Development of an Indirect ELISA with Artificially Synthesized N Protein of PPR Virus

Guo-rui Zhang^a Jiang-yong Zeng^c Yu-min Zhu^b Shi-juan Dong^b Se Zhu^c
Rui-song Yu^b Ciren Duoji^c Zhi-hai Lei^a Zhen Li^b

^aCollege of Veterinary Medicine, Nanjing Agricultural University, Nanjing, ^bInstitute of Animal Sciences and Veterinary Medicine and Shanghai Key Laboratory of Agricultural Genetics and Breeding, Shanghai Academy of Agricultural Sciences, Shanghai, and ^cTibet Livestock Research Institute, Tibet Academy of Agricultural and Animal Sciences, Lhasa, China

Key Words

Indirect ELISA • Peste des petits ruminants virus •
Nucleocapsid protein • Nucleocapsid gene •
Artificial gene synthesis

Abstract

The full-length gene encoding the nucleocapsid (N) protein of the virus (PPRV) responsible for an outbreak of peste des petits ruminants in Tibet in 2007 was synthesized in two stages using overlapping PCR without the need for viral genomic cDNA as template. The full-length N gene was successfully expressed in *Escherichia coli*, and the purified gene product bound to monoclonal antibody raised against PPRV N protein. Furthermore, it was able to replace recombinant B-N antigen as the coating antigen in a commercial ELISA kit prepared with another PPRV strain. Recombinant protein was employed as the coating antigen to develop an indirect ELISA for PPRV antibody detection in the sera of infected small ruminants. Antibody detection was optimal at a 1:200 serum dilution and an antigen concentration of 3.2 µg/ml, and the positive threshold (cutoff) value of the assay was 2.18. Analysis of 697 serum samples revealed the sensitivity and specificity of the indirect ELISA to be 96.7 and 96.1%, respectively, compared with a commercially available ELISA test.

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Introduction

Peste des petits ruminants (PPR) is an acute, highly contagious and economically important viral disease with morbidity and mortality rates as high as 100 and 90%, respectively [1]. The disease, a contagion in goats and sheep, is characterized by severe pyrexia, oculonasal discharges, necrotizing and erosive stomatitis, enteritis and pneumonia [2–4]. PPR was initially epidemic in the Ivory Coast, West Africa [5], and later spread across sub-Saharan Africa, the Arabian Peninsula and south-west Asia [6]. In July 2007, a PPR outbreak in the southwestern Ngari region of Tibet resulted in heavy goat mortality and huge economic losses to local farmers [7]. PPR is classified as a rapidly spreading and transboundary disease, and is compulsorily notifiable to the Office International des Epizooties [8].

PPR virus (PPRV), the causative agent of PPR, belongs to the genus *Morbillivirus* of the family Paramyxoviridae [9], has a single-stranded, negative-sense RNA genome encoding eight proteins in the order 3'-N-P/C/V-M-F-H-L-5', and is antigenically related to rinderpest virus (RPV) [10]. Although only one PPRV serotype is recognized, PPRV isolates have been classified into four lineages (lineages 1–4) based on molecular epidemiological studies.

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Prof. Zhen Li
Institute of Animal Sciences and Veterinary Medicine and Shanghai Key Laboratory of Agricultural Genetics and Breeding, Shanghai Academy of Agricultural Sciences
Shanghai 201106 (China)
Tel. +86 21 6220 0389, Fax +86 21 6220 7858, E-Mail zhenli60@163.com

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Lineages 1–3 have been reported in Africa and lineage 4 in Arabia, Southern Asia and the Middle East. The PPR vaccine viruses Nigeria75/1 and Sungri/96 belong to lineages 1 and 4, respectively [11, 12]. Phylogenetic analysis has confirmed that the Tibetan PPRV strain is a lineage 4 virus and is closely related to viruses currently circulating in neighboring countries of southern Asia [7].

Materials and Methods

Table 1. Sequences of primers used for DNA synthesis

Primer	Number of bases	Sequence (5' to 3')
P1	54	ATGGCTACTTTATTAAAAATCTTTAGCTTTATTTAAACGTAATAAAGATAAAGCT
P2	60	AATACCACGAATAGCACCACCAGAACCAGAAGCAGTAGGAGCTTTATCTTTATTACGTTT
P3	60	GGTGGTGCTATTCGTGGTATTAAAAATGTTATTATTGTTCTATTCCTGGTGATTCTTCT
P4	60	ACGAACTAAACGATCTAATAAACGAGAACGAGTAGTAATAGAAGAATCACCAGGAATAGG
P5	60	TTATTAGATCGTTTAGTTTCGTTTAGCTGGTGATCCTGATATTAATGGTTCTAAATTAAC
P6	60	AGATTCAACAAATAAAGATAACATAGAAATCATAACACCAGTTAATTTAGAACCATTAAT
P7	60	TTATCTTTATTTGTTGAATCTCCTGGTCAATTAATTCACCGTATTACTGATGATCCTGAT
P8	60	ACGAGTAGATTGAACAACTTCAACTAAACGAATAGAAACATCAGGATCATCAGTAATACG
P9	60	GAAGTTGTTCAATCTACTCGTTCTCAATCTGGTTTAACTTTTGCTTCTCGTGGTGCTGAT
P10	60	ACCTTCAGTAGAAAAATACATATCAGCTTCATTATCTAAATCAGCACCACGAGAAGCAAA
P11	60	ATGTATTTTCTACTGAAGGTCTTCTTCTGGTGGTAAAAACGTATTAATTGGTTTGAA
P12	60	TTCAGGATCTTGAACCTCAATATCAATAATTCACGATTTTCAAACCAATTAATACGTTT
P13	60	ATTGAAGTTCAGATCCTGAAGAATTTAATATGTTATTAGCTTCTATTTTAGCTCAAGTT
P14	60	AGTATCAGGAGCAGTAACAGCTTTAGCTAATAAAATCCAACTTGAGCTAAAATAGAAGC
P15	60	GCTGTTACTGCTCCTGATACTGCTGCTGATTCTGAATTACGTCGTTGGGTAAATATACT
P16	60	TTTATCTAAACGAAATTCACCAATAACACGACGTTGTTGAGTATATTTAACCCAACGACG
P17	60	GGTGAATTTTCGTTTAGATAAAAGTTGGTTAGATGCTGTTTCGTAATCGTATTGCTGAAGAT
P18	60	ATCTAAAATTAAGAAACCATAAAACGACGTAAGATAAAATCTTCAGCAATACGATTACG
P19	60	ATGGTTTCTTTAATTTTAGATATTAACGTAATCCTGGTAATAAACCTCGTATTGCTGAA
P20	60	ACCAGCTTCAACAATATAATTATCAATATCACAAATCATTTTCAGCAATACGAGGTTTATT
P21	60	AATTATATTGTTGAAGCTGGTTTAGCTTCTTTATTTTAACTATTAAATTTGGTATTGAA
P22	60	ACCAGCAAATTCATGTAAACCTAAAGCAGGATACATAGTTTCAATACCAAATTTAATAGT
P23	60	GGTTTACATGAATTTGCTGGTGAATTATCTACTATTGAATCTTTAATGAATTTATATCAA
P24	60	TTCTAAAATAACCATATAAGGAGCAACTTCACCTAATGTTGATATAAATTCATTAAAGA
P25	60	CCTTATATGGTTATTTTAGAAAATTTCTATTCAAATAAAATTTTCTGCTGGTGCTTATCCT
P26	60	TAATTCAACACCAACACCCATAGCATAAGACCATAATAAAGGATAAGCACCAGCAGAAAA
P27	60	ATGGGTGTTGGTGTGAATTAGAAAATTCTATGGGTGGTTTAAATTTTGGTCGTTCTTAT
P28	60	AACCATTTCTTGACCTAAACGAAAATAAGCAGGATCAAATAAGAACGACCAAAATTTAA
P29	60	CGTTTAGGTCAAGAAATGGTTCGTCGTTCTGCTGGTAAAGTTTCTTCTGTTATTGCTGCT
P30	60	AGAACTAATTTAGCTTCTTCAGCAGTAATACCTAATTCAGCAGCAATAACAGAAGAAAC
P31	60	GAAGAAGCTAAATTAGTTTCTGAAATTGCTTCTCAAGCTGGTGATGAACGTACTGCTCGT
P32	60	TTGTAAAAAAGAACTTGAGCTTGACGAGGACCAGTACCACGAGCAGTACGTTTCATCACC
P33	60	GCTCAAGTTTCTTTTTTACAACATCAAACCTGGTGGTGGTGAATCTTCTGCTCCTGCTACT
P34	60	TTCAGAACCATTAGGAATAGCAGCTTTAACACCTTCACGAGTAGCAGGAGCAGAAGATTC
P35	60	GCTATTCCTAATGGTTCTGAAGAACGTGATCGTAAACAACTCGTCCTGGTCGTCCTCGT
P36	60	AGGCATAATTTCTAATAATAATTGACCAGGAGTTTACCACGAGGACGACCAGGACGAGT
P37	60	TTATTATTAGAAATTATGCCTGAAGATGAAGTTTCTCGTGAATCTGGTCAAAATCCTCGT
P38	60	TTGTAAACGAAATAAAGCTTCAGCAGAACGTTGAGCTTCACGAGGATTTTGACCAGATTC
P39	60	GAAGCTTTATTTTCGTTTACAAGCTATGGCTAAAATTTTAGAAGATCAAGAAGAAGGTGAA
P40	63	TTAACCTAATAAATCTTTATCATTATAAACTTGATTATTATCTTCACCTTCTTCTTGATCTTC

30 s, then a final extension at 72° for 10 min. Amplified fragments were purified with a Gel Extraction Kit (Axygen China). In the second stage, the full-length RN gene was obtained using overlapping PCR with the 405–414 bp fragments prepared above. The PCR reaction system consisted of: PrimeSTAR HS DNA polymerase (Takara) 1 μ l, 5 $\times$ PrimeSTAR Buffer (Mg²⁺ plus; Takara) 10 μ l, outer (primer) oligonucleotides (30 μ M; primer 1 and primer 40) 1 μ l $\times$ 2, fragment 1–4 (100 μ g/ml) 2 μ l $\times$ 4, and ddH₂O 29 μ l. Reaction conditions were as follows: 94° for 10 min, 25 cycles of 94° for 30 s, 58° for 15 s, and 72° for 100 s, followed by a final extension of 72° for 10 min. Amplified fragments were purified as above, cloned into the simple pMD19-T vector (Takara) and sequenced (Shanghai Shenggong Bio, Co., Ltd.). The vector carrying the targeted fragment was designated as pMD19-T-RN'.

Site-Directed Mutation Correction

Point mutations in RN were corrected with a set of new complementary primers (N1–N8) containing the modified nucleotide. Firstly, five fragments were amplified by PCR using primer pairs P1/N2, N1/N3, N4/N6, N5/N8 and N7/P40, and pMD19-T-RN' as the template (table 2). PCR was then performed with primers P1/P40 using the products above as the templates, to synthesize the full-length corrected sequence which was then cloned into the simple pMD19-T vector and sequenced.

Construction of the Expression Vector

The RN gene was fused with the expression vector pET-32a using innovative homologous recombination technology (CloneEZ™ Kit, GenScript Corporation). Homologous recombination primers carried tags of fifteen bases at their 5' ends complementary to either side of the vector linearization site. The primers were: PPRNF – 5'AAGGCCATGGCTGATATGGCTACTTTATTAAAATC3' and PPRNR – 5'GAATTCGGATCCGATTAAACCTAATAAATCTTTAT3'. Amplification conditions were: 4° for 10 min, 25 cycles at 94° for 30 s, 58° for 15 s and 72° for 2 min, with a final extension of 72° for 10 min. The amplicon and linearized vector were purified using the AxyPrep™ PCR Cleanup Kit (Axygen China). The expression construct with the RN gene sequence was designated as pET-32a-RN.

Prokaryotic Expression of the Synthesized Recombinant PPRV N Gene

Fresh Luria-Bertani medium (containing 50 μ g/ml penicillin) was inoculated with an overnight culture of BL21-pET-32a-RN and incubated at 37° with shaking (200 rpm) until the cells had reached mid-log phase (OD₆₀₀ = 0.6–0.8). Induction of recombinant protein synthesis was initiated by addition of isopropyl β -D-1-thiogalactopyranoside to a final concentration of 1 mM, after which the cell suspension was maintained at 30° for 4–6 h.

SDS-PAGE and Western Blotting

Proteins were separated by SDS-PAGE (5% w/v stacking gel, 10% w/v resolving gel) and transferred onto a polyvinylidene fluoride membrane (JingKeHongDa Biotechnology, Co., Ltd.) [14], which was then blocked with 3% (w/v) skimmed milk powder in phosphate-buffered saline (PBS) for 1 h at room temperature. Expressed recombinant proteins on the membrane were stained by incubation with anti-PPRV N monoclonal antibody (mAb) and anti-mouse HRPO conjugate (Abgent Primary Antibody Company) with diaminobenzidine as the chromogen. mAb against N protein was donated by the China Animal Health and Epidemiology Center.

Table 2. Primers for site-directed correction of mutations

Primer	Sequence (5' to 3')	Number of bases
N1	CTTTATTTGTTGAATCTCCTGGTC	24
N2	GACCAGGAGATTCAACAAATAAAG	24
N3	ATTGCTGAAGATTTATCTTTACGTC	25
N4	GACGTAAAGATAAATCTTCAGCAAT	25
N5	ATTTTGATCCTGCTTATTTTCGTTT	25
N6	AAACGAAAATAAGCAGGATCAAAAT	25
N7	CTCAAGTTTCTTTTTTACAACATCA	25
N8	TGATGTTGTAAAAAAGAAACTTGAG	25

Purification of Recombinant N Protein

LB medium (50 ml) was harvested 4 h after induction and centrifuged at 6,000 g for 10 min. The pellet was resuspended in 4 ml of guanidium lysis buffer (4 M guanidium hydrochloride, 10 mM NaH₂PO₄, 10 mM NaH₂PO₄, 500 mM NaCl, pH 7.8) by gentle rocking at room temperature for 15 min, followed by three 5-second bursts of ultrasound (with 10-second pauses in between) while immersed in ice. The lysate was centrifuged at 10,000 g for 15 min at 4°, and the supernatant was loaded onto a column containing nickel chelated agarose (Sigma) equilibrated with binding buffer (6 M urea, 10 mM NaH₂PO₄, 10 mM NaH₂PO₄, 500 mM NaCl, pH 7.8). The column was washed with binding buffer and washing buffer (20 mM imidazole, 10 mM NaH₂PO₄, 10 mM NaH₂PO₄, 500 mM NaCl, pH 7.8), and non-targeted proteins were removed by sequential elution with buffer (10 mM NaH₂PO₄, 10 mM NaH₂PO₄, 500 mM NaCl, pH 7.8) containing 100, 200 and 300 mM imidazole. Recombinant N protein was eluted with 500 mM imidazole buffer, and the final concentration of purified protein was estimated colorimetrically with a Micro BCA Protein Assay Kit (Pierce, Rockford, Ill., USA) at 550 nm using an ELISA reader.

Commercial c-ELISA Kit for Detection of Antibodies against PPRV

The c-ELISA kit for determining antibodies against PPRV was obtained from Cirad Bios, UPR15, TA A-15/G, Campus International Baillarguet, Montpellier, France. Manipulation of the c-ELISA was based on the method described by Libeau et al. [15], in which recombinant B-N antigen was used as the coating antigen, and serum sample and mAb competed to combine with B-N antigen. Percentage inhibition (PI) values were obtained by detecting the amount of labeled secondary antibody. Samples with c-ELISA PI values of ≤ 50 and > 50 were considered to be PPRV antiserum negative and positive, respectively.

Serum Samples

Based on the above criteria, 290 serum samples (87 goat and 203 sheep) collected from Tibetan farms were PPRV negative, while 209 samples (98 goat and 111 sheep) were PPRV positive. The majority of positive samples (n = 150: 76 goat, 64 sheep) with PI values of 50–96 were collected from animals raised in an area where vaccination was carried out with live-attenuated PPRV vaccine. Other positive samples (n = 59; PI ≥ 55) were collected from goats (n = 22) and sheep (n = 37) from nonvaccination areas. Se-

rum samples ($n = 198$; $PI \leq 40$) taken from healthy goats raised on Chongming Island (near Shanghai), where there has been no recorded outbreak of PPR, served as negative controls, while samples with a PI value of 91 were designated as strong positive controls. Samples designated as weak positive were prepared by diluting strong positives with 4 volumes of negative serum.

Modified c-ELISA Using RN instead of B-N Antigen

The antigenic specificity of the recombinant N protein was determined using a modified c-ELISA in which recombinant PPRV N protein replaced B-N. The protocol was otherwise identical to that used for the commercial c-ELISA kit, and wells with no serum served as the mAb control (zero percent competition). Serum controls included strong positive, weak positive and negative samples.

Indirect ELISA Using Artificially Synthesized Recombinant PPRV N Protein as the Coating Antigen

Antigen was dissolved in carbonate/bicarbonate buffer solution (0.05 M, pH 9.6) and coated onto 96-well plates (Maxisorp F96; Nunc, Kamstrup, Denmark) by incubating overnight at 4°. After washing with PBS-T buffer (0.002 M PBS containing 0.05% Tween 20), the wells were blocked with 250 μ l blocking buffer (PBS-T containing 3% lactalbumin hydrolysate) for 60 min. After further washing, 50 μ l diluted serum was added and the wells were again incubated for 60 min to allow the antigen and first antibody to combine. The plates were washed three times and, following addition of either anti-goat-HRPO (Immunology Consultants Laboratory) or anti-sheep-HRPO (Santa Cruz Biotechnology) conjugate (diluted 1:8,000 with blocking buffer in both cases), were incubated at 37° for 30 min to allow the primary and secondary antibodies to combine. Substrate solution (1 mg/ml TMB containing 4 μ l 3% H_2O_2) was dispensed into each well, and the color reaction was allowed to develop for 10 min in the dark. After termination of the reaction, absorbance values at 450 nm were measured with the FAO/IAEA EDI software [16].

Optimization of the Prototype Indirect ELISA

Dilutions of antigen and antibodies for optimization of the prototype indirect ELISA (i-ELISA) were determined by the standard checkerboard titration procedure. Antigen concentrations were 12.8, 6.4, 3.2, 1.6, 0.8 and 0.4 μ g/ml, and serum dilutions (negative serum: c-ELISA $PI \leq 20$; positive serum c-ELISA $PI \geq 75$ from PPR-vaccinated animals) were 1:50, 1:100, 1:200 and 1:400. Antigen and serum dilutions that gave the maximum positive/negative (P/N) ratio were selected. The reaction temperature, time and other conditions were selected from the index of P/N values [17].

Determination of Cutoff Values

Cutoff values of the prototype i-ELISA were determined as the mean + 2 standard deviations (SDs) and the mean + 3 SDs, which were derived from the P/N values of 198 negative Chongming samples. Diagnostic sensitivity and specificity were calculated using the two cutoff values. Positive and negative controls for i-ELISA usually gave absorbance values of ≥ 0.6 and ≤ 0.2 , respectively, and the test was repeated when values fell outside this range.

Evaluation of i-ELISA Sensitivity and Reproducibility

The sensitivity of the i-ELISA was evaluated using strongly positive sera ($PI = 91$) serially diluted (twofold) with negative goat serum ($PI \leq 15$). Operating conditions were selected on the basis of the optimization data obtained above.

Twenty sera were selected to evaluate the reproducibility of the assay. For each sample, the coefficient of variation (CV) was calculated between plates (inter-assay variation) and within the same plate (intra-assay variation). Each sample was tested using four different plates on different occasions to determine the inter-assay CV, and four replicates within each plate were used to calculate the intra-assay CV.

Results

Cloning of the RN Gene and Mutation Correction

Partial and full-length RN genes were obtained by overlapping PCR, which yielded four smaller (405, 411, 411 and 414 bp) and one larger (1,578 bp) DNA fragments.

Sequence analysis of the RN revealed four point mutations generated during amplification, which were 245 G redundancy, 679 C→T, 1079 C→A, and 1256 T→C. A full-length corrected sequence was prepared using the two-stage procedure described in 'Materials and Methods', and successful elimination of the mutations was confirmed by cloning and sequencing.

Expression and Purification of PPRV N Protein

A protein with a molecular weight of 75 kDa, which corresponded well with the presumed recombinant PPRV N, was obtained through isopropyl β -D-1-thiogalactopyranoside induction of transformed *Escherichia coli* cells and was confirmed as PPRV N antigen by Western blotting (fig. 2). The protein was located mainly in the soluble phase of the cell lysate and, after elution from nickel-chelated agarose columns, was ~95% pure (fig. 3). Yields of up to 10.9 mg/l culture were achieved.

Modified c-ELISA Substituting B-N with RN

Twelve samples were tested in parallel by using the two antigens. The corresponding PI values differed by less than 5% in seven sample sets, and between 5 and 15% in the remaining five (fig. 4).

Working Concentrations of Antigen and Serum for i-ELISA

In the checkerboard titration tests, the maximum difference in the OD values for positive and negative sera was obtained with a 1:200 dilution of serum at an antigen concentration of 3.2 μ g/ml. Under these conditions, the P/N value was 5.37 at a 1:8,000 dilution of anti-goat/sheep-HRPO conjugate (fig. 5).

Fig. 2. Western blot analysis of soluble lysate. Lane 1: soluble lysate of BL21-32a-RN (5 h). Lane 2: lysate from induced BL21 which carried the empty vector. Lane M: prestained molecular weight protein markers.

Fig. 3. SDS-PAGE analysis of expressed proteins. Lane 1: soluble lysate of BL21-32a. Lane 2: soluble lysate of BL21-32a-RN (0 h). Lane 3: lysate of BL21-32a-RN (5 h). Lanes 4-7: protein eluted from the nickel-chelated agarose column by 100, 200, 300 and 500 mM imidazole, respectively. M: low-molecular-weight protein markers.

Table 3. Effect of different cutoff values on sensitivity and specificity of i-ELISA

	Cutoff value	
	2.18 (mean + 2 SDs)	2.33 (mean + 3 SDs)
Sensitivity	96.7% (202/209)	94.7% (198/209)
Specificity	96.1% (469/488)	97.8% (477/488)

Validation and Repeatability of i-ELISA

For the twenty selected sera, the inter-assay CV ranged from 1.9 to 5.8%, and the intra-assay CV ranged from 1.4 to 5.0%.

Cutoff Values, Specificity and Sensitivity of i-ELISA

The mean P/N value for the 198 PPRV-negative control sera taken from healthy goats raised on Chongming Island was 1.875 with an SD of 0.152. Therefore, cutoff values were set to 2.18 (mean + 2 SDs) and 2.33 (mean + 3 SDs).

Sensitivity and specificity values were 96.7 and 96.1%, respectively, at the 2.18 cutoff level, and 94.7 and 97.8%, respectively, at the 2.33 cutoff level compared with a commercially available ELISA test (table 3).

The i-ELISA P/N values of 697 serum samples that had been designated as either positive or negative by c-ELISA are shown in figure 6.

P/N values of a strong positive serum sample gradually decreased with increasing dilution with negative serum and were negatively correlated with the log of the dilution (fig. 7). The maximum dilution giving a positive result was 1:12,800.

Discussion

PPR is recognized as a major constraint in augmenting small ruminant productivity in regions and countries, including parts of Africa, the Middle East, India and China, where the disease is epidemic. In view of the economic importance of the disease, priority has been given to effective serological surveillance of PPRV. Since ELISA is widely accepted as a highly sensitive and efficient method for detecting different kinds of antigen or antibody, our aim was to develop an i-ELISA for the serological surveillance of PPRV in China.

Among the structural proteins of morbilliviruses, the N protein is strongly immunogenic and has a highly conserved structure [18]. The protein is expressed at high levels in infected cells [19], and has different epitopes that can be used for identifying PPRVs of different lineages.

Fig. 4. Comparison of c-ELISA P/N values using B-N or R-N as coating antigen. 1–12 = goat serum samples; Cm = mAb control; C++ = strong positive serum control; C+ = weak positive serum control; C- = negative serum control. Differences in the two PI values were <5% for samples 2–4, 6, 8, 10 and 11, and between 5 and 15% for the remainder.

Fig. 5. Checkerboard titration of antigen and sera. The maximum P/N value of 5.37 was obtained between positive and negative serum samples at a coating antigen concentration of 3.2 µg/ml and a 1:200 dilution of the serum sample.

Fig. 6. Reassessment of 209 positive and 488 negative samples with i-ELISA. Note: Data are based on a cutoff value of 2.18.

Fig. 7. Effect of dilution with negative serum on the reactivity of a convalescent serum with antigen. Dotted line shows positive/negative cutoff.

Therefore, we have employed the N protein of PPRV/China/Tibet/Geg/07-30 to develop an i-ELISA for detecting antibody against PPRV. Previously, recombinant N proteins of the virulent Kabete O strain of RPV [20] and Nigeria75/1 PPRV [3] were expressed in baculovirus, and used in ELISAs to distinguish between vaccinated and

RPV-infected animals, and for serological diagnosis of PPR, respectively.

Genes encoding N protein have been isolated from several different PPRV strains and used in the development of PPRV serological detection methods [8, 19, 21]. However, since access to these genes also required ma-

nipulation of the PPRV, we have adopted a novel approach in order to reduce the potential risks associated with using viral material. In our research, we have used information relating to PPRV/China/Tibet/Geg/07-30 accessible in the databases and advanced molecular biological tools to artificially synthesize the entire N gene of this PPRV strain. Several oligonucleotide-based methods for the synthesis and assembly of long DNA sequences have been described in recent years including the PCR-based thermodynamically balanced inside-out procedure [22], successive PCR [23], PCR-based two-step DNA synthesis (PTDS) [24], and PCR-based accurate synthesis [25]. In this study, we used the PTDS method to successfully synthesize the entire N gene of PPRV. However, as this is a PCR-based procedure, DNA polymerase proofreading defects are inherent and four point mutations occurred during synthesis. These were readily corrected adopting a similar experimental approach and using the initially acquired but defective full-length 1,578-bp DNA fragment as the template.

Different strategies were adopted for effective R-N expression and gene product purification, including the use of prokaryotic preferred codons and addition of a 6-his tag at the N-terminal of the recombinant protein. Substitution of B-N with R-N in tests conducted with a commercial ELISA kit resulted in similar P/N values (<5% difference) for seven of the test samples, but bigger differences (5–15%) in the case of five other sera. This discrepancy may be attributable to the fact that the two N proteins (from the Tibetan strain and Nigeria75-1 strain, respectively), although 95.6% homologous in terms of

amino acid sequence, had different epitopes with different affinities for the serum sample.

Using the cutoff value obtained using the mean of 198 negative samples plus $2 \times$ the SD, the assay exhibited a high degree of specificity and sensitivity with inter-assay and intra-assay CVs less than 4.3 and 5.0%, respectively. These data were confirmed in a twofold serial dilution test in which a strong positive serum sample was serially diluted with PPR-negative goat serum (PI = 17) prior to testing with i-ELISA. Positive results were recorded with dilutions as high as 1:12,800.

In conclusion, we describe an efficient procedure for obtaining coating antigen for use in an i-ELISA of PPRV antibody among goats or sheep that eliminates the need for virus culture and reduces the bio-risk posed by virus-dependent manipulation. Our data confirmed the similar antigenic nature of the native and recombinant proteins, and the suitability of the latter for use in i-ELISA-based PPRV surveillance.

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