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RECOMBINANT LUMPY SKIN DISEASE VIRUS PROTEINS LSDV095 AND LSDV103 AS POTENTIAL ANTIGENS FOR DIFFERENTIAL DIAGNOSIS OF CAPRIPOXVIRUS INFECTIONS

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ABSTRACT

Capripoxviruses cause infections in ruminants that are a significant threat to animal husbandry in many countries worldwide. Though the problem is sufficiently studied, to date there are no high-producing serological diagnostic tests available that can differentiate between capripoxvirus infections. To develop efficient serological tests, two recombinant lumpy skin disease virus proteins (LSDV095 and LSDV103) were expressed in *Escherichia coli* and purified using affinity chromatography. It was shown that both proteins were antigenically active against control sera, using western blotting and an indirect ELISA. As antigens, the recombinant proteins LSDV095 and LSDV103 detected antibodies only in the sera of animals infected with virulent capripoxvirus strains. The LSDV103 protein reacted only with the sera of animals infected with the virulent sheep pox and goat pox viruses. The results of this study allow the preliminary conclusion that the recombinant protein LSDV103 can be used for interspecies differentiation of capripoxvirus infections.

Key words: capripoxvirus, lumpy skin disease virus, sheep pox virus, goat pox virus, recombinant antigens, ELISA, western blot

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INTRODUCTION

Capripoxvirus genus of Poxviridae family unites viruses of sheep pox, goat pox and lumpy skin disease [1]. Sheep pox virus (SPPV) and goat pox virus (GTPV) induce diseases in sheep and goats characterized by fever, generalized papules or nodules, vesicles (rare), inner lesions (especially in lungs) and death. Most of the studied strains of SPPV and GTPV cause more severe clinical disease just in sheep or goats, but there are some strains that are equally pathogenic to both animal species. Sheep pox and goat pox are endemic in African countries north of the equator, in the countries of Near East and Asia. From 2010 through 2018, the diseases were intermittently recorded in Bulgaria, Chinese Taipei, Israel, Kazakhstan, Kyrgyzstan, Mongolia, Morocco, Greece and in Russia [2].

Mainly cattle is affected by the lumpy skin disease virus (LSDV) provoking fever, nodules on the skin, on the mucosa and on inner organs, exhaustion, and enlargement of lymph nodes, skin edema, and sometimes death. LSDV strains are identical antigenically to the virus strains causing sheep pox and goat pox, but are different genetically. The geography of the lumpy skin disease partially differs from the same of the sheep pox and goat pox. The disease is endemic in the majority of African and Near Eastern countries. However, in 2015 and 2016, it spread to the

south-eastern Europe, to Balkans and to the Caucasus, and in 2016 the lumpy skin disease was for the first time recorded also in Kazakhstan [3].

Capripoxvirus infections are diagnosed on the basis of clinical signs. Laboratory confirmation is based on classical virological methods including animal bioassay and electron microscopy [2, 3]. PCR can be used for rapid identification of the agent [4, 5].

The virus neutralization test (VNT) is the only validated serological test available. However, immunity to capripoxvirus infections is mainly mediated by the cellular immune response and this test is not sensitive enough for identification of animals that have been in contact with the virus and have low level of neutralizing antibodies. Immunofluorescence assay and diffuse precipitation test are less specific due to cross-reactions with antibody to other poxviruses. Western blot with use of the structural viral protein P32 is a sensitive and specific method for serological diagnosis of capripoxviruses but is difficult and expensive to carry out.

An enzyme-linked immunosorbent assay (ELISA) is convenient, cost-effective and high-performance for serodiagnosis. The use of suitable recombinant capripoxvirus antigens for ELISA is a promising direction in the development of standardized serological test [6, 7].

This paper presents the results of obtaining recombinant proteins LSDV095 and LSDV103 and their antigenic characterization to determine the possibility of using in serological tests to identify and differentiate capripoxvirus infections

Materials and methods

Viruses. The LSDV strain Dermatitis nodulares/2016/Atyrau/KZ isolated in 2016 during the outbreak in Atyrau region and SPPV strain A (Microbial Collection of the Research Institute for Biological Safety Problems RK ME&S – Science Committee) were used. For virus cultivation, a primary lamb testis cells (LT) maintained by the Laboratory of Cellular Biotechnology of the Research Institute for Biological Safety Problems RK ME&S – Science Committee was used.

Sera. Susceptible animals were experimentally infected with the virulent strains of capripoxviruses to obtain capripoxvirus-positive sera. The sera were collected on days 14 and 21 post infection (dpi). Sera from bull immunized with the attenuated LSDV vaccine strain Neethling-RIBSP and with the commercial vaccine LUMPIVAX (KEVEVAPI, Kenia) were also used as well as sera from sheep immunized with the SPPV vaccine strain NISKhI.

Normal sera were obtained from clinically healthy animals from capripoxvirus-free regions.

Plasmid Production. The LSDV genes LSDV095 (GeneID:921567) and LSDV103 (GeneID:921542) were amplified with the genomic viral DNA using pairs of primers LSDV095_F ctaccatggacttcatgaaaaaatatac and LSDV095_R gtactcgagtttgctgttattatcatcca, LSDV103_F ccgccatggaaaaattatctcgaagcag and LSDV103_R ccggcggccgccataccatcgtcgatagagtcat, respectively. Viral DNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA). The obtained products were digested with respective enzymes and cloned into the plasmid vectors pET26b and pET28b (containing a HIS6-tag on the C-terminus), and giving rise to pET26/LSDV095 and pET28/LSDV103. Plasmids were sequenced to verify the integrity of the inserts.

Gene Expression and Protein Purification. Plasmids pET26/LSDV095 and pET28/LSDV103 were transformed into *Escherichia coli* strain ER2566 (NEB, USA) according to the manufacturer's instructions. Bacterial cells were grown in 100 mL LB-kan (Luria-Bertani broth, containing 50 μg/mL of kanamycin) at 37°C to optical density OD600 = 0.6–1.0. Gene expression was induced by addition IPTG to final concentration 1 mM to the bacterial suspension with subsequent incubation for 2 h at 37°C. Proteins LSDV095 and LSDV103 were formed inclusion bodies. The cells were harvested by centrifugation at 5000× g for 15 min and resuspended in lysis buffer (100 mM Tris HCl pH 8.0, 150 mM NaCl, 1% [vol/vol] Triton X100, 1% [wt/vol] sodium deoxycholate). Cells were lysed in the presence of lysozyme (1 mg/mL) by

double freezing and thawing. Protein inclusions were thrice rinsed with lysis buffer without detergents and dissolved in a solubilization buffer consisting 50 mM Tris HCl pH 8.0, 150 mM NaCl, 8 M urea. Additional protein purification was performed using agarose *HisPur Cobalt Superflow* (Invitrogen) under denaturating conditions according to the manufacturer's protocol.

Western blot. For immunodetection, the purified recombinant proteins were separated in 15% SDS-PAAG and transferred onto nitrocellulose membrane (Invitrogen, USA). After protein transfer, the nitrocellulose membrane was incubated for 1 h at room temperature (RT) in blocking buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1 % (vol/vol) Tween-20, 5% non-fat dry milk). The membrane was probed with primary antigen-specific serum for two hours at RT, washed three times with TBS-Tween 20 (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1 % (vol/vol) Tween-20) and incubated in alkaline phosphatase-conjugated rabbit anti-bovine or donkey anti-sheep antibody for 1 h at RT. After three washes with TBS-Tween 20, the membrane was developed by utilizing the BCIP/NBT Phosphatase Substrate Kit (Invitrogen, USA), according to the manufacturer's instructions

ELISA. 96-wells plates were coated with the 100 ng of affinity purified recombinant proteins LSDV095 or LSDV103 per well in 100 μL of 0.05 M carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. Plates were washed four times with TBS-Tween 20 and blocked with TBS-Tween 20 containing 5% skimmed dry milk for 1 h at 37°C. Plates were again washed four times with TBS-Tween 20. Test sera, diluted 1/100 in the blocking buffer, were added to wells (100 μL/well). Plates were incubated for 1 h at 37°C and washed four times with TBS-Tween 20. Rabbit anti-bovine immunoglobulin IgG conjugated to horseradish peroxidase, diluted 1:10000 (or donkey anti-sheep immunoglobulin in dilution 1:30000) was added (100 μL/well) and the plates were incubated for 1 h at 37°C. After washing 100 μL of the substrate (ABTS) was added into each well. The plates were incubated for 20 min at RT, and optical density of reaction product was read at 405/630 nm on ELISA plates reader. Cut-off values were determined using the mean optical density values from negative control sera plus three standard deviations.

RESULTS

The target genes LSDV095 and LSDV103 were amplified with genomic DNA of the lumpy skin disease virus Dermatitis nodulares/2016/Atyrau/KZ and cloned into the expressing bacterial vectors pET26b and pET28b, respectively. The resulted plasmids pET26/LSDV095 and pET28/LSDV103 (fig. 1) encoded the recombinant protein LSDV095 (protein_ID:AAK85056.1) containing protein LSDV095 – 1-161 a.a., N-terminal peptide MKYLLPTAAAGLLLLAAQPAMA (pelB) and C-terminal peptide LEHHHHHH; and recombinant protein LSDV103 (protein_ID:AAK85064.1), containing protein LSDV103 – 4-189 a.a., and C-terminal oligopeptide AAALEHHHHHH, respectively. Plasmids were transformed into *E.coli* cells, strain ER2566.

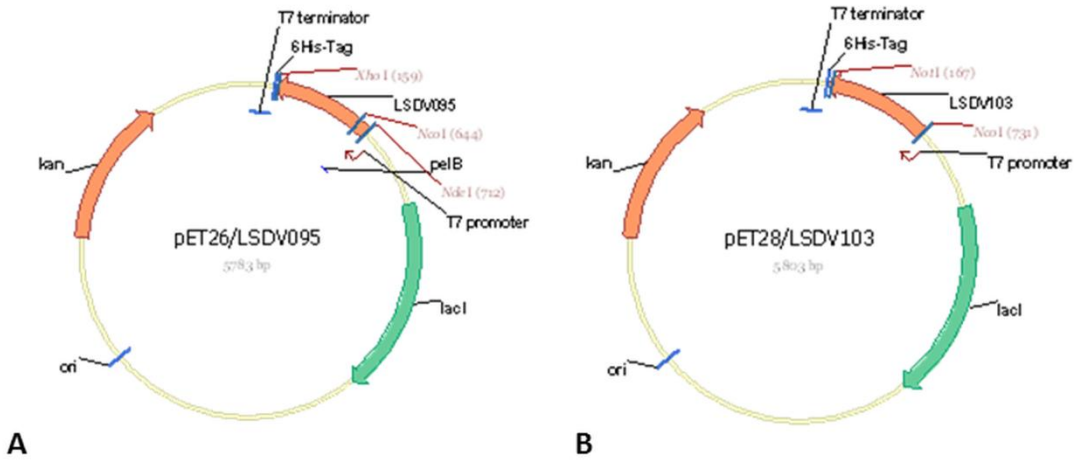
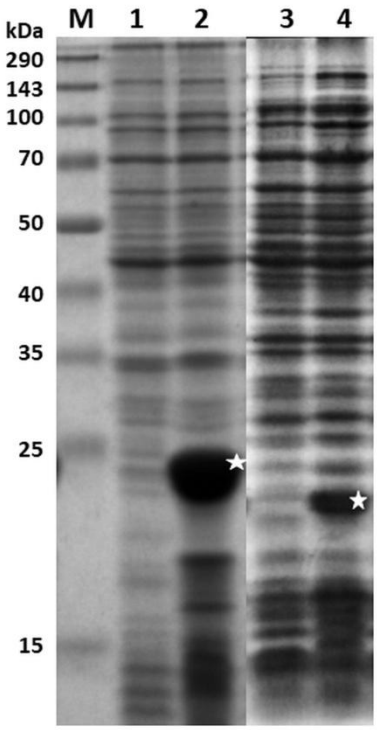


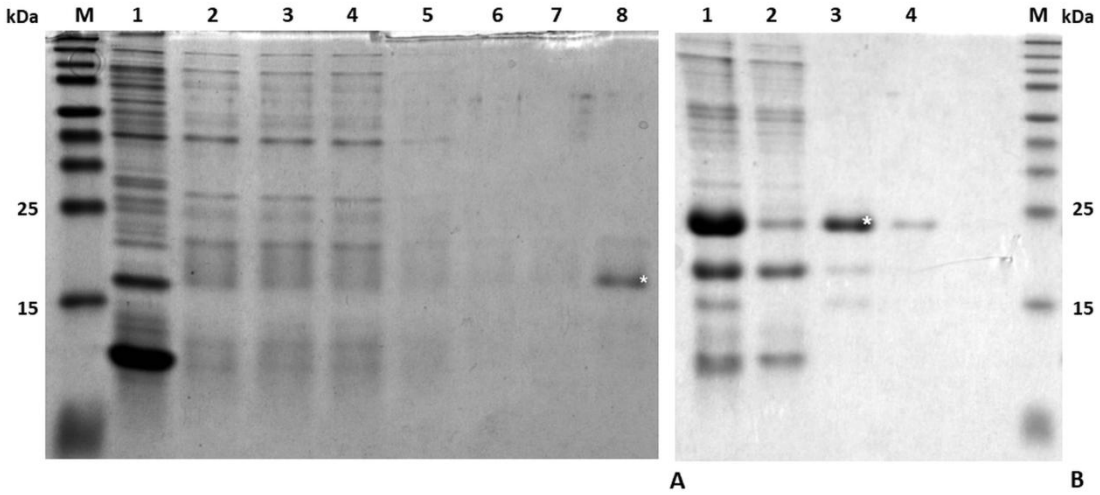
Fig. 1. Maps of expressing plasmids

Induction of protein expression in bacterial cells using IPTG resulted in the production of recombinant proteins LSDV095 and LSDV103 with molecular weight ~ 20 and 22 kDa that corresponded to predicted molecular masses (fig. 2). Presence of the polyhistidine sequence in the recombinant proteins at the C-terminus made it possible to purify them using metal chelate chromatography. This method enables obtaining recombinant proteins purified up to 80% and higher (fig. 3).



LSDV103 protein: 1 – total proteins prior to IPTG induction, 2 – protein fraction after IPTG induction; LSDV095 protein: 3 – total proteins prior to IPTG induction, 4 – protein fraction IPTG induction. Recombinant proteins are marked with asterisks.

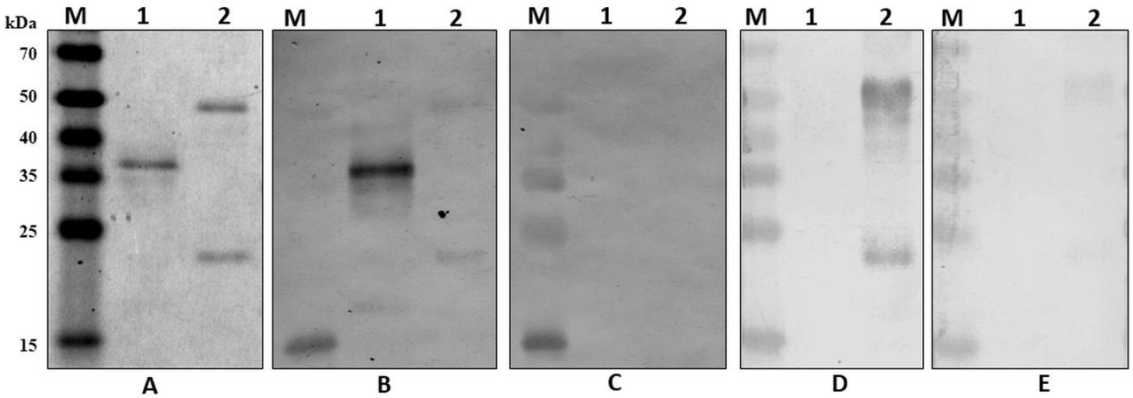
Fig. 2. Electrophoretic analysis of the protein fractions produced in *E. coli* transformed by pET26/LSDV095 and pET28/LSDV103



A - LSDV095protein: 1 – total protein after IPTG induction, 2, 3 – cell lysate, 4 – flow-through, 5, 6, 7 – wash; 8 – elution; B - LSDV103 protein: 1 – cell lysate; 2 – flow-through, 3, 4 – elution.
M –protein molecular weight marker. Recombinant proteins are marked with asterisks.

Fig.3. Purification of recombinant proteins

Antigenic characteristics of the recombinant proteins were evaluated by Western blot (fig. 4) and ELISA (table 1) using sera from reconvalescent animals, from experimentally infected and vaccinated against capripoxviruses animals.



A - electrophoresis of affinity purified proteins in SDS-PAAG under non-reducing conditions (without 2-mercaptoethanol); B - membrane was probed with blood serum of the calf experimentally infected with LSDV; C - membrane was probed with normal bovine serum; D - membrane was probed with serum of the sheep experimentally infected with the SPPV; E - membrane was probed normal ovine serum.
M – molecular weight marker of proteins, 1 – LSDV095 protein, 2 – LSDV103 protein.

Fig. 4. Western blot analysis of recombinant proteins LSDV095 and LSDV103

It was shown that in Western blotting the sera from cattle experimentally infected with the LSDV reacted with the protein LSDV095, while in the course of interaction with LSDV103 these sera reacted much weaker (fig. 4B). Sera from the sheep experimentally infected with the SPPV worked positively only with LSDV095 protein.

Table 1. Characterization and immunoreactivity of recombinant LSDV proteins in ELISA

Protein	Blood sera (positive/total)				
	bovine			ovine	
	reconvalescent	experimentally infected, 21 days	vaccinated	experimentally infected, 14 days	vaccinated
LSDV095	0/7	4/5	2/14	0/1	0/4
LSDV103	0/7	0/5	0/14	1/1	0/4
SPPV095	0/7	4/5	2/14	1/1	0/4

In ELISA the sera from the sheep and cattle vaccinated against capripoxvirus infections, naïve and experimentally infected animals were used. Except LSDV095 and LSDV103 proteins, the earlier obtained protein SPPV095 was used [8]. As the Table data show, 80% of specimens (4 of 5) of sera from cattle experimentally infected with LSDV reacted positively to proteins LSDV095 and SPPV095. The serum from the sheep experimentally infected with the SPPV demonstrated positive reaction only with SPPV095, and failed to work with LSDV095 (it conforms to the results of Western blotting). Protein LSDV103 was positive only with the serum of the sheep experimentally infected with the SPPV. In the sera of the vaccinated (85.7% of cattle and 100% of sheep) and reconvalescent animals (100%) antibodies to the proteins under study were not detected.

DISCUSSION

The virus neutralization test is considered the gold standard method for detecting of neutralizing antibodies to capripoxviruses. However, it cannot differentiate between GTPV, SPPV and LSDV and is time consuming, requires BSL-3 containment in disease-free countries, and. It should also be noted that serological methods based on using cell cultures are expensive, and their reproducibility depends on the quality of the cell batch [9]. Babiuk et al. [10] developed an indirect ELISA for detection of antibodies to capripoxviruses with use of heat-inactivated Nigerian SPPV as an antigen. Diagnostic sensitivity and specificity of ELISA was 96% and 95%, respectively, while diagnostic sensitivity of the virus neutralization test was 96% at 100% diagnostic specificity. It was also shown that the antigen based on the inactivated virus detected specific antibodies in animals infected with virulent viruses.

Recombinant capripoxvirus antigens were also proposed to be used in indirect ELISA. When recombinant proteins 095 and 103 served as antigens, sensitivity and diagnostic specificity of ELISA ranged within 95-97% for sera from sheep and goats experimentally infected with virulent strains of capripoxvirus, but they failed to detect antibodies in vaccinated sheep or goats [7]. Moreover, cross-reactivity with antibodies to orf-viruses was not observed. It was found that the GTPV protein P32 expressed in *Pichia pastoris* can be used in indirect ELISA as a diagnostic antigen. The standardized indirect ELISA based on P32 protein demonstrated relative specificity (84.2%) and sensitivity (94,2-100%) as compared to the virus neutralization test and indirect ELISA based on the whole virus [6]. Orlova et al. obtained bacterially expressed recombinant proteins P17 and P18 of the SPPV, and on their basis indirect ELISA for detection of antibodies to the SPPV and GTPV was developed [11]. Testing of 55 deliberately negative ovine and caprine blood sera demonstrated 96.3% specificity of P17-ELISA and 100% specificity of P18-ELISA.

The recombinant antigens produced in the result of our studies have high specificity. Protein LSDV095 detects antibodies only in animals infected with epizootic strains of the LSDV. LSDV103 protein detected antibodies in small ruminants infected with SPPV. This antigen can probably be used for interspecies differentiation of capripoxvirus infections. The obtained results show the perspective of developing a high-productive standardized test ELISA that will not require usage of infectious agents and will be suitable for epizootological monitoring of capripoxvirus infections.

CONCLUSION

Bacterially expressed recombinant LSDV proteins LSDV095 and LSDV103 were produced. In reactions with control sera they demonstrated antigenic activity and specificity. The results of the study allow preliminarily concluding that the recombinant protein LSDV103 can be used in interspecies differentiation of capripoxvirus infections.

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