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Development and evaluation of multi-epitope protein p72 (MeP72) for the serodiagnosis of African swine fever

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Summary. – African swine fever (ASF) is an acute and severe infectious disease that seriously endangers the global porcine industry. In order to develop ASF serodiagnostic reagents with high specificity and sensitivity, in the present study, the antigenic epitopes of P72 protein of African swine fever virus (ASFV) were analyzed, and the ASFV multi-epitope fusion gene MeP72 in tandem with the dominant linear epitopes was constructed. The recombinant multi-epitope fusion MeP72 (reMeP72) was prepared in *Escherichia coli*. A colloidal gold-based immunochromatographic assay (CGIA) based on reMeP72 was developed for the detection of antibodies against ASFV. A total of 139 pig clinical serum samples were used for assessment of the potential diagnostic value of reMeP72. The results showed that CGIA did not cross-react with positive sera of viruses, such as classical swine fever virus (CSFV), porcine circovirus type 2 (PCV2), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV) and swine influenza virus (SIV), showing high specificity. Sensitivity analysis showed that CGIA could detect ASFV-positive serum at a dilution of 1:64. Compared with commercial ASFV kits, the sensitivity and specificity of ASFV CGIA based on reMeP72 protein were 85.7% and 97.6%, respectively. The agreement rate of the two methods was 96.4%, showing a good detection performance. The results indicated that the reMeP72 was of potential value for the serodiagnosis of ASF.

Keywords: African swine fever virus; P72 gene; antigenic protein; colloidal gold-based immunochromatographic assay

Introduction

African swine fever (ASF), an acute, high-contact infectious disease in pigs, is caused by African swine fever virus (ASFV) (Galindo and Alonso, 2017; Olesen *et al*., 2017; Sánchez-Cordón *et al*., 2018), which is characterized by high fever, anorexia, cyanosis of the skin, ataxia, neurological symptoms, extensive bleeding in various tissues and organs (Blome *et al*., 2013; Cisek *et al*., 2016), and high mortality rate. As a double stranded DNA virus, ASFV belongs to the family *Asfarviridae* and the genus Asfivirus (Galindo and Alonso, 2017), which is currently the only DNA arbovirus that can be transmitted by soft ticks (Sánchez-Vizcaíno *et al*., 2015; Cisek *et al*., 2016). ASFV is classified as a c lass A animal pathogenic microorganism by World Organization for Animal Health (OIE) and has been listed as the first class of animal pathogenic microorganisms in China (Li *et al*., 2018; Sánchez-Cordón *et al*., 2018; Ge *et al*., 2018).

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Abbreviations: ASF = African swine fever; ASFV = African swine fever virus; reMeP72 = recombinant multi-epitope fusion MeP72; CSFV = classical swine fever virus; PCV2 = porcine circovirus type 2; PRV = pseudorabies virus; PRRSV = porcine reproductive and respiratory syndrome virus; SIV = swine influenza virus; CGIA = colloidal gold-based immunochromatographic assay

In the recent years, ASFV has spread from the African continent to many countries in Europe, Asia and Africa, causing severe economic losses to the global porcine industry (Cisek *et al*., 2016; Śmietanka *et al*., 2016; Rock, 2017; Brown *et al*., 2018; Ge *et al*., 2018; Li *et al*., 2018). At present, as there is no effective ASF vaccine available in the world (Zakaryan and Revilla *et al*., 2016; Arias *et al*., 2018; Revilla *et al*., 2018), the prevention and control of ASF can only rely on such measures as timely diagnosis and culling of infected animals (Hamdy *et al*., 1981; Pan *et al*., 1982; Haines *et al*., 2013; Bellini *et al*., 2016; Giménez-Lirola *et al*., 2016; Bergeron *et al*., 2017). At the present, the diagnosis of ASF is mainly based on etiological and serological diagnostic methods (Hutchings *et al*., 2006; Cubillos *et al*., 2013; Randriamparany *et al*., 2016; Carlson *et al*., 2018). However, the isolation and identification of ASFV need to be conducted in laboratories with biosafety level III or higher level. In terms of serological diagnosis, although ELISA detection kits for ASFV have been developed in other countries, their widespread uses in basic units have been limited due to their high-cost use. Therefore, it is of great significance to develop fast and simple ASF serological diagnostic methods with high specificity and high sensitivity for the prevention and control of ASFV infection (Gallardo *et al*., 2009).

The main purposes of this study were to prepare ASFV MeP72 multi-epitope fusion protein, and to analyze and evaluate its specificity and sensitivity through CGIA, aiming for the development of serological reagents for rapid diagnosis of ASFV infection.

Materials and Methods

Predictive analysis of ASFV P72 protein epitopes. The amino acid sequence encoded by the P72 gene (B646L) was selected from the standard strain BA71V (GenBank Acc. No. U18466.2) of ASFV, and the software ABCpred, BepiPred 1.0 Server, Immunomedicine Group, and DNAStar-Protean were applied to predict the linear epitopes of the P72 protein. Based on the prediction results (Table 1), we screened out the dominant linear epitopes and used them for the construction of the MeP72 multi-epitope fusion gene in the next step.

Construction of ASFV multi-epitope fusion gene MeP72. The nucleotide sequences corresponding to the amino acid sequences of different epitopes were ligated with a flexible peptide (GPG) coding sequence (GGCCCGGGC, nine nucleotides) in a following order: M-EP1-GPG-EP2-GPG-EP3-GPG-EP4-GPG-EP5-GPG-EP6-GPG-EP7-* (Supplemental Fig. S1), and a multi-epitope fusion gene MeP72 was obtained. The codon prediction software RACC was used to analyze the rare codons of *E. coli* with the multi-epitope fusion gene MeP72. Optimalisation of the rare codons of *E. coli* in the multi-epitope fusion gene MeP72 to the preferred codons was done according to the principle of synonymous mutation. The gene synthesis was done by Shenzhen Gene Technology Co., Ltd. (Huada, China).

Generation and identification of recombinant expression vectors. The synthetic gene MeP72 was ligated in to the vector pMD19-T (Takara, Japan) at 4°C overnight to gain recombinant plasmid pT-MeP72. Primer 6.0 software was used to design the following specific primers FP1-RP1, FP1: 5'-CGGAATTCATGGAAGAAACACATTTG-3'; RP1: 5'-CGCTCGAGTTAGTTAATAGCAGATGCC-3. Using pT-MeP72 as a template, PCR of MeP72 gene was performed under the conditions: 95°C, 5 min; 94°C, 45 s; 58°C, 45 s; 72°C, 40 s; 30 cycles; and extension at 72°C, 10 min. The PCR product was double digested with *Eco*RI and *Xho*I enzymes, and the target gene fragment was recovered and ligated in to the expression vector pET-32a (+) (Invitrogen, USA) with T4 DNA ligase (Takara, Japan) at 4°C overnight. The recombinant expression plasmid pET-P72 was screened by PCR and double-enzyme digestion.

Preparation and purification of recombinant protein MeP72. The constructed recombinant expression plasmid pET-P72 was transformed into competent *E. coli* BL21(DE3) cells (Invitrogen, USA), and positive transformants were selected and incubated on rotation shaker overnight at 37°C at 180 rcf/min. When OD600 nm was about 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG; Takara, Japan) at a final concentration of 1.0 mmol/l was added

Table 1. List of dominant linear epitope sequences selected from P72 protein

Epitope name	Sequence of dominant linear epitope	Position in *P72* gene	Length
EP1	EETHLVHFNAHFKPY	157–201	15
EP2	PRNGYDWDNQTPL	412–450	13
EP3	LYENVRFDVNGNSLDEYSSDVTTLVRKFCIP	559–651	31
EP4	KPHQSKPILTDENDTQR	748–798	17
EP5	ITPITDATYLDIRRNVH	883–933	17
EP6	KFGHVVNA	1465–1488	8
EP7	TTADLVVSASAIN	1855–1893	13

to induce gene expression. Recombinant bacteria that were induced for 0 h, 2 h, 4 h, 6 h, and 8 h were collected and analyzed by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE). ASFV-positive serum was used as the primary antibody and HRP-labeled rabbit anti-pig IgG (Abnova, China) was used as the secondary antibody. Western blot was performed to analyze the reactivity of reMeP72. The expressed reMeP72 protein was purified according to the instruction manual of Ni-NTA Spin kit (Qiagen, Germany).

Development of colloidal gold immunochromatographic assay. The structure of the colloidal gold-based immunochromatographic assay (CGIA) strip is shown in the Fig. 2. The treated (immersed in 0.01M PBS buffer pH 7.2 containing 5 % BSA, and then dried at 37 °C) nitrocellulose (NC) membrane (Sigma, USA) was affixed to a PVC board (Abnova, China). One end of the NC membrane was affixed with a gold label pad and a sample pad while the other end was affixed with a water absorption pad. The test line (T-line) was coated with reMeP72 protein at 0.5 mg/ml, 0.75 mg /ml, 1 mg/ml, 1.25 mg/ml, 1.5 mg/ml, 1.75 mg/ml, and 2 mg/ml. On the quality control line (C-line), 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 1.25 mg/ml, 1.5 mg/ml, 1.75 mg/ ml, and 2 mg/ml of goat anti-pig IgG were applied. ASFV standard positive serum was added to the test strip, and incubated for 10 min. The clearest color and the lowest protein concentration were observed and taken as the optimal T-line and C-line antigen coating concentrations. Each experiment was repeated 3 times.

Assessment of CGIA specificity, sensitivity and agreement. The established CGIA method was used to detect the positive sera against classical swine fever virus (CSFV), porcine circovirus type 2 (PCV2), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV) and swine influenza virus (SIV), and its detection performance was evaluated with ASFV-positive serum at 1:4, 1: 8, 1:16, 1:32, 1:64, 1:128, 1:256-fold dilutions. The CGIA and commercial ASFV indirect ELISA kit (INGENASA, Spain) were tested simultaneously with 139 pig clinical serum samples. The tested sera were collected from commercial pig farms with outbreak records of ASF in Xinjiang province. The detection results of the two methods were compared, and the specificity, sensitivity and agreement were analyzed.

Results

The dominant antigenic epitopes of P72 protein were predicted, and a MeP72 multi-epitope fusion gene was successfully generated. Then, the *E. coli* rare codons in the fusion gene MeP72 were replaced by favored codon and further confirmed by sequencing (Fig. 1). The recombinant plasmid pET-MeP72 was double digested with *Eco*RI and *Xho*I, and a 402 bp target fragment was obtained, indicating that the pET-MeP72 was successfully generated (Supplemental Fig. S2).

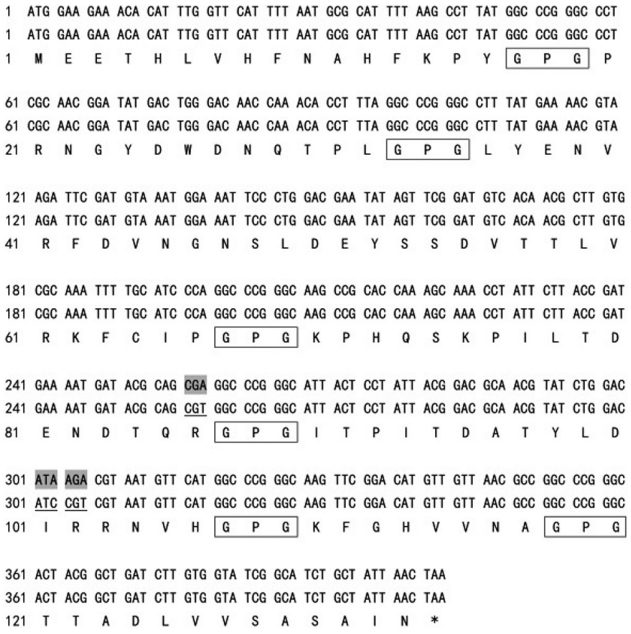

Fig. 1

The original and optimized nucleotide sequence of *MeP72* gene and its encoding amino acid sequence

Line 1: Original nucleotide sequence of *MeP72* gene; Line 2: Optimized nucleotide sequence of *MeP72* gene; Line 3: Amino acid sequence of MeP72 protein. Note: Flexible peptides are boxed. Rare codons in *E. coli* are shadowed, while preference codons are underlined.

SDS-PAGE results showed that the reMeP72 protein was displayed as a band at 32.74 kDa, as expected, indicating that reMeP72 is correctly expressed in *E. coli*. The highest expression level was at 8 hours after induction with IPTG (Supplemental Fig. S3). Western blot analysis showed that the expressed reMeP72 protein was specifically recognized by ASFV-positive sera (Supplemental Fig. S3), indicating its good reactogenicity.

A CGIA method for the detection of ASFV antibodies was established using the purified reMeP72 (Fig. 2). The results of the optimization test of CGIA reaction conditions showed that the T-line band was the clearest when the coating concentration of reMeP72 protein was 1 mg/ml, and the C-line band was the clearest when the goat anti-pig IgG concentration was 1.25 mg / ml, so the above parameters were used to establish the CGIA method.

CGIA test results showed that CGIA established with reMeP72 did not react with the positive sera of CSFV, PCV2, PRV, PRRSV, and SIV (Fig. 3), indicating that the established CGIA is highly specific. When testing multiple dilutions of ASF-positive sera by CGIA the results showed that the best dilution of positive sera was with a dilution factor of 1:64 (Fig. 4). This indicated that the CGIA detection method is highly sensitive.

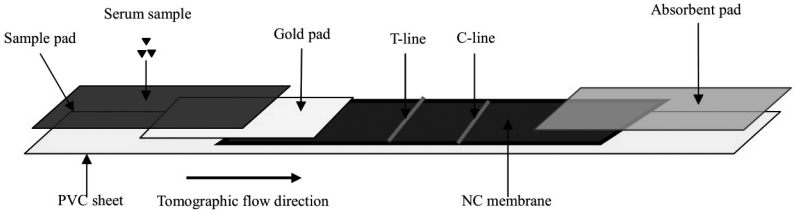

Fig. 2

Schematic representation of the structure of CGIA strip for the detection of anti-ASFV antibody

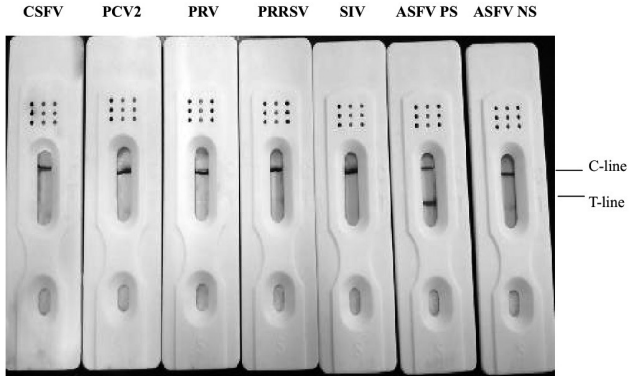

Fig. 3

Evaluation of the performance of CGIA in sera samples
Note: CSFV, classical swine fever virus; PCV2, porcine circovirus type 2; PRV, pseudorabies virus; PRRSV, porcine reproductive and respiratory syndrome virus; SIV, swine influenza virus; PS, positive sera; NS, negative sera; C-line, control line; T-line, test line.

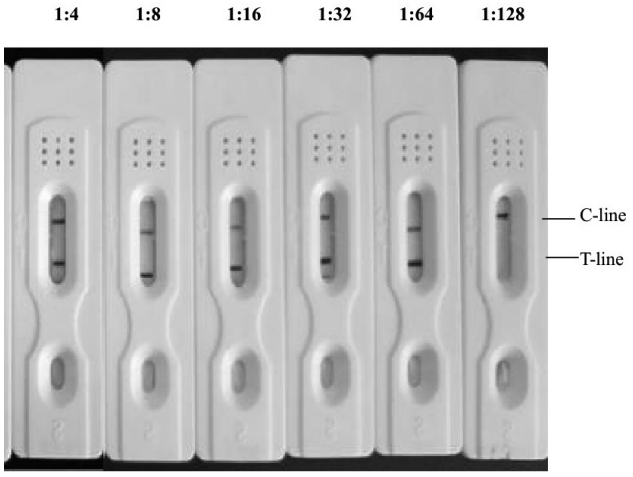

Fig. 4

Detection of the anti-ASFV antibodies at different dilutions of positive sera by CGIA
Note: C-line, control line; T-line, test line.

Comparing the commercial ASFV ELISA kit, the sensitivity and specificity of the CGIA method were 85.7% and 97.6%, respectively, and the agreement rate of these two methods was 96.4%, indicating that the CGIA method exhibits a quite good detection performance (Table 2).

Discussion

With the continuous outbreak of the global ASF epidemic, serological diagnosis has become an important technical guarantee for the prevention and control of the ASF epidemic (Pastor *et al*., 1992; Gallardo *et al*., 2009; Cubillos *et al*., 2013; Bellini *et al*., 2016; Sastre *et al*., 2016; Carlson *et al*., 2018). Currently, the conventional serological detection methods of ASFV include indirect immunofluorescence test and ELISA methods (Pan *et al*., 1982; Giménez-Lirola *et al*., 2016; Bergeron *et al*., 2017). The immunofluorescence test has the advantage of high sensitivity and rapid speed, but it is difficult to achieve large-scale detection of samples (Pan *et al*., 1982). ELISA method is a widely used serological detection method, which has the advantage of fast, simple and easy to standardize the carrier (Hamdy *et al*., 1981). The method can be used for detection of specific ASFV antibodies or antigens in the serum samples. The commonly used ELISA methods include indirect ELISA, competitive ELISA and double-antibody sandwich ELISA (Bergeron *et al*., 2017). However, the imported ELISA kits are expensive and

Table 2. Comparison of results between CGIA and ASFV indirect-ELISA kit for the detection of ASFV antibodies

		ASFV indirect ELISA kit		
		Positive	**Negative**	**Total**
CGIA	Positive	12	3	15
	Negative	2	122	124
	Total	14	125	139

require certain equipment and professional operations, which limit their use in grassroots units.

The currently existing researches have shown that the ASFV genome contains 151 to 167 open reading frames (ORFs), which can encode more than 150 proteins (Galindo and Alonso, 2017). Among them, ASFV P72, P54, and P30 proteins constitute the structural proteins of ASFV virus particles, which play an important role in infecting host cells (Barderas *et al*., 2000; Neilan *et al*., 2004; Cubillos *et al*., 2013; Giménez-Lirola *et al*., 2016). Furthermore, the above proteins have strong antigenicity and are important target antigen molecules that can be used for the development of ASF-specific diagnostic methods (Gallardo *et al*., 2009). P72 protein is produced in the late stage of viral infection and is encoded by the ORF B646L gene (Galindo and Alonso, 2017). Sastre *et al*. (2016) used the P72 protein to establish a dual lateral flow assay for simultaneous detection of antibodies against ASFV and CSFV with good sensitivity and specificity. Specific monoclonal antibody against the p72 protein for detection of viral antigens is used in the INGENASA sandwich ELISA kit developed in Spain (Heimerman *et al*., 2018). It is designated by the OIE as the reference ASFV diagnostic kit and has good specificity and sensitivity.

The development of rapid and specific diagnostic methods with high specificity and sensitivity is of great significance for the prevention and control of the ASF epidemic (Giménez-Lirola *et al*., 2016; Arias *et al*., 2018; Carlson *et al*., 2018). As a fast, simple and intuitive detection method, colloidal gold-based immunochromatographic assay (CGIA) has been used for the serological diagnosis of many animal infectious diseases (Nurulfiza *et al*., 2011; Lu *et al*., 2015; Sastre *et al*., 2016). Given the large molecular weight of P72 protein, it is difficult to prepare a complete P72 recombinant protein. Here, the MeP72 multi-epitope fusion gene was expressed in *E. coli*. The reMeP72 recombinant protein was used in CGIA method for detecting ASFV antibodies. Compared with those of the commercial ASFV ELISA detection kit, the established CGIA method displayed higher specificity and sensitivity in the clinical sera. These two methods have a high coincidence rate, suggesting that reMeP72 is a potential antigen for the serodiagnosis of ASF.

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Supplemenraty information is available in the online version of the paper.

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