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Preparation of a monoclonal antibody against recombinant LSDV034 protein and its application in detecting lumpy skin disease virus through a competitive enzyme-linked immunosorbent assay (cELISA)

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Abstract

Lumpy skin disease (LSD) is a highly contagious disease caused by lumpy skin disease virus (LSDV) in bovines. Rapid and accurate diagnosis is very important to controll it. However, current commercial detection kits need to be improved in terms of sensitivity or specificity. This study aimed to develop a novel diagnostic competitive enzyme-linked immunosorbent assay (cELISA) based on the newly identified antigen gene *LSDV034*. The rLSDV034 protein was identified as a potential diagnostic antigen, and it was expressed, purified, and used to immunize BALB/c mice. Using laboratory-prepared indirect ELISA (iELISA), the positive cell lines were screened, and their blocking activity was further verified by competitive ELISA (cELISA). The cell line, 1H7, was chosen to produce mouse ascites, which were purified for a monoclonal antibody (mAb, 5.395 mg/mL). The heavy chain type of the 1H7 mAb was identified as IgG1a, and its light chain subtype was identified as κ. Furthermore, cELISA was developed to detect bovine serum antibodies, with rLSDV034 (4 μg/mL) as the coating antigen and HRP-1H7 mAb (1:300) as the competitive antibody. The cutoff value of cELISA was 55%, based on 32 negative bovine serum samples. The analytical sensitivity was 1:8, and no cross-reaction was detected with bovine viral diarrhea virus (BVDV), infectious bovine rhinotracheitis virus (IBRV), *Pasteurella multocida* (*P. multocida*), or *Mycoplasma bovis* (*M. bovis*) from the serum samples. The diagnostic sensitivity and specificity of cELISA were 98.46% (95% confidence interval, CI: 91.7–100) and 100% (95% CI: 89.1–100), respectively, based on the analysis of 30 LSDV-infected bovine serum samples, 35 GTPV-vaccinated samples, and 32 negative samples. The overall coincidence of the cELISA with the virus neutralization test (VNT) reached 98.97% (95% CI: 94.4–100). Furthermore, we used cELISA to analyze 230 clinical bovine serum samples (including 59 infected and 171 vaccinated samples) and found that the serum positivity rates of the immunized samples (on d 60 postimmunization) and infected samples were 77.78% (95% CI: 70.8–83.8%) and 71.19% (95% CI: 57.9–82.2), respectively.

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These results indicate that the developed cELISA is promising for detecting serum antibodies in naturally infected or vaccinated cattle.

Keywords Lumpy skin disease, Lumpy skin disease virus, rLSDV034 protein, monoclonal antibody (mAb), cELISA, Goat pox vaccine

Introduction

Lumpy skin disease (LSD) is an infectious disease caused by lumpy skin disease virus (LSDV) in bovines, and it is primarily transmitted through insects. LSDV can infect multiple animals, such as giraffes (Dao et al. 2022). LSD is primarily characterized by widespread nodules on the skin, and it can penetrate the subcutaneous layer, reaching the muscles (Abutarbush et al. 2015). In August 2019, LSD broke out for the first time in the Ili region of Xinjiang, China. Subsequently, LSDV spread nationwide, affecting a wide range of cattle, including beef, dairy, and yak. The incidence of LSD ranges from 5 to 45%, with an average mortality of approximately 5%, and LSD has been listed as a Class II animal infection disease in China (Yuan et al. 2023). Due to its high infectivity and severe harm to cattle, LSD poses a significant threat to the healthy and sustainable development of China's cattle industry (Lu et al. 2021).

LSDV belongs to the *Poxviridae* family and the *Capripoxvirus* (CaPV) genus. It is a linear and double-stranded DNA virus encompassing 156 open reading frames (ORFs). Genetically, this virus is relatively stable, presenting only one serotype (Tulman et al. 2001). The nucleotide sequence of LSDV closely resembles that of sheep pox virus and goat pox virus, with over 97% similarity, resulting in cross-protection during vaccination and serological cross-reaction. Since the live attenuated goat pox vaccine has been widely used for the prevention and control of LSD in China, there is an urgent need to develop a detection method for the clinical diagnosis and evaluation of vaccine effects (Tulman et al. 2002; Tuppurainen et al. 2021).

At present, the World Organization for Animal Health (WOAH) has recommended several serological detection methods for LSDV, mainly including the virus neutralization test (VNT), Western blot analysis (WB), indirect immunofluorescence assay (IFA), and enzyme-linked immunosorbent assay (ELISA) (WOAH 2023). Though regarded as the gold standard for detecting *capripoxvirus* serum antibodies, the complexity and time-consuming nature of VNT renders it unsuitable for large-scale serum sample screening, as well as Western blot analysis. And IFA can result in nonspecific staining, leading to misjudgment (Zeedan et al. 2019). Several kinds of ELISA methods, such as the ID-Vet Screen® Capripox Double Antigen multispecies test kit (ID-Vet, Grabels, France) and polyclonal antibodies (Babiuk et al. 2009; Bowden et al. 2009; Venkatesan et al. 2018), have been developed to detect serum antibodies. The specific antigens and polyclonal antibodies exhibit multiple advantages, such as high production efficiency, low production cost, and low technical requirements (Lipman et al. 2005).

Consequently, they are widely applied in the clinical diagnosis, epidemiological surveillance, prevention and control of LSD. However, a significant drawback of specific antigens or polyclonal antibodies is that they tend to lack sensitivity to low concentrations of serum antibodies. In contrast, mAb-based cELISA displays high sensitivity to target antibodies in complex serum samples, even to specific antibodies in relatively small amounts of serum samples (Dobrovolskaia et al. 2006). Thus, it is essential to develop an ELISA diagnostic method using mAbs against LSDV for detecting serum antibodies in cattle naturally infected with LSDV and vaccinated with GTPV.

This study aimed to develop a new cELISA method to detect serum antibodies in cattle infected with LSDV or vaccinated with attenuated GTPV based on identification of a new target, recombinant LSDV034, through Western blot analysis and mass spectrometry. Subsequently, we prepared a mAb against the recombinant LSDV034 protein and established a 1H7 mAb-based cELISA, which exhibits promising application potential for the prevention and control of LSD.

Results

Recombinant protein expression

The expression of the purified rLSDV034 protein was detected by SDS-PAGE (Fig. 1A, B) and Western blotting (Fig. 1). Protein bands were detected using an commercial anti-His-tagged mAb and an HRP-conjugated goat anti-mouse IgG (H + L) antibody, which indicated that the protein was successfully expressed (Fig. 1A, C). A protein band with a molecular weight of ~ 30 kDa was observed, indicating the desirable purification effect of the rLSDV034 protein (Fig. 1B). Western blot analysis using an LSDV-positive serum antibody and an HRP-conjugated rabbit anti-bovine IgG (H + L) antibody revealed the presence of the target protein bands, suggesting the reactogenicity of the rLSDV034 protein (Fig. 1D).

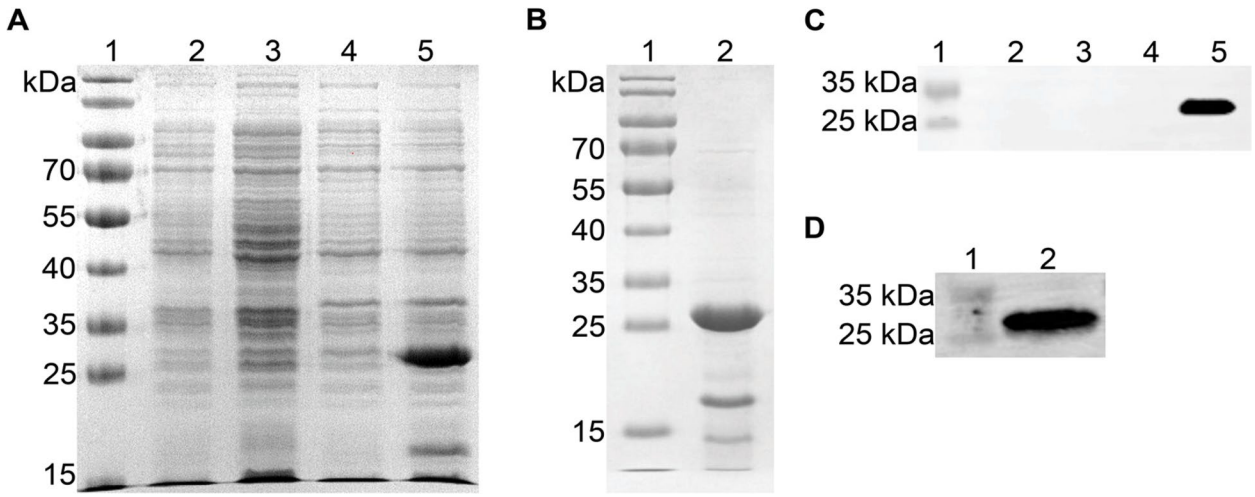

Fig. 1 Expression, purification, and reactogenicity of the rLSDV034 protein as determined by SDS-PAGE (**A**, **B**) and Western blot analysis (**C**, **D**). In (**A**) and (**C**), Lane 1, molecular weight marker; Lane 2, pET-28a (+); Lane 3, pET-28a + IPTG; Lane 4, rLSDV034; Lane 5, rLSDV034 + IPTG. In (**B**) and (**D**), Lane 1, molecular weight marker; Lane 2, purified rLSDV034 protein

Determination of mouse serum titers

SPF BALB/c mice were immunized with the purified rLSDV034 protein three times, with an additional SPF BALB/c mouse serving as a negative control. Tail venous blood samples were collected after the third immunization with the rLSDV034 protein (Fig. 2A). The results showed that the mouse serum antibody titers after three immunizations met the fusion standard (1:12,800) (Fig. 2B). The highest positive serum titer was observed in mouse 3, reaching 1:204,800, with an OD_{450} value of

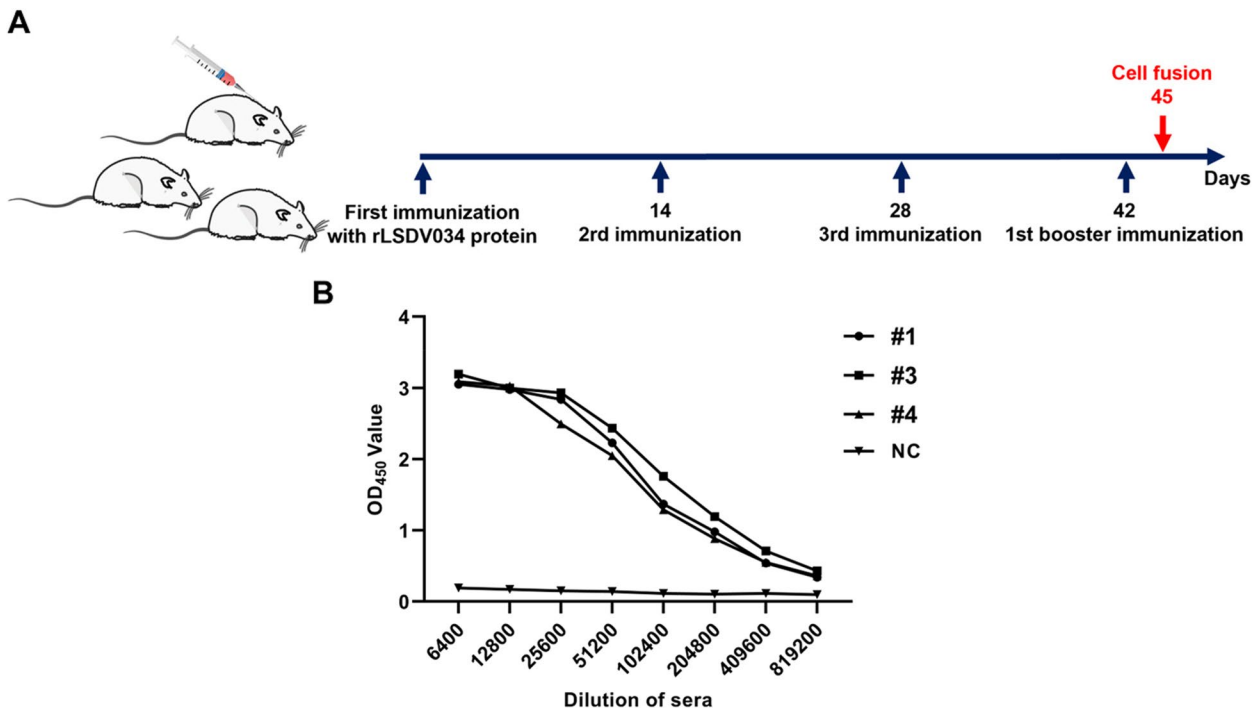

Fig. 2 Immunization of SPF BALB/c mice with the rLSDV034 protein. **A** Flowchart of 4 mouse immunizations with purified rLSDV034. #1, #3, #4, and the negative control (NC) represent different SPF BALB/c mice with and without immunization (NC). **B** OD_{450} values of immunized mouse sera at various dilutions ranging from 1:100 to 1:819,200

1.196. Consequently, mouse 3 was selected for booster immunization and subsequent cell fusion.

Generation and verification of the hybridoma cell lines

To obtain positive hybridoma cell lines stably secreting mAbs against the rLSDV034 protein, we performed a homemade iELISA with the rLSDV034 protein and control protein as coating antigens. After three rounds of subcloning, positive hybridoma cell lines were obtained (Fig. 3A). The chosen hybridoma cell line was termed 1H7 and stored in the China Center for Type Culture Collection (CCTCC) at Wuhan University (Wuhan City, China) under the preservation number CCTCC NO: C2023160.

The 1H7 mAb was 2-fold serially diluted from 1:100 to 1:204,800, and its titer reached 102,400 (Fig. 3B). Subsequently, the purification of the 1H7 mAb and the presence of its heavy and light chains were confirmed using SDS-PAGE and Coomassie Brilliant Blue staining (Fig. 3C). Additionally, Western blot analysis revealed that the protein band had a molecular weight of ~ 30 kDa, which was consistent with the predicted value of the rLSDV034 protein (Fig. 3D).

Preparation of mouse ascites and identification of mAb subtypes

The 1H7 hybridoma cell line was intraperitoneally injected to produce a large amount of mAb. The heavy chain of the 1H7 mAb was identified as the IgG1a type, and its light chain was identified as the κappa (κ) type (Table 1).

Optimization of cELISA conditions

As shown in the supplementary materials, the optimal conditions determined by cELISA included an antigen coating concentration of 4 μg/mL for the rLSDV034 protein, a serum dilution ratio of 1:2, an HRP-conjugated 1H7 mAb dilution ratio of 1:300, and an one-hour of

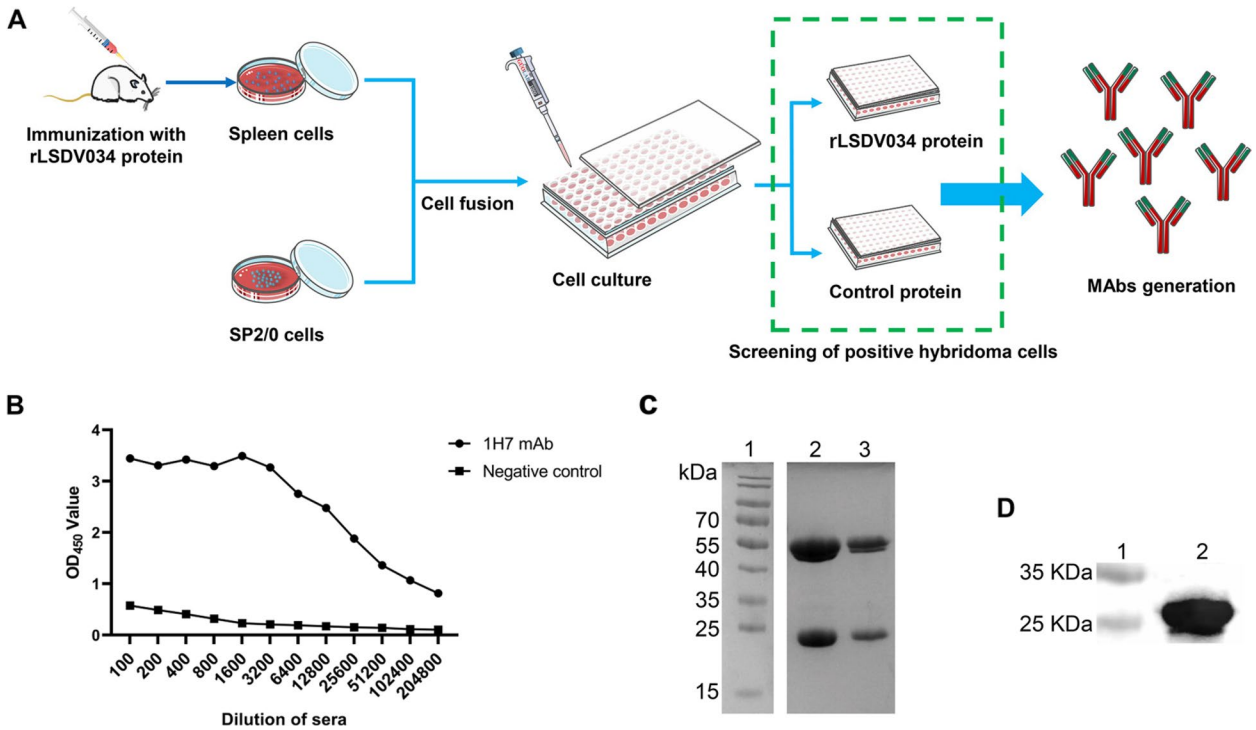

Fig. 3 Generation, purification, and verification of the 1H7 mAb. **A** Schematic diagram of the 1H7 mAb preparation; Control protein: *E. coli* lysate transformed with the pET-28a vector. **B** Antibody titers of the purified 1H7 mAb. **C** The molecular weight of the purified 1H7 mAb was determined by SDS–PAGE analysis. **D** Reactivity of the 1H7 mAb against the rLSDV034 protein, as determined by Western blot analysis

Table 1 Identification of 1H7 mAb subtypes

Subtypes	IgG1a	IgG2a	IgG2b	IgG3	IgM	IgA	κ	γ
OD_{450} values	1.309	0.079	0.048	0.048	0.046	0.063	1.008	0.079

ELISA reaction. The chromogenic reaction conditions were optimized at 37°C for 15 min.

Determination of the cutoff value

The PI values of infected, immunized, and negative serum samples were calculated. The results showed that the PI values of infected serum and immunized serum samples were significantly greater than those of the negative serum samples ($P < 0.0001$) (Fig. 4), indicating the excellent clinical applicability of the 1H7 mAb-based cELISA. The 32 negative bovine serum samples from uninfected or unvaccinated individuals were used to determine the ELISA cutoff value. The mean PI value of these 32 negative samples was 34.52%, with a standard deviation (SD) of 8.20%. Furthermore, the cutoff value was 50.92 (mean + 2 SD), and 59.12 (mean + 3 SD), respectively. Therefore, the final cutoff value (mean + 2.5 SD) of cELISA was 55%.

Analytical specificity and sensitivity of cELISA

Only a specific reaction with LSDV-positive sera was detected, and no cross-reactions were detected with BVDV-, IBRV-, *P. multocida*- or *M. bovis*-positive sera, indicating the excellent specificity of the cELISA. At a positive serum dilution of 1:8, the PI value was 67.92% (a PI ≥ 55% was considered positive). Thus, the lowest limit of detection (LOD) for positive serum was 1:8, which is greater than the optimal dilution ratio of 1:2 recommended by cELISA.

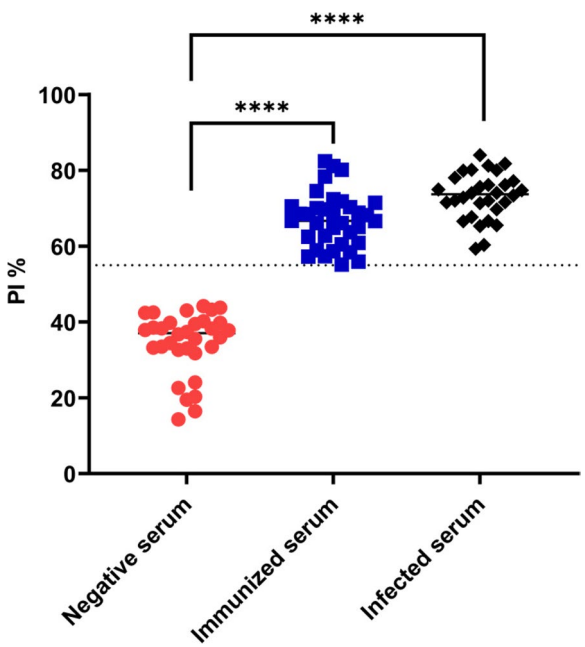

Fig. 4 ELISA detection of clinical serum samples with known backgrounds (30 LSDV-infected serum samples, 35 GTPV-immunized serum samples, and 32 negative serum samples)

Repeatability, reproducibility, and thermal stability of cELISA

The intrabatch coefficient of variation of cELISA ranged from 2.01% to 7.49%, which were all below 10% (Table 2), and the interbatch coefficient of variation of 3 batches of cELISA ranged from 0.97% to 9.57%, also below 10% (Table 3), indicating the desirable repeatability and reproducibility of cELISA. The difference in OD_{450} values was less than 0.2 among 3 replicates of the same serum sample incubated at 37°C for 3 d, and the PI value of positive sera was > 55%, indicating that the cELISA could withstand 3 d of high-temperature damage, exhibiting high thermal stability.

Application of cELISA

cELISA using the rLSDV034 protein and HRP-conjugated 1H7 mAb and iELISA (indirect ELISA) using the ID-vet Screen® Capripox Double Antigen Multispecies test kit were used to detect serum antibodies in three cattle immunized with the live attenuated goat pox vaccine at 0, 15 and 30 dpi (d postimmunization). cELISA successfully detected 3/3 of the serum antibodies at 15 dpi, while iELISA detected only 2/3 of the serum antibodies

Table 2 Repeatability of cELISA

Reference serum	Repeatability (OD_{450} values)			Coefficient of variation (CV %)
	Test 1	Test 2	Test 3	
1 (+)	0.507	0.463	0.502	4.910
2 (+)	0.482	0.516	0.444	7.493
3 (+)	0.377	0.340	0.341	5.977
4 (-)	1.819	1.853	1.780	2.010
5 (-)	1.110	0.964	1.003	7.370
6 (-)	0.955	1.034	0.993	3.975

"+" indicates that the serum antibody was positive; "-" indicates that the serum antibody was negative

Table 3 Reproducibility of cELISA

Reference serum	Reproducibility (CV %)		
	Test 1	Test 2	Test 3
1 (+)	7.493	5.428	5.094
2 (+)	5.977	7.424	9.568
3 (+)	4.910	0.973	8.160
4 (-)	2.010	9.356	4.226
5 (-)	7.370	3.906	7.541
6 (-)	3.975	5.826	5.698

"+" indicates that the serum antibody was positive; "-" indicates that the serum antibody was negative

at 15 dpi (Fig. 5). Additionally, 230 clinical bovine serum samples were tested by cELISA, including 171 GTPV-vaccinated serum samples and 59 LSDV-infected serum samples. The serum positivity of the GTPV-vaccinated individuals was 77.78% (95% CI: 70.8–83.8) at 60 dpi, and that of the LSDV-infected individuals was 71.19% (95% CI: 57.9–82.2).

Discussion

In this study, we employed Western blot analysis to investigate the reactogenicity of the rLSDV034 protein in bovine serum samples infected with LSDV and vaccinated with GTPV, revealing its potential as a novel diagnostic target protein for cELISA. Previous research has identified the P32 protein of LSDV as a promising diagnostic target, but the hydrophobicity in its transmembrane region makes it challenging to induce P32 protein highly expressed (Heine et al. 1999). Thus, the expression of truncated P32 protein was considered incomplete. In addition, the P32 protein also exhibited a particularly low full-length expression in the eukaryotic Sf9 insect expression system. The absence of a transmembrane structure in the LSDV034 protein allowed its high-level expression in prokaryotic systems after codon optimization. The *LSDV034* gene is annotated as an RNA-binding protein, and it has been reported to be involved in the regulation of gene expression and the RNA molecule life cycle. This RNA-binding protein plays an important role in the regulation of RNA splicing, maintenance of cellular function, and environmental adaptation (He et al. 2023). Given its association with RNA-binding proteins, the functions of the *LSDV034* gene remain to be further investigated.

Currently, the establishment of ELISA for the detection of serum antibodies against LSDV is primarily based on the principle of antigen–antibody binding or the use of polyclonal antibodies. For example, Bowden et al. (2009) used core proteins such as the ORF095 protein and ORF103 protein as detection targets to establish iELISA methods, and the corresponding diagnostic specificity and sensitivity ranged from 95-97%. Additionally, Venkatesan et al. (2018) employed the GTPV P32 protein as a coating antigen to establish an iELISA, which exhibited a diagnostic specificity of 100% and a diagnostic sensitivity of 97.1%. Jing et al. (2021) used the ORF117 protein as an ELISA detection target for clinical application. However, mAb-based ELISA exhibits greater specificity and sensitivity than polyclonal antibody- or specific protein-based ELISA (Weiner 2015). MAbs have promising applications in ELISA, immunochromatographic strip detection, and other serological detection methods (Milovanović et al. 2020; Tang et al. 2022). In this study, based on the robust reactogenicity and immunogenicity of the rLSDV034 protein, we prepared a mAb targeting this protein. Furthermore, based on the rLSDV034 protein and HRP-1H7 mAb, a cELISA detection method was developed for detecting bovine serum antibodies. The World Organization for Animal Health (WOAH) recommends VNT as the gold standard for detecting serum antibodies against LSDV (WOAH 2023). Based on VNT data, 1H7 mAb-based cELISA exhibited a diagnostic sensitivity of 98.46% (95% CI: 91.7–100), which was greater than the 96.92% (95% CI: 89.3–99.6) sensitivity of iELISA using the ID-Vet Screen® Capripox Double Antigen Multispecies test kit, indicating the promising potential of our established cELISA.

The live attenuated goat pox vaccine originating from a field-isolated GTPV strain is commonly used for LSD prevention and control. However, the risk of recombining GTPV with wild-type LSDV strains has raised wide concern over its biosafety (Tuppurainen et al. 2014). Recently, the Chinese Ministry of Agriculture evaluated an inactivated goat pox vaccine aimed at immunizing

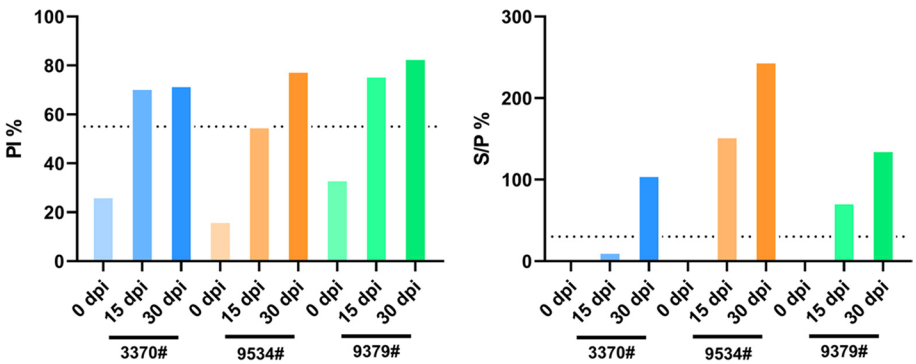

Fig. 5 Results of a cELISA using the rLSDV034 protein and HRP-conjugated 1H7 mAb and an iELISA using the ID-Vet Screen® Capripox Double Antigen Multispecies test kit for serum antibodies in three cattle immunized with GTPV at 0, 15 and 30 dpi (d postimmunization) to determine the earliest detection time

cattle to control LSD spread (http://www.moa.gov.cn/govpublic/xmsyj/202305/t20230517_6427790.htm). Our developed cELISA is also expected to be useful for detecting bovine serum antibodies after immunization with an inactivated vaccine. In our study, cELISA exhibited the ability to sensitively detect immunized serum antibodies as early as 15 dpi, highlighting its application value in LSDV serum antibody detection. However, it cannot distinguish naturally LSDV-infected serum samples from GTPV-vaccinated serum samples. Future studies are suggested to detect bovine serum samples with known backgrounds using cELISA extensively and to compare the effects of cELISA with those of VNT for further verification of this method.

Conclusion

The rLSDV034 protein was obtained via prokaryotic expression, and its 1H7 mAb was prepared. Using the rLSDV034 protein and an HRP-conjugated 1H7 mAb, a cELISA was developed for the detection of serum antibodies in cattle infected with LSDV and immunized with GTPV. Its diagnostic sensitivity and specificity were 98.46% (95% CI: 91.7–100) and 100% (95% CI: 89.1–100), respectively, indicating its promising clinical application prospects.

Methods

Experimental animals

Four- to six-week-old and 10-week-old SPF BALB/c mice were obtained from the Experimental Animal Center of Huazhong Agricultural University and used to develop the mAbs. Naturally, infected beef cattle, immunized cattle, and LSDV-negative cattle from one farm in Qinghai Province and another farm in Hubei Province, China, were sampled with the consent of the farm owners.

Sample collection

We collected 30 serum samples from individuals naturally infected with lumpy skin disease virus (LSDV), 35 serum samples from individuals vaccinated with the live attenuated goat pox vaccine (AV41 strain, 0, 15, 30, 45 and 60 dpi), and 32 negative serum samples from individuals not infected with LSDV or unvaccinated with GTPV. The backgrounds of the serum samples were confirmed with an ID Screen® Capripox Double Antigen Multispecies test kit (ID-Vet, Grabels, France). The neutralizing antibody titers of infected and immunized sera were tested by VNT. A total of 230 clinical serum samples with known backgrounds of natural infection or vaccination were collected for clinical cELISA detection.

Positive serum samples infected with bovine viral diarrheal virus (BVDV), infectious bovine rhinotracheitis virus (IBRV), *Pasteurella multocida* (*P. multocida*), and *Mycoplasma bovis* (*M. bovis*) were stored in the laboratory for subsequent analysis.

LSDV034 gene synthesis, expression, and protein identification

The high-abundance peptide segments in the recombinant LSDV034 protein were identified through Western blot analysis and mass spectrometry using goat pox vaccine virus and antibodies against bovine serum naturally infected with LSDV. Moreover, the high reactogenicity of *LSDV034*, which has high nucleotide homology (98.31%) to AXA19927.1 in the goat pox vaccine virus (GTPV), made the rLSDV034 protein a novel target for establishing a cELISA detection method.

The 576-bp sequence from the LSDV strain China/GD01/2020 (accession number: MW355944.1) was retrieved from GenBank. Subsequently, the codon of the nucleotide sequence was optimized, and the recombinant plasmid *pET28a-LSDV034* was synthesized. Then, the strains were transformed into *E. coli* BL21(DE3) cells, and the resulting positive recombinant bacteria were incubated with continuous shaking. The expression of the rLSDV034 protein was induced with 0.5 $mmol^{-1}$ isopropyl-β-D-thiogalactoside (IPTG, Biosharp, China) at 37°C for 5 h. The positive bacterial cells were harvested, resuspended in 1% (*v*/*v*) imidazole phosphate buffer (0.01 M, pH 7.4) and crushed using a high-pressure crusher (Life Technologies, Carlsbad, USA). The rLSDV034 protein in the supernatant was purified and eluted using a high-affinity Proteinlso® Ni–NTA Resin His Bind Purification Filler (TransGen, Beijing, China). The eluate containing the rLSDV034 protein was collected and concentrated using a 10 kDa ultrafiltration membrane. The protein concentration was determined using a bicinchoninic acid kit (Cowin, Jiangsu, China).

The rLSDV034 protein was separated by denaturing SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (Biosharp, Beijing, China). Subsequently, the separated protein was electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Merck, Germany) for Western blot analysis. The membrane was then blocked with 5% (w/v) skim milk in TBST buffer (TBS containing 0.05% (*v*/*v*) Tween-20) for 2 h at 25°C. After blocking, the membrane was incubated with a commercial mAb (Abbkine, Wuhan, China) specifically targeting the six His tags, which were diluted at a proportion of 1:5,000 in advance for 12 h at 4°C. The other membrane was incubated for 12 h at 4°C with LSDV-positive serum (diluted in advance at a ratio of 1:200). The two obtained membranes were washed three times with TBST and then incubated with 1:5000 diluted HRP-conjugated goat anti-mouse IgG (H+L) (Abbkine, Wuhan, China) and 1:5000 diluted HRP-conjugated rabbit anti-bovine IgG (H+L) (Abbkine, Wuhan, China) for

1 h at 25 °C. After being washed with TBST three times, the protein bands were visualized using enhanced chemiluminescence (ECL) reagents and a chemiluminescence imaging system (Bio-Rad, Richmond, USA).

Development of monoclonal antibodies against the rLSDV034 protein

The purified rLSDV034 protein was used to immunize 4- to 6-week-old SPF BALB/c mice. Specifically, mice received subcutaneous injections of 100 μg of protein and were immunized at 14-day intervals. After the third immunization, mouse tail venous blood was collected, and the mouse serum antibodies were determined using a homemade iELISA. The determination criteria for serum antibodies included an OD_{450} of the immunized mouse serum/OD_{450} of the unimmunized mouse serum > 2.1 and an OD_{450} of the positive serum antibody > 1. The mice with the highest serum titers received a booster immunization via intraperitoneal injection of 200 μg of rLSDV034 protein without emulsified adjuvants.

Cell fusion was performed by a previously reported method (Ossipow and Fischer 2014). Hybridoma cells were generated by fusing myeloma cells ($1–2\times10^7$ cells/ml) and immunization with spleen cells (1×10^8 cells/ml) using polyethylene glycol (PEG 4000, Sigma, USA). Positive hybridoma cell lines were screened using homemade iELISA with the rLSDV034 protein and control protein as coating antigens. Hybridoma cells with a high P/N ratio were subcloned three times by a limiting dilution method to ensure stable mAb secretion (Greenfield 2019). The selection criteria for positive hybridoma cells were an OD_{450} of mAb against the rLSDV034 protein/OD_{450} of mAb against the control protein > 2.1 and an OD_{450} > 1 (Xiao et al. 2023). Subtypes of positive monoclonal antibodies were identified using a mAb subclass identification kit (Biodragon, Suzhou, China).

Subsequently, mouse ascites were collected. Briefly, 10-week-old SPF BALB/c mice were injected with 500 μL of Freund's incomplete adjuvant (IFA, Sigma, USA), and after 5–7 d, they were intraperitoneally injected with antibody-positive hybridoma cells ($5\times10^5–1\times10^6$) to obtain mouse ascites. The mouse ascites were purified using rProtein G Beads 4FF columns (Smart Life Sciences, Changzhou, China) to obtain the mAb. The mAb titer was determined using homemade iELISA and stored at -80°C.

Establishment of a competitive ELISA (cELISA)

The optimal coating concentration of the rLSDV034 protein and the serum dilution ratio were determined using the checkerboard titration method (Li et al. 2024). HRP-conjugated 1H7 mAbs were diluted at ratios of 1:50, 1:100, 1:200, 1:300, 1:400 and 1:500, while the other variables remained constant. ELISA was performed, and the OD_{450} values of the HRP-conjugated 1H7 mAb at various dilution concentrations were measured. The percent inhibition (PI) was calculated to determine the optimum dilution for the HRP-conjugated 1H7 mAb according to the following formula: PI = (1-OD_{450} value of positive serum sample/OD_{450} value of negative serum control) × 100%.

The optimal chromogenic reaction time for the cELISA based on the rLSDV034 protein and HRP-conjugated 1H7 mAb was determined by the above method, and the PI was calculated under four chromogenic reaction conditions: 37°C for 5, 10, 15 and 20 min.

Finally, the optimal cELISA reaction time was determined. Specifically, 50 μL of LSDV-positive serum (1:2) or negative control serum (1:2) was incubated with 50 μL of the HRP-conjugated 1H7 mAb (1:300) at 37°C for 30, 45 or 60 min, and the PI concentration was calculated to determine the optimal cELISA reaction time. The chromogenic reaction procedure was as follows: 100 μL/well of 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB, Solarbio, Beijing, China)/H_2O_2 was added, the mixture was incubated, the reaction was terminated by the addition of 50 μL of ELISA stop solution, and the OD_{450} was measured using a microplate reader (BMG LABTECH, Offenburg, Germany).

The validity test criteria for the serum samples included a PI > 60%, an OD_{450} > 1.0, and an OD_{450} < 0.1. Serum samples with PI values ≥ 55% were considered positive, and those with PI values < 55% were considered negative.

Determination of the cutoff value for cELISA

Thirty LSD-naturally-infected serum samples, 35 live attenuated goat pox vaccinated serum samples and 32 clinically negative serum samples were detected to determine the cutoff value of the cELISA. ELISA was conducted, OD_{450} values were recorded, and PI values were calculated. The mean and standard deviation (SD) of the PI values were calculated, and the cutoff value was determined by the mean ± 3 SD to cover 100% of the serum samples (Han et al. 2015).

Evaluation of cELISA

The analytical specificity of the cELISA for the positive sera of LSDV, BVDV, IBRV, *P. multocida* and *M. bovis* was evaluated using the rLSDV034 protein and an HRP-conjugated 1H7 mAb. The sensitivity of cELISA to different dilutions of LSDV-positive serum (from 1:2 to 1:64) was assessed. OD_{450} values were measured, and PI values were calculated to determine the analytical specificity and sensitivity of cELISA.

The repeatability and reproducibility of cELISA were assessed. Reference serum samples ($n=6$) were analyzed using three batches of cELISA (LSDV001, LSDV002 and LSDV003) with the rLSDV034 protein and HRP-conjugated 1H7 mAb, and the PI was calculated. For the repeatability analysis, 6 different serum samples were tested with 3 replicates within a single batch of cELISA. For reproducibility analysis, six different serum samples were tested via three batches of cELISA.

In addition, the stability of the cELISA was evaluated. Briefly, the rLSDV034 protein-coated plates were incubated at 37°C for 1, 3, 5 or 7 d. LSDV-positive serum and negative serum samples were tested with three biological replicates. OD_{450} values were recorded, and PI values were calculated to evaluate the stability of the cELISA under high-temperature conditions.

Application of the cELISA

In this study, cELISA was employed to detect bovine serum antibodies using the rLSDV034 protein and an HRP-conjugated 1H7 mAb. Three bovine serum samples from pigs immunized with the live attenuated goat pox vaccine were collected at 3 different time points (0, 15 and 30 dpi) to access the earliest detection timeline of cELISA. Additionally, 230 clinical bovine serum samples with known backgrounds were examined.

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism V. 8.3.0 (San Diego, CA, USA). The data were tested for normality, and if the criteria were met, Student's *t* test was applied to compare significant differences in PI values among different serum samples (naturally infected with LSDV, vaccinated with GTPV, and negative). $P<0.05$ was considered as statistical significance.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44149-024-00126-x.

Supplementary Material 1.

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Authors' contributions

A.G. and C.H. conceived the project. X.Y., H.Z., W.J., X.Y., Z.X., L.Y., and Y.G. performed the experiments and organized the data. A.G., C.H., Y.C., J.C., X.C., and L.Z. offered helpful discussions and technical assistance. A.G. and C.H. revised the manuscript. All authors have read and approved the final version of the manuscript.

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Availability of data and materials

The data supporting the findings of this study are available within the article, and further inquiries can be directed to the corresponding author.

Declarations

Ethics approval and consent to participate

This study was conducted in strict accordance with the recommendations provided in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The Hubei Administrative Committee for Laboratory Animals approved the animal experiments (Approval Nos. HZAUMO-2022–0180 and HZAUCA-2023–0022).

Consent for publication

All the authors approved and provided their consent for publication of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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