Chen *et al. Animal Diseases* (2024) 4:9
https://doi.org/10.1186/s44149-024-00114-1

Animal Diseases

ORIGINAL ARTICLE

Open Access

Two nanobody-based immunoassays to differentiate antibodies against genotype 1 and 2 porcine reproductive and respiratory syndrome virus

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) infection causes significant economic loss to the global pig industry. Genotype 1 and 2 PRRSV (PRRSV-1 and -2) infections have been reported in China, Europe and America. For accurate prevention, nanobodies were first used as diagnostic reagents for PRRSV typing. In this study three nanobodies targeting both PRRSV-1 and -2, two targeting PRRSV-1 and three targeting PRRSV-2, were screened and produced. To develop two competitive ELISAs (cELISAs), the g1-2-PRRSV-Nb3-HRP nanobody was chosen for the g1-2-cELISA, to detect common antibodies against PRRSV-1 and -2, and the g1-PRRSV-Nb136-HRP nanobody was chosen for the g1-cELISA, to detect anti-PRRSV-1 antibodies. The two cELISAs were developed using PRRSV-1-N protein as coating antigen, and the amounts for both were 100 ng/well. The optimized dilution of testing pig sera was 1:20, the optimized reaction times were 30 min, and the colorimetric reaction times were 15 min. Then, the cut-off values of the g1-2-cELISA and g1-cELISA were 26.6% and 35.6%, respectively. Both of them have high sensitivity, strong specificity, good repeatability, and stability. In addition, for the 1534 clinical pig sera, an agreement rate of 99.02% (Kappa values = 0.97) was determined between the g1-2-cELISA and the commercial IDEXX ELISA kit. For the g1-cELSIA, it can specifically detect anti-PRRSV-1 antibodies in the clinical pig sera. Importantly, combining two nanobody-based cELISAs can differentially detect antibodies against PRRSV-1 and -2.

Keywords PRRSV, Competitive ELISA, Nanobody, Antigen epitope

Handling Editor: Fang He.

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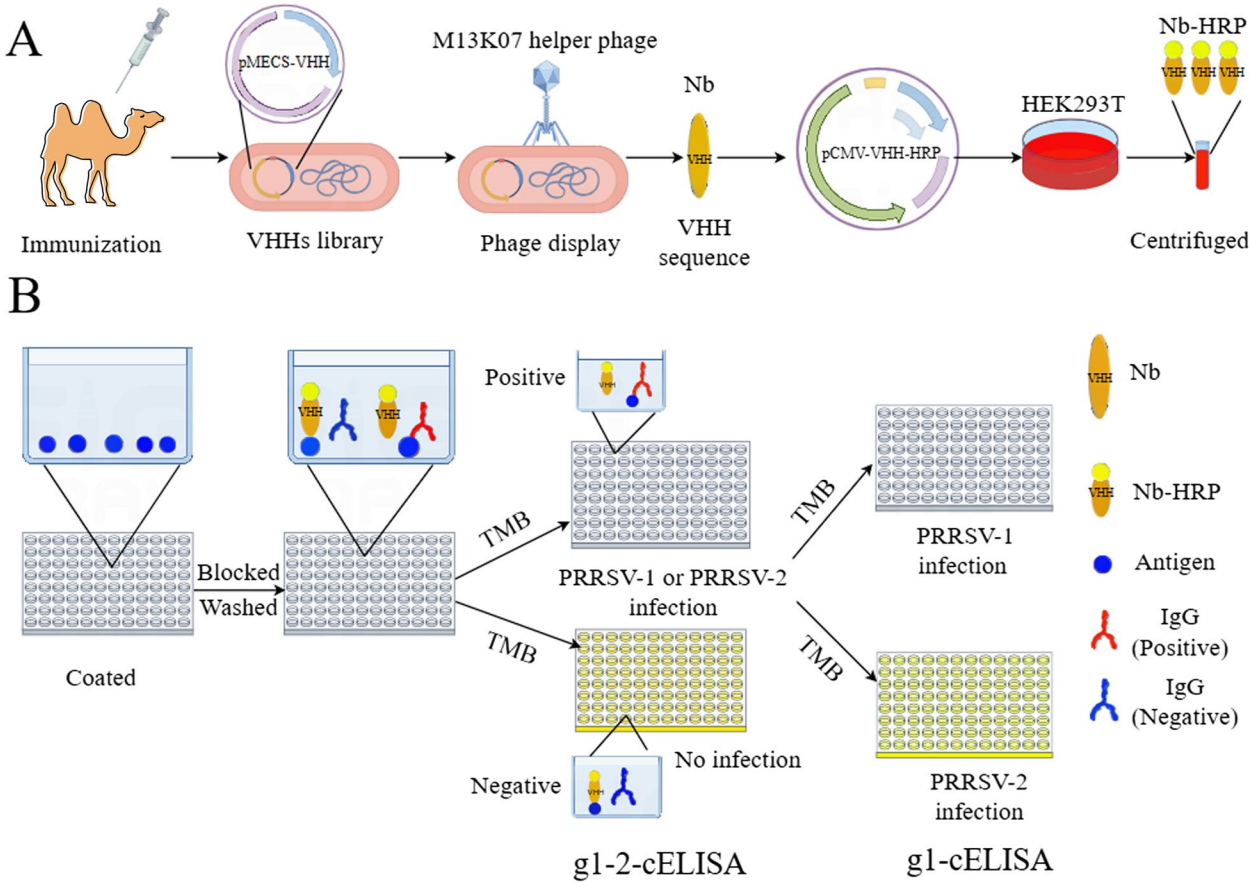
Graphical abstract



Introduction

Porcine reproductive and respiratory syndrome (PRRS), caused by the PRRS virus (PRRSV), is a major disease threatening large-scale pig farms worldwide (Nan et al. 2017). The disease is characterized by reproductive disorders in sows and respiratory diseases in all aged pigs, especially piglets (Chang et al. 2002; Sun et al. 2023). PRRSV continues to cause considerable economic loss to the global pig industry, specifically up to US$ 640 million annually in the United States (Du et al. 2017). The virus, an enveloped and positive-stranded RNA virus, belongs to *Nidovirales*, family *Arteriviridae*, and the genus *Betaarterivirus* (Brinton et al. 2021). The viral genome contains 11 open reading frames (ORFs), including ORF1a, ORF1b, ORF2a, ORF2b, ORF3-7 and ORF5a (Lunney et al. 2016). The ORF7 encodes the nucleocapsid (N) protein with a molecular weight of 14–17 kDa (Nelson et al. 1994). The protein accounts for 40% of the viral particles and induces strong immune responses after PRRSV infects the pigs (Han and Yoo 2014). Anti-PRRSV N protein antibodies can be detected 7 d after viral infection (Plagemann 2006). Then, the N protein was universally used as the antigen for serological diagnosis of PRRSV infection (Yu et al. 2010).

All different PRRSV isolates are mainly divided into two genotypes, namely *Betaarterivirus suid 1* (genotype 1 PRRSV, PRRSV-1) and *Betaarterivirus suid 2* (genotype 2 PRRSV, PRRSV-2) (Fiers et al. 2023). The two genotypes share only approximately 60% nucleotide identities and do not produce cross-protection (Stadejek et al. 2008). A previous study reported that PRRSV-1 is popular in Europe, and PRRSV-2 is mainly popular in America and Asia (Stadejek et al. 2002). However, along with the global trade of pigs in recent years, Stadejek et al. reported the PRRSV-1 infection in Asia, especially in China (Stadejek et al. 2013; Wang et al. 2023; Xu et al. 2023). Therefore, accurately controlling and preventing PRRSV infection

is important for differential diagnosis of PRRSV-1 and PRRSV-2 infection in pig farms. Enzyme-linked immunosorbent assay (ELISA) is commonly used to diagnose PRRSV infection and evaluate vaccination (Yahara et al. 2002). The most available commercial ELISA kits are the indirect ELISA (iELISA) using the N protein as the coating antigen and traditionally secondary antibodies as the reagents (Seo et al. 2016). However, the iELISA requires high-purity antigen and enzyme-labeled secondary antibodies, resulting in high production costs and complicated production methods of commercial kits (Sattler et al. 2015). Additionally, it is not easy to differentiate antibodies against PRRSV-1 and -2 with the iELISA using the complete N protein as a coating antigen because the two genotypes of PRRSV N proteins have common epitopes (Chu et al. 2009). As we know, neither the commercial ELISA kit nor the existing developed ELISA can distinguish the different genotypes of PRRSV infection (Ge et al. 2019).

Nanobodies derived from the variable region of heavy chain antibody (VHH) in camelids have attractive advantages, such as small size, ease of genetic manipulation, and high specificity (Vanlandschoot et al. 2011). Now, nanobodies have more promising applications in disease diagnosis and drug development (Xiang et al. 2020). In particular, nanobodies have been universally used as reagents for developing immunoassays to detect antibodies, small molecules, and toxins from different samples (Sheng et al. 2019). Previously, a platform for developing competitive ELISA (cELISA) to detect antibodies in the sera has been established using the nanobody with horse radish peroxidase (HRP) tag (nanobody-HRP). Several cELISAs have been developed using the platform with advantages, including easy operation, good stability, simple production, and low-cost production (Sheng et al. 2019). For example, a cELISA for specifically detecting anti-PRRSV-2 antibodies in the pig sera (g2-cELISA) has been established by us with the platform (Duan et al. 2021). The assay showed 98% agreement with the IDEXX commercial ELISA kit to detect antibodies against PRRSV-2 (Duan et al. 2021). However, the nanobody-based cELISAs have yet to be developed to detect common antibodies against PRRSV-1 and -2 and specific antibodies against PRRSV-1. So, based on the advantages of nanobodies, nanobodies against common epitopes of PRRSV-1 and -2 and specific epitopes of PRRSV-1 were screened in the present study. Subsequently, nanobody-HRP fusion proteins were produced, and two nanobody-based cELISAs were developed to separately detect the common anti-PRRSV-1 and -2 antibodies (g1-2-cELISA) and specific anti-PRRSV-1 antibodies (g1-cELISA) in the pig sera. The two cELISAs can be used to detect anti-PRRSV-1 and -2 antibodies differentially and to meet the monitoring of different genotypes of PRRSV infection.

Results

Expression and purification of the recombinant PRRSV-1 and -2-N proteins

After expressing and purifying the two recombinant PRRSV N proteins by the *E. coli* system, SDS-PAGE analysis showed that they were both successfully expressed in the soluble forms with an expected size of 17 kDa and purified with the Ni-Resin (Fig. 1A and B). Western blotting analysis revealed that two recombinant proteins could react with the positive pig sera for anti-PRRSV antibodies, indicating they have good antigenicity (Fig. 1C and D). Then, the purified recombinant PRRSV-1-N and PRRSV-2-N (PRRSV-1 and -2-N) proteins were used as the coating antigens to screen nanobodies and develop the two cELISAs for detecting anti-PRRSV antibodies.

Construction of a phage display VHH library against PRRSV-N proteins

After the last immunization, the titers against PRRSV-1 and -2-N proteins in the immunized camels were detected separately with the iELISA. The results showed that the titers both reached up to $1{:}10^6$ (Fig. 1E). After the *VHH* genes were amplified by the nested RT-PCR, an approximately 700 bp band was obtained in the first round of PCR (Fig. 1F) and a 400 bp band was successfully amplified in the second round of PCR (Fig. 1G). Subsequently, the phage displays VHH library was successfully constructed, containing approximately 4.5×10^8 individual transformant colonies.

Screening of nanobodies against PRRSV-1 and -2-N proteins

After three rounds of panning, the specific VHHs phages against PRRSV-1 and -2-N proteins were 1.3 and 16.1 in the first round and 138 and 1268 in the third round, respectively, indicating that the specific phages were successfully enriched (Fig. 2A). The results of iELISA using 192 periplasmic extracts from the third round panning plates showed that 66 extracts could react with PRRSV-1-N protein or PRRSV-2-N protein. Then, the 66 colonies were sequenced. According to the amino acids of CDR3 from the 66 sequences, the alignments showed that three nanobodies (named g1-2-PRRSV-Nb3, -Nb13 and -Nb36) were against both PRRSV-1 and -2-N proteins, two nanobodies (named g1-PRRSV-Nb90 and -Nb136) specifically against PRRSV-1-N protein, and three nanobodies (named g2-PRRSV-Nb5, -Nb14 and -Nb27) specifically against PRRSV-2-N protein (Fig. 2B). Amino

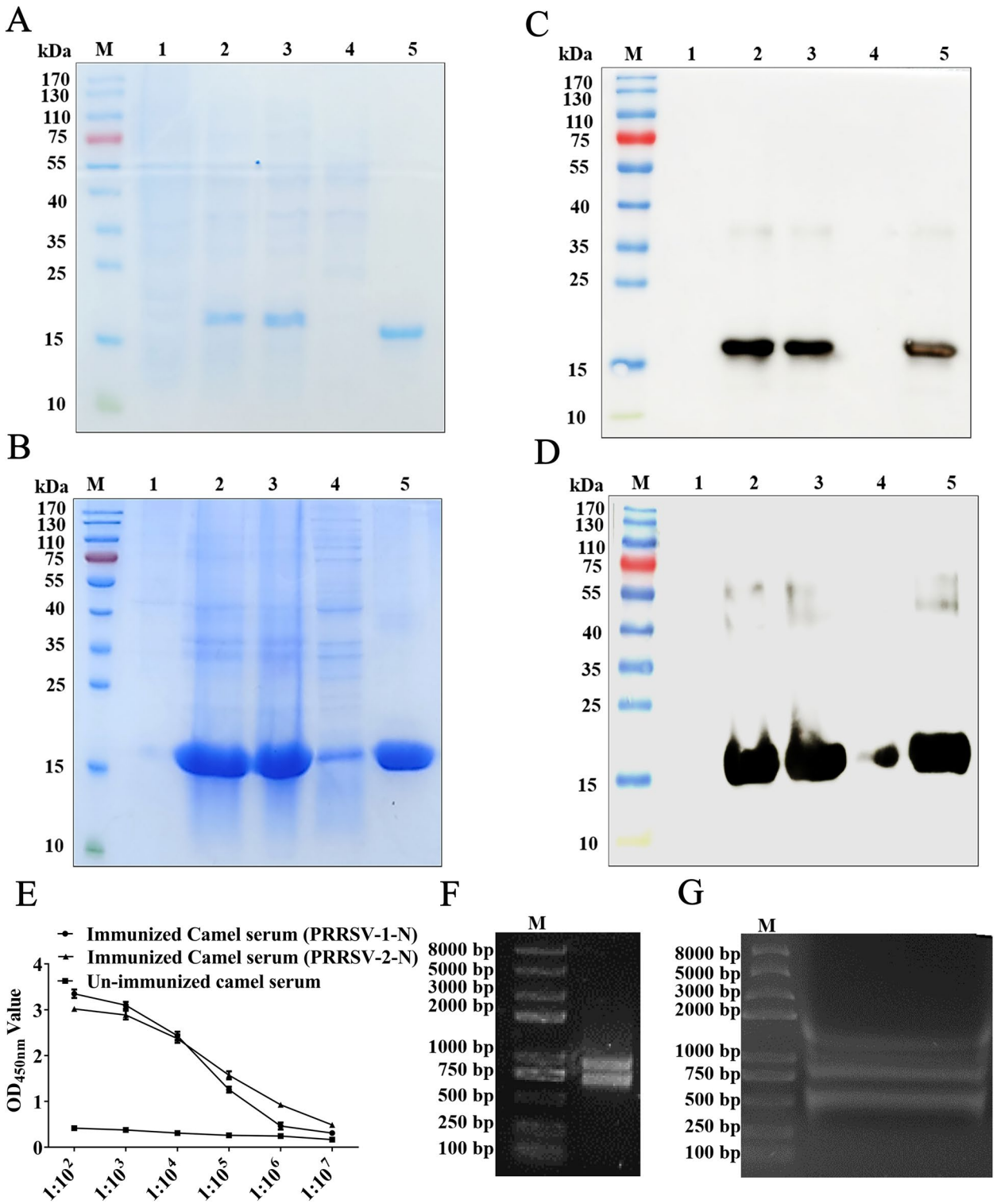


Fig. 1 Immunization of camels and construction of the VHH library. **A** SDS-PAGE analysis of the recombinant PRRSV-1-N protein. **B** SDS-PAGE analysis of the recombinant PRRSV-2-N protein. **C** Antigenic analysis of the recombinant PRRSV-1-N protein using the positive pig sera for PRRSV as the first antibody. **D** Antigenic analysis of the recombinant PRRSV-2-N protein using the positive pig sera for PRRSV as the first antibody. **E** Titers of antibodies against anti-PRRSV-1 and -2-N proteins in the immunized camel by indirect ELISA. **F** The VHH genes were amplified by the first round of PCR. **G** The VHH genes were amplified by the second round of PCR.M: Molecular weight markers; Lane 1: pET28a vector control; Lane 2: Induction with 0.1 mM IPTG; Lane 3: Inclusion body in precipitation after sonication; Lane 4: Soluble protein in supernatant after sonication; Lane 5: Purified PRRSV-1 or -2-N protein

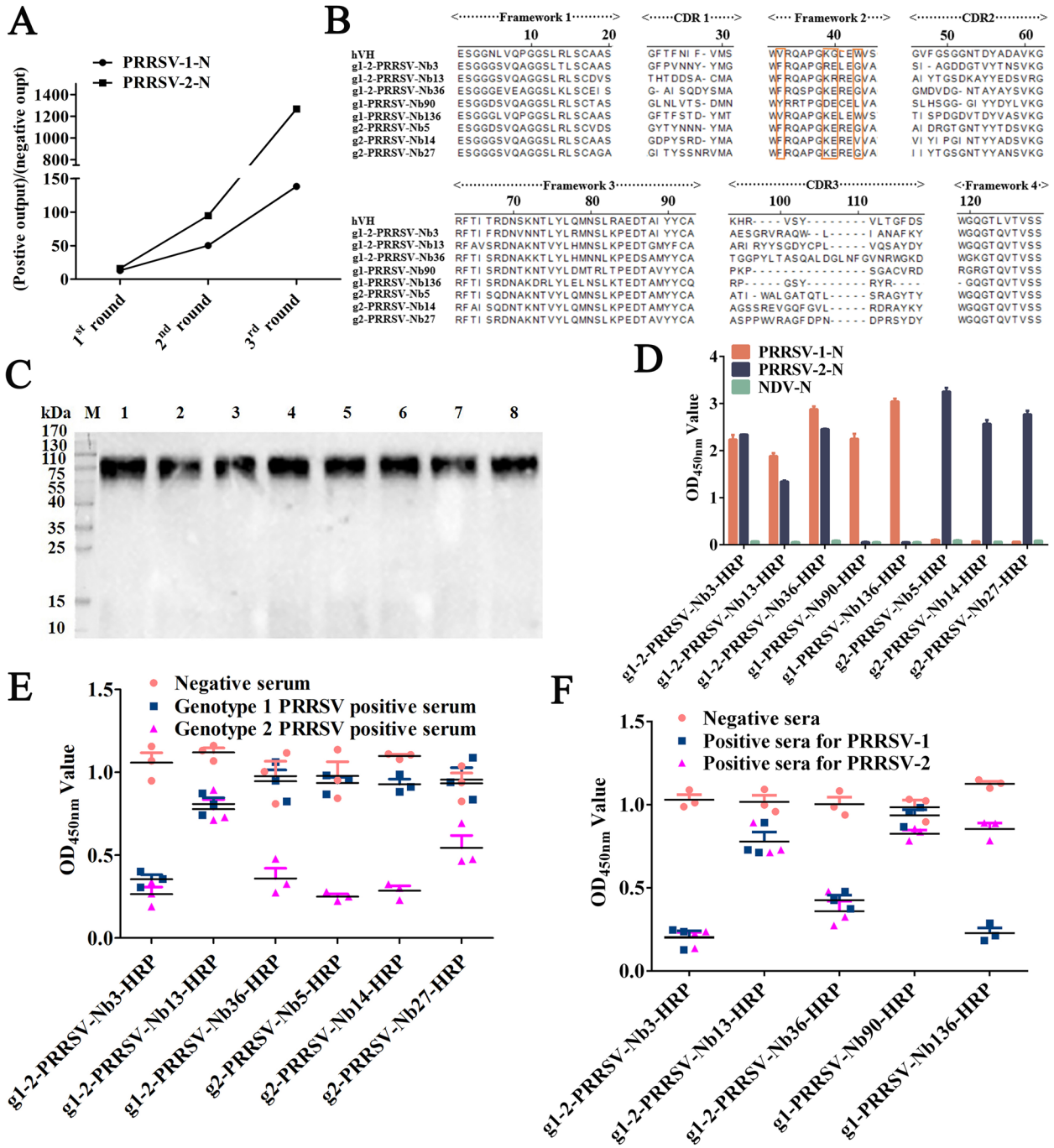


Fig. 2 Screening and identification of specific nanobodies from the VHH library. **A** Titers of antibodies against anti-PRRSV-1 and -2-N proteins in the immunized camel by indirect ELISA. **B** Amino acid sequence alignment of eight nanobodies against anti-PRRSV-1 and -2-N proteins. **C** Western blotting analysis of eight nanobody-HRP fusion proteins secreted into the medium of HEK293T cells. Anti-HA monoclonal antibody was used as the first antibody, and HRP labeled goat anti-mouse antibody as the second antibody. M: Molecular weight markers; Lane 1: g1-2-PRRSV-Nb3-HRP; Lane 2: g1-2-PRRSV-Nb13-HRP; Lane 3: g1-2-PRRSV-Nb136-HRP; Lane 4: g1-PRRSV-Nb90-HRP; Lane 5: g1-PRRSV-Nb136-HRP; Lane 6: g2-PRRSV-Nb5-HRP; Lane 7: g2-PRRSV-Nb14-HRP; Lane 8: g2-PRRSV-Nb27-HRP. **D** Analysis of the binding ability of eight nanobody-HRP fusion proteins against PRRSV-1 and -2-N proteins by ELISA. **E** Comparisons of positive pig sera for PRRSV blocking five nanobody HRP fusion proteins to react with PRRSV-1-N protein. **F** Positive pig sera for PRRSV blocking six nanobody HRP fusion proteins to bind to PRRSV-2-N protein

acid sequence alignment of these eight nanobodies with human VH revealed typical hydrophilic amino acid substitutions including Val33, Gly40 and Trp43 in the framework 2 region of the nanobodies (Fig. 2B).

Production of nanobody-HRP fusion proteins against PRRSV-1 and -2-N proteins

Based on the previous descriptions, all the screened nanobodies were designed for expression with HRP, HA and His tags (Sheng et al. 2019). After the positive recombinant plasmids containing the genes encoding the nanobodies and HRP were transfected into HEK293T cells. Western blotting showed that the eight fusion proteins were successfully secreted into the supernatant (Fig. 2C). These nanobody-HRP fusion proteins were named g1-2-PRRSV-Nb3-HRP, -Nb13-HRP, -Nb36-HRP, g1-PRRSV-Nb90-HRP, -Nb136-HRP and g2-PRRSV-Nb5-HRP, -Nb14-HRP, -Nb27-HRP. Meanwhile, these fusion proteins cannot react with Newcastle disease virus (NDV) N protein, expressed using the same expressing vector and the bacterial system as the two recombinant PRRSV N proteins, indicating that the g1-PRRSV-Nb90-HRP and -Nb136-HRP could specifically recognize the PRRSV-1-N protein (Fig. 2D). The g2-PRRSV-Nb5-HRP, -Nb14-HRP, and -Nb27-HRP could specifically recognize the PRRSV-2-N protein, and the g1-2-PRRSV-Nb3-HRP, -Nb13-HRP and -Nb36-HRP could recognize both PRRSV-1-N and PRRSV-2-N proteins (Fig. 2D).

Development of two competitive ELISAs for detecting anti-PRRSV antibodies

In a previous study, a cELISA to detect anti-PRRSV-2 antibodies was developed using the nanobody-HRP fusion protein as a reagent (Duan et al. 2021). Then, two cELISAs to detect both anti-PRRSV-1 and -2 antibodies and only anti-PRRSV-1 antibody were developed using the above nanobody-HRP fusion proteins in the study. For the selection of best nanobody-HRP fusion proteins to develop two cELISAs, blocking ELISAs showed that the blocking rate was the highest when the g1-2-PRRSV-Nb3-HRP was used as the blocked antibody for detecting anti-PRRSV-1 and -2 antibodies and g1-PRRSV-Nb136-HRP was for detecting anti-PRRSV-1 antibodies (Fig. 2E and F). Then, the g1-2-PRRSV-Nb3-HRP was selected as the reagent for developing the g1-2-cELISA, and g1-PRRSV-Nb136-HRP was for the g1-cELISA.

For the optimized amounts of coating antigen and nanobody-HRP fusion proteins, PRRSV-1-N protein as coating antigen was both 100 ng/well for the two cELISAs (Table 1). The optimized dilution of g1-2-PRRSV-Nb3-HRP was 1:100 for the g1-2-cELISA, and the one of g1-PRRSV-Nb136-HRP was 1:10 for the g1-cELISA (Table 1).

The optimized dilutions of testing pig sera in the two cELISAs showed that they were both 1:20 because the P/N values were the lowest for the two assays testing positive and negative pig sera (Table 2).

Finally, for determining the optimized times of incubation and colorimetric reaction, the two cELISAs' P/N values were the lowest when the incubating times of the mixtures with PRRSV-1-N protein were 30 min, and the colorimetric reaction time was 15 min (Table 3).

Cut-off values, sensitivities, specificities, reproducibility, and stabilities of the two competitive ELISAs

When 187 negative pig sera for anti-PRRSV antibodies were tested with the two cELISAs, the average percent inhibition (PI) value of g1-2-cELISA was 4.7%, and the SD was 7.3%. Then, the cut-off value of the g1-2-cELISA was 26.6% (mean PI + 3 × SD). For the g1-cELISA, the

Table 1 Optimized amounts of PRRSV-1-N protein as the coating antigen and dilutions of g1-2-PRRSV-N-Nb3-HRP and g1-PRRSV-Nb136-HRP fusion protein using the direct ELISA

Nanobody-HRP fusion protein	Amount of PRRSV-1-N protein (ng/well)	Dilutions of nanobody-HRP fusion proteins					
		1:10	1:50	1:100	1:200	1:400	1:800
g1-2-PRRSV-N-Nb3-HRP	50	2.008	1.127	0.88	0.463	0.212	0.143
	100	2.199	1.443	1.018	0.597	0.318	0.208
	200	2.339	1.709	1.349	0.8	0.423	0.246
	400	2.387	2.079	1.742	1.26	0.618	0.711
	800	2.384	2.121	1.818	1.402	0.731	0.911
g1-PRRSV-N-Nb136-HRP	50	0.751	0.334	0.243	0.161	0.108	0.091
	100	1.025	0.433	0.305	0.242	0.17	0.128
	200	1.551	0.763	0.509	0.351	0.23	0.173
	400	1.856	1.144	0.799	0.518	0.361	0.254
	800	2.088	1.491	1.101	0.704	0.527	0.408

Table 2 Optimized dilution of testing pig sera separate for the g1-2-cELISA and g1-cELISA

cELISA	Sample No	Sera type	Dilutions of the pig serum samples					
			1:10	1:20	1:40	1:80	1:160	1:320
g1-2-cELISA	1	Positive	0.14	0.15	0.24	0.31	0.54	0.51
		Negative	1.09	1.06	1.08	1.09	1.05	1.11
		P/N	0.12	0.14	0.22	0.29	0.52	0.46
	2	Positive	0.28	0.28	0.41	0.50	0.68	0.82
		Negative	1.09	1.06	1.08	1.09	1.05	1.11
		P/N	0.25	0.27	0.38	0.46	0.64	0.74
	3	Positive	0.20	0.24	0.40	0.56	0.69	0.81
		Negative	1.09	1.06	1.08	1.09	1.05	1.11
		P/N	0.19	0.22	0.37	0.51	0.66	0.73
	4	Positive	0.07	0.09	0.14	0.18	0.25	0.36
		Negative	1.09	1.06	1.08	1.09	1.05	1.11
		P/N	0.07	0.09	0.13	0.16	0.24	0.33
g1-cELISA	5	Positive	0.19	0.20	0.37	0.54	0.80	0.88
		Negative	0.97	0.92	0.95	0.96	0.93	0.95
		P/N	0.20	0.22	0.39	0.56	0.86	0.92
	6	Positive	0.25	0.25	0.36	0.46	0.50	0.66
		Negative	0.97	0.92	0.95	0.96	0.93	0.95
		P/N	0.26	0.28	0.38	0.48	0.54	0.70
	7	Positive	0.30	0.29	0.36	0.54	0.69	0.98
		Negative	0.97	0.92	0.95	0.96	0.93	0.95
		P/N	0.31	0.32	0.38	0.57	0.74	1.04
	8	Positive	0.08	0.09	0.23	0.34	0.39	0.47
		Negative	0.97	0.92	0.95	0.96	0.93	0.95
		P/N	0.08	0.10	0.24	0.36	0.42	0.50

Table 3 Optimized incubation time of the mixture containing pig sera and nanobody-HRP fusion proteins with the antigen and color reaction times using a checkerboard assay for the g1-2-cELISA and g1-cELISA

Color reaction time (min)	Sera type	Incubation time (min) of antigens, sera and Nbs-HRP fusion proteins					
		g1-2-PRRSV-Nb3-HRP			g1-PRRSV-Nb136-HRP		
		20	30	40	20	30	40
10	Negative	0.229	1.002	1.089	0.187	0.862	0.989
	Positive	0.174	0.211	0.219	0.114	0.241	0.263
	P/N	0.760	0.211	0.201	0.610	0.280	0.266
15	Negative	0.298	1.108	1.105	0.195	0.942	1.005
	Positive	0.172	0.198	0.207	0.115	0.243	0.267
	P/N	0.577	0.179	0.187	0.590	0.258	0.266
20	Negative	0.299	1.112	1.18	0.209	0.978	1.012
	Positive	0.177	0.212	0.221	0.127	0.251	0.272
	P/N	0.592	0.191	0.187	0.608	0.257	0.269

average PI value was 9.5%, SD was 8.7%, and the cut-off value was 35.6%. Therefore, the PI value of the pig sera, being ≥ 26.6% for the g1-2-cELISA, was considered positive for anti-PRRSV antibodies. Conversely, it was negative. Similarly, the PI value of ≥ 35.6% for the g1-cELISA was considered positive for anti-PRRSV-1 antibodies.

For the specificity of two cELISAs, PI values of g1-2-cELISA to detect positive pig sera for anti-PRRSV-1 and

-2 antibodies were all greater than 52%. However, for the positive pig sera for antibodies against PEDV, TGEV, PCV2, PRV, and PPV, all the PI values were lower than 20% (Fig. 3A). For the g1-cELISA, the PI values of only positive pig sera for antibodies against PRRSV-1 were greater than 50%. The others, including against PRRSV-2, PEDV, TGEV, PCV2, PRV and PPV, were lower than 23% (Fig. 3B). These results indicated that the two cELISAs have good specificities.

To evaluate the sensitivity of the two cELISAs, the sera from the pre and post-infected pigs with PRRSV-2 SD16 strain, PRRSV-2 NADC30-like strain, and PRRSV-1 GZ11-G1 strain were tested. The results showed that the positive seroconversions in the pigs for the first time were at 7 dpi (day post infection) and continued positive until 28 dpi (Fig. 3C, D, and E). Different dilutions of strongly and weakly positive pig sera were tested with the two cELISAs. Results showed that the dilution of weakly positive serum was still positive at 1:80, and the strongly positive serum was positive at 1:320 (Fig. 3F). The above data showed that the two cELISAs have strong sensitivity.

After six positive and negative pig sera were tested in different plates and on occasions by the two cELISAs, the results showed that the intra-assay CV of the PI values for the g1-2-cELISA was 1.37–8.59% with a median value of 5.23%, while the range for the inter-assay CV was 2.01–9.79% with a median value of 6.15% (Table 4). For the g1-cELISA, the results showed that the intra-assay CV was 0.23–3.19% with a median value of 1.78%, and the inter-assay CV was 1.12–7.67% with a median value of 4.92% (Table 4). Therefore, the two cELISA exhibits good repeatability.

Stability analysis showed that CV of OD_{450nm} for g1-2-PRRSV-Nb3-HRP and g1-PRRSV-Nb136-HRP fusion proteins were separately 6.61% and 7.03% separately when the coating plates and nanobody-HRP fusion proteins were stored at 4°C for 0, 1, 2, 3, 4, 5, 6, 7 and 8 months (Fig. 3G). The results suggested that the coated plates and fusion proteins as reagents of the two cELISAs have a long validity period when stored at 4°C. The two cELISAs with the stored plates and fusion proteins were also used to test four positive and negative pig sera. The results showed that the CV of the PI values for the g1-2 -cELISA ranged from 2.75% to 8.02%, with a median value of 5.02%. The g1-cELISA ranged from 7.41% to 2.70%, with a median value of 5.69% (Fig. 3H). These results indicated that the stored plates and fusion proteins maintained good competitive effects for 8 months. Therefore, the two cELISAs to detect antibodies against PRRSV exhibit good stabilities for following the production of commercial kits.

Agreements between two competitive ELISAs and commercial ELISA kit

To evaluate the clinical applications of the two cELISAs, 123 sera from the challenged pigs (0–28 dpi) and 1534 clinical pig sera were tested with the two cELISAs and a commercial IDEXX ELISA kit. The commercial kit can detect antibodies against PRRSV-1 and -2 but cannot distinguish two genotypes. The results showed that the g1-2-cELISA showed an agreement rate of 100% with the commercial ELISA kit to detect the sera from the challenged pigs (Kappa values = 1), for the g1-cELISA coincided in 123 (30 + /43-), an agreement rate of 59.35% (Kappa values = 0.30). Meanwhile, for the 1534 clinical pig sera collected from various farms in Shannxi, an agreement rate of 99.02% (Kappa values = 0.97) was determined between the g1-2-cELISA and commercial kit and the agreement rate of 24.84% was between g1-cELISA and commercial kit (Table 5).

Among the 1534 clinical pig sera, the 15 inconsistent sera between the g1-2-cELISA and commercial kit were further tested by IFA and g2-ELISA, which we have developed in a previous study (Duan et al. 2021). Of these, 11 were negative with g1-2-cELISA, g1-cELISA, and positive with a commercial kit. The results showed that five sera were positive and 6 ones were negative by IFA and four were positive and seven were negative by g2-cELISA (Fig. 4). Another four sera were positive by g1-2-cELISA and negative by commercial kit. The IFA results showed that all four were positive by IFA. One was positive for g1-cELISA and negative for g2-cELISA, indicating that the sera were from the pig infected by the

(See figure on next page.)

Fig. 3 Sensitivity, specificity and stability of two cELISA using the g1-2-PRRSV-Nb3-HRP and g1-PRRSV-Nb136-HRP fusion proteins as reagents, respectively. **A** Specificity analysis of g1-cELISA to detect antibodies against swine viruses. **B** Specificity analysis of g1-2-cELISA to detect antibodies against swine viruses. **C** Serum samples from five challenged SPF pigs with SD16 strains were detected using g1-2-cELISA and g1-cELISA. **D** Serum samples from five challenged SPF pigs with NADC30-like strains were detected using g1-2-cELISA and g1-cELISA. **E** Serum samples from six challenged SPF pigs with GZ11-G1 strains were detected using g1-2-cELISA and g1-cELISA. **F** Determination of the largest dilution of positive pig serum samples for anti-PRRSV-1 and -2 antibodies. **G** Stability analysis of the two cELISA using direct ELISA to determine the nanobody-HRP fusion protein still binding to the coated plates at different times. **H** Stability analysis of the two cELISA to still detect the positive pig sera for PRRSV. The coated ELISA plates with PRRSV-1-N protein and nanobody-HRP fusion proteins were stored at 4°C for 1, 2, 3, 4, 5, 6, 7 and 8 months. Statistical analysis was performed using GraphPad Prism V. 9.0. Comparisons between groups were considered statistically significant at $P < 0.05$

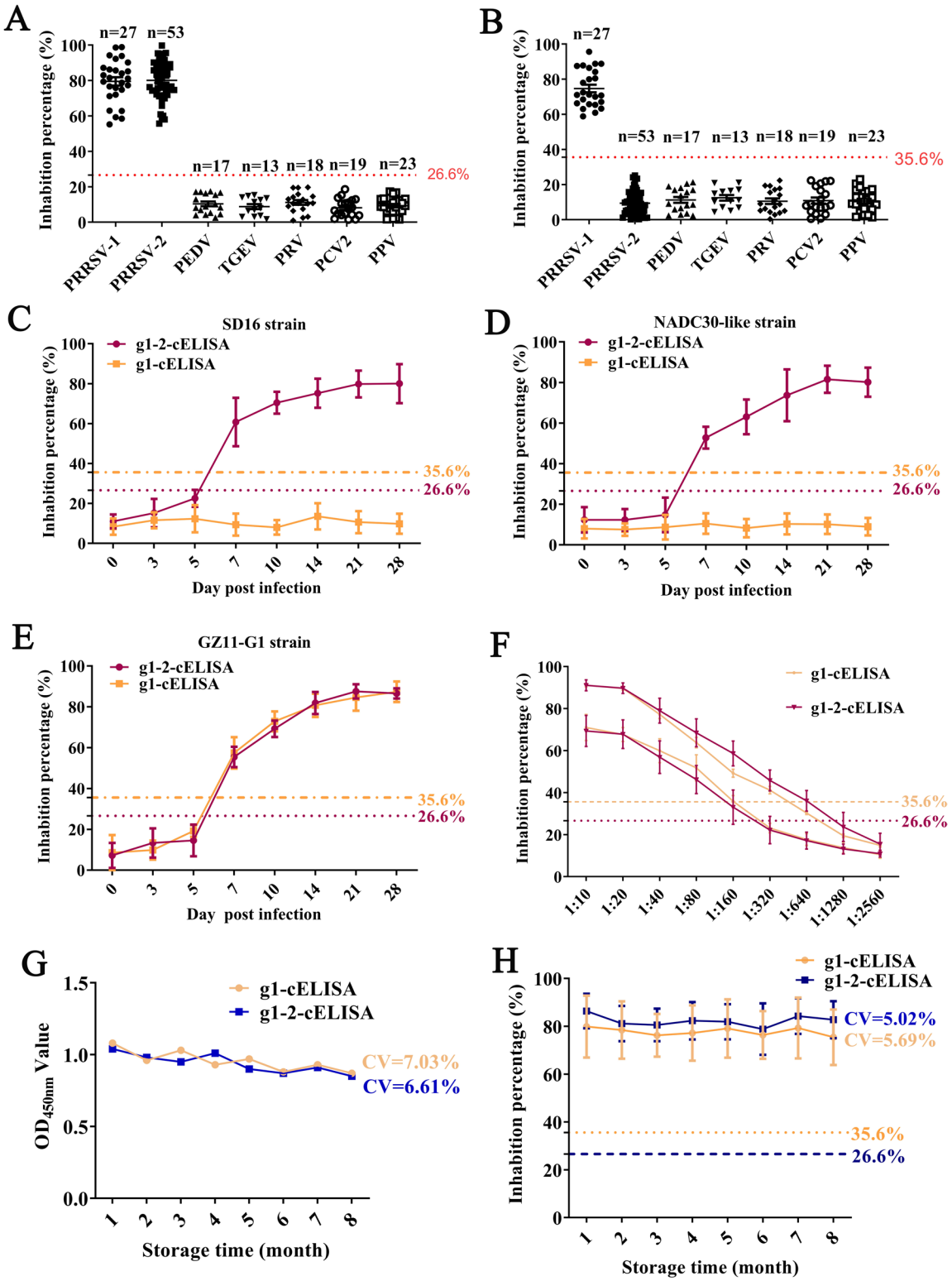


Fig. 3 (See legend on previous page.)

Table 4 Reproducibility of the g1-2 cELISA and g1-cELISA determined by CV % value of intra- and inter-assay

cELISA	Intra- or Inter-assay	CV % of 6 sera	Median value
g1-2- cELISA	Intra-assay	1.37–8.59	5.23
	Inter-assay	2.01–9.79	6.15
g1- cELISA	Intra-assay	0.23–2.19	1.78
	Inter-assay	1.12–7.67	4.92

PRRSV-1 (Fig. 4). Statistical analysis indicated that the g1-2-cELISA had a high level of agreement with the commercial IDEXX ELISA kit (Kappa values = 0.97), and the g1-cELISA was able to detect anti-PRRSV-1 antibodies specifically.

Discussion

PRRSV is considered one of the major infectious diseases causing economic losses in the global pig industry (Jakab et al. 2022). The disease cannot be differentially diagnosed by clinical symptoms from other swine diseases and sometimes manifests as subclinical symptoms (Oh et al. 2019; Lin et al. 2020; Risser et al. 2021). Laboratory diagnosis assays, including virus isolation, RT-PCR, and ELISA, are necessary to identify PRRSV infection (Zhao et al. 2021). ELISA is the main method, and various commercial ELISA kits for detecting anti-PRRSV antibodies have been developed (Biernacka et al. 2018; Antunes et al. 2015). However, the commercial ELISA kit is usually developed based on the model of iELISA, which requires HRP-conjugated secondary antibodies as the important reagents (Yu et al. 2018). The procedures for HRP-conjugated secondary antibodies are complex, resulting in poor batch stability for large-scale production. In the present study, the two developed nanobody-based cELISAs innovatively used the nanobody-HRP fusion proteins as the reagents and have high agreements with the commercial IDEXX PRRS X3 Ab Test Kit.

In contrast, the nanobody-HRP fusion protein for the cELISA has achieved a 1:1 pairing of nanobody and HRP, overcoming the shortcomings of producing HRP-conjugated secondary antibodies. To overcome each instantaneous transfection, the HEK293T cells stably secreting and expressing nanobody-HRP fusion proteins can also be developed. The fusion proteins can then be produced in large quantities using stable cells, which can further reduce the cost and simplify the procedures for following the production of commercial ELISA kits. Therefore, in this study, the developed cELISA can effectively replace the existing commercial kits to detect anti-PRRSV antibodies in pigs and be applied to monitor PRRSV infection in pigs.

Initially, PRRSV-1 was an epidemic only in Europe (Stadejek et al. 2013). With the increase in global trade, PRRSV-1 infection has been reported in Asia and North America (Wang et al. 2023; Xu et al., 2023). However, the available commercial ELISA kit cannot differentiate antibodies against PRRSV-1 and -2 (Qiu et al. 2019). Previously, we established a nanobody-based g2-cELISA to specifically detect anti-PRRSV-2 antibodies in the pig sera (Duan et al. 2021). To further accurately diagnose PRRSV-1 or -2 infection in pigs, especially in Asia and North America, a simple serological method for detecting anti-PRRSV-1 antibodies must be established. This study developed the two nanobody-based cELISAs to separate common anti-PRRSV and specific anti-PRRSV-1 antibodies. The three nanobody-based cELISAs can detect antibodies against PRRSV-1 and -2 in different scenarios and requirements and quickly discriminate between antibodies against PRRSV-1 and -2. If the pigs were co-infected by two genotypes of PRRSV, the g1-cELISA and g2-cELISA can differentiate the combined antibodies

Table 5 Comparisons of the developed g1-2-cELISA and g1-cELISA with commercial IDEXX ELISA kit by detecting challenged and clinical pig serum samples

cELISA	Samples	Positive (+) or negative (-)	Number	Commercial ELISA kit		Agreement	Kappa value	Positive rate
				+	-			
g1-2-cELISA	Challenged sera	+	80	80	0	100%	1.00	65.04%
		-	43	0	43			
	Clinical sera	+	1126	1122	4	99.02%	0.97	73.40%
		-	408	11	397			
g1-cELISA	Challenged sera	+	30	30	0	59.35%	0.30	24.39%
		-	93	50	43			
	Clinical sera	+	3	2	1	24.84%	0.0004	0.13%
		-	1531	1122	379			

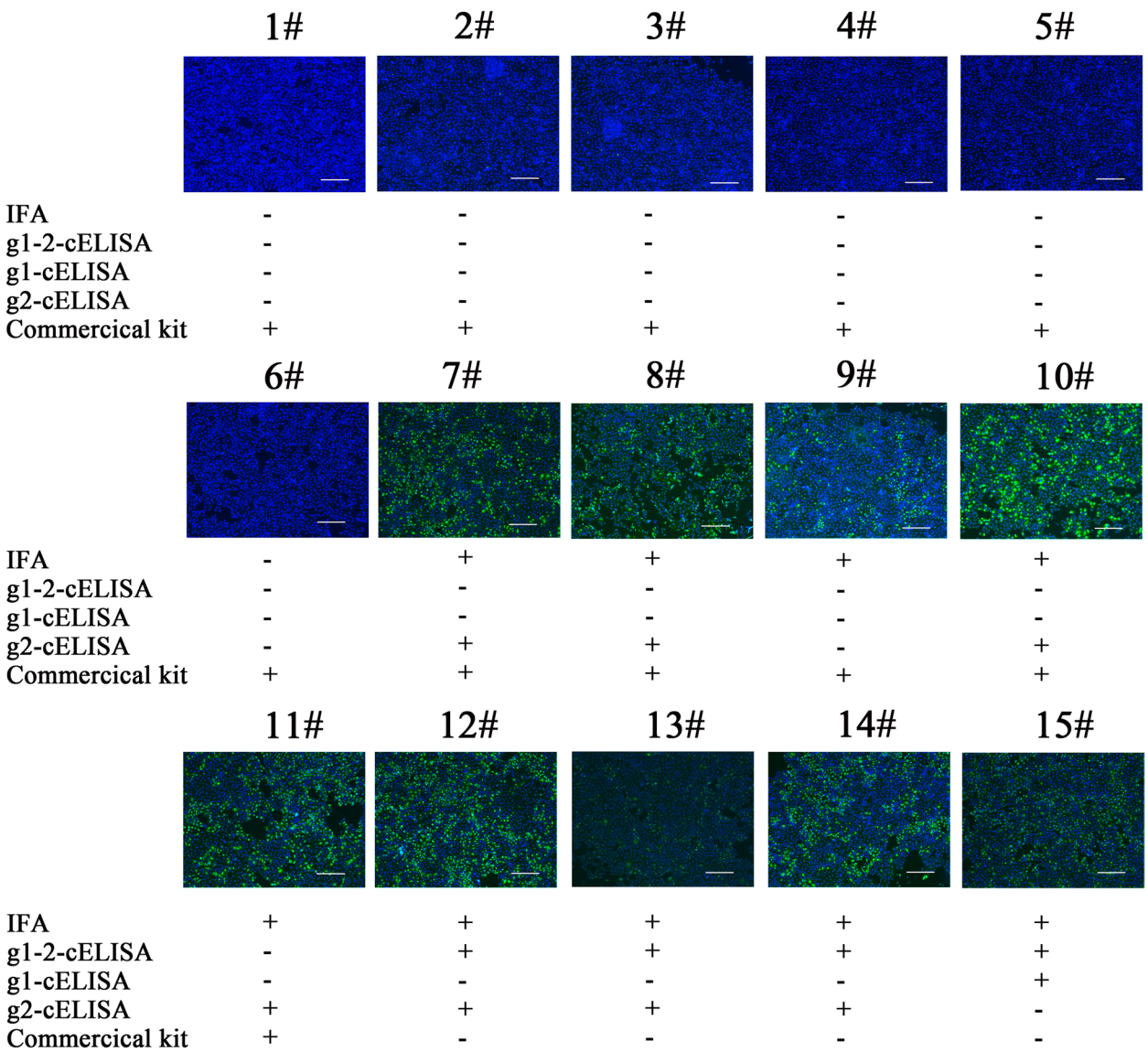


Fig. 4 Detecting the pig serum samples with inconsistent results between the g1-2-cELISA and commercial IDEXX X3 ELISA kit by IFA and g2-cELISA. The remaining 11 sera negative using g1-2-cELISA and positive using the commercial IDEXX ELISA kit and g2-cELISA were tested by IFA. Four clinical pig sera are positive for g1-2-cELISA, and negative for the commercial IDEXX ELISA kit were tested by IFA. "+" represented positive, and "-" represented negatively

from the two genotypes. However, the titers of the two antibodies in the sera from the co-infected pigs cannot be determined with the two cELISAs.

It is well known that PRRSV-N protein is a structural protein encoded by ORF7 and is a relatively conserved region among all PRRSV ORFs. The amino acids of PRRSV-N protein share about 96% and 100% identities within PRRSV-1 and -2, respectively. However, the inter-genotype homology of PRRSV-N protein was about 60%, indicating common or specific epitopes between PRRSV-1 and -2-N proteins (Nelsen et al. 1999). Previous studies showed that monoclonal antibodies determined the common epitopes in the aa 50–66 region of N proteins between PRRSV-1 and -2 (Le et al. 1998; Sun et al. 2023). In the present study, the g1-2-PRRSV-Nb3-HRP was used as the blocked antibody for detecting anti-PRRSV-1 and -2 antibodies, and g1-PRRSV-Nb136-HRP was for detecting anti-PRRSV-1 antibodies. In the future, we will accurately identify epitopes recognized by the two nanobodies and analyze the biological function and conservation of epitopes.

Several cELISAs have been developed for detecting antibodies against different animal viruses using nanobody-HRP fusion proteins (Sheng et al. 2019; Duan et al. 2021; Mu et al. 2021; Ma et al. 2019). These assays showed easy operations, low cost for commercial production, and good stability of different batches. For the PRRSV, we developed three nanobody-HRP fusion protein-based cELISA to detect separate common or specific antibodies against PRRSV-1 and -2. The g1-2-cELISA for detecting common antibodies showed high agreement with the commercial IDEXX ELISA kit being universally used in the field. However, the sensitivity of g1-2-cELISA was from 1:80 to 1:320. The dilution of testing pig sera with a commercial ELISA kit can reach up to 1:500, and its sensitivity is higher than the g1-2-cELISA. In the commercial ELISA kit, enzyme-labeled secondary antibodies can amplify the signals for antigen binding to antibodies. Therefore, we attempted to further improve the sensitivity of the nanobody-based cELISA by constructing a poly-nanobody.

Conclusion

In summary, two nanobody-based cELISAs (g1-2- and g1-cELISAs) to separately detect common or specific antibodies against PRRSV-1 and -2 have been developed in the study. Subsequently, the g1-2-PRRSV-Nb3 and g1-PRRSV-Nb136 with horse radish peroxidase fusion proteins were separate as reagents for developing two cELISAs. The g1-2-PRRSV-Nb3-HRP was selected for developing the g1-2-cELISA to detect common antibodies against PRRSV-1 and -2, and g1-PRRSV-Nb136-HRP was for the g1-cELISA to detect anti-PRRV-1 antibodies specifically. The co-use of two cELISAs can differentiate the antibodies against PRRSV-1 and -2. Both cELISAs have high sensitivity, strong specificity, good repeatability, and stability. Our study provided practical tools to accurately monitor PRRSV infection in the pig farms.

Materials and methods

Serum samples

One hundred and twenty-three serum samples were collected at 0, 3, 5, 7, 10, 14, 21 and 28 dpi of 16 4-week-old piglets infected with PRRSV to evaluate the sensitivity of the developed two cELISA (Duan et al. 2021). Five pigs were for the PRRSV-2 SD16 strain, five were for the PRRSV-2 NADC30-like strain, and the other six were for the PRRSV-1 GZ11-G1 strain (Duan et al. 2021). Totally 187 negative pig sera for anti-PRRSV antibodies were obtained from the healthy pigs and verified negative *via* a commercial ELISA kit (IDEXX, Westbrook, ME, USA). Ninety positive pig sera for antibodies against other swine viruses, including porcine epidemic diarrhea virus (PEDV) ($n=17$), transmissible gastroenteritis virus (TGEV) ($n=13$), porcine parvovirus (PPV) ($n=23$), porcine circovirus type 2 (PCV2) ($n=19$), and porcine pseudorabies virus (PRV) ($n=18$) were used to determine the specificity of the developed two cELISA. These positive pig sera for antibodies against other swine viruses were also confirmed by the commercial ELISA kit (IDEXX, Westbrook, ME, USA). Thirteen strongly and six weakly positive pig sera for antibodies against PRRSV were used to determine the sensitivity of the two developed cELISA. These pig sera were also detected with the commercial ELISA kit (IDEXX, Westbrook, ME, USA). A total of 1534 clinical pig sera were collected from the pig farms in Shaanxi province, China, and were tested using the commercial ELISA kit and the two developed cELISAs in the study.

Cells, viruses, and vectors

Human embryonic kidney cells (HEK293T) and African green monkey kidney cells (MARC-145) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Hangzhou Putai Biotechnology Co., LTD) supplemented with 10% fetal bovine serum (FBS, Hangzhou Putai Biotechnology Co., LTD) at 37°C under 5% CO_2. PRRSV-2 SD16 strains (GenBank ID: JX087437) and PRRSV-1 GZ11-G1 strain (GenBank ID: KF001144) were propagated and titrated in MARC-145 cells in DMEM supplemented with 3% FBS. Based on the previous descriptions, the pCMV-HRP vector was constructed using the commercial pEGFP-N1 vector (Clontech, Japan) as the backbone (Sheng et al. 2019). The pET-28a (+) plasmid (Novagen, Darmstadt, Germany), containing the sequences for a 6-histidine (6×His) tag downstream of the gene insertion site, was used as the expression vector.

Expression and purification of the recombinant PRRSV-N proteins

The PRRSV-1 and -2-N proteins were separately expressed and purified according to the previous descriptions (Plagemann 2006; Sattler et al. 2015; Duan et al. 2021). Briefly, the two genes separately encoding PRRSV-1 and -2-N proteins were synthesized and ligated into the commercial vectors pET-28a (+) by the Azenta Life Sciences Company. Then, the positive recombinant plasmids were transfected into *Escherichia coli* (*E. coli*) strain BL21 (DE3) cells for expressing the recombinant proteins. After the conditions were optimized, the expression of PRRSV-1-N protein was induced with 0.1 mM isopropyl-β-thiogalactopyranoside (IPTG) at 16°C for 18 h. The PRRSV-2-N protein was induced with 0.1 mM IPTG at 37°C for 8 h. After induced, the bacterial cells were centrifuged at 12,000 *g* for 30 min at 4°C and were ultrasonicated. After centrifuging again, the

supernatants were collected and loaded onto 1 mL Ni-Resin (Shanghai Hengyuan Biological Technology Co., LTD). Then, the recombinant proteins were purified based on the manual instructions. Finally, the expression, purification, and antigenicity of two recombinant PRRSV-N proteins were analyzed by SDS-PAGE and Western blotting with the positive pig sera for PRRSV as first antibody and the goat anti-pig IgG (H&L) HRP (Bioss, Beijing, China) as second antibody.

Bactrian camel immunization and library construction

A 4-year-old male Bactrian camel was immunized subcutaneously five times using the purified PRRSV-1 and -2-N proteins. For the first time, the two recombinant proteins (1 mg/mL) were emulsified with an equal volume of Freund's Complete Adjuvant. The emulsifications were used in Freund's Incomplete Adjuvant for the other four times. The immunization was given every two weeks. After five immunizations, the serum samples from the immunized camel were collected and tested for anti-PRRSV-N antibodies using the iELISA. On the 7th d after the last immunization, the peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples by the Leucosep tubes (Greiner Bio-One, Germany). Then, total RNA was extracted from the PBMCs, and the cDNA was synthesized by reverse transcribed. Briefly, the 8 μL (4.5 μg) of total RNA, 1 μL of Oligo-dT_{20} primer, and 2 μL of RNase-free water in an RNase-free tube were mixed and incubated for 5 min at 65°C. The mixture was immediately on ice for 2 min and added with 2 μL of 10 × RT buffer, 4 μL of 25 mM $MgCl_2$, 2 μL of 0.1 mM DTT, 1 μL of RNase OUTTM (40 U), and 1 μL of SuperScriptT-MIII Reverse transcriptase (200 U). The *VHH* genes were amplified using the cDNA as templates by nested PCR, as described previously. The first amplification was briefly performed with the primer pairs CALL001 and CALL002 (Duan et al. 2021). Then, the expected 700 bp fragments of PCR products were extracted according to the instructions of the EasyPure Quick Gel extraction kit (TransGen Biotech, Beijing, China) and used as the templates for the second amplification. The second amplification used VHH-FOR and VHH-REV primer pairs (Duan et al. 2021). After the second round of PCR products (400 bp) were purified, the products and phagemid pMECS vector with HA tag were digested by *Not* I and *Pst* I enzymes (NEB, Ipswich, MA, USA). After the PCR products were cloned into the pMECS vector using the T4 ligation enzyme (NEB, Ipswich, MA, USA), the positive plasmids were electro-transformed into competent *E. coli* TG1 cells at 1.8 kV, 2.5 μF and 200 Ω by ten 5 mm cuvettes on ice. The transformed bacterial cells were cultured on Luria–Bertani (LB) agar plates with 2% glucose and 100 mg/ml ampicillin at 37°C for 8 h. After the recombinant bacteria were collected from the plates, the 100 μL of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} diluted aliquot of the library was used to calculate the library size (library size = dilution multiple × bacterial colonies × volume). The 95 monoclonal bacteria were randomly selected from the plates and detected with primer pairs VHH-FOR and VHH-REV (Duan et al. 2021) by PCR to determine the transformation efficiency.

Screening of nanobodies against PRRSV-N protein

To screen the nanobodies against PRRSV-N protein, phage display technology was used for three rounds of panning, as described previously (Sheng et al. 2019; Duan et al. 2021; Mu et al. 2021; Ma et al. 2019). First, the VHH phage library (1 mL) was inoculated into 200 mL of LB/AMP-GLU medium, which shook at 37°C for 1 h. Then, the 20 MOI of M13K07 helper phages were added to infect the *E. coli* cells. The cells were inoculated into 400 ml of 2 × YT/AMP-KAN medium. After centrifuging at 4°C for 30 min at 2,200 *g*, the supernatant was added with 1/5 volume of PEG/NaCl solution to precipitate the particles of the recombinant phages. The 96-well ELISA plates (Beijing Solarbio Science & Technology Co., Ltd) were coated separately with PRRSV-1 and -2-N proteins (200 μg/mL) using PBS (0.1 M, pH 7.4) at 4°C for 16 h. Then, the plates were blocked with blocking buffer [PBS with 0.5% Tween-20 containing 10% (w/v) skim milk] at 37°C for 1 h. After the plates were washed with PBS'T (PBS containing 0.05% Tween 20), the rescued recombinant phage particles (5×10^{11} PFU/mL) were added to the plates and incubated at 25°C for 1 h. After being washed with PBS'T again, the plates were added with 100 μL Glycine–HCl buffer (pH 2.2) to elute the recombinant phage. Then, the eluted phages were immediately neutralized with the same volume of 1 M Tris buffer (pH 9.0). Subsequently, the eluted phages were used to infect *E. coli* TG1 and further amplified. After three rounds of panning, the enrichment of specific phage particles was calculated. Then, 192 colonies were randomly selected from the third round panning plates and cultured in 1 mL TB medium. Until the OD_{600nm} reaches 0.6, 10 μL of 1 M IPTG was added to culture overnight at 37°C. After the cell pellets were frozen and thawed, the recombinant soluble periplasmic extracts were collected and detected by the iELISA described below. Finally, the positive clones were sequenced and classed into groups according to high variable complementary determination region 3 (CDR3) sequences.

Expression of nanobody-HRP fusion proteins with HEK293T cells

The nanobody-HRP fusion proteins were expressed with the HEK293T cells as previously described (Sheng et al.

2019; Duan et al. 2021; Mu et al. 2021; Ma et al. 2019). Briefly, the genes encoding nanobodies were obtained from positive recombinant pMECS vector by digestion with *Not* I and *Pst* I enzymes. Then, they were cloned into the pCMV-HRP vector with C-His and N-HA tags, which were also digested with the same enzymes. After being sequenced, the positive recombinant plasmids were transfected into the HEK293T cells using polyetherimide reagent (PEI, Polysciences Inc. Warrington, USA). At 3 d-post-transfected, the supernatants containing nanobody-HRP fusion proteins were collected and centrifuged at 3,000 *g* for 15 min. The expression of a nanobody-HRP fusion protein in the medium was identified by Western blotting, ELISA with the medium or PRRSV-1, and -2-N proteins as the coated antigen. For Western blotting, after transfection 72 h, the supernatants containing nanobody-HRP fusion proteins were analyzed by Western blotting with the mouse anti-His antibodies as the first antibody and the goat anti-mouse IgG (H&L) HRP (Bioss, Beijing, China) as second antibody.

ELISA

To determine the titration of antibodies in the immunized camel, the periplasmic extracts containing specific nanobodies and the supernatants containing nanobody-HRP fusion proteins reacting with PRRSV-1 and -2-N proteins, the ELISAs were performed as previous descriptions, respectively (Sheng et al. 2019; Duan et al. 2021; Mu et al. 2021; Ma et al. 2019). The 96-well plates were coated separately with the purified PRRSV-1 and -2-N proteins (400 ng/well) at 4°C for 16 h. Then, the plates were blocked with the blocking buffer at 37°C for 2 h. After the plates were washed with PBS'T three times, they were added with the different dilutions of serum samples from immunized camel, periplasmic extracts from the 192 colonies of the third round panning plates, or the supernatant from the transfected HEK293T cells and incubated at 37°C for 1 h. For the serum samples, the plates were then incubated with rabbit anti-camel polyclonal antibodies for 1 h at 37°C and followed with HRP-labelled goat-rabbit IgG. The plates were incubated with HRP-labelled HA monoclonal antibodies (GenScript, Biotech Corp., China) for 1 h at 37°C for the periplasmic extracts. Then, after being washed three times with PBST, the plates were added with 100 µL of tetramethyl benzidine (TMB, Beijing Solarbio Science & Technology Co., Ltd) and incubated for 15 min at 37°C for color reaction. Finally, each well's optical density at 450 nm (OD_{450nm}) was read using an automatic microplate reader (Bio-Rad, USA) after the reaction was stopped by 50 µl of 3 M H_2SO_4. The TMB was directly added for a color reaction for the supernatant, and the OD_{450nm} values were read after the reaction was stopped.

Indirect immunofluorescence assay

To determine the inconsistent pig sera in the virus-infected cells, an indirect immunofluorescence assay (IFA) was performed as described previously (Duan et al. 2021). Briefly, MARC-145 cells were infected separately with the PRRSV-2 SD16 strain at 0.1 MOI. At 36 h post-infection (hpi), the infected cells were fixed with 4% paraformaldehyde at 4°C for 30 min. After being washed twice with PBS, the cells were blocked with 1% BSA in PBS for 1 h. After being washed three times with PBS again, the cells were incubated with the clinical pig serum samples at 37°C for 1 h. Then, after being washed again, the cells were incubated with goat anti-pig IgG (H&L)-Alexa Fluor 488 (Life-iLab, Shanghai, China) at 37°C for 1 h. The cells were also stained with 2-(4-Amidinophenyl)-6-indolecarbamidine (DAPI) dihydrochloride (Beyotime, Shanghai, China) at 25°C for 15 min. Finally, the cells were observed under fluorescence microscopy (Leica AF6000, Germany).

Development of two competitive ELISAs using nanobody-HRP fusion proteins

Previously, a cELISA was established using nanobody-HRP fusion protein as the reagent to detect antibodies against PRRSV-2 (g2-cELISA) (Duan et al. 2021). Based on the previous procedures, two cELISAs to separately detect common antibodies against PRRSV-1 and -2 (g1-2-cELISA) and specific antibodies against PRRSV-1 (g1-cELISA) were developed in the study.

To optimize the dilutions of nanobody-HRP fusion proteins and the coating antigens amount for the two cELISAs, the direct ELISAs were performed as the above descriptions. The different amounts of PRRSV-1-N protein for the coating antigens were 50, 100, 200, 400, and 800 ng/well. For the nanobody-HRP fusion proteins, the different dilutions of 1:10, 1:50, 1:100, 1:200, 1:400, and 1:800 were used. Finally, the best dilutions of the fusions and amounts of coating antigens were selected when the OD_{450nm} values were approximately 1.0 in the direct ELISAs.

To determine the optimal dilutions of testing pig sera in the two cELISAs, three positive and negative pig sera for antibodies were detected separately against PRRSV-1 and -2. These sera were diluted with 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320 for detection. The optimal dilution of testing pig sera was selected when the cELISAs' P/N values were the lowest.

In addition, the incubation times between the mixture of testing pig sera and nanobody-HRP fusion proteins with the coated antigen and the times of colorimetric reaction were also optimized. The incubation times of 20, 30, and 40 min and the color reaction times of 10, 15, and 20 min were separately used in the cELISAs.

Then, the optimal times for incubation and color reaction were selected when the P/N values of the cELISAs were the lowest.

After optimizing the above conditions, the procedure of the two cELISAs was as follows. The 96-well plates were coated with the optimal concentration of PRRSV-1-N protein (100 μg/well) with PBS and incubated at 4°C for 16 h. After being washed three times with PBS'T, the plates were blocked with a blocking buffer at 37°C for 2 h. After being washed three times with PBS'T, each well was added into the mixture containing optimal diluted pig serum sample and nanobody-HRP fusion protein against PRRSV-1-N protein or PRRSV-1- and -2-N proteins (100 μL/well) and incubated optimal time at 37°C. After being washed three times with PBST again, the plates were added with TMB (100 μL/well) and incubated at 25°C for the optimized time. After the colorimetric reactions were stopped with 3 M H_2SO_4 (50 μL/well), the OD_{450nm} values were read with an automatic microplate reader. Finally, the percent inhibition (PI) values of testing pig sera were calculated with the following formula: PI (%) = [1-(OD_{450nm} value of testing pig serum sample/OD_{450nm} value of negative sample)] × 100%.

Determination of cut-off values, sensitivities, specificities, reproducibility, and stabilities of two cELISAs

To determine the cut-off values of the two cELISAs, 187 negative pig sera for anti-PRRSV antibodies were tested with the two assays. Then, the cut-off values for the developed two cELISAs were set at the mean PI of these negative pig sera plus 3 standard deviations (SD), which could give 95–99% confidence for the negative pig sera falling within the defined range.

The sensitivities of two cELISAs were evaluated separately with 13 strongly and 6 weakly positive pig sera for anti-PRRSV antibodies diluted from 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280 and 1:2560. The pig sera from the pigs challenged with PRRSV-2 SD16 and NADC-like strains and PRRSV-1 GZ11-G1 strain were also detected with the two cELISAs.

The specificities of two cELISAs were assessed using 90 positive pig sera for antibodies against other swine viruses, including PEDV ($n = 17$), TGEV ($n = 13$), PPV ($n = 23$), PCV2 ($n = 19$) and PRV ($n = 18$). Additionally, for the cELISA to specifically detect 27 positive pig sera for antibodies against PRRSV-1, 53 positive pig sera for antibodies against PRRSV-2 were also tested with the assays.

The reproducibility of the developed two cELISAs was determined with 6 positive and negative pig serum samples. Then, the coefficient of variation (CV) was used to evaluate the inter- and intra-plate differences. Each sample was tested with three plates on different occasions to determine the inter-assay CV, and three replicates within each plate were used to calculate the intra-assay CV.

The stabilities of two cELISAs were also evaluated for the following development of commercial kits. Briefly, the purified recombinant PRRSV-1-N protein was coated into ELISA plates using 100 ng/well at 4°C for 16 h. After being washed three times with PBST, the plates were blocked with a blocking buffer at 37°C for 2 h. The plates were washed again, dried in a fume hood, and vacuumed. Then, the dried plates and optimized dilution of nanobody-HRP fusion were stored at 4°C. The direct ELISA and cELISA were performed respectively with the procedure mentioned above at 0, 1, 2, 3, 4, 5, 6, 7 and 8 month-post-stored to evaluate the stability of the two cELISAs.

Comparisons between two cELISAs and commercial ELISA kit

To determine the agreements of the two cELISAs with the commercial ELISA kit, 1657 pig sera were tested simultaneously with the three assays. Out of which, 85 were from the different dpi of 10 challenged pigs with different PRRSV-2 strains SD16 and NADC30-like, 48 were from six challenged pigs with PRRSV-1 strain GZ11-G1 and 1534 were from the 6 pig farms in Shaanxi Province. The testing results of the three assays were analyzed *via* SPSS software. The IFA was subsequently performed based on the above description with minor modifications for the inconsistent pig sera.

Acknowledgements

We thank Pro. Hanchun Yang from China Agricultural University for providing PRRSV-1 GZ11-G1 strains.

Authors' contributions

Xu Chen: Conceptualization, Validation, Data curation, Writing – original draft. Yueting Chang: Data curation, Formal analysis, Validation. Lu Zhang: Investigation, Data analysis. Xinyu Zhao: Investigation, Data analysis. Zhihan Li: Homologous modeling and molecular docking. Zhijie Zhang: Validation. Pinpin Ji: Validation. Jiahong Zhu: Methodology. Qingyuan Liu: Validation. Jiakai Zhao: Methodology. Baoyuan Liu: Investigation, Data analysis. Xinjie Wang: Homologous modeling and molecular docking. Yani Sun: Methodology, Writing – review & editing. Qin Zhao: Conceptualization, Methodology, Writing – review & editing, Funding acquisition.

Funding

The research is funded by grants from the National Key R&D Program of China (2023YFD1800304), the National Natural Science Foundation of China to QZ (32273041), the Natural Science Foundation of Shaanxi Province of China (2022JC-12), and the Central Public-interest Scientific Institution Basal Research Fund, National Data Center of Animal Health.

Availability of data and materials

Statistical analysis was performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA) using a one-way analysis of variance (one-way ANOVA). Comparisons between groups were considered statistically significant at $P < 0.05$. Repeatability was assessed using CV (CV = SD/Mean), where a CV value of less than 10% for the intra-plate assay was considered acceptable repeatability. Kappa index values were calculated to estimate the coincidence between two cELISAs and the commercial ELISA kit. These calculations were performed using SPSS software (Version 20, http://www.spss.com.cn).

Declarations

Ethics approval and consent to participate

Animal experiments were performed based on the Guidance for Experimental Animal Welfare and Ethical Treatment by the Ministry of Science and Technology of China. The protocols of animal experimental procedures were carried out following the guidelines of the Northwest A&F University Institutional Committee for the Care and Use of Laboratory Animals and were approved by the Committee on Ethical Use of Animals of Northwest A&F University (approval no. 20220036/03).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Author Qin Zhao was not involved in the journal's review or decisions related to this manuscript.

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Received: 14 December 2023 Accepted: 22 February 2024
Published online: 28 March 2024

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