Establishment of High-sensitivity Rapid Fluorescence Quantitative Detection Method for Antibody against Peste des Petits Ruminants Virus

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Abstract [**Objectives**] This study was conducted to establish a rapid quantitative method for detecting antibody against Peste des Petits Ruminants Virus (PPR V) in sheep serum. [**Methods**] Soluble N protein and NH fusion protein were obtained in *Escherichia coli* prokaryotic expression system by optimizing codons and expression conditions of *E. coli*. Furthermore, based on the purified soluble N protein and NH fusion protein, a high-sensitivity fluorescence immunoassay kit for detecting the antibody against PPR V was established. [**Results**] The method could quickly and quantitatively detect PPR V antibody in sheep serum, with high sensitivity and specificity, without any cross reaction to other related sheep pathogens. The intra-batch and inter-batch coefficients of variation were less than 10% and 15%, respectively, and the method had good repeatability. Through detection on 292 clinical serum samples, it was compared with the French IDVET competitive ELISA kit, and the coincidence rate of the two methods reached 93.84%. Compared with the serum neutralization test, the detected titer value of the high-sensitivity rapid fluorescence quantitative detection method was basically consistent with the tilter value obtained by the neutralization test on the standard positive serum (provided by the WOAH Brucellosis Reference Laboratory of France). [**Conclusions**] This method can realize rapid quantitative detection of PPR V antibody on site, and has high practical value and popularization value.

Key words Peste des Petits Ruminants; N protein; NH fusion protein; Soluble expression and purification; Rapid quantitative detection

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Peste des Petits Ruminants (PPR) is an acute contact infectious disease caused by Peste des Petits Ruminants Virus (PPR V) in goats, sheep and wild ruminants, characterized by fever, stomatitis, diarrhea and pneumonia. It is an animal disease that must be reported according to the World Organization for Animal Health (OIE), and China lists it as a class I animal infectious disease[1-2]. The epidemic situation of PPR will not only bring great influence to the development of animal husbandry, but also bring huge economic losses to society, and it may also lead to the extinction of endangered wild animals and bring irreparable social losses. PPR V belongs to Morbillivirus of paramyxoviridae. The genome of the virus is a minus-strand RNA, including six genes, N-P-M-F-H-L, from 3 to 5, encoding six structural proteins and two non-structural proteins. Among them, the antigenicity of N protein and H protein is stable, and the antibodies of N protein and H protein are dominant in the serums of infected animals, so they serve as main target proteins for the development of diagnostic reagents[3-6]. In July, 2007, PPR was first introduced into Ali, Tibet, China, and the epidemic situation was quickly controlled. At the end of November, 2013, PPR was introduced into China again, and the epidemic spread to many provinces. In December 2015, the Ministry of Agriculture issued the *National Plan for the Eradication of Peste des Petits Ruminants* (2016 - 2020), which proposed that by 2020, except for the land border counties (regiments) adjacent to the countries with the epidemic situation of PPR or the immune isolation belts within 30 km along the border, we should strive to achieve the national prevention and control goal of non-immune epidemic-free areas[7-10]. In recent years, in order to achieve the goal of prevention and control and provide scientific and technological support, research on new vaccines and rapid diagnosis techniques has become the focus. Based on the high-sensitivity rapid lanthanide fluorescence quantitative detection system, a rapid detection method for antibody against PPR V was established. The sensitivity and specificity of this method are similar to those of commercial ELISA kit, but it has the advantages of on-site, rapid and quantitative detection. This method is very suitable for rapid and large-scale screening and detection of PPR by veterinary departments at all levels at the grass-roots level, and provides important technical means for the prevention and control of PPR, showing strong application and popularization value.

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Materials and Methods

Materials

Expression strain BL21 (DE3) was purchased from Beijing TransGen Biotech Co., Ltd. PET30a fusion expression vector was purchased from Novagen. Enzymes and reagents used: T4DNA ligase, restriction endonucleases *Nde* I, *Xho* I, 2000 DNA Marker, IPTG, SDS and Taq PCR Master Mix, were all purchased from Takara Biotechnology (Dalian) Co., Ltd. DNA Extraction Kit,

agarose, plasmid rapid extraction kit and DNA rapid purification and recovery kit were all purchased from Beijing TransGen Biotech Co., Ltd. Pre-stained protein Marker was purchased from Fermentas. The synthesis of the target gene sequence of the fusion protein was completed by Beijing Genomics Institute (BGI). Competition ELISA kit was purchased from the French company IDVET. Lanthanide fluorescent microspheres and rapid highly-sensitive fluorescence quantitative detection system were all developed by Chengdu Weirui Biotechnology Co., Ltd. Negative and positive serum samples were preserved by the China Animal Disease Control Center (Veterinary Diagnosis Center of the Ministry of Agriculture and Rural Affairs). Standard positive serum was purchased from the OIE Brucellosis Reference Laboratory of France. Standard negative serum was negative sheep serum purchased from Thermo Fisher Scientific.

Methods

Acquisition of target gene fragments According to the N and H genes of PPRV on NCBI website, the target sequences of N and H genes were optimized to preferred codon sequences of *Escherichia coli*, and target gene sequences, *i.e.*, N and NH tandem fusion expression gene sequences of PPRV, were synthesized by chemical synthesis methods, respectively.

Amplification and fusion of target genes A pair of primers was designed according to the sequence of *N* gene of PPRV, and *Nde* I and *Xho* I cutting sites were added to the 5' end and 3' end of the primers respectively for amplification of the target fragment.

Forward primer PPRV-NF: catatg GCGACCTTACTGAAAAG CCTGGCG;

Reverse primer PPRV-NR: ctcgag ACCCAGCAGGTCTTTG TCGTTGT.

A pair of primers was designed according to the sequence of the NH gene of PPRV, and *Ned* I and *Xho* I cutting sites were added to the 5' end and 3' end of the primers respectively for amplification of the target fragment.

Forward primer PPRV-NHF: catatg GCGACCTTACTGAAA AGCCTGGCG;

Reverse primer PPRV-NHR: ctcgag AACCGGGTTGCAGGT-GACTTCAAT.

A linker sequence (GGTGGCGGTGGAATCGGAGGTGGTG-GAAGCGGAGGAGGTGGAAGC) was added between N and H gene sequences to amplify NH tandem fusion sequence.

Construction and identification of recombinant plasmids pET30a vector was digested with *Ned* I and *Xho* I, and the enzyme digestion system contained 10 × enzyme digestion buffer 40 μl, *Ned* I and *Xho* I 1 μl each, vector 24 μl, and deionized water 8 μl. The enzyme digestion product was recovered by gel recovery kit after 1% agarose electrophoresis. Next, the enzyme-digested vector was ligated with the amplified N or NH coding gene sequence in a 10 μl ligation system, which contained N or NH coding gene fragment 5.5 μl, pET30a vector 1.5 μl, T4 DNA ligase 1 μl and T4 DNA ligation buffer 2 μl. The tube was gently tapped on the wall, inverted to mix evenly, and then immediately put in a ligation instrument for ligation at 22 ℃ for 4 h. The recombinant plasmids were identified by restriction enzyme digestion and sequenced for target fragments. The plasmids identified to be positive by sequencing were named pET30a-N and pET30a-NH.

Construction of recombinant *E. coli* strains pET-30a-(N)-BL21(DE3) and pET-30a-(NH)-BL21(DE3) The ligation products of pET30a-N and pET30a-NH were transformed into BL21(DE3) cells, respectively. Under aseptic conditions, a proper amount of BL21 bacterial liquid was added into plates containing LB(Kan+) solid culture medium and evenly coated. After the bacterial liquid was completely absorbed, the plates were marked, and inverted for static culture in a constant temperature incubator at 37 ℃ for 16 h. Monoclonal colonies were selected for bacterial liquid identification, enzyme digestion identification and bacterial liquid sequencing respectively, to confirm that N fragment and NH fragment were transformed into pET30a vector, respectively.

Establishment of soluble induced expression conditions of recombinant proteins The recombinant expression plasmids identified above were transformed into the competent strain *E. coli* BL21(DE3), and positive clones were selected and cultured at 37 ℃ overnight. The bacterial liquids were inoculated into liquid LB medium containing kanamycin (50 μg/ml) at a ratio of 1∶100, and cultured at 37 ℃ and 200 r/min until the OD value was 0.4 -0.6. Next, 1 ml of uninduced bacterial liquid was taken out as a control, and isopropyl β-D-thiogalactoside (IPTG) was added to the rest of each liquid to induce protein expression. After optimizing the conditions such as temperature, time and IPTG concentration, it was finally determined that the high-efficiency soluble induced expression conditions of proteins were: IPTG concentration of 0.75 mM, overnight induction for 13 h, and centrifugation at 4℃ and 8 000 r/min for 30 min, and bacteria were collect after induced expression. After high-pressure large-scale cell crushing, the supernatant was collected by centrifugation at 16 000 r/min and 4 ℃ for 30 min.

Expression and purification of recombinant proteins N and NH

Pretreatment of resin First, 1 ml of Nisepharosetm 6 Fast Flow resin was washed using pure water with a volume 10 times of the resin, for 3 times. Next, the resin was washed using 10 mmol/L imidazole buffer with a volume 10 times of the resin, for 3 times. Finally, the resin settled naturally, and the waste liquid was discarded.

Loading to column The supernatant prepared in "Pretreatment of resin" was loaded to a resin column to let it pass through the column. The operation was repeated for 5 to 7 times.

Impurity removal and elution of recombinant proteins Each recombinant protein was subjected to washing and elution with 10 mmol/L imidazole buffer, 20 mmol/L imidazole buffer, 40 mmol/L imidazole buffer, 60 mmol/L imidazole buffer, 80 mmol/L imidazole buffer, 100 mmol/L imidazole buffer, 200 mmol/L imidazole buffer and 500 mmol/L imidazole buffer in turn.

Acquisition of proteins The filtrates generated by elution with eluent concentrations of 100, 200 and 500 mmol/L were collected, and the filtrate generated by elution with 500 mmol/L eluent was further purified through Superdex200 gel column molecular sieve produced by GE Company. Finally, the purified protein was added into a dialysis bag (8 kd) for dialysis in 2 L of dialysate overnight, and then stored in a refrigerator below −70 ℃.

Antigenicity analysis (Dot-ElISA) of purified proteins The expressed and purified N and NH proteins were incubated with serum samples negative and positive to PPR V antibody by the DAB staining method, respectively. It was observed that the NC membranes incubated with serum showed no obvious spots in the molecular weight regions corresponding to the antigens.

Establishment and optimization of rapid and quantitative method for detecting PPR V antibody in sheep serum The optimum coating concentration of N protein and the optimum lanthanide fluorescent microsphere labeling concentration of NH protein were determined by checkerboard square titration with a high-sensitivity rapid fluorescence quantitative detection system. The optimal concentration of sealing solution was determined by sealing the chromatographic test strips with different concentrations of skim milk, and the optimal serum dilution ratio and the optimal working time were also determined. Meanwhile, according to the comparison of the titer results of the serum neutralization test with the serum titer results of quantitative system detection, the optimal coating concentration of N protein and the optimal labeling concentration of lanthanide fluorescent microspheres of NH protein were continuously optimized. Criterion for judging the best detection method: The best reaction condition of this method was that the consistency between the titer value of neutralization test on positive and negative serum and the detected titer value of the quantitative system (*T/C* value) was the best.

Determination of sample dilution ratio First, 80 μl of diluted sheep serum samples were added to the prepared chromatographic cards for rapid quantitative detection, respectively, and chromatography was performed while avoiding exposure to strong light for 15 min. The detection cards were put into a Wellray® WR-1608 fluorometer for detection, and the tilter value (*T/C* value) of samples was read after detection. The serum dilution ratio which resulted in the best consistency between the titer value (*T/C* value) of quantitative detection on samples and the titer value of the serum neutralization test was selected.

Determination of critical value for positive and negative samples in the rapid quantitative detection method and determination of quantitative curve Four hundred sheep serum samples which were negative by IDVET kit in our laboratory were tested quickly and quantitatively, and the average value (X) and standard deviation (SD) of the 400 serum samples were calculated. *T/C* value > X + 3SD was judged as positive, and *T/C* value $< \overline{X}$ + 3SD was judged as negative. Meanwhile, a quantitative curve was established by combining the serum *T/C* value obtained by diluting strongly-positive serum and determining the diluted serum with the titer value of the neutralization test.

Specificity test O-type foot and mouth disease virus-positive sheep serum, A-type foot and mouth disease virus-positive sheep serum, Asia-I-type foot and mouth disease virus positive-sheep serum, brucellosis positive serum, caprine arthritis encephalitis-positive serum, *E. coli*-positive sheep serum and PPR V-negative serum were diluted with a sample diluent according to 1 ∶ 200, respectively.

Sensitivity test The serum strongly positive to PPR V antibody (purchased from the WOAH PPR V Reference Laboratory) was diluted at ratios of 1 ∶ 50, 1 ∶ 100, 1 ∶ 200, 1 ∶ 400, 1 ∶ 800, 1 ∶ 1 600, 1 ∶ 3 200, 1 ∶ 6 400, 1 ∶ 12 800 and 1 ∶ 25 600 by the method of doubling dilution using a sample diluent. The established indirect ELISA method was used for detection, and the IDVET antibody detection kit was adopted for comparison, so as to determine the largest dilution ratio at the critical value for positive samples.

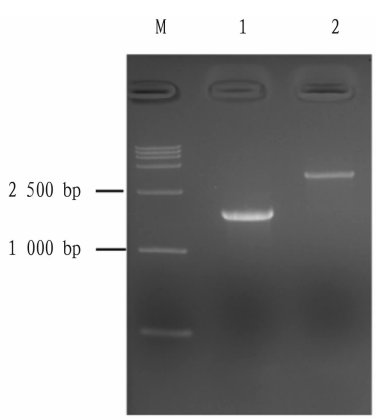
Repeatability test The established rapid quantitative detection method was used to detect 10 sheep serum samples on the same batch of plates and different batches of plates, respectively, and parallel determination was carried out for 5 times. The intra-batch and inter-batch coefficients of variation (CV) were calculated.

Coincidence test The established rapid quantitative detection method and the PPR V competition law antibody diagnostic kit produced by IDVET company were used to detect 292 serum samples, and the coincidence rate was calculated. Meanwhile, compared with the neutralization test of 100 serum samples, the coincidence rate of neutralization titer was calculated.

Results and Analysis

Amplification and fusion of objective genes

The amplification products of target genes N and NH were detected by agarose gel electrophoresis. The results showed that the products were consistent with expected sizes, at about 1 587 bp and 3 456 bp, respectively, as shown in Fig. 1.

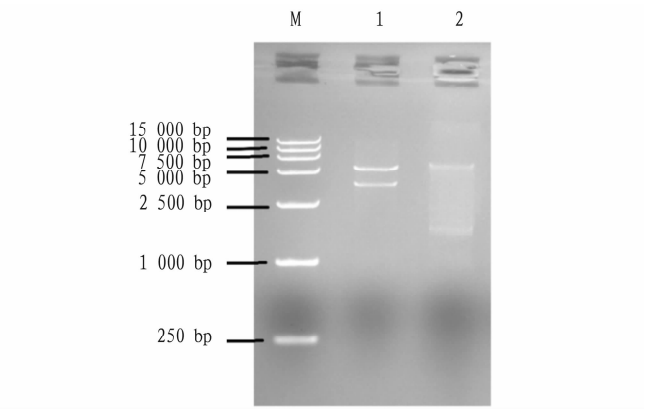


M. DNA Marker; 1. PCR amplification results of N gene; 2. PCR amplification results of NH gene.

Fig. 1 PCR amplification of N and NH genes

Construction and identification of recombinant plasmids

PET-30a-(N) and pET-30a-(NH) plasmids were, respectively, identified by digestion using *Nde* I and *Xho* I. PET-30a-(n) and pET-30a-(NH) showed a vector fragment of about 5Kb both, as well as respective fragments with target fragment sizes, as shown in Fig. 2. The foreign fragments N and NH in the recombinant plasmids pET-30a-(N) and pET-30a-(NH) were subjected to sequencing and splicing, and then aligned with corresponding gene fragments of N and NH registered in Genbank. The results showed that the two target fragments were almost identical to the expected sequences.

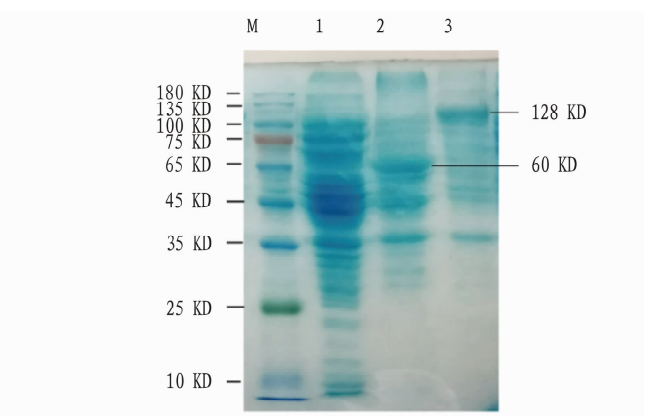


M. DNA Marker; 1. Enzyme digestion results of pET-30a-(N); 2. Enzyme digestion results of pET-30a-(NH).

Fig. 2 Identification of pET-30a-(N) and pET-30a-(NH) plasmids by enzyme digestion

SDS-PAGE analysis

Treated samples of the experimental groups, control groups and protein molecular weight Marker were taken for SDS-PAGE electrophoresis test. The results showed that recombinant *E. coli* strains pET-30a-(N)-BL21 (DE3) and pET-30a-(NH)-BL21 (DE3) could induce the production of N protein and NH protein, respectively, as shown in Fig. 3.

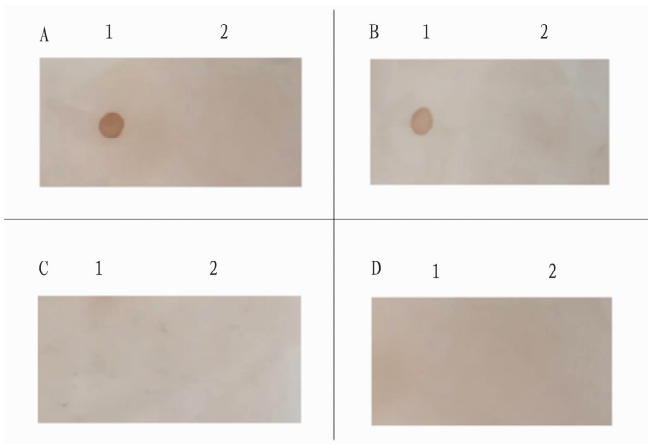


A. M. Protein Marker; 1. Negative control strain supernatant lysate; 2. pET-30a-(N)-BL21 (DE3) strain supernatant lysate; 3. pET-30a-(NH)-BL21 (DE3) strain supernatant lysate.

Fig. 3 Supernatant lysates of pET-30a-(N)-BL21 (DE3) and pET-30a-(NH)-BL21 (DE3) strains

Detection results of antigenicity analysis (Dot-ElISA) of purified proteins

After DAB staining, the NC membranes of recombinant *E. coli* pET-30a-(N)-BL21(DE3) and pET-30a-(NH)-BL21(DE3) strain proteins incubated with positive serum showed an obvious spot in the corresponding molecular weight region of the antigen, respectively, but no spot in other regions. The NC membrane incubated with negative serum exhibited no obvious spots in the corresponding molecular weight regions of antigens, as shown in Fig. 4.



A. Results of N recombinant protein incubated with positive serum; 1. pET-30a-(N)-BL21 (DE3) strain protein incubated with positive serum; 2. Negative control incubated with positive serum; B. NH recombinant protein incubated with positive serum; 1. pET-30a-(NH)-BL21 (DE3) strain protein incubated with positive serum; 2. Negative control incubated with positive serum; C. The results of N recombinant protein incubated with negative serum; 1. pET-30a-(N)-BL21 (DE3) strain protein incubated with negative serum; 2. Negative control incubated with negative serum; D. The results of NH recombinant protein incubated with negative serum; 1. pET-30a-(NH)-BL21 (DE3) strain protein incubated with negative serum; 2. Negative control incubated with negative serum.

Fig. 4 Results of antigenicity analysis (Dot-ELISA) of recombinant proteins N and NH

Establishment and optimization of high-sensitivity rapid quantitative method for detecting PPR V antibody in sheep serum

After gradient dilution, the purified N protein was used for coating chromatographic test strips after dilution. Meanwhile, the NH protein labeled with lanthanide fluorescent microspheres was subjected to doubling dilution, and the coating amount of protein N, the concentration of NH protein labeled with lanthanide fluorescent microspheres and the dilution ratio of serum were determined by chessboard square titration. Moreover, other detection conditions of the rapid quantitative detection method were optimized on the basis of the optimal antigen coating concentration, concentration of NH protein labeled by lanthanide fluorescent microspheres and serum dilution ratio. The results showed that when the coating concentration of N protein was 2 μg/ml, the concentration of NH protein labeled by lanthanide fluorescent microspheres was 0.5 μg/ml, and the dilution ratio of serum was

1∶200, the consistency between the detected titer value (*T/C* value) of PPR-positive and- negative serum and the titer value of the serum neutralization test was the best, and the P/N value between positive and negative serum was the largest. Therefore, it was determined that the optimum coating concentration of N antigen was 2 μg/ml, the concentration of NH protein labeled by lanthanide fluorescent microspheres was 0.5 μg/ml, and the dilution ratio of serum was 1∶200. Meanwhile, it was determined that the best detection steps included adding a 80 μl of diluted serum sample into the sample hole of a card for 15 min of chromatography while avoid exposure to strong light, then putting the detection card into a fluorometer for detection, and finally reading the titer value of the kit after detection.

Determination of critical value for positive and negative samples in the rapid quantitative detection method and determination of quantitative curve

Four hundred negative sheep serum samples were detected by the established high-sensitivity rapid fluorescence quantitative detection method to determine their *T/C* values. The average *T/C* value of 400 negative serum samples was 0.168, and the 3SD was 0.05, so the critical *T/C* value for positive and negative samples, $\overline{X}+3SD$, was 0.218. A quantitative curve was established by combining the serum *T/C* value obtained by diluting the strongly-positive serum and determining the diluted serum, with the titer value of the neutralization test. The critical *T/C* value (0.218) corresponded to 10 titer value of the serum neutralization test, that is, when the titer value of the kit was equal to or greater than 10 titer value, the sample was judged to be positive; and when the titer value of the kit was lower than 10 titer value, the sample was judged to be negative. Other serum *T/C* values were quantified using the quantitative curve by corresponding to corresponding neutralizing titer values in the serum neutralization test.

Specificity test

Type O foot and mouth disease virus-positive sheep serum, type A foot and mouth disease virus-positive sheep serum, type Asia-I foot and mouth disease virus-positive sheep serum, brucellosis-positive serum, caprine arthritis encephalitis-positive serum, *E. coli*-positive sheep serum and PPR V-negative serum were detected by the high-sensitivity rapid fluorescence quantitative detection method. The results showed that the results of this method were negative for all serum samples.

Sensitivity test

The established high-sensitivity rapid fluorescence quantitative detection method was used to detect serum samples strongly positive, positive and weakly positive to PPR V with different dilution ratios, respectively. The results showed that the minimum detection limits of three batches of kits were 1∶800 for strongly-positive serum, 1∶400 for positive serum and 1∶200 for weakly-positive serum.

Repeatability test

After diluting serum samples with different titers by 200 times, parallel repeated detection was made. The results showed that the intra-batch coefficient of variation was less than 10%, and inter-batch coefficient of variation was less than 15%. The results showed that the established high-sensitivity rapid fluorescence quantitative detection method had good repeatability.

Coincidence test

Self-made and commercial kits were used to detect 292 clinical samples, respectively. The results showed that 140 positive samples and 152 negative samples were detected by the self-made kit, and 146 positive samples and 146 negative samples were detected by the commercial kit. The coincidence rates of positive samples and negative samples from the two kinds of test kits were 91.78% and 95.89%, respectively, and the overall coincidence rate was 93.84%. Meanwhile, compared with the titer of the neutralization test on standard positive serum (provided by the OIE Brucellosis Reference Laboratory of France), the detected titer value of the high-sensitivity rapid fluorescence quantitative detection method was consistent with that of the neutralization test on standard positive serum.

Conclusions and Discussion

The routine laboratory detection methods of PPR are relatively mature. RT-PCR, QRT-PCR, virus isolation experiments and antigen capture ELISA can be used for pathogen detection, and neutralization test, competitive ELISA and agar diffusion test can be used for antibody detection. Among them, RT-PCR and QRT-PCR methods are suitable for clinical case confirmation, antigen capture ELISA is suitable for monitoring epidemic-free population, and competitive ELISA is the most widely used serum antibody detection method at present, with mature commercial detection kits. In recent years, the development of new diagnostic techniques for PPR with higher sensitivity and simpler detection operation has become a research hotspot. High-sensitivity rapid fluorescence quantitative detection method is a new detection method with high sensitivity and specificity developed in recent years. This method uses lanthanide fluorescent microspheres as a coating medium, and makes full use of the characteristics of lanthanide elements such as wide excitation spectrum, narrow emission spectrum and large Stokes shift to realize rapid and sensitive detection. Compared with ELISA, the high-sensitivity rapid fluorescence quantitative detection method is more sensitive, specific, and has lower detection background value, higher signal-to-noise ratio and simpler operation, so it is suitable for the rapid detection of a large number of samples and more suitable for the diagnosis of diseases at the grassroots level. Applying lanthanide fluorescent microspheres to develop accurate, sensitive, simple and new rapid PPR V diagnostic techniques can better meet the needs of prevention and control of PPR[11-13].

The high-sensitivity rapid fluorescence quantitative detection method for PPR requires small equipment and can be carried around, so the cloud platform can also realize on-site mobile detection + remote diagnosis + timely data analysis. The establishment of this method provides a very good research exploration for the construction of the Internet of Things for animal disease monitoring.

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