein expression. Following the sonographic fragmentation of the bacteria, the ultrasound supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. Next, the ASF-positive reference serum was used as the primary antibody and horseradish peroxidase-labeled rabbit anti-pig IgG as the secondary antibody for western blot analysis to detect whether the expressed product was antigenic. The Ni-NTA Fast Start Kit was used to purify the recombinant protein of the bacterial lysate supernatant to obtain the ASFVp54 recombinant protein. After its concentration was measured, it was stored at −80°C for later use.

Eukaryotic expression of the ASFV p54 protein

The recombinant plasmid pMD18T-vp54 containing the full-length p54 gene was amplified, after which the PCR products were recovered, purified, ligated to the pFastBac/NT-TOPO vector, and transferred into the *E. coli* competent cell DH5α. After being identified as positive by sequencing, the pFastBac-p54 recombinant plasmid was obtained and subsequently transformed into DH10Bac competent cells. After that, the recombinant cosmid Bacmid-p54 was obtained through blue–white screening and plasmid extraction. Recombinant *baculovirus* AcNPV-p54 was obtained by using a liposome-mediated transfection of Sf9 insect cells with Bacmid-p54. Following 72 h of the infection of Sf9 cells and the sonication of normal Sf9 cells, the cell supernatant was collected and the protein concentration determined. Using standard ASFV21-positive serum to perform western blot detection on the supernatant, the expression and biological activity of the recombinant protein was analyzed, and the protein was stored at −80°C for later use.

Secondary structure of the ASFV p54 protein and predicting the antigen epitopes with B cells

The specific amino acid sequence of the ASF p54 protein (GenBank: ADD62408) is MYTILIA IVVLVIIIIVLIYLFSSRKKKAAAAIEEEDIQFINPYQDQQWAEVTPQPGTSKPAGATTASAGKPVT GRPATNRPATNKPVTDNPVTDRLVMATGGPAAAPAAASAHPTEPYTTVTTQNTASQTMSAI ENLRQRNTYTHKDLENSL.

Table 1. Amino acid sequences of regions of the synthetic predicted epitope polypeptide

Region	Amino acid sequence
A51–65	EVTPQPGTSKPAGAT
A23–29	SSRKKKA
A72–94	PVTGRPATNRPATNKPVTDNPVT
A114–120	HPTEPYT
A134–130	TQNTASQ
A137–150	NLRQRNTYTHKDLE

The Carnier–Robson, Chou–Fasman, and Karplus–Schulz methods along DNA Star software were used to predict the secondary structure of the ASF p54 protein [9]. Meanwhile, the Kyte–Doolittle, Jameson–Wolf and Emini methods were used to predict the protein's hydrophilicity, antigenic index, and surface probability in order to infer potential epitopes of p54 protein B cells [10].

Validation of the ASF epitope serum for predicted epitopes using indirect ELISA

Six predicted epitope peptides were synthesized—A23–29, A36–45, A72–94, A114–120, A124–130, and A137–150—along with a negative control, the A51–65 peptide, in the p54 protein. Excluding the predicted epitope peptide sequence, 15 consecutive amino acid peptides were used as another negative control. The specific amino acid sequence is shown in **Table 1**.

The six mentioned peptides were coated on the microtiter plate while using the standard ASF-positive and -negative sera as the primary antibodies and rabbit anti-pig HRP-IgG as the secondary antibody. Indirect ELISA method was used to compare the P/N value of each antigen peptide upon interacting with the negative and positive sera, as well as to compare the P/N value when the standard positive serum interacted with the six synthetic peptides and the negative control peptide, A51–65.

Preparation of a mAb for the ASFV p54 protein

The BALB/c mice were immunized with a purified prokaryotic expression of the recombinant p54 protein, after which the spleen cells of immunized mice with high titers were mixed with SP2/0 myeloma cells at a ratio of 10:1 to obtain hybridoma cells. Three screening antigens were coated with indirect ELISA plate plates, prokaryotic and eukaryotic expressed p54 recombinant proteins, and synthetic amino acid polypeptide A with the sequence 23SSRKKKAAAAIEEEDIQFINPYQDQQWAEV52. The wells with strong positive results in the indirect ELISA that met the three screening antigens were selected, and the limiting dilution method was used for subcloning until all of the wells with a positive rate reached 100%. After the positive hybridoma cells were injected into the 10-wk-old mice, a large amount of ASF p54 mAb 2E4 was obtained. Last, the subclass, specificity, and affinity of the mAbs were identified.

Validation of the predicted antigen epitopes according to mAbs with indirect ELISA

The six peptides and the negative control peptide were coated on the ELISA plate. mAb 2E4 and negative control IgG were used as the primary antibodies and goat anti-mouse IgG-HRP as the secondary antibody. After the substrate was developed, its OD_{450} and P/N value were determined in order to judge the antigenicity of the peptide corresponding to mAb 2E4.

Verification of the predicted antigen epitopes according to mAbs with blocking ELISA

According to the experimental procedure for blocking ELISA, fold ratio-diluted ASF-positive and -negative sera were used as blocking agents, mAb 2E4 as the detection antibody, and

sheep anti-mouse IgG-HRP as the secondary antibody. After the substrate was developed, the OD_{450} value was determined, and the percentage inhibition (PI) was calculated to determine whether the ASF-positive serum had a blocking effect when the polypeptide bound to the mAb in order to verify the antigenicity of the polypeptide corresponding to mAb 2E4.

Localization of the antigen site on the surface of the ASF p54 protein B cells

To obtain the necessary amino acid sequence included in the specific binding site of mAb 2E4, we designed 19 amino acid peptides. In the ProteOn XPR36 macromolecular interaction system, mAb 2E4 interacted with the predicted antigen site polypeptide, and its exact sequence was found by changing the number of amino acids at both ends of the polypeptide. The 19 peptides were coated with the ELISA plate, and indirect ELISA was performed to verify the antigen antibody reaction with Mab 2E4.

Development of a competitive ELISA method for detecting ASFV antibodies

The purified ASFV p54 recombinant protein from the prokaryotic expression was used as the coating antigen, and the obtained mAb labeled with HRP IgG was used as a competitive antibody. Phalanx titration was used to determine the working concentration of the coating antigen and the working concentration and reaction time of the mAb-HRP IgG. First, the ELISA plate was coated with the antigen diluted with a carbonate buffer solution (pH 9.6) and kept at 4°C overnight. After the liquid was discarded, the plate was washed 3 times with 0.05% phosphate-buffered saline with Tween 20 (PBST), the blocking solution was added and incubated at 37°C for 1 h, and the washing steps were repeated. The serum samples were diluted 20-fold with PBST and placed onto the ELISA plate. After incubation at 37°C for 1 h and washing 3 times, the prediluted mAb-HRP IgG was added and incubated at 37°C for 30 min. The ELISA plate was washed 3 times with PBST, after which the chromogenic substrate solution was added. After incubation at 37°C for 10 min, a stop solution was added to terminate the reaction, and the OD values were read using an ELISA plate reader. The test allowed configuring a positive and negative comparison and calculating the inhibition percentage.

RESULTS

Immunogenicity of the prokaryotically expressed ASFV p54 protein

The ASFV p54 protein was expressed using the prokaryotic expression system and its antigenicity examined. The results of western blot analysis showed a special hybridization band in the expected 28-kDu fragment, as consistent with the positive control. The non-induced negative control had no specific hybridization band, which indicated that our method successfully expressed the ASFV p54 recombinant protein and had good reactivity with anti-ASFV antibodies (**Fig. 1**).

Immunogenicity of the eukaryotic expression of the ASFV p54 protein

The ASFV p54 protein was expressed using the Bac-to-Bac baculovirus expression system and its antigenicity tested. The results of western blot analysis revealed that 72 h after infection with AcNPV-p54 in lane 3, the Sf9 cell lysate showed a clear, specific band at 25 kDu, while the normal Sf9 cell lysate in lane 2 did not, thereby indicating that the p54 protein can not only be expressed in Sf9 cells but also has good reactivity with standard positive serum (**Fig. 2**).

https://doi.org/10.4142/jvs.21134

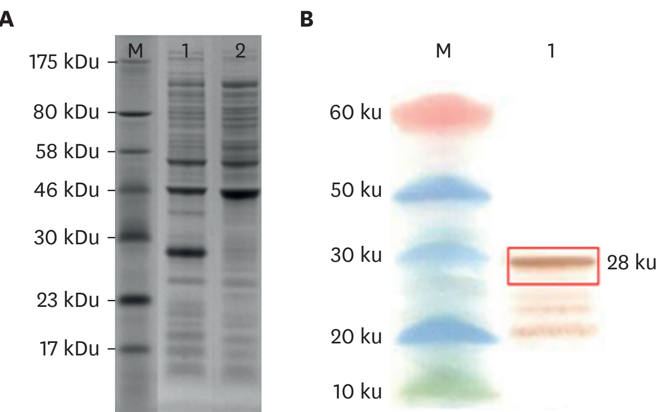

Fig. 1. Results of SDS-PAGE and western blot analysis for detecting the prokaryotic expression of the ASFV p54 protein. (A) SDS-PAGE analysis (M: protein marker; 1: positive control; 2: negative control). (B) Western blot positive hybridization.

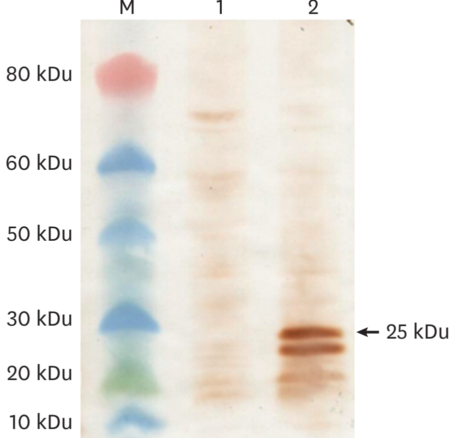

Fig. 2. Results of a western blot analysis to detect the eukaryotic expression of the ASFV p54 recombinant protein (M: protein marker; 1: normal Sf9 cell protein; 2: p54 recombinant protein).

Analysis of the secondary structure of the ASFV p54 protein and predicting the antigen epitope

The α-helical regions jointly predicted by the Carnier–Robson and Chou–Fasman methods were 27–39, 105–111, 134–140, and 149–153, while the β-sheet regions were 1–20, 92–98, 120–124, and 130–132 (**Fig. 3**).

The flexible regions of the secondary structure of the p54 protein include corner regions, unregulated coiled regions, and flexible structure regions. The angular regions of the protein's secondary structure were predicted by using the Chou–Fasman method and determined to be 23–27, 42–50, 55–59, 60–64, 68–72, 78–82, 89–93, 100–104, 114–118, 126–130, and 142–146. Meanwhile, the uncoiled regions of p54 protein, predicted by the Garnier–Robson method, were 41–43, 47–48, 54–56, 58–60, 70–71, 102–105, 113–118, and 126–130, while the flexible structural regions of the p54 protein, predicted by the Karplus–Schulz method, were 23–29, 34–38, 43–48, 53–95, 101–105, 115–131, and 138–151.

After analyzing the protein's hydrophilicity, antigen index, and surface accessibility, amino acid fragments with hydrophilic properties much greater than 0 were 22–65, 67–95, 112–131,

https://doi.org/10.4142/jvs.21134

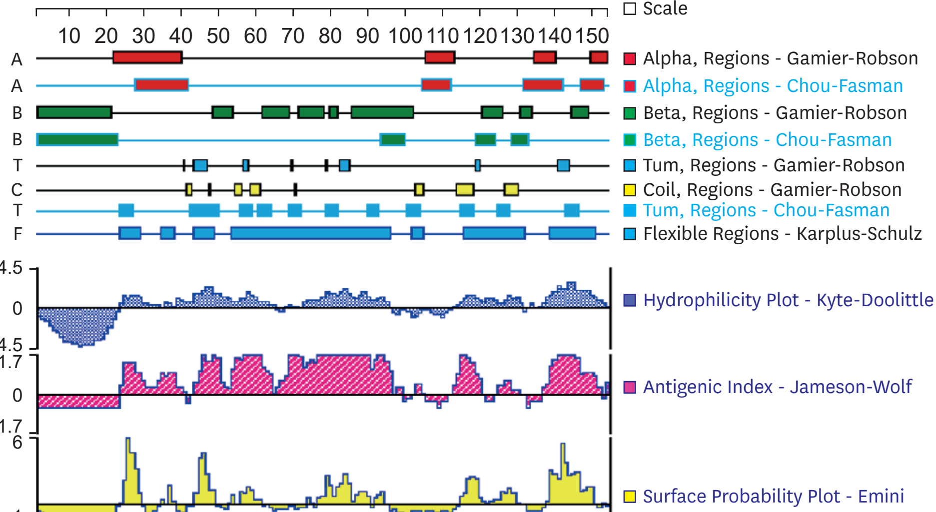

Fig. 3. Analysis of the characteristics of the p54 protein's secondary structure.

and 133–153. Amino acid fragments with an antigenic index greater than 0 were 23–40, 43–99, 102–104, 111–120, 124–131, and 136–153, while amino acid fragments with protein surface accessibility greater than 1 were 24–29, 33–38, 42–49, 53–61, 72–74, 76–94, 114–130, and 137–150.

As shown in **Fig. 3** and by the comprehensive analysis of the results of predicting the secondary structure, the peptides most likely to form B cell epitopes in the ASF p54 protein were A23–29, A33–38, A72–94, A114–120, A124–130, and A137–150. Those peptides have better hydrophilicity, surface accessibility, and high antigen exponential, as well as corners and random coil structures that facilitate the formation of epitopes either inside or nearby.

In our study, the average antigen index of the amino acid residues potentially formed by the p54 protein was calculated by using the antigenic index method. The results revealed that the regions A23–29, A33–38, A72–94, A114–120, and A137–150 had a higher antigenic index, which is also the dominant epitope region.

Results of indirect ELISA of ASF serum for predicted antigenic epitopes

We used indirect ELISA to compare the OD_{450} values of the six peptides with negative and positive sera, the ratio (i.e., P/N value) of the peptides, and the OD_{450} of the negative and positive sera. As shown in **Table 2**, the six predicted antigenic epitopes had P/N values greater than 2.5, while the negative control peptide had a value of 2.0, thereby confirming that

Table 2. Results of an ELISA of the reactions of predicted antigen epitopes with positive and negative sera

Region	OD price		P/N price
	Positive serum	Negative serum	
A23–29	0.75	0.20	3.75
A36–45	1.11	0.28	3.96
A72–94	0.73	0.16	4.50
A114–120	0.96	0.24	2.86
A124–130	0.57	0.22	2.59
A137–150	0.83	0.29	4.00
A51–65 (negative)	0.36	0.18	2.00

ELISA, enzyme-linked immunosorbent assay; OD, optical density; P/N, positive/negative.

A23–29, A36–45, A72–94, A114–120, A124–130, and A137–150 were indeed antigen sites on the surface of the ASF p54 protein B cells.

We used the same standard positive serum in interaction with the predicted six peptides and the A51–65 negative control peptide and compared the OD_{450} values. As shown in **Table 3**, the results revealed that except for the A124–130 peptide, all other P/N values exceeded 2.0, thereby indicating that the predicted peptides A23–29, A36–45, A72–94, A114–120, and A137–150 had good antigenicity and can be used as antigenic epitopes of the ASF P54 protein. In addition, the P/N value of the A124–130 polypeptide was only 1.58, which indicates that its antigenicity is average, as consistent with the average result on the antigenicity index.

Acquisition and identification of mAbs

We used three methods of screening antigen binding to obtain a stable, highly specific, clear background and the specific antigen epitope A23–52 hybridoma cell line 2E4. At present, the cell line has been collected by the China Type Culture Collection (No. C2014212). We identified that the mAb secreted by that strain of hybridoma cells was of subclass IgG1. As shown by indirect ELISA, mAb 2E4 had no cross-reactivity with other common swine disease pathogens. Using the ProteOn XPR36 macromolecular interaction system, the affinity between mAb 2E4 and the specific epitope polypeptide A in the ASF p54 protein was examined. The results showed that the binding affinity of the two was strong and is suitable for detecting and diagnosing (data not shown).

Results of indirect ELISA with mAbs on predicted antigen epitopes

The indirect ELISA of the obtained mAb 2E4 with six predicted peptides showed that the mAb could only bind to 36EDIQFINPYQ45 (**Table 4**).

Results and analysis of blocking ELISA to predict the antigen epitopes

Using the synthetic peptide 36EDIQFINPYQ45 as the coating antigen, the ASFV-negative and -positive reference sera as the blocking agent, and mAb 2E4 as the detection antibody, the predicted antigen epitope was blocked in an ELISA. The results showed that the binding of mAb 2E4 to the 36EDIQFINPYQ45 peptide antigen was blocked by the ASFV-positive reference serum, and the inhibition rate decreased along with the concentration of the positive reference serum (**Fig. 4**).

Table 3. Results of an ELISA of positive serum reactions with predicted antigen epitopes

Region	A23–29	A36–45	A72–94	A114–120	A124–130	A137–150	A51–65 (negative)
OD price	0.75	1.11	0.73	0.96	0.57	0.83	0.36
P/N price	2.08	3.08	2.03	2.67	1.58	2.31	-

ELISA, enzyme-linked immunosorbent assay; OD, optical density; P/N, positive/negative.

Table 4. Results of indirect ELISA of mAbs' reaction with the predicted antigen epitopes

mAb	Values of OD_{450} nm of the predicted antigen epitopes						
	A23–29	A36–45	A72–94	A114–120	A124–130	A137–150	A51–65[a]
mAb 2E4	0.077	0.616	0.071	0.078	0.068	0.083	0.075
mAb(−)[b]	0.072	0.080	0.066	0.078	0.075	0.069	0.071

ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; OD, optical density.
[a]The ASFV p54 protein contains amino acid sequences without the predicted epitope that can be used as negative control antigens.
[b]mAbs against swine influenza virus can be used as a negative control antibody.

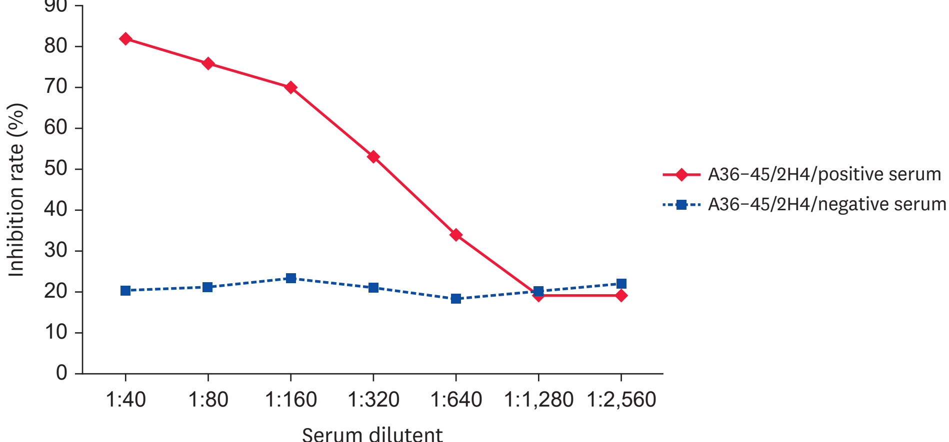

Fig. 4. Results of a blocking ELISA for polypeptide binding reactions with mAb.
ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody.

Localization of the antigen site on the surface of the ASF p54 protein B cells

To obtain the necessary amino acid sequences included in the specific binding site of mAb 2E4, the 19 synthesized peptides were combined and dissociated with mAb 2E4 by using a macromolecule interaction instrument. According to the results, the antigen site should include at least the amino acid sequence 37DIQFINPY44.

Meanwhile, 19 amino acid peptides were used to coat the ELISA plate, and indirect ELISA was used to verify the reaction of those peptides with the antigen–antibody of mAb 2E4 (**Table 5**), which confirmed that the sequence of the minimum essential amino acids, contained at the site was 37DIQFINPY44.

Preliminary establishment of the competitive ELISA for detecting ASFV antibodies

Phalanx titration revealed that the best coating concentration of the ASFV p54 recombinant protein and the best working concentration of HRP-mAb were 2.5 and 15 μg/mL, respectively. The best reaction time in the sample was 30 min. Based on those optimized conditions, a competitive ELISA was established for detecting ASFV antibodies. Following the established procedure, 150 negative serum samples were tested, and the PI, mean PI, and SD of each sample were calculated using the formula PI (%) = [1 – (tested sample OD_{450}/standard negative OD_{450})] × 100%. The sum of the PI means and 3 times the SD was defined as the

Table 5. Results of an indirect ELISA of peptides and mAb 2E4

Region	OD value	Result (+/−)	Region	OD value	Result (+/−)
A32–45	2.02	+	A37–43	0.33	−
A33–45	0.97	+	A37–42	0.55	−
A34–35	0.52	+	A39–48	2.27	+
A35–45	0.90	+	A38–47	2.26	+
A36–45	1.77	+	A37–45	2.24	+
A37–45	2.24	+	A36–45	1.77	+
A38–45	0.18	−	A35–44	2.73	+
A39–45	0.06	−	A34–43	0.57	−
A40–45	0.05	−	A33–42	0.62	−
A37–44	2.92	+			

ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; OD, optical density.

https://doi.org/10.4142/jvs.21134

critical threshold of the positive results, whereas the sum of the PI means and 2 times the SD was defined as the critical threshold of the negative results. Based on the samples that we examined, ones with PI values less than 45% were negative, ones with PI values exceeding 50% were positive, and ones with PI values in between were suspicious.

To evaluate the specificity of the developed competitive ELISA, serum samples for ASFV, swine fever virus, porcine reproductive and respiratory syndrome, foot and mouth disease, swine influenza virus, and pseudorabies virus were used. The results showed no cross-reaction with any of the non-ASFV positive sera, thereby indicating that the developed assay was specific for ASFV. Next, intra- and inter-batch repeatability tests were conducted using the 36 samples. The results indicated the good repeatability of the method. When the assay was further evaluated with 107 serum samples, the results showed that 11 samples were positive, while 96 samples were negative. In comparison with the ASFV testing kit made by the INIA, the accordance rate was 99.06%.

ELISA-detected field samples

A total of 1,000 clinical samples were used to test the detection methods applied in our study along with other detection methods, and the coincidence rate of the two types was calculated. A total of 1,000 samples were tested in the clinical trial, 26 of which were positive, with a positive rate of 2.6% (**Tables 1** and **2**). All positive samples and another 74 negative samples were selected and verified by commercial ELISA kits of the ASF antibody. The positive coincidence rate of the two kits was 100%, the negative coincidence rate was 97.3%, and the total coincidence rate was 98% (**Table 6**). Clinical trials have proven that the method has good sensitivity, strong specificity, and a high coincidence rate and can be applied to detect ASF antibodies in a large number of clinical samples.

DISCUSSION

The only DNA virus of the Asfarviridae family, ASFV can encode 150–200 kinds of proteins, 54 of which are structural proteins [11]. The p54 structural protein, approximately 25 kDu in size, is encoded by the ASFV E183L gene, located within the virus's capsid, and can directly bind to the 8-kDu light chain of microtubule motor dynein in the cytoplasm, thereby allowing the virus to enter the replication site in the host cell [12,13]. Compared with other structural proteins, p54 exists in the early stage of virus replication and plays an important role in viral replication, viral transfection, and structural stability maintenance. It is also the primary immunogenic protein of ASFV [14,15]. According to amino acid sequence analysis, the p54 protein is a specific protein of ASFV, and there is no crossover with other viral protein sequences.

A series of post-transcriptional processes, including folding, signal peptide cleavage, glycosylation, acylation and phosphorylation, can be performed on the expressed exogenous proteins after the transfection of insect cells with recombinant baculovirus. The Bac-to-Bac baculovirus expression system has the advantages of good stability, high expressiveness, and good protein solubility, but due to the low surface amount, it is difficult to achieve purification.

Table 6. Results of a comparison of 100 clinical samples

Method	Positive rates	Negative rates	Total coincidence rate
Blocking ELISA	26% (26/100)	72% (72/100)	98% (98/100)
Commercial ELISA	26% (26/100)	74% (74/100)	

ELISA, enzyme-linked immunosorbent assay.

 https://doi.org/10.4142/jvs.21134

The prokaryotic expression system has the characteristics of simple operability and high expression volumes, but most of the expressed products exist in the form of non-bioactive or insoluble inclusion bodies. The form of existence, expressed amount, and stability of the expressed product relate to many factors, including strain, toxicity of the expressed product to strain, expressed form, temperature, concentration of inducer, induction time, and expression vector. The expression vector used in our study can be highly expressed in BL21 (DE3) bacteria, and a large number of target proteins can be detected in the lysate supernatant. Therefore, the purification procedure is greatly simplified, while the possible loss of activity caused by the denaturation and renaturation of the inclusion body is avoided. The target protein can be effectively purified by commercial HIS label protein purification kits.

The secondary structure of a protein has important effects on its function. Because the bond energy of α-helix and β-sheet is relatively high, the structure is relatively stable, does not easily bind with antibodies, and rarely forms B cell epitopes alone. Corner and random coil structures are more prone to torsion and are generally located on the surface of the protein; their structure protrudes outwards and easily binds to antibodies, thereby increasing the possibility of forming B cell epitopes [16]. In our study, the Carnier–Robson, Chou–Fasman, and Garnier–Robson methods were used to predict the α-helix and β-sheet and to analyze the corner area, random coil area, and flexible structure area [17]. The Kyte–Doolittle, Jameson–Wolf, and Emini methods were used to analyze the hydrophilicity, antigenic index, and surface accessibility of the protein and to further analyze the amino acid residues that may form the epitope in the protein. Comprehensive analysis revealed that six regions of the p54 protein (i.e., A23–29, A36–45, A72–94, A114–120, A124–130, and A137–150) are more likely to form epitopes and were tested with indirect ELISA, which verified that the six regions can indeed form B cell epitopes.

At present, genetic engineering is commonly used to express the ASF p54 protein in order to prepare antigens. However, that method is time-consuming and costly. Artificially synthesized epitope peptides have high specific epitope concentrations, strong specificity, a low production cost, and shorter production cycles, which make them good raw materials for clinical detection methods and kits. Our study showed by using indirect ELISA and blocking ELISA that the predicted A36–45 peptides not only reacted with the ASF standard positive serum but also specifically bound to the mAb 2E4 prepared in our study with good antigenicity. We also used the protein macromolecule interaction system to interact with the acquired mAb and the possible antigenic sites of the ASFV p54 protein. As a result, an antigenic site in the p54 protein of the ASF virus was obtained and verified, and the minimum number of essential amino acids constituted by the site was analyzed. The ultimate sequence was DIQFINPY. We searched all 197 gene sequences of the ASF virus P54 protein from the NCBI database, analyzed their corresponding amino acid sequences, and found that the amino acid sequence is highly conservative. Therefore, our method can be used to prepare mAb to predict different epitope polypeptides and screen out mAbs against different epitopes, which lays a solid foundation for developing not only immunological detection methods but peptide vaccines as well.

REFERENCES

1. Emini EA, Hughes JV, Perlow DS, Boger J. Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. J Virol. 1985;55(3):836-839.
PUBMED | CROSSREF

2. Rahimi P, Sohrabi A, Ashrafihelan J, Edalat R, Alamdari M, Masoudi M, et al. Emergence of African swine fever virus, northwestern Iran. Emerg Infect Dis. 2010;16(12):1946-1948.
PUBMED | CROSSREF

3. Ito S, Bosch J, Martínez-Avilés M, Sánchez-Vizcaíno JM. The evolution of African swine fever in China: a global threat? Front Vet Sci. 2022;9:828498.
PUBMED | CROSSREF

4. Alonso C, Miskin J, Hernáez B, Fernandez-Zapatero P, Soto L, Cantó C, et al. African swine fever virus protein p54 interacts with the microtubular motor complex through direct binding to light-chain dynein. J Virol. 2001;75(20):9819-9827.
PUBMED | CROSSREF

5. Glazunova AA, Korennoy FI, Sevskikh TA, Lunina DA, Zakharova OI, Blokhin AA, et al. Risk factors of African swine fever in domestic pigs of the Samara region, Russian Federation. Front Vet Sci. 2021;8:723375.
PUBMED | CROSSREF

6. Kollnberger SD, Gutierrez-Castañeda B, Foster-Cuevas M, Corteyn A, Parkhouse RM. Identification of the principal serological immunodeterminants of African swine fever virus by screening a virus cDNA library with antibody. J Gen Virol. 2002;83(Pt 6):1331-1342.
PUBMED | CROSSREF

7. Chenais E, Depner K, Guberti V, Dietze K, Viltrop A, Ståhl K. Epidemiological considerations on African swine fever in Europe 2014-2018. Porcine Health Manag. 2019;5(1):6.
PUBMED | CROSSREF

8. Dietze K, Beltran-Alcrudo D, Khomenko S, Seck B, Lubroth J, Martin V, et al. African swine fever (ASF) recent developments - timely updates. Rome: Food and Agriculture Organization; 2012.

9. Hernáez B, Díaz-Gil G, García-Gallo M, Ignacio Quetglas J, Rodríguez-Crespo I, Dixon L, et al. The African swine fever virus dynein-binding protein p54 induces infected cell apoptosis. FEBS Lett. 2004;569(1-3):224-228.
PUBMED | CROSSREF

10. Hernaez B, Escribano JM, Alonso C. Visualization of the African swine fever virus infection in living cells by incorporation into the virus particle of green fluorescent protein-p54 membrane protein chimera. Virology. 2006;350(1):1-14.
PUBMED | CROSSREF

11. Garnier J, Osguthorpe DJ, Robson B. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J Mol Biol. 1978;120(1):97-120.
PUBMED | CROSSREF

12. Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. J Mol Biol. 1982;157(1):105-132.
PUBMED | CROSSREF

13. Kokh DB, Wenzel W. Flexible side chain models improve enrichment rates in in silico screening. J Med Chem. 2008;51(19):5919-5931.
PUBMED | CROSSREF

14. Reis AL, Parkhouse RM, Penedos AR, Martins C, Leitão A. Systematic analysis of longitudinal serological responses of pigs infected experimentally with African swine fever virus. J Gen Virol. 2007;88(Pt 9):2426-2434.
PUBMED | CROSSREF

15. Rodríguez JM, García-Escudero R, Salas ML, Andrés G. African swine fever virus structural protein p54 is essential for the recruitment of envelope precursors to assembly sites. J Virol. 2004;78(8):4299-4313.
PUBMED | CROSSREF

16. Salas ML, Andrés G. African swine fever virus morphogenesis. Virus Res. 2013;173(1):29-41.
PUBMED | CROSSREF

17. Takamatsu HH, Denyer MS, Lacasta A, Stirling CM, Argilaguet JM, Netherton CL, et al. Cellular immunity in ASFV responses. Virus Res. 2013;173(1):110-121.
PUBMED | CROSSREF