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DEVELOPMENT OF FLUORESCENCE IMMUNOCHROMATOGRAPHIC ASSAY KIT FOR AFRICAN SWINE FEVER VIRUS FAST TESTING

Summary

The continuing spread of African swine fever (ASF) outside Africa in the whole world, has heightened awareness of the threat posed by this devastating disease to the global pig industry and food security. Fast and accurate African swine fever virus (ASFV) detection method is very important for ASF prevention. A double antibody sandwich FICA method was optimized and established, and FICA strips (FICAS) were assembled and then evaluated. The sensitivity of the FICAS kit was 0,044 ng/mL. The recoveries ranged from 102,07 % to 108,60 %, and all CVs were below 7 %. The common pigs infectious disease samples, positive samples and healthy control samples did not lead to false negative and false positives. The kit can be stably stored at 37 °C for 7 days without significant decrease in fluorescence value.

The prepared FICAS kit is rapid, feasible and effective for testing ASFV within 15 min. This study provides a new method for rapidly screening ASFV infection in pigs industry.

Keywords: *african swine fever virus, fluorescence immunochromatographic assay, fast testing, kit.*

Резюме

Продолжающееся распространение африканской чумы свиней (АЧС) за пределами Африки во всем мире повысило осведомленность об угрозе, которую представляет это разрушительное заболевание для мировой свиноводческой отрасли и продовольственной безопасности. Быстрый и точный метод обнаружения вируса африканской чумы свиней очень важен для профилактики болезни. Метод FICA с двойным сэндвичем антител был оптимизирован и внедрен, а полоски FICA (FICAS) были собраны и затем оценены. Чувствительность набора FICAS составила 0,044 нг/мл. Возвраты варьировались от 102,07 % до 108,60 %, а все CV были ниже 7 %. Обычные образцы инфекционных заболеваний свиней, положительные образцы и здоровые контрольные образцы не привели к ложноотрицательным и ложноположительным результатам. Набор можно стабильно хранить при температуре 37 °C в течение 7 дней без существенного снижения значения флуоресценции.

Подготовленный набор FICAS является быстрым, осуществимым и эффективным способом для тестирования вируса АЧС в течение 15 минут. Это исследование предлагает новый метод быстрого скрининга инфекции вируса АЧС в свиноводстве.

Ключевые слова: *вирус африканской чумы свиней, флуоресцентный иммунохроматографический анализ, быстрое тестирование, набор.*

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INTRODUCTION

African swine fever virus (ASFV) is a double stranded DNA virus, can cause approaching 100 % mortality in domestic pigs, severely threatening the global pig industry [1]. ASFV infection of domestic pigs leads to a lethal hemorrhagic fever, leading to death and virus transmission [2]. This swine disease caused by ASFV infection is called African swine fever (ASF). To date, ASFV has spread throughout Africa, Asia and Europe [3]. As there is no effective vaccine, surveillance and diagnosis play key roles in ASFV control. However, current technique for the surveillance and diagnosis of ASFV is insufficient and inconvenient. So far, PCR and ELISA are the most commonly used testing method in clinical practice, and more convenient and fast immunological methods are urgently needed [4, 5].

Fluorescence immunochromatographic assay (FICA) is regarded as a promising diagnostic tool for fast testing, and it has been widely used in the detection of pathogens, pesticide residue, biomarkers, etc [6–8]. FICA has many advantages, including rapid test procedure, convenient operational equipment, and low cost testing. In this study, we aims to establish a fast detection method for ASFV antigen using FICA method. At present, we have already prepared P54 recombinant antigen and its paired monoclonal antibodies (MAb) [9]. Using these validated antigen and antibodies, we prepared a fast and accurate FICA kit, meanwhile evaluated its sensitivity, accuracy, specificity and stability.

This study aims to establish a fast detection method for ASFV antigen using fluorescence immunochromatographic assay (FICA).

MATERIALS AND METHODS

Antigen, antibody, reagents and clinical samples

P54 recombinant antigen (*Escherichia coli*) and its paired MAb (3E2 and 4B2) of ASFV were obtained from Guangzhou Youdi Biotechnology Co., Ltd. Europium (III) (Eu^{3+}) labeling kit was purchased from PerkinElmer (Norwalk, USA). The buffers used in this study are all self-prepared. 23 clinical nasal discharge samples with ASFV positive, and 100 healthy ral/nasopharyngeal swab samples came from South China Agricultural University. The positive samples of transmissible gastroenteritis virus (TGEV, 6 cases), porcine respiratory coronavirus (PRCV, 8 cases), porcine hemagglutinating encephalomyelitis virus (PHEV, 12 cases), and porcine circovirus (PCV, 10 cases) also came from South China Agricultural University. All samples were stored at -80 °C .

Conjugation of 3E2 antibodies to fluorescent microspheres

The Eu^{3+} fluorescent microspheres were activated by the classical EDC/NHS (1-(3-Dimethylaminyl)-3-ethylenediamine hydrochloride/N-hydroxysuccinimide) method and then coupled with 3E2 antibodies. After centrifugal washing, activated fluorescent microspheres were added into 3E2 antibodies (1 mg), and gently shaken at room temperature for 3 h. Then, blocked (5 % BSA, 1h, at room temperature) the fluorescent microspheres, resuspended them in buffer (25 mmol/L Tris-base, 1 % BSA, 0,05 % Tween 20, pH 7.5), and stored in the dark at 4 °C . Conjugation of DNP-BSA to fluorescent microspheres was also carried out according to the above steps.

Coating of NC membrane

4B2 antibodies were coated in Test line (T line), and DNP antibodies were coated in control line (C line). Coating was performed by the XYZ3060 3D spraying platform at a spraying speed of 1,0 μL/cm, and at the concentration of 1 mg/mL and 0,8 mg/mL. After coating, NC membranes were dried in a 37 °C air drying oven for 2 h, and then used for the preparing of FICAS kit.

Preparing of sample pads and bonding pads

3E2 antibodies-fluorescent microspheres conjugates and DNP-BSA-fluorescent microspheres conjugates were dissolved in bonding pads buffer (60 mmol/L $Na_2HPO_412H_2O$, 1 % Trition-X100, 0,5 % polyvinyl alcohol, pH 7.4), and then simultaneously coated onto the bonding pads through soaking. After draining, cut them into 6 mm wide strips for the preparing of FICAS kit. The sample pads were soaked in a buffer (20 mmol/L sodium tetraboric acid, 0,2 % sodium casein, 1 % Trition-X100, 0,5 % polyvinyl pyrrolidone, 0,1 % ProClin 300, pH 7.8) for 1 h. After draining, cut them into 18 mm wide strips for the preparing of FICAS kit.

Preparing of FICAS kit

The FICAS consist of 5 component: PVC baseboard, sample pad, bonding pad, NC membrane, and absorbent paper. After pre-treatment, the sample pad, bonding pad, NC membrane and absorbent paper (16 mm) are sequentially fixed on the PVC baseboard, and then the whole board was cut into 3 mm wide strips. The strips were slotted into the plastic cards, sealed and stored in a dark bag. These strips, along with the sample buffer and FICA analyzer, constitute the kit.

Test procedure

After optimization, the test procedure was finally established: Sample dissolved into 100 μL sample buffer, and dropped into the sample well, and then inserts the strip into a FICA analyzer (Youdi, #YDT023). After 15 min, the analyzer will automatically perform the test and calculate the concentration of ASFV antigen, which was calculated using the built-in standard curve (Figure 1).

Figure 1 – FICAS kit component and Schematic diagram of test procedure

Reference interval

A total of 100 healthy control samples were used to determinate the reference interval. FICTS kit tested the above samples, and one-sided upper limit of the 95 % reference interval range was calculated using the following formula: mean + 1,64SD.

Sensitivity

The FICAS to detect the serial dilutions of P54 antigen (0, 0,1, 1, 10, 100 and 500 ng/mL), and the concentrations of P54 antigen were plotted as X axis, the ratio of T line' fluorescence values/C line's fluorescence values as the Y axis, performed a linear fit and obtained the standard curve. Three replicates in each concentration. 0 ng/mL was used as a sample for 20 tests, and the 20 T/C values obtained were substituted into the standard curve to calculate the mean and SD. The sensitivity of this FICAS kit is (mean+ 2*SD).

Accuracy assay

Dilute P54 antigen to 1 ng/mL, 10 ng/mL, and 500 ng/mL using healthy control swab samples, and test their concentrations using the FICAS kits. Coefficient of variation (CV, SD/mean×100 %) and recovery ((determined concentration-basal concentration)/spiked concentration×100 %) were calculated to evaluate accuracy. Five replicates in each test.

Specificity assay

The positive samples of TGEV, PRCV, PHEV and PCV, 23 positive samples, 100 healthy control samples, as the samples, were tested using these FICAS kits. According to the reference interval, we determined the negative or positive of these samples and finally obtained the specificity results.

Stability assay

The FICAS and sample buffer were stored at 37 °C for 7 consecutive days. The high, medium and low concentration P54 antigen (1 ng/mL, 100 ng/mL, and 500 ng/mL) as the samples to performed the daily testing, recorded their fluorescence values, and plotted the curves to evaluate the stability of FICAS kit.

Statistical methods

Data were statistically analyzed using SPSS 19.0. All date is shown as the mean ± SD or mean. Standard curve was plotted using GraphPad Prism 5 (GraphPad Software, USA).

RESULTS AND DISCUSSION

Standard curve and sensitivity

The standard curve of the FICAS kit is shown in Figure 2. Curves equation is: y=0,1407x + 0,6218 (R^2 = 0,9964), and the curve exhibit a well-defined linear relationships in the 0–500 ng/mL concentration ranges. The calculated sensitivity of the FICAS kit was 0,044 ng/mL.

Reference interval

Normality test confirmed that 100 values belong to normality distribution, and the one-sided upper limit of 95 % reference interval range is 0,051 ng/mL (calculated using: mean + 1,64*SD*), indicating when the sample concentration measured using this FICTS kit is greater than 0,051 ng/mL, it may be ASFV positive, and when it is below 0,051 ng/mL, it may be ASFV negative.

Accuracy assay

The accuracy of the FICAS kit was evaluated by detecting the healthy control sample that had been added with P54 antigen (1, 10 and 500 ng/mL). As listed in Table 1, the recoveries of three concentrations ranged from 102,07 % to 108,60 %, and all *CVs* were below 7 %. The accuracy results indicated that the accuracy of the FICAS kit was high.

Table 1 – Accuracy results of the FICAS kit

Added concentration (ng/mL)	mean±SD	Recovery (%)	CV (%)
1	1,11±0,070	108,60	6,35
10	10,49±0,33	104,72	3,10
500	510,36±4,48	102,07	0,88

Specificity results

Specificity results are presented in Table 2, indicating the common pigs infectious disease samples do not lead to cross-reactivity.

And, 23 positive samples and 100 healthy control samples also did not lead to false negative and false positives. Specificity results means that the FICAS kit had high specificity to ASFV.

Table 2 – Specificity results of the FICAS kit

Interferents	n	Detected concentration	P/N
ASFV	23	43,88±29,53	All P
TGEV	6	0,028±0,012	All N
PRCV	8	0,040±0,0081	All N
PHEV	12	0,030±0,010	All N
PCV	0	0,026±0,0077	All N
Healthy control	100	0,03±0,013	All N

Stability assay

After 7 consecutive days of 37 °C storage, the T line'- and C line' fluorescence values of the the high, medium and low concentration P54 antigen (1 ng/mL, 100 ng/mL, and 500 ng/mL) (Figure 3) has no significantly changes, and their ratio T/C has no markedly changes, indicating that the stability of the FICAS kit is good, the expiration date of this kit is more than 12 months at room temperature.

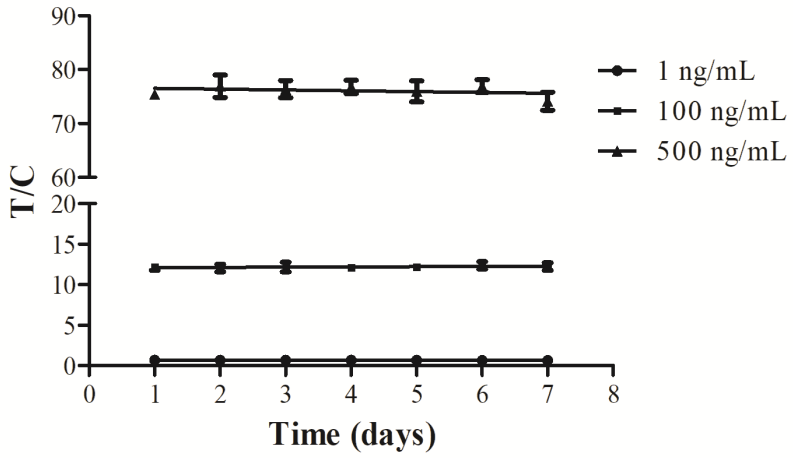

Figure 3 – Stability results of the FICAS kit (7 days, 37 °C). T/C: fluorescence values of T line/fluorescence values of C line

ASF, as a highly contagious disease of pigs, its transmission speed is very fast, so daily monitoring of pig farms at the grassroots level is very necessary. Immunological testing is more suitable for daily monitoring in pig farms due to its simplicity and rapidity. In addition to ELISA method, some new detection technologies are being developed [5, 10, 11]. Li C et al. established a reliable and sensitive suspension microarray technology-based multiplexing method for ASFV antibody detection with a 16-fold improvement in detection sensitivity compared to commercial ELISA kits [5]. Li C et al. prepared a FICTS using the truncated P54 protein as an antigen and Eu-doped fluorescent microspheres as tracers to specifically detect anti-ASFV antibodies [11]. Currently, most commonly used commercial immunological methods are used to detect ASFV antibodies [4, 5, 12]. Differently, our FICAS kit directly detects ASFV pathogens. In this study, we established a double antibody sandwich FICA method and assembled FICAS kit to detect the ASFV pathogen. This rapid, feasible and effective FICAS kit provides more options for rapidly screening ASFV infection in pigs industry.

P54 protein is a main structural protein of ASFV, a 25-kDa polypeptide encoded by the E183L gene. P54 protein is main binding site of serum antibodies and has good immunogenicity. Importantly, antibodies against P54 protein can be detectable early after ASFV infection of domestic pigs [13]. Gallardo C et al. found recombinant ASFV protein P54 is a sensitive and specific target for the detection of antibodies in European and West African domestic pigs and warthogs [14]. Cao Y et al. prepared a strain of mAb against the P54 protein, which could successfully recognize the exogenously expressed P54 protein and the chimeric virus constructed in our laboratory [15]. A FICTS using the truncated P54 protein as an antigen and commercial ELISA kits showed high consistency to specifically detect anti-ASFV antibodies [11]. Similarly, we have also prepared recombinant P54 protein and its mAb, which has been used for the detection of anti-ASFV antibodies [9]. In our this further study, we prepared a FICAS kit using P54 protein and its mAb to detect the ASFV pathogen, which has high sensitivity, accuracy, specificity and stability, and there was a high degree of specificity/sensitivity with 23 positive samples and 100 healthy control samples.

The performance of the prepared fluorescence probes plays an important role in the sensitivity and stability of the FICA system. Eu^{3+}-microspheres are one of the most commonly used fluorescence microspheres in FICA system [16, 17]. The surface of the microspheres is modified with carboxyl groups (- COOH) or streptavidin (- SA), making the formed conjugates more stable [18]. Eu^{3+} nanoparticles-based FICAS has high sensitivity and stability. The FICAS was stable and could be stored for up to one year at room temperature [19]. Research has shown that combining a europium-based ELISA can be applicable to achieve high specificity and sensitivity in an ELISA format [20, 21]. In this study, we chose Eu^{3+} label-microspheres as the fluorescence probes, and also established a high sensitivity, accuracy, specificity and stability FICA method. Our FICAS kit realized the quantitative analysis of ASFV antigen within 15 min, the sensitivity were 0,044 ng/mL with strong specificity, the expiration date of this kit was more than 12 months at room temperature, all recoveries ranged from 102,07 % to 108,60 %, and all *CVs* were below 7 %. Compared with the sensitivity, specificity, precision of reported time-resolved fluorescence immunoassay method (sensitivity 0.015mg/mL, recovery 92,00 to 103,62 %, CV 5,20 to 11,96 %, detection time 45 min) [22], the FICTS exhibited a comparable detection performance with the additional advantages of the detection time (15 min). Therefore, this Eu^{3+} nanoparticles-based FICAS can be a rapid, feasible and effective method in ASFV screening.

In conclusion, the prepared FICAS kit is rapid, feasible and effective for testing ASFV within 15 min, which may play a great role in serological diagnosis and epidemiological monitoring of ASF in the future. The establishment of this FICA method foreshadows FICA has great implementation promise in detecting animal infectious diseases.

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