

Rapid Communication
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A rapid and quantitative fluorescent microsphere immunochromatographic strip test for detection of antibodies to porcine reproductive and respiratory syndrome virus

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ABSTRACT

A fluorescent microsphere-based immunochromatographic strip test (FICT) was developed for the rapid, sensitive, and quantitative detection of porcine reproductive and respiratory syndrome virus (PRRSV) antibodies at the pen-side. The assay was based on the formation of a sandwich immune-complex (anti-pig IgG-PRRSV antibodies-NSP7/N), which was validated by a comparison with IDEXX-ELISA using 3325 clinical specimens. The diagnostic specificity, sensitivity, and accuracy of FICT were 97.28, 93.41, and 94.95%, respectively. FICT showed a good correlation with the virus neutralization assay. Overall, a promising pen-side diagnostic tool was developed for the rapid and quantitative detection of PRRSV antibodies within 15 min.

Keywords: Fluorescence; immunochromatographic assay; PRRSV; immunochromatography; pen-side detection

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is one of the most devastating swine diseases worldwide, causing reproductive failure in pregnant sows, respiratory distress in populations of piglets and growing pigs, and a delayed and inadequate adaptive immune response. In China, the PRRSV has been reported since 1995, with CH-la isolated from an aborted embryo of a sow in 1996 [1]. The virus has since spread rapidly through China and on other continents around the world [2]. An outbreak of highly pathogenic PRRS (HP-PRRS) occurred in China in 2006 [3], which was characterized by severe symptoms (fever, asitia, dyspnea, 50%–100% illness rates, and 20%–100% death rates) that distinguished it from other diseases. The PRRSV has two lineages (European and North American) according to nucleotide sequence analysis [4], and most isolated PRRSVs in China belong to the North American genotype. The PRRSV is a cause of severe economic loss to the pig farming

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Yang L, Chen C; Data curation: Duan Y; Methodology: Wei Y; Project administration: Yang B, Li Y; Supervision: He B; Validation: Tian D; Writing - original draft: Wei Y, Yang B; Writing - review & editing: Yang L, Liu W.

industry [5,6], which highlights the need for rapid and sensitive diagnostic tools that can be implemented pen-side and distributed to various pandemic regions.

Several diagnostic methods have been used to assess PRRSV antibodies, including virus neutralization (VN) assays [5], immunoperoxidase monolayer assays, immunofluorescence assays, multiplexed fluorescent microsphere immunoassay [7], and enzyme-linked immunosorbent assays (ELISA) [8,9]. These assays, however, require expensive instrumentation, well-trained technicians, and are time-consuming [10,11]. In addition, they cannot be deployed outside laboratories. In recent years, the lateral flow immunoassay (LFIA) has been applied in clinical medicine and can quickly detect infectious diseases of humans and animals, including PRRSV [12]. The most common type of LFIA is gold nanoparticle-based LFIA, but this can only be used for qualitative detection, and the sensitivity is unsatisfactory. In recent years, a variety of different labels, including fluorescein, quantum dots, nanozyme, and colloidal carbon, have been used for LFIA to develop more sensitive and quantitative detection assays. Several innovative LFIAs have been used to detect a wide range of pathogens, such as influenza virus [13], plasmodium falciparum [14], Ebola virus [15,16], and Peste des petits ruminants virus [17]. These LFIAs have shown good sensitivity and quantitative characteristics. To establish a sensitive and quantitative pen-side technology to detect PRRSV antibodies, an innovative lateral flow chromatography strip was developed using fluorescent microspheres as a labeling probe instead of gold particles. The system was found to be more suitable for the efficient detection of antibodies against the PRRSV outside laboratories.

MATERIALS AND METHODS

Recombinant antigens purification and characterization

PRRSV nucleocapsid protein (N) and nonstructural protein 7 (NSP7) have conserved and divergent epitopes that are recognized by virus-specific antibodies. Therefore, they have been used as diagnostic antigen candidates for the detection of virus-specific antibodies and disease diagnosis [8,18,19]. In this study, recombinant PRRSV NSP7 and N proteins were expressed and purified by cloning the NSP7 or N gene of a North American PRRSV strain (GenBank: EF536003.1) into the pET28a vector (Novagen, USA) and transforming it to *Escherichia coli*. Briefly, *E. coli* cells harboring the PRRSV-NSP7 or PRRSV-N expression plasmid were induced by isopropyl- β -D-thiogalactopyranoside (IPTG), followed by growth at 37°C for 6 h. The over-expressed recombinant protein was then obtained from an AKTA prime plus system equipped with a HisTrap HP column (GE Healthcare, Sweden) for affinity chromatography, eluted at a gradient from 50 to 300 mM imidazole, and dialyzed against a PBS buffer. The purified rNSP7 and rN proteins were identified by SDS-PAGE and WB (Western blotting). WB analysis was performed using an anti-His-tag antibody, followed by blotting with HRP-conjugated secondary antibody and detection by chemiluminescence.

Preparation of fluorescent microsphere probe

Fluorescent microsphere-labeled goat anti-pig IgG was prepared and concentrated in the conjugate pad to assemble the test strips. Briefly, 100 μ L of a 300 nm diameter fluorescent microsphere suspension (Nanodot, China) was dissolved in 500 μ L of BS-T2 (25 mM boric acid and 0.15% Tween-20, pH 8.5) and centrifuged. Microspheres were resuspended by the addition of 300 μ L BS-T1 (25 mM boric acid and 0.25% Tween-20, pH 8.5). Subsequently, 10 μ L of 2 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) (Thermo, China) and 10 μ L of 4 mg/mL N-hydroxysuccinimide (NHS) (Solarbio, China) were resuspended,

mixed, and incubated on a rotating reaction frame in the dark for 15 min. Microspheres were resuspended twice in 500 μ L of BS-T1, centrifuged, and resuspended in 500 μ L BS-T2. Subsequently, 100 μ g of goat anti-pig IgG (Solarbio) was added and mixed on the rotating reaction frame for 2 h and rotated twice with 15 μ L of 9% casein for 30 min. Finally, the precipitated bioconjugates were resuspended in BS-T1 and stored at 4°C.

FICT-strip assemble

The sample pad and conjugate pad were saturated with Tris-HCl buffer 1 (0.02 M Tris-HCl, 1% Triton X-100, 0.3% BSA, pH8.0) and Tris-HCl buffer 2 (0.02 M Tris-HCl, 0.3% BSA, and 0.2% Tween-20, pH8.0), respectively, and air-dried for 2 h. The conjugate pad was then coated with a fluorescent antibody (30 μ L/cm), cut into 300 \times 20 mm pieces, and stored at room temperature. The purified rNSP7 and rN proteins were mixed at a molar ratio of 1:2 and diluted with Tris-HCl to 1.5 mg/mL as the test line (T). Rabbit anti-goat IgG was used at 2 mg/mL as the control line (C). The test line and control line were then dispensed (2 μ L/cm) along a nitrocellulose membrane (Millipore, USA) sheet pasted to a backing card using an HM3030 Draw membrane dispenser (Kinbio, China). A membrane strip was arranged, from left to right, with a sample pad, conjugate pad, nitrocellulose membrane, and absorbent pad pasted onto a 300 \times 80 mm backing card. The card was cut longitudinally, divided into 4.0 \times 80 mm strips using a strip cutter, and stored in sealed bags under dry conditions at room temperature.

FICT-strip test

Samples (80 μ L) were added to the sample pad for the reaction over 15 min. The test strip was then inserted into a portable fluorescence immunoassay analyzer (Helmen, China) with excitation and emission at 365 and 615 nm, respectively, and the result was quantified using a fluorescence immunoassay analyzer (**Fig. 1A**). The test results could also be transmitted to the remote terminal through the built-in network module to allow the storage and analysis of data. The test results of the FICT strips could be visualized under UV light by the naked eye (**Fig. 1B**).

RESULTS

Preparation of diagnostic antigens

E. coli cells harboring the NSP7 or N expression plasmid were induced with IPTG, and the subsequent growth media was purified using a HisTrap FF 5-mL column. Finally, the rNSP7 and rN proteins were confirmed by SDS-PAGE and a WB assay (**Fig. 2**). The concentrations of the two proteins were determined using a Bradford assay with bovine serum albumin (BSA, Amresco, USA) as the standard and stored at -80°C.

Preparation of FICT-strip

Fluorescent microsphere-labeled goat anti-pig IgG was concentrated in the conjugate pad. The diagnostic antigens (rNSP7, rN) and rabbit anti-goat IgG were immobilized on a nitrocellulose membrane as test and control lines, respectively. The sample application pad, conjugate release pad, nitrocellulose membrane, and absorbent pad were assembled into a lateral flow test strip. To obtain the detection cutoff values, a commercial ELISA kit (IDEXX-ELISA PRRS) and FICT were used to detect 285 swine serum samples (China Animal Disease Prevention and Control Center, CDC) to acquire the P/N and T/C values. The data were analyzed using ROCR (v3.3.3) to obtain positive predictive values as the Y-axis, and false-positive rates as the X-axis. The receiver operating characteristic (ROC) curves were then drawn. An AUC below the ROC curve was treated as the evaluation criteria. An improved

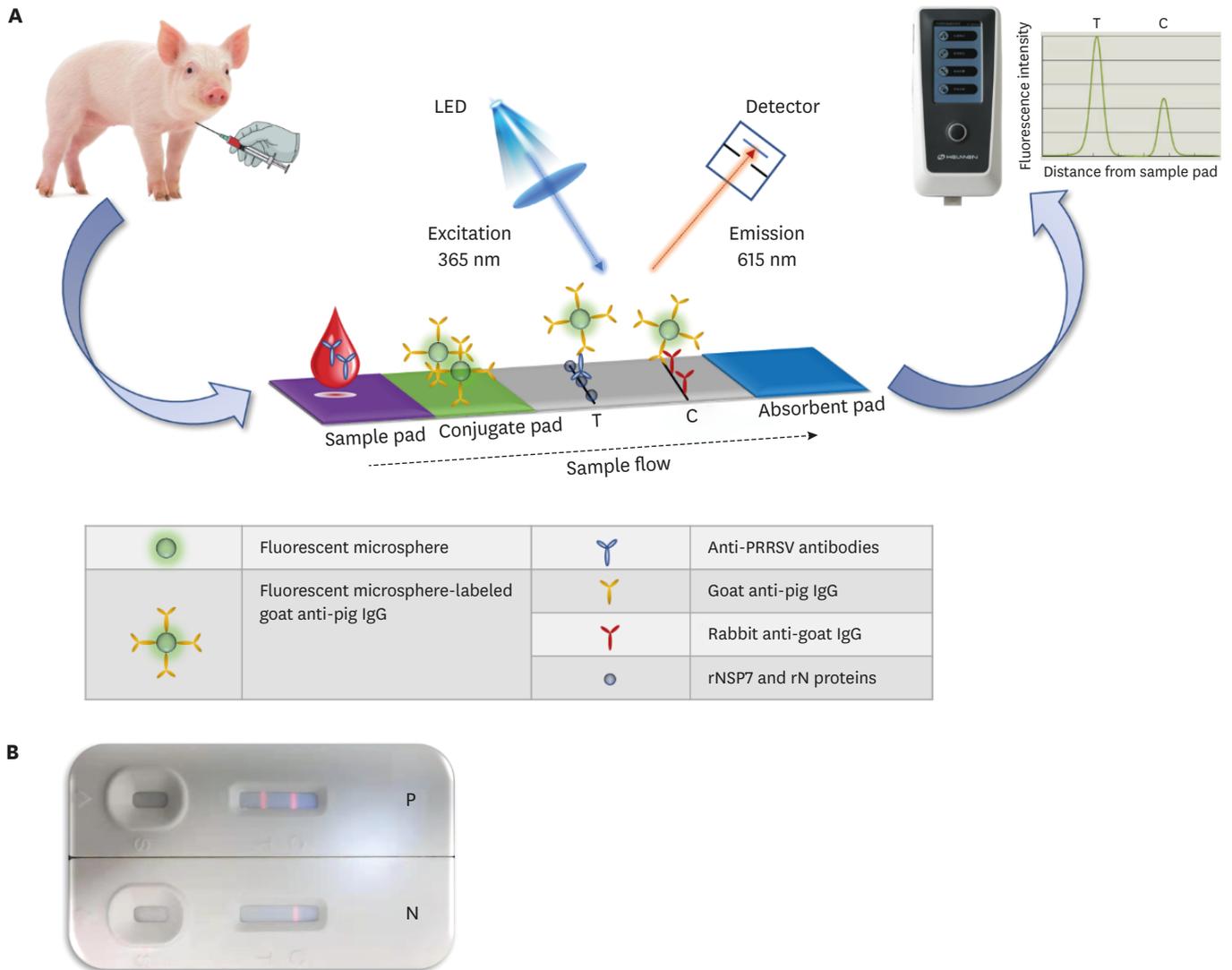


Fig. 1. Schematic illustration of the principle of FICT. (A) Schematic diagram of FICT. The pig serum sample was dropped onto the sample pad, and the fluorescent microsphere-labeled anti-pig IgG captured IgG in the serum. When the complexes migrated on the NC membrane, porcine reproductive and respiratory syndrome virus specific antibodies were captured by the rNSP7 and rN proteins immobilized on the test line, resulting in a fluorescent band (T line). The excess conjugates were captured by rabbit anti-goat IgG immobilized on the control line, resulting in the other fluorescent band (C line). After 15 min, the strip was used to detect the fluorescent light using a portable fluorescence immunoassay analyzer. (B) Positive and negative detection visualized under UV light by the naked-eye was achieved.

FICT, fluorescent microsphere-based immunochromatographic strip test; IgG, immunoglobulin G; NC, nitrocellulose.

diagnosis effect was noted when the AUC was close to one; the detection of this strip was 0.906, demonstrating high accuracy. When calculating the Youden index = sensitivity + specificity-1 (sensitivity: 87.879%, specificity: 85.621%), the corresponding T/C value (0.101) of the maximum Youden (0.735) was taken as the cutoff value. Therefore, T/C ratios ≥ 0.101 and ratios < 0.101 were considered positive and negative, respectively.

Sensitivity and specificity

The analytical sensitivity of the FICT assay was determined using two-fold serial dilutions of the standard reference serum (Institute of Veterinary Drugs Control, China) with a neutralizing antibody (NA) titer of 24. The NA titer is expressed as the reciprocal of the highest dilution ratio of serum that inhibited 90% virus replication, ranging from 24 to

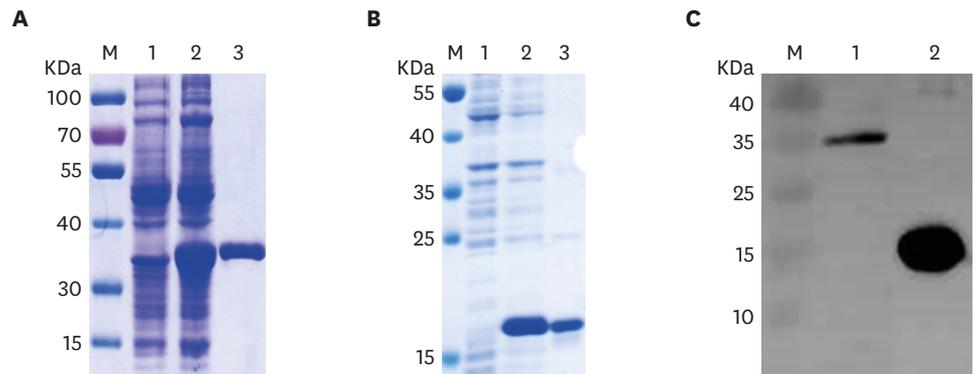


Fig. 2. Expression and characterization of PRRSV rNSP7 and rN proteins. (A, B) SDS-PAGE analysis of PRRSV protein expression. M: protein marker; lane 1 and 2: pET28a-PRRSV-NSP7 or pET28a-PRRSV-N bacteria lysates before and after induction; lane3: purified rNSP7 (33KD) or rN (17KD) proteins through NI-NTA affinity chromatography. (C) Western blot analysis of PRRSV proteins. M: protein marker; lane 1 and 2: purified rNSP7 and rN proteins immunoblotted using swine PRRSV-positive sera. PRRSV, porcine reproductive and respiratory syndrome virus.

0.75. IDEXX-ELISA was also used for parallel testing. Each relative T/C ratio was plotted as a calibration curve for the PRRSV antibody titers, in which the antibody titer can be obtained using a four-parameter sigmoidal (logistic) model in GraphPad Prism 7 ($y = (A-D)/[1 + (x/C)^B] + D$, $R^2 = 0.99875$, $A = 2.49356$, $B = -0.89421$, $C = 3.90963$, $D = -0.42843$). Excellent linearity was observed in the antibody titer range of 0.75–24 (**Fig. 3A**). Therefore, the fluorescent values of the standard clinical samples were incorporated into the standard curve, and the FICT could detect PRRSV antibody titers quantitatively. The detection limits of FICT and IDEXX-ELISA were 0.73 (cutoff: $T/C \geq 0.101$) and 1.75 (cutoff: $S/P \geq 0.4$), respectively. The sensitivity of the FICT assay was two times higher than the commercial IDEXX-ELISA kit. The specificity of the FICT was also examined using the antisera of other closely related porcine viruses, including classical swine fever virus, transmissible gastroenteritis virus, pseudorabies virus, and porcine circovirus 2). The results showed that the FICT could detect PRRSV-positive serum but had no cross-reaction with other antisera.

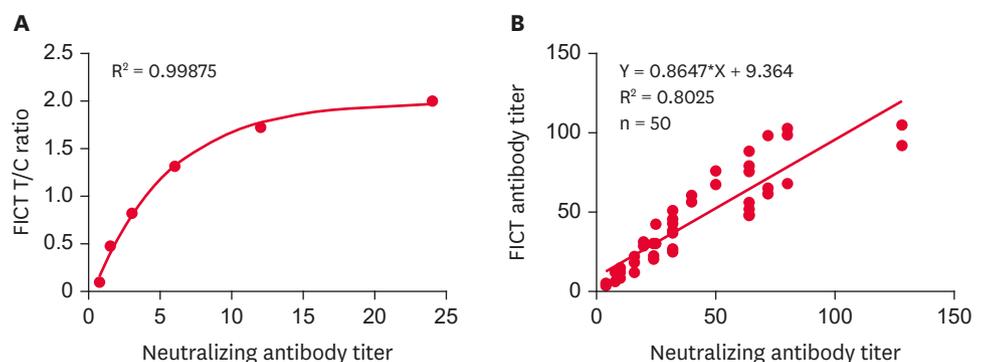


Fig. 3. FICT strip has a good correlation with the virus neutralization assay. (A) The standard curve. Serial dilutions of the PRRSV standard reference serum with an NA titer of 24 (24, 12, 6, 3, 1.5, and 0.75) were tested with FICT. With a four-parameter sigmoidal (logistic) model in GraphPad Prism 7, each relative value of the T/C ratio was plotted as a calibration curve for the PRRSV antibody titers. (B) Correlation between FICT and neutralization assay on 50 vaccinated serum samples. The T/C ratios of each serum sample tested by FICT were converted to antibody titers via a standard curve. The NA titers were expressed as the reciprocal of the highest dilution ratio of serum that inhibited 90% virus replication. The least-squares linear regression analysis was carried out, which showed a strong correlation between the two assays. FICT, fluorescent microsphere-based immunochromatographic strip test; PRRSV, porcine reproductive and respiratory syndrome virus.

Accuracy and stability

To evaluate the repeatability of FICT within and between runs, the T/C values of the three standard serum samples were detected with three batches of strips. Ten replicates for each sample were tested. The coefficients of variation (CV) were analyzed by $SD/\bar{X} \times 100\%$. The CV (%) values of the batches within and between were less than 14.2%, which met the requirement of precision. Accelerated stability tests of the FICT were also performed by storing the strips at 37°C for 15 days and examining the T/C ratio of a standard reference serum. The CV(%) was < 15%, and the sensitivity and specificity of FICT of the treated and untreated strips were similar, proving their excellent stability.

Good correlation between FITC and VN assay

To assess the correlation of the FICT and VN assay, 50 positive sera (Herds were vaccinated twice with the live attenuated vaccine at a four-week interval, and the sera were collected after 14 days of the second immunization) were tested in parallel using these two assays. The VN assay was performed on MARC-145 cells and PRRSV strain VR-2332, as described previously [20], and the NA titers were expressed as the reciprocal of the highest dilution ratio of serum that inhibited 90% virus replication. The FICT test results were converted to antibody titers using the standard curve. The results were then compared using least-squares linear regression analysis. The correlation coefficient between the two assays was 0.80. **Fig. 3B** presents a scatter diagram of FICT and NA titers. Therefore, FICT is promising for evaluating the immunity of vaccinated herds at the pen-side.

Clinical evaluation

Finally, the clinical utility of the FICT assay was validated by detecting 3325 clinical samples from five provinces in China (Liaoning, Hebei, Shandong, Sichuan, and Guangdong). These samples were also tested simultaneously using an IDEXX-ELISA kit. Of the 3,325 clinical samples, 1,419 were detected as PRRSV antibody-negative, and 1,906 samples were determined to be positive by the FICT. The IDEXX-ELISA yielded 2,202 PRRSV antibody-positive samples and 1,323 negative samples (**Table 1**). Therefore, the diagnostic specificity, sensitivity, and accuracy of FICT were 97.28% (1,287/1,323), 93.41% (1,870/2,002), and 94.95% (3,157/3,325), respectively. These results show that the FICT could detect the PRRSV antibody with high sensitivity, specificity, and accuracy.

DISCUSSION

PRRS is recognized as one of the most important infectious diseases, causing serious economic losses in the swine industry worldwide. A rapid, sensitive, and accurate diagnosis could prevent the spread of PRRSV disease in pigs. In recent years, the FICT has been used to detect the presence of antigens and antibodies in animal diseases. This study established a highly sensitive, rapid diagnostic reagent based on the use of fluorescent microsphere-

Table 1. Comparison of the FICT assay with IDEXX-ELISA using 3,325 clinical serum samples

| Assays | IDEXX-ELISA | | Total |
|----------|-------------|----------|-------|
| | Positive | Negative | |
| FICT | | | |
| Positive | 1,870 | 36 | 1,906 |
| Negative | 132 | 1,287 | 1,419 |
| Total | 2,002 | 1,323 | 3,325 |

FICT, fluorescent microsphere-based immunochromatographic strip test.

labeled goat anti-pig IgG as a detection antibody, and rNSP7 and rN proteins that serve as capture antigens. The fluorescent signal was detected using a portable FIA meter that uses an LED as the excitation light source. The simplicity and accuracy of a PRRS diagnosis are very important for minimizing PRRSV disease. In general, the relatively high sensitivity that characterizes the use of the FICT for the detection of PRRSV antibodies makes it a good alternative for the immunodiagnosis of the disease that can be applicable for use in the field.

This study established a fluorescent microsphere immunochromatographic assay with enhanced sensitivity for the rapid and quantitative detection of PRRSV antibodies. The FICT assay combines the advantages of ELISA and colloidal gold test strip technology, enabling the quantitative detection of PRRSV antibodies in less than 15 min with an easy-to-access portable fluorescence immunoassay analyzer. This assay is slightly more sensitive than the ELISA method that has a good correlation with the VN assay, which was detected one month after vaccination. In addition, considering that neutralizing antibodies will not be detected in herds until one month after vaccination, an evaluation of the NA by FICT is conditional and limited; the test result will be useful only one month after vaccination. The detection of specific antibodies by the FICT is more convenient than other tests. Therefore, the FICT detection platform is expected to be complementary to existing ELISA and colloidal gold test strip techniques for the detection of PRRSV antibodies. Given the excellent correlation between the FICT and IDEXX kits for the detection of a large number of clinical samples, this product can be used for a rapid evaluation of the PRRSV infection pressure and PRRSV replication level in pigs. In addition, the diagnostic specificity, sensitivity, and accuracy of FICT were 97.28, 93.41, and 94.95%, respectively. The performance evaluation for the accuracy, specificity, and stability tests showed that the indicators are in line with requirements.

In summary, the novel FICT-strip test technology established in this study can detect antibodies against the PRRSV rapidly, ultra-sensitively, and quantitatively. This test provides a new promising pen-side method for the rapid screening of PRRSV infections or for monitoring the vaccine immune efficacy in external laboratories and resource-limited settings.

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