Establishment of Double-antigen Sandwich Time-resolved Fluorescence Immunoassay for Detection of Pest des Petits Ruminants Virus

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Abstract [**Objectives**] This study was conducted to explore rapid and large-scale screening and detection of peste des petits ruminants (PPR), so as to provide important technical means for prevention, control and purification of PPR. [**Methods**] Soluble N protein and NH fusion protein were successfully obtained in an *Escherichia coli* expression system by optimizing *E. coli* codon and expression conditions. Furthermore, based on purified soluble N protein and NH fusion protein, a double-antigen sandwich time-resolved fluorescence immunoassay method for detection of peste des petits ruminants virus (PPRV) was established. [**Results**] The method has high sensitivity and specificity and can specifically detect the antibody against PPRV in sheep serum, and it has no cross reaction with other related diseases. The method was used to detect 292 clinical samples, and compared with French IDVET competition ELISA kit. The coincidence rates of positive samples and negative samples from the two kinds of test kits were 92.47% and 97.26%, respectively, and the overall coincidence rate was 94.86%. The intra-group and inter-group coefficients of variation in the repeatability test were less than 10%. [**Conclusions**] Compared with the traditional ELISA method, the double-antigen sandwich time-resolved fluorescence immunoassay for detection of PPRV has equivalent sensitivity and specificity, and simple and rapid operation, and thus high application and popularization value.

Key words Peste des petits ruminants; N active protein; NH fusion protein; Soluble expression and purification; Time-resolved fluorescence immunoassay

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Peste des petits ruminants (PPR) is an acute, febrile and highly contagious disease caused by peste des petits ruminants virus (PPRV), which has the characteristics of high incidence rate and mortality. 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[1-3]. The disease first occurred in Côte d'Ivoire, Africa in 1942, and is currently prevalent in the Middle East, West Africa, the Arabian Peninsula and South Asia. 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[4-8]. The control and elimination of epidemic diseases cannot be separated from effective vaccines and rapid and sensitive detection methods as technical support. Time-resolved fluorescence immunoassay (TRFIA) is a diagnostic and detection method with high sensitivity, strong specificity and simple and rapid operation. In this method, rare earth ion chelates are usually used as labels of antigens or antibodies. For example, europium (Eu) is used for labeling antigens or antibodies. Based on the luminescent characteristics of rare earth element chelates, the time-resolved technique is adopted to measure fluorescence, realizing simultaneously detection of wavelength and time parameters for signal resolution, which can effectively eliminate the interference of non-specific fluorescence and greatly improve the analytical sensitivity. At present, this method is widely used in the field of human infectious disease diagnosis and detection, but there is very little research in the field of animal disease diagnosis and detection, and there are no registered products on the market. Accelerating the launch of related products can provide strong technical support for the implementation of the national plan for the elimination of PPR in China, and has high practical and promotional 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. Recombinant *Escherichia coli* strains pET-30a-(N)-BL21 (DE3) and pET-30a-(NH)-BL21(DE3) were identified, preserved and supplied by Center for Disease Control and Prevention, P. R. China. Skim milk powder was purchased from Oxoid, USA. ELISA plates were purchased from Costar Group, USA. Europium (Eu) labeling element (DTTA-Eu NA) was purchased from Guangzhou Darui Biotechnology Co., Ltd. Negative and positive serum samples were preserved by the Chinese Center for Disease Control and Prevention (Veterinary Diagnosis Center of the Ministry of Agriculture and Rural Affairs).

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 *E. 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: catatgGCGACCTTACTGAAAAG CCTGGCG;

Reverse primer PPRV-NR: ctcgag ACCCAGCAGGTCTTTGT CGTTGT.

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: catatgGCGACCTTACTGAA AAGCCTGGCG;

Reverse primer PPRV-NHR: ctcgag AACCGGGTTGCAGGT-GACTTCAAT.

A linker sequence (GGTGGGTGGATCGGGGGGGGGGAAG CGGGGGGGGGGGAGC) 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 rpm 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 rpm for 30 min, and bacteria were collected after induced expression. After high-pressure large-scale cell crushing, the supernatant was collected by centrifugation at 16 000 rpm 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 three times. Next, the resin was washed using 10 mmol/L imidazole buffer with a volume 10 times of the resin, for two 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 five to seven 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 beaten 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 PPRV 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.

Preparation of Eu-labeled antigen conjugate

Pretreatment of antigen A certain amount of NH fusion antigen was added into an ultrafiltration tube with molecular weight cut off 50 KD, and centrifuged at 9 000 rpm for 5 min, and the filtrate was discarded. Next, 200 μl of labeling buffer was added, and centrifugation was performed at 9 000 rpm for 5 min. After discarding the filtrate, 200 μl of labeling buffer was added again, and centrifugation was performed as described above (at 9 000 rpm for 5 min). This step was repeated for six times. After the last centrifugation, the final volume was controlled to 150 − 200 μl. Subsequently, the ultrafiltration tube was taken out, and the filtrate was discarded. Finally, the ultrafiltration tube was turned over in a new collection tube, which was then centrifuged at 8 000 rpm for 2 min to collect the filtrate.

Labeling of antigen According to the requirement of 0.2 mg of DTTA-Eu NA labeling reagent for labeling of 1 mg of antigen, the antibody to be labeled and the labeling reagent were fully mixed according to the mass ratio of 5∶1, and the mixture was incubated overnight at 25 ℃ on a shaker.

Purification of Eu-labeled antigen The Eu-labeled antigen was purified by SephadexTM G-50 column gel chromatography. The specific steps were as follows: (1) Sephadex TM G-50 packing was mixed well with water, and loaded into a column. The column was treated with a purification column equilibrium buffer. In specific, the column was washed with five column volumes of buffer while controlling the flow rate at 1 ml/min, making the pH of the column reach 7.8. At this time, sample loading could be carried out. (2) After the column was equilibrated, the valve port at the upper end of the column was removed. When the liquid level in the column dropped to the plane of the gel column, the sample could be drawn and slowly added into the column. The plane of the column should not be destroyed. After the liquid level settled slightly, 1 ml of purification column eluent was added, and the column was reinstalled. (3) Elution was performed with eluent while controlling the flow rate at about 1 ml/min. The collection of sample was started when the protein detection showed that there was a protein peak ($OD_{280\ nm}$), according to 1 ml/tube. (4) After the collection of the labeled conjugate was completed, 5 μl was taken from each tube and added to a 96-well ELISA plate, and then, 200 μl of enhancement liquid was added. The fluorescence value was measured after shaking on an oscillator for 5 min, and several tubes with higher fluorescence values were collected.

Preparation of Eu label diluent Tris-base 30.3 g, NaCl 45 g, NaN_3 5 g, Tween-20 0.5 ml, skimmed milk powder 25 g and casein 16 g (pH = 7.8) were fully mixed to obtain a mixture, which was diluted to 500 ml. The obtained liquid was filtered with a 0.22 μm filter to obtain a filtrate, which was stored at 4 ℃.

Preparation of Eu-labeled conjugate The Eu-labeled anti-NH antigen was diluted to 9 μg/ml with the Eu label diluent. The obtained liquid was quantitatively packaged in a sterile condition according to different specifications and labeled.

Preservation of labeled conjugate After combining the collected labeled conjugate, the concentration of labeled antibody was detected by the BCA method. Next, 10% BSA was added to a final concentration of 0.1%, and the concentration was measured after mixing well. The name of the labeled conjugate, the date of labeling and other information were recorded, and it was then stored at −20 ℃. Three batches were labeled continuously by the same process, and the batch numbers were 4F12E-01, 4F12E-02 and 4F12E-03.

Coating and sealing of N antigen Coating: The purified N antigen of PPR was diluted from 32 to 0.125 μg/ml with carbonate coating solution with pH 9.6, and according to 100 μl/well, the obtained liquids were added to a Costa ELISA plates, which were placed at 2 − 8 ℃ for 12 − 16 h. Sealing: The coated plates were taken out and washed with PBST for 3 times, and sealed with 5% skim milk according to 350 μl/well. After standing at 37 ℃ for 2 h, washing after sealing was performed as above. Negative control and positive control were detected by prepared reaction plates with different concentrations, and diluted enzyme-labeled monoclonal antibody was added for reaction. Finally, the fluorescence detection values and *P/N* values were compared, and the coating concentration with the largest *P/N* value was selected as the best coating concentration.

Determination of optimal working concentration of Eu-labeled antigen Negative control and positive control were added into coated N antigen plates, and the Eu-labeled conjugate was diluted from 36 to 0.6 μg/ml by the method of doubling dilution with the Eu label diluent, and the diluted liquids were added into reaction plates, respectively. Other steps were carried out according to routine operation. The fluorescence detection values and P/N values were compared, and the working concentration of Eu-labeled conjugate with the detection value of the standard positive control at about 50 000 and the largest *P/N* value was selected as the optimal working concentration of Eu-labeled antigen.

Determination of sample dilution ratio Into prepared reaction plates, PPRV antibody-negative and positive sheep serum samples subjected to doubling dilution were added. Under the same other

conditions, time-resolved fluorescence immunoassay was carried out to select the dilution ratio with the highest positive value and the largest *P/N* value.

Selection of optimal reaction time for antigen Four groups of negative and positive controls were added to prepared reaction plates, and they were oscillated at room temperature for 30, 60, 90 and 120 min, respectively, according to established procedures. Under the same other conditions, time-resolved fluorescence immunoassay was carried out, and the action time giving the highest *P/N* value while being the shortest was determined as the optimal reaction time.

Selection of optimal reaction time for Eu-labeled conjugate Four groups of negative and positive controls were added to prepared reaction plates, and according to established procedures, diluted Eu-labeled conjugate solution was added, and the plates were oscillated at room temperature for 30, 60, 90 and 120 min, respectively. Under the same other conditions, time-resolved fluorescence immunoassay was carried out, and the action time giving the highest *P/N* value while being the shortest was determined as the optimal reaction time.

Determination of critical value for positive and negative samples The established time-resolved fluorescence immunoassay method was used to detect 400 negative sheep serum samples to obtain the fluorescence detection values, the average value X and standard deviation SD of the 400 negative serum samples. The fluorescence value of the critical value $\overline{X}+3SD$ for positive and negative samples was calculated. The *S/N* value of the critical value for determining positive and negative samples was calculated according to following *S/N* formula: *S/N* = (Critical fluorescence value-Fluorescence value of negative quality control serum)/(Fluorescence value of positive quality control serum-Fluorescence value of negative quality control serum).

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, PPRV-negative serum and 30 known uninfected animal serum samples were diluted with a sample diluent according to 1∶200, respectively.

Sensitivity test The serum strongly positive to PPRV antibody 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.

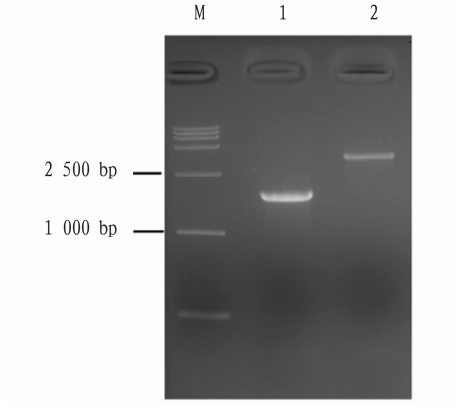
Repeatability test The established rapid quantitative detection method was used to detect 10 sheep serum samples on the same batch of plate and different batches of plates, respectively, and parallel determination was carried out for 5 times. The intra-batch and inter-batch coefficients of variation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

Compliance test The established rapid quantitative detection method and the PPRV competition law antibody diagnostic kit produced by IDVET company were used to detect 292 serum samples, and the compliance rate 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 the expected sizes, about 1 587 bp and 3 456 bp, respectively, as shown in Fig. 1.



M. DNA Marker DL 15 000; 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.

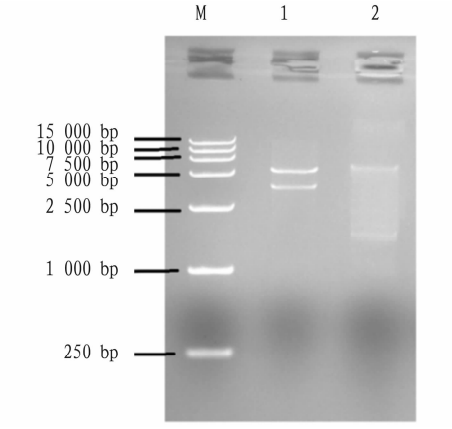
SDS-PAGE analysis

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.

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

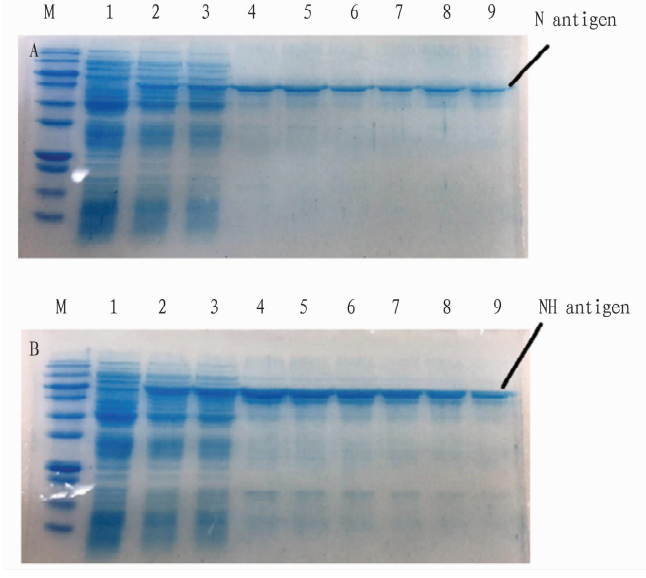
After DAB staining, the NC membranes of recombinant *E. coli* pET-30a-(N)-BL21 (DE3) and pET-30a-(NH)-BL21 (DE3) strain proteins incubated with positive serum showed an obvious spot in the corresponding molecular weight region of the antigen, respectively, but no spot in other regions. The NC membrane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

the corresponding molecular weight regions of antigens, as shown in Fig. 4.



M. DNA Marker DL 15 000; 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

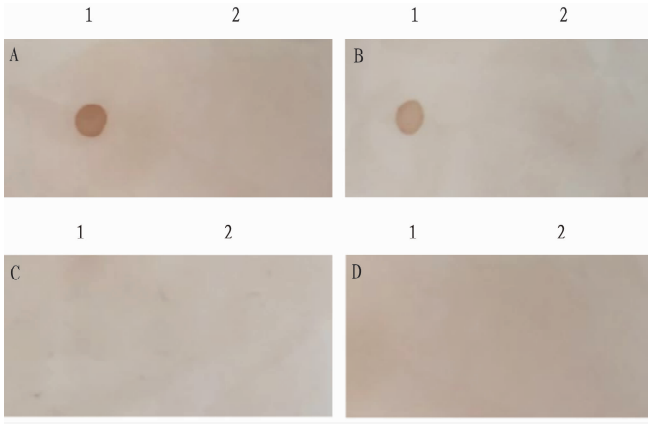


A. M. Protein molecular weight Marker; 1. Negative control; 2,3. pET-30a-(N)-BL21 (DE3) strain supernatant lysate; 4 – 8. Gradient purified N antigen; 9. Final purified N antigen. B. M. Protein molecular weight Marker; 1. Negative control; 2,3. pET-30a-(NH)-BL21 (DE3) strain supernatant lysate; 4 – 8. Gradient purified NH antigen; 9. Final purified NH antigen.

Fig. 3 Expressions and purification of N and NH antigens

Selection of optimal coating concentration for N antigen and optimal dilution concentration for Eu-labeled NH antigen

When the coating concentration of N antigen was 1 μg/ml and the concentration of Eu-labeled NH antigen was 9 μg/ml, the P/N value of the standard negative and positive controls was the largest. Therefore, the optimal coating concentration for N antigen was 1 μg/ml, and the optimal concentration of Eu-labeled NH antigen was 9 μg/ml.



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

Sample dilution ratio

It was finally determined by comparing the results of different dilution ratios for the three kinds of samples that the sheep serum sample diluted by 200 times showed the highest positive value and the largest negative and positive difference (*P/N* value).

Selection of reaction time for antibody to be detected

It was finally determined by comparing the results of different reaction time for the antibody to be detected that the best reaction time was 60 min of room temperature oscillation, with which the *P/N* was close to the maximum, and the reaction time was relatively short.

Selection of reaction time for Eu-labeled antigen

It was finally determined by comparing the results of different reaction time for Eu-labeled antigen that the best reaction time was 30 min of room temperature oscillation, which achieved the largest *P/N* and short reaction time.

Determination of critical value for positive and negative samples by time-resolved fluorescence immunoassay

The established time-resolved fluorescence immunoassay method was used to detect 400 negative sheep serum samples. The fluorescence detection values, the average value X and standard deviation SD of the 400 negative serum samples were determined, and the fluorescence value of the critical value $\overline{X} + 3SD$ for positive and negative samples was calculated. According to the S/N formula: S/N = (Critical fluorescence value-Fluorescence value of negative quality control serum)/(Fluorescence value of positive quality control serum-Fluorescence value of negative quality control serum), the S/N value of the critical value for positive and

negative samples was 0.2. That is, when the *S/N* value of the sample was equal to or greater than 0.2, the sample was determined to be positive, and when the *S/N* value was less than 0.2, the sample was judged to be negative.

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 PPRV-negative serum were detected by time-resolved fluorescence immunoassay. The results showed that the results of this method were negative for all serum samples.

Sensitivity test

The established time-resolved fluorescence immunoassay method was used to detect serum samples strongly positive, positive and weakly positive to PPRV with different dilution ratios, respectively. The results showed that the minimum detection limits of the three batches of kits were 1 ∶ 1 600 for strongly positive serum, 1 ∶ 800 for positive serum and 1 ∶ 400 for weakly positive serum.

Repeatability test

After diluting serum sample with different titers by 200 times, parallel repeated detection was made. The results showed that the intra-batch and inter-batch coefficients of variation were less than 10%. The results showed that the established time-resolved fluorescence immunoassay method had good repeatability.

Coincidence test

Self-made and commercial kits were used to detect 292 clinical samples, respectively. The results showed that 139 positive samples and 153 negative samples were detected by time-resolved fluorescence immunoassay, and 146 positive samples and 146 negative samples were detected by the commercial kits. The coincidence rates of positive samples and negative samples from the two kinds of test kits were 92.47% and 97.26%, respectively, and the overall coincidence rate was 94.86%.

Conclusions and Discussion

With the development of molecular biology, material science, information science and other technologies, rapid and sensitive new diagnostic techniques have become a research hotspot in recent years. Time-resolved fluorescence immunoassay is a special fluorescence analysis method, which makes use of the difference between the wavelength of fluorescence and its excitation wavelength, overcomes the influence of variegated light in ordinary ultraviolet-visible spectrophotometry, and improves the specificity of detection. Meanwhile, the photoelectric receiver of time-resolved fluorescence is not on the same straight line as the excitation light, so the excitation light cannot directly reach the photoelectric receiver, thus improving the sensitivity of optical analysis. Moreover, different from the traditional detection method based on HRP enzyme-labeled antigens or antibodies, this method usually uses rare earth elements to label antigens or antibodies, and relies on the luminescent characteristics of rare earth element chelates to simultaneously detect wavelength and time parameters for signal resolution, thereby effectively eliminating the interference of non-specific fluorescence. In this study, the rare earth element Eu was selected as the label. Eu chelates are chelating agents with a bifunctional group structure, one end of which can be connected with Eu, and the other end can be connected with the free amino group on an antibody/antigen molecule to generate a kind of Eu-labeled antibody/antigen, which forms an immune complex after immune reaction. Meanwhile, in order to overcome the weak fluorescence intensity of Eu-labeled antigens in water, an enhancer was added to the reaction system to dissociate Eu from the complex, and free Eu chelate with the enhancer to form a colloidal molecular group, which could emit strong fluorescence under the excitation of ultraviolet light, and the signal was enhanced by millions of times. Time-resolved fluorescence immunoassay has mature application in the detection of hormones, viral hepatitis markers and tumor-associated antigens.

Conventional detection methods of PPR are relatively mature. Etiological detection methods include traditional pathogen isolation methods, RT-PCR methods, and commercial antigen capture ELISA kits. Serological detection methods include competition ELISA, indirect ELISA, and commercial kits. However, at present, the detection of PPR lacks sensitive and rapid diagnostic techniques. The genome of PPR encodes six structural proteins, and includes N gene, which is 1 575 bp in length, encoded by 525 amino acids and has a molecular weight of 58 KD. H gene has a full length of 1 852 bp, which is encoded by 609 amino acids, and its molecular weight is 68 KD[9-10]. The antigenicity of N and H proteins is stable, and the antibodies against N and H proteins are dominant in the serum of animals infected by the virus, so they are good target genes serving as diagnosis antigens[11-15]. In this study, N and H proteins with good antigenicity of PPR were used, and in order to improve the sensitivity of the detection kit, an NH fusion protein was synthesized by chemical synthesis, and used as the raw material for the double-antigen sandwich time-resolved fluorescence immunoassay detection kit together with N protein. The antibody detection kit established in this study has high sensitivity and simple and rapid operation, and can be used as an alternative method of ELISA for antibody detection of PPR. This method is suitable for rapid screening and detection of PPR by all levels of veterinary departments at the grassroots level and entry-exit inspection and quarantine bureaus, and provides important technical means for prevention, control and purification of PPR.

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successfully solves the key technical problems of comprehensive prevention and control of new SCWL in large area in sugarcane production. A cooperative extension model of "sugar factory + scientific research + agricultural department + farmers" was constructed and has been successfully applied in a large area, realizing the scientific prevention and control of SCWL, preventing new SCWL phytoplasmas from invading from abroad with introduction from the source. These measures have effectively curbed the spread of SCWL phytoplasmas in domestic inter-provincial sugarcane areas, and provide key technical support and safety guarantee for high-quality development, loss reduction and efficiency improvement of sugar industry in major producing areas such as Yunnan, Guangxi and Guangdong.

Popularization and Application of Prevention and Control Techniques has Achieved Remarkable Social and Economic Benefits

Over the years, based on "enterprise as the main body and industry as the guidance", the collaborative promotion model of "sugar factory + scientific research + agricultural department + farmers" has been built for demonstration and promotion. Prevention and control techniques of new SCWL have been widely promoted and applied in nine counties and cities of three main producing prefectures and cities in Yunnan Province, where SCWL occurs. The harm has been controlled and the prevention and control effect is significant. From 2021 to 2023, the techniques were promoted and applied in 151 khm^2 of producing areas totally, saving 1.81 Mt of sugarcane harvest from pest control and reducing 233 kt of "sugar loss". The sales revenue was increased by 2.156 billion yuan, and the profit was increased by 805 million yuan, and the tax was increased by 93 million yuan. The promotion and application of these achievements have successfully solved the bottleneck of comprehensive prevention and control of new SCWL in sugarcane production, achieved scientific prevention and control of SCWL, and effectively curbed the spread of SCWL, making significant contributions to the development of border ethnic groups, frontline economy, farmers' income increase, enterprise efficiency improvement, and rural revitalization.

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