A novel multi-epitope recombinant protein for diagnosis of African swine fever

ZHAN GAO

LVRI <https://orcid.org/0000-0003-4847-6537>

JUNJUN SHAO

lvri

GUANGLEI ZHANG

lvri

SUDAN GE

Lanzhou Veterinary Research Institute

YANYAN CHANG

LVRI

LEI XIAO

lvri

HUIYUN CHANG (✉ changhuiyun@caas.com)

Research article

Keywords: African Swine Fever Virus, an Indirect Enzyme-Linked Immunosorbent Assay, Recombinant protein, Multi-epitope

Posted Date: May 22nd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-29580/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

A novel multi-epitope recombinant protein for diagnosis of African swine fever

Zhan Gao, Jun-jun Shao, Guang-lei Zhang, Su-dan Ge, Yan-yan Chang, Lei-Xiao,
Hui-yun Chang

State Key Laboratory of Veterinary Etiological Biology, OIE/National Foot-and-Mouth Disease Reference Laboratory, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, 730046, Gansu, China

*Correspondence: Hui-yun Chang (changhuiyun@caas.cn)

Address: No.1 Xujiaping, Yanchangbao, Chengguan District, Lanzhou 730046, Gansu, China.

Abstract

Background: African swine fever (ASF) is an acute, severe and highly fatal infectious disease of pigs. The disease spreads rapidly, causing huge economic losses to the pig industry in infected areas. The structural proteins p30 and p54 in African swine fever virus (ASFV) have been verified as diagnostic antigens.

Methods: In this study, we constructed a novel multi-epitope fusion antigen gene based on P30 and P54 proteins, induced expression in a prokaryotic expression system and analyzed the reactivity of the recombinant fusion protein. The purified recombinant protein m35 was used as the coating antigen to establish an indirect enzyme-linked immunosorbent assay (ELISA) detection method for ASFV. 116 serum samples and positive sera of other swine diseases were detected by indirect ELISA.

Results: Our results indicate that the m35 gene fragment with a length of 558bp was successfully constructed. SDS-PAGE and Western Blotting analysis showed that the protein had a band at 22kDa, proving its good reactogenicity. ROC analysis was performed to validate the assay, the area under the ROC curve is 0.9738 (95% confidence interval, 0.9336 to 1.014), and does not cross-react with other swine diseases.

Conclusion: Our results show that its sensitivity and specificity were highly accurate. It is feasible to use this recombinant protein as a diagnostic antigen to distinguish ASFV infection.

Keywords: African Swine Fever Virus; an Indirect Enzyme-Linked Immunosorbent Assay; Recombinant protein; Multi-epitope

Background

ASF is an acute, highly contagious swine infection caused by ASFV, with a fatality rate of up to 100%[7]. The pig industry in the affected area caused huge economic losses. The World Health Organization (OIE) included it in the list of animal diseases that must be notified.

The ASF was first discovered in Kenya, Africa in 1921, and had been outbreaks in Africa only until 1957, and then spread to Europe and the Americas, but the epidemic was controlled except Sardinia. In 2007, ASF was introduced into Georgia, and then through Georgia to Armenia, Azerbaijan, Russia and other countries[10]. And it was introduced to China in August 2018, where it is a major type of animal disease prevention and control. Based on next-generation sequencing and comparison with related European p72 genotype II strains, the Chinese strain 2018 / AnhuiXCGQ genome is 189,393 bp long and encodes 181 open reading frames, with 54-107

variants between other gene type II strain This strain has the highest similarity to the POL / 2015 / Podlaskie strain(99.972%)[5]. No commercial vaccines are currently available, so establishing effective detection methods is of great significance for the prevention and control of ASF.

ASFV is composed of five parts: nucleoid, core shell, inner envelope, capsid, and external envelope. It encodes 54 structural proteins and more than 100 non-structural proteins[4]. Both P30 and P54 proteins in structural proteins can induce specific immune responses. The P30 protein encoded by the open reading frame CP204L is mainly involved in virus internalization and plays an important role in the process of the virus entering the host cell[13]. And a large number appeared in the early stage of virus infection, inducing the body to produce neutralizing antibodies[1]. The P54 protein encoded by the E183L gene is present in the inner envelope of the virions and is involved in the adsorption and entry of the virus. Therefore, P30 and P54 proteins are ideal antigens for serological diagnosis and immunological detection[11, 15].

In this study, we predicted the predominant epitopes of P30 and P54 proteins to synthesize the synthetic gene m35 with small molecular weight and easy expression. The prokaryotic expression of the gene was purified, and the immunological characteristics of the recombinant protein were analyzed by SDS-PAGE and western blotting, and an indirect ELISA detection method based on the recombinant protein was established; this provided an effective way for the timely diagnosis of ASFV.

Methods

Computer prediction of epitopes and construction of multi-epitope fusion genes

The sequences of p30 and p54 proteins of ASFV (GenBank: MK128995.1) were obtained from NCBI (<http://www.ncbi.nlm.nih.gov>). Four online epitope prediction tools DNASTar, ABCpred Prediction (http://crdd.osdd.net/raghava/abcpred/ABC_submission.html), Scratch(<http://scratch.proteomics.ics.uci.edu/>) and IEDB (<http://www.iedb.org/>) were used to screen the most immunogenic cell epitopes. The epitopes are linked together by the linker "GGGGS" and added to its 3' end the 6 × His tag, whose codons were optimized for pET-28a (+), was synthesized by Nanjing genscript.

Expression, and purification of recombinant protein

The synthetic recombinant plasmid was transformed into E. coli BL21(Sangon Biotech Co., Ltd Shanghai China). The transformed E. coli BL21 cells were grown in a shaker at 220 rpm and 37 °C, inoculated into 4 L of LuriaBertani (LB, containing 30 µg/ml kanamycin) at a dilution of 1:100. When the optical density at 600 nm (OD₆₀₀) of the culture reached approximately 0.6, the culture was induced by 1M IPTG. Induction at 37°C for four hours, the cell culture was collected by centrifugation, and aliquots before and after induction were analyzed by SDS-PAGE. The collected cell culture was resuspended in buffered lysate and then sonicated on ice. The lysate was centrifuged at 5000g and 4°C for 20 minutes, and binding buffer containing 8 M urea was added to the precipitate (inclusion body form) for overnight dissolution at 4°C. After centrifugation, the recombinant proteins containing the His - tag were purified by affinity chromatography using Ni Sepharose™ excel (GE). The purified proteins were refolded in dialysate (0.25 M NaCl, 50 mM Tris, 0.5 mM EDTA, 2 mM GSH,

and 0.2 mM GSSG; pH 8.0) containing different concentrations of urea (6 M to 0 M in 1 M decrements) at 4 °C. Finally, the proteins were dialyzed in PBS (pH 7.4) twice to obtain the completely renatured proteins. The concentration of m35 protein was determined using the Bradford Protein Assay Kit, respectively, according to the manufacturer's instructions.

SDS-PAGE, Western blot analysis

The purified proteins m35 were analyzed by 10% SDS-PAGE and stained with Coomassie blue R250 or transferred to a polyvinylidene fluoride (PVDF) membranes for Western blot analysis using a mouse anti-His monoclonal antibody (mAb; Abcam, MA) and the ASFV positive serum. The membranes were blocked in PBST containing 5% skim milk at 37 °C for 1 hour, and then incubated overnight at 4 °C with diluted mAb or positive serum. The membranes were then washed three times with PBST and incubated with the diluted goat antimouse and rabbit anti-pig second antibody conjugated with horseradish peroxidase (HRP; Abcam) at 37 °C for 1 hour. After three washes in PBST, protein detection was visualized with the BeyoECL Plus Kit (Beyotime, China), following the manufacturer's instructions, and exposed to X-ray film. Each band was analyzed using a Gel Image System (Tanon, China).

iELISA

Ninety-six-well microtiter plates (Corning, USA) were coated with 0.25 µg per well of the recombinant protein in coating buffer (0.01 M PBS, pH 7.4) and incubated overnight at 4 °C. Incubate overnight. After washing 3 times with PBST, blocking with 5% skimmed milk powder at 37 °C for 2 h, and then washing 3 times, incubated with serum 1:100 in serum dilution (100 µL per well) at 37 °C for 1 hour, then wash the wells 3 times, and dilute anti-swine IgG antibody to 1: 20000, add 100 µL per well, incubate for 30 minutes at 37 °C. After washing 3 times with PBST, 100 µL of TMB substrate was added to each well. After reacting at 37 °C for 15 minutes, 100 µL per well of stop solution (2M H₂SO₄) was added. ODs were measured at 450 nm using an ELISA plate reader (BioTek, USA).

Dot plot and receiver-operating characteristic (ROC) were performed using the GraphPad Prism version 7.0 for Windows.

116 serum samples from Professional laboratory in Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu, China. Serum samples were classified as negative or positive according to Western blot results, with 87 negative samples and 29 positive samples.

Cross-reaction analysis

The examination of the analytical specificity by m35 ELISA was conducted for determining the cross-reactive degree among PRV, PRRSV, PCV, PDCoV, CSFV, and FMDV sera samples. The experiment contained negative control and positive control.

Results

Construction of multiple epitope genes

The Chinese strain 2018 / AnhuiXCGQ genome's genebank is MK128995.1. Then integrate multiple epitope prediction programs to select the cell epitope with the best overlap result, as shown in Table1.

Protein	Start-end position	Sequence
p30	15-28	FKTDLRSSSQVVFH
	24-33	QVVFHAGSLY
	73-80	GYTEHQAQ
	115-123	CTSSFETLF
	173-181	TPLKEEKE
	182-193	VVRLMVIKLLKK
P54	17-26	CLSPVTTPSF
	145-161	HPAEPYTTVTQTASQ
	160-169	SQTMSAIENL
	167-181	ENLRQRNTYTHKDLE

Table 1 Epitope sequences selected in p30 and p54

Recombinant protein identification

we inserted the synthetic gene in the bacterial expression vector pET-28a and transformed it into *E. coli* BL21 (DE3) cells. Then, the induced cells were sonicated and analyzed by SDS-PAGE. A specific band representing the recombinant product was obtained (Fig. 1A and B). Ni-NTA affinity purification was used, and the purified products were analyzed by SDS-PAGE (Fig. 1c). The concentration of the product was 1.4 mg/ml when measured with a protein assay kit. Western blot analysis showed that m35 could react with anti-His mAb (Fig. 2W1), as well as ASFV positive serum (Fig.2W2).

Fig. 1 Preparation of recombinant protein. **A** SDS-PAGE of m35-induced expression (M, marker; Lane 1, uninduced cells; Lane 2, IPTG-induced cells for 4h.) **B** Analysis of m35 expression induced by SDS-PAGE (M, marker; Lane 3, supernatant of IPTG-induced cells; Lane 4, deposition of IPTG-induced cells) **C** SDS-PAGE of m35 purification (M, marker; Lane 5, purified recombinant protein.)

Fig. 2 Western blotting analysis of the purified recombinant protein using the anti-His-tag polyclonal antibody (M, marker; Lane W1, purified recombinant protein) Western blotting analysis of the purified recombinant protein using the ASFV-positive serum (M, marker; Lane W2, purified recombinant protein),

iELISA

To evaluate the analysis, 116 serum samples were tested by indirect ELISA and Western blot, including positive serum (29 samples) and negative serum (87 samples). The dot plot summarizes the OD values of these samples (Fig. 3a). ROC analysis was performed to assess the best sensitivity and specificity (Fig. 3b). According to ROC analysis, the AUC for this test was 0.9738 (95% confidence interval (CI) 0.9336 to 1.014). In addition, a diagnostic sensitivity of 93.1% (95% CI, 77.23 to 99.15) and a specificity of 96.55% (95% CI, 90.25 to 99.28) were obtained from the optimal cut-off value (0.3435).

Fig. 3 IELISA analysis of serum samples. **a** Dot plot of the m35 IELISA assay. **b** ROC analysis of m35 iELISA assay results.

Cross-reaction analysis

There were no cross reactions in the ELISA using PRV, PRRSV, PCV, PDCoV, CSFV, and FMDV positive sera. The OD values of the disease-positive sera were less than their critical values (Fig. 4).

Fig. 5 IELISA analysis of other swine disease serum samples.

Discussion

The current laboratory diagnosis of African swine fever includes animal inoculation, virus isolation, viral nucleic acid DNA detection, and specific antibody detection. Among them, animal vaccination, virus isolation, and viral nucleic acid detection require professional personnel in laboratories above Level 3. The operation is proceeding, it is difficult to meet the needs of the grassroots. Therefore, the serological detection method is one of the commonly used diagnostic methods for ASFV due to the simple operation steps and low cost. It is one of the important

methods for diagnosing and monitoring pigs infected with African swine fever virus[14].

In serological diagnosis, In 1979, ELISA was used for the first time to identify ASFV antigens and antibodies. The whole virus antigens were used. The reproducibility was good and a limited antigen concentration of 50-500 HAD₅₀ / ml could be detected[19]. p72, p30, p54, pp62 are the major diagnostic proteins[6, 9]. P72 protein is the main capsid protein, mainly involved in virus binding cells[13]. Since the semi-purified P72 protein was prepared in 1981 to establish an ELISA method, the protein has been used for detection research[17]. There are currently commercial detection kits based on this protein. PP62 protein is mostly identified as a late-stage protein. After proteolytic processing, two main structural proteins, p35 and p15, are produced[16]. The expression of pp62 using the baculovirus expression system and the establishment of an ELISA detection method show that the sensitivity and specificity are improved compared to traditional diagnostic methods [8]. P30 and p54 proteins are the most antigenic proteins[2]. Both proteins are incorporated into virus particles and mediate vaccine neutralization[12]. Interestingly, p54 protein has better reactivity than p30 in protein imprinting, but recombinant p30 protein is more effective in detecting antibodies by ELISA. This may be related to the conformational epitope included in p30[15]. Since then, these two proteins have been widely used in the diagnosis of African swine fever[3, 15].

The B cell epitope refers to the chemical group on the surface of the antigen molecule that can be specifically recognized and bound by the b cell surface receptor or antibody Fab part, which is the material basis for humoral immunity. Is the focus of research in immunodiagnosis and immunotherapy[18]. In recent years, with the development of bioinformatics, the bioinformatics prediction of epitopes has gradually replaced the traditional methods of epitope research. At present, the prediction of B cell linear epitopes is mainly based on the physical and chemical properties, structural characteristics, and statistical significance of amino acids. In our research, we selected the highly immunoreactive p30 and P54 proteins. We used DNASTar, ABCpred, Scratch, and IEDB to perform linear b-cell epitope analysis and prediction of these two protein sequences. In the end, we chose 10 epitopes as a result. The GGGGS flexible linker is used to connect each epitope in series, and the research proves that the flexible linker is more conducive to the function of each epitope. This is used as a new antigen sequence for detecting ASFV for prokaryotic expression and purification. The Western blotting analysis results show that m35 protein has good antigenicity and is suitable for diagnostic antigens

ROC analysis was performed to validate the assay, and its sensitivity and specificity were highly accurate($0.9 < \text{AUC} < 1$). And after testing, m35 iELISA does not react with other swine diseases. Therefore, it is feasible to use m35 recombinant protein as a diagnostic antigen to distinguish ASFV infection.

However, although the Elisa method is simple and suitable for large-scale epidemiological surveys, it is not suitable for the diagnosis of ASFV during the outbreak period, many influencing factors will appear false positive.

Conclusions

In this article, we analyzed and predicted the structural proteins p30 and p54 of the African swine fever virus with software analysis, selected the dominant epitope fragments, and connected them with flexible peptides to obtain the recombinant gene m35. And confirmed that the indirect ELISA method based on m35 protein has high sensitivity and specificity, which provides a choice for the development of the ASFV Elisa serological detection kit.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Funding

LVRI Enterprise Cooperation (LSY-2018-138)

Acknowledgments

Not applicable.

Availability of data and materials

All the data supporting the conclusions of this article is available and included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests

Authors' contributions

Z-G, H-YC conceived and designed this study. The ELISA method is performed by Z-G, GL-Z, S-DG, Y-YC and L-X. J-JS and H-YC provided constructive comments and suggestions, reviewed, and improved the manuscript. Z-G wrote this paper. The final manuscript read and approved by all authors.

References

1. Afonso CL, Alcaraz C, Brun A, Sussman MD, Onisk DV, Escibano JM, Rock DL (1992) Characterization of P30, a highly antigenic membrane and secreted protein of African Swine Fever Virus. *Virology* 189:368-373
2. Alcaraz C, De Diego M, Pastor MJ, Escibano JM (1990) Comparison of a radioimmunoprecipitation assay to immunoblotting and ELISA for detection of antibody to African swine fever virus. *Journal of veterinary diagnostic investigation: official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 2:191-196
3. Alcaraz C, Rodriguez F, Oviedo JM, Eiras A, De Diego M, Alonso C, Escibano JM (1995) Highly specific confirmatory Western blot test for African swine fever virus antibody detection using the recombinant virus protein p54. *Journal of Virological Methods* 52:111-119
4. Alejo A, Matamoros T, Guerra M, Andres G (2018) A Proteomic Atlas of the African Swine Fever Virus Particle. *J Virol* 92
5. Bao J, Wang Q, Lin P, Liu C, Li L, Wu X, Chi T, Xu T, Ge S, Liu Y, Li J, Wang S, Qu H, Jin T, Wang Z (2019) Genome comparison of African swine fever virus China/2018/AnhuiXCGQ strain and related European p72 Genotype II strains. *66:1167-1176*

6. Cubillos C, Gomez-Sebastian S, Moreno N, Nunez MC, Mulumba-Mfumu LK, Quembo CJ, Heath L, Etter EM, Jori F, Escribano JM, Blanco E (2013) African swine fever virus serodiagnosis: a general review with a focus on the analyses of African serum samples. *Virus Res* 173:159-167
7. Galindo I, Alonso CJV (2017) African swine fever virus: a review. 9:103
8. Gallardo C, Blanco E, Rodríguez JM, Carrascosa AL, Sanchez-Vizcaino JM (2006) Antigenic properties and diagnostic potential of African swine fever virus protein pp62 expressed in insect cells. *J Clin Microbiol* 44:950-956
9. Gallardo C, Soler A, Nieto R, Carrascosa AL, De Mia GM, Bishop RP, Martins C, Fasina FO, Couacy-Hymman E, Heath L, Pelayo V, Martín E, Simón A, Martín R, Okurut AR, Lekolol I, Okoth E, Arias M (2013) Comparative evaluation of novel African swine fever virus (ASF) antibody detection techniques derived from specific ASF viral genotypes with the OIE internationally prescribed serological tests. *Veterinary Microbiology* 162:32-43
10. Gogin A, Gerasimov V, Malogolovkin A, Kolbasov DJVr (2013) African swine fever in the North Caucasus region and the Russian Federation in years 2007-2012. 173:198-203
11. Gomez-Puertas P, Rodriguez F, Oviedo JM, Ramiro-Ibanez F, Ruiz-Gonzalvo F, Alonso C, Escribano JM (1996) Neutralizing antibodies to different proteins of African swine fever virus inhibit both virus attachment and internalization. *J Virol* 70:5689-5694
12. Gomez-Puertas P, Rodriguez F, Oviedo JM, Brun A, Alonso C, Escribano JM (1998) The African swine fever virus proteins p54 and p30 are involved in two distinct steps of virus attachment and both contribute to the antibody-mediated protective immune response. *Virology* 243:461-471
13. Gómez-Puertas P, Rodríguez F, Oviedo JM, Ramiro-Ibáñez F, Ruiz-Gonzalvo F, Alonso C, Escribano JM (1996) Neutralizing antibodies to different proteins of African swine fever virus inhibit both virus attachment and internalization. *J Virol* 70:5689-5694
14. Jurado C, Martinez-Aviles M, De La Torre A, Stukelj M, de Carvalho Ferreira HC, Cerioli M, Sanchez-Vizcaino JM, Bellini S (2018) Relevant Measures to Prevent the Spread of African Swine Fever in the European Union Domestic Pig Sector. *Frontiers in veterinary science* 5:77
15. Oviedo JM, Rodriguez F, Gómez-Puertas P, Brun A, Gómez N, Alonso C, Escribano JM (1997) High level expression of the major antigenic African swine fever virus proteins p54 and p30 in baculovirus and their potential use as diagnostic reagents. *Journal of Virological Methods* 64:27-35
16. Simón-Mateo C, Andrés G, Almazán F, Viñuela E (1997) Proteolytic processing in African swine fever virus: evidence for a new structural polyprotein, pp62. *J Virol* 71:5799-5804
17. Tabares E, Fernandez M, Salvador-Temprano E, Carnero ME, Sanchez-Botija C (1981) A reliable enzyme linked immunosorbent assay for African swine fever using the major structural protein as antigenic reagent. *Arch Virol* 70:297-300
18. Van Regenmortel MH (2009) What is a B-cell epitope? *Methods in molecular biology* (Clifton, NJ) 524:3-20
19. Wardley R, Abu EE, Crowther J, Wilkinson PJTJoh (1979) A solid-phase enzyme linked immunosorbent assay for the detection of African swine fever virus antigen and antibody. 83:363-369

Figures

Figure 1

Preparation of recombinant protein . A SDS PAGE of m35 induced expression (M marker Lane 1 uninduced cells; Lane 2, IPTG induced cells for 4h. 4h.) B Analysis of m35 expression induced by SDS PAGE (M marker Lane 3, supernatant of IPTG induced cells; Lane 4, deposition of IPTG induced cells) C SDS PAGE of m35 purification (M, marker; Lane 5 , purified recombinant protein.)

Figure 2

Western blotting analysis of the purified recombinant protein using the anti His tag polyclonal antibody (M, marker; Lane W1, purified recombinant protein) Western blotting analysis of the purified recombinant protein using the ASFV positive serum (M, marker; Lane W2, purified recombinant protein),

Figure 3

IELISA analysis of serum samples. a Dot plot of the m35 IELISA assay. b ROC analysis of m35 i ELISA assay results.

Figure 4

IELISA analysis of other swine disease serum samples.