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Evaluation of an In-House ELISA for Detection of Antibodies Against the Lumpy Skin Disease Virus in Vaccinated Cattle

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ABSTRACT

Lumpy skin disease (LSD) causes the death and premature culling of animals, resulting in great economic losses in animal husbandry practice. The primary methods for preventing its spread are the vaccination of susceptible livestock and timely and accurate diagnoses. Considering the high risk of LSD virus (LSDV) spread in Kazakhstan, the development of a sensitive and specific enzyme-linked immunosorbent assay (ELISA) would be crucial in ensuring the veterinary safety of the country. Thus, in this study, we developed an indirect ELISA based on the P32 antigen of LSDV for the serological diagnosis of cattle and evaluated its effectiveness. In this indirect ELISA, recombinant P32 (rP32) protein was used, which was produced using the DNA of an LSDV isolated from Kazakhstan. The protein was purified using metal affinity chromatography and examined using a mass spectrometer. The optimal concentrations of rP32 for immobilization on the plate were observed to be 0.6 and 0.3µg/mL. The serum dilutions with the lowest background values were 1:400 and 1:200. As a blocking buffer, 3% skimmed milk in phosphate-buffered saline-Tween (pH 7.4) was used. Secondary antibodies were used at a dilution of 1:20,000. In studies using an in-house P32/ELISA, 71% of bovine serum samples that were collected 28d after vaccination tested positive. Overall, the indirect ELISA showed high potential for epizootological monitoring and mass screening of animals vaccinated with a domestic vaccine. However, to obtain more reliable results, continuing this study using a larger number of serum samples from vaccinated cows is necessary.

Key words: Lumpy Skin Disease, Recombinant Antigen, Indirect ELISA, Antibodies, Vaccination.

INTRODUCTION

Lumpy skin disease virus (LSDV) belongs to the *Poxviridae* family of the *Capripoxvirus* genus and is the causative agent of lumpy skin disease (LSD), a dangerous transboundary disease that poses a serious threat to livestock breeding (Namazi and Tafti 2021; Anwar et al. 2022). The World Organization for Animal Health (OIE) lists LSD as a notifiable disease due to its significant economic impact (OIE 2021). The disease results in serious losses in animal productivity, abortions and complications during subsequent insemination, infertility of sires, skin damage, body weight reduction, and increased healthcare expenses (Khan et al. 2021; Namazi and Tafti 2021; Amin et al. 2021). The transmission of the virus from sick to healthy animals primarily occurs through blood-sucking insects (Tuppurainen et al. 2017; Khan et al. 2022; Hussien et al. 2022). Vaccination is the main method of combating the spread of the LSDV. To date, live attenuated vaccines against LSDV are mainly being used (Tuppurainen et al. 2021; Ratyotha et al. 2022; Whittle et al. 2023). Vaccines based on other members of the *Capripoxvirus* family, such as sheeppox and goatpox viruses, also can induce cross-immunity (Kitching 1983; Tuppurainen et al. 2012). In Kazakhstan, where LSD was first identified in 2016 on the western territory (Mathijs et al. 2020), a full vaccination of susceptible cattle was performed to prevent the spread of the virus to the entire

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territory. At first, the homologous Kenyan vaccine Lumpivax™ (KEVEVAPI) was used. Later, at the Research Institute of Biological Safety Problems in Kazakhstan, a vaccine was developed from the attenuated strain termed "Neethling-RIBSP" which has been successfully used in most regions of the country since 2020 (Ospanov et al. 2022).

Confirmation using laboratory diagnostic methods is necessary to evaluate the effectiveness of the vaccination (Haegeman et al. 2021). The main methods of detecting antibodies against the LSDV include the virus neutralization test, enzyme-linked immunosorbent assay (ELISA), and western blot analysis (OIE 2021). The virus neutralization test is the "gold standard" and is considered the most specific method for detecting antibodies against the virus. However, a significant disadvantage of this method is the complexity of setting and interpreting the results, duration of the reaction, and expensive laboratory requirements (Awad et al. 2010). Western blotting is a laborious and costly method that is unsuitable for screening large numbers of animals (Berguido et al. 2022). In contrast, ELISA can be used to examine a large number of samples with high sensitivity and specificity in a short time (Samojlović et al. 2019; Zeedan et al. 2019). This method has been successfully used to screen many livestock for various diseases. Since the spread of capripoxviruses, numerous studies have been performed with an aim to develop effective ELISA systems (Bowden et al. 2009; Tian et al. 2010; Chervyakova et al. 2018; Samojlović et al. 2019; Sthitmatee et al. 2023), however, only a few commercial tests are currently available. To date, only two ELISA kits are commercially available: the ID Screen® Capripox Double Antigen Multi-species from IDvet (France) and the Sheep/Goat Anti-Lumpy Skin Disease virus (LSDV) IgG ELISA kit from Life Technologies (India). Both kits were designed to detect antibodies against capripoxviruses.

The extensive and, most crucially, rapid spread of LSD in cattle to new territories emphasizes the critical need for improved diagnostic serological methods, such as ELISA and lateral flow assay.

The purpose of this study was to develop an indirect ELISA based on the P32 antigen of the LSDV and evaluate its effectiveness. This assay may allow for effective serological monitoring of cattle vaccinated against LSD as well as screening susceptible animals imported into Kazakhstan.

MATERIALS AND METHODS

Ethical Statement

This study was supervised and approved by the Local Ethical Committee of the National Center for Biotechnology, Astana, Kazakhstan (Protocol No. 3 dated August 7, 2020).

Serum Samples

Serum samples were obtained from 38 cattle vaccinated against LSDV using the Neethling-RIBSP vaccine manufactured in Kazakhstan from a farm in the Akmola region. Fifty serum samples from unvaccinated animals from prosperous farms in the East Kazakhstan region were used as negative controls.

Recombinant P32 (rP32) Protein Preparation

The sequence of the gene encoding the protein P32 of the LSDV was obtained according to our previously described method (Tursunov et al. 2022). Briefly, using the primers, we synthesized LSDV genomic DNA (provided by the Laboratory of Applied Genetics, "National Center for Biotechnology," Astana, Kazakhstan); the gene (780bp) was amplified using polymerase chain reaction (PCR). The resulting sequence was cloned into a pGEM-TEasy intermediate vector for production and selection. Following that, the DNA fragment was cloned into the expression vector pET28b+ using EcoRI/XhoI and T4 DNA ligase. After transformation of the resulting recombinant vector using the heat shock method, a preparative amount accumulated in the *Escherichia coli* DH5α strain. The recombinant construct was isolated using the HiPure Plasmid Midiprep Kit (Vilnius, Lithuania) and transformed into the expression strain *E. coli* BL21(DE3) via electrophoresis. For gene expression, the isopropyl-β-D-1-galactopyranoside (IPTG) inducer was added at various concentrations (0.1–1mM). The transformed expression strain was cultivated at two distinct temperatures (25 and 37°C) with stirring at 170rpm. To determine the optimal cultivation time, 5mL samples were taken every 2h after the addition of the inducer (2, 4, 6, and 18h). The obtained fractions were analyzed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant protein was purified via metal affinity chromatography using HisTrap Columns (GE Healthcare Life Sciences, Cardiff, UK) according to the manufacturer's instructions. The amino acid sequence of the purified rP32 protein was determined via nanoscale liquid chromatography and tandem mass spectrometry (nano LC-MS/MS). The obtained spectra were analyzed using the MASCOT database (http://www.matrixscience.com/).

Optimization of the Main Steps of Indirect ELISA

The rP32 protein was immobilized onto plates in 0.05M bicarbonate buffer (pH 9.6). Different concentrations (5–0.15μg/mL) were used to determine the optimal antigen concentration. Antigen incubation was performed at 4°C overnight. All stages of washing the plate with unbound components were performed three times with phosphate-buffered saline (PBS) containing Tween 20 (PBS-Tw) (pH 7.4). Several blocking buffers (1% bovine serum albumin, 1% and 3% skimmed milk, and 1% gelatin) were used to block free zones in the plate wells. All blocking buffers were added at 200μL/well and incubated at 37°C for 40min. After washing, the control serum was added at a dilution of 1:100, followed by double titration to 1:12800. After repeated incubation for 1 h, the optimal dilution of the secondary antibodies labeled with horseradish peroxidase was determined by adding various dilutions (1:5,000, 1:10,000, 1:15,000, 1:20000, and 1:30,000). Following incubation and washing, the plates were washed three times with PBS without Tween. The reaction was performed by adding 100μL of orthophenylenediamine substrate solution and incubating at 25°C in a dark place for 10min. The reaction was stopped by adding 100μL of 2M H_2SO_4. The optical

density (OD) at 490nm was determined using a spectrophotometer (Bio-Rad).

To determine the cutoff value for differentiating positive and negative samples, the average OD of the sera of healthy, unvaccinated animals was used. Sera were considered positive if their OD exceeded twice the average OD of negative sera (Erdenebaatar et al. 2003).

Comparing Indirect ELISA Based on rP32 With Commercial ELISA

The previously optimized parameters were used to assess the diagnostic potential of the developed ELISA. A total of 88 sera samples were tested, of which 38 were from vaccinated animals and 50 were from healthy, unvaccinated animals. Additionally, all samples were examined in parallel using a commercial ELISA kit, in accordance with the manufacturer's instructions.

RESULTS

rP32 Protein Preparation

Genomic DNA was used to amplify the sequence encoding the P32 protein of the LSDV. The resulting fragment was cloned into the pET28b+ expression vector and single colonies after transformation into *E. coli* BL21(DE3) cells were examined via PCR. Electrophoresis revealed that all the selected colonies showed a band of approximately 1,000bp, which corresponded to the expected size (Fig. 1).

For rP32 expression, the optimal IPTG concentration was 0.5mM. Purification of the recombinant protein via metal affinity chromatography revealed that the protein was eluted from the column by the addition of a buffer containing 500mM imidazole (Fig. 2A). To confirm the presence of the target proteins in the samples, western blotting with monoclonal antibodies (mAbs) against the 6His Tag was performed. The results indicated that the mAbs specifically bound to a protein with a molecular weight of approximately 32kDa.

Nano LC-MS/MS Findings

Analysis of the peptides in the MASCOT database revealed that they belonged to the P32 protein of LSDV (Fig. 3).

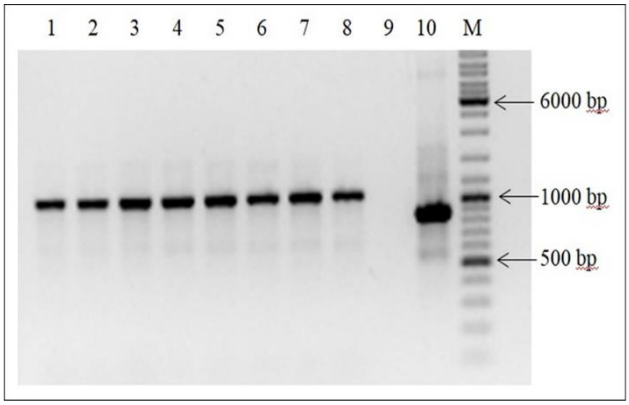

Fig. 1: PCR products on a 1% agarose gel. Lanes 1–8, Tested clones; Lane 9, Negative control; Lane 10, Positive control; and Lane M, DNA ladder (#SM0333, Thermo Fisher Scientific).

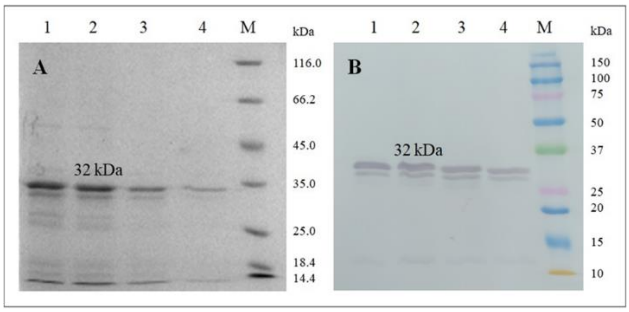

Fig. 2: SDS-PAGE (a) and western blotting (b) of the rP32/pET28 protein. Lines 1–4, purified P32 protein (500 mM imidazole); Lane M, Molecular weight markers (#1610375, Bio-Rad).

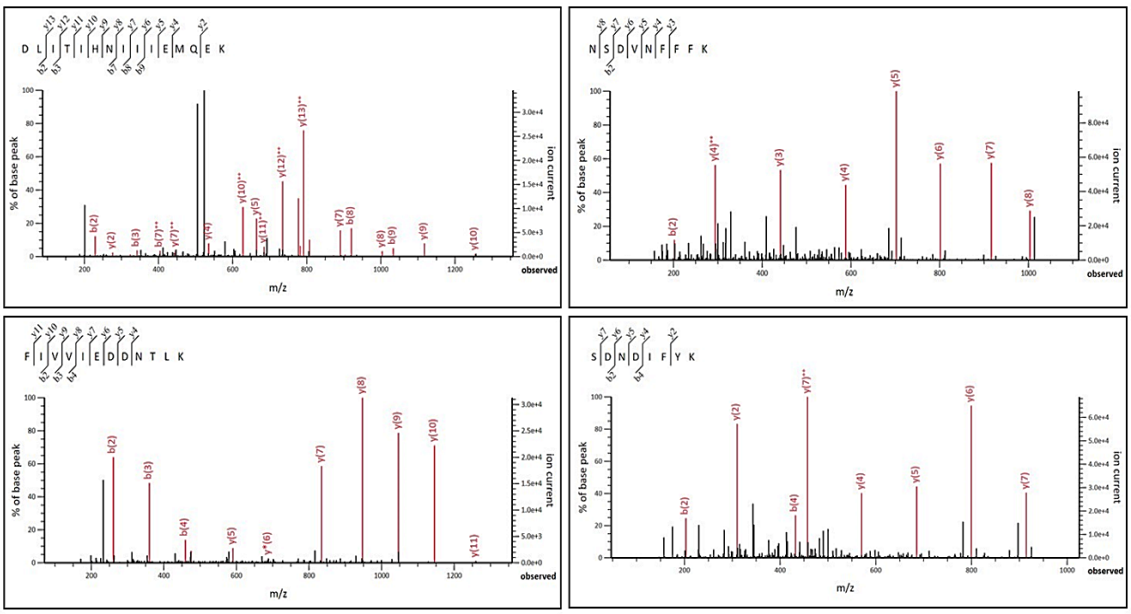

Fig. 3: LC-MS/MS of rP32/pET28 peptides.

Main Steps of Indirect ELISA

The optimal concentrations of rP32 for immobilization on plates were 0.6 and 0.3μg/mL (Table 1). The antigen was diluted in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The serum dilution at which the lowest background values were observed was 1:400 at a protein concentration of 0.6μg/mL and 1:200 for 0.3μg/mL. Optimal performance was observed when 3% milk in PBS-Tw (pH 7.4) was used as a blocking buffer. Secondary antibodies were used at a dilution of 1:20,000.

Effectiveness of rP32-Based Indirect ELISA

In studies using P32/ELISA, 27 (71%) serum samples from vaccinated cows showed positive results, and 11 (29%) were negative (Table 2). The mean titer of the positive sera was 0.358. All serum samples tested using a commercial ELISA kit showed negative results. The average titer of sera from vaccinated cows was 0.049, while that from negative cows was 0.05. The titers of the positive control (PC) of the commercial kit were 0.273 (PC1) and 0.295 (PC2), while those of the negative control (NC) were 0.056. When testing negative sera to determine the cutoff value, both tests showed 100% specificity. The cutoff value for P32/ELISA was 0.334 (0.167 × 2).

Table 1: Antigen concentrations and serum dilutions

Serum dilution	Antigen (P32) concentration (μg/mL)					
	5	2.5	1.2	0.6	0.3	0.15
1:100	1.61	2.06	2.35	2.76	2.72	2.05
1:200	1.13	2.35	2.38	3.11	3.35*	2.12
1:400	1.43	2.61	2.66	3.62*	2.78	2.49
1:800	1.57	2.62	3.14	3.60	3.21	2.48
1:1600	1.74	2.28	3.19	2.71	3.15	2.87
1:3200	1.52	2.33	2.96	1.95	3.29	2.76
1:6400	1.30	2.36	2.88	1.50	2.96	2.17
1:12800	1.25	1.96	1.94	1.26	2.58	2.26

*Optimum antigen concentration and serum dilution.

Table 2: Comparative analysis of in-house and commercial ELISA on the sera of vaccinated cows

Test variant	P32/ELISA (Positive)	P32/ELISA (Negative)	Total
Commercial ELISA (Positive)	0	0	0
Commercial ELISA (Negative)	27 (71)	11 (29)	38 (100)
Total	27 (71)	11 (29)	38 (100)

Values in parentheses are percentages.

DISCUSSION

A key factor in the prevention of LSD in cattle is the vaccination of susceptible livestock. Therefore, the development of a diagnostic kit to assess the effectiveness of vaccination and monitor unvaccinated livestock plays a critical role in ensuring veterinary safety. To date, many ELISAs have been developed for the detection of capripoxviruses. One of the first was an indirect ELISA test based on inactivated sheep pox virus. The specificity and sensitivity of the test were 97 and 88%, respectively (Bowden et al. 2009). One of the latest developments is the use of indirect ELISA to detect antibodies against LSDV. ELISA was performed on purified and inactivated LSDV isolated from skin nodules of infected animals with obvious clinical signs (Sthitmatee et al. 2023). The diagnostic potential of the developed ELISA was determined using field sera, and a commercial ELISA kit was used for comparison. The results of these studies revealed that the sensitivity and specificity of the developed test were not inferior to those of the commercial test, amounting to 95% and 89.8%, respectively. Notably, developing an ELISA based on an entire virus may lead to certain difficulties associated with its sufficient production (Tuppurainen et al. 2012). In addition, when working with the virus, certain laboratory conditions, nutrient media, and trained personnel are required (Ebrahimi-Jam et al. 2021; Berguido et al. 2022). This complicates the process of producing ELISA tests and leads to increased prices for the final product.

The technologies used to obtain recombinant antigens overcome these limitations. Based on recombinant proteins, many ELISAs have been developed to diagnose various animal diseases, including capripoxviruses. For example, the use of the recombinant structural protein P32 as an antigen, which includes the main immunogenic determinants and is present in all capripoxviruses (Tian et al. 2010). Based on the P32 antigen of the LSDV, an ELISA was developed by analogy with diagnostic tests against sheep pox and goat pox viruses (Babiuk et al. 2009). In Kazakhstan, studies have been conducted to develop an ELISA based on recombinant LSDV095 and LSDV103 proteins of the LSDV capable of differentiating capripoxvirus infections (Chervyakova et al. 2018).

In our study, the rP32 protein used to develop the ELISA test was obtained using the DNA of the LSDV isolated in Kazakhstan. The recombinant protein was produced using a prokaryotic system in *E. coli*. This method allowed us to obtain a preparative amount of protein and standardize all the main steps (Pouresmaeil and Azizi-Dargahlou 2023). The protein was purified using metal affinity chromatography and examined using mass spectrometry. The results revealed that the protein belonged to the LSDV.

The optimal concentrations of the P32 antigen for immobilization on the plate were found to be 0.6 and 0.3mg/mL. In the first case, at a serum dilution of 1:400, the difference between the ODs of vaccinated and unvaccinated animals was 3.62, and in the second case, 3.35. The optimal dilution of the conjugate was 1:20,000, and the lowest background was observed when 3% milk was used.

In the examination of the sera of vaccinated livestock using an ELISA based on the recombinant P32 antigen, 71% of the sera showed a positive result. The titers of these sera were two or more times higher than the mean values of the negative sera. Notably, when these sera were tested using a commercial ELISA kit, all showed negative results. Simultaneously, the commercial kit controls showed reliable results, indicating that the kit functioned properly. These results may be related to the fact that the sera of animals immunized with the Neethling-RIBSP vaccine were collected on the 28th day after vaccine administration. The negative reaction observed with the commercial kit was possibly due to insufficient time for antibody isolation. This finding was also evidenced by the titers of sera from vaccinated cows showing negative

results in the P32/ELISA. The titers of the 11 serum samples ranged from 0.314 to 0.332, which is close to the threshold cutoff value. Another reason may be the mutation of the viruses circulating in a particular area. The Neethling-RIBSP vaccine was obtained from a strain that was 99.96% identical to the Kubash/Kaz/16 strain of the virus, which was isolated from pathological material from a cattle population with lumpy dermatitis in the Atyrau region (GenBank: MN642592) (Ospanov et al. 2022).

Based on the obtained data, it can be assumed that domestic diagnostic tests should be performed in countries where domestic vaccines are used. The effectiveness of the test is likely to be low when foreign commercial tests are used to assess the effectiveness of vaccination.

Conclusion

The indirect ELISA based on the rP32 protein showed high efficiency in the examination of the serum of vaccinated animals. Considering the high risk of LSD spread in Kazakhstan and the serious economic losses associated with it, the development of a sensitive and specific ELISA is crucial for ensuring the veterinary safety of the country. However, despite these results, continuing this study using a larger number of serum samples from vaccinated cows is necessary to determine the suitability of rP32 of the LSDV for serological diagnosis. In addition, these studies will help determine the duration of immunity in vaccinated animals.

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Author's Contribution

KT and KM: Conceptualization, study design, and writing of the original draft. KT, LT, DK: Investigation (gene amplification, cloning, protein expression and purification, western blot, preparation of samples for mass spectrometry). PT: Recombinant protein analysis by LC-MS/MS spectrometry and data analysis. RM: Collection of data and serum samples. KT, KM: Optimization of the main ELISA parameters, ELISA setting, and data analysis. RM, KM: Reviewing and editing the manuscript.

Conflicts of Interest: The authors declare no competing interests.

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