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Preparation and preliminary application of fluorescent microsphere test strips for feline parvovirus antibodies

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Abstract

This study introduces a novel diagnostic modality for the detection of feline panleukopenia virus (FPV) antibodies in feline serum by using fluorescent microsphere immunochromatographic test strips (FM-ICTS). Leveraging the inherent specificity of antigen-antibody interactions, the FM-ICTS approach demonstrates considerable potential for efficient and accurate FPV antibody detection within a short timeframe. The FM-ICTS method demonstrates strong diagnostic performance, with consistent accuracy and stability over time. PBS buffer dilution enables detection across the range of FPV antibody haemagglutination inhibition (HI) titres in both healthy and immunized or infected cats. A high correlation ($R^2 = 0.9733$) between the T/C ratio and FPV antibody titres confirms the method's effectiveness in quantifying these titres. Clinical validation with 84 samples supports its reliability by matching results with HI assays. Additionally, stability tests show that the test strips maintain performance during storage, with a coefficient of variation (CV) below 12% over three months at 25℃. This innovative FM-ICTS framework emerges as a promising avenue for expedient and dependable disease diagnosis within the realm of veterinary science, offering implications for timely disease management and surveillance.

Keywords Fluorescence microsphere, Serodiagnosis, Antibody assay, Feline panleukopenia virus, Immunochromatography

Introduction

Feline panleukopenia virus (FPV) is a single-stranded DNA virus that severely affects felids. It belongs to the family *Parvoviridae* and shares a close genetic relationship with canine parvovirus and porcine parvovirus [1]. The transcription of the genome of FPV is regulated by two primary open reading frames (ORFs) situated downstream of the p4 and p38 promoters. These ORFs encode essential proteins crucial for viral processes: nonstructural proteins (NS1, NS2) and capsid viral proteins (VP1, VP2), which constitute the structural component of the viral capsid. VP2, prominently featured among the capsid proteins, accounts for approximately 90% of the total nuclear capsid proteins [2]. Beyond its structural role,

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VP2 also serves as a pivotal immunogenic protein, eliciting the production of antibodies [3]. FPV is primarily transmitted to other cats through direct contact with infected sources, such as bodily fluids, faeces, and vomit. It replicates in the digestive, respiratory, lymphatic, and bone marrow systems of the cat, causing significant damage to these organs. Infected cats exhibit typical clinical symptoms, including high fever, anorexia, vomiting, diarrhoea, weight loss, weakness, and leukopenia. FPV can impair the immune system of the cat, leading to a drastic reduction in white blood cell count (known as leukopenia), making the cat more susceptible to secondary infections. Vaccination is crucial for preventing FPV infection. Regular vaccination effectively prevents FPV infection and reduces the spread of the virus within cat populations. Antibody testing is commonly used to assess vaccine efficacy. Several diagnostic techniques have been utilized for the evaluation of viral antibodies, including virus neutralization (VN) assays, immunofluorescence assays, haemagglutination inhibition (HI) assays, and enzyme-linked immunosorbent assays (ELISA) [4]. The HI test is considered the gold standard for detecting FPV antibodies [5]. Generally, an HI antibody titre of 1:20 to 1:40 is considered protective against FPV [6, 7].

Immunochromatography is an innovative diagnostic tool based on the specific interaction between antigens and antibodies. The markers commonly employed in this method include colloidal gold, quantum dots, and time-resolved fluorescent microspheres. Compared to traditional laboratory diagnostic methods such as ELISA and PCR, immunochromatography offers the distinct advantage of shortened detection times, with all reactions achievable within 30 min. Additionally, its results are easily and swiftly interpreted, making it highly suitable for onsite applications. Consequently, immunochromatography holds substantial potential for rapid disease diagnosis, which showcases its immense utility in the field. The substitution of traditional colloidal gold with fluorescent microspheres (FM) in immunochromatography has extended the shelf life and enhanced the sensitivity of the test strips. The purpose of this study was to develop an immunochromatographic strip using FM labelling for the detection of FPV antibody in cat serum [8]. Levels of analytes were assessed using a portable fluorescence detector containing an ultraviolet (UV) light source, which is amenable to use in both clinical and laboratory settings (Fig. 1A).

Materials and methods

Recombinant antigen expression, purification and characterization

The VP2 protein contains a conserved main epitope that stimulates the production of antibodies. Therefore, the VP2 protein has been used to produce subunit vaccines. Amplification of FPV preserved in the laboratory with primers (FPV-VP2-Primer-F: CGCGGATCCATGAGTG ATGGAGCAGTTCAAC; FPV-VP2-Primer-R: GCTCTA GATTAATGATGATGATGATGATATAATTTTCTAGG TGCTAGTTGAG) to obtain the recombinant VP2 protein coding sequence with His tag. The recombinant VP2 protein coding sequence was cloned and inserted into the p*Cold*-I vector and transformed into BL21(DE3). The FPV-VP2 expression plasmid was induced by IPTG, followed by growth at 16 °C for 30 h. BL21(DE3) cells were harvested and lysed by sonication to release proteins. The supernatant of the lysed cells was purified by a HisPur Ni NTA kit (Smart-Lifesciences, China). Through elution steps, imidazole in the buffer was used to dissociate the binding of the VP2 protein and nickel ions on the purification column. Then, ammonium sulphate was added to a final concentration of 20%, and the mixture was incubated at 4 °C for 8 h. The mixture was centrifuged, and the precipitate was resuspended in PBS. Then, a 10 kDa dialysis bag was used to remove salt. The quality of the purified VP2 protein was evaluated by protein concentration determination, SDS–PAGE analysis, WB and other experimental methods to confirm the purity and integrity of the protein. The purified VP2 protein was loaded onto a 10% gradient Tris-glycine SDS-polyacrylamide gel, and proteins were transferred to a 0.2 μm PVDF membrane. The membrane was blocked with 5% nonfat milk in PBS containing 0.05% Tween-20 for one hour at room temperature, followed by incubation with primary feline serum positive for FPV antibodies at 4 °C overnight at a 1:1000 dilution. The secondary anti-feline IgG-HRP was diluted at 1:5000 in blocking buffer and incubated for 1 h at room temperature. Proteins were visualized using an Ultra-sensitive ECL chemiluminescent detection kit (Beyotime).

Preparation of fluorescent microsphere probes

First, 100 μL of a 200 nm diameter fluorescent microsphere (PS-COOH Europium Chelate Microspheres, excitation: 340 nm; emission: 620 nm) suspension (Bangs Laboratories, Inc-FCEU002) was added to 900 μL of pure water, centrifuged at 14,000 rpm for 25 min at 9 °C and washed. The supernatant was removed, and the microspheres were resuspended in 1000 μL of pure water, centrifuged at 14,000 rpm for 25 min at 9 °C and washed for a second time. The supernatant was removed, and the microspheres were resuspended in 1000 μL of 20 mM pH 5.3 2-(Nmorpholino)-ethanesulfonic acid (MES) (Sigma–Aldrich, China). If the microspheres aggregated after centrifugation, they were dispersed by ultrasonic dispersion for 5 min. Then, 25 μL of 10 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Thermo, China) was added to the washed microspheres and mixed quickly. Then, 25 μL of 10 mg/

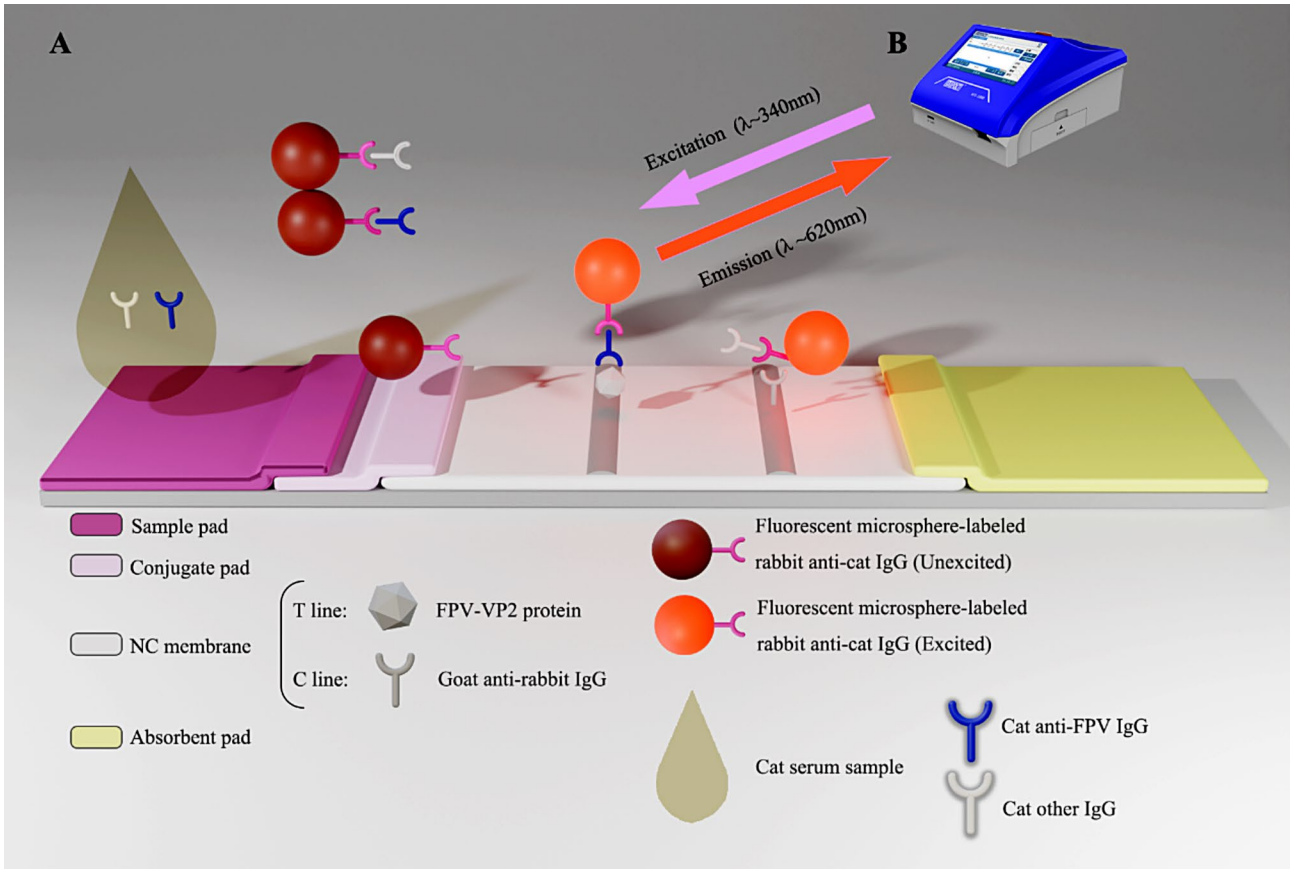

Fig. 1 (**A**) Schematic illustration of the principle of FM-ICTS. For positive samples, FM-anti-cat IgG. (source: rabbit) binds specifically to the FPV antibody and forms an immune complex (FM-anti-cat IgG-FPV antibody), which in turn is captured by the VP2 on the T-line and produces a fluorescent signal, and excess FM-anti-cat IgG continues to migrate and is captured by the anti-rabbit IgG immobilized on the C-line and produces a fluorescent signal. For negative samples, FM-anti-cat IgG can only be captured by anti-rabbit IgG immobilized on the C-wire, and excess FM-anti-cat IgG migrates to the absorbent pad. (**B**) Dry immuno-fluorescence analyser AF-1000 (LAB, China)

ml N-hydroxysulfosuccinimide sodium salt (NHS) (OKA, China) was added to the microspheres, mixed quickly, ultrasonicated for 5 min, and incubated at 37 °C for 30 min. The mixture was centrifuged at 14,000 rpm for 25 min at 9 °C, and the supernatant was removed. The microspheres were resuspended in 1000 μL coupling buffer (20 mmol/L Tris-HCl, pH 8.0). If the microspheres aggregated after centrifugation, they were dispersed by ultrasonic dispersion for 5 min. The effects of two different conjugation buffers (Tris buffer (20 mmol/Lol/L Tris-HCl, pH=8.0) and HEPES (4-hydroxyethylpiperazine-1-ethanesulfonic acid) buffer (20 mmol/Lol/L HEPES, pH=8.0)) were compared.

Conjugation of fluorescent microsphere probes

Then, 20, 40, 60, and 80 μg of rabbit anti-cat antibody (Biodragon, China) were separately added to 1000 μg of activated microspheres and incubated at 37 ℃ for 2 h (resulting in 2, 4, 6, and 8 μg/100 μg of fluorescent microspheres), to compare the optimal conjugation ratio between antibodies and microspheres. Bovine serum albumin (BSA) was added to a final concentration of 1%, mixed quickly and incubated at 37 ℃ for 1 h. The microsphere-antibody mixture was centrifuge at 14,000 rpm for 25 min. The supernatant was removed and the microspheres were resuspended with resuspension solution (0.12 g Tris; 5 g sucrose; 2.5 g trehalose; 1.5 g BSA; 40.88 g water; pH 8.5). If the microspheres aggregated after centrifugation, they were dispersed by ultrasonic dispersion for 5 min.

FM-ICTS assembly

The sample pad and conjugate pad were saturated with Tris-HCl buffer 1 (20 mmol/L Tris-HCl, 1% Triton X-100, 0.3% BSA, pH 8.0) and Tris-HCl buffer 2 (20 mmol/L Tris-HCl, 0.3% BSA, and 0.2% Tween-20, pH 8.0), respectively, and air-dried for 2 h. The conjugate pad was then coated with a fluorescent antibody (10 μL/cm), and the purified rVP2 proteins were diluted with 0.01 M PBS to 0.9 mg/mL as the test line (T). Goat anti-rabbit IgG (Sangon Biotech, China) was used at 0.02 mg/mL as the control line (C). The test line and control line were then

dispensed (1 μL/cm) along a nitrocellulose membrane. A membrane strip was arranged, from left to right, with a sample pad, conjugate pad, nitrocellulose membrane, and absorbent pad pasted onto a 300×80 mmol/L backing card. The card was cut longitudinally, divided into 4.0×80 mmol/L strips using a strip cutter, and stored in sealed bags under dry conditions at room temperature.

Sensitivity and specificity

Utilizing a serial dilution of two-fold increments of feline serum with a determined FPV anticoagulant inhibitory titre of 1:1024, the sensitivity of FM-ICTS was assessed. A calibration curve was constructed by plotting each relative T/C value against the titres of FPV antibodies. Additionally, the specificity of the FM-ICTS was evaluated by examining serum samples containing closely related feline viruses (feline herpesvirus, feline calicivirus).

Accuracy and stability

To assess the intra- and inter-batch reproducibility of FM-ICTS, three batches of test strips were used to determine the T/C values of serum samples. Five replicates were performed for each sample. Additionally, a stability assessment of the FM-ICTS was conducted by storing the test strips at 25 °C for 1, 3 months and evaluating the T/C ratio using standard reference sera.

Clinical evaluation

Finally, a total of 84 clinical samples from Jilin, China were analysed. The serum samples used in this study were primarily obtained from our laboratory's serum repository. Clinical serum samples were diluted 100-fold using 10 mmol/L PBS. Subsequently, 100 μL of the diluted sample was dispensed onto the FM-ICTS sample pad. Following a 20 min incubation period, quantitative analysis was performed by employing a dry immuno-fluorescence analyser to measure the fluorescence intensity at the T-line and C-line of the test strip. This validation aimed to confirm the clinical utility of the FM-ICTS. Additionally, these samples underwent HI test simultaneously for comparative assessment.

Results and conclusions

Preparation of diagnostic antigens

The *E. coli* cells containing the FPV-VP2 expression plasmid were induced with isopropyl-l-D-throgalactopyranoside (IPTG), followed by purification from subsequent growth culture using a 3 mL HisPur Ni NTA kit. Finally, analysis was conducted through SDS–PAGE and Western blotting (WB) to characterize the purified protein (Fig. 2).

Selection of the coupling buffer

The hydrophobic nature of FM makes them prone to aggregation under certain conditions, e.g., microspheres with neutralizing charged groups. The combination of antibodies and microspheres necessitates suitable pH and ion strength conditions. The appropriate pH range typically lies between 6 and 9. Probe precipitation becomes likely when the ion strength surpasses 0.2 M. Thus, an inadequate ionic strength of the conjugation buffer may lead to FM aggregation, resulting in suboptimal coupling efficiency. The particles employed for conjugation should maintain stability and dispersion. To explore optimal working conditions, a comparison of two distinct commonly used conjugation buffer solutions (20 mmol/L Tris-HCl, pH 8.0; 20 mmol/L HEPES, pH 8.0) was conducted. When 20 mmol/L Tris-HCl solution (pH 8.0) was applied, the brightest fluorescent signal and T/C value were obtained (Fig. 3A). Therefore, 20 mmol/L Tris-HCl

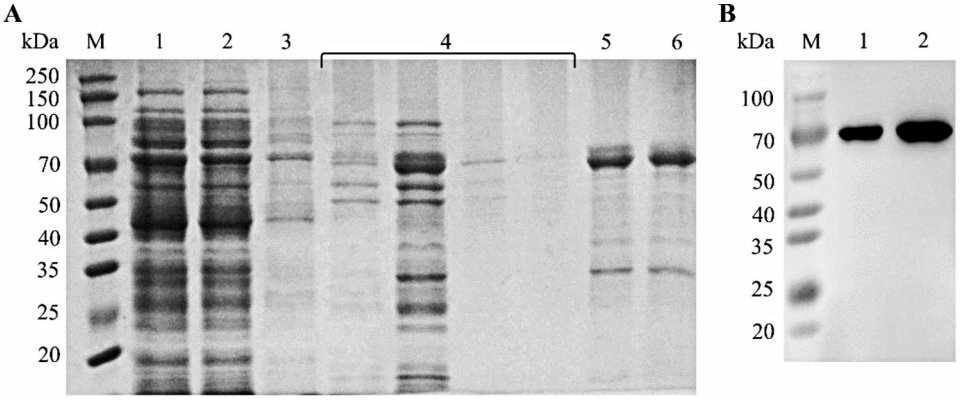

Fig. 2 Expression and characterization of recombinant FPV VP2 protein from *E. coli*. The fusion protein was expressed from pCold-VP2-His/BL21(DE3). (**A**) SDS–PAGE analysis of FPV VP2 protein expression and purification. M: protein marker; lane 1: the supernatant obtained after sonication of BL21(DE3) cells expressing VP2 protein; lane 2: the liquid after passing through the His-Tag Ni^{2+} affinity column of the supernatant; lane 3: wash buffer; lane 4: elution buffer; lane 5: the eluate after first purification with ammonium sulphate; lane 6: the eluate after second purification with ammonium sulphate. (**B**) Western blot analysis of FPV VP2 proteins. M: protein marker; lane 1: unpurified VP2 protein immunoblotted using feline FPV-positive sera; lane 2: purified VP2 proteins immunoblotted using feline FPV-positive sera

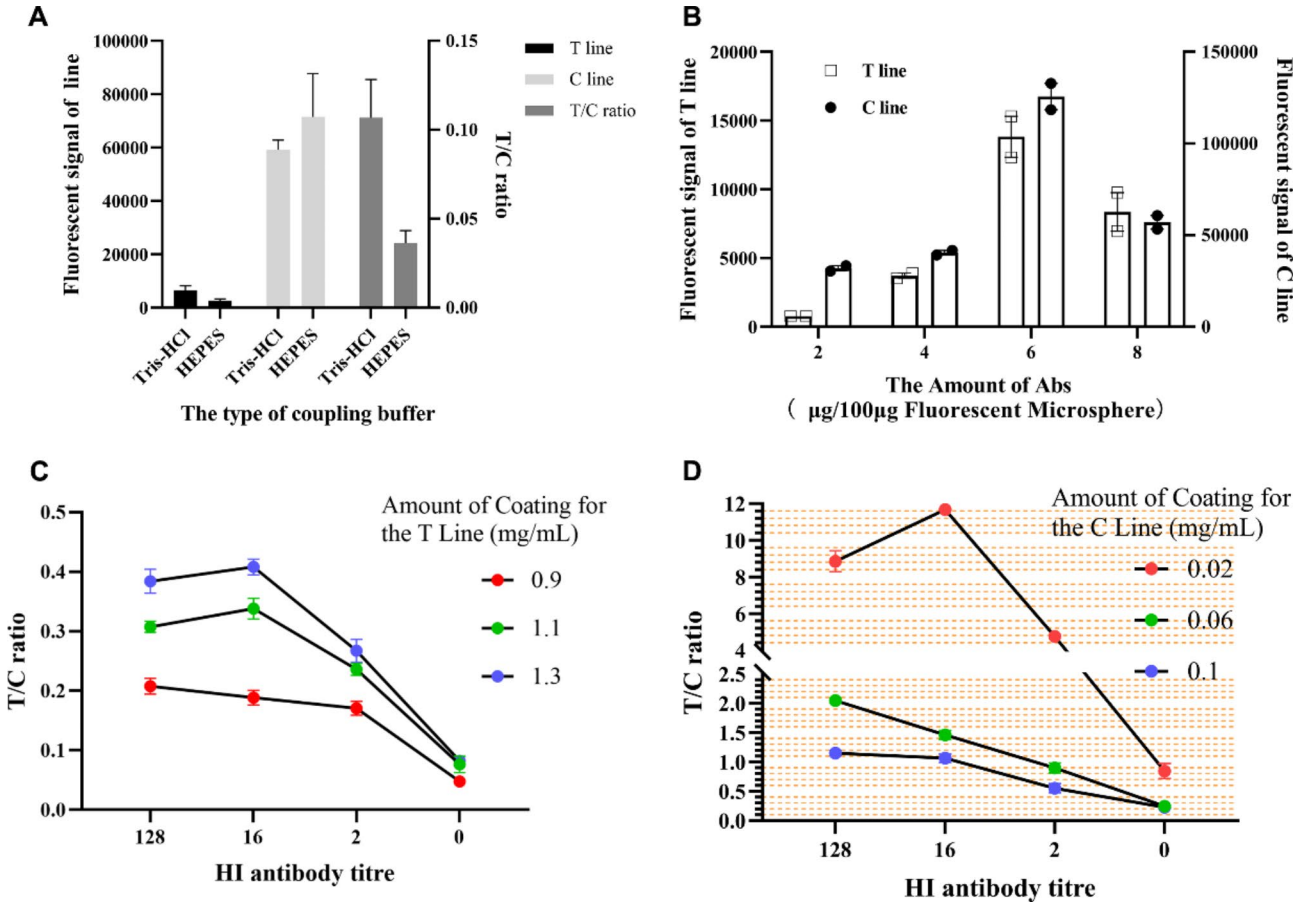

Fig. 3 (**A**) The conjugation buffer. (**B**) Concentration of the rabbit anti-cat antibody. (**C**) Optimal encapsulation amount for the T line. (**D**) Optimal encapsulation amount for the C line

solution (pH 8.0) was chosen as the optimal coupling buffer in this study.

Optimal conjugation amount

The amount of antibody coupled to FM is a critical component of making FM-ICTS. It impacts coupling efficiency and analytical performance. Insufficient surface antibodies can result in inadequate probe affinity to the antigen, while excessive antibodies can lead to protein congestion, thereby reducing sensitivity. FM (100 μg) was prepared and coupled with varying ratios of rabbit anti-cat IgG (2, 4, 6, and 8 μg) to assess the optimal antibody quantity for coupling. As shown in Fig. 3B, when coupled at a ratio of 6 μg of rabbit anti-feline antibodies to 100 μg FM, the most robust fluorescence signal was observed at both the T-line and C-line. Consequently, we adopted this optimal coupling proportion of 6 μg rabbit anti-feline antibodies per 100 μg of fluorescent microspheres.

Optimal encapsulation amount

The performance of the test strip was enhanced by fine-tuning the quantities of substances encapsulated within the T and C lines. It was observed that an encapsulation quantity of 0.9 mg/mL for the VP2 protein on the T line showed the strongest correlation between the T/C ratio with the serum hemagglutination inhibition (HI) titre (Fig. 3C). Subsequently, adjusting the encapsulation quantity on the C line to 0.06 mg/mL ensured that the T/C ratio for positive samples exceeded 1, while for negative samples, the T/C ratio remained below 0.5, and also maintained a strong correlation between the T/C ratio and the HI titre, aiding in reliable interpretation (Fig. 3D). This effect is attributed to the influence of the encapsulation quantities on the T and C lines, which affect the capture efficiency of the fluorescent microspheres and the intensity of the background signal. Encapsulation quantities that are too high or too low compromise the assay's sensitivity and specificity. Therefore, the selected encapsulation quantities of 0.9 mg/mL for the VP2 protein and 0.06 mg/mL for the goat anti-rabbit antibody represent the optimal conditions for the application of the test strip.

The test of sensitivity and specificity

As shown in Fig. 4A, the standard curve of antibody titres was determined using the variable slope model

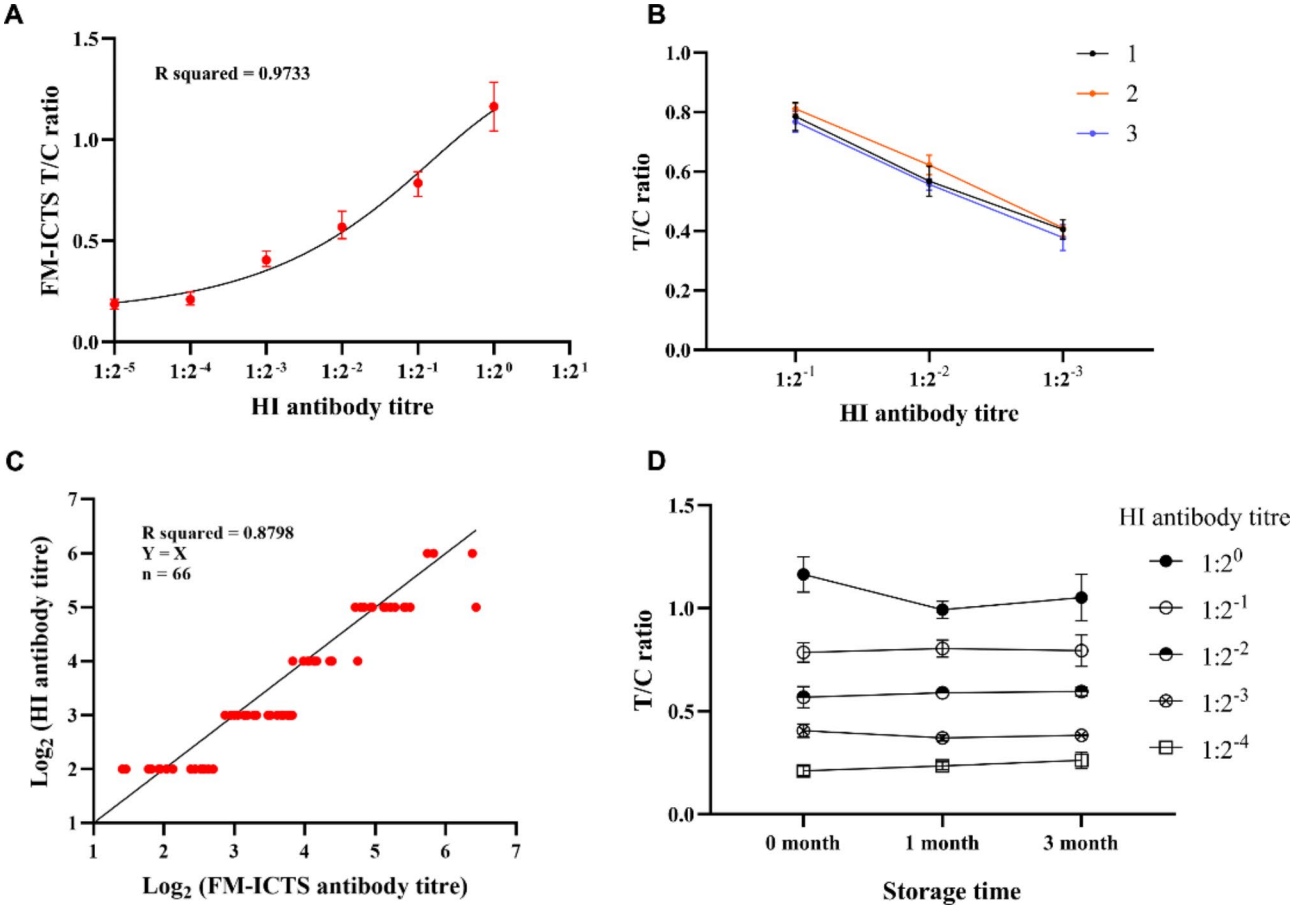

Fig. 4 (**A**) The standard curve. (**B**) The intra- and inter-batch reproducibility of FM-ICTS. (**C**) Correlation between FM-ICTS and HI test on 66 positive serum samples. The T/C ratios of each serum sample tested by FM-ICTS were converted to antibody titres via the standard curve. (**D**) Stability assessment of FM-ICTS

Table 1 The data of specificity test

Serum sample	T/C value					CV value
FHV antibody +	0.1559	0.1419	0.1404	0.1377	0.1208	8.05%
FCV antibody +	0.0836	0.0979	0.0887	0.0942	0.0669	12.55%

in GraphPad Prism 9 (Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope)), R^2=0.9733, Bottom = -2.354, Top=1.311, LogEC50 = -0.3265, HillSlope=1.000). The specificity experiments of the FM-ICTS were conducted using five samples each of feline herpesvirus and feline calicivirus sera. The results indicated that the FM-ICTS can detect FPV-positive sera while exhibiting no cross-reactivity with other sera (Table 1).

The test of accuracy and stability

Three batches of test strips were used to assess the T/C ratio of three standard serum samples. Five replicates were performed for each sample, and the coefficient of variation (CV) was calculated as SD/X × 100%. Both intra-batch and inter-batch CV values were below 11%, meeting precision requirements (Fig. 4B). Furthermore, a stability test of FM-ICTS packaged in aluminium foil bags was conducted by storing the test strips at 25 °C for 1, 3 months and evaluating the T/C ratio using standard reference sera, yielding a CV < 12% (Fig. 4D). Comparable sensitivity and specificity were observed between processed and unprocessed test strips, confirming its favourable stability.

Clinical evaluation

As shown in Fig. 4C, among the 84 clinical samples tested, 66 were identified as FPV antibody-positive, and 18 samples were determined to be negative. The coagulation inhibition assay detected a total of 66 FPV antibody-positive samples and 18 negative samples. R-squared value of 0.8798 suggests that approximately 87.98% of the variance in the dependent variable can be explained by the independent variable(s). This indicates

a strong relationship between the variables in the regression model. Consequently, FM-ICTS demonstrates commendable diagnostic specificity, sensitivity, and accuracy.

Discussion

In this study, the ratio of fluorescence values between the T line and C line was used as the detection result, which can compensate for the inherent heterogeneity of lateral flow test strips and sample matrices. This approach is more reliable and reproducible than using only the T line for signal quantification. Initially, two different coupling buffers, Tris and HEPES buffers, were compared for their effects on the coupling efficiency between fluorescent microspheres and rabbit anti-cat antibodies. It was found that Tris buffer better maintained the stability and activity of fluorescent microspheres, while HEPES buffer was less suitable for the binding between fluorescent microspheres and rabbit anti-cat antibodies. This resulted in lower T line fluorescence values and T/C ratios when using HEPES buffer for coupling, compared to when using Tris buffer. The study also investigated the impact of different coupling amounts on fluorescent microsphere test strips. Both T line fluorescence values and T/C ratios decreased when the coupling amount was either too high or too low. Insufficient rabbit anti-cat antibodies on the fluorescent microspheres hindered effective binding with FPV VP2 antigen when the coupling amount was too low. Conversely, an excessive coupling amount led to either interference with fluorescence detection by the instrument due to an excess of rabbit anti-cat antibodies on the fluorescent microspheres or aggregation of fluorescent microspheres, affecting fluorescence signal output. Finally, optimization of the encapsulation amounts of the T line and C line on the test strips was performed. The encapsulation amounts of the T line and C line influenced the capture efficiency of fluorescent microspheres and the intensity of background signals. Encapsulation amounts that were too high or too low decreased the sensitivity of the test strips.

The FM-ICTS exhibits a limit of detection (LOD) for FPV HI titres in feline serum at a dilution of $1{:}2^{-5}$ and a quantification range (LOQ) from $1{:}2^{-4}$ to $1{:}2^{0}$. Dilution with PBS buffer allows for coverage of the range of FPV antibody HI titres found in healthy cats, as well as elevated antibody levels due to infection or immunization. The obtained standard curve regression model showed a strong correlation between the T/C ratio and the FPV antibody titres ($R^2=0.9733$). There was an acceptable linear relationship between the concentrations detected by the two methods, indicating that the developed FM-ICTS possesses good analytical performance and can effectively quantify the FPV antibody titres in feline serum. Finally, we conducted stability tests on the test strips by storing them at 25 °C for 0, 1, and 3 months and testing them with sera of different HI titres. The results showed that the coefficient of variation (CV) of the test strips was less than 12%, indicating that there was no significant performance degradation during storage, and the test strips maintained good stability. However, due to time constraints, longer-term stability testing could not be carried out.

Conclusions

FM-ICTS stands out for its rapidity, user-friendly operation, cost efficiency, remarkable specificity, and sensitivity, rendering it ideally suited for swift diagnostic applications. This innovative technology has found widespread utilization in diverse fields, including biomedical sciences, food safety, environmental surveillance, and beyond [9–12]. Simultaneously, FMs have also been applied in various forms within the field of analyte detection [13]. In the domain of veterinary medicine, FM-ICTS plays a pivotal role in enhancing our ability to diagnose and manage diseases in animals.

In this study, we developed a rapid and straightforward fluorescent microsphere test strip assay based on lateral flow immunoassay (LFIA) technology, integrated with Eu(III) chelate particles, for the quantitative determination of feline panleukopenia virus (FPV) antibody titres in serum. The assay operates through a direct competitive immunoassay on a lateral flow strip, with a detection time of 20 min. The fluorescent microsphere test strip, leveraging Eu(III) chelate particles, enables semi-quantitative detection of anti-FPV antibody titres in serum. The FM-ICTS have satisfactory repeatability sensitivity and stability. Clinical trial results affirmed the assay's consistency with traditional standard methods, validating its reliability and effectiveness for on-site monitoring of FPV antibodies in cats. This FM-ICTS offers significant clinical application value. It does not require expensive equipment, complex operations, or preprocessing and its exceptional sensitivity enables early and accurate FPV detection, even when antibody levels are low, facilitating prompt intervention and treatment. The quantitative nature of FM-ICTS allows precise measurement of FPV-specific antibody concentrations, aiding in the evaluation of the strength and duration of the immune response post-vaccination. Although the assay's performance characteristics may not match methods like ELISA, its simplicity, sensitivity, and accuracy make it highly suitable as a primary clinical diagnostic tool. The quantitative information provided aids veterinarians in making informed decisions concerning booster vaccinations and the comprehensive management of infected cats.

Abbreviations

FPV	Feline panleukopenia virus
ORFs	Open reading frames
NS	Nonstructural protein

VP	Viral protein
VN	Virus neutralization
HI	Haemagglutination inhibition
ELISA	Enzyme-linked immunosorbent assays
FM	Fluorescent microspheres
UV	Ultraviolet
FM-ICTS	Fluorescent microsphere immunochromatographic test strips
IPTG	Isopropyl-l-D-throgalactopyranoside
WB	Western blotting
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12896-024-00900-2.

Supplementary Material 1

Supplementary Material 2

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Author contributions
JY.S. conceived and designed the experiments, conducted data analysis, and wrote the manuscript with adding from GL.L., EK.F. and YN.C. supported the research with funding acquisition. MP.Y., XH.Z., W.L. and W.S. provided critical insights into the study design, contributed to data interpretation, and revised the manuscript. ZJ.W., L.Y. and CX.W. provided expertise in the technical aspects of the study, contributed to data analysis, and critically reviewed the manuscript. All the authors have read and approved the manuscript.

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Data availability
All data supporting the findings of this study are available within the paper. The FPV-VP2 amplification primer sequences are provided in the Methods section. Other experimental data are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This study was approved by the Animal Ethics Committee of Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences (approval number No.ISAPSAEC-22-82).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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