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Justas

LAZUTKA

THE DEVELOPMENT AND EVALUATION OF DETECTION SYSTEMS FOR SCHMALLENBERG VIRUS SPECIFIC ANTIBODIES

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Technological sciences,

chemical engineering (05T)

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LAZUTKA

ANTIKŪNŲ PRIEŠ SCHMALLENBERG VIRUSĄ DETEKCIJOS SISTEMOS SUKŪRIMAS IR ĮVERTINIMAS

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List of abbreviations

293T	Human Embryonic Kidney Cells 293
Aa	amino acid
Ac	acetate
AINV	Aino virus
AKAV	Akabane virus
BFAE	Bovine Foetal Aorta Endothelium Cells
BHK-21	Baby Hamster Kidney Cells 21
BSR	a derivative of BHK cells
BUNV	Bunyamwera virus
CCHFV	Crimean Congo Haemorrhagic Fever virus
CI	confidence interval
CPT-Tert	sheep choroid plexus cells immortalized with the simian virus 40 (SV40) T antigen
cRNA	copy RNA
EDTA	ethylenediaminetetraacetic acid
eIF2α	elongation initiation factor 2 alpha
ELISA	enzyme-linked immunosorbent assay
GP	glycoprotein
HFRS	Haemorrhagic Fever with Renal Syndrome
HPS	Hantavirus Pulmonary Syndrome
IFA	immunofluorescence analysis
LAMP	loop-mediated isothermal amplification
LB (Miller)	lysogeny broth, Miller formulation
MAb	monoclonal antibody
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MDCK	Madin-Darby Canine Kidney Epithelial Cells

mRT-qPCR	multiplex reverse-transcriptase quantitative PCR
NSDV	Nairobi Sheep Disease virus
NSm	Non-structural protein M
NSs	Non-structural protein S
PAA	polyacrylamide
PBS	phosphate buffer saline
PBST	phosphate buffer saline with Tween-20
PMSF	phenylmethylsulfonyl fluoride
PRNT	plaque reduction neutralization test
RdRp	RNA dependent RNA polymerase
RPA	recombinase polymerase amplification
RT	room temperature
RT-qPCR	reverse transcription real time quantitative PCR
RVFV	Rift Valley Fever virus
S/P	single-to-positive signal ratio
SBV	Schmallenberg virus
SDS	sodium dodecyl sulphate
TSWV	Tomato Spotted Wilt virus
UTR	untranslated region
UUKV	Uukuniemi virus
Vero	kidney epithelial cells extracted from African green monkey
VNT	virus neutralization test
vRNA	viral RNA
YPD	yeast extract, peptone, dextrose medium
YPGal	yeast extract, peptone, galactose medium

INTRODUCTION

A disease affecting cattle was discovered in Germany and the Netherlands in 2011. Its clinical signs were fever, decreased milk production, and diarrhoea. Metagenomic analysis revealed a new Orthobunyavirus as a cause of the infection. The virus was named Schmallenberg virus (SBV) as it was isolated from a farm near German town of Schmallenberg (Hoffmann et al., 2012). Since then, SBV has spread across most European countries (Doceul et al., 2013). The viraemic period of SBV is short, lasting one to six days. However, the virus is dangerous to animal foetuses. The usual symptoms are head, trunk or limb malformations of newborn cattle (Hoffmann et al., 2012). It was discovered that SBV can also replicate successfully in goat, sheep and even dogs (Sailleau et al., 2013).

SBV belongs to the *Peribunyaviridae* family of viruses and is assigned to genus *Orthobunyavirus*. Biting midges are thought to be the main vectors for spreading the SBV infection as the virus can be carried by *Culicoides* insect vectors (De Regge et al., 2012). Bunyaviruses have three single-stranded RNA genome segments which code structural and non-structural viral proteins. The most abundant protein in virions and infected cells is the nucleocapsid (N) protein. Its main function is to protect the viral RNA form degradation (Schmaljohn & Nichol, 2007).

A vaccine against SBV was developed in 2013 (Wernike, Nikolin, et al., 2013). However, to evaluate the prevalence of SBV and to determine the need for cattle vaccination fast and cheap SBV detection methods are needed. One of these detection methods is the enzyme-linked immunosorbent assay (ELISA).

Research aim:

To develop an enzyme-linked immunosorbent assay by using recombinant SBV structural proteins synthesized in *S. cerevisiae* and evaluate its ability to detect SBV-specific antibodies in bovine serum, saliva and milk samples.

Research objectives:

- To investigate possibility of SBV nucleocapsid, glycoprotein 2 and chimeric nucleocapsid-glycoprotein 2 protein production in *S. cerevisiae* yeast.
- To evaluate produced antigens for optimal reactivity with bovine serum samples.
- To develop assays for detecting SBV-specific antibodies in bovine serum, saliva and milk.
- To compare newly developed assays with available alternative assays.
- To test bovine serum samples from Lithuania and Ukraine for the presence of SBV-specific antibodies and evaluate the prevalence of SBV virus in these countries.

Novelty and relevance

Schmallenberg virus is an emerging virus that has spread across Europe in several years. The estimated impact of SBV infection on cows range from €23 to €43 per cow per year and €19 to €37 per ewe per year (Waret-Szkuta et al., 2017). Because SBV rapidly spreads and has economic impact on dairy farms, fast and accurate SBV detection systems are needed. No commercial assay was available at the beginning of this research. However, a commercial ELISA was developed by other researchers using an *E. coli* based SBV N protein in 2013 (Bréard et al., 2013). It can detect SBV antibodies in bovine serum and milk. There have been no reports on system that could analyse bovine saliva samples.

The developed system would provide an alternative tool for scientists to evaluate the state of SBV infection in cows while using non-invasive sample collection methods. A non-invasive test to detect antibodies in individual bovine saliva samples could be used to test calves as well as adult cattle. As the level of antibodies in saliva is lower than in serum, a sensitive detection system is needed. Yeast-derived SBV protein synthesis platform could provide a highly reactive protein for SBV detection system as proteins synthesized in yeast tend to retain their antigenic properties (Razanskiene et al., 2004). Finally, the epidemiology of a novel virus is also important, thus testing of biologic samples from local farms provide information about the spread of the virus in the Lithuania and Ukraine.

THESIS STATEMENTS

1. Yeast *Saccharomyces cerevisiae* is a suitable organism to produce recombinant structural Schmallenberg nucleocapsid protein.
2. Recombinant Schmallenberg virus nucleocapsid protein is recognized by Schmallenberg virus-specific antibodies in bovine samples.
3. Recombinant Schmallenberg virus nucleocapsid protein is suitable for use in systems that detect Schmallenberg virus-specific antibodies in bovine serum, milk or saliva.
4. Schmallenberg virus specific antibodies can be found in blood, milk and saliva in Lithuanian and Ukrainian cattle.
5. Bovine saliva is a valid specimen to detect Schmallenberg virus-specific antibodies and evaluate the prevalence of SBV.

1. REVIEW OF KNOWN LITERATURE

1.1. *Bunyavirales* order of viruses

Schmallenberg virus belongs to the group of single stranded, enveloped, negative sense RNA viruses. When initially discovered it was assigned to the *Bunyaviridae* family of viruses, genus *Orthobunyavirus*. In 2017 the taxonomy of bunyaviruses was revised. A new order was formed and called *Bunyavirales*, which consisted mostly of families formed from genera of the previous *Bunyaviridae* family. The *Bunyaviridae* family ceased to exist and its genera *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus* were moved to *Peribunyaviridae, Hantaviridae, Nairoviridae, Phenuiviridae* and *Tospoviridae* families accordingly. SBV shares many common features with *Bunyavirales* viruses, or bunyaviruses, thus it is advantageous to review characteristics of main families of *Bunyavirales* order.

Bunyaviruses share a common genome organization and structure. Their RNA genome is divided into three segments – small (S), medium (M), and large (L). (Elliott, 1990). Bunyaviruses can infect humans, animals, plants and insects. They can cause encephalitis, haemorrhagic fever with renal syndrome, thrombocytopenia, pulmonary syndrome and other diseases in humans (Schmaljohn & Nichol, 2007). Viruses of *Hantavirus* family are carried and transmitted by rodents, while the rest of the viruses of the order are carried and transmitted by arthropod vectors. Assignment to the families is based on a lack of serologic cross-reactivity with members of other families, the patterns of sizes of virion proteins and genome segments, gene expression strategy and conserved terminal nucleotide sequences of the genomic RNAs (Schmaljohn & Nichol, 2007).

1.1.1. Family *Peribunyaviridae*

Family *Peribunyaviridae* consists of *Orthobunyavirus* and *Herbevirus* genera. Herbeviruses were discovered in 2013 in mosquitoes in Africa. However, it is

not known if they infect vertebrate hosts as they were not shown to replicate in animal cell lines (Marklewitz et al., 2013). The genus *Orthobunyavirus* consists of more than 170 viruses that are transmitted by mosquitoes and infect vertebrate hosts (Schmaljohn & Nichol, 2007). These viruses can be assigned into 18 serogroups according to their antigenic or haemagglutination inhibition properties with the Simbu serogroup being the largest one (Saeed et al., 2001). However, viruses that belong to one serogroup can differ by their prevalence and host. The Simbu serogroup includes such pathogens as Oropouche and Akabane virus. Oropouche virus is spread in South America and is known to cause dengue-like illness in people. Akabane virus is spread in Australia, Japan and the Middle East and is able to cause epizootic birth defects (Saeed et al., 2001; Conraths et al., 2012).

There is also variety among viruses of the same serogroup at the molecular level. In the Simbu serogroup nucleocapsid proteins have a 40 % amino acid sequence identity, but G2 glycoproteins share only 17 % of amino acid identity in the same serogroup. This may be due to the different properties and functions of these proteins. The viral glycoproteins are responsible for haemagglutination, virulence and virus entry into the host cell. The G2 protein is situated on the outside of the virion surface and reacts with the receptors of the host cell. The N protein is found inside the viral particle where it forms a ribonucleocapsid with the viral RNA. The changes in G2 protein can lead to more diverse types of receptors that the virus can attach to. Bunyaviruses are also able to exchange the M segment of their genome, increasing the types of hosts they can infect. Thus it is clear that evolutionary processes can create differences in a closely related group of viruses (Saeed et al., 2001; Yanase et al., 2012; Schmaljohn & Nichol, 2007).

1.1.2. Family *Phenuiviridae*

The other major group of the bunyaviruses is the family *Phenuiviridae.* Its largest genus *Phlebovirus* contains 70 viruses. Most virus members of the genus are associated with phlebotomine sandflies. However, the most researched

species of the genus is Rift Valley Fever Virus (RVFV), which is associated with mosquitoes. It can cause abortions in infected livestock and mild febrile illness in humans. It is common to observe outbreaks of these symptoms in livestock-producing areas in humans and animals alike (Schmaljohn & Nichol, 2007). When infected, human patients can sometimes develop neurological disorders, haemorrhagic fever or thrombosis. Newborn lambs suffer from severe RVFV infection symptoms, which in 95–100 % cases result in death.

Major role in spreading the virus is played by *Culex pipens* and other mosquitoes, which are vectors for the pathogen. Interestingly, mice, rats, dogs, cats, camels, ferrets, hamsters, gerbils and Rhesus macaques are also susceptible to RVFV. However, it is not known if the virus can be transmitted directly from one animal species to another (Ikegami & Makino, 2011).

1.1.3. Family *Nairoviridae*

The main hosts of nairoviruses are ticks. Ticks feed on vertebrate blood, which results in nairoviruses infecting animals and humans. In addition, vertebrates infected with nairoviruses can further help spread the virus if uninfected ticks feed on vertebrate blood. Nairobi Sheep Disease (NSDV) and Crimean Congo Haemorrhagic Fever (CCHFV) viruses are two of the most important viruses in the *Nairovirus* genus (Schmaljohn & Nichol, 2007).

NSDV causes haemorrhagic enteritis with diarrhoea in sheep. It can mostly be found throughout East Africa. Abortions of infected animals are frequent and an up to 90 % death rate can be observed (Modrow et al., 2013; Marczinke & Nichol 2002). CCHFV is spread in Africa, Asia, Eastern Europe, and the Middle East (Appannanavar & Mishra, 2011). Its infection causes haemorrhagic fevers in humans. Oedema, necrosis, congestion and haemorrhage are seen in major organs (Schmaljohn & Nichol, 2007). The mortality rate can vary from 3 to 30 %, depending on the region (Ergonul, 2012).

1.1.4. Family *Hantaviridae*

Hantaviruses form an exception in the *Bunyavirales* order as they are transmitted to humans by rodents instead of arthropods. Direct contact with rodents is not necessary for infection to occur as viruses can be acquired by the respiratory route through inhalation of virus particle-containing aerosols of rodent excreta (Schmaljohn & Nichol, 2007; Lednicky, 2003).

Hantaviruses have been found in Europe, Asia and the Americas. They can cause Haemorrhagic Fever with Renal Syndrome (HFRS) or Hantavirus Pulmonary Syndrome (HPS) in humans depending on the virus type. No cytopathic effect is seen in human cells during the infection. However, HFRS or HPS can result in kidney failure or acute respiratory illness, which can sometimes be fatal. The mortality rate for HFRS is about 7 % of cases, while HPS can result in the death of about 40 % of patients (Lednicky, 2003).

Interestingly, hantavirus infected rodents show no symptoms of illness. Only New York and Sin Nombre hantaviruses are known to cause pathology in rodents. This, and the fact that the infection may last several months or years, makes rodents a reservoir for the virus. The transmission of hantaviruses among rodents is horizontal and is thought to occur through direct contact and infectious aerosols (Schmaljohn & Nichol, 2007; Lednicky, 2003).

1.1.5. Family *Tospoviridae*

Tospoviruses are plant-pathogenic thrips-transmitted bunyaviruses. There are 14 species assigned to the *Tospovirus* genus, *Tospoviridae* family with Tomato Spotted Wilt Virus (TSWV) being one of them. TSWV was first discovered in 1930 after it was isolated from infected tomatoes. TSWV and other tospoviruses infect more than 1,000 plant species, however TSWV alone can infect more than 800 different species. TSWV causes various symptoms, like necrotic/chlorotic rings and flecking on leaves, stems and fruits, with early infections leading to one-sided growth, drooping leaves reminiscent of vascular wilt, stunting or

death. The annual losses due to the virus are estimated to be around 1 billion USD (Scholthof et al., 2011; Rotenberg et al., 2015).

1.2. Morphology and biochemical properties of the bunyavirus virion

Virions of bunyaviruses are spherical in shape, 80 to 120 nm in diameter with 5 to 10 nm surface glycoprotein (GP) projections embedded in a lipid bilayer envelope (Figure 1.1). Orthobynyaviruses can have 270 to 1,400 glycoproteins per virion (Schmaljohn & Nichol, 2007). GPs most likely form dimers consisting of G1 and G2, which later form penton or hexon structures of 840 and 1,100 kDa respectively (Sherman et al., 2010). The form of the virion is also pH-dependent. Uukuniemi (UUKV) virus shows tall GP spikes of 13 nm height at pH 7 and flat, lower spikes of 8 nm height at pH 6. This property might be related to the pH-dependent viral entry into the cell, as this conformational change reveals the hydrophobic parts of GPs and enhances the fusion between the viral membrane and the host endosomal membrane (Overby et al., 2008).

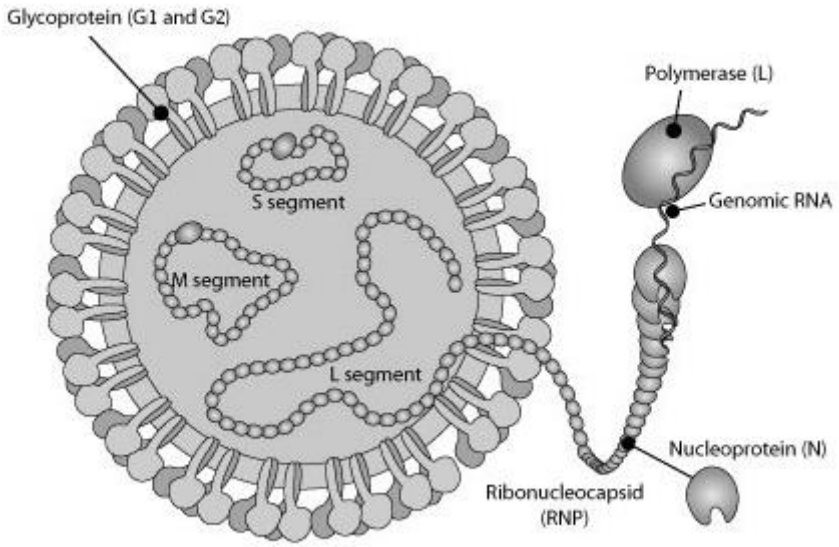


Figure 1.1. Structure of the virion of bunyaviruses. The outer layer of the virion is formed of glycoproteins. The nucleocapsid proteins bind to the segmented viral genome and form ribonucleocapsids. The picture was taken from http://viralzone.expasy.org/

Virions of UUKV contain about 2 % RNA, 58 % protein, 33 % lipid and 7 % carbohydrate. Their sedimentation coefficient ranges from 400 to 500 S, while densities range from 1.16 to 1.18 g/cm^3 in sucrose and from 1.20 to 1.21 g/cm^3 in CsCl. Treatment with lipid solvents or detergents remove the viral envelope (Schmaljohn & Nichol, 2007).

1.3. Genomic structure of bunyaviruses

Virions of bunyaviruses contain three single-stranded RNA genome segments, which vary in size. The segments are named according to their relative size: L (large), M (medium) and S (small) (Schmaljohn & Nichol, 2007). The terminal nucleotide sequences of each segment are complementary and form panhandle-like structures (Kohl et al., 2004). The segmented genomic RNA of bunyaviruses associates with the nucleocapsid protein to form S, M and L ribonucleoproteins or ribonucleocapsids. At least one of each ribonucleocapsids is needed for the virion to be infectious (Schmaljohn & Nichol, 2007).

The L segment of bunyaviruses has only one open reading frame in its negative-sense RNA. It codes the L protein – the viral RNA-dependent RNA polymerase. The protein can also act as an endonuclease, transcriptase and helicase. The L protein varies in size from 250 kDa in *Orthobyniavirus*, *Hantavirus* and *Phlebovirus* to 450 kDa in *Nairovirus* genera (Guu et al., 2012; Schmaljohn & Nichol, 2007). Similarly, the L segment size can differ from ~6,500 nucleotides (hantaviruses, orthobunyaviruses, phleboviruses) to ~9,000 (tospoviruses) or even 12,000 nucleotides (nairoviruses). All viruses of the order code the L protein in a negative-sense in its cRNA (Schmaljohn & Nichol, 2007).

The M segment of bunyaviruses ranges from ~3,600 to ~4,900 nucleotides. It encodes two envelope glycoproteins G1 and G2 in a single open reading frame derived from viral RNA. However, the polyprotein precursor of G1 and G2 has not been observed *in vivo* (Ulmanen et al., 1981; Schmaljohn & Nichol, 2007). Some bunyaviruses can code the non-structural protein NSm in this segment.

The M segment of phleboviruses has five in-frame translation initiation codons upstream of the G2 coding sequence. It was shown that the fourth or fifth AUG codon is used for G1-G2 synthesis, while translation from the second AUG codon results in NSm synthesis. Translation from the second AUG forms an uncleaved NSm and G2 78-kDa polypeptide, from which NSm originates (Kakach et al., 1989). The exception is topsoviruses as their NSm proteins are translated from ambisense mRNA (Schmaljohn & Nichol, 2007).

The S segment varies from 900 nucleotides in orthobunyaviruses to 3,000 nucleotides in tospoviruses. Hantaviruses and nairoviruses code only the nucleocapsid protein in their S segment. Orthobunyaviruses code a second protein NSs within a nucleocapsid protein ORF which has an alternative start codon (Figure 1.2). A phenomenon called "leaky scanning" aids in the translation of NSs. Phleboviruses and tospoviruses, however, use a different strategy. Their N protein is translated from mRNA which is complementary to vRNA, while NSs is translated from mRNA which is of the same sense as vRNA (Modrow et al., 2013; Schmaljohn & Nichol, 2007).

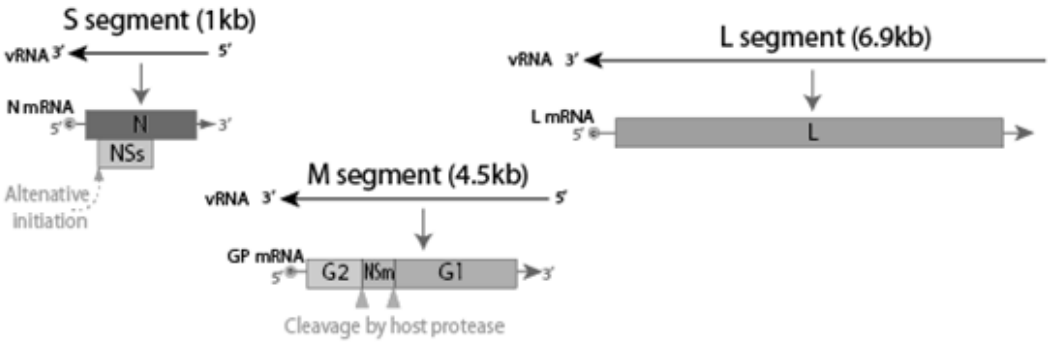


Figure 1.2. The organisation of the genome of orthobunyaviruses. The alternative initiation site within the N gene results in NSs protein synthesis during leaky ribosome scanning events. The M segment is cleaved by host proteases to form the G2, G1 and NSm proteins. The L segment codes viral RNA-dependent RNA polymerase. The picture was taken from http://viralzone.expasy.org/

1.4. Translation, modifications and functions of bunyavirus proteins

The most abundant protein in virions and infected cells is the nucleocapsid (N) protein. The size of the N protein varies according to the S segment size of the virus. Its molecular weight can be 19–30 kDa in *Orthobunyavirus*, *Phlebovirus*, *Tospovirus* genera or 48–54 kDa in *Hantavirus* and *Nairovirus* genera (Guu et al., 2012). The main function of the N protein is to protect viral RNA from degradation. It also interacts with L, G1 and G2 proteins and can help form panhandle-like structures of RNA, thus acting as an RNA chaperone (Schmaljohn & Nichol, 2007; Mir & Panganiban, 2006). N proteins bind preferentially to the 5' end of vRNA and are also able to oligomerize. It has been shown that Hantavirus N proteins can form trimers through interaction between the C-terminal residues of N protein molecules (Kaukinen et al., 2004; Osborne & Elliott, 2000). Orthobunyaviruses and tospoviruses use a different oligomerization mechanism by adding one N molecule at a time instead of associating preformed trimers (Leonard et al., 2005; Uhrig et al., 1999).

Some bunyaviruses code the non-structural S protein in their S segment. The size of NSs proteins can vary from 10 kDa for orthobunyaviruses to 50 kDa for tospoviruses. The NSs of RVFV can inhibit the transcription of host mRNAs, including interferon-β mRNAs. It can also induce post-transcriptional downregulation of the dsRNA-dependent protein kinase to prevent phosphorylation of eIF2α thus promoting viral translation in infected cells (Schmaljohn & Nichol, 2007; Ikegami et al., 2009).

The viral envelope glycoproteins are translated from a single mRNA. It is likely that the polyprotein precursor is cleaved by host signalases, as both G1 and G2 are preceded by signal sequences. The hantavirus G1 and G2 precursor, for example, is cleaved at the pentapeptide motif WAASA, which is conservative among hantaviruses (Löber et al., 2001).

Proteins coded at the M segment have a high cysteine content of 4 to 7 percent and positions of these residues are conservative among related

viruses. This may suggest that disulfide bridge formation is critical for correct folding of these proteins. The amino end of the proteins is typically exposed at the outside of the virion, while the carboxy-terminus is anchored into the membrane. It was also discovered that most of the bunyaviruses have glycoproteins with high-mannose glycans. This is different with orthobunyaviruses, as their G2 contains complex oligosaccharides. Nairoviruses are also an exception as their glycoproteins have N-linked and O-linked carbohydrates (Schmaljohn & Nichol, 2007). Glycoproteins of bunyaviruses are responsible for virus entry into the host cell. It was shown that G1 and G2 are targeted by neutralizing antibodies (Hofmann et al., 2013).

Another protein that is coded by M segment in some bunyaviruses, the NSm, is a membrane bound protein and can be found in the membrane of the Golgi complex. NSm of tospoviruses can be found in cell walls and plasmodesmata. Its function could be to facilitate cell-to-cell movement of the tospovirus. However, the function of NSm for the rest of the viruses remains unknown (Schmaljohn & Nichol, 2007).

The L proteins of bunyaviruses vary in size from 237 kDa for phleboviruses to 459 kDa for nairoviruses. It is not known if L proteins are modified after translation. Structural analysis of the La Crosse virus L protein revealed that it has to undergo conformational changes in order to initiate RNA synthesis, as the 3' end of the matrix RNA is often found attached to the outside of the polymerase, rather than the active site. It was also noticed that the L protein has distinct sites for binding single stranded 3' and 5' ends of vRNA. This prevents the forming of RNA panhandle secondary structure (Gerlach et al., 2015).

1.5. Transcription and replication of bunyaviruses

The transcription of vRNA to mRNA is performed by viral polymerase. The N protein is also necessary as it prevents the forming of RNA secondary

panhandle-like structure and makes the 3' end of vRNA accessible for RNA dependent RNA polymerase (Schmaljohn & Nichol, 2007). The transcription is also dependent on translation. There are transcription termination signals within the segment coding sequence that interact with newly synthesized mRNA and inhibits its transcription. Ribosomes attached to the newly synthesized mRNA prevent these interactions and the termination signals within the coding sequence are suppressed. However, ribosomes do not perform translation at the UTRs so the transcription termination signals at the UTRs are not supressed (Barr, 2007).

Bunyaviruses do not have a cap-synthesizing mechanism for their mRNA. Thus, the 7-metylguanosine cap is stolen from host cell mRNAs. This process involves the viral L polymerase and the N protein. The polymerase attaches to the host mRNA and cuts it several nucleotides downstream from the cap structure. This capped RNA is then used as a primer in the viral mRNA synthesis. The uncapped cellular mRNAs are then degraded by the host nucleases (Decroly et al., 2011).

To generate copies of bunyaviral genomic RNAs, antigenomic RNAs (cRNAs) must first be synthesized. cRNAs then serve as templates for genome synthesis, which is performed by the viral L polymerase. It is known that complementary 3' and 5' UTR sequences are needed for successful synthesis of vRNA. The synthesis may be induced because a double stranded RNA structure is formed (Barr & Wertz, 2004). Additionally, it was noticed, that the S, M and L segments of bunyaviruses are replicated at a different speed. The M segment is replicated the most rapidly compared to the other segments. This may be due to differences in the 3' and 5' UTR sequences of the segments. When the UTRs of the S and L segments were replaced with the UTR sequence of the M segment an enhanced replication ability of the S and L segments was observed (Barr et al., 2003).

The cRNAs of bunyaviruses are unmodified and lack the 5' cap structure or non-viral sequences. This shows that viral mRNA transcription and vRNA replication happens at a different time in the host cell. One possible inductor of vRNA replication is the NSs protein, as was shown in the experiments with RVFV. The

NSs interacts with cellular general transcription factor II H (TFIIH), reducing the mRNA synthesis. This may create favourable conditions for uncapped cRNAs to be replicated by viral polymerases (Le May et al., 2004).

The replication of the genome itself is thought to occur without any primers. A prime-and-realign model is suggested as a mode of replication. In this case, a guanosine triphosphate (pppG) would align with the third nucleotide (cytosine) of the template RNA. After several nucleotides are synthesized, the polymerase would slip backwards and realign the newly generated RNA in such a way that the pppG would overhang the RNA template. The polymerase would then cut the pppG overhang which would result in a monophosphorylated uridine at the 5' end of the molecule (Schmaljohn & Nichol, 2007).

Errors made during replication or other RNA manipulation can be repaired by the viral polymerase. In the case of Bunyamwera virus (BUNV), viral RNA-dependent RNA polymerase can repair up to 15 missing nucleotides at the 5' end of the matrix RNA. One of the possible explanations could be a homologous recombination event occurring between UTRs. RdRp can also remove additional nucleotides from RNA, however it does so only with cRNA, but not with genomic RNA and the mechanism of this process is still unclear (Walter & Barr, 2010).

1.6. Biology of Schmallenberg virus

1.6.1. Discovery of Schmallenberg virus

A disease affecting cattle was observed in Germany and the Netherlands in 2011. Its clinical signs were fever, decreased milk production, and diarrhoea. Pestiviruses, bovine herpesvirus type 1, foot-and-mouth disease virus, bluetongue virus, epizootic haemorrhagic disease virus, Rift Valley fever virus and bovine ephemeral fever virus were excluded as the causative agents of the disease. Metagenomic analysis performed after next generation sequencing revealed a new Orthobunyavirus closely related to Shamonda and Sathuperi

viruses. The new virus was named Schmallenberg virus (SBV) as it was isolated from a farm near the German town of Schmallenberg (Hoffmann et al., 2012). Electron microscopy experiments revealed that SBV forms spherical enveloped 80–120 nm diameter particles (Figure 1.3), that are typical to the bunyaviruses (Wernike, Conraths, et al., 2014).

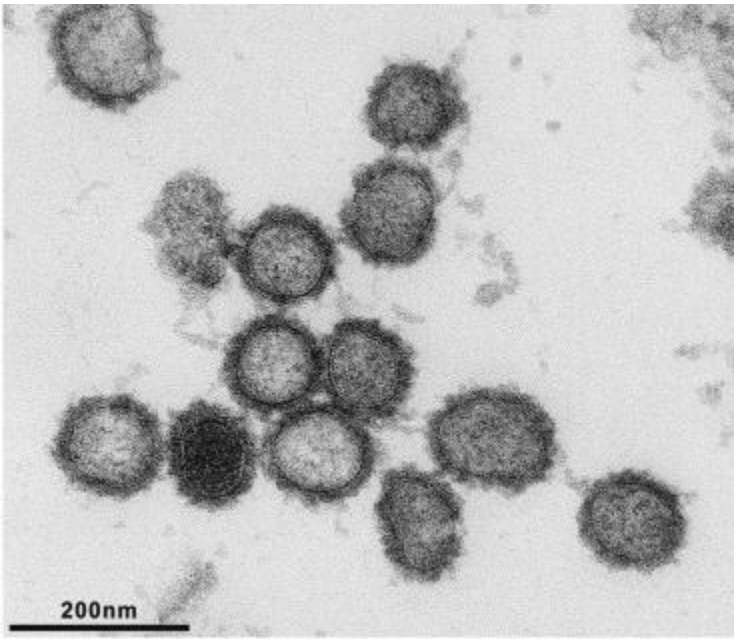


Figure 1.3. Electron microscopy photograph of a Schmallenberg virus (Wernike, Conraths, et al., 2014).

The origins of SBV are still unknown. Initial research suggested that SBV could be a product of the reassortment of genome segments between Shamonda and Sathuperi viruses (Yanase et al., 2012). However, further research revealed that SBV could be much older and, in fact, an ancestor of other bunyaviruses, such as Shamonda virus (Goller et al., 2012).

1.6.2. Clinical symptoms of the SBV associated disease

Several clinical symptoms associated with SBV have been described. The acute infection of SBV in adult cows results in fever following a drop in milk

production and diarrhoea. These signs can last for 2–3 weeks. However, the viraemic period is short lasting one to six days (Hoffmann et al., 2012). Cows seem to be affected the most by the virus as sheep or goats show no or very little clinical signs (Rodríguez-Prieto et al., 2014; Wernike et al., 2013; Laloy et al., 2015). On the other hand, SBV poses the most substantial threat to animal foetuses. SBV nucleic acid has been found in stillborn, aborted and newborn lambs and calves. Both male and female newborns are affected by the virus. The usual symptoms are head, trunk or limb malformations; other signs may include psychological, sensory, motor or neurovegetative disorders (Garigliany, Bayrou, Kleijnen, Cassart, Jolly, et al., 2012; Garigliany, Bayrou, Kleijnen, Cassart & Desmecht, 2012).

1.6.3. Pathogenesis of Schmallenberg virus

Neurons are the primary cells for SBV to replicate. The analysis of brain and spinal cord of 8 naturally SBV infected lambs and calves revealed lesions and tissue damage. SBV was detected in the cell bodies and processes of neurons in both the brain and spinal cord (Varela et al., 2013). Moreover, inflammation was detected in lambs, calves and goat kids naturally infected with the virus. The most frequently inflamed brain region was the mesencephalon. Axonal density reduction was observed near the SBV-created lesions in the tissue. However, SBV was also found in brains without inflammation (Herder et al., 2013). It was shown that SBV can successfully replicate in sheep CPT-Tert, bovine BFAE, human 293T, dog MDCK, hamster BHK-21 and BSR cells. The virus could reach titers of 10^6 PFU/ml at 48 h post-infection and induce a cytopathic effect (CPE) in most cell lines. It could replicate very efficiently in the CPT-Tert cell line. Also, mice inoculated with natural and synthetic SBV died 8 days post-inoculation. This confirms that mice are susceptible and permissive to SBV. The virus was also found in the grey matter of the brain of mice (Varela et al., 2013).

1.6.4. Vectors, prevalence and vaccines

The SBV related Aino and Akabane viruses are known to be transmitted by biting midges belonging to the *Culicoides* genus (Yanase et al., 2005). The same is true for SBV, as its nucleic acid was amplified in *C. obsoletus complex*, *C. dewulfi* and *C. chiopterus* samples collected in Belgium (De Regge et al., 2012). SBV was also found in *Culicoides* vectors in Denmark, the Netherlands, Sweden, Norway and Poland (Rasmussen et al., 2012; Elbers et al., 2013; Larska et al., 2013; Doceul et al., 2013). Recently, serologic evidence has shown a potential SBV infection in China and Ethiopia (Zhai et al., 2017; Sibhat et al., 2017), which could lead to a world-wide spread of the virus.

A study has shown that SBV nucleic acid can be found in *Culicoides* midges caught in 2012 in Germany, while midges caught in 2011 had no traces of SBV. In addition, no SBV nucleic acid was found in *Simuliidae* black flies collected during an SBV epidemic (Kameke et al., 2016).

While SBV can replicate successfully in goats, sheeps, cows and even dogs (Sailleau et al., 2013), antibodies against SBV were also found in many wild animal species. The ability of SBV to replicate in wild species may also contribute greatly to the spread of SBV. Wild ruminants *Dama dama, Cervus elaphus, Ovis aries musimon, Capreolus capreolus, Bison bonasus* were seropositive during the autumn/winter of 2013 in Poland. Their serum anti-SBV antibody levels were lower than those of domestic ruminants, which suggests the SBV spread from domestic to wild fauna (Larska et al., 2013). *Cervus elaphus* was also seropositive in the Alpine region in Italy in 2012. Together with *Capreolus capreolus* it was also seropositive in Belgium in the autumn of 2011 (Chiari et al., 2014; Linden et al., 2012). Another study showed that wild boars *Sus scrofa* caught in Germany in 2011-2013 had SBV-specific antibodies (Mouchantat et al., 2015).

One other way of infection can be through animal semen. SBV nucleic acid was found in some seropositive bull semen several months after infection (Ponsart et al., 2014).

In order to protect cattle and control the spread of SBV, a vaccine against SBV was developed in 2013. Five different prototype vaccine formulations were produced and tested. These prototypes were made out of inactivated SBV grown in different cell line cultures. Saponin and aluminium hydroxide were used as vaccine adjuvants. No side effects and no viral RNA were observed after the vaccination of animals. An increase of average neutralizing antibodies titer after the second immunization was observed (Wernike Nikolin et al., 2013). It was also shown that SBV vaccine completely protects sheep from SBV infection after a single immunization (Hechinger et al., 2014).

1.7. Generation of viral proteins in yeast

To be able to study viral proteins sufficient quantities (milligrams) of these proteins are needed. Most of the recombinant proteins used for research are produced in *E. coli* or yeast cells (Bill, 2014). Moreover, 30 % to 50 % of commercial proteins are produced in *E. coli* (Mattanovich et al., 2012; Martínez et al., 2012). These microorganisms are unicellular so their culture is cheap and they are easy to maintain, compared to mammalian or insect cells. However, bacterial cells often struggle to produce eukaryotic proteins due to inclusion body formation or low yields. They are unable to achieve some post-translational modifications such as N-glycosylation. Moreover, proteins produced by bacteria often require additional *in vitro* modifications, such as refolding (Sørensen, 2010).

Some of these problems can be avoided by utilizing eukaryotic cells for recombinant protein production. Yeast have a eukaryotic protein modification system in comparison to prokaryotes like *E. coli*. This means that eukaryotic proteins are likely to be folded and modified in a natural way. Yeast can perform

other post-translational modifications of proteins, including proteolytic processing of signal peptides, disulphide bond formation, subunit assembly, acylation and glycosylation (Hou et al., 2012). However, *S. cerevisiae* tend to incorporate more mannose into proteins than other eukaryotes, which could shorten the half-life of some biopharmaceutical proteins (Wildt & Gerngross, 2005). An alternative approach to produce recombinant viral proteins is to use other eukaryotic protein synthesis systems, like baculovirus infected insect cells or mammalian cells. However, longer time is needed to prepare the cells for recombinant protein synthesis and the medium for the cells is usually expensive (Demain & Vaishnav, 2009).

Many therapeutic proteins are successfully produced using yeast based systems – human interleukin 6, insulin precursor, human serum albumin and others (Martínez et al., 2012). They are often approved for therapeutic use (Çelik & Çalık, 2011). For example, a human hepatitis B vaccine was produced by recombinant yeast already in 1984 and licensed for general use in 1986 (McAleer et al., 1984; Hilleman, 1987). Proteins used in diagnostics can also be obtained by *S. cerevisiae* based protein synthesis systems. Yeast were used to produce hantavirus nucleocapsid proteins which were highly immunogenic, stable over a long time and contained almost no nucleic acids (Razanskiene et al., 2004). These proteins can be used to produce monoclonal antibodies and develop hantavirus-specific antibody detection systems (Sandmann et al., 2005; Petraitytė et al., 2008; Kucinskaite-Kodze et al., 2011).

Yeast have been used to produce antigens of other viruses such as measles, mumps, rubella. These antigens were later used in developing diagnostic systems for these viruses. Measles virus nucleocapsid was successfully synthesized in *S. cerevisiae* and *P. pastoris* yeast and formed stable nucleocapsid-like structures in the absence of viral RNA (Slibinskas et al., 2004). Yeast-derived recombinant measles nucleocapsid proteins together with monoclonal antibodies against these viral antigens were utilized to create a measles-specific IgM ELISA for use with serum and oral fluid samples (Samuel

et al., 2003). A mumps virus nucleocapsid was produced in *P. pastoris* yeast and was used to create a rapid immunochromatographic test for mumps-specific IgM in oral fluid specimens (Warrener et al., 2010). A rubella virus glycoprotein E1 was produced in *S. cerevisiae* and used as a coating antigen for detecting Rubella virus E1 antibodies in an indirect ELISA (Wen & Wang, 2005).

Yeast are also great for producing virus-like particles (VLPs). The VLPs of a virus resemble an actual virion. However, they lack viral genetic material and cannot replicate. They are interesting as potential vaccine candidates and as protein, DNA or drug carriers (Kim & Kim, 2016).

More than 30 different VLPs have been produced in yeast (Kim & Kim, 2016). Some of the VLPs can display heterologous epitopes on their surface by modifying the VLP coding genes. Hepatitis B surface antigen VLPs were stable while carrying a 19 amino acid HVP E7 epitope (Pumpens et al., 2002). VLPs formed from hamster polyomavirus VP1 could display epitopes ranging from 9 to 120 amino acids. These particles have four potential regions for epitope surface display and are highly immunogenic (Gedvilaite et al., 2000; Zvirbliene et al., 2006).

These examples show that *S. cerevisiae* is efficient, cheap and easy to prepare cell factory. It can be used to create recombinant viral proteins that are usable as drugs or as tools for creating monoclonal antibodies and virus-specific antibody detection systems. Until 2014 no serology tests were known to utilize SBV proteins produced by *S. cerevisiae*. The monoclonal antibodies against SBV were obtained using SBV N proteins synthesized by *E. coli* (Zhang et al., 2013). This suggests that *S. cerevisiae* was generally overlooked by researchers as a potential tool to produce recombinant SBV proteins that could be used in SBV diagnostics.

1.8 Virus detection systems

1.8.1 Viral nucleic acid detection systems

Viral nucleic acids can be purified from the sample and amplified using enzymes, which results in detectable amounts of viral genetic information. This relatively simple procedure made a polymerase chain reaction (PCR) one of the favourite tools in viral diagnostics. Nearly all known human viruses have a dedicated nucleic acid amplification assay (Valones et al., 2009).

Modifications of conventional PCR are real-time PCR (q-PCR) or reverse transcription qPCR (RT-qPCR). These methods utilize fluorescent probes and detector systems that analyse an increase in fluorescence while nucleic acid is being amplified. This allows quantification of nucleic acid and adds sensitivity to the test. However, qPCR requires additional equipment and technical expertise to perform correctly (Kubista et al., 2006).

A real time quantitative reverse transcription PCR (RT-qPCR) SBV nucleic acid detection method was developed in the Friedrich Loeffler Institut in Germany. It was used to detect SBV nucleic acid in sheep placental fluid, umbilical cord, cerebrum and spinal cord (Bilk et al., 2012) and in the cerebrum and the cerebellum of calves (De Regge et al., 2013). The same detection system was also used to find SBV nucleic acid in insect vectors. *C. obsoletus complex, C. obsoletus s.s., C. dewulfi* and *C. chiopterus* species were positive for SBV (De Regge et al., 2012). A similar test called one-step multiplex reverse-transcriptase quantitative PCR (one-step mRT-qPCR) was developed for the simultaneous detection and differentiation of SBV, AKAV and AINV (Lee et al., 2015).

Multiplex assays enable simultaneous detection of multiple different viruses. This can be beneficial when symptoms caused by different viruses cannot be reliably distinguished. A triplex real-time RT-PCR assay can detect Zika, Chikungunya and Dengue viruses in patients with arboviral infection symptoms. Serum, plasma, urine, placental tissue, brain tissue and amniotic fluid are suitable sources of viral nucleic acid for this assay (Pabbaraju et al., 2016).

Similar assays exist for animal virus detection. Four triplex qPCR and qRT-PCR protocols were developed to distinguish swine viruses: 1) Indiana serotype of Vesicular Stomatitis Virus (VSV-IN), New Jersey serotype of Vesicular Stomatitis Virus (VSV-NJ), Swine Vesicular Disease Virus (SVDV), 2) Foot and Mouth Disease Virus (FMDV), Classical Swine Fever Virus (CSFV) or African Swine Fever Virus (ASFV), 3) Porcine Circovirus type 2 (PCV2), Porcine Parvovirus (PPV), Porcine Pseudorabies Virus (PRV), 4) European type (type 1) Porcine Reproductive and Respiratory Syndrome Virus (PRRSV-EU), North American type (type 2) Porcine Reproductive and Respiratory Syndrome Virus (PRRSV-NA), Swine Influenza Virus (SIV) (Shi et al., 2016). The arboviral and swine virus assays are 100 % specific and have a level of detection of 10–15 copies of target nucleic acid molecules.

Isothermal amplification of nucleic acids uses enzymes that allow primer binding to the matrix without the change in reaction temperature. A variety of enzymes can be used depending upon technique – DNA polymerases, RNA polymerases, reverse transcriptases, ligases, helicases, recombinases, primases. Optimal temperature for the reaction ranges from 37 °C to 65 °C. Usually a set of 2 or more primers are used in a reaction with few exceptions when nuclease or nickase forms 3' overhangs in a matrix. Up to 10^9 copies of desired amplicon can accumulate at the end of isothermal amplification (Zhao et al., 2015). This allows visual interpretation of a successful reaction by adding DNA-binding dye such as SYBR-Green or ethidium bromide. Alternatively, accumulation of a reaction by-product magnesium pyrophosphate results in turbidity of the reaction mixture, which can reduce the cost of the procedure even more. While isothermal amplification is often cheaper than conventional PCR due to simpler equipment needed, it has its own limitations. Like PCR, virus detection using isothermal amplification is limited to a set of known viruses, as primers have to be designed before the test. What is more, isothermally amplified nucleic acid cannot usually be used in downstream molecular applications or experiments,

such as cloning, as short DNA fragments (150 bp) or long branched DNA is formed during amplification (Boonham et al., 2014; Zhang & Tanner, 2017).

A highly specific loop-mediated isothermal amplification (LAMP) assay was developed to detect SBV. In comparison, the recombinase polymerase amplification (RPA) assay was less specific as it produced false-negative results and showed cross-reactivity with several viruses from the Simbu serogroup. Thus RPA is not recommended as a diagnostic tool for SBV infection by the authors while LAMP is suitable for use in the field or where PCR or qPCR equipment is not available (Aebischer et al., 2014).

Next generation sequencing is a powerful tool that allows discovery of new viruses. This technique detects pyrophosphate molecules or hydrogen ions that are released after incorporation of a nucleotide in DNA polymerization process (Rothberg et al., 2011; Ronaghi et al., 1998). One of the early uses of pyrosequencing was the discovery of an unknown arenavirus transmitted through solid-organ transplantation (Palacios et al., 2008). Also, this technology enabled genotyping of the Human papillomavirus (Barzon et al., 2011).

SBV was detected by pyrosequencing 3 samples from cows with clinical signs of unknown infection. The obtained nucleic acid sequences were assembled into full genome. However, there were some sequence gaps which had to be filled by using Sanger sequencing or pyrosequencing cell culture isolate (Hoffmann et al., 2012). The downside of amplification techniques is that nucleic acid of the virus of interest has to be purified before amplification. This becomes a challenge when testing animals for acute infections. For example, the viremic period of SBV infection lasts up to 6 days post infection. Viral RNA cannot be found in the body of the animal after the infection (Hoffmann et al., 2012). This limits the time frame when the virus can be detected. In such cases serology testing becomes an attractive alternative approach for virus detection.

1.8.2 Antigen-antibody detection systems

A foreign material in an animal body causes an increase of immunoglobulins. Thus, detecting specific antibodies in blood is widely used application in virus diagnostics. Serology also helps when screening blood donations or during vaccination process. Immunoassays and neutralisation tests are the commonly used serology techniques.

Immunoassays are based on formation of immuno-complex between the antibody and the antigen, either of which can be present in a patient's body during or after the infection. Immunoassays usually use labelled antibodies or antigens to generate signal after the immuno-complex is formed (Souf, 2016).

One of the earliest examples of an immunoassay is a radioimmunoassay (RIA) which uses radioisotopes as labels. ELISA method was later developed by replacing radioisotopes with enzymes such as alkaline phosphatase or horseradish peroxidase (Gan & Patel, 2013). There are several types of ELISA prevailing today.

Direct ELISA is the simplest immunoassay. The sample containing the antigen of interest is adsorbed to the 96-well plate and after that a specific labelled antibody is added. If the antibody-antigen complex is formed, the colour of the mixture will change after adding the substrate for the enzyme. Direct ELISA is, however, less sensitive than other ELISAs, as many different antigens bind to the plate well (Grange et al., 2014).

Indirect ELISA relies on adhesion of an antigen to a solid phase, usually a 96-well plate well. A primary antibody is then added so it can bind to the antigen. A labelled secondary antibody is later added following the introduction of the substrate for the enzyme, which results in a colour change of the reaction mix. More than one secondary antibody can bind to the primary antibody, which increases the intensity of the reaction colour and sensitivity of the assay.

Sandwich ELISA uses immobilised specific antibodies. The antigen from the serum is captured by the antibodies and "sandwiched" by adding primary antibodies. The rest of the procedure is the same as with the indirect ELISA. Sandwich ELISA is more sensitive than direct ELISA as only antibody-antigen complex is present in the plate wells in the signal detection phase.

Competitive ELISA is used when the antigen is small or does not have multiple epitopes, thus making sandwich ELISA impossible. In competitive ELISA the antigen is immobilised in a plate well. A primary antibody is incubated with the same antigen and antigen-antibody complex is poured in the antigen-coated well. The more antigen was added in the sample, the less free antibody will be left to bind with the coated antigen, resulting in reduced intensity of the colour. A modification where there is a competition between different antibodies who bind the same antigen is also possible (Gan & Patel, 2013).

Other type of serology testing is the virus neutralisation test (VNT). VNT evaluates the ability of the antibodies that are present in the sample to inhibit the viral infection of the cells. If an antibody is present in the sample, it will attach to the virus and will prevent its entry to the host cell, thus limiting the toxic effect of the virus to the cells (Thullier & Sesardic, 2010).

VNT and immunofluorescence analysis (IFA) that can detect SBV-specific antibodies have been developed for SBV. They confirmed infections for RT-PCR SBV positive cows (Loeffen et al., 2012). A similar assay to VNT is a plaque reduction neutralization test (PRNT), which also successfully detected anti-SBV antibodies in bovine sera (Mansfield et al., 2013).

The downsides of VNT and PRNT assays are that they need several days to be performed, cannot be applied to large scale analysis of samples and require facilities suitable for work with live viruses. Recently, an indirect ELISA for fast detection of anti-SBV antibodies in bovine serum has been developed and evaluated. It is not as sensitive as the neutralization tests – in 98.9 % of cases the commercial ELISA test shows the same results as a virus neutralization test.

However, ELISA is suitable for large scale serum analysis, while VNT or PRNT cost more money per sample and take longer to perform (Bréard et al., 2013). The difference in the sensitivity between commercial ELISA and neutralization tests may arise because VNT can also utilize IgM antibodies which are present at the end of SBV viremia. IgG antibodies against SBV detected by ELISA exist only in low quantities at the end of SBV viremia. However, IgG is a useful marker to determine the infection status of an animal, as it was shown that SBV-specific IgM immunoglobulins persist in sheep only for about 2 weeks post infection. IgG antibodies provide a long-term protection and can be found in serum specimens at least 12 months post infection (Poskin et al., 2015). More differences between the types of assays come from the fact that commercial ELISA is based on an *E. coli* synthesized His-tagged recombinant SBV N protein, while the VNT and PRNT tests can potentially utilize antibodies against other viral proteins, such as surface glycoproteins.

1.8.3 Mass spectrometry detection techniques

Recent advances in mass spectrometry (MS) techniques, namely the development of matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), has led to an increased application of these tools in virus research. MALDI and ESI are techniques that use high electrical field or laser impulse to generate ions from a sample (Ho & Reddy, 2010). The emitted ions are then caught in mass analyser where their mass-to-charge ratio (m/z) is calculated (Singhal et al., 2015). Viral proteins are mainly used as samples in MS, although lipid analysis of the virus envelope is also possible as was shown with mumps virus (Brgles et al., 2016).

MS is capable of identifying single amino acid changes in mutant virus proteins. A single amino acid change in a peptide can result in a drug-resistant strain of a virus, as is the case of Human rhinovirus 14 (HRV 14) (Heinz et al., 1989). A MALDI-MS technique was applied to differentiate between HRV14 and its

naturally occurring drug-resistant mutant HRV14-Cys1199Tyr, as well as Tobacco mosaic virus (TMV) and genetically engineered mutants of TMV (Lewis et al., 1998). However, virus identification with the help of MS from clinical samples remains limited, as virus cultures have to be grown before the MS analysis to produce enough testing material (Majchrzykiewicz-Koehorst et al., 2015).

1.8.4 Other virus detection techniques

Electron microscopy (EM) is a tool allowing visualisation and identification of live virus samples. It is based on virus adsorption on hydrophilic grids and negative staining of the sample with heavy metal salts. The samples can be prepared and the results can be obtained in a few hours. Human, plant and animal viruses have been successfully identified using transmission EM (Gentile & Gelderblom, 2014; Zechmann & Zellnig, 2009). Viruses can be analysed from cell-cultures, urine, feces, vesicule fluid (Biel & Gelderblom, 1999). However, this type or research is limited only to the laboratories which can afford the expensive equipment needed to perform the experiments.

Recently, lab-on-chip approach in virus diagnostics gained a lot of interest. Lab-on-chip uses amplification or immunoassay methods in a smaller scale allowing great throughput of samples or detection of multiple viruses during the same assay. A multiplex *in situ* RNA analysis system was developed, where influenza or rhinovirus could be detected and identified within 15 minutes. Mutants with one base pair difference could be distinguished from wild-type viruses using this assay. The limit of detection was 10–50 of viruses per sample (Shaffer et al., 2015). A microfluidic nucleic acid microarray hybridization platform was developed for simultaneous detection of 26 pathogens, including Dengue and Chikugunya viruses. This test had an 85 % and 90 % agreement for Dengue and Chikugunya viruses accordingly, compared to the reference tests (Tan et al., 2014).

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Buffers and solutions

2.1.1.1. Solutions for DNA extraction, electrophoresis and DNA modifying enzymes

NaOH-SDS solution: 1 % SDS, 0.2 M NaOH (Reachim, Russia).

NaAc (pH 4.8): 1.425 M sodium acetate, pH 4.8 (Sigma Aldrich, USA).

NaAc (pH 7.0): 3 M sodium acetate, pH 7.0 (Sigma Aldrich, USA).

RNase A solution: 10 mg/ml pancreatic ribonuclease A (ThermoFisher Scientific Baltics, Lithuania).

TAE buffer solution (pH 8.3): 0.04 M Tris acetate (Amresco, USA), 1 mM EDTA (Sigma Aldrich, USA).

Agarose (1 %): 1 % agarose (Lonza, Switzerland) in TAE.

Ethidium bromide solution: 10 mg/ml ethidium bromide (Sigma Aldrich, USA).

DNR dye: 0.04 % bromophenol blue, 0.04 % xylolcyanol, 30 % glycerol (Roth, Germany).

Taq Polymerase buffer (ThermoFisher Scientific Baltics, Lithuania).

DNA digestion B, G, O, R, Y endonuclease buffers (ThermoFisher Scientific Baltics, Lithuania).

T4 DNA ligase buffer (ThermoFisher Scientific Baltics, Lithuania).

FastAp Thermosensitive Alkaline Phosphatase buffer (ThermoFisher Scientific Baltics, Lithuania).

2.1.1.2. Solutions for preparing *E. coli* and *S. cerevisiae* competent cells

Solution A: 5 mM Tris-HCl pH 7.4–8.0, 5 mM $MgCl_2$ (Sigma Aldrich, USA), 100 mM NaCl (Amresco, USA).

Solution B: 5 mM Tris-HCl pH 7.4–8.0, 5 mM $MgCl_2$, 100 mM $CaCl_2$ (Merck, USA).

TE: 10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA (Sigma Aldrich, USA).

TE-LiAc: 0.1 M CH_3COOLi (Sigma Aldrich, USA) solution in TE.

TE-PEG: 50 % (w/v) PEG 4000 (Sigma Aldrich, USA) solution in TE.

2.1.1.3. Solutions for protein purification under denaturing conditions with Ni-NTA resin

A: 6 M Guanidine hydrochloride, 0.1 M NaH_2PO_4 (Applichem, Germany), 0.5 % glycerol, 1 % Tween-20, (Sigma Aldrich, USA), 10 mM imidazole, 2 mM PMSF (Roth, Germany), 10 mM Tris-HCl pH 8.0;

B: 8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris-HCl (pH 8.0);

C: 8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris-HCl (pH 6.3);

D: 8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris-HCl (pH 5.9);

E: 8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris-HCl (pH 4.5).

Protein renaturation solution: 50 mM sodium acetate buffer (pH 5.0), 100 mM sodium chloride.

2.1.1.4. Solutions for protein purification under native conditions with sucrose gradient centrifugation

Disruption buffer: PBS (0.137 M NaCl, 0.028 M KCl, 0.01 M Na_2HPO_4, 0.238 M KH_2PO_4, pH7.2), 2 mM EDTA, 2mM PMSF (Roth, Germany).

Sucrose solutions for gradient formation: 70 %, 60 %, 50 %, 40 %, 30 % sucrose (Roth, Germany) w/v in disruption buffer.

2.1.1.5. Solutions for protein electrophoresis

Acrylamide/bisacrylamide solution: 30 % (w/v) acrylamide (Sigma Aldrich, USA), 0.8 % N, N'-methylbisacrylamide (Sigma Aldrich, USA).

4xTris-HCl/SDS (pH 6.8): 0.5 M Tris-HCl (pH 6.8), 0.4 % (w/v) SDS (BioRad, USA).

4xTris-HCl/SDS (pH 8.8): 1.5 M Tris-HCl (pH 8.8), 0.4 % (w/v) SDS.

Ammonium persulfate solution: 10 % $(NH_4)_2S_2O_8$ (Roth, Germany).

Concentrating gel solutions: 13 % (v/v) Acrylamide/bisacrylamide solution, 25 % 4xTris-HCl/SDS (pH 6.8), 0.5 % ammonium persulfate solution, 0.1 % TEMED (N, N, N', N'- tetramethylendiamine) (Sigma Aldrich, USA).

Separating gel solutions: 40 % (v/v) Acrylamide/bisacrylamide solution, 25 % 4xTris-HCl/NDS (pH 8.8), 0.33 % ammonium persulfate solution, 0.066 % TEMED.

2x protein dye buffer solution: 25 % 4xTris-HCl/NDS (pH 6.8), 20 % (w/v) glycerol, 4 % (w/v) SDS, 0.001 % (w/v) bromophenol blue, 10 % (v/v) mercaptoethanol.

Tris-glycine/SDS electrophoresis buffer solution (pH 8.3): 25 mM Tris, 0.2 M glycine (Fisher Scientific, USA), 0.1 % SDS.

Coomassie blue solution: 50 % (v/v) ethanol, 0.05 % (v/v) *Coomassie Brilliant Blue R-250*, 10 % (v/v) acetic acid (Roth, Germany).

Gel washing solution: 5 % (v/v) acetic acid.

Gel fixing solution I: 40 % (v/v) ethanol, 10 % (v/v) acetic acid.

Gel fixing solution II: 10 % (v/v) ethanol, 5 % (v/v) acetic acid.

2.1.1.6. Solutions for immuno-enzymatic reactions

Semi-dry blot transfer solution: 25 mM Tris (Sigma Aldrich, USA), 0.2 M glycine (Fisher Scientific, USA), 20 % (v/v) ethanol (Vilniaus degtinė, Lithuania).

TBS: 20 mM Tris, 8 g/l NaCl (pH 7.6).

TBS-T: 0.1 % Tween-20 (v/v) (Fisher Scientific, USA) solution in TBS.

Blocking solution: Roti Block solution (Roth, Germany)

Developer solution: mixed A and B solutions:

A: 16.8 mM 4-chlor-1-naphtol in ethanol;

B: H_2O_2 0.018 % (v/v) in 25 ml TBS.

Coating buffer: 66.7 mM $NaHCO_3$, 30 mM Na_2CO_3 (pH 9.4–9.5).

Wash buffer PBS-T: PBS, 0.1 % Tween-20 (pH 7.2).

Roti-Block: 1:10 Roti-Block concentrate in water (Carl Roth, Germany).

TMB: 3,3',5,5' tetramethylbenzidine solution (Invitrogen, Frederick, USA)

Chicken serum: 1 % chicken serum in wash buffer (Gibco/Invitrogen, UK).

2.1.2. Antibodies for immunoblot and immunosorbent assays

Anti-6His monoclonal, from mouse. ThermoFisher Scientific, USA.
Anti-mouse IgG (H+L) polyclonal HRP, from goat. BioRad, USA.
Anti-bovine IgG polyclonal HRP, from rabbit. Sigma Aldrich, USA.
Anti-bovine IgA polyclonal HRP, from sheep. AbD Serotec/BioRad, USA.
Anti-bovine F(ab')2 IgG polyclonal HRP, from rabbit. LifeSpan BioSciences, USA.

2.1.3. Media for microorganisms

LB (Miller) medium: LB Broth, Germany 25 g/l (Fisher Scientific, USA).
LB agar medium: LB Broth, Germany 25 g/l, 2 % agar (Merck, USA).
YPD yeast medium: 1 % yeast extract (Merck, USA), 2 % soy peptone (Applichem, Germany), 2 % glucose (Roth, Germany).
YPD agar yeast medium: YPD yeast medium, 2 % agar (Merck, USA).
YPGal yeast medium: 1 % yeast extract, 2 % soy peptone, 2.5 % galactose.
Ampicillin solution (50 mg/ml ampicillin Roth, Germany, 50 % ethanol): 50 µg/ml in LB medium.
Formaldehyde solution (37 % formaldehyde) (Sigma, USA): 0.3–1.2 µl/ml in YPD medium.

2.1.4. Oligonucleotides for PCR DNA amplification

All oligonucleotides were produced by Metabion International, Germany.
Notation of oligonucleotides is in the 5'→3' direction.
SchN_5': TGCCTAGGACAATGTCAAGCCAATTCATTTTTG
SchN_3L: ATATCTAGAACCTCCAGATCCACCAGAGATGTTGATACCGA ATTGCTGCAAG
SchN_3X: GCACCTAGGATCGATGTTGATACCGAATTGCTG
SchOG2_5P: GACACTAGTACAATGCTTCTAAATATTGTTTTGA

SchOG2_3L: ATATCTAGAACCTCCAGATCCACCAGATCTAGTTTTTGG
CAAAGCTTTATAACCA
SchOG2_3S: GACACTAGTTTATCTAGTTTTTGGCAAAGCTTTATAACCA
SchOG2-5: TAACTAGTACAATGGGCACTAGAGGATCGAGGT
SchOG2-3: GGACTAGTTTAATGATGATGATGATGATGTCTAGT
SchOG2S_5P: GCACTAGTACAATGTTGTTGAATATCGTCTT
SchOG2S_3L: TATCTAGAACCTCCAGATCCTCCACTTCTGGTCTTTGGT
TGCCTTGT
SchOG2S_3S: GCACTAGTTCATCTGGTCTTTGGTAATGCCTTGT
SchOG2S_5t: GCACTAGTATAATGGCTTTGCCATTGAAGGAAGGT
SchOG2S_3tL: TATCTAGAACCTCCAGATCCACCAGACATCAATTCAG
GCAATATACTACC
SchOG2S_3tS: GCACTAGTTCACATCAATTCAGGCAATATACTACC
SchOG2S_3tN: GCACTAGTCATCAATTCAGGCAATATACTACC
PYK5: TATTCATTCTTTTTCATCCTTTGG
PGK3: TCCTTACCTTCCAATAATTCCAAAG

2.1.5. ELISA antigens

Andes hantavirus nucleocapsid protein was obtained from the collection of the Department of Genetic Engineering of Eukaryotes, Institute of Biotechnology, Vilnius university (Schmidt et al., 2006).

2.1.6 Microorganisms

All strains of microorganisms were obtained from the collections of the Departament of Genetic Engineering of Eukaryotes, Institute of Biotechnology, Vilnius university.
Escherichia coli strain DH5α: F^- *gyrA96* (NaI^r) *recA1 relA1 endA1 thi*$^{-}$*1 hsdR17* (r_k^- m_k^+) *glnV44 deoR* Δ*(lacZYA-argF)U169* [ϕ80dΔ*(lacZ)M15*].
Escherichia coli strain GM119: F^- *dam*$^{-}$*3 dcm*$^{-}$*6 lacY1 galK2 galT22 tonA31 tsx*$^{-}$*78 supE44 mtl*$^{-}$*1(thi*$^{-}$*1).*
S. cerevisiae strain AH22-214: MATa, *leu2-3,112, his4-519*.

2.2. Methods

2.2.1. DNA deproteination and ethanol/isopropanol precipitation

Equal volume of phenol (pH 7.5) was added to DNA solution. The mixture was shaken for 1 min and spun for 2 min in a table-top centrifuge at maximum speed. The upper water phase was collected into a new separate centrifuge tube. The process was repeated one more time. 3 M NaAc (pH 7.0) was added to the DNA solution up to a final concentration of 0.3 M NaAc. Later, 2 volumes of ethanol or 0.7 volumes of isopropanol were added. The mixture was placed in –20 °C temperature for 30 min or –70 °C for 10 min. The mixture was then centrifuged in a table-top centrifuge at maximum speed at 0 °C for 15 min. The supernatant was discarded. The pellets were washed with ice-cold 70 % ethanol and diluted in water.

2.2.2 Purification of DNA plasmids

Bacteria carrying a plasmid of interest were grown for 16 h at 37 °C in LB medium with 50 μg/ml ampicillin. The cells were centrifuged at 2,000 × g for 10 min and supernatant was discarded. 200 μl of water was added to the debris and the cells were lysed with the double volume (400 μl) of lysis NaOH-SDS solution. Equal volume (400 μl) of NaAc (1.425 M, pH 4.8) was added, the mixture was shaken and cooled at –20 °C for 10 min. After that, 1/3 of the mixture volume (300 μl) of chloroform was added and the mixture was centrifuged at 2,000 × g for 20 min. The upper water layer was collected into a separate centrifuge tube and 0.8 volumes of isopropanol was added into it. After 5 min the mixture was spun in a table-top centrifuge at maximum speed for 3 min. The pellets were dissolved in water with 1 μg of RNAse A (5 U) and incubated at 37 °C for 1h. The DNA was then purified using ethanol/isopropanol. The concentration of a plasmid was determined using NanoDrop (ThermoFisher, USA) spectrophotometer.

2.2.3. DNA electrophoresis

DNA electrophoresis was performed in 1 % agarose gels with 0.1 μg/ml ethidium bromide. The gel was put into TAE buffer solution and 10 V/cm current was applied for 30 min. The gels were analysed under UV light using Herolab EASY (Herolab, Germany) gel documentation system.

2.2.4. Other DNA manipulation reactions

Purification of DNA fragments from agarose gel was performed using GeneJET Gel Extraction Kit according to manufacturer's recommendations.

DNA digestion was performed using conventional or FastDigest restriction enzymes according to manufacturer's recommendations (ThermoFisher Scientific, USA).

DNA 5' phosphate cleavage was performed using FastAP alkaline phosphatase according to manufacturer's recommendations (ThermoFisher Scientific, USA).

Ligation of DNA was performed using T4 DNA ligase in T4 DNA ligase buffer according to manufacturer's recommendations (ThermoFisher Scientific, USA). The ratio of plasmid to DNA fragment was 1:1 to 1:5. Ligated plasmids were used for *E. coli* transformation.

2.2.5. Preparation of competent *E. coli* cells

E. coli recipient cells were grown in LB medium for 16 hours in test tubes. 1/100 volume of recipient cells were transferred from each test tube and grown in the same volume of LB medium as before at 37 °C until 0.3 optical density units at 550 nm wavelength were achieved by the cell culture. Later procedures were performed in an ice bath or at 0-4 °C temperature. The cells were collected by spinning the tubes at 2,000 × g for 10 min. The cells were washed with ice-cold solution A and centrifuged once more. The supernatant was removed and the cells were incubated in an equal volume of ice-cold $CaCl_2$ solution for 60 min. After that the cells were collected using the same centrifugation procedures, suspended in 1/10 volume of solution B and used for transformation.

2.2.6. Transformation of competent *E. coli* cells

Ligation reaction mix or 0.2 μg of purified plasmid was used with competent *E. coli* cells (see 2.2.5). The suspension of cells and DNA was incubated for 30 min on ice, then for 90 s at 42 °C. After the thermal shock the cells were placed back onto ice and 3–5 volumes of LB medium were added. The cells were incubated at 37 °C for 1 h, centrifuged at 2,000 × g for 10 min, suspended in LB and spread over LB agar medium plates with ampicillin. The plates were kept at 37 °C for 18 h.

2.2.7. Transformation of *S. cerevisiae* cells

S. cerevisiae recipient cells were grown in test tube for 16 h. 1/10 of *S. cerevisiae* recipient cells were transferred to a new separate test tube with YPD medium and grown at 30 °C until 0.4-1.0 optical density units at 600 nm wavelength were reached by the cell culture. The cells were collected by centrifugation at 2,000 × g for 10 min, washed with TE buffer solution and collected using the same conditions. The cells were suspended in TE-LiAc (0.1 M) and incubated at 30 °C for 1 h and collected using the same conditions. Cells were suspended in TE-LiAc. 1/4 of suspended cells were mixed with 2–5 μg of plasmid DNA for a single transformation. The transformation mix was incubated for 20 min at 30 °C. Two volumes of 50 % TE-PEG were added and the mixture was incubated for 60 min at 30 °C. An incubation of 15 min at 42 °C followed. The cells were then collected by centrifuging them at 2,000 × g for 5 min in a table-top centrifuge, washed with TE solution, suspended in YPD medium and incubated overnight at 30 °C. The next day, the cells were collected by centrifuging them at 2,000 × g for 5 min, spread over YPD agar medium plates containing 0.4 μl/ml to 0.8 μl/ml formaldehyde and incubated at 30 °C for two days.

2.2.8. Induction of protein synthesis in yeast

Transformed yeast cells were grown in YPD medium containing 0.4 μl/ml concentration of formaldehyde in 2 litre Erlenmeyer flasks at 30 °C with

180 rpm shaking. After the 24 h of growth a 25 % galactose solution with 4 µl/ml concentration of formaldehyde was added into the YPD medium until it reached a final 2.5 % galactose concentration. The growth was continued for another 16–24 h.

2.2.9. Purification of SBV N proteins from *S. cerevisiae* under native conditions

At 16–24 h after the induction of recombinant protein synthesis, yeast cells were collected by centrifuging them at 2,000 × g for 5 min. Yeast cells were suspended in equal volume of disruption buffer PBS and another equal volume of glass beads (0.5 mm diameter, Sigma-Aldrich, USA) was added to the mixture. Cells were disrupted mechanically by vortexing at 4 °C for 7 min. The lysates were cleared from debris by centrifugation at 2,000 × g for 3 min. The insoluble protein fraction was separated by centrifugation at 10,000 × g for 30 min at 4 °C. The supernatant was collected and placed on top of a 70 % / 60 % / 50 % / 40 % sucrose solution in PBS in an ultracentrifuge tube. The proteins were centrifuged at 110,396 × g for 15 h. The 70 % and 60 % sucrose solutions containing the SBV N protein were collected and concentrated by centrifugation for 2 h at 110,396 × g through 30 % sucrose solution. The protein pellet containing the SBV N protein was suspended in PBS. Glycerol was added to the final concentration of 40 % and the protein was kept at –20°C until further use.

2.2.10. Purification of SBV N proteins from *S. cerevisiae* under denaturing conditions

At 16–24 h after the induction of recombinant protein synthesis, yeast cells were collected by centrifuging them at 2,000 × g for 5 min. Yeast cells were suspended in equal volume of disruption buffer A and another equal volume of glass beads (0.5 mm diameter, Sigma-Aldrich, USA) was added to the mixture. Usually, 8 g of wet yeast were used for high yield protein purification and 1 g was used for small-batch protein purification. The cells were disrupted by vortexing them for

periods of 30 s. In between these periods the cells were cooled at 4 °C for 30 s. Total vortexing time was 5 min. The cell debris was sedimented by centrifuging the obtained yeast lysates at 3,000 × g for 5 min. Insoluble proteins were spun down by centrifugation at 10,000 × g for 10 min at 4 °C. The Ni–NTA resin with protein binding capacity of 10–20 mg/ml was equilibrated in disruption buffer and mixed with the supernatant. Binding was performed by shaking for 1 h at room temperature (RT). N protein purification was performed on a polypropylene column, according to manufacturer's recommendations for denaturing purification of insoluble proteins (Qiagen, Germany). The main portion of the protein was eluted in buffer E (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 4.5). To evaluate the purity of the eluted recombinant N protein SDS-PAGE with Coomassie Brilliant Blue staining and Western blot with the mAb against 6His-tag epitope were performed. Eluted protein was dialyzed against sodium acetate buffer (50 mM sodium acetate, 100 mM sodium chloride, pH 5.0) and stored at –20 °C with 40 % of glycerol.

2.2.11. SDS-PAGE analysis

Protein solution was mixed with an equal volume of protein dye buffer solution and heated for 10 min at 95–100 °C. Polyacrylamide gel was made from two layers containing 4 % and 12 % PAA. After loading samples into PAA gel wells the vertical electrophoresis was performed at 200 V 30 mA in Tris-glycine/SDS electrophoresis buffer solution.

2.2.12. Western-blot

After protein gel electrophoresis the PAA-SDS gel was soaked in transfer buffer and transferred onto a PVDF membrane. The semi-dry protein transfer was done at 12 V for 30 min. The membrane was then blocked using RotiBlock or 1 % skim milk solution for 1 h. The membrane was washed with TBS-T buffer ant placed into a container with serum or primary antibodies diluted in TBS-T buffer. Dilutions were determined empirically for each experiment. After incubating the

membrane for 2 h at RT, it was washed and incubated with secondary antibody conjugated with HRP diluted in TBS-T for 1 h. After washing the membrane with TBS, it was developed using developing solutions A and B.

2.2.13. Indirect IgG ELISA for detection of SBV N protein-specific antibodies in bovine serum

Microtiter plates (Nerbe Plus GmbH) were coated with 400 ng per well of recombinant SBV N-His protein or 200 ng per well of recombinant SBV N protein in 100 µl of 0.05 M carbonate-bicarbonate coating buffer (pH 9.6) and incubated for 16 h at 4 °C. Plates were washed three times with PBS-T and then blocked by the addition of 150 µl of blocking buffer reagent per well (1x Roti-Block). The plates were incubated at RT for 1 h. After blocking, the plates were washed three times with PBS-T, and 100 µl aliquots of serum specimens, diluted 1:200 in PBST buffer with 10 % Roti-Block, were added to the wells. Plates were incubated for 1 h at 37 °C and washed five times with PBST. 100 µl aliquots of rabbit anti-bovine IgG conjugated to HRP, diluted 1:10,000 (v/v) in PBS with 5 % Roti-Block, were added to each well and the plates were incubated for 1 h at 37 °C. After washing five times with PBS-T, 100 µl of TMB substrate was added to each well and the enzyme reaction was stopped with an equal volume of 1 M H_2SO_4 solution. The optical density at 450 nm was determined for each sample using an ELISA plate reader (Sunrise Tecan, Switzerland).

2.2.14. Indirect IgG ELISA for the detection of SBV N protein-specific antibodies in cow's milk and saliva, and indirect IgA ELISA for saliva

Microtiter plates (Nerbe Plus GmbH, Winsen/Luhe, Germany) were coated with 2 µg/ml of recombinant SBV N protein in 100 µl of 0.05 M carbonate-bicarbonate coating buffer (pH 9.6) and incubated overnight at 4 °C. Plates were washed three times with PBS-T and then blocked by the addition of 150 µl of blocking buffer per well (1x Roti-Block). The plates were incubated at room temperature for 1 h. After blocking, the plates were washed three times with

PBST and 100 µl aliquots of milk specimens diluted 1:10 in PBST were added to the wells. Plates were incubated for 1 h at 37 °C and washed five times with PBST. 100 µl aliquots of rabbit anti-bovine IgG conjugated to HRP, diluted 1: 20,000 (v/v) in PBST, were added to each well and the plates were incubated for 1 h at 37 °C. After washing five times with PBST, 100 µl of TMB substrate was added to each well and the enzyme reaction was stopped with an equal volume of 1 M H_2SO_4 solution after 10 min of incubation. The optical density at 450 nm was determined for each sample using an ELISA plate reader (Sunrise Tecan, Switzerland).

In the case of saliva anti-SBV IgA ELISA, saliva samples were diluted 1:3 in PBST and sheep anti-bovine IgA, conjugated to HRP, were diluted 1: 20,000 in PBST with 5 % RotiBlock and 1 % chicken serum. For the saliva anti-SBV IgG ELISA, 4 µg/ml of the SBV N protein was used per well. Nunc Maxisorp microtiter plates (Thermo Scientific, Denmark) were blocked with 150 µl of 5 % chicken serum in PBS per well. Saliva samples were diluted 1:3 and rabbit anti-bovine F(ab'2) IgG conjugated to HRP were diluted 1:30,000 in PBST with 5 % Roti-Block and 1 % chicken serum.

2.2.15. Determining the cut-off values of the different ELISAs

To define the positive/negative threshold of the SBV N-His based indirect IgG SBV ELISA, 11 serum samples previously determined as SBV IgG-negative by commercial ID Screen test were used. The OD values of ELISA were corrected for non-specific reactivity and reported as sample-to-positive (S/P) values $SP = \left(\frac{OD\ sample}{OD\ positive\ control}\right) \times 100$, according to Breard et al. (2013). To calculate the cut-off value, the reactivity of positive and negative reference serum samples from the ID Screen test kit with the recombinant SBV N protein were analysed. The cut-off value was calculated as the mean of S/P values of negative samples plus 2 × SD (22+10) with 95 % confidence:

$Cut - off = \frac{x_1+x_2+\cdots+x_n}{n} + 2 \times SD(x_1, x_2, \ldots, x_n)$, where x is an S/P value of the individual sample, n is the number of samples and SD is the standard deviation of the set of samples. The tests were run in duplicate.

The cut-off value of our serum anti-SBV IgG system based on the SBV N protein was calculated as follows: 27 serum samples that were negative according to the commercial indirect SBV ELISA test were tested against the SBV N protein. The optical density of a sample was divided by the optical density of a reference serum sample and a sample-to-positive (S/P) value in percent was obtained for each sample as described above. The calculation of S/P values was performed according to Bréard et al (Bréard et al., 2013). S/P values for milk and saliva experiments were calculated after incubating milk or saliva samples with the closely related hantavirus Andes nucleocapsid protein (Schmidt et al., 2006).

The cut-off value for the serum, milk, saliva IgG and saliva IgA systems were determined using the following formula:

$Cut - off = \frac{x_1+x_2+\cdots+x_n}{n} + 3 \times SD(x_1, x_2, \ldots, x_n)$, where x is an S/P value of the individual sample, n is the number of samples and SD is the standard deviation of the set of samples. The samples that showed reactivity within the average plus 2×SD and average plus 3×SD range were considered doubtful.

2.2.16. Electron microscopy

Electron microscopy was performed using a Morgagni 268 (D) transmission electron microscope (FEI, USA). 0.2 μg of protein of interest was poured on a copper grids coated with carbon. After 1 minute the grids were dried with filter paper, washed with water and dried again using filter paper. 2 % uranyl acetate solution was applied to the grid for 2 minutes. The grid was dried with filter paper and additionally dried for 5 minutes in air.

2.2.17. Statistical analysis

Differences between observed groups were evaluated by performing Chi-square contingency table test. A correlation coefficient was calculated to measure the relationship of serologic status between different samples collected from same cattle. Sensitivity and specificity were calculated to measure the agreement in serologic status between same samples collected from same cattle but tested with different assays. Correlation coefficients were calculated using Microsoft Excel software. Confidence intervals were calculated with 95 % confidence level using MedCalc software (MedCalc software, Belgium).

3. RESULTS

3.1. Synthesis of SBV N protein in yeast *Saccharomyces cerevisiae*

Proteins that react with SBV-specific IgG antibodies can be used to develop an anti-SBV IgG detection system. Yeast *Saccharomyces cerevisiae* strain AH22-214 was used as a platform for SBV nucleocapsid protein production. The SBV nucleocapsid gene for plasmid construction was synthesized by "GenScript", USA (GenBank: HE649914.1, Figure 3.1). The expression vectors pFX7-6HisN and pFD3 were obtained from the collections of the Departament of Genetic Engineering of Eukaryotes, Institute of Biotechnology, Vilnius university (Sasnauskas et al., 1999; Razanskiene et al., 2004).

Sticky ends of the DNA coding nucleocapsid protein were formed using *XmaJI* restriction nuclease. The yeast expression vector pFX7-6HisN was linearized using *XbaI* restriction endonuclease and was used to create SBV N gene variant coding 6 His amino acid sequence at the N-terminus of the protein. SBV N coding DNA sequence was ligated with pFX7-6HisN plasmid sequence and amplified using *E. coli* cells (Figure 3.1, C). The integrity of the gene was proven by sequencing the plasmid using PYK5 and PGK3 expression vector PCR primers.

A

ATG*CACCATCACCATCACCAT*TCTAGGACAATGTCAAGCCAATTCATTTTTGAAGATGTACCACAACG
GAATGCAGCTACATTTAACCCGGAGGTCGGGTATGTGGCATTTATTGGTAAGTATGGGCAACAACTCA
ACTTCGGTGTTGCTAGAGTCTTCTTCCTCAACCAGAAGAAGGCCAAGATGGTCCTACATAAGACGGCA
CAACCAAGTGTCGATCTTACTTTTGGTGGGGTCAAATTTACAGTGGTTAATAACCATTTTCCCCAATA
TGTCTCAAATCCTGTGCCAGACAATGCCATTACACTTCACAGGATGTCAGGATATCTAGCACGTTGGA
TTGCTGATACATGCAAGGCTAGTGTCCTCAAACTAGCTGAAGCTAGTGCTCAGATTGTCATGCCCCTT
GCTGAGGTTAAGGGATGCACCTGGGCCGATGGTTATACAATGTATCTTGGATTTGCACCTGGGGCCGA
AATGTTCCTTGATGCTTTTGACTTCTATCCACTAGTTATTGAAATGCATAGGGTCCTCAAGGACAATA
TGGATGTAAATTTTATGAAAAAAGTCCTCCGCCAACGCTATGGAACAATGACTGCTGAAGAATGGATG
ACTCAGAAAATAACAGAAATAAAAGCTGCTTTTAATTCTGTTGGACAGCTTGCCTGGGCCAAATCTGG
ATTCTCTCCTGCTGCTAGAACCTTCTTGCAGCAATTCGGTATCAACATCTAATCTAGG

B

MHHHHHHSRT MSSQFIFEDV PQRNAATFNP EVGYVAFIGK YGQQLNFGVA
RVFFLNQKKA KMVLHKTAQP SVDLTFGGVK FTVVNNHFPQ YVSNPVPDNA
ITLHRMSGYL ARWIADTCKA SVLKLAEASA QIVMPLAEVK GCTWADGYTM
YLGFAPGAEM FLDAFDFYPL VIEMHRVLKD NMDVNFMKKV LRQRYGTMTA
EEWMTQKITE IKAAFNSVGQ LAWAKSGFSP AARTFLQQFG INI

C

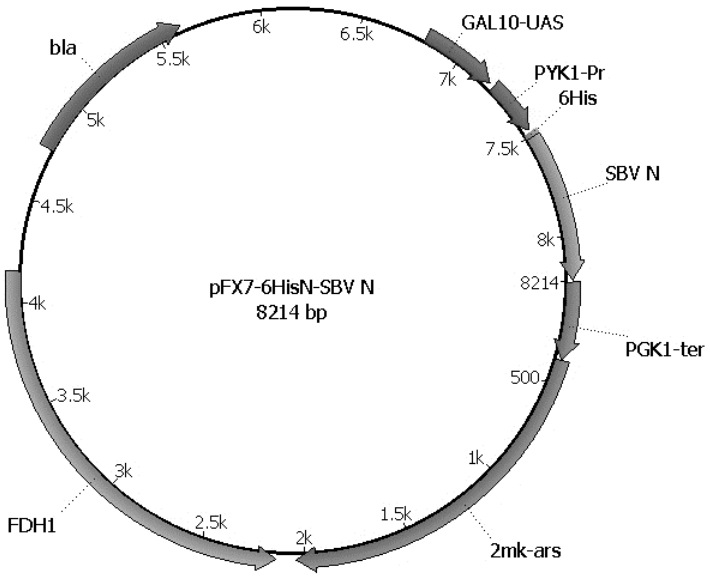


Figure 3.1. Schmallenberg virus N gene (A) and N-His amino acid (B) sequences and pFX7-6HisN-SBV N plasmid chart (C). (A) Schmallenberg virus N gene sequence (GenBank: HE649914.1). 6His coding sequence is marked in *italic*; (C) 2mk-ars – 1.74 kb fragment of yeast 2µm plasmid; FDH1 – *FDH1* gene of *Candida maltosa*, conferring resistance to formaldehyde; bla – beta-lactamase gene, grants resistance to *E. coli* against ampicillin; GAL10-UAS/PYK1-Pr – yeast hybrid galactose inducible gene promoter; PGK1-Ter – fragment of *S. cerevisiae PGK1* gene transcription terminator sequence. SBV N – Schmallenberg virus nucleocapsid protein coding sequence; 6His– 6 histidine amino acids coding sequence.

Yeast cells were transformed with the pFX7-6HisN-SBV N plasmid. The recombinant gene expression was induced by adding sucrose to the yeast growth medium. The synthesis efficiency of the recombinant SBV N-His protein was proven both by electrophoresis and immunoblotting (Figure 3.2). SDS-PAGE analysis of crude lysates of *S. cerevisiae* harbouring the pFX7-6HisN-SBV-N plasmid revealed the presence of an additional protein band after induction with galactose. This band of approximately 26 kDa was present in the lysates of yeast transformed with pFX7-6HisN-SBV-N. As evaluated by SDS-PAGE, the synthesis level of the SBV N-His protein was approximately 2 % of the total cellular protein (Figure 3.2, A – lane 2). Meanwhile, no additional band of the corresponding molecular size was observed in crude lysates of *S. cerevisiae* harbouring the pFX7-6HisN vector used as a negative control (Figure 3.2, A – lane 1). As the main quantity of the SBV N-His protein was found to be insoluble after cell lysis, the protein was purified using denaturing solutions to increase its solubility. The yield of the 6HisN-tagged N protein using nickel-chelate chromatography under denaturing conditions was about 3.0–3.5 mg/g wet weight of yeast as determined by SDS-PAGE (Figure 3.2 C) and Bradford assay. The purified recombinant SBV N-His protein was also insoluble and formed precipitate during and after dialysis in PBS buffer. 50 mM sodium acetate buffer (pH 5.0) containing 100 mM sodium chloride was a better choice for SBV N-His refolding as less precipitate formed after the dialysis. The purified SBV N-His protein was visible as a single protein band in Coomassie Brilliant Blue-stained SDS-PAGE gels (Figure 3.2, A – lane 3; Figure 3.2, C). Western blot analysis with mAbs against 6His-tag epitope showed that the protein of interest had His-tag and was successfully purified (Figure 3.2, B – lanes 2 and 3).

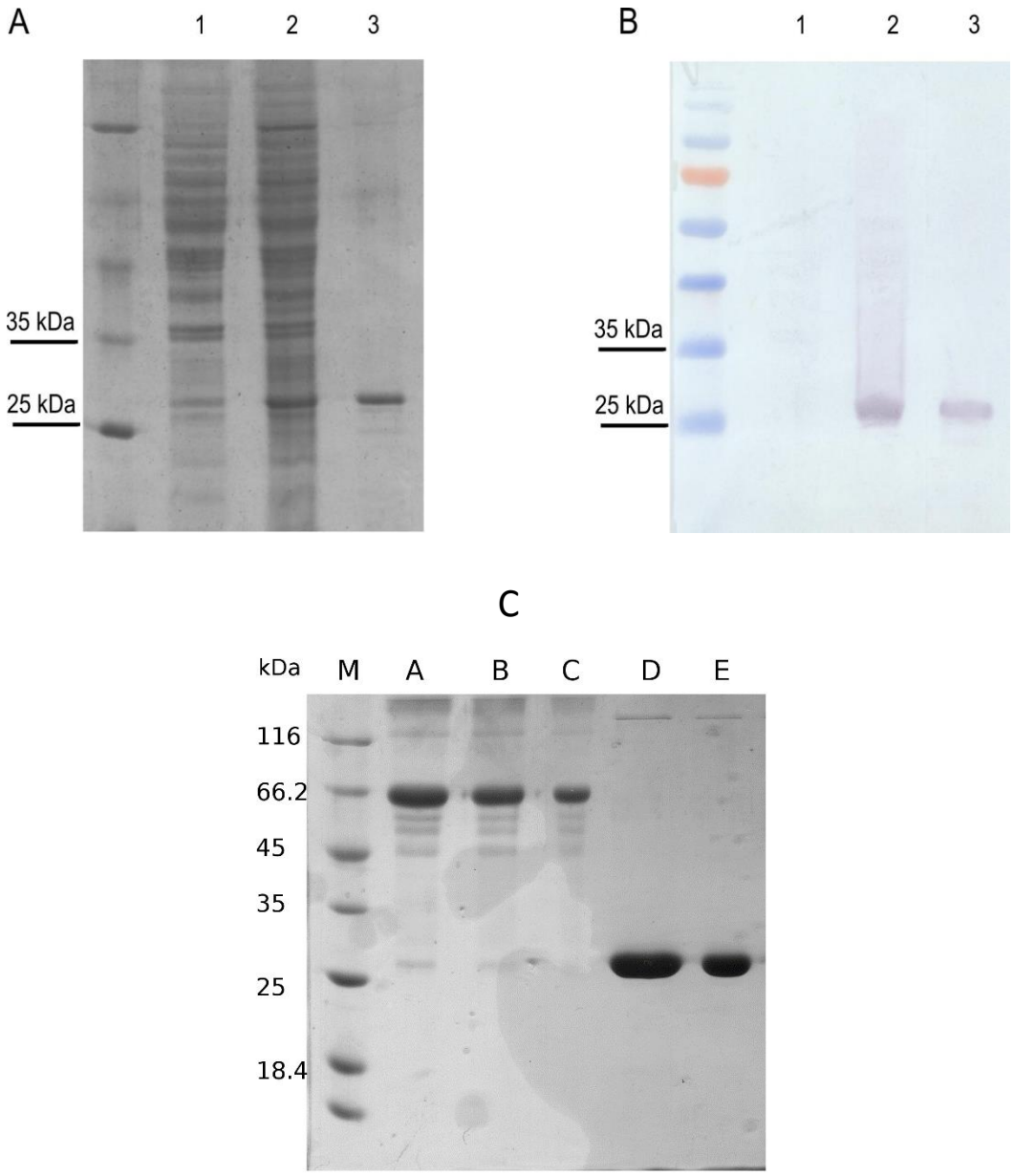


Figure 3.2. Analysis of yeast cell lysates and purified SBV N-His protein by SDS-PAGE (A, C) and Western blot (B). (A, B) In lanes: 1 – lysate of mock- transformed *S. cerevisiae* pFX7-6His; 2 – lysate of *S. cerevisiae* transformed with a plasmid pFX7-6His-N encoding SBV N protein; 3 – Ni-chelate resin-purified SBV N protein. Western blotting was performed using the mAb against 6His-tag epitope; (B) In lanes: PageRuler Prestained Protein Ladder was used. (C) Lanes A, B, C – 4 μg, 2 μg and 1 μg of BSA protein, accordingly. Lanes D and E – 8 μg and 4 μg of purified SBV N-His protein, accordingly. M – PageRuler Unstained Protein Ladder.

In order to confirm the sequence identity of the full-length recombinant SBV N-His protein a mass spectrometry analysis was performed by the scientists of the Institute of Biochemistry. Enzymatic digests were performed using trypsin, chymotrypsin and endopeptidase-AspN to generate internal peptides for detailed mass spectrometry analysis. Peptide analysis confirmed the primary structure of the recombinant SBV N-His protein predicted from DNA sequence.

A pFD3 plasmid does not have 6HisN-tag sequence thus it was used to express SBV N gene without the His-tag. *XmaJI* restriction enzyme was used to hydrolyse the SBV N DNA, while *XbaI* was used to prepare the pFD3 plasmid. The insert SBV N DNA was ligated with the pFD3 plasmid. The resulting pFD3-SBV N plasmid was amplified using *E. coli* bacteria. Later, pFD3-SBV N was used to transform *S. cerevisiae* cells. The transformed yeast was grown in YPD medium and recombinant protein synthesis was induced by adding galactose into the medium. The yeast cells were disrupted mechanically and SBV N protein was purified using centrifugation in sucrose solution. It was found mainly at 60 % and 70 % of sucrose gradient fraction (Figure 3.3). The protein containing fractions were concentrated by centrifugation in 30 % sucrose solution and sucrose was later removed by dialysis against PBS. The yield of the SBV N protein was 1.3 mg/g wet weight of yeast. Nucleocapsid-like structures were not observed during the transmission electron microscope analysis of the purified SBV N or SBV N-His proteins.

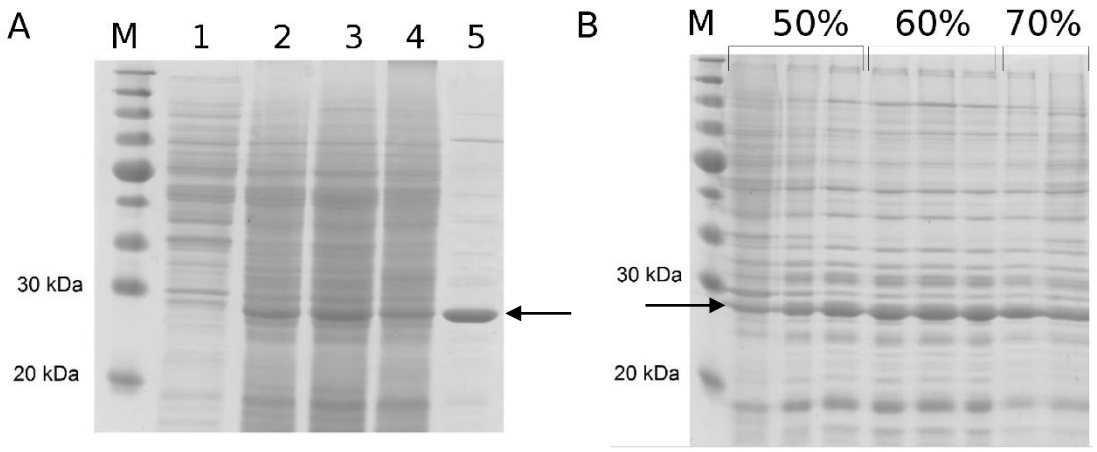


Figure 3.3. SDS-PAGE analysis of SBV N protein ultracentrifugation. (A) In lanes: 1 – lysate of *S. cerevisiae* yeast transformed with mock pFD3 plasmid, 2 – lysate of SBV N protein-producing *S. cerevisiae* yeast, 3 – supernatant of SBV N protein-producing yeast, 4 – precipitate of SBV N protein-producing yeast, 5 – SBV N protein after ultracentrifugation in sucrose solution. (B) SBV N protein samples collected after ultracentrifugation in sucrose solution. Fractions corresponding to 50 %, 60 % and 70 % concentration of sucrose solution were collected and analysed in PAA gel. Lane M – PageRuler Prestained Protein Ladder marker. Arrows mark SBV N protein.

3.2. Synthesis of SBV G2 protein in yeast *Saccharomyces cerevisiae*

An SBV-specific IgG detection system can be improved by utilizing other SBV structural proteins, such as glycoprotein G2 (G2). Several strategies were used for the G2 gene expression in yeast. A full length SBV gene coding G2 was chosen as a reference (Figure 3.4, A).

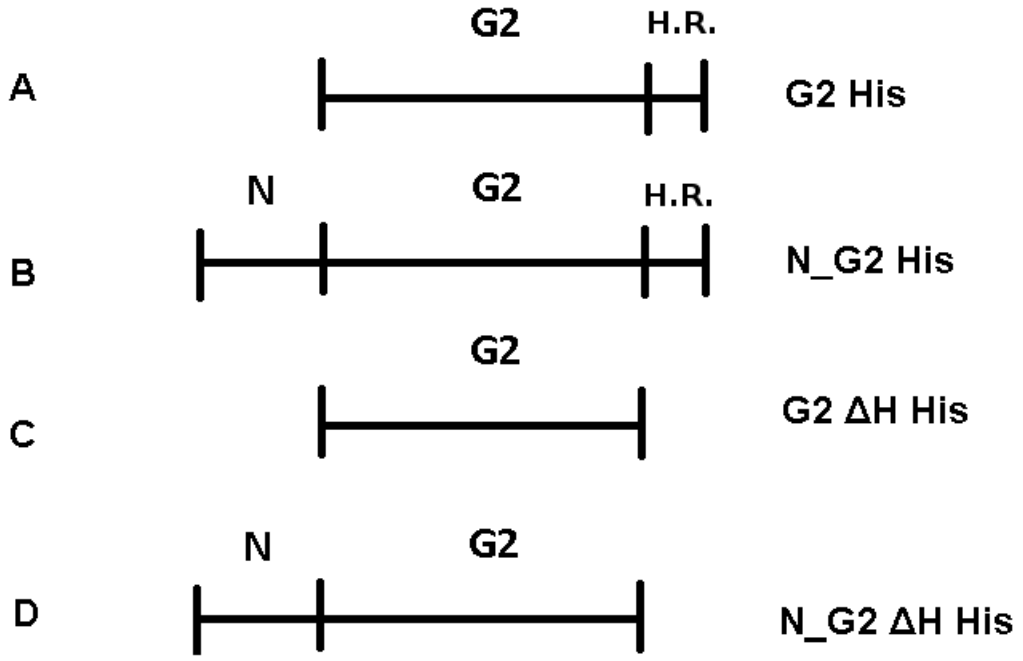


Figure 3.4. Schematic representation of SBV G2 protein variants. A – full length G2 protein, B – full length G2 protein fused with full length nucleocapsid protein at the N terminus, C – G2 protein without hydrophobic region, D – G2 protein without hydrophobic region fused with full length nucleocapsid protein at the N terminus. H.R. – hydrophobic region of SBV G2 protein.

The nucleotide sequence of the G2 gene (GenBank: CCF55030.1) was changed to be optimal for *S. cerevisiae* codon usage bias without changing the amino acid sequence of the G2 protein (Figure 3.5). The other version of the gene was constructed using SchOG2S_3tS and SchOG2S_5P primers and called G2ΔH (Figure 3.4 C). It had the predicted hydrophobic region of 203-308 amino acids at the C-terminus removed (Figure 3.5).

A

ATG*CACCATCACCATCACCAT*TCTAGTACAATGTTGTTGAATATCGTCTTAATCAGTAACTT
AGCCTGCTTAGCTTTCGCTTTGCCATTGAAGGAAGGTACCAGAGGTTCCAGATGCTTTTTGA
ATGGTGAATTAGTCAAGACAGTAAACACCTCTAAAGTTGTCTCAGAATGTTGCGTTAAGGAT
GACATCTCCATTATTAAGAGTAACGCTGAACATTACAAGTCAGGTGACAGATTGGCTGCAGT
AATTAAATACTACAGATTGTACCAAGTTAAGGACTGGCATTCTTGTAATCCAATCTATGATG
ACCACGGTTCATTCATGATTTTGGATATAGACAACACTGGTACATTAATTCCTAAAATGCAC
ACCTGTAGAGTCGAATGCGAAATTGCATTGAACAAGGATACTGGTGAAGTAATATTGAACTC
CTACAGAATTAATCATTACAGAATCTCTGGTACAATGCACGTTTCAGGTTGGTTCAAGAATA
AGATCGAAATCCCATTGGAAAACACTTGTGAATCTATTGAAGTCACTTGCGGTTTGAAGACA
TTGAACTTCCATGCCTGTTTCCATACCCACAAATCCTGCACTAGATACTTCAAGGGTAGTAT
ATTGCCTGAATTGATGATCGAATCTTTCTGTACTAACTTGGAATTAATCTTGTTAGTTACAT
TCATTTTGGTTGGTTCAGTCATGATGATGATCTTGACCAAGACTTACATCGTATACGTTTTT
ATACCAATCTTTTACCCTTTCGTTAAGTTGTATGCCTACATGTACAATAAGTACTTCAAGTT
GTGTAAGAACTGCTTGTTAGCTGTTCATCCATTCACAAATTGTCCTTCCACCTGTATCTGCG
GTATGATCTATACTACAACCGAAAGTTTGAAGTTGCACAGAATGTGTAACAACTGCTCAGGT
TACAAGGCATTACCAAAGACCAGATAA

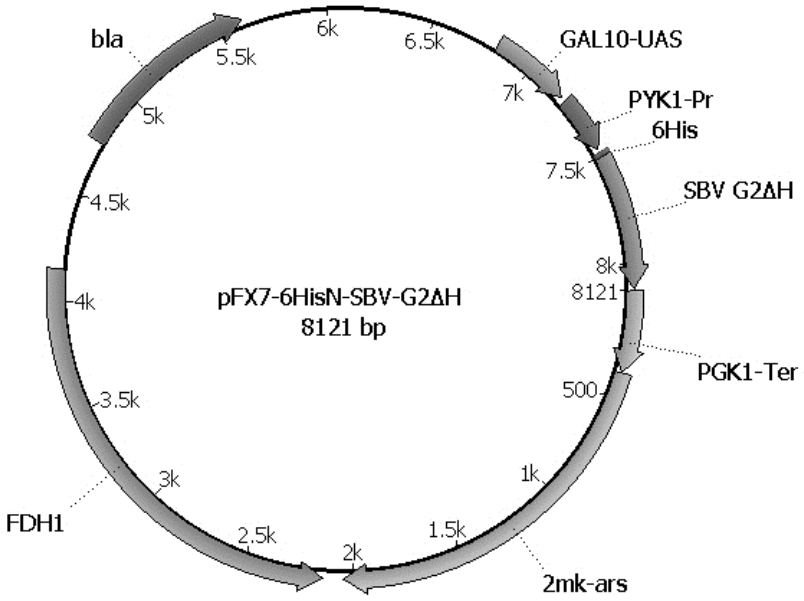
B

MHHHHHHSSTMLLNIVLISNLACLAFALPLKEGTRGSRCFLNGELVKTVNTSKVVSECCVKD
DISIIKSNAEHYKSGDRLAAVIKYYRLYQVKDWHSCNPIYDDHGSFMILDIDNTGTLIPKMH
TCRVECEIALNKDTGEVILNSYRINHYRISGTMHVSGWFKNKIEIPLENTCESIEVTCGLKT
LNFHACFHTHKSCTRYFKGSILPELM

Figure 3.5. Schmallenberg virus G2 gene sequence (A) and G2ΔH protein amino acid sequence (B). (A) Schmallenberg virus G2 gene sequence (GenBank: CCF55030.1) optimized for *S. cerevisiae* codon usage bias. 6His coding sequence is marked italic. Underlined regions mark primer-binding sites to create SBV G2ΔH gene variant. (B) 6-His sequence is located at the N-terminus of the protein.

Both gene variants were inserted into pFX7-6HisN vectors by ligating sticky DNA ends created by *XbaI* and *BcuI* restriction enzymes. The resulting plasmids pFX7-6HisN-SBV-G2 and pFX7-6HisN-SBV-G2ΔH (Figure 3.6) were constructed. The plasmids were amplified in *E. coli* and used to transform *S. cerevisiae*. The yeast cells were grown in YPD medium and recombinant protein synthesis was induced by adding galactose into the yeast growth medium. SDS-PAGE analysis revealed an increased production of a protein that was approximately 25 kDa in weight. This is similar to theoretical weight of 24 kDa of SBV-G2ΔH protein. A small sample of protein was purified using Ni-NTA resin (Figure 3.6, B). The synthesis of SBV G2 protein in yeast was not observed.

A



B

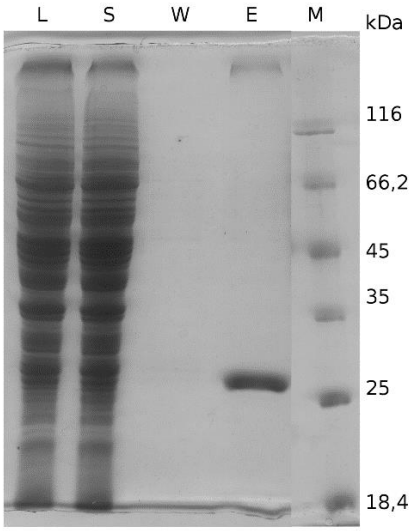


Figure 3.6. pFX7-6HisN-SBV-G2ΔH plasmid chart (A) and SDS-PAGE analysis of SBV-G2ΔH protein (B). (A) 2mk-ars – 1.74 kb fragment of yeast 2 µm plasmid; FDH1 – *FDH1* gene, conferring resistance to formaldehyde; bla – beta-lactamase gene, grants resistance to *E. coli* against ampicillin; GAL10-UAS/PYK1-Pr – yeast hybrid galactose inducible gene promoter; PGK1-Ter – fragment of *S. cerevisiae PGK1* gene transcription terminator sequence. SBV G2ΔH – Schmallenberg virus G2 hydrophilic protein coding sequence; 6His – 6 histidine amino acids coding sequence. (B) In lanes: L – lysate of SBV-G2ΔH producing yeast, S – supernatant of SBV-G2ΔH producing yeast, W – sample collected after Ni-NTA resin washing, E – sample collected after Ni-NTA resin elution, M – molecular protein mass marker.

However, western-blot analysis did not show a reaction with the purified protein using mouse anti-6His HRP antibodies (data not shown). Batch purification of the protein resulted in almost undetectable levels of the protein.

DNA that codes fused nucleocapsid and G2 proteins was constructed in order to increase the chances of obtaining the G2 protein. The chimeric proteins were designed to consist of SBV N protein and a full length SBV G2 or of SBV N protein and a shorter version of G2 protein – the G2ΔH protein (Figure 3.4, B and D). The N and G2 proteins were linked using SGGSGGSST amino acids (Figure 3.7), which were coded by our designed primers SchN_3L (see Materials and Methods).

A nucleocapsid-coding gene was amplified using SBV N_5 and SBV N_3L primers. The amplified DNA was ligated with pFD3 or pFX7-6HisN plasmids. The resulting plasmids were then hydrolysed with the *XmaJI* restriction enzyme to allow insertion of G2 coding sequence. SBV-G2 and SBV-G2ΔH DNA was amplified using primers SchOG2_5P and SchOG2_3S or SchOG2S_5t and SchOG2S_3ts and sticky ends were created by hydrolysing the DNA with *BcuI* nucleases.

A

ATGTCAAGCCAATTCATTTTTGAAGATGTACCACAACGGAATGCAGCTACATTTAACCCGGA
GGTCGGGTATGTGGCATTTATTGGTAAGTATGGGCAACAACTCAACTTCGGTGTTGCTAGAG
TCTTCTTCCTCAACCAGAAGAAGGCCAAGATGGTCCTACATAAGACGGCACAACCAAGTGTC
GATCTTACTTTTGGTGGGGTCAAATTTACAGTGGTTAATAACCATTTTCCCCAATATGTCTC
AAATCCTGTGCCAGACAATGCCATTACACTTCACAGGATGTCAGGATATCTAGCACGTTGGA
TTGCTGATACATGCAAGGCTAGTGTCCTCAAACTAGCTGAAGCTAGTGCTCAGATTGTCATG
CCCCTTGCTGAGGTTAAGGGATGCACCTGGGCCGATGGTTATACAATGTATCTTGGATTTGC
ACCTGGGGCCGAAATGTTCCTTGATGCTTTTGACTTCTATCCACTAGTTATTGAAATGCATA
GGGTCCTCAAGGACAATATGGATGTAAATTTTATGAAAAAAGTCCTCCGCCAACGCTATGGA
ACAATGACTGCTGAAGAATGGATGACTCAGAAAATAACAGAAATAAAAGCTGCTTTTAATTC
TGTTGGACAGCTTGCCTGGGCCAAATCTGGATTCTCTCCTGCTGCTAGAACCTTCTTGCAGC
AATTCGGTATCAACATC**tctggtggatctggaggttctagtaca**ATGCTTCTAAATATTGTT
TTGATATCAAACTTAGCATGTTTAGCTTTTGCACTCCCTCTTAAGGAAGGCACTAGAGGATC
GAGGTGCTTCCTAAATGGTGAGTTGGTCAAAACGGTTAACACATCAAAGGTCGTTTCAGAGT
GCTGTGTTAAAGACGACATATCTATCATTAAATCAAATGCTGAACATTATAAATCTGGTGAT
AGATTGGCTGCTGTTATTAAATATTATAGATTGTATCAAGTAAAAGATTGGCATTCTTGTAA
TCCAATTTATGATGATCATGGTTCTTTTATGATTTTGGATATTGATAATACTGGTACTTTGA
TTCCAAAAATGCATACTTGTAGAGTTGAATGTGAAATTGCTTTGAATAAAGATACTGGTGAA
GTTATTTTGAATTCTTATAGAATTAATCATTATAGAATTTCTGGTACTATGCATGTTTCTGG
TTGGTTTAAAAATAAAATTGAAATTCCATTGGAAAATACTTGTGAATCTATTGAAGTTACTT
GTGGTTTGAAAACTTTGAATTTTCATGCTTGTTTTCATACTCATAAATCTTGTACTAGATAT
TTTAAAGGTTCTATTTTGCCAGAATTGATGATTGAATCTTTTTGTACTAATTTGGAATTGAT
TTTGTTGGTTACTTTTATTTTGGTTGGTTCTGTTATGATGATGATTTTGACTAAAACTTATA
TTGTTTATGTTTTTATTCCAATTTTTTATCCATTTGTTAAATTGTATGCTTATATGTATAAT
AAATATTTTAAATTGTGTAAAAATTGTTTGTTGGCTGTTCATCCATTTACTAATTGTCCATC
TACTTGTATTTGTGGTATGATTTATACTACTACTGAATCTTTGAAATTGCATAGAATGTGTA
ATAATTGTTCTGGTTATAAAGCTTTGCCAAAAACTAGA*CATCATCATCATCATCATCAT*TAA

B

MSSQFIFEDVPQRNAATFNPEVGYVAFIGKYGQQLNFGVARVFFLNQKKAKMVLHKTAQPSV
DLTFGGVKFTVVNNHFPQYVSNPVPDNAITLHRMSGYLARWIADTCKASVLKLAEASAQIVM
PLAEVKGCTWADGYTMYLGFAPGAEMFLDAFDFYPLVIEMHRVLKDNMDVNFMKKVLRQRYG
TMTAEEWMTQKITEIKAAFNSVGQLAWAKSGFSPAARTFLQQFGINI**sggsggsst**MLLNIV
LISNLACLAFALPLKEGTRGSRCFLNGELVKTVNTSKVVSECCVKDDISIIKSNAEHYKSGD
RLAAVIKYYRLYQVKDWHSCNPIYDDHGSFMILDIDNTGTLIPKMHTCRVECEIALNKDTGE
VILNSYRINHYRISGTMHVSGWFKNKIEIPLENTCESIEVTCGLKTLNFHACFHTHKSCTRY
FKGSILPELMIESFCTNLELILLVTFILVGSVMMMILTKTYIVYVFIPIFYPFVKLYAYMYN
KYFKLCKNCLLAVHPFTNCPSTCICGMIYTTTESLKLHRMCNNCSGYKALPKTRHHHHHH

Figure 3.7. DNA (A) and amino acid (B) sequences of chimeric SBV N_G2 protein. Linker sequence between peptides N and G2 is marked in lowercase bold font.

The amplified G2 gene variants were inserted into previously constructed pFD3-SBV N or pFX7-6HisN-SBV N plasmids. This resulted in plasmids containing chimeric SBV-N_G2 and SBV-N_G2ΔH coding sequences with 6His tags at N or C terminus (Figure 3.8). All plasmids were used to transform yeast.

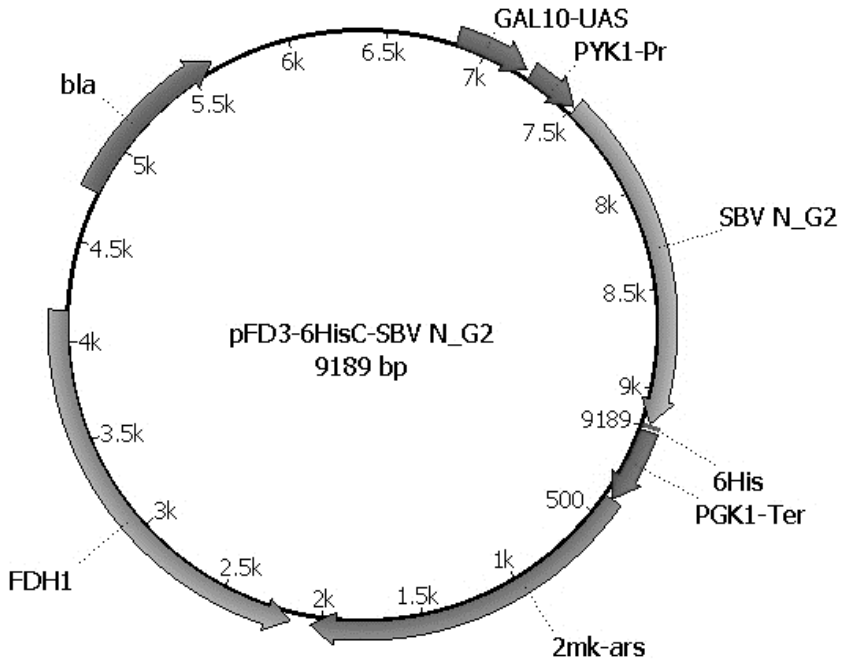


Figure 3.8. pFD3-6HisC-SBV N_G2 plasmid chart. 2mk-ars – fragment of yeast 2 μm plasmid; FDH1 – *FDH1* gene of *Candida maltose,* conferring resistance to formaldehyde; bla – beta-lactamase gene, grants resistance to *Escherichia coli* against ampicillin; GAL10-UAS/PYK1-Pr – yeast hybrid galactose inducible gene promoter; PGK1-Ter – terminator sequence. SBV N-G2 – Schmallenberg virus chimeric N-G2 protein coding sequence; 6His – 6 histidine amino acids coding sequence.

The expression of recombinant DNA was induced by adding sucrose to the yeast growth medium. The SBV N_G2ΔH-NHis protein was successfully purified using Ni-NTA under denaturing conditions with a yield of 0.15 mg from 1 g of yeast. The SBV N_G2ΔH-CHis and SBV N_G2-NHis proteins were not visible in SDS-PAGE gels (data not shown). The protein SBV N_G2-CHis with a full length G2 peptide was also purified using Ni-chelate chromatography (Figure 3.9). The yield of purified SBV N_G2-CHis protein after dialysis was 0.3 mg from 1 g of wet yeast as determined by SDS-PAGE analysis (Figure 3.9, B) and Bradford assay. The purification of chimeric protein under native conditions was not performed as this protein was mostly found in the precipitate fraction of cell lysates (Figure 3.9, A).

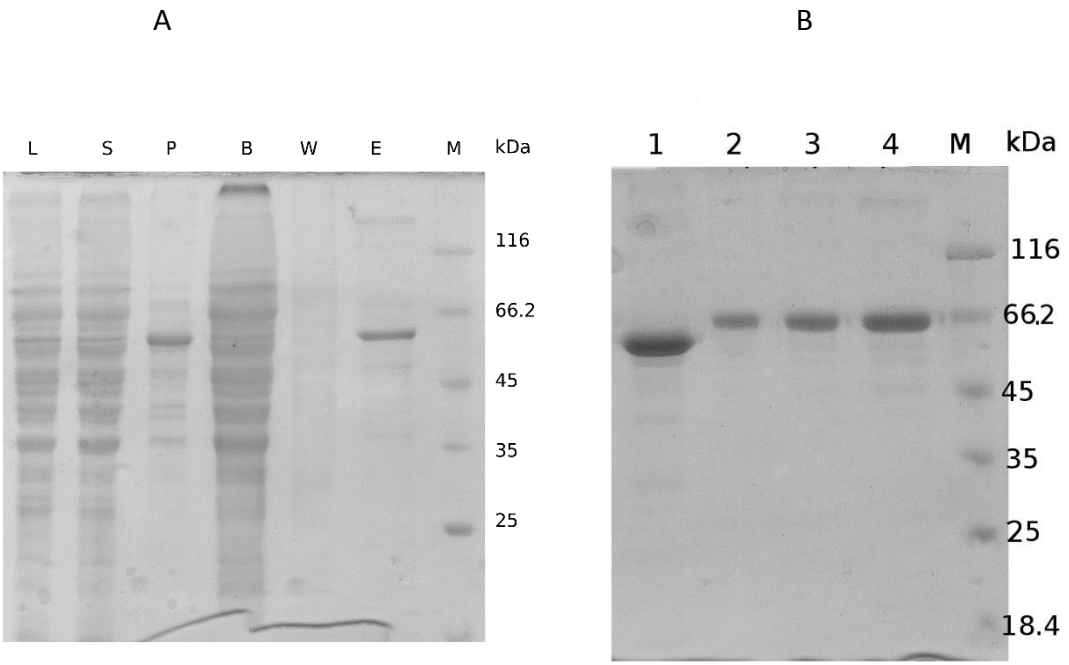


Figure 3.9. SDS-PAGE analysis of SBV N_G2-CHis proteins. (A) In lanes: L – lysate of SBV N_G2-CHis producing yeast, S – supernatant of SBV N_G2-CHis producing yeast cell lysate, P – precipitate of cell lysates, B – sample collected after Ni-NTA resin incubation with cell lysates, W – sample collected after Ni-NTA resin washing, E – sample collected after Ni-NTA resin elution, M – molecular protein mass marker. (B) Lane 1 – 8 μg of purified SBV N_G2-CHis protein. Lanes 2, 3 and 4 – 1 μg, 2 μg and 4 μg of BSA protein, respectively.

3.3. Antigenicity analysis of recombinant SBV N protein using monoclonal antibodies

Monoclonal antibodies (mAbs) against the SBV N protein can serve neutralization of SBV infections or help improve the detection of SBV or SBV-related virus antibodies in samples.

Purified SBV N-His protein was used to make monoclonal antibodies in mice. The immunization, purification and evaluation of mAbs were performed by the scientists of the Department of Immunology and Cell Biology, Institute of Biotechnology, Vilnius University. They were able to obtain four stable hybridoma cell lines producing IgG antibodies: 4F3, 7B6, 8G10 and 11C10. These mAbs were sent to Friedrich-Loeffler Institut (FLI) in Germany for further evaluation.

The immunofluorescence test was performed using SBV-infected BHK cells, clone BRS5 (L194, Collection of Cell Lines in Veterinary Medicine, Greifswald-Insel Riems) as antigen matrix in 96-well plates. A cell suspension was seeded, incubated for 24 h at 37°C, and subsequently infected with SBV strain BH80/11. Forty-eight hours after infection the medium was removed and the cells were fixed using heat treatment (2 h at 80°C). Both, infected and uninfected cells were incubated with each mAb (1:10 diluted hybridoma supernatants) for 1 h at RT. After washing with Tris-buffered saline with 0.1% Tween-20 (TBST), a fluorescein isothiocyanate (FITC-) conjugated goat antimouse IgG (Sigma-Aldrich Co.) was added and incubated for 1 h at RT. Thereafter, the cells were washed, embedded with Dabco fluorescence conservation buffer (Sigma-Aldrich Co.), and analyzed using an inverted fluorescence microscope (Nikon Eclipse Ti-U, Nikon Instruments Inc., Melville, NY, USA).

All mAbs were reactive with SBV-infected BHK cells. No immunoreactivity of the mAbs with uninfected BHK cells used as a negative control was observed (Figure 3.10).

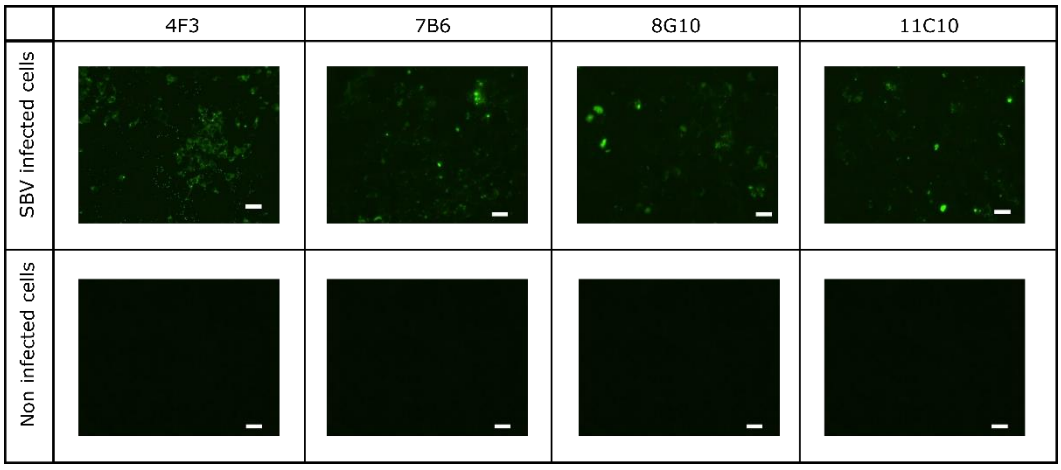


Figure 3.10. Fluorescence microphotographs showing the reactivity of the mAbs with BHK cells infected with SBV BH80/11 strain (upper panel). Non-infected BHK cells were used as a negative control (lower panel). Hybridoma culture supernatants were used at a dilution 1/10. MAbs codes are indicated on the top of each picture. Scale bar 100 µm. This experiment was performed by the researchers of FLI. Photography courtesy of FLI.

Western Blot analysis using 4F3 cell line monoclonal antibodies revealed that mAbs against recombinant SBV N-His protein can also recognize recombinant SBV N protein (Figure 3.11).

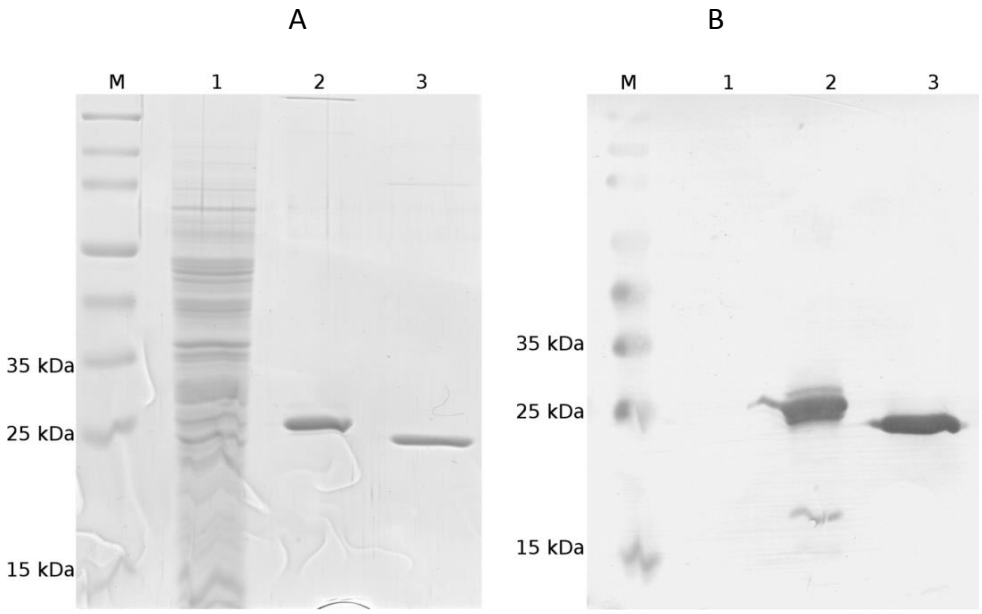


Figure 3.11. Analysis of purified SBV N-His and SBV N proteins by SDS-PAGE (A) and Western Blot (B). In lanes: M – Spectra Broad Range Prestained Protein Ladder, 1 – lysate of *S. cerevisiae* yeast transformed with mock pFD3 plasmid, 2 – SBV N-His protein after nickel-affinity chromatography purification, 3 – SBV N protein after ultracentrifugation in sucrose solution. Western blotting was performed using mouse 4F3 monoclonal anti-SBV N antibodies and anti-mouse IgG HRP antibodies.

3.4. Development of ELISAs that detect anti-SBV antibodies

An indirect ELISA model was chosen for the detection of SBV-specific antibodies in bovine sera, saliva and milk. The purified antigens were tested against SBV positive milk or serum samples. Positive serum samples were obtained from the Friedrich-Loeffler Institut, while positive milk samples were determined by testing them with commercial SBV indirect IgG assay. Negative milk and serum samples were determined by testing them with commercial SBV indirect IgG assays.

The reactivity of SBV-positive bovine sera was tested with yeast-derived recombinant SBV N, SBV N-His and control Andes N proteins. Positive serum samples revealed higher reactivity with the SBV N protein compared to the SBV N-His protein. The control antigen Andes N protein showed very low levels of reactivity (Figure 3.12).

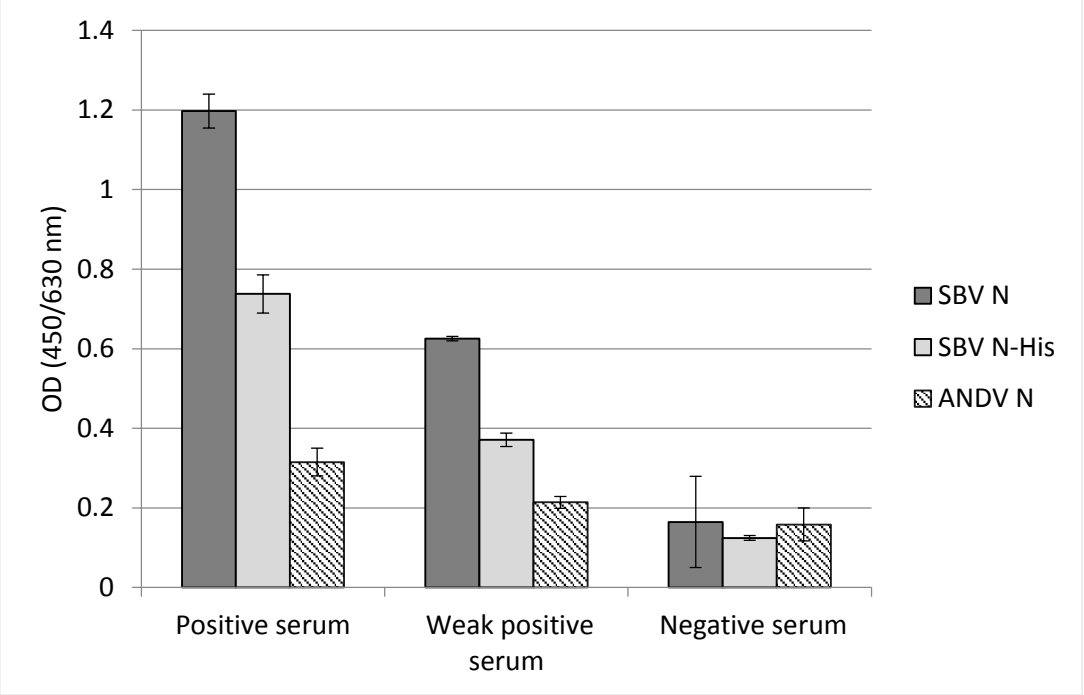


Figure 3.12. Comparison of reactivity of SBV N proteins with bovine sera. SBV N-His – Schmallenberg virus nucleocapsid protein with 6His-tag purified under denaturing conditions. SBV N – Schmallenberg virus nucleocapsid protein purified under native conditions. ANDV N – Andes virus nucleocapsid protein used as a control. Sera were diluted 1/200 in wash buffer. Error bars represent standard error of mean.

Similarly, the milk samples from SBV-positive cows, as determined by commercial serum and milk IgG tests, reacted with SBV N protein stronger than with SBV N-His protein (Figure 3.13).

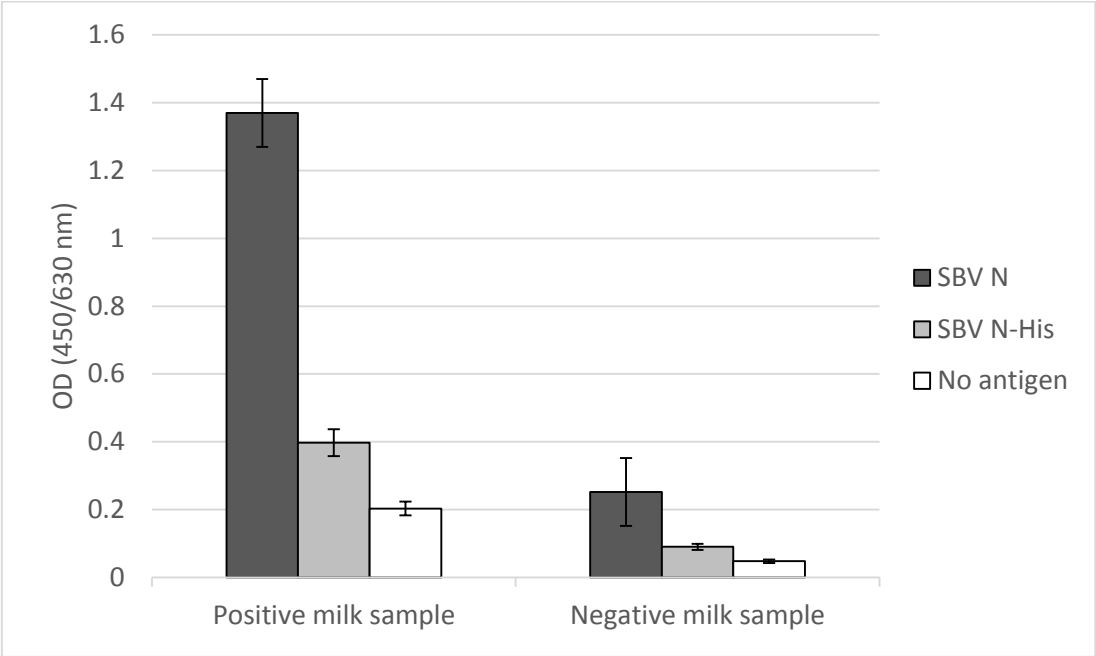


Figure 3.13. Comparison of reactivity of SBV N proteins with bovine milk. SBV N-His – Schmallenberg virus nucleocapsid protein with 6His-tag purified under denaturing conditions. SBV N – Schmallenberg virus nucleocapsid protein purified under native conditions. Error bars represent standard error of mean.

An attempt was made to utilize a purified SBV N_G2-CHis protein as an antigen in SBV ELISA. However, the protein failed to discriminate between positive and negative bovine serum samples and was discarded from further experiments. In contrast, SBV N and SBV N-His proteins showed high reactivity with SBV positive serum sample (Figure 3.14).

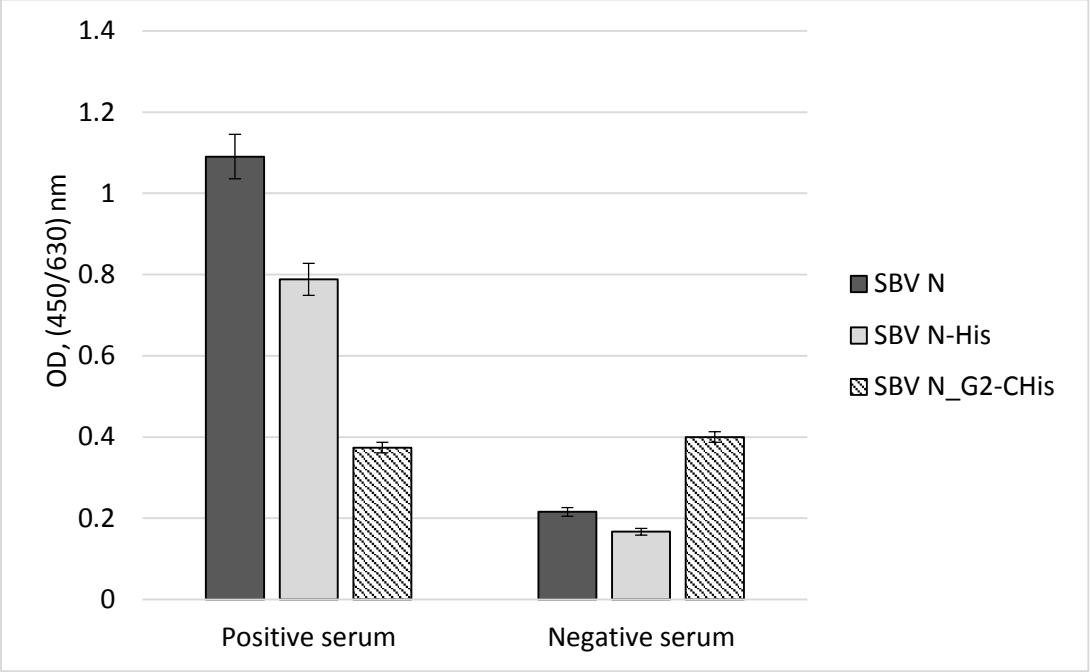
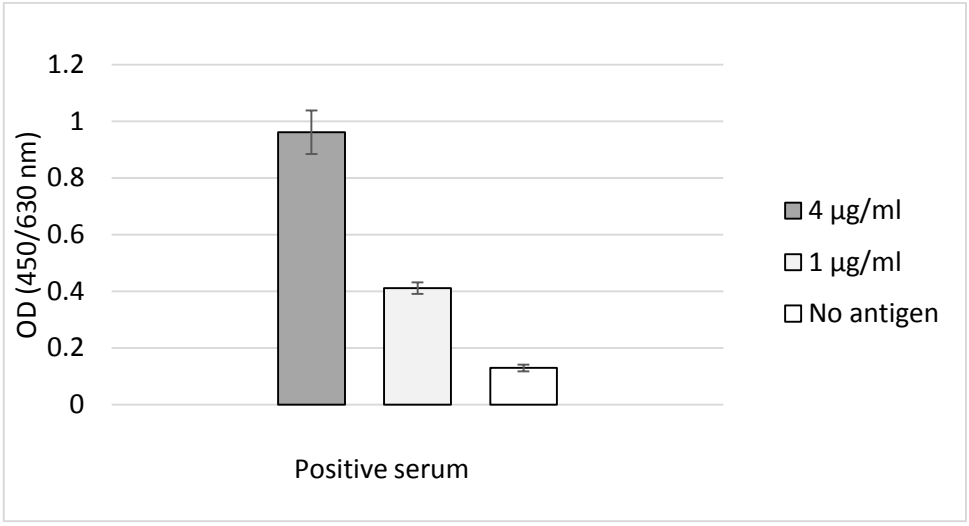


Figure 3.14. Comparison of reactivity of SBV proteins with bovine sera. SBV N – Schmallenberg virus nucleocapsid protein purified under native conditions. SBV N-His – Schmallenberg virus nucleocapsid protein with 6His-tag purified under denaturing conditions. SBV N_G2-CHis – chimeric SBV N and G2 protein with 6His tag purified under denaturing conditions. Sera were diluted 1/200 in wash buffer. Error bars represent standard error of mean.

As recombinant SBV N protein showed high reactivity with SBV-positive milk and serum samples it was decided to use it in developing SBV milk, serum and saliva antibody detection systems. SBV N-His was chosen to be used only in SBV serum antibody detection system.

The conditions of various parameters had to be evaluated in order to create SBV serum antibody detection system. Bovine serum analysis revealed an optimal 4 μg/ml SBV N-His antigen concentration (Figure 3.15, A) and 1/200 serum dilution with the buffer solution (Figure 3.15, B) for the SBV N-His based ELISA.

A



B

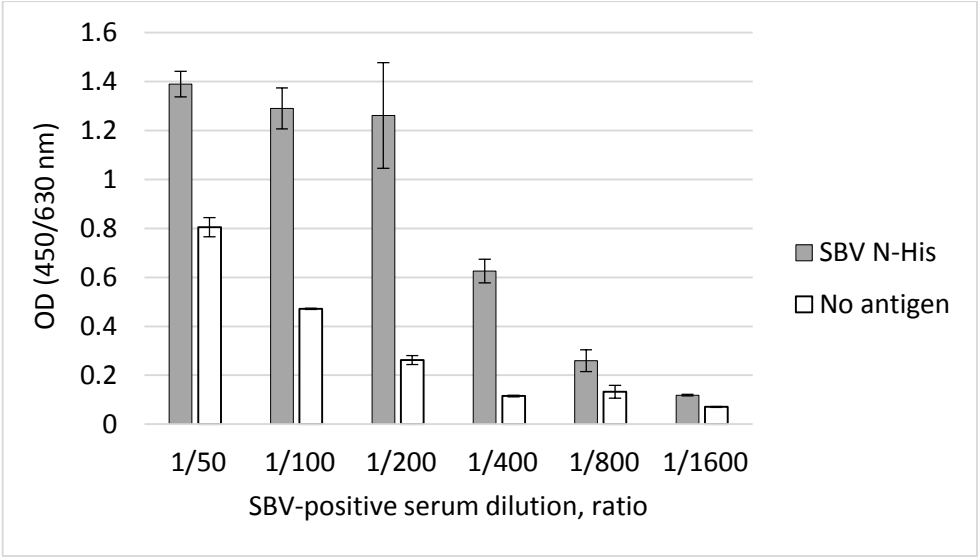


Figure 3.15. Influence of SBV N-His antigen concnetration (A) and SBV-positive serum dilutions (B) on the optical density in SBV IgG ELISA. 2 µg/ml (A) or 4 µg/ml (A and B) of SBV N-His protein was used as an antigen. Serum samples were diluted 1/200 (A). Secondary mouse anti-bovine IgG antibodies diluted 1/10,000 in wash buffer were used. Error bars represent standard error of mean.

A 1/100 serum dilution resulted in the same average signal strength as 1/200 serum dilution (OD 1.28 compared to 1.26), however the background average signal value was increased by 1.9 fold (OD 0.26 compared to 0.47) (Figure 3.15). The positive average signal was 4.8 times stronger than the

background average signal using the 1/200 serum dilution, the positive average signal was 2.5 times stronger than the background average signal while using 1/100 serum dilution. Also, 1/200 dilution allowed economic use of serum samples.

A positive signal threshold (cut-off) was determined for indirect SBV N-His assay by using a set of 11 SBV-negative serum samples. A serum sample was considered positive in indirect SBV N-His assay when its S/P value was greater than 32. An S/P cut-off value was determined for the system as described in the Methods section (2.2.14.).

SBV N protein without His-tag was used as an antigen while developing other indirect ELISAs. The optimal antigen concentrations and sample dilution ratios were determined by checkerboard titrations. Titrations revealed the optimal protein concentration of 2 µg/ml for serum IgG (Figure 3.16), 2 µg/ml for milk IgG and saliva IgA and 4 µg/ml for saliva IgG assay (Figure 3.18).

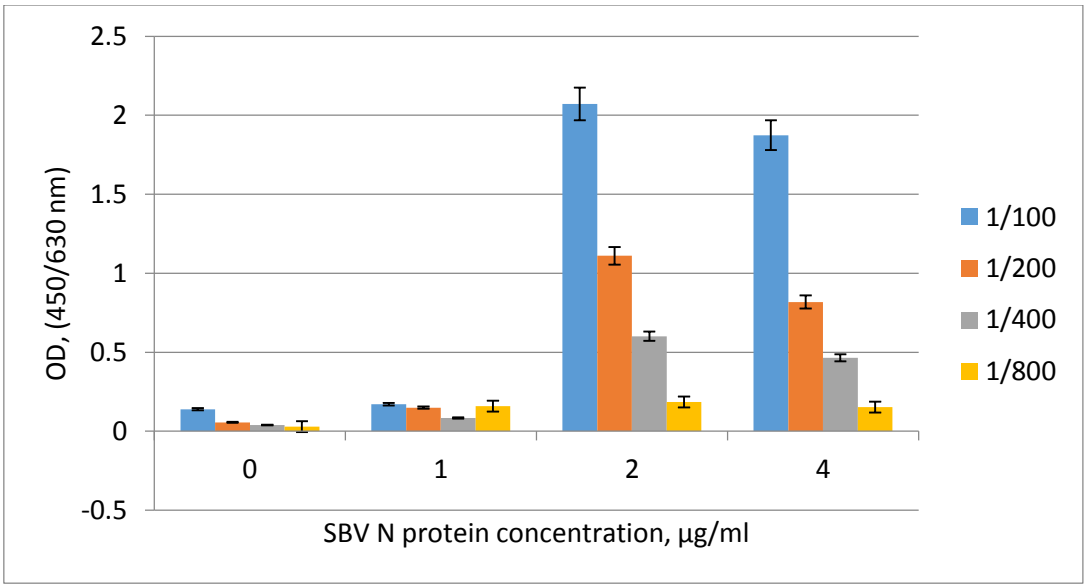


Figure 3.16. Dependence of an optical density on the antigen concentration and serum dilution in an indirect ELISA. SBV N concentration is displayed on the X axis, optical density units, as measured at 450 nm wavelength, are displayed on the Y axis. Blue, orange, grey and yellow bars represent serum dilutions with PBS buffer 100, 200, 400 and 800 times accordingly. Sigma anti-bovine IgG HRP antibodies diluted 1/10,000 in PBS were used. Error bars represent standard error of mean.

The optimal serum sample dilution was determined to be 1/200 in wash buffer. The best milk and saliva sample dilutions were 1/10 and 1/3, accordingly. These sample dilutions gave a good discrimination between positive and negative reactions and were more economical in the use of the sample (Figure 3.16, Figure 3.17 and Figure 3.18). Nerbe plus plates were used for serum IgG, milk IgG and saliva IgA assays, but for the saliva IgG assay the Nunc Maxisorp plate was chosen in order to increase the sensitivity of the assay.

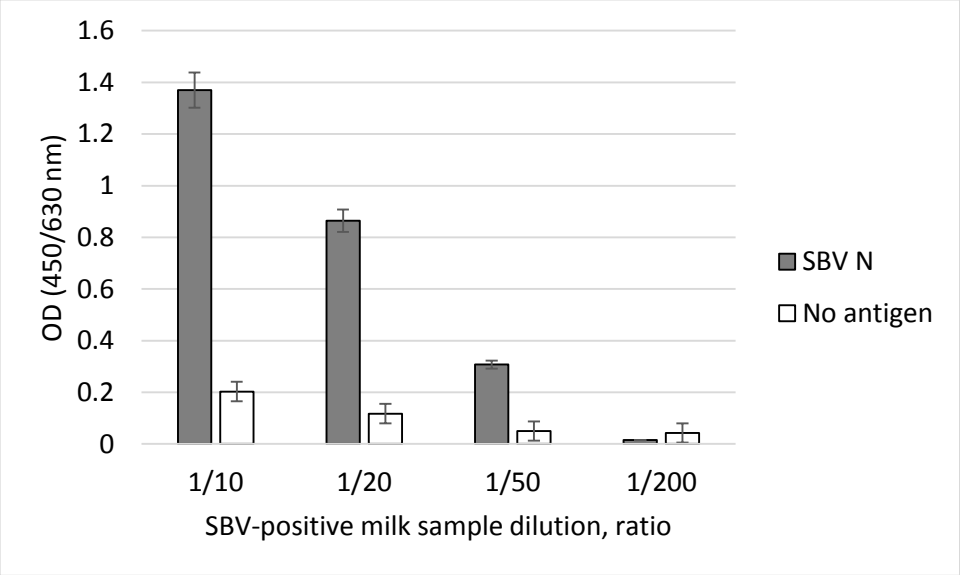
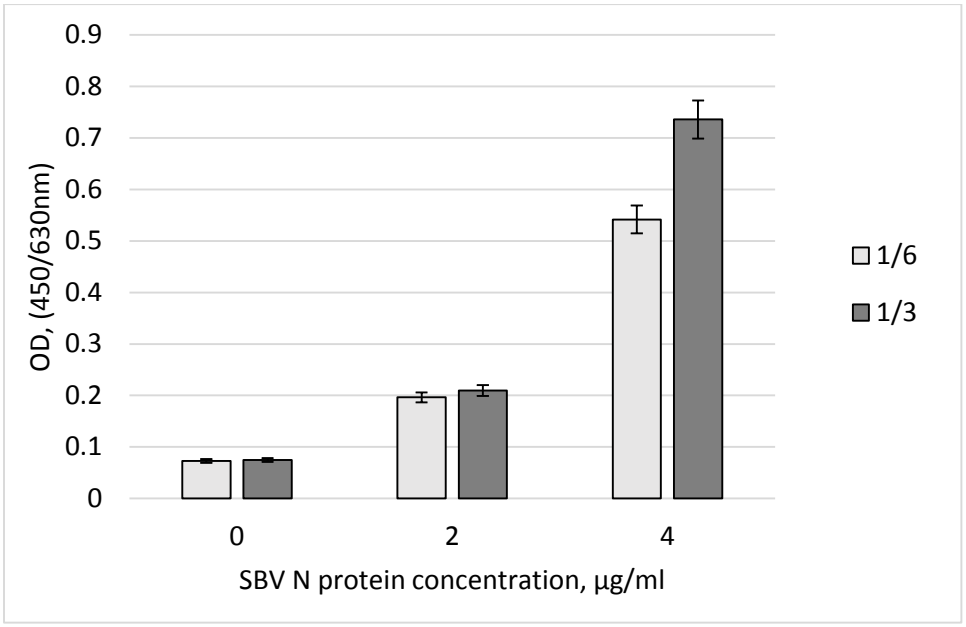


Figure 3.17. Influence of SBV-positive milk sample dilution on optical density in indirect SBV N milk IgG assay. Milk sample dilution ratio is displayed on the X axis. Rabbit anti-bovine IgG antibodies conjugated to HRP, diluted 1:20,000 (v/v) in PBST were used. Error bars represent standard error of mean.

Maximum reactivity was achieved in saliva IgG assay when Nunc Maxisorp plates were used for antigen coating. Nerbe plus plates were not sufficient to reliably detect specific IgG in saliva with low antibody titers, therefore Nunc Maxisorp plate was used to compensate for low IgG levels in saliva (Figure 3.19).

A



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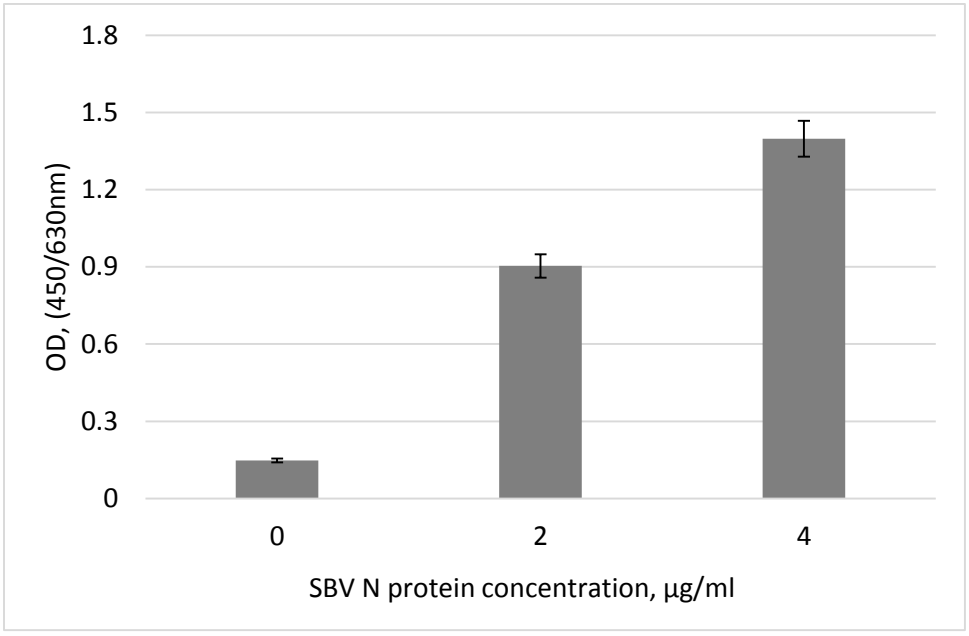


Figure 3.18. Influence of antigen concentration on reactivities of saliva samples in saliva IgG (A) and IgA (B) assays. Secondary bovine anti-IgG HRP antibodies were diluted in 1/30,000 ratio (A), while secondary bovine anti-IgA HRP antibodies were diluted in 1/20,000 ratio (B). Saliva samples were diluted in 1/6 or 1/3 (A) ratio. The plates were coated with the SBV N antigen at concentration of 2 µg/ml or 4 µg/ml. Error bars represent standard error of mean.

A 1/30,000 dilution of anti-bovine IgG HRP conjugate and a 1/20,000 dilution of anti-bovine IgA-HRP conjugate gave the greatest discrimination between reactivity of positive samples with the SBV N antigen and the control antigen – hantavirus Andes N protein. The saliva sample and anti-bovine IgG/IgA conjugates were diluted in PBS with 5 % Roti-block and 1 % chicken serum solution. This buffer solution was found to be more effective in blocking non-specific binding, leading to lower OD values for the negative control and therefore higher sample-to-positive ratios (Figure 3.19).

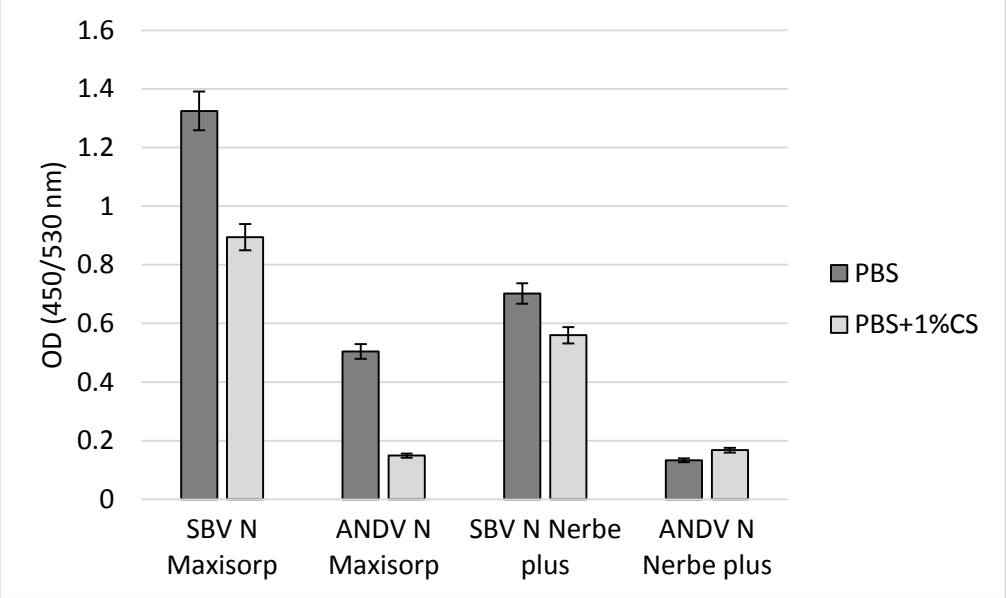


Figure 3.19. Influence of different types of 96-well plates and dilution buffer composition on reactivities of saliva IgG positive samples in SBV saliva IgG assay. Saliva samples and secondary Sigma anti-bovine IgG-HRP antibodies were diluted in PBS (A and B) or PBS+1 %CS (B) – PBS with 5 % Roti-block and 1 % chicken serum solution. Secondary antibodies were diluted in 1/30,000 ratio, saliva samples were diluted in 1/6 (A) or 1/3 (A and B) ratio. Nerbe plus (A and B) or Maxisorp (B) 96-well plates were used. The plates were coated with the SBV N antigen at concentration of 2 µg/ml (A) or 4 µg/ml (A and B). Error bars represent standard error of mean.

The cut-off values for the systems were determined as described in the Methods section (2.2.14). The S/P cut-off values for milk IgG, serum IgG, saliva IgA and saliva IgG were 30 %, 28 %, 15 % and 10.5 %, respectively.

In conclusion, five indirect SBV IgG ELISA systems were developed using SBV nucleocapsid proteins. SBV N-His protein was used to create ELISA that could

detect anti-SBV IgG antibodies in bovine serum, while SBV N protein was used to create ELISAs that could detect anti-SBV IgG antibodies in bovine milk, serum and saliva as well as anti-SBV IgA antibodies in bovine saliva.

3.5. Detection of anti-SBV antibodies in bovine samples

Commercial Schmallenberg ELISA IgG tests were used to evaluate the presence of anti-SBV antibodies in bovine serum and milk. The newly developed ELISAs were evaluated by comparing their results to the results obtained with commercial tests. Newly developed saliva tests were compared to commercial serum ELISA, as there was no commercially available Schmallenberg saliva test.

3.5.1. Detection of anti-SBV IgG antibodies in bovine sera

97 serum samples collected in Lithuanian farms in spring 2013 were tested with commercial IDVet Schmallenberg IgG ELISA kit and in parallel using newly developed indirect SBV IgG SBV N-His ELISA. 82 positive serum samples were detected by the commercial test. 78 of these serum specimens were also described as positive by SBV N-His ELISA, 4 of them were described as negative (Figure 3.20). Therefore, the sensitivity of indirect IgG SBV N-His ELISA was 95 % (78 / 82 x 100) compared to the commercial test. 15 serum specimens were determined as negative by the commercial test. 14 of these serum samples were described as negative and one as positive by the indirect IgG SBV ELISA. Thus, the specificity of the newly developed indirect IgG SBV ELISA was 93 % (14 / 15 x 100). The correlation coefficient between the optical densities of both tests was 0.703061.

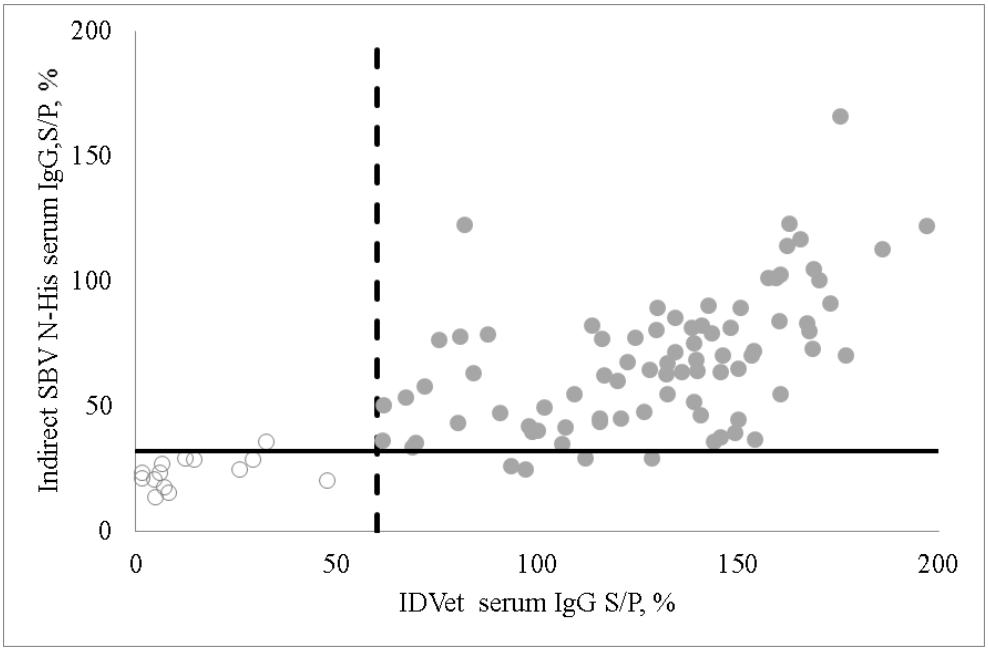


Figure 3.20. Comparison of S/P values of bovine serum samples that were tested with indirect SBV N-His serum IgG ELISA and commercial IgG IDVet serum ELISA. Same bovine serum samples were tested with commercial and in-house SBV IgG serum ELISA. Grey dots represent positive serum samples determined by the commercial test, while negative samples are represented by white dots. The solid horizontal line represents the cut-off value of the newly developed indirect IgG SBV N-His ELISA, the dashed vertical line represents the cut-off value of the commercial test.

58 bovine serum samples were tested with the commercial IDVet SBV ELISA kit and our detection system based on SBV N without His-tag. These serum samples were collected in 2014 in Lithuania and also had their matching milk and saliva samples collected from the same cows. The commercial test revealed that 54 serum samples were positive, one was negative and 3 were doubtful. Doubtful samples were excluded from further testing. 52 of these positive serum samples were described as positive by the newly developed system that based on the SBV N protein without 6HisN-tag. The remaining two positive samples were described as doubtful by the new assay. The negative serum sample was recognized in both tests (Figure 3.21). The sensitivity of the indirect serum IgG SBV assay was 96.30 % (95 % Confidence Interval: 87.25 % – 99.55 %). The correlation coefficient between the optical densities of the same serum samples in indirect IgG SBV ELISA and in commercial ELISA was 0.375436.

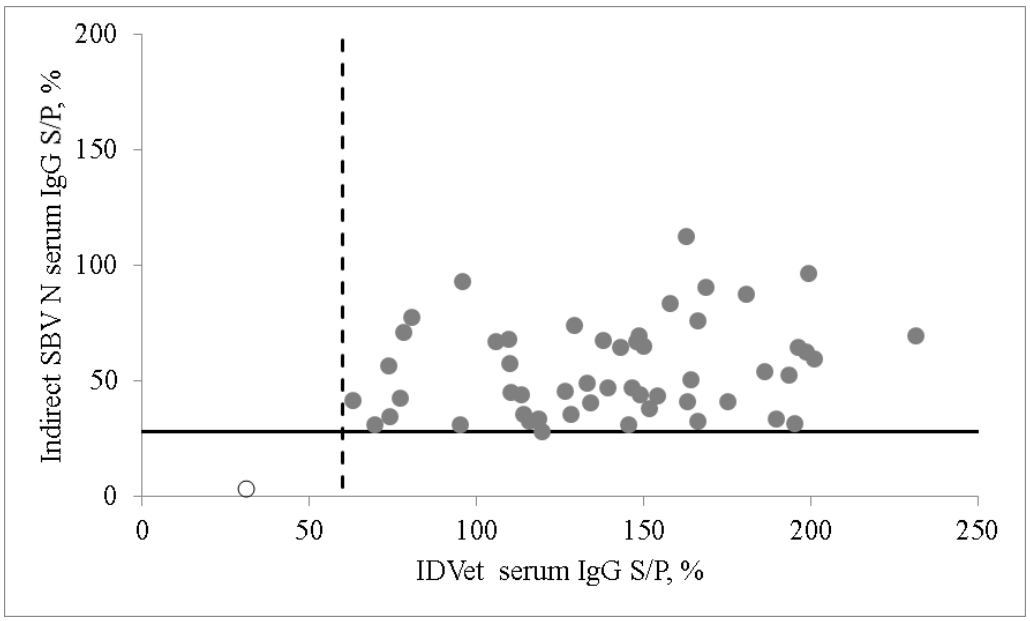


Figure 3.21. Comparison of S/P values of bovine serum samples that were tested with indirect SBV N serum IgG ELISA and commercial IgG IDVet serum ELISA
Same bovine serum samples were tested with commercial and in-house SBV IgG serum ELISA. Positive serum samples determined by the commercial test are represented by grey dots, while negative samples are represented by white dots. The solid horizontal line represents the cut-off value of the in-house indirect IgG SBV N ELISA, the dashed vertical line represents the cut-off value of the commercial test.

A large-scale analysis of bovine sera collected in Lithuanian and Ukrainian farms was performed. Three hundred and twenty-one serum samples were collected from farms in Lithuania in autumn in 2013 by colleagues at The Institute of Microbiology and Virology, Veterinary Academy, Lithuanian University of Health Sciences. The samples were tested using a commercial IDVet SBV IgG ELISA kit following manufacturer's recommendations in order to evaluate the prevalence of SBV in Lithuania. Two hundred and eighty-four samples were described as positive, 28 were negative and 9 were doubtful according to this test (Table 1). This shows that 88.47 % of samples had anti-SBV antibodies.

Table 1. Distribution of SBV infected cows among Lithuanian farms in 2013.

Region	Number of samples			Number of farms
	Positive	Negative	Total	
Kaišiadorys	73	16	89	4
Marijampolė	95	3	98	5
Jonava	27	2	29	2
Tauragė	51	6	57	3
Kėdainiai	19	0	19	1
Prienai	19	1	20	1
Total	284	28	312	16

A set of 490 bovine serum samples was collected in Lithuania in 2014. These samples were tested with newly developed indirect IgG SBV N based ELISA. 82 samples (16.73 %) were shown to be positive, while 398 samples (83.27 %) were shown to be negative (Table 2).

Table 2. Distribution of SBV infected cows among Lithuanian farms in 2014.

Region	Number of samples			Number of farms
	Positive	Negative	Total	
Šakiai	26	64	90	2
Panevėžys	2	38	40	1
Pakruojis	10	70	80	2
Marijampolė	23	57	80	2
Kėdainiai	4	36	40	1
Kalvarijos	2	38	40	1
Jonava	2	38	40	1
Šiauliai	3	37	40	1
Šalčininkai	10	30	40	1
Total	82	408	490	12

Additionally, significant differences were found in age-specific prevalence of antibodies against SBV in cattle ($x^2 = 22.4$ %, df = 3, $p < 0.001$). It was shown that older cows had higher rates of SBV infection than younger cows and calves (Table 3).

Table 3. Distribution of SBV-specific antibodies among different age groups of cows in Lithuanian farms in 2014.

Age	Number of samples		Total	Percent positive
6-12 months	Positive	8	125	6.40
	Negative	117		
12-24 months	Positive	15	119	12.61
	Negative	104		
First-calf heifers	Positive	25	122	20.49
	Negative	97		
Cows	Positive	34	124	27.42
	Negative	90		

A set of 522 serum samples was collected in Ukraine in 2014 by researchers of the Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine. Samples were tested with our developed indirect IgG SBV N based ELISA. Sixty-two cows had antibodies against SBV, while we could not detect these antibodies in the remaining 460 serum samples. The test showed that 11.87 % of cows were seropositive. No infected cows were found in Sumy, Lviv and Dnipropetrovsk regions (Table 4).

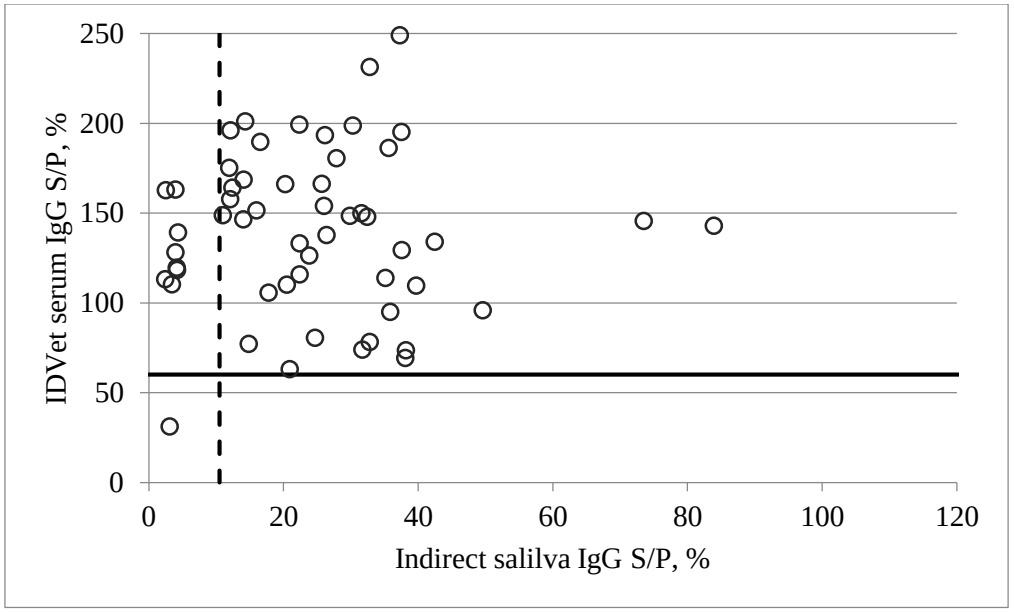
Table 4. Distribution of SBV infected cows among Ukrainian farms in 2014.

Region	Number of samples			Number of farms
	Positive	Negative	Total	
Vinnytsia	2	31	33	3
Zaporozhye	4	6	10	1
Chernihiv	4	37	41	3
Poltava	15	79	94	8
Zhytomyr	5	46	51	4
Kirovohrad	8	21	29	1
Cherkasy	4	74	78	6
Khmelnytsky	1	19	20	2
Sumy	0	39	39	3
Kharkiv	1	29	30	1
Rivne	3	12	15	2
Mykolaiv	7	3	10	1
Ivano-Frankivsk	1	20	21	2
Ternopil	7	24	31	3
Lviv	0	10	10	1
Dniproptrovsk	0	10	10	1
Total	62	460	522	42

3.5.2. Detection of anti-SBV IgG and IgA antibodies in bovine saliva

Fifty-five cows from Lithuania were tested for SBV-specific IgG and IgA antibodies in their saliva samples. These cows had their matching serum samples tested earlier (see 3.5.1). Forty-six individuals were positive for SBV IgG antibodies in saliva. Eight cows with no IgG antibodies in their saliva had SBV IgG antibodies in the sera (Table 5, B). The IgG antibody titer was lower in the saliva than in the serum in all cows tested. The only sample that was negative in the serum assay was also negative in the saliva assay (Figure 3.22, A). The sensitivity and specificity of saliva IgG test was 85.19 % (95 % CI: 72.88 % – 93.38 %) and 100 % (95 % CI: 2.5 % – 100 %), respectively. However, more seronegative samples are required to evaluate specificity reliably. There was no correlation (correlation coefficient r = 0.025967) between the saliva/serum IgG pairs.

A



B

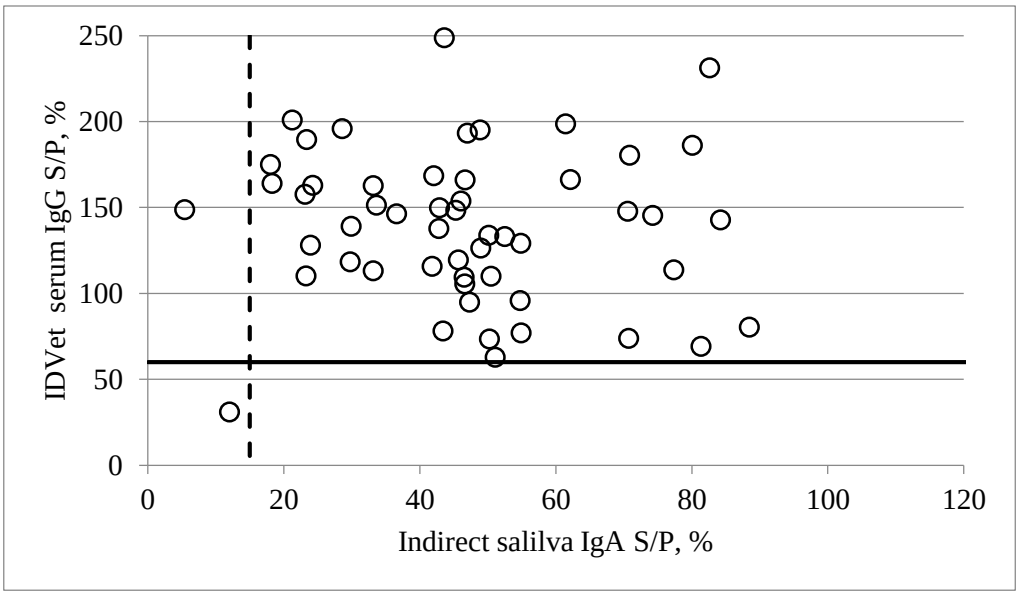


Figure 3.22. Comparison of S/P values of saliva and serum samples that were tested with indirect saliva IgG ELISA (A), indirect saliva IgA ELISA (B) and commercial IgG IDVet serum IgG ELISA (A and B). Bovine saliva samples were tested with in-house indirect IgG (A) and IgA (B) ELISAs, while bovine serum samples form same cows were tested with commercial IgG IDVet ELISA (A and B). The horizontal line represents the cut-off value of the serum IgG IDVet ELISA (A and B), while the vertical line represents the cut-off value of the saliva IgG (A) and IgA (B) test.

Fifty-three cows tested positive in the saliva IgA test compared to fifty-four positive cows in the IDVet IgG test. One cow tested negative in both serum IgG and saliva IgA assays and one saliva sample was described as false-negative (Table 5, C; Figure 3.22, B). The sensitivity of the saliva IgA ELISA to the commercial serum IgG ELISA was 98.15 % (95 % CI: 90.11 % – 99.95 %).

Forty-five saliva samples were positive for the presence of SBV-specific IgG's out of fifty-three saliva IgA positives. Eight saliva samples displayed reactivity below the cut-off value in the IgG assay while showing a positive reaction in an anti-SBV IgA ELISA (Figure 3.23). One saliva IgG positive sample was negative in the saliva IgA assay, while one sample was negative in both assays (Table 5, D). The saliva IgG test was able to detect 84.91 % (95 % CI: 72.41 % – 93.25 %) of samples that were identified as saliva IgA positive. Correlation analysis estimated the relationship between the antibody concentration in saliva IgG and saliva IgA with a correlation coefficient r = 0.665204 (Figure 3.23).

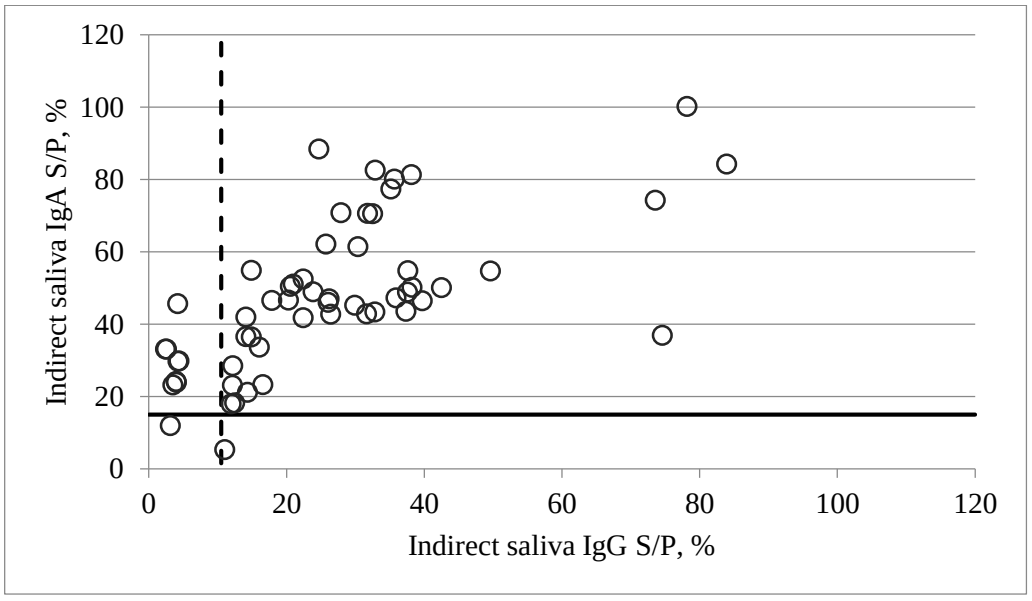


Figure 3.23. Comparison of S/P values of saliva samples that were tested with indirect saliva IgG and IgA ELISAs. Same bovine saliva samples were tested with in-house indirect IgG and IgA ELISAs. The horizontal line represents the cut-off value of saliva IgA test, while the vertical line represents the cut-off value of saliva IgG test.

3.5.3. Detection of anti-SBV IgG antibodies in bovine milk

Milk samples collected form Lithuanian farms in 2014 were tested with a commercial milk IgG ELISA kit and with the newly developed SBV milk IgG ELISA detection system.

Fifty-eight milk samples were screened with the commercial IDVet IgG ELISA test. Fifty-one positive and one negative milk sample was determined by commercial assay. Six milk samples were determined as doubtful and were excluded from further examination. The remaining fifty-two samples were tested with the newly developed indirect milk SBV IgG ELISA test. Forty-eight out of 52 samples were described as positive and one sample was described as negative. Three samples that were determined as positive in the commercial test were doubtful in newly developed test (Figure 3.24; Table 5, A).

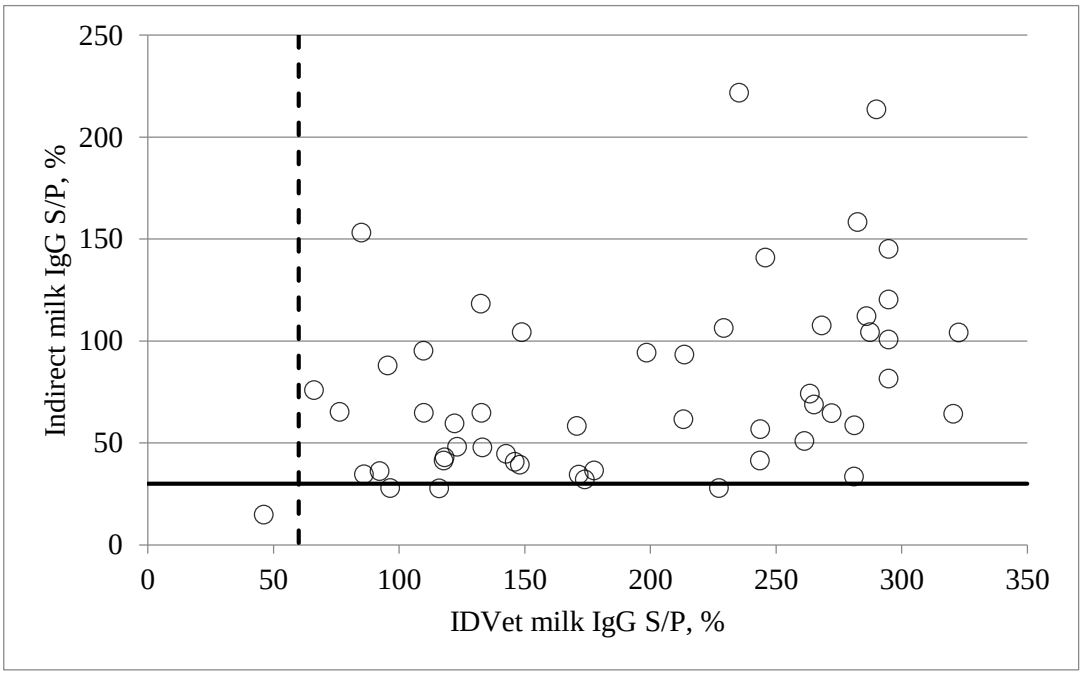


Figure 3.24. Comparison of S/P values of bovine milk samples that tested with SBV milk IgG ELISA and commercial "IDVet" IgG milk ELISA. Same bovine milk samples were tested with in-house indirect IgG and with commercial "IDVet" milk ELISA. The horizontal line represents the cut-off value of the milk IgG test, while the vertical line represents the cut-off value of the commercial milk IgG test.

The sensitivity of milk SBV IgG ELISA was 94,12 % (95 % CI: 83,76 % - 98,77 %). The correlation coefficient between the optical densities of the same milk samples in indirect IgG SBV ELISA and in commercial ELISA was 0,387307.

A relationship was revealed between the presence of SBV-specific antibodies in serum and milk samples. Cows that had SBV infection markers in their blood were more likely to also carry SBV infection markers in their milk (Figure 3.25). A correlation coefficient of r = 0.659339 also indicates that cows with high serum S/P ratio tend to have high milk S/P ratio.

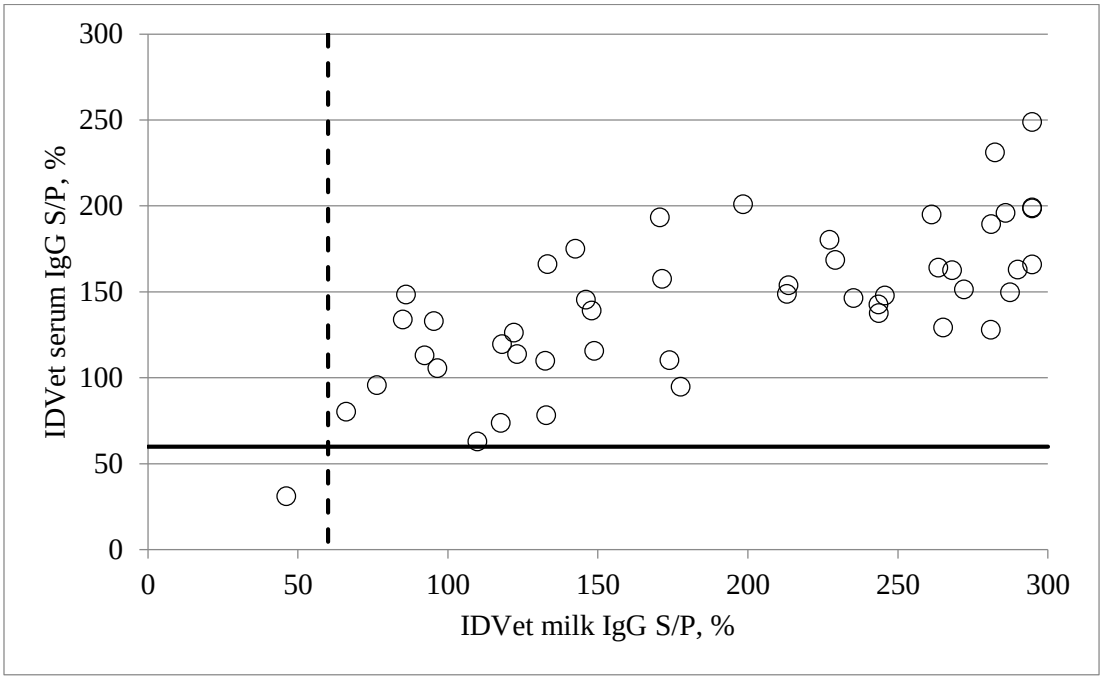


Figure 3.25. Comparison of S/P values of bovine milk and serum samples that were tested with commercial "IDVet" milk and serum IgG ELISAs. Milk and serum samples collected from the same cows were tested using commercial serum or milk assays. The horizontal line represents the cut-off value of the commercial serum IgG test, while the vertical line represents the cut-off value of the commercial milk IgG test.

Table 5. A comparison of bovine serum, milk and saliva samples analysed with the in-house indirect SBV N ELISA tests and the commercial IDVet serum or milk indirect IgG ELISA tests.

(A)				
		„ID Screen® SBV Milk Indirect“		
		Positive	**Negative**	**Total**
Indirect milk IgG ELISA	Positive	48	0	48
	Negative	3	1	4
	Total	51	1	52
(B)				
		„ID Screen® SBV Indirect“		
		Positive	**Negative**	**Total**
Indirect saliva IgG ELISA	Positive	46	0	46
	Negative	8	1	9
	Total	54	1	55
(C)				
		„ID Screen® SBV Indirect“		
		Positive	**Negative**	**Total**
Indirect saliva IgA ELISA	Positive	53	0	53
	Negative	1	1	2
	Total	54	1	55
(D)				
		Indirect saliva IgA ELISA		
		Positive	**Negative**	**Total**
Indirect saliva IgG ELISA	Positive	45	1	46
	Negative	8	1	9
	Total	53	2	55

4. DISCUSSION

Earlier research has shown that recombinant His-tagged hantavirus N proteins purified under denaturing conditions allowed the development of highly specific and sensitive serological assays (Petraityte et al., 2007). A similar yeast vector system was exploited during this work to produce N proteins of SBV virus. The purification yield was 3.0–3.5 mg from 1 g of yeast for the recombinant His-tagged SBV N protein and 1.0 mg from 1 g of yeast for the non-His-tagged SBV N protein. The yields were similar to the yield of hantavirus N proteins (Razanskiene et al., 2004). For comparison, 2.1 mg of mumps nucleocapsid protein was purified from 1 g of *P. pastoris* yeast (Slibinskas et al., 2003), while human polyomavirus VP1 proteins can be purified with a yield of 0.44–1.05 mg/g from *S. cerevisiae* cells (Norkiene et al., 2015). On the other hand, the yield of 0.15 mg of SBV N_G2ΔH-NHis protein and 0.3 mg of SBV N_G2-CHis protein from 1 g of yeast is considered to be low. The synthesis levels of the proteins in yeast could also be influenced by the position of His-tag peptides at N- or C-terminus of the protein. The C-tagged N_G2ΔH protein and the N-tagged N_G2 protein were not seen in yeast cell lysates in SDS-PAGE gels. His-tag could affect the stability of recombinant proteins, as was shown with an ubiquitin-conjugating enzyme E2 from *Agrocybe aegerita.* The C-terminus tagged E2 protein was less stable than the N-tagged one. It was speculated that the tag made the C-terminus of the E2 protein more available to proteases (Li et al., 2013).

It is known that His-tagged hantavirus N proteins are more immunogenic in bank vole than native nucleocapsids (Dargeviciute et al., 2002). Thus, the SBV N-His protein was used to produce monoclonal antibodies against SBV N protein. SBV N-His protein was purified under denaturing conditions and was renatured using acetate buffer. The denaturation-renaturation cycle could have influenced SBV N-His structure due to the lack of chaperone proteins after purification, as SBV

N protein has three cysteine amino acid residues, which can form disulphide bonds.

The immunofluorescence assay data confirmed that the mAbs raised against yeast-expressed SBV N protein recognize native viral nucleocapsids. This data proves antigenic similarity between yeast-expressed N protein and virus-derived N protein. It is hard to tell which epitopes are recognized by the antibodies as the immunotyping of the antibodies was not performed. It is known that anti-SBV mAbs react with SBV N proteins during Western Blot analysis. This could indicate that these mAbs recognize linear epitopes of the protein. Neither SBV N-His nor SBV N proteins seem to form nucleocapsid-like structures, as was shown by electron microscopy experiments. It is therefore likely that mAbs generated during this research recognize epitopes that originate from single protein rather than protein oligomers. However, research by other authors showed that SBV N protein can form tetramers or hexamers *in vitro* (Dong et al., 2013).

The virus neutralization test (VNT) is the "gold" standard in virus serology diagnostics. However, there was no possibility during the preparation of presented work to perform this kind of test. Therefore, it was chosen to compare our developed assay with commercial one, as a commercial test was proven to be 98.9 % agreeable with the VNT (Bréard et al., 2013). The one drawback of commercial ELISA is the fact that it can sometimes show reactivity with antibodies against other Simbu serogroup viruses like Aino, Akabane, Douglas, Peaton and others (Mathew et al., 2015). However, it is worth mentioning that Simbu viruses are mainly widespread in Africa, Asia or Oceania.

One could predict that an in-house ELISA could have potential application in SBV diagnostics if our test would show agreeable results to the commercial test. Indeed, the indirect SBV ELISA serum test results showed comparable agreement with the commercially available test based on an *E. coli*-expressed SBV N protein (Bréard et al., 2013). Yeast-expressed SBV N protein may be a

suitable antigen for serological diagnosis of SBV infections or infections, caused by other Simbu group viruses in cows as the high number of serum samples from SBV-infected cows were found to be positive in the newly developed indirect IgG SBV ELISA. However, not all cows that were determined as positive by commercial test were also defined as positive in newly developed IgG SBV ELISA. Newly developed assay should be further evaluated and optimized using more cow serum specimens collected at variable intervals during the course of the disease. Mansfield and colleagues (Mansfield et al., 2013) have shown that the commercial ELISA test could not recognize all SBV-positive serum samples. They conducted a plaque reduction neutralization test (PRNT) that appeared to be more sensitive than the commercial ELISA. The essential difference between the PRNT assay and the SBV N protein-based ELISA is that the PRNT assay allows detection of antibodies against all structural viral proteins. These results suggest that serologic ELISA tests might be improved by incorporating other viral structural proteins in the test. This was utilized in creating a whole virus antigen based ELISA (Näslund et al., 2014). It was attempted to develop an antibody detection system based on the G2 protein of SBV in this work. However, the SBV N_G2-CHis protein, which had higher production levels in yeast than truncated SBV N_ G2ΔH-NHis, did not have the ability to reliably discriminate between positive and negative samples. SBV N_ G2ΔH-NHis protein was not considered as a candidate antigen due to the low yield. It was decided not to include SBV N_G2 proteins in further testing and rely on the SBV N protein purified under native conditions, which had high reactivity values with positive sera and low reactivity values with negative sera. Furthermore, it was later discovered that mice that were inoculated with SBV infected BHK cells, produced monoclonal antibodies that were specific to N and G1 proteins or G1/G2 complexes (Wernike et al., 2015).

This study showed significant difference in seroprevalence of SBV/Simbu group viruses between different age groups in 2014. Older cows tend to be SBV/Simbu group virus seropositive more often than the younger ones. This may be

explained by the time spent in the fields as young calves are often kept indoors and have less exposure to the vectors that carry SBV/Simbu group viruses. These results are in line with seroprevalence observations in Ethiopia (Sibhat et al., 2018), while no age-related differences were found by other authors (Elbers et al., 2012). However, Elbers and colleagues were investigating the prevalence of SBV in cattle during the first SBV outbreak. It was shown that anti-SBV and other maternal antibodies against bunyaviruses reside in calves for only six months (Elbers et al., 2012; Elbers et al., 2014). This means that part of the young cattle observed after the initial outbreak of the disease will have no antibodies against SBV/Simbu group viruses in their sera. In contrast, our results show a possible outbreak of SBV in Lithuania in 2013 rather than 2014. Eighty-seven percent of tested cows had antibodies against SBV/Simbu group viruses in 2013 and 17 % had antibodies against SBV/Simbu group viruses in 2014. In comparison, 11.87 % of tested Ukrainian cows in 2014 were seropositive. A large proportion of cows carrying SBV antibodies was also observed by other scientists in 2012 in Germany, Belgium, Holland, France. Up to 80 % of tested animals in various regions of these countries were considered seropositive (Garigliany, Bayrou, Kleijnen, Cassart & Desmecht, 2012; Zanella et al., 2015; Wernike, Silaghi, et al., 2014; Elbers et al., 2012). Less infected animals were observed in these countries in later years (Wernike, Conraths, et al., 2014). This effect can in part be attributed to the number of circulating vectors that carry SBV. The number of *Culicoides* biting midges decreased in the Netherlands in 2012 (Elbers et al., 2013). This could mean that the circulation of SBV decreases after initial epidemic and the virus does not cause a lot of new infections at the same farms. This and the tendency of maternal antibodies to degrade rapidly suggest that a few years after the epidemic a lower proportion of infected animals will be observed. There is no data about SBV infection in Lithuania in 2011–2012. It is possible that SBV was carried over to Lithuania and Ukraine only after the initial outbreak was observed in Western Europe, thus a latency of infection in the region is observed.

Antibodies against SBV/Simbu group viruses can be found in bovine serum, milk and saliva after SBV infection. Thus, the use of non-invasive sample extraction methods is recommended when possible, as antibodies against SBV/Simbu group viruses are present in milk and saliva. Milk and saliva sample collection is cheap, non-invasive and animal welfare-friendly. However, milk and saliva samples are not sterile and are subject to bacterial degradation over time. Furthermore, salivary composition is influenced by the method of collection and the degree of stimulation of salivary flow. Stimulation of salivation before sample collection may lower antibody concentration in saliva. The dilution effect of fluids from the salivary glands requires extremely sensitive tests that are able to detect small quantities of the antibody. Saliva contains antibodies in concentrations that are 1,000-fold less than those in blood. Sensitive detection systems are thus needed to reveal the utility of saliva as a diagnostic medium (Parry et al., 1987; McKie et al., 2002).

A commercially available antibody ELISA developed in 2013 (Bréard et al., 2013; Mansfield et al., 2013) was able to detect antibodies against SBV/Simbu group viruses in serum and milk samples collected in Lithuania. This allowed to evaluate the antibody screening assays developed in this work for use on both individual milk and saliva samples. This was done by comparing the results of individual milk and saliva antibody testing to individual milk and serum antibody testing. The relationship between individual serum and milk antibodies for SBV was also established by others (Daly et al., 2015). This work also shows that animals who have antibodies against SBV/Simbu group viruses in their sera usually have corresponding antibodies in their milk samples.

Bovine milk and saliva samples were tested for IgG and IgA antibodies against SBV/Simbu group viruses. As SBV N purified under native conditions showed greatest reactivity with SBV-positive serum and milk samples, it was chosen as an antigen for the new systems. Initial experiments optimized the concentration of the SBV N protein used as a coating antigen, the selection of the micro-test plates, the dilutions of the milk and saliva samples and the dilution of the HRP

conjugated secondary antibody to provide the best discrimination between known positive and negative specimens. Maxisorp plates were used in saliva IgG assay to increase sensitivity, as IgG levels in saliva is low. According to the manufacturer, Maxisorp plates have protein binding capabilities of 600–650 ng/cm^{2,} while Nerbe plus plates have 250 ng/cm^2 protein binding capabilities. Higher antigen concentration on plate can create favourable conditions for antibodies in a sample to bind to the antigen. Also, addition of 5 % of Roti-block and 1 % chicken serum in saliva and secondary antibody dilution buffers reduced the non-specific binding of the antibodies. The proteins of chicken serum in the buffer most likely have reacted with the low-specificity antibodies in a saliva sample, thus lowering the non-specific reactions, as the formed complexes would have been washed away during the washing steps. Roti-block is a protein free polymer-based blocking agent that contains Tween-20 detergent. The mechanism of action of this reagent is not disclosed, however it is possible that additional amount of Tween-20 contributed to decreased non-specific binding of saliva antibodies to the SBV N antigen.

A control antigen had to be used to determine the cut-off values for milk and saliva assays as not enough negative milk or saliva samples were available. A nucleocapsid of an Andes hantavirus was used as a control antigen in our assay. Andes hantavirus belongs to genus *Hantavirus, Hantaviridae* family and it was the closest relative to SBV, whose nucleocapsid protein was readily available at the time in our laboratory. Andes hantavirus has 47 % amino acid sequence identity with SBV N protein. While use of this ANDV N antigen does not inform about the ability of the assays to discriminate between SBV and other Simbu group viruses, it is suitable in determining the cut-off values of the test. Other authors also used different antigens for determination of cut-off values. Starkey and co-authors (Starkey et al., 1995) report the use of glutathione *S*-transferase (GST) tagged proteins in enzyme immunoassays (EIAs). They have used GST as a control antigen to permit the estimation of background OD in EIAs. Lin and co-authors (Lin et al., 2008) used a bovine bocavirus protein as a control antigen

to define the cut-off in ELISAs established to detect human bocavirus specific antibodies. Nucleocapsid proteins of Shamonda or Sathuperi viruses could be considered candidates as a control antigen as they are closely related to SBV. These viruses are considered by some authors to be ancestors of SBV (Yanase et al., 2012), while others try to prove SBV to be an ancestor of Shamonda virus (Goller et al., 2012). However, calculation of the cut-off value for the assay becomes complicated due to high cross reactivity of bovine antibodies against Simbu serogroup viruses.

Few samples were described as doubtful while screening the bovine serum and milk samples with commercial SBV IgG tests, according to the manufacturer's instructions. Three serum samples and six milk samples were excluded from the study for being doubtful. Some authors suggest repeating the tests with longer incubation times to define the antibody status of doubtful samples, or they exclude these samples from their calculations (Hartter et al., 2000; Bouche et al., 1998). The doubtful samples were excluded from further study, as samples taken over a period of time from the same bovine were not available for the study.

The sensitivity of the newly developed milk IgG assay with the commercial test was 94 %. These results suggest that the purified recombinant SBV N protein can be used to detect antibodies against SBV/Simbu group viruses in milk samples in laboratory setting without relying on commercial assays. The sensitivity of 96 % for the serum IgG assay with the commercial serum IgG ELISA was achieved while using the SBV N protein. This is similar to the SBV N-His based serum IgG assay, where the sensitivity of the assay was 95 %. The slight improvement might be due to a differently folded nucleocapsid protein. A low degree of correlation between optical densities of the same serum samples in commercial and our tests was observed and may suggest that different epitopes on the nucleocapsid protein are recognized in these assays.

This work shows for the first time that IgA is a reliable marker in diagnosing past infection of SBV/Simbu group viruses using bovine saliva samples. The detection of IgA antibodies against SBV/Simbu group viruses in saliva was

achieved in 98 % of the cows that were determined as positive by commercial SBV test. High sensitivity shows that saliva IgA assay can be used to identify potential prevalence of SBV/Simbu group viruses in cattle without the need of invasive sample acquisition techniques. However, the saliva IgG assay is less sensitive than the saliva IgA assay, probably because of a lower IgG concentration in saliva (Duncan et al., 1972). There are few studies demonstrating the production of detectable levels of viral-specific antibodies in bovine saliva samples. Most research on cattle saliva as a diagnostic sample has been carried out with regard to foot and mouth disease virus (FMDV) diagnostics to this date (Archetti et al., 1995; Prickett & Zimmerman, 2010). However, saliva IgA testing system is a novelty in SBV research as only milk and serum ELISA is commercially available. The described assay further demonstrates the applicability of saliva samples for the diagnosis of viral infections.

CONCLUSIONS

1. The Schmallenberg virus nucleocapsid and chimeric nucleocapsid-glycoprotein 2 protein can be produced in *Saccharomyces cerevisiae* cells.
2. Monoclonal antibodies against the recombinant Schmallenberg virus nucleocapsid protein recognize SBV-infected cells.
3. Yeast-derived recombinant Schmallenberg virus nucleocapsid proteins are suitable for the detection of bovine antibodies against SBV/Simbu group viruses in serum, milk and saliva.
4. The developed assays for the detection of bovine IgG antibodies against SBV/Simbu group viruses in bovine sera and milk are comparable to commercial SBV detection systems.
5. The prevalence of SBV or SBV-related Simbu serogroup viruses can be determined by using bovine saliva IgA antibodies.
6. SBV or SBV-related Simbu serogroup viruses were present in Lithuanian and Ukrainian cows in the years 2013 and 2014.

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SUMMARY

SANTRUMPŲ SĄRAŠAS

ANDV	Andes hantavirusas
BHK	(angl. *Baby Hamser Kidney*) žiurkėno jauniklio inkstų ląstelės
CI	(angl. *Confidence Interval*) patikimumo intervalas
HRP	(angl. *Horse-Radish Peroxidase*) krienų peoksidazė
IFA	imunofementinė analizė
IgA	A klasės imunoglobulinas
IgG	G klasės imunoglobulinas
JSA	jaučio serumo albuminas
MAk	monokloniniai antikūnai
NDS-PAGE	natrio dodecilsulfato-poliakrilamido gelio elektroforezė
OD	(angl. *optical density*) optinis tankis
PAA	poliakrilamidas
PAk	polikloniniai antikūnai
PBS	(angl. *Phosphate Buffer Saline*) fosfatinis buferis
PMSF	fenilmetilsulfonilfluoridas
RNAzė A	ribonuklеazė A
S/P santykis	(angl. *sample-to-positive*) tiriamo mėginio ir teigiamo pavyzdžio
SBV N	Schmallenberg viruso nukleokapsidės baltymas
SBV	Schmallenberg virusas

ĮVADAS

2011 metais Vokietijoje ir Olandijoje buvo aptikta nauja galvijams kenkianti liga. Jos požymiai yra karščiavimas, viduriavimas, pieno kiekio sumažėjimas. Po metagenominės analizės paaiškėjo, jog ligą sukelia iki šiol nežinomas ortobunyavirusas, pavadintas Schmallenberg virusu (SBV) pagal Šmalenbergo miestelį, kuriame buvo aptiktas (Hoffmann ir kt., 2012). Per trumpą laiką šis virusas paplito didžiojoje dalyje Europos valstybių (Doceul ir kt., 2013). Virusas yra labai pavojingas galvijų vaisiams nėštumo metu. Viruso paveiktam vaisiui gali pasireikšti galvos, liemens ar galūnių deformacijos (Hoffmann ir kt., 2012). Taip pat pastebėta, jog SBV gali daugintis ožkose, avyse ir šunyse (Sailleau ir kt., 2013). SBV viremijos periodas trunka vidutiniškai šešias dienas. Manoma, jog *Cullicoides* kraujasiurbiai vabzdžiai yra pagrindiniai SBV platintojai (De Regge ir kt., 2012).

SBV priklauso *Peribunyaviridae* šeimos virusams, *Orthobunyavirus* genčiai. Bunyavirusų genomas sudarytas iš trijų viengrandės RNR segmentų, kurie koduoja struktūrinius ir nestruktūrinius virusinius baltymus. Gausiausiai virione ir ląstelėse aptinkamas yra nukleokapsidės (N) baltymas. Jo pagrindinė funkcija yra saugoti virusinę RNR nuo degradacijos (Schmaljohn ir kt., 2007).

2013 metais sukurta vakcina, apsauganti nuo SBV infekcijos (Wernike ir kt., 2013). Greitų ir pigūs SBV infekcijos aptikimo metodai galėtų padėti nustatyti, kuriuos galvijus reikia vakcinuoti. Vienas tokių metodų yra imunofermentinė analizė (IFA).

Tyrimo tikslas:

Sukurti rekombinantiniais mielėse susintetintais Schmallenberg viruso struktūriniais baltymais pagrįstą imunofermentinės analizės sistemą ir įvertinti jos galimybes seilėse, piene ir serumuose atpažinti prieš Schmallenberg virusą susidariusius antikūnus.

Tyrimo uždaviniai:

- Ištirti galimybę mielėse *S. cerevisiae* susintetinti SBV nukleokapsidės, glikoproteino 2 ir chimerinio nukleokapsidės-glikoproteino 2 baltymus.
- Įvertinti išgrynintų antigenų savybes reaguoti su galvijų serumo antikūnais, specifiškais SBV.
- Sukurti testus, aptinkančius SBV specifiškus antikūnus galvijų serume, piene ir seilėse.
- Palyginti naujai sukurtas SBV detekcijos sistemas su kitomis SBV detekcijos sistemomis.
- Ištirti Lietuvoje ir Ukrainoje surinktus galvijų serumus dėl galimos SBV infekcijos.

Naujumas ir aktualumas

Schmallenberg virusas yra naujas gyvūnų virusas, per keletą metų paplitęs didžiojoje Europos dalyje. Numanoma šio viruso žala gali būti nuo €23 iki €43 vienai karvei per metus (Waret-Szkuta ir kt., 2017). Dėl spartaus paplitimo ir daromos tiesioginės žalos gyvulininkystei tyrėjams reikalingi greiti ir tikslūs SBV infekcijos aptikimo įrankiai. Tokių komerciškai gaunamų įrankių šio tyrimo pradžioje rinkoje nebuvo. Vėliau, 2013 metais, buvo pristatyta komercinė SBV antikūnų aptikimo galvijų serumuose ir piene sistema, paremta rekombinantiniu *E. coli* susintetintu N baltymu (Bréard ir kt., 2013). Disertacijos tyrimų metu nebuvo žinoma apie bandymus sukurti antikūnų prieš SBV aptikimo sistemą naudojant SBV baltymus, pagamintus *Saccharomyces cerevisiae* ląstelėse. Taip pat nebuvo žinoma apie testus, kurie atpažintų SBV antikūnus galvijų seilėse. Tokia sistema leistų panaudoti neinvazinius galvijų mėginių rinkimo būdus, tiriant SBV paplitimą tiek jaunuose, tiek suaugusiuose galvijuose. Tiriant galvijų seiles reikalinga labai jautri detekcijos sistema, nes seilėse antikūnų koncentracija yra mažesnė nei serume. Mielėse susintetintas SBV baltymas galėtų tikti tokiai sistemai, nes yra žinoma, jog mielėse

susintetinti virusiniai baltymai puikiai išlaiko savo antigenines savybes (Razanskiene ir kt., 2004). Ne mažiau svarbu yra stebėti ir paties viruso paplitimą regione, todėl Lietuvos ir Ukrainos ūkių galvijų mėginių ištyrimas prisidėtų prie SBV paplitimo tyrimų.

Ginamieji teiginiai:

1. Mielės *S. cerevisiae* yra tinkamas šeimininkas SBV struktūrinio nukleokapsidės baltymo gavimui.
2. Rekombinantinį struktūrinį SBV nukleokapsidės baltymą atpažįsta SBV specifiški antikūnai galvijų mėginiuose.
3. Rekombinantinis struktūrinis SBV nukleokapsidės baltymas yra tinkamas SBV specifiškų antikūnų testavimui galvijų piene, seilėse bei serumuose.
4. SBV specifiškų antikūnų galima aptikti Lietuvos ir Ukrainos galvijų mėginiuose.
5. Seilės yra tinkamas mėginys SBV specifiškiems antikūnams aptikti.

TYRIMŲ METODIKA

Reagentai, fermentai. Visi naudoti reagentai buvo aukščiausio grynumo laipsnio. Fermentai: restrikcijos endonukleazės, DNR polimerazės, FastAP šarminė fosfatazė, T4DNR ligazė, RNAzė A bei DNR ir baltymų masės standartai, nukleotidų mišiniai, elektroforezės buferiniai tirpalai buvo įsigyti iš ThermoFisher Scientific (USA). Mitybinių terpių komponentai įsigyti iš Sigma Aldrich (Vokietija), Applichem (Vokietija) ir Carl Roth GmbH (Vokietija).

Pagrindiniai molekulinės biologijos metodai. DNR ir baltymų elektroforezė (NDS-PAGE), DNR ligavimas, hidrolizavimas, 5‘-fosfatų pašalinimas, PGR, Western imunoblotingas, kompetentinių *E. coli* ir mielių ląstelių paruošimas ir transformacija plazmidėmis buvo atliekami kaip aprašyta Green ir Sambrook (Green ir kt., 2012). DNR fragmentų išskyrimas iš PGR mišinio ir agarozės gelio, plazmidinės DNR išskyrimas buvo atlikti su atitinkamais ThermoFisher Scientific Baltics pagamintais rinkiniais, pagal gamintojo rekomendacijas.

Mielių auginimas, rekombinantinių baltymų sintezės indukcija ir ląstelių lizatų paruošimas. Mielių kultūra auginama skystoje 200 ml YEPD terpėje 2 L tūrio Erlenmejerio kolbose 24 val. Ląstelės indukuojamos pridėjus pusę tūrio 2xYEPD terpės ir galaktozės tirpalo iki galutinės 2,5 % koncentracijos. Indukcija vyksta 16-20 val. Visos mitybinės terpės turi 0,3 – 0,4 μg/ml formaldehido. Lizato paruošimui ląstelės surenkamos centrifuguojant 10 min. 2000×g, biomasė praplaunama vandeniu ir užpilama tokiu pat tūriu ardymo buferio. Ardymui imama dvigubai didesnė stiklo rutuliukų (Ø=212-300 μm) masė nei ląstelių biomasė. Vorteksuojama 8-10 kartų po 30 s grąžinant mėgintuvėlius į ledą. Ląstelių nuolaužos nusodinamos centrifuguojant 2000×g 5 min.

Ultracentrifugavimas sacharozės tirpale. Mielės, sintetinančios rekombinantinį baltymą, suardomos PBS buferyje ir centrifuguojamos šaltai 10000×g 30 min. Sacharozės (30 % - 70 %) tirpalai ardymo buferiniame tirpale yra supilstomi į ultracentrifuginius mėgintuvėlius. Ląstelių lizato supernatantas

yra atsargiai užnešamas ant tirpalų paviršaus. Ultracentrifugavimas sacharozės tirpale vyksta 20-48 val. 37000 aps./min. greičiu, esant 4°C temperatūrai (Beckman ultracentrifuga L8-70, rotoriaus tipas 70 TI). Po centrifugavimo surenkamos frakcijos po 1 ml ir analizuojamos NDS-PAGE.

Rekombinantinių baltymų gryninimas denatūruojančiomis sąlygomis. Mielės, sintetinančios rekombinantinį baltymą, suardomos buferyje, turinčiame 6 M GuCl, 0,1 M NaH_2PO_4, 0,5 % glicerolio, 1 % Tween-20, 10 mM imidazolo, 2 mM PMSF. Netirpūs baltymai pašalinami centrifuguojant 10000×g 10 min. Supernatantas užpilamas ant 10-20 mg/ml baltymo surišimo talpą turinčios Ni-NTA agarozės ir inkubuojamas 1 val. kambario temperatūroje. Tolimesnis gryninimas atliekamas pagal gamintojo rekomendacijas (Qiagen, Hilden, Germany). Eliucijos frakcija dializuota acetatiniame buferyje (pH 5,0) ir užšaldyta pridėjus glicerolio iki 40 % galutinės koncentracijos. Rekombinantinio baltymo grynumas analizuotas NDS-PAGE metodu.

Imunofermentinė analizė (IFA). Į šulinėlius pilama 2 µg/ml koncentracijos antigeno 50 mM natrio karbonato (pH 9,3) tirpale ir inkubuojama 4 ºC temperatūroje per naktį. Kiekvienas šulinėlis plaunamas praplovimo (PBS su 0,1 % Tween-20) tirpalu tris kartus. Tada blokuojama Roti-Block (Carl Roth GmbH, Vokietija) po 150 µL kiekviename šulinėlyje, 1 val. kambario temperatūroje. Blokuota plokštelė plaunama tris kartus plovimo tirpalu. Inkubuojama su serumo tirpalu (100 µL šulinėliui) 2 val. 37 ºC temperatūroje, serumai skiedžiami PBS tirpalu su 0,1 % Tween-20. Po to plaunama 5 kartus ir inkubuojama su antriniais antikūnais 1 val. 37 ºC. Tada plaunama 5 kartus ir ryškinama pridedant po 100 µL 3,3',5,5'-tetrametilbenzidino (TMB) substrato (DakoCytomation, Danija) į kiekvieną šulinėlį, palaikoma 10 min. tamsoje, kambario temperatūroje. Reakcija sustabdoma įpilant po 100 µL 10 % sieros rūgšties. Spektrofotometru išmatuojama sugertis 450 nm bangos ilgyje (lyginant su sugertimi 620 nm).

Elektroninė mikroskopija. Baltymų mėginiai stebimi transmisiniu elektroniniu mikroskopu Morgagni 268 (D) (FEI, JAV). 0,2 μg baltymų užpilama ant varinės plokštelės, turinčios anglimi padengtą paviršių. Po 1 min. plokštelė nusausinama ir nuplaunama vandeniu. Užpilamas 2 % uranilo acetato tirpalas, kuris po 2 min. nusausinamas filtriniu popieriumi.

Naudoti pradmenys 5‘ ⇨3‘ kryptis:

SchN-5‘: TGCCTAGGACAATGTCAAGCCAATTCATTTTTG
SchN_3L: ATATCTAGAACCTCCAGATCCACCAGAGATGTTGA
TACCGAATTGCTGCAAG
SchN_3X: GCACCTAGGATCGATGTTGATACCGAATTGCTG
SchOG2_5P: GACACTAGTACAATGCTTCTAAATATTGTTTTGA
SchOG2_3L: ATATCTAGAACCTCCAGATCCACCAGATCTAGTTTTT
GGCAAAGCTTTATAACCA
SchOG2_3S: GACACTAGTTTATCTAGTTTTTGGCAAAGCTTTATAACCA
SchOG2-5: TAACTAGTACAATGGGCACTAGAGGATCGAGGT
SchOG2-3: GGACTAGTTTAATGATGATGATGATGATGTCTAGT
SchOG2S_5P: GCACTAGTACAATGTTGTTGAATATCGTCTT
SchOG2S_3L: TATCTAGAACCTCCAGATCCTCCACTTCTGGTCT
TTGGTAATGCCTTGT
SchOG2S_3S: GCACTAGTTCATCTGGTCTTTGGTAATGCCTTGT
SchOG2S_5t: GCACTAGTATAATGGCTTTGCCATTGAAGGAAGGT
SchOG2S_3tL: TATCTAGAACCTCCAGATCCACCAGACATCAATT
CAGGCAATATACTACC
SchOG2S_3tS: GCACTAGTTCACATCAATTCAGGCAATATACTACC
SchOG2S_3tN: GCACTAGTCATCAATTCAGGCAATATACTACC
PYK5: TATTCATTCTTTTTCATCCTTTGG
PGK3: TCCTTACCTTCCAATAATTCCAAAG

Naudoti antikūnai:

MAk prieš 6His epitopą, išskirti iš pelės. ThermoFisher Scientific (JAV).
PAk prieš pelės IgG (H+L), žymėti krienų peroksidaze, išskirti iš ožkos. BioRad (JAV).
PAk prieš jaučio IgG, žymėti krienų peroksidaze, išskirti iš triušio. Sigma Aldrich (JAV).
PAk prieš jaučio IgA, žymėti krienų peroksidaze, išskirti iš avies. AbD Serotec/Biorad (JAV).
PAk prieš jaučio F(ab')2 IgG, žymėti krienų peroksidaze, išskirti iš triušio. LifeSpan BioSciences (JAV).

Mikroorganizmų kamienai:

Escherichia coli kamienas DH5α: F^- *gyrA96* (NaI^r) *recA1 relA1 endA1 thi*$^{-}$*1 hsdR17* ($r_k^- m_k^+$) *glnV44 deoR* Δ*(lacZYA-argF)U169* [ϕ80dΔ*(lacZ)M15*].
Escherichia coli kamienas GM119: F^- *dam*$^{-}$*3 dcm*$^{-}$*6 lacY1 galK2 galT22 tonA31 tsx*$^{-}$*78 supE44 mtl*$^{-}$*1(thi*$^{-}$*1).*
S. cerevisiae kamienas AH22-214: MATa, *leu2-3,112, his4-519.*

Statistinė analizė:

Skirtumai tarp tiriamų grupių buvo įvertinti Chi kvadrato testu. Koreliacijos koeficientas skaičiuotas norint nustatyti tų pačių galvijų, tačiau skirtingų jų mėginių tarpusavio ryšį. Jautrumas buvo skaičiuojamas norint nustatyti serologinės būklės panašumą tarp tų pačių mėginių, tačiau tirtų skirtingais metodais. Koreliacijos koeficientas ir jautrumas buvo skaičiuoti Microsoft Excel programine įranga. Patikimumo intervalai (CI, 95%) skaičiuoti naudojant MedCalc programinę įrangą (MedCalc software, Belgija).

REZULTATAI

SBV struktūrinių baltymų sintezė *S. cerevisiae* ląstelėse

SBV struktūriniai baltymai yra reikalingi norint sukurti IFA sistemą, atpažįstančią SBV specifiškus antikūnus. Mielėse buvo bandomi susintetinti du SBV nukleokapsidės baltymo variantai – su histidinų inkarine seka ir be jos. Rekombinantinio SBV N baltymo, turinčio N-gale 6His uodegą sintezei (1 pav. A) buvo naudojamas pFX7-6His N vektorius (Razanskiene ir kt., 2004). SBV N geną koduojanti DNR seka (GenBank Nr. HE649914.1; 1 pav. B) buvo liguota į pFX7-His N vektorių naudojant BcuI, XmaJI nukleazes ir T4 DNR ligazę (2 pav.).

A

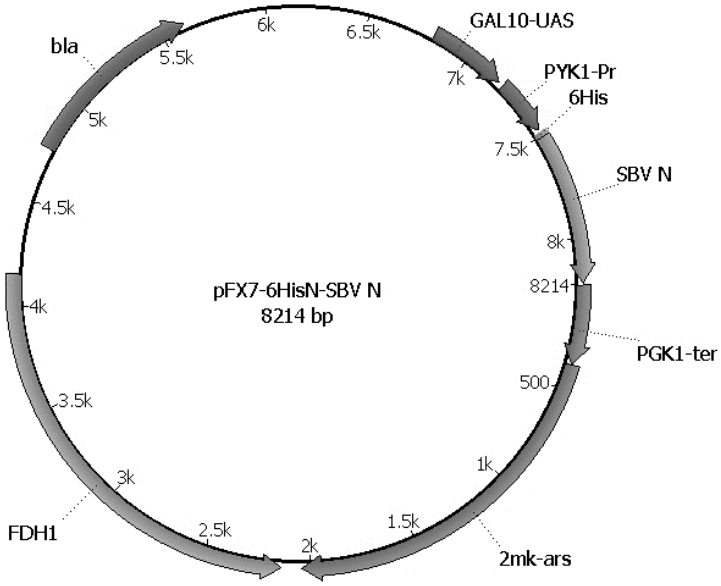
MHHHHHHSRTMSSQFIFEDVPQRNAATFNPEVGYVAFIGKYGQQLNFGVARVFFLNQKKAKM
VLHKTAQPSVDLTFGGVKFTVVNNHFPQYVSNPVPDNAITLHRMSGYLARWIADTCKASVLK
LAEASAQIVMPLAEVKGCTWADGYTMYLGFAPGAEMFLDAFDFYPLVIEMHRVLKDNMDVNF
MKKVLRQRYGTMTAEEWMTQKITEIKAAFNSVGQLAWAKSGFSPAARTFLQQFGINI

B

ATG*CACCATCACCATCACCAT*TCTAGGACAATGTCAAGCCAATTCATTTTTGAAGATGTACC
ACAACGGAATGCAGCTACATTTAACCCGGAGGTCGGGTATGTGGCATTTATTGGTAAGTATG
GGCAACAACTCAACTTCGGTGTTGCTAGAGTCTTCTTCCTCAACCAGAAGAAGGCCAAGATG
GTCCTACATAAGACGGCACAACCAAGTGTCGATCTTACTTTTGGTGGGGTCAAATTTACAGT
GGTTAATAACCATTTTCCCCAATATGTCTCAAATCCTGTGCCAGACAATGCCATTACACTTC
ACAGGATGTCAGGATATCTAGCACGTTGGATTGCTGATACATGCAAGGCTAGTGTCCTCAAA
CTAGCTGAAGCTAGTGCTCAGATTGTCATGCCCCTTGCTGAGGTTAAGGGATGCACCTGGGC
CGATGGTTATACAATGTATCTTGGATTTGCACCTGGGGCCGAAATGTTCCTTGATGCTTTTG
ACTTCTATCCACTAGTTATTGAAATGCATAGGGTCCTCAAGGACAATATGGATGTAAATTTT
ATGAAAAAAGTCCTCCGCCAACGCTATGGAACAATGACTGCTGAAGAATGGATGACTCAGAA
AATAACAGAAATAAAAGCTGCTTTTAATTCTGTTGGACAGCTTGCCTGGGCCAAATCTGGAT
TCTCTCCTGCTGCTAGAACCTTCTTGCAGCAATTCGGTATCAACATCTAA

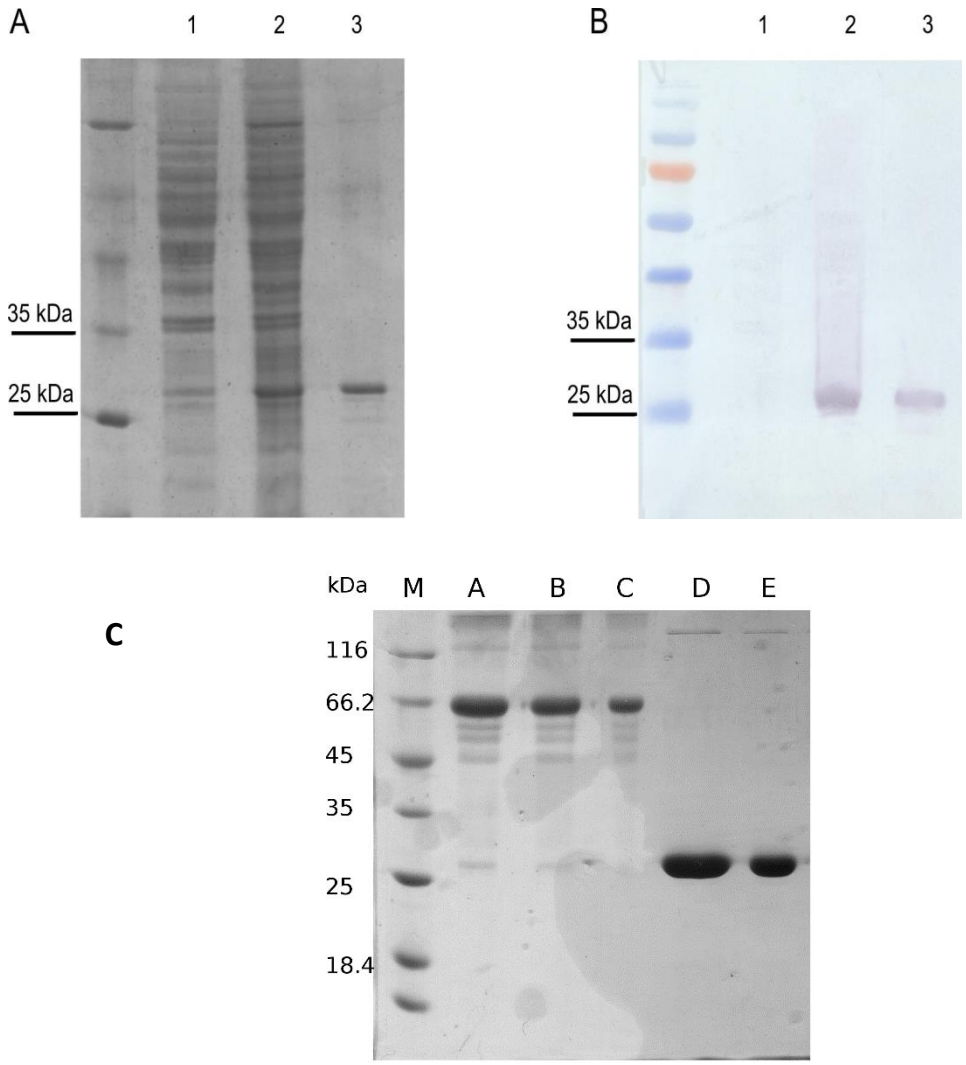
1 paveikslas. Schmallenberg viruso N geno seka (A) ir N baltymo aminorūgščių seka (B). (A) Schmallenberg viruso N geno seka (GenBank Nr. HE649914.1). 6His koduojanti seka pažymėta kursyvu. (B) SBV N-His baltymo aminorūgščių seka.

Geno integralumas patikrintas nusekvenavus klonuotą geną. Po baltymo sintezės indukcijos išanalizavus transformuotų mielių ląstelių lizatus NDS-PAGE geliuose ties 26 kDa žyme buvo pastebėtas SBV N-His baltymas (3 pav. A). Gryninant šį baltymą jis pasilikdavo netirpių mielių baltymų frakcijoje, todėl jis buvo gryninamas denatūruojančiomis sąlygomis naudojant nikelio afininės chromatografijos kolonėlę.



2 paveikslas. Mielių raiškos vektoriaus pFX7-6HisN-SBV N schema. 2mk-ars – mielių 2 μm plazmidės fragmentas; FDH1 – *FDH1 Candida maltosa* genas, suteikia atsparumą formaldehidui; bla – beta-laktamazės genas, suteikia atsparumą ampicilinui; GAL10-UAS/PYK1-Pr – mielių hibridinis galaktoze indukuojamas promotorius; PGK1-Ter – mielių PGK1 geno transkripcijos terminatorius. SBV N – Schmallenberg viruso nukleokapsidės baltymą koduojanti seka; 6His – 6 histidino aminorūgštis koduojanti seka.

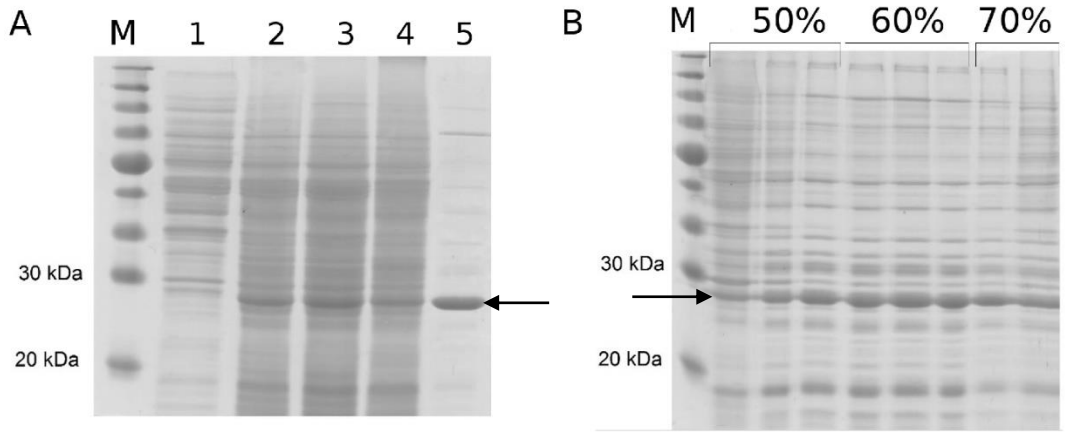
Denatūruojantys karbamido ir guanidino hidrochlorido gryninimo tirpalai buvo naudojami norint padidinti SBV N-His baltymo tirpumą. Po gryninimo baltymas dializuotas 50 mM natrio acetato buferyje (pH 5,0). Šio rekombinantinio baltymo pavyko gauti 3,0–3,5 mg iš 1 g mielių (3 pav. C). Vilniaus universiteto Biochemijos institute atlikta masių spektrometrijos analizė patvirtino pilno ilgio rekombinantinio SBV N-His baltymo seką.



3 paveikslas. Mielių lizatų ir išgryninto SBV N-His baltymo NDS-PAGE (A, C) ir imunoblotingo (B) analizė. (A) Takeliuose: Išgrynintas SBV N-His baltymas ir mielių lizatai frakcionuoti 12% NDS-PAGE geliuose. Naudotas PageRuler Unstained Protein Ladder molekulinės masės žymuo. (B) Imunoblotingo analizė atlikta naudojant MAk prieš 6His-tag epitopą. Naudotas PageRuler Prestained Protein Ladder molekulinės masės žymuo. (A ir B) 1 – *S. cerevisiae*, transformuotų pFX7-6His vektoriumi lizatas; 2 – *S. cerevisiae*, transformuotų pFX7-6His N-SBV N plazmide, koduojančia SBV N-His baltymą, lizatas; 3 – Ni-NTA sorbentu išgrynintas SBV N baltymas. (C) Šulinėliai A, B ir C – 4 µg, 2 µg ir 1 µg JSA baltymo. Šulinėliai D ir E – 8 µg ir 4 µg išgryninto SBV N-His baltymo.

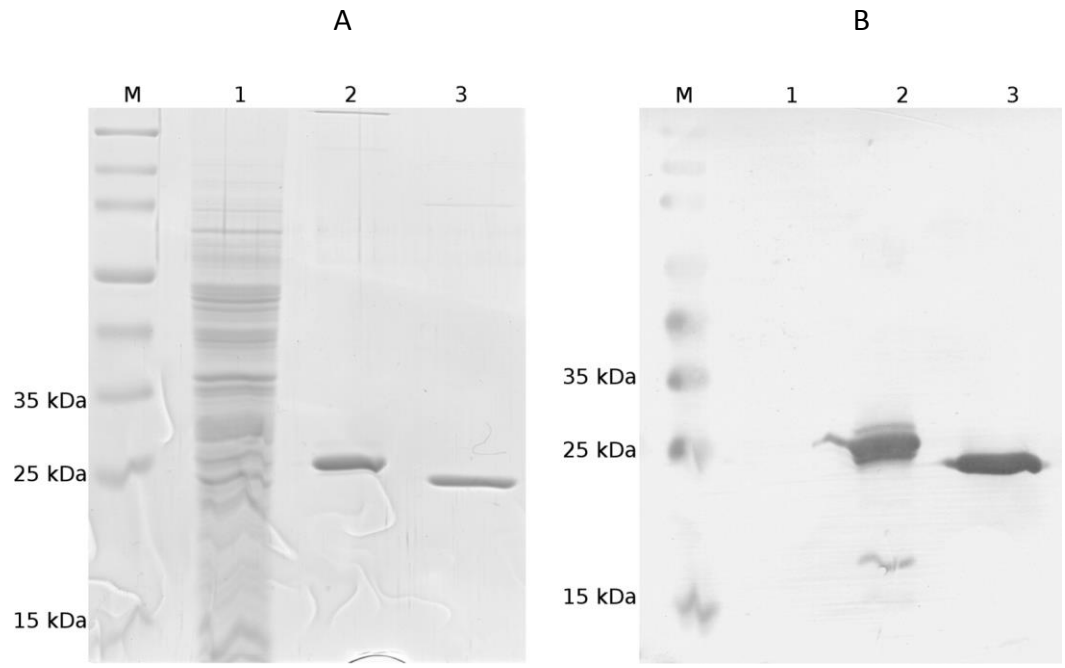
Natyvaus SBV N baltymo gavimui tas pats nukleokapsidės baltymą koduojantis genas buvo klonuotas į pFD3 vektorių (Sasnauskas ir kt., 1999). Po mielių transformacijos plazmide ir baltymo sintezės indukcijos pavyko mielėse susintetinti apie 25 kDa dydžio SBV N baltymą (4 pav. A). Šis baltymas neturėjo

histidinų liekanų, todėl buvo grynintas sacharozės tirpale ultracentrifuguojant. Daugiausiai SBV N baltymo susikaupė tarp 60 % ir 70 % sacharozės koncentracijos tirpalų (4 pav. B).



4 paveikslas. Mielių lizatų ir išgryninto SBV N baltymo NDS-PAGE analizė. (A) Takeliuose: 1 – *S. cerevisiae*, transformuotų pFD3 vektoriumi lizatas; 2 – *S. cerevisiae*, transformuotų pFD3-SBV N plazmide, koduojančia SBV N baltymą, lizatas; 3 – SBV N baltymą produkuojančių mielių lizato supernatantas; 4 – SBV N baltymą produkuojančių mielių lizato nuosėdos; 5 – išgrynintas SBV N baltymas. (B) Takeliuose SBV N baltymo mėginiai, surinkti po ultracentrifugavimo sacharozės tirpale. PAA gelyje buvo tirtos 50 %, 60 % ir 70 % koncentracijos sacharozės tirpalą atitinkančios frakcijos. Rodyklės žymi SBV N baltymo vietą gelyje. Takelis M – PageRuler Prestained Protein Ladder žymuo.

Po baltymo sukoncentravimo 30 % sacharozės tirpale, sacharozė buvo pašalinta dializuojant SBV N baltymą PBS buferyje. Analizuojant SBV N-His ir SBV N baltymų grynumą NDS-PAGE geliuose papildomų baltymų priemaišų nepastebėta (5 pav. A). SBV N baltymo išeiga – 1,3 mg iš 1 g mielių. Elektroninės mikroskopijos būdu analizuojant SBV N ir N-His baltymų mėginius nepastebėta į nukleokapsides panašių struktūrų.



5 paveikslas. Mielių lizato ir išgrynintų SBV N baltymų analizė NDS-PAGE (A) ir imunoblotingo (B) metodais. M – Spectra Broad Range Prestained Protein žymuo, 1 – *S. cerevisiae* mielių, transformuotų nemodifikuota pFD3 plazmide, lizatas, 2 – SBV N-His baltymas po nikelio afininės chromatografijos gryninimo, 3 – SBV N baltymas po ultracentrifugavimo sacharozės tirpale. Imunoblotingo analizei naudoti iš pelių išskirtų monokloninių antikūnų, specifiškų SBV, klonas 4F3 ir komerciniai antikūnai prieš pelės IgG, žymėti HRP.

Konstruojant raiškos plazmidę, koduojančią kitą struktūrinį SBV baltymą glikoproteiną 2 (G2), buvo naudota GenBank duomenų bazės seka Nr. CCF55030.1. Seka buvo optimizuota *S. cerevisiae* palankiam kodonų rinkiniui (6 pav. A). Taip pat buvo sukonstruota raiškos plazmidė, turinti trumpesnį G2 variantą – be hidrofobinės C dalies (ΔH) baltymo C-gale (6 pav. B ir C, 7 pav. C).

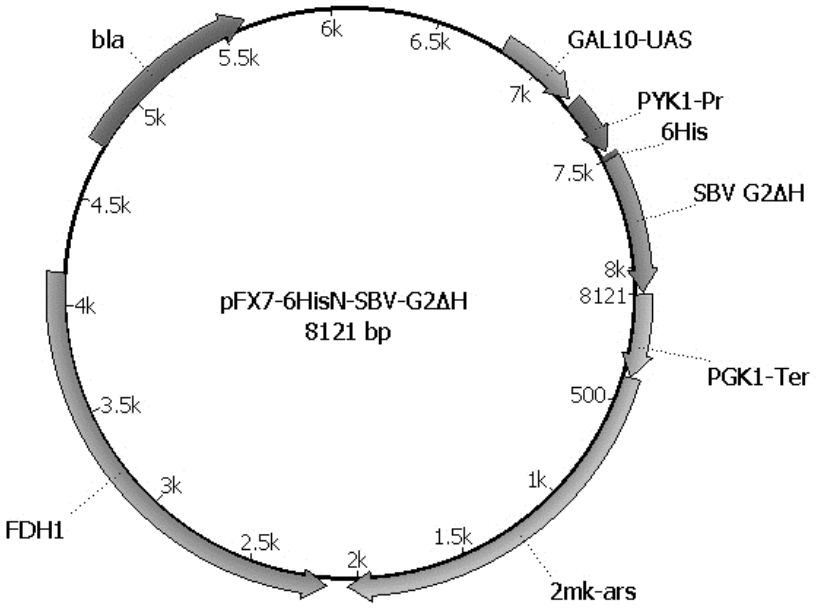
A

ATG*CACCATCACCATCACCAT*TCTAGTACAATGTTGTTGAATATCGTCTTAATCAGTAACTTAGCCTGCTTAGCTT
TCGCTTTGCCATTGAAGGAAGGTACCAGAGGTTCCAGATGCTTTTTGAATGGTGAATTAGTCAAGACAGTAAACAC
CTCTAAAGTTGTCTCAGAATGTTGCGTTAAGGATGACATCTCCATTATTAAGAGTAACGCTGAACATTACAAGTCA
GGTGACAGATTGGCTGCAGTAATTAAATACTACAGATTGTACCAAGTTAAGGACTGGCATTCTTGTAATCCAATCT
ATGATGACCACGGTTCATTCATGATTTTGGATATAGACAACACTGGTACATTAATTCCTAAAATGCACACCTGTAG
AGTCGAATGCGAAATTGCATTGAACAAGGATACTGGTGAAGTAATATTGAACTCCTACAGAATTAATCATTACAGA
ATCTCTGGTACAATGCACGTTTCAGGTTGGTTCAAGAATAAGATCGAAATCCCATTGGAAAACACTTGTGAATCTA
TTGAAGTCACTTGCGGTTTGAAGACATTGAACTTCCATGCCTGTTTCCATACCCACAAATCCTGCACTAGATACTT
CAAGGGTAGTATATTGCCTGAATTGATGATCGAATCTTTCTGTACTAACTTGGAATTAATCTTGTTAGTTACATTC
ATTTTGGTTGGTTCAGTCATGATGATGATCTTGACCAAGACTTACATCGTATACGTTTTTATACCAATCTTTTACC
CTTTCGTTAAGTTGTATGCCTACATGTACAATAAGTACTTCAAGTTGTGTAAGAACTGCTTGTTAGCTGTTCATCC
ATTCACAAATTGTCCTTCCACCTGTATCTGCGGTATGATCTATACTACAACCGAAAGTTTGAAGTTGCACAGAATG
TGTAACAACTGCTCAGGTTACAAGGCATTACCAAAGACCAGATAA

B

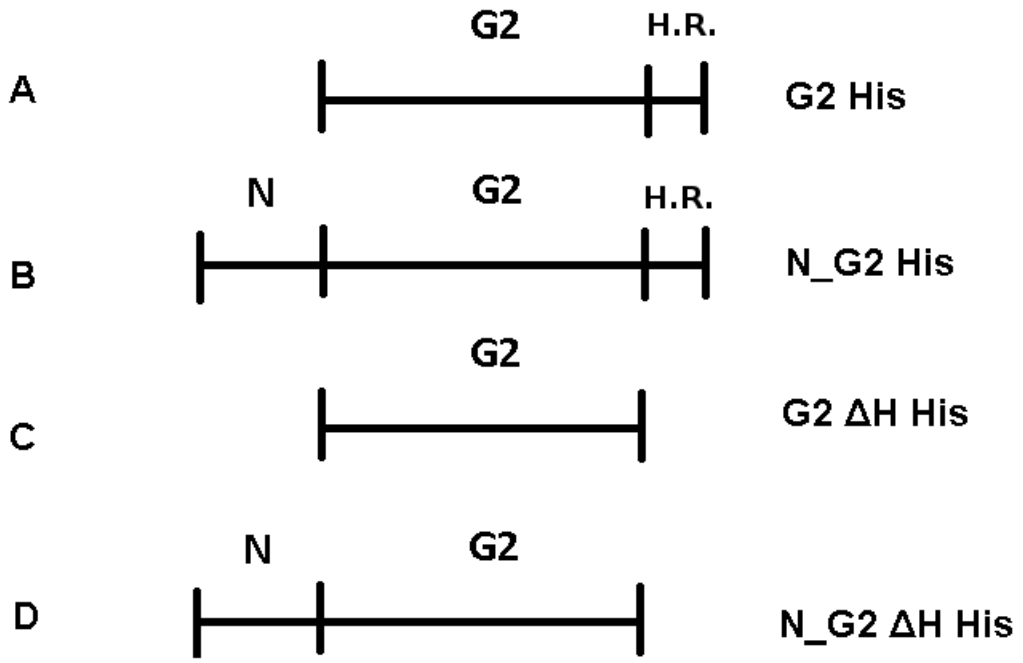
MHHHHHHSSTMLLNIVLISNLACLAFALPLKEGTRGSRCFLNGELVKTVNTSKVVSECCVKDDISIIKSNAEHYKS
GDRLAAVIKYYRLYQVKDWHSCNPIYDDHGSFMILDIDNTGTLIPKMHTCRVECEIALNKDTGEVILNSYRINHYR
ISGTMHVSGWFKNKIEIPLENTCESIEVTCGLKTLNFHACFHTHKSCTRYFKGSILPELM

C



6 paveikslas. Schmallenberg viruso G2 geno DNR seka (A), SBV G2ΔH baltymo aminorūgščių seka (B) ir mielių raiškos vektoriaus pFX7-6HisN-SBV-G2ΔH schema (C). (A) SBV G2 geno DNR seka (GenBank Nr. CCF55030.1), 6His koduojanti seka pažymėta kursyvu. Pabraukti regionai žymi pradmenų, skirtų G2ΔH geno variantui sukurti, hibridizacijos vietas. (B) SBV G2ΔH baltymo aminorūgščių seka. 6His seka matoma baltymo N galinėje dalyje. (C) Mielių raiškos vektoriaus pFX7-6HisN-SBV-G2ΔH schema. 2mk-ars – mielių 2µm plazmidės fragmentas; FDH1 – *FDH1 Candida maltosa* genas, suteikia atsparumą formaldehidui; bla – beta-laktamazės genas, suteikia atsparumą ampicilinui; GAL10-UAS/PYK1-Pr – mielių hibridinis galaktoze indukuojamas promotorius; PGK1-Ter – mielių PGK1 geno transkripcijos terminatorius. SBV G2ΔH – Schmallenberg viruso glikoproteino 2 be hidrofobinės C dalies baltymą koduojanti seka; 6His – 6 histidino aminorūgštis koduojanti seka.

Abi plazmidės buvo kurtos pFX7-6His N pagrindu. Po mielių transformacijos šiomis plazmidėmis ir baltymų sintezės indukcijos buvo pabandyta išgryninti nedidelius kiekius rekombinantinių baltymų. G2 baltymo sintezė mielėse nebuvo stebima, bet buvo išgrynintas savo dydžiu į SBV G2ΔH baltymą (10 pav. A) panašus baltymas. Western-blot metodu, naudojant antikūnus prieš 6His inkarinę seką, SBV baltymų nepavyko aptikti (duomenys nepateikti). Didelio kiekio SBV G2ΔH baltymo denatūruojančiomis sąlygomis gryninimas taip pat buvo nesėkmingas.



7 paveikslas. Schematinis SBV G2 ir chimerinių N_G2 baltymų variantų vaizdas. A – pilno ilgio SBV G2 baltymas, B – pilno ilgio SBV G2 baltymas sulietas su pilno ilgio SBV N baltymu, C – SBV G2 baltymas be hidrofobinio regiono C-gale, D – SBV G2 baltymas be hidrofobinio regiono C-gale sulietas su pilno ilgio SBV N baltymu. H.R. – hidrofobinis G2 baltymo regionas.

Buvo nuspręsta G2 geną sulieti su N baltymo genu norint pagerinti G2 baltymo sintezę mielėse. Konstruojant plazmides buvo naudota pilno ilgio N geno seka ir pilno ilgio arba sutrumpintos G2 geno sekos, nekoduojančios hidrofobinės baltymo dalies (7 pav. B ir D). Tarp N ir G2 genų sekų buvo įterpta SSGSSI peptidą koduojanti seka – jungtukas (8 pav. A).

A

MSSQFIFEDVPQRNAATFNPEVGYVAFIGKYGQQLNFGVARVFFLNQKKAKMVLHKTAQPSV
DLTFGGVKFTVVNNHFPQYVSNPVPDNAITLHRMSGYLARWIADTCKASVLKLAEASAQIVM
PLAEVKGCTWADGYTMYLGFAPGAEMFLDAFDFYPLVIEMHRVLKDNMDVNFMKKVLRQRYG
TMTAEEWMTQKITEIKAAFNSVGQLAWAKSGFSPAARTFLQQFGINI**sggsggsst**MLLNIV
LISNLACLAFALPLKEGTRGSRCFLNGELVKTVNTSKVVSECCVKDDISIIKSNAEHYKSGD
RLAAVIKYYRLYQVKDWHSCNPIYDDHGSFMILDIDNTGTLIPKMHTCRVECEIALNKDTGE
VILNSYRINHYRISGTMHVSGWFKNKIEIPLENTCESIEVTCGLKTLNFHACFHTHKSCTRY
FKGSILPELMIESFCTNLELILLVTFILVGSVMMMILTKTYIVYVFIPIFYPFVKLYAYMYN
KYFKLCKNCLLAVHPFTNCPSTCICGMIYTTTESLKLHRMCNNCSGYKALPKTRHHHHHH

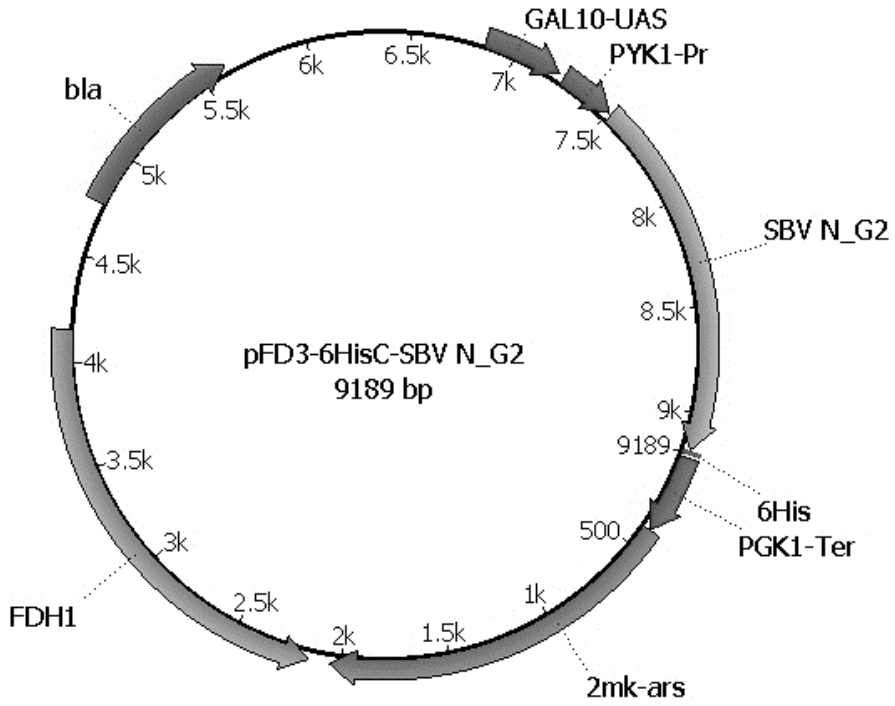
B

ATGTCAAGCCAATTCATTTTTGAAGATGTACCACAACGGAATGCAGCTACATTTAACCCGGA
GGTCGGGTATGTGGCATTTATTGGTAAGTATGGGCAACAACTCAACTTCGGTGTTGCTAGAG
TCTTCTTCCTCAACCAGAAGAAGGCCAAGATGGTCCTACATAAGACGGCACAACCAAGTGTC
GATCTTACTTTTGGTGGGGTCAAATTTACAGTGGTTAATAACCATTTTCCCCAATATGTCTC
AAATCCTGTGCCAGACAATGCCATTACACTTCACAGGATGTCAGGATATCTAGCACGTTGGA
TTGCTGATACATGCAAGGCTAGTGTCCTCAAACTAGCTGAAGCTAGTGCTCAGATTGTCATG
CCCCTTGCTGAGGTTAAGGGATGCACCTGGGCCGATGGTTATACAATGTATCTTGGATTTGC
ACCTGGGGCCGAAATGTTCCTTGATGCTTTTGACTTCTATCCACTAGTTATTGAAATGCATA
GGGTCCTCAAGGACAATATGGATGTAAATTTTATGAAAAAAGTCCTCCGCCAACGCTATGGA
ACAATGACTGCTGAAGAATGGATGACTCAGAAAATAACAGAAATAAAAGCTGCTTTTAATTC
TGTTGGACAGCTTGCCTGGGCCAAATCTGGATTCTCTCCTGCTGCTAGAACCTTCTTGCAGC
AATTCGGTATCAACATC**tctggtggatctggaggttctagtaca**ATGCTTCTAAATATTGTT
TTGATATCAAACTTAGCATGTTTAGCTTTTGCACTCCCTCTTAAGGAAGGCACTAGAGGATC
GAGGTGCTTCCTAAATGGTGAGTTGGTCAAAACGGTTAACACATCAAAGGTCGTTTCAGAGT
GCTGTGTTAAAGACGACATATCTATCATTAAATCAAATGCTGAACATTATAAATCTGGTGAT
AGATTGGCTGCTGTTATTAAATATTATAGATTGTATCAAGTAAAAGATTGGCATTCTTGTAA
TCCAATTTATGATGATCATGGTTCTTTTATGATTTTGGATATTGATAATACTGGTACTTTGA
TTCCAAAAATGCATACTTGTAGAGTTGAATGTGAAATTGCTTTGAATAAAGATACTGGTGAA
GTTATTTTGAATTCTTATAGAATTAATCATTATAGAATTTCTGGTACTATGCATGTTTCTGG
TTGGTTTAAAAATAAAATTGAAATTCCATTGGAAAATACTTGTGAATCTATTGAAGTTACTT
GTGGTTTGAAAACTTTGAATTTTCATGCTTGTTTTCATACTCATAAATCTTGTACTAGATAT
TTTAAAGGTTCTATTTTGCCAGAATTGATGATTGAATCTTTTTGTACTAATTTGGAATTGAT
TTTGTTGGTTACTTTTATTTTGGTTGGTTCTGTTATGATGATGATTTTGACTAAAACTTATA
TTGTTTATGTTTTTATTCCAATTTTTTATCCATTTGTTAAATTGTATGCTTATATGTATAAT
AAATATTTTAAATTGTGTAAAAATTGTTTGTTGGCTGTTCATCCATTTACTAATTGTCCATC
TACTTGTATTTGTGGTATGATTTATACTACTACTGAATCTTTGAAATTGCATAGAATGTGTA
ATAATTGTTCTGGTTATAAAGCTTTGCCAAAAACTAGA*CATCATCATCATCATCATCAT*TAA

8 paveikslas. SBV N_G2 sulieto baltymo aminorūgščių seka (A) ir baltymą koduojanti DNR seka (B). N ir G2 koduojančias sekas jungiantis jungtukas pažymėtas mažosiomis paryškintomis raidėmis.

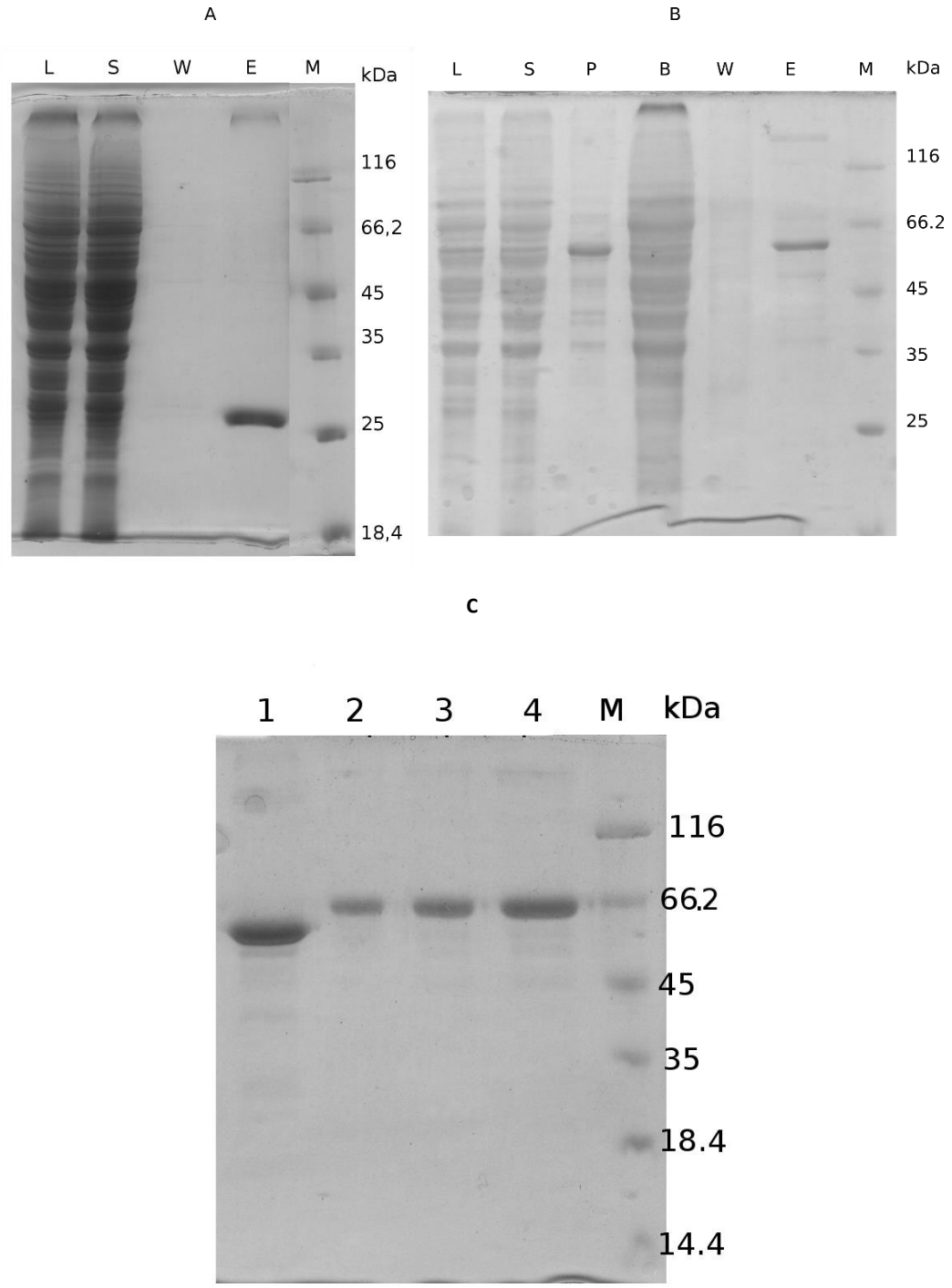
Plazmidės buvo kurtos pFD3 plazmidės pagrindu (9 pav.). Po mielių transformacijos ir baltymo sintezės indukcijos, SBV N_G2ΔH-NHis baltymo nikelio afininės chromatografijos būdu pavyko išgryninti tik nedidelį kiekį –

0,15 mg baltymo iš 1 g mielių. Tuo tarpu pilno ilgio SBV N_G2-CHis baltymo išeiga buvo 0,3 mg iš 1 g mielių (10 pav. C).



9 paveikslas. Mielių raiškos vektoriaus pFD3-6HisC-SBV N_G2 schema. 2mk-ars – mielių 2 µm plazmidės fragmentas; FDH1 – genas, suteikia atsparumą formaldehidui; bla – beta-laktamazės genas, suteikia atsparumą ampicilinui; GAL10-UAS/PYK1-Pr – mielių hibridinis galaktoze indukuojamas promotorius; PGK1-Ter – mielių PGK1 geno transkripcijos terminatorius. SBV N_G2– Schmallenberg viruso hibridinį nukleokapsidės ir glikoproteino 2 baltymą koduojanti seka; 6His – šešias histidino aminorūgštis koduojanti seka.

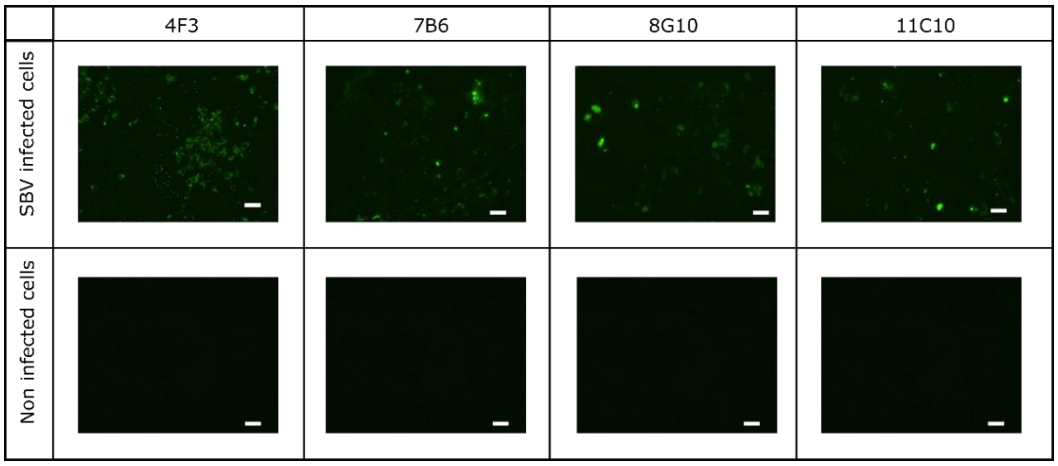
SBV N_G2-CHis baltymas nebuvo gryninamas natyviomis sąlygomis, nes didžioji jo dalis pasilikdavo netirpioje mielių lizato frakcijoje (10 pav. B).



10 paveikslas. Mielių lizatų ir išgrynintų SBV-G2ΔH (A) ir SBV N_G2-CHis (B,C) baltymų NDS-PAGE analizė. (A ir B) L –SBV-G2ΔH ir SBV N_G2-CHis baltymą produkuojančių mielių lizatas. S – SBV-G2ΔH ir SBV N_G2-CHis baltymą produkuojančių mielių lizato supernatantas. P – mielių ląstelių lizato nuosėdos. B – baltymai surinkti Ni-NTA inkubacijos mielių lizatais. W – Ni-NTA sorbento praplovimo frakcija. E – Ni-NTA sorbento eliucijos frakcija. M – baltymų molekulinės masės žymuo. (C) 1 šulinėlis – 8 µg išgryninto SBV N_G2-CHis baltymo. 2, 3 ir 4 šulinėliai – 1 µg, 2 µg ir 4 µg JSA baltymo.

Rekombinantinio SBV N baltymo antigeninių savybių tyrimas panaudojant monokloninius antikūnus

Monokloniniai SBV specifiški antikūnai (MAk) buvo sukurti Vilniaus universiteto Biotechnologijos instituto Imunologijos ir ląstelės biologijos skyriaus darbuotojų, naudojant išgrynintą SBV N-His baltymą. Gautos keturios MAk gaminančios hibridomos ląstelių linijos: 4F3, o 7B6, 8G10 ir 11C10. Friedrich-Loeffeler institute (FLI, Vokietija) šie MAk buvo patikrinti su SBV užkrėstomis BHK ląstelėmis. Imunofluorescencinė analizė buvo atlikta naudojant šių ląstelių kloną BRS5. Ląstelių suspensija buvo laikyta 96 šulinėlių plokštelėse 24 valandas, o tada užkrėsta SBV BH80/11 štamu. Po 48 val. ląstelės buvo užfiksuotos karščiu. Užkrėstos ir kontrolinės ląstelės buvo 1 val. inkubuotos su kiekvienu iš MAk. Nuplautos ląstelės buvo inkubuotos fluoresceino izotiocianatu žymėtais anti-pelės IgG antikūnais, gautais iš ožkos. Ląstelės analizuotos invertuotu fluorescenciniu miksroskopu (Nikon Eclipse Ti-U, Nikon Instruments Inc., USA). Visi minėti antikūnai reagavo su užkrėstomis BHK ląstelėmis ir nereagavo su kontrolinėmis ląstelėmis (11 pav.).

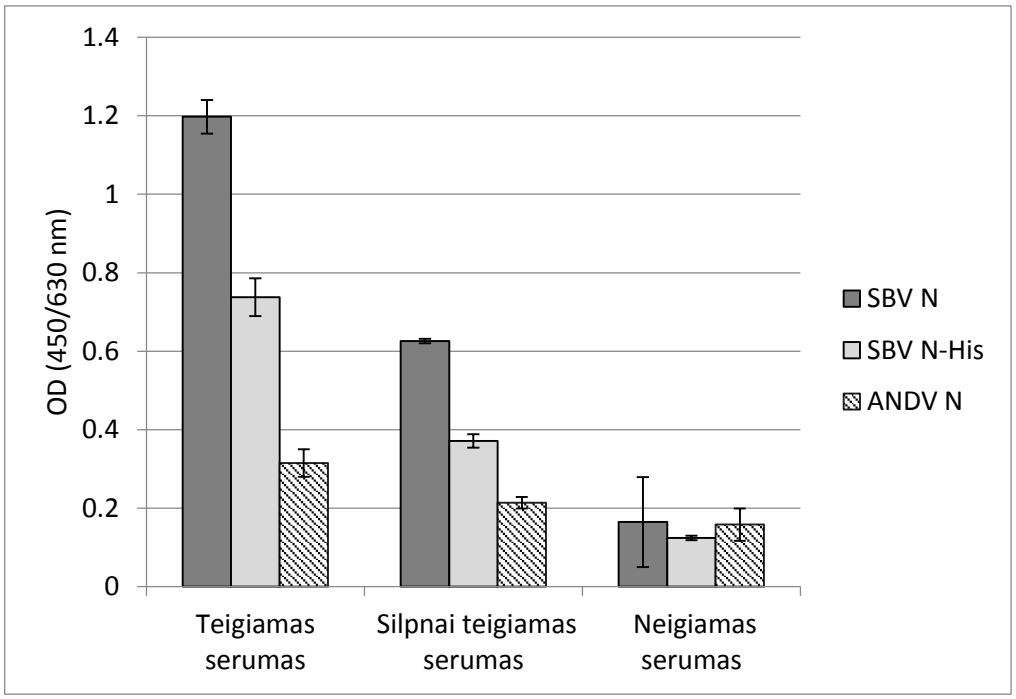


11 paveikslas. Fluorescencijos nuotraukos, rodančios MAk reakciją su SBV BH80/11 štamu užkrėstomis BHK ląstelėmis (viršutinė eilė). Neužkrėstos BHK ląstelės buvo naudojamos kaip kontrolinės (apatinė eilė). Hibridomos ląstelių kultūros supernatantas buvo skiestas 1/10. MAk žymėjimas pateiktas virš paveikslėlių. Mastelis – 100 μm. Eksperimentas atliktas Friedrich-Loeffler instituto Vokietijoje darbuotojų.

SBV specifiškų antikūnų testavimo sistemų kūrimas

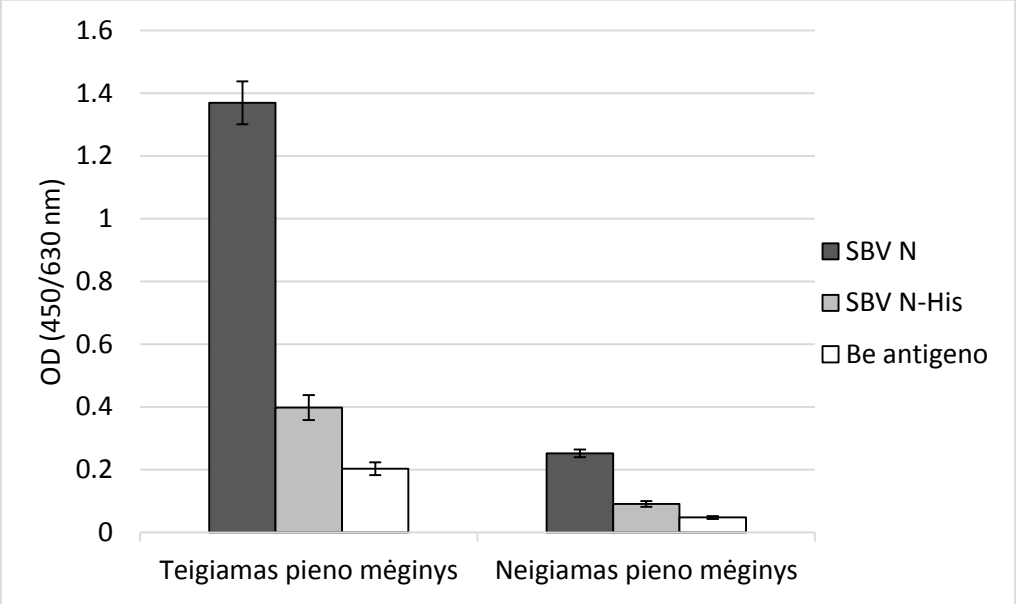
SBV specifiškiems antikūnams karvių seilių, serumo ir pieno mėginiuose aptikti buvo kuriamos netiesioginės IFA sistemos. Anksčiau išgryninti antigenai buvo patikrinti su SBV užsikrėtusių karvių pieno ir serumo mėginiais. Teigiami serumo mėginiai buvo gauti iš Friedrich-Loeffler instituto (Vokietija), o teigiami pieno mėginiai buvo nustatyti komerciniu SBV IgG testu (IDVet, Prancūzija). Neigiami pieno ir serumo mėginiai tai pat buvo nustatyti komerciniu SBV IgG testu.

Atliktas SBV N ir SBV N-His baltymų palyginimas naudojant kontrolinius sveikų ir užkrėstų SBV karvių serumus. Užkrėstų karvių serumai stipriau reagavo su SBV N nei su SBV N-His baltymu, o su kontroliniu Andes viruso N baltymu serumų reakcija buvo labai žemo lygio (12 pav.).



12 paveikslas. Rekombinantinių SBV N ir SBV N-His baltymų reakcijos su karvių serumo IgG antikūnais IFA teste palyginimas. SBV N – natyviomis sąlygomis grynintas SBV N baltymas. SBV N-His – denatūruojančiomis sąlygomis grynintas SBV N baltymas, ANDV N – kontrolinis Andes viruso nukleokapsidės baltymas. Ribos kiekvieno stulpelio viršuje žymi standartinę vidurkio paklaidą. Antriniai antikūnai prieš jaučio IgG, žymėti HRP, buvo skiesti 1/10000 PBS buferyje.

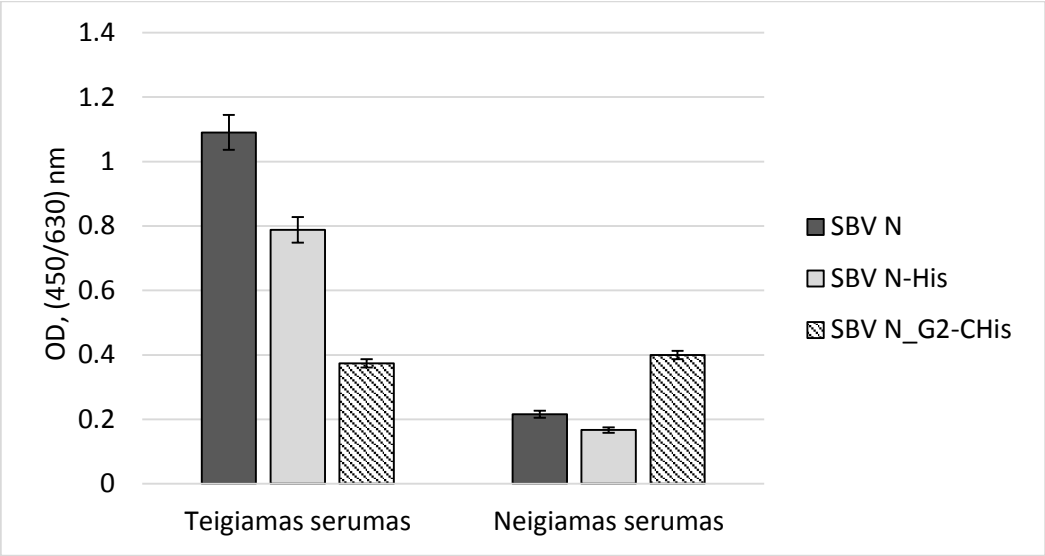
Rekombinantinis SBV N baltymas pasižymėjo didžiausiu reakcijų optinio tankio skirtumu tarp SBV specifiškų antikūnų turinčio ir tokių antikūnų neturinčios karvės serumų. Rekombinantinius SBV N baltymus patikrinus su SBV antikūnų turinčių karvių pieno mėginiais paaiškėjo, kad SBV specifiškų IgG antikūnų turintys pieno mėginiai taip pat stipriau reagavo su SBV N nei su SBV N-His baltymu (13 pav.).



13 paveikslas. Rekombinantinių SBV N ir SBV N-His baltymų reakcijos su karvių pieno IgG antikūnais IFA teste palyginimas. Antigenais naudoti SBV N ir SBV N-His baltymai. Antriniai antikūnai prieš jaučio IgG, žymėti HRP, buvo skiesti 1/10000 PBS buferyje. Ribos kiekvieno stulpelio viršuje žymi standartinę vidurkio paklaidą.

Šio darbo metu kuriamą IFA norėta patobulinti joje su SBV N baltymu naudojant ir SBV G2 baltymą, tačiau imuofermentinės analizės sistemos kūrimas naudojant chimerinį SBV N_G2-CHis baltymą nebuvo sėkmingas. Nepavyko nustatyti tokių sąlygų, kurias taikant aiškiai matytųsi skirtumas tarp teigiamo ir neigiamo serumo pavyzdžio (14 pav.), todėl SBV N_G2-CHis baltymas tolimesniuose tyrimuose naudotas nebuvo. SBV N_G2ΔH-NHis

baltymas reakcijos su karvių serumo antikūnais nebuvo tyrinėjamos dėl mažos jo išeigos.

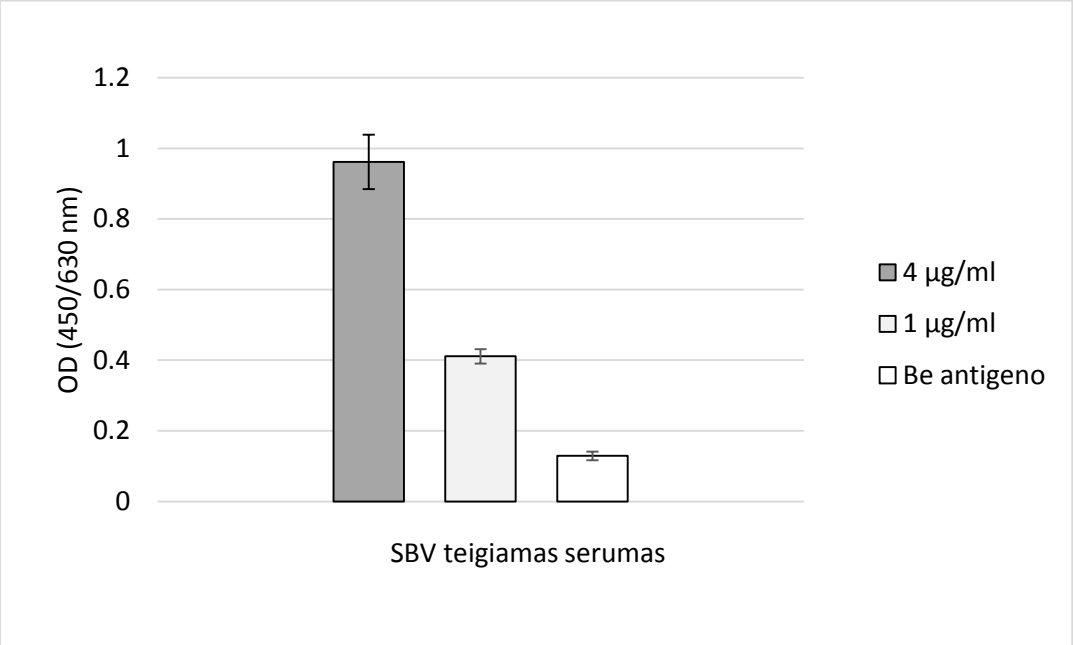


14 paveikslas. Rekombinantinių SBV N, N-His ir N_G2 baltymų reakcijų su SBV teigiamais ir SBV neigiamais galvijų serumais IFA teste palyginimas. SBV N-His – denatūruojančiomis sąlygomis išgrynintas SBV nukleokapsidės baltymas su 6His inkarine ar. seka. SBV N – natyviomis sąlygomis išgrynintas SBV nukleokapsidės baltymas. SBV N_G2-CHis – chimerinis iš SBV N ir SBV G2 baltymų sudarytas baltymas, turintis 6His inkarinę aminorūgščių seką, grynintas denatūruojančiomis sąlygomis. Antriniai antikūnai prieš jaučio IgG, žymėti HRP, buvo skiesti 1/10000 PBS buferyje. Ribos kiekvieno stulpelio viršuje žymi standartinę vidurkio paklaidą.

Rekombinantinis SBV N baltymas leido pasiekti didesnes optinio tankio vertes naudojant kontrolinius karvių pieno ir serumo mėginius, todėl nuspręsta jį naudoti kuriant piene, serume ir seilėse SBV specifiškus antikūnus aptinkančias sistemas. SBV N-His baltymas dėl savo žemesnio reaktyvumo su SBV specifiškais antikūnais naudotas tik kuriant karvių serume SBV specifiškus antikūnus aptinkančią sistemą.

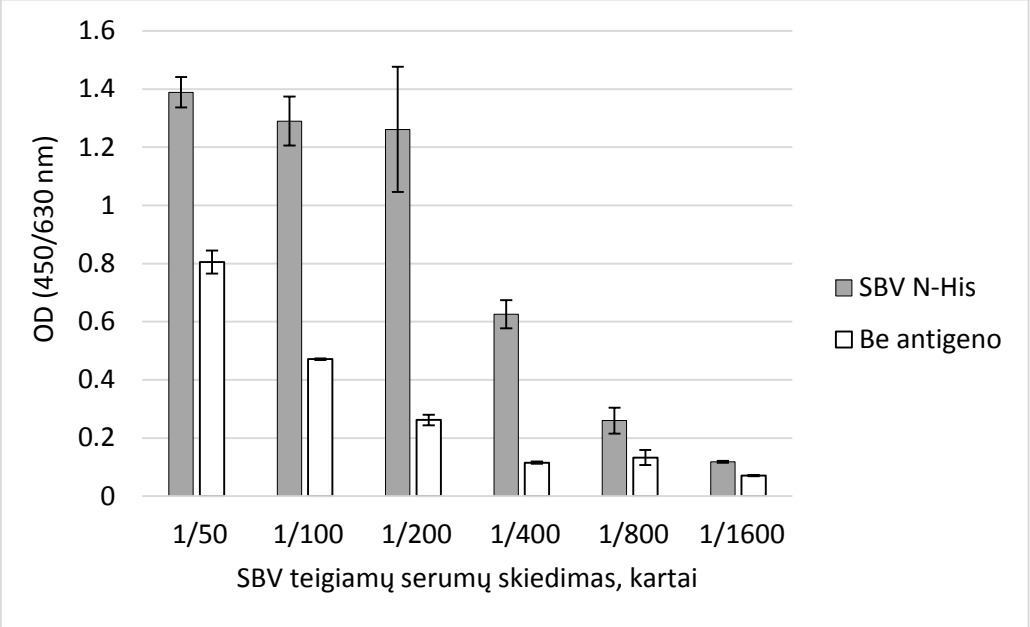
Kuriant netiesioginę IFA, skirtą aptikti SBV specifiškus antikūnus karvių serumuose, buvo pabandyta išsiaiškinti optimalią SBV N-His antigeno koncentraciją, reikalingą analitinės plokštelės padengimui. Naudojant 4 μg/ml

SBV N-His baltymo koncentraciją (15 pav.) nustatytas optimalus 200 kartų serumų skiedimas plovimo buferiu (16 pav.). Santykiu 1/100 skiesti serumai lėmė tokio pačio stiprumo aptikimo signalą, kaip ir 1/200 santykiu skiesti serumai, tačiau 1,9 karto smarkesnį foninį signalą, nei 1/200 santykiu skiesti serumai (16 pav.).



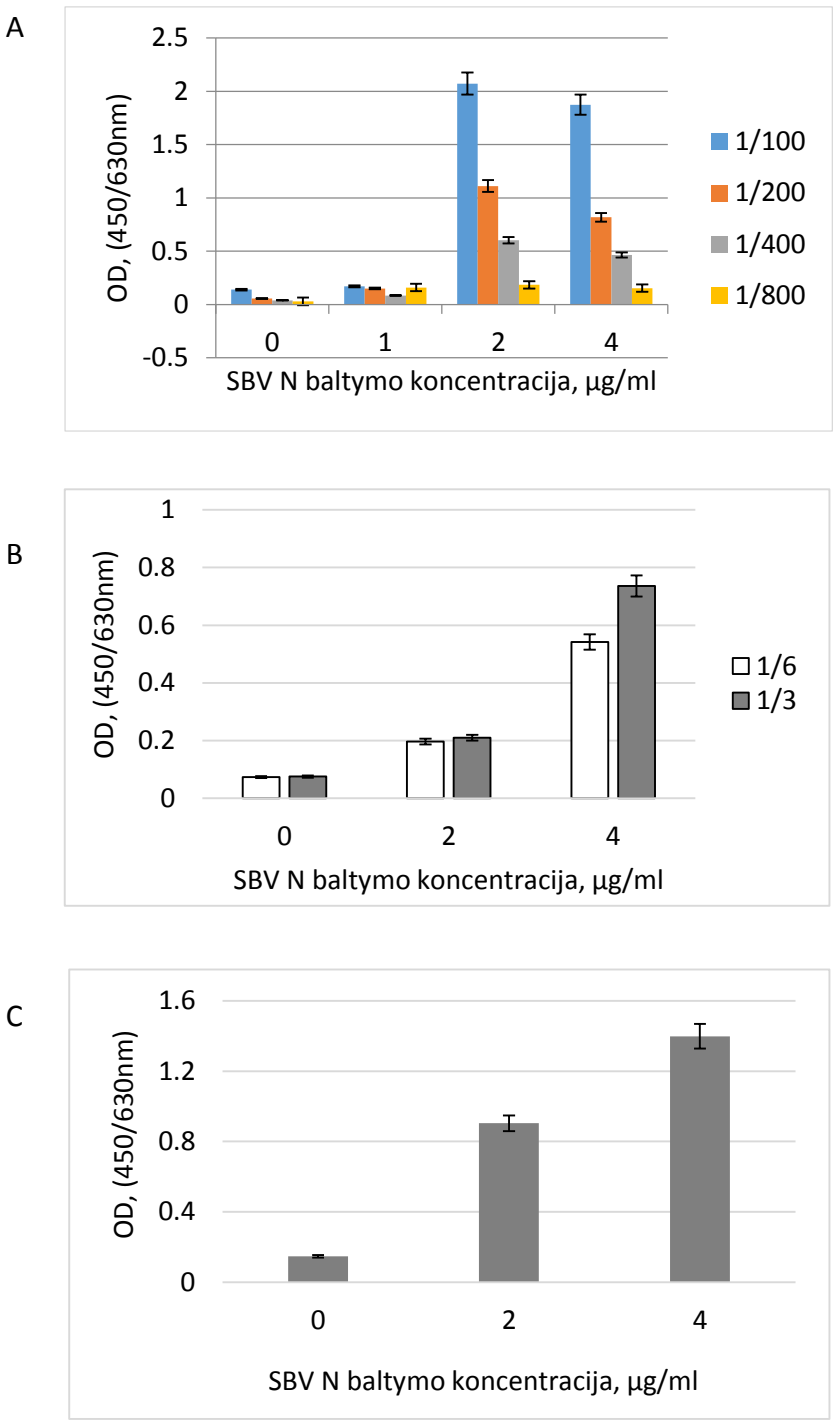
15 paveikslas. Optinio tankio priklausomybė nuo SBV N-His koncentracijos SBV IgG IFA teste. Antigenu naudotas 4 µg/ml arba 1 µg/ml koncentracijos SBV N-His baltymas. Antriniai antikūnai prieš jaučio IgG, žymėti HRP, buvo skiesti 1/10000 PBS buferyje. Serumo mėginys skiestas 200 kartų. Ribos kiekvieno stulpelio viršuje žymi standartinę vidurkio paklaidą.

Riba, skirianti teigiamus ir neigiamus mėginius, buvo nustatyta įvertinus 11 neigiamų serumų reakciją su SBV N-His baltymu IFA testo metu. Šių mėginių parodyti optinių tankių vienetai padalinti iš teigiamo mėginio optinio tankio ir gautas S/P rodiklis, išreikštas procentais (Bréard ir kt., 2013). Prie visų S/P verčių vidurkio pridėjus du šių verčių standartinius nuokrypius gauta S/P=32 % riba, virš kurios esantys mėginiai yra laikomi teigiamais.



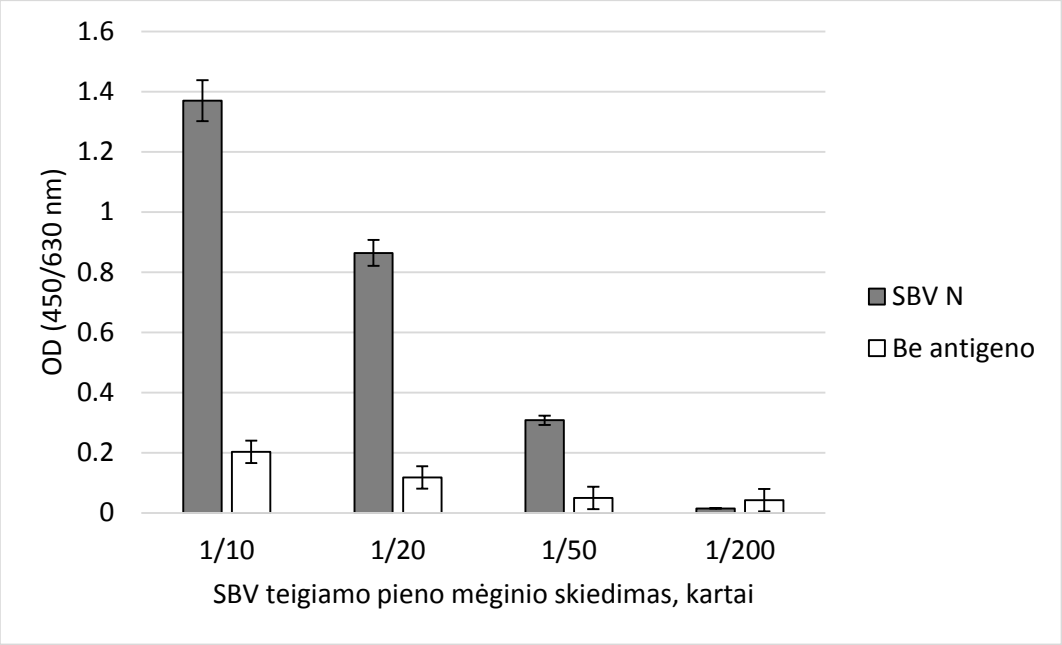
16 paveikslas. Optinio tankio priklausomybė nuo SBV N-His koncentracijos ir SBV teigiamų serumų skiedimo kartų SBV IgG IFA teste. Antigenu naudotas SBV N-His baltymas. Antriniai antikūnai prieš jaučio IgG, žymėti HRP, buvo skiesti 1/10000 PBS buferyje. Ribos kiekvieno stulpelio viršuje žymi standartinę vidurkio paklaidą.

Kuriant naujas imunofermentinės analizės sistemas reikėjo išsiaiškinti optimalias tiriamų mėginių ir antigenų koncentracijas. Nustatyta optimali 2 μg/ml SBV N baltymo koncentracija SBV pieno IgG, serumo IgG ir seilių IgA testams ir 4 μg/ml SBV N koncentracija seilių IgG SBV testui (17 pav.).



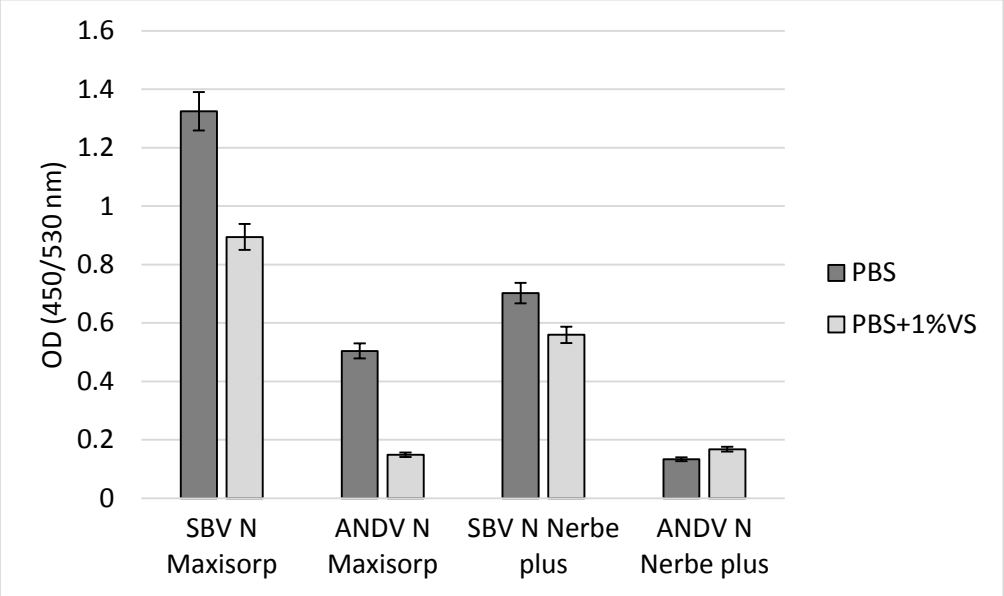
17 paveikslas. Optinio tankio priklausomybė nuo antigeno koncentracijos serumo IgG (A), seilių IgG (B) ir seilių IgA (C) IFA testuose. SBV N baltymo koncentracija pažymėta X ašyje, reakcijų optinių tankių vertės pažymėtos Y ašyje. (A) Mėlyna, oranžinė, pilka ir geltona stulpelių spalva žymi 100, 200, 400 ir 800 kartus skiestą serumą. Antriniai antikūnai prieš jaučio IgG, žymėti HRP, buvo skiesti 1/10000 PBS buferyje. (B) Šviesiai pilka ir tamsiai pilka stulpelių spalva žymi 6 ir 3 kartus skiestus seilių mėginius. Antriniai antikūnai prieš jaučio IgG, žymėti HRP, buvo skiesti 1/30000 plovimo buferyje. (C) Antriniai antikūnai prieš jaučio IgA, žymėti HRP, buvo skiesti 1/20000 plovimo buferyje. Ribos kiekvieno stulpelio viršuje žymi standartinę vidurkio paklaidą.

Kuriamose analizės sistemose serumo mėginiai buvo skiedžiami santykiu 1/200, pieno mėginiai buvo skiedžiami 1/10, o seilių – 1/3 (17 pav. A ir B, 18 pav.).



18 paveikslas. Optinio tankio priklausomybė nuo mėginio skiedimo kartų pieno IgG IFA teste. SBV N – natyviomis sąlygomis grynintas SBV N baltymas. SBV N-His – denatūruojančiomis sąlygomis grynintas SBV N baltymas. Ribos kiekvieno stulpelio viršuje žymi standartinę vidurkio paklaidą.

Nerbe Plus 96 šulinėlių plokštelės buvo pasirinktos seilių IgA, serumų IgG ir pieno IgG testams, o Nunc MaxiSorp plokštelės seilių IgG testui (19 pav.). Seilių mėginiai ir antriniai antikūnai buvo skiesti PBS buferyje, turinčiame 5 % RotiBlock blokavimo reagento ir 1 % viščiuko serumo. Šie priedai leido sumažinti nespecifinių reakcijų su kontroliniais baltymais kiekį ir gauti didesnį skirtumą tarp teigiamų ir neigiamų mėginių optinių tankių (19 pav.).



19 pav. Skirtingų plokštelių ir skiedimo buferių įtaka seilių antikūnų reaktyvumui su SBV N baltymu SBV seilių IgG teste. Naudotos Nerbe plus ir Maxisorp plokštelės. Seilių mėginiai ir antriniai antikūnai prieš jaučio IgG konjuguoti HRP skiesti PBS – fosfatiniame buferyje, arba PBS+1 % VS – PBS su 5 % Roti-block ir 1 % viščiuko serumu. Ribos kiekvieno stulpelio viršuje žymi standartinę vidurkio paklaidą.

Testų riba, skirianti teigiamus ir neigiamus mėginius, buvo išreikšta kaip tiriamojo mėginio ir teigiamo mėginio santykis „S/P“ procentais. Šiai ribai suskaičiuoti SBV N serumo IgG sistemoje buvo naudoti 27 neigiami serumai. Jų optiniai tankiai buvo padalinti iš teigiamo mėginio optinio tankio ir gautos S/P reikšmės panaudotos formulėje:

$$Riba = \frac{x_1 + x_2 + \cdots + x_n}{n} + 3 \times SD(x_1, x_2, \ldots, x_n),$$

kur x yra S/P mėginio reikšmė, n – mėginių kiekis, SD – mėginių S/P reikšmių standartinis nuokrypis.

Pieno ir seilių IFA testams ribinių verčių nustatymui buvo naudojamas kontrolinis Andes hantaviruso antigenas. Jo reakcijos su pieno ar seilių mėginiais optiniai tankiai buvo padalinti iš teigiamų mėginių, reagavusių su SBV N baltymu, optinio tankio. Tokiu būdu gautos S/P reikšmės, kurios

naudotos aukščiau aprašytoje formulėje. S/P ribinės vertės pieno IgG, serumų IgG, seilių IgA ir seilių IgG buvo atitinkamai 30 %, 28 %, 15 % ir 10,5 %.

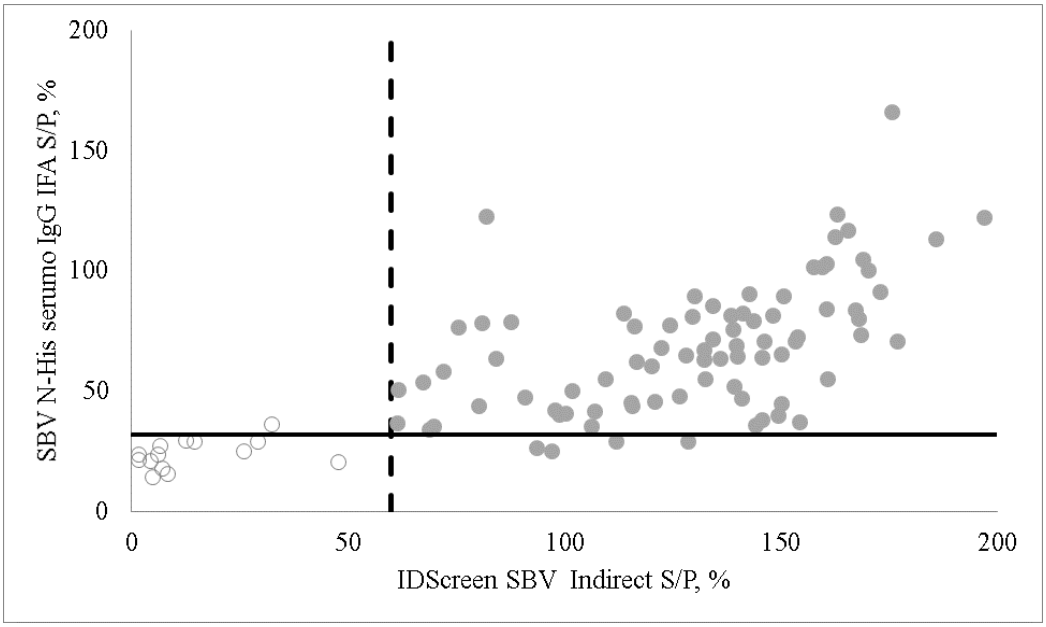
Naujos SBV specifiškus antikūnus aptinkančios sistemos buvo sukurtos naudojant rekombinantinius SBV nukleokapsidės baltymus. Viena IFA sistema buvo sukurta naudojant SBV N-His baltymą ir buvo skirta SBV specifiškų antikūnų aptikimui karvių serumuose. Likusios IFA sistemos buvo sukurtos naudojant SBV N baltymą ir naudotos SBV specifiškų antikūnų aptikimui karvių serumuose, seilėse bei piene.

SBV specifiškų antikūnų aptikimas galvijų mėginiuose

Komerciniai anti-SBV antikūnų aptikimo rinkiniai buvo naudojami įvertinti SBV paplitimą galvijuose. Naujai sukurtų IFA sistemų rezultatai buvo palyginami su komerciniais rinkiniais gautais rezultatais. Naujai sukurtų seilių IFA rezultatai buvo palyginami su komerciniu serumų SBV IgG rinkiniu, nes rinkoje nėra komercinio anti-SBV IgG ir IgA galvijų seilėse aptinkančio analogo.

SBV specifiškų IgG antikūnų aptikimas karvių serumuose

2013 metų pavasarį Lietuvos sveikatos mokslų universiteto Veterinarijos akademijos darbuotojai surinko 97 karvių kraujo serumus iš įvairių Lietuvos ūkių. Šie mėginiai ištirti komerciniu „ID Screen® Schmallenberg virus Indirect“ testu. Nustatyta, jog 82 serumų mėginiai turėjo SBV specifiškų antikūnų (20 pav.). Tie patys 97 mėginiai buvo patikrinti naujai sukurta SBV N-His IFA sistema. 78 iš 82 teigiamų serumų buvo nustatyti kaip teigiami SBV IFA metu, o 4 buvo neigiami (20 pav.). Šios IFA sistemos jautrumas yra 95 % (78 ÷ 82 × 100 %). Iš 15 neigiamų serumų IFA sugebėjo aptikti 14, o vieną serumą įvertino kaip teigiamą, todėl šios IFA sistemos specifiškumas yra 93 % (14 ÷ 15 × 100 %). Koreliacijos koeficientas tarp šių testų optinių tankių buvo 0,703061.



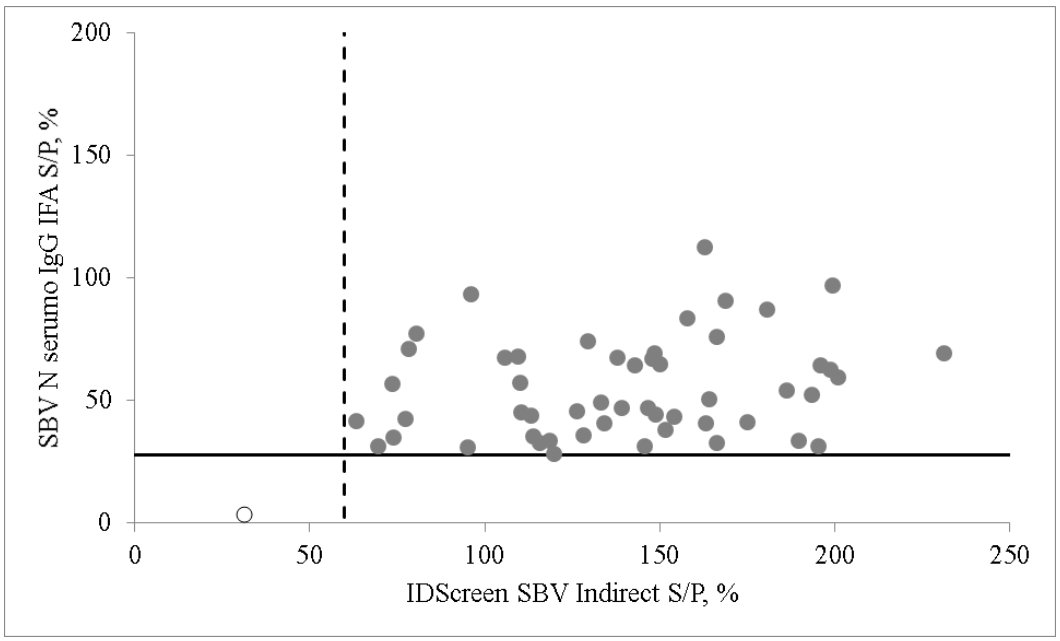
20 paveikslas. Karvių serumų mėginių, tirtų SBV N-His serumo IgG IFA testu ir „ID Screen SBV Indirect“ IFA testu, S/P reikšmių palyginimas. Komercinio testo nustatyti teigiami mėginiai pažymėti pilkais skrituliais, o neigiami – tuščiaviduriais. Ištisinė horizontali linija žymi laboratorinio testo ribą tarp teigiamų ir neigiamų reikšmių, brūkšninė linija – ribą tarp komercinio testo teigiamų ir neigiamų reikšmių.

2013 metų rudenį surinkti 312 karvių kraujo serumai iš įvairių Lietuvos ūkių ištirti anksčiau minėtu komerciniu rinkiniu, kuriuo nustatyta, jog 284 serumų mėginiai turėjo SBV specifiškų antikūnų – tai sudaro 88,47 % visų mėginių (1 lentelė).

1 lentelė. SBV užsikrėtusių karvių pasiskirstymas Lietuvos ūkiuose 2013 metais.

Rajonas	Mėginių skaičius			Ūkių skaičius
	Teigiami	Neigiami	Viso	
Kaišiadorių	73	16	89	4
Marijampolės	95	3	98	5
Jonavos	27	2	29	2
Tauragės	51	6	57	3
Kėdainių	19	0	19	1
Prienų	19	1	20	1
Viso	284	28	312	16

Naudojant natyviai grynintu SBV N baltymu pagrįstą testą buvo tirti 2013 metais surinkti karvių serumų mėginiai. 58 karvių serumo mėginius patikrinus komerciniu „ID Screen® Schmallenberg virus Indirect“ rinkiniu nustatyta, jog 54 mėginiai buvo teigiami, 1 buvo neigiamas, o likę 3 – abejotini. Abejotini mėginiai tolimesniuose tyrimuose nebuvo naudoti. Vėliau likę 55 serumo mėginiai patikrinti natyviai išgrynintu SBV N baltymu pagrįstu testu. 52 iš 54 teigiamų mėginių taip pat buvo nustatyti kaip teigiami, vienas mėginys abiejuose testuose buvo neigiamas, o du komercinio testo metu identifikuoti teigiamas mėginiai SBV N testo buvo nustatyti kaip abejotini (21 pav.). SBV N testo jautrumas lyginant su komerciniu testu yra 96,30 % (95 % patikimumo intervalas CI: 87,25 % - 99,55 %). Tų pačių mėginių optinių tankių koreliacija skirtinguose testuose buvo r = 0,375436.



21 paveikslas. Karvių serumų mėginių, tirtų SBV N serumo IgG IFA testu ir „ID Screen SBV Indirect“ IFA testu, S/P reikšmių palyginimas. Komercinio testo nustatyti teigiami mėginiai pažymėti pilkais skrituliais, o neigiami – tuščiaviduriais. Ištisinė horizontali linija žymi laboratorinio testo ribą tarp teigiamų ir neigiamų reikšmių, brūkšninė linija – ribą tarp komercinio testo teigiamų ir neigiamų reikšmių.

Natyviai grynintu SBV N baltymu pagrįstu testu buvo ištirti ir 2014 metais surinkti 490 karvių serumai. Šiuo atveju tik 82 mėginiai buvo teigiami (16,73 %), o 398 (83.27 %) – neigiami (2 lentelė).

2 lentelė. SBV užsikrėtusių karvių pasiskirstymas Lietuvos ūkiuose 2014 metais.

Rajonas	Mėginių skaičius			Ūkių skaičius
	Teigiami	Neigiami	Viso	
Šakių	26	64	90	2
Panevėžio	2	38	40	1
Pakruojo	10	70	80	2
Marijampolės	23	57	80	2
Kėdainių	4	36	40	1
Kalvarijų	2	38	40	1
Jonavos	2	38	40	1
Šiaulių	3	37	40	1
Šalčininkų	10	30	40	1
Viso	82	408	490	12

Šio eksperimento metu pastebėta, jog suaugusios karvės buvo dažniau užsikrėtę SBV (27,4 %), nei pirmametės karvės (6,4 %) (3 lentelė). Įvertinus gautus rezultatus Chi kvadrato dažnių lentelės testu nustatyta, jog skirtumai tarp amžiaus grupių yra reikšmingi ($X^2 = 22,4\%$, df = 3, $p < 0,001$).

3 lentelė. SBV infekcijos paplitimas skirtingose karvių amžiaus kategorijose Lietuvoje 2014 metais.

Amžius	Mėginių skaičius		Viso	Teigiami mėginiai, %
6-12 mėn.	Teigiami	8	125	6,40
	Neigiami	117		
12-24 mėn.	Teigiami	15	119	12,61
	Neigiami	104		
Pirmaveršės karvės	Teigiami	25	122	20,49
	Neigiami	97		
Suaugusios karvės	Teigiami	34	124	27,42
	Neigiami	90		

Naujai sukurta SBV IFA buvo ištirti Molekulinės biologijos ir genetikos instituto Ukrainoje darbuotojų 2014 metais iš Ukrainos ūkių surinkti 522 karvių serumai. Iš jų 62 serumuose pavyko aptikti SBV specifiškus antikūnus, tai sudaro 11,87 % visų mėginių (4 lentelė).

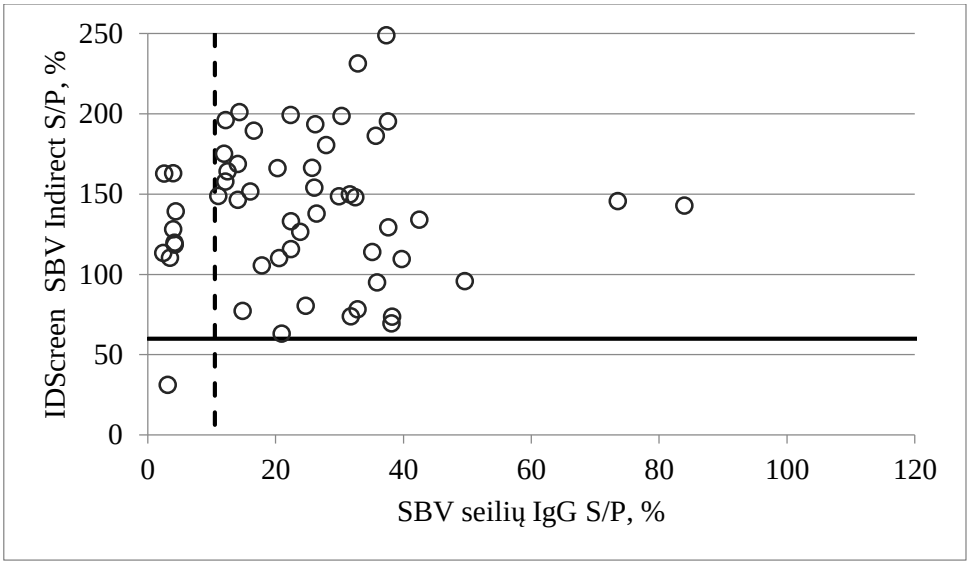
4 lentelė. SBV užsikrėtusių karvių pasiskirstymas Ukrainos ūkiuose 2014 metais.

Rajonas	Mėginių skaičius			Ūkių skaičius
	Teigiami	Neigiami	Viso	
Vinnytsia	2	31	33	3
Zaporozhye	4	6	10	1
Chernihiv	4	37	41	3
Poltava	15	79	94	8
Zhytomyr	5	46	51	4
Kirovohrad	8	21	29	1
Cherkasy	4	74	78	6
Khmelnytsky	1	19	20	2
Sumy	0	39	39	3
Kharkiv	1	29	30	1
Rivne	3	12	15	2
Mykolaiv	7	3	10	1
Ivano-Frankivsk	1	20	21	2
Ternopil	7	24	31	3
Lviv	0	10	10	1
Dniproptrovsk	0	10	10	1
Viso	62	460	522	42

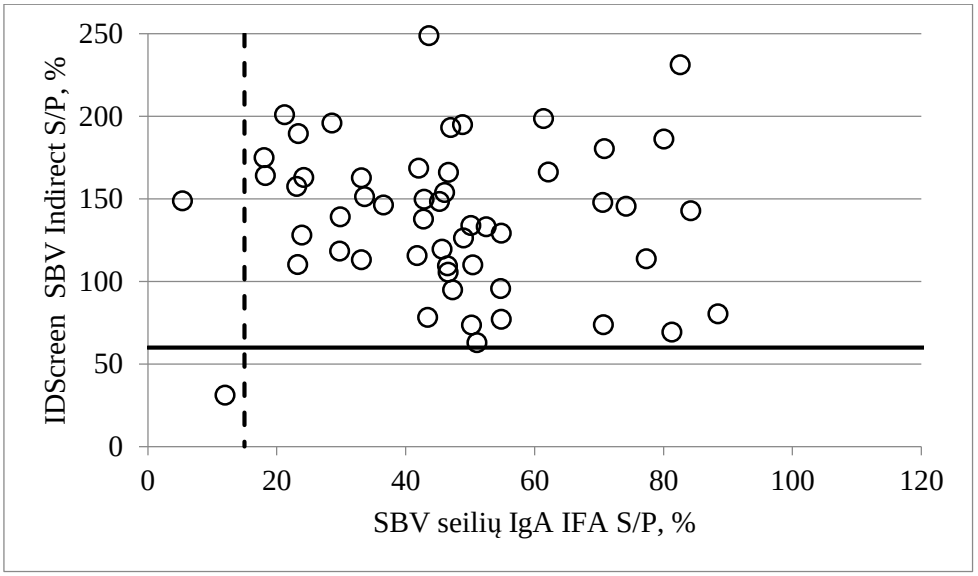
SBV specifiškų IgG ir IgA antikūnų aptikimas karvių seilėse

55 karvių serumai buvo patikrinti komerciniu „ID Screen® Schmallenberg virus Indirect“ testu. 54 karvių serumai turėjo antikūnų prieš SBV, o vienas serumas jų neturėjo. Tų pačių karvių seiles patikrinus seilių IgG testu, SBV specifiškus IgG antikūnus pavyko aptikti tik 46 seilių pavyzdžiuose (5 lentelė B). 9 karvių seilių mėginiuose SBV specifiškų antikūnų aptikti nepavyko, nors 8 iš šių karvių turėjo SBV specifiškų antikūnų kraujyje (22 pav. A). SBV seilių IgG testo jautrumas buvo 85,19 % (95 % CI: 72,88 % - 93,38 %). Testo specifiškumas buvo 100 %, tačiau tiksliam specifiškumo nustatymui reikėtų didesnio sveikų karvių mėginių skaičiaus. Tiriant tų pačių seilių IgA antikūnus, paaiškėjo, jog 53 karvės turi SBV specifiškus IgA antikūnus. Du seilių mėginiai nerodė SBV IgA antikūnų, nors viena iš šių karvių turėjo SBV specifiškų antikūnų kraujyje (5 lentelė C, 22 pav. B). SBV seilių IgA testo jautrumas buvo 98,15 % (95 % CI: 90,11 % - 99,95 %).

A



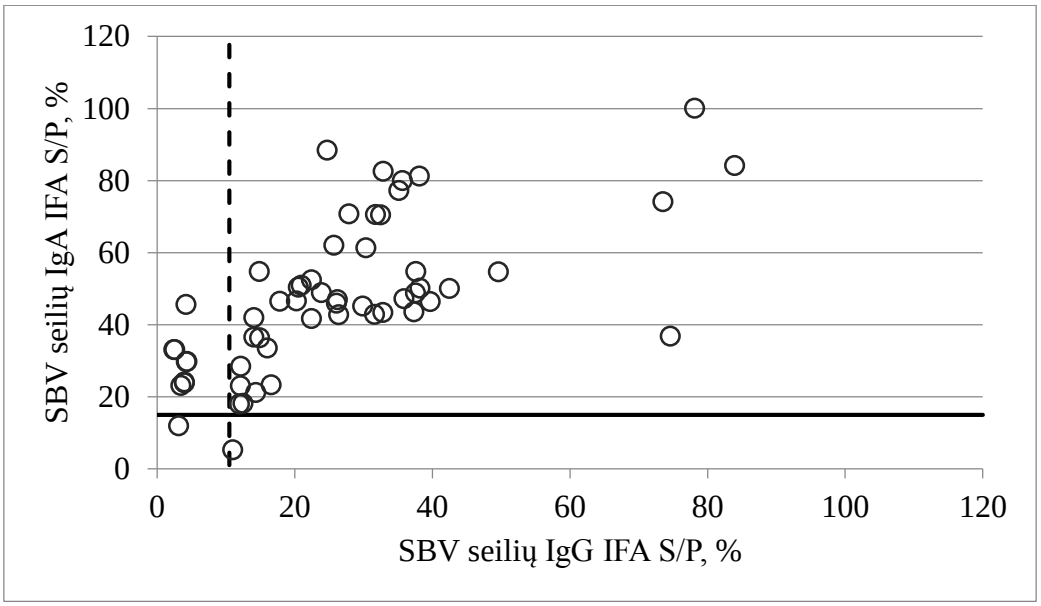
B



22 paveikslas. Galvijų seilių ir serumų mėginių, tirtų SBV seilių IgG (A), IgA (B) IFA testais bei „ID Screen SBV Indirect“ IFA testu (A ir B), S/P verčių palyginimas. Vertikali linija žymi SBV seilių IgG (A) arba IgG (B) IFA ribą tarp teigiamų ir neigiamų verčių, o horizontali (A ir B) – komercinės IFA ribą.

Palyginus tarpusavyje SBV seilių IgG ir IgA testus paaiškėjo, kad seilių IgG testas atitinka seilių IgA testą 84,91 % (95 % CI: 72,41 % - 93,25 %). Tarp

seilių IgG ir IgA testų optinių tankių verčių nustatyta koreliacija – koreliacijos koeficientas lygus 0,665204 (23 pav.).



23 paveikslas. Galvijų seilių mėginių, tirtų SBV seilių IgG ir IgA IFA testais, S/P verčių palyginimas. Vertikali linija žymi SBV seilių IgG IFA ribą tarp teigiamų ir neigiamų verčių, o horizontali – seilių SBV IgA IFA ribą.

Viename seilių mėginyje neaptikti SBV specifiški IgA antikūnai, nors SBV specifiškus IgG antikūnus jame aptikti pavyko (5 lentelė D).

5 lentelė. Sukurtų SBV netiesioginių IFA sistemų palyginimas su komerciniais testais.

(A)

		„ID Screen® SBV Milk Indirect“		
		Teigiami	**Neigiami**	**Viso**
Pieno IgG netiesioginė IFA	Teigiami	48	0	48
	Neigiami	3	1	4
	Viso	51	1	52

(B)

		„ID Screen® SBV Indirect“		
		Teigiami	**Neigiami**	**Viso**
Seilių IgG netiesioginė IFA	Teigiami	46	0	46
	Neigiami	8	1	9
	Viso	54	1	55

(C)

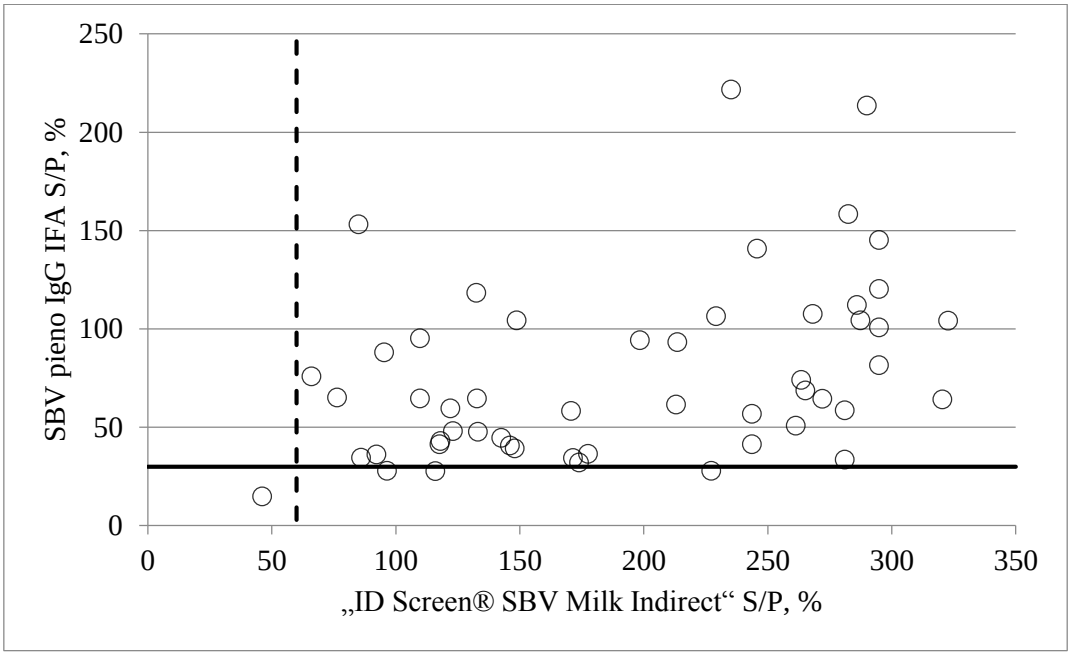
		„ID Screen® SBV Indirect“		
		Teigiami	**Neigiami**	**Viso**
Seilių IgA netiesioginė IFA	Teigiami	53	0	53
	Neigiami	1	1	2
	Viso	54	1	55

(D)

		Seilių IgA netiesioginė IFA		
		Teigiami	**Neigiami**	**Viso**
Seilių IgG netiesioginė IFA	Teigiami	45	1	46
	Neigiami	8	1	9
	Viso	53	2	55

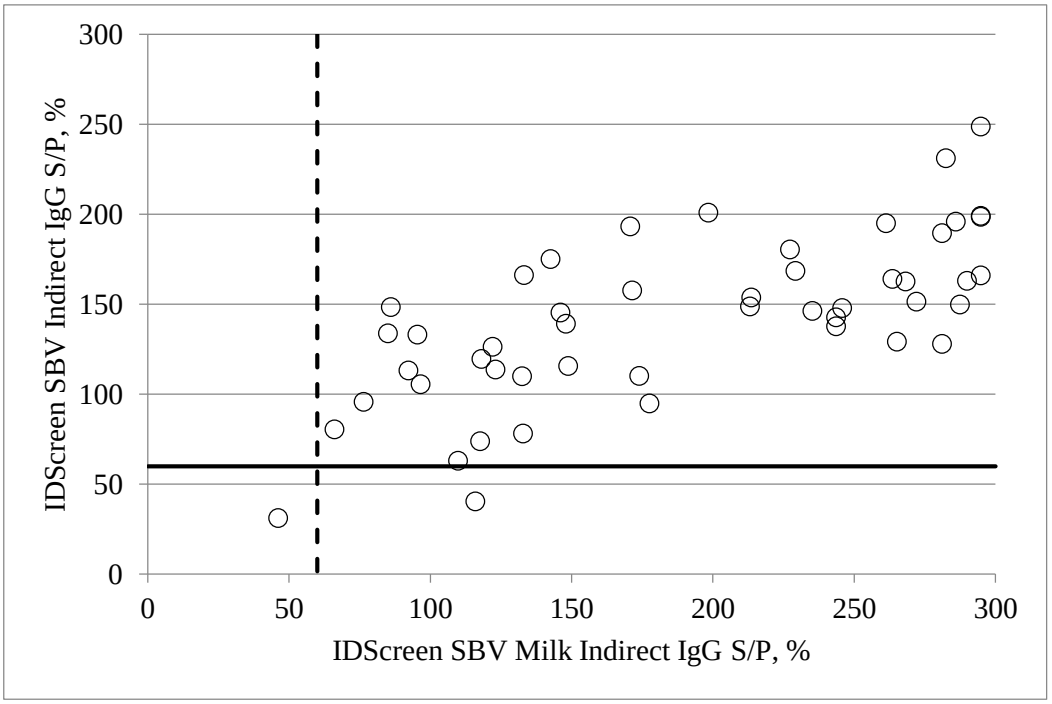
SBV specifiškų IgG antikūnų aptikimas karvių piene

58 karvių pieno mėginiai buvo ištirti komerciniu „ID Screen® Schmallenberg virus Milk Indirect“ testu. 6 mėginiai identifikuoti kaip abejotini ir tolimesniuose eksperimentuose nenaudoti. 51 mėginys buvo teigiamas, o vienas – neigiamas. Ištyrus tuos pačius mėginius SBV N baltymo pagrindu sukurta IFA, SBV specifiškų antikūnų pavyko aptikti 48 mėginiuose, viename mėginyje IgG nerasta, o 3 mėginiai buvo abejotini. Visi 48 teigiami mėginiai komercinio testo taip pat buvo identifikuoti kaip teigiami (5 lentelė A). SBV N pieno mėginių IFA testo jautrumas siekė 94,12 % (95 % CI: 83,76 % - 98,77 %), o SBV N pieno mėginių IFA testo optinių tankių priklausomybė nuo komercinio „ID Screen® Schmallenberg virus Milk Indirect“ testo optinių tankių yra nedidelė – koreliacijos koeficientas 0,387307 (24 pav.).



24 paveikslas. Galvijų pieno mėginių, tirtų SBV pieno IgG IFA testu ir „ID Screen SBV Milk Indirect“ IFA testu, S/P verčių palyginimas. Horizontali linija žymi SBV pieno IgG IFA ribą tarp teigiamų ir neigiamų verčių, o vertikali – komercinės IFA ribą.

Komerciniais pieno ir serumo IgG rinkiniais nustatyta, jog galvijai, turintys kraujyje SBV specifiškų IgG antikūnų, yra linkę jų turėti ir piene. Iš tų pačių galvijų surinkti pieno ir serumo mėginiai koreliavo savo reakcijos intensyvumu abiejuose testuose – nustatytas koreliacijos koeficientas r = 0,659339 (25 pav.).



25 paveikslas. Galvijų serumo ir pieno mėginių S/P reikšmių, apskaičiuotų naudojant komercinius pieno ir serumo „IDScreen“ testus, palyginimas. Horizontali linija žymi komercinio serumo rinkinio ribą tarp teigiamų ir neigiamų verčių, o vertikali – komercinio pieno rinkinio ribą tarp teigiamų ir neigiamų verčių.

REZULTATŲ APTARIMAS

Ankstesni rekombinantinių virusinių baltymų tyrimai atskleidė, jog mielėse susintetinti hantavirusų N baltymai, sujungti su His inkarine seka, yra tinkami jautrioms antikūnų prieš hantavirusus aptikimo sistemoms sukurti (Petraityte ir kt., 2007). Panaši mielių baltymų sintezės sistema panaudota šiame darbe siekiant pagaminti Schmallenberg viruso rekombinantinius N baltymus. Mielių raiškos sistemos yra pajėgios pagaminti rekombinantinius baltymus, kurių išeiga siekia kelis miligramus iš gramo mielių. Šiame darbe iš 1 g mielių pavyko išgryninti 3,0–3,5 mg SBV N-His baltymo ir 1,3 mg SBV N baltymo. Tai atitinka hantavirusų rekombinantinių N baltymų išeigą (Razanskiene ir kt., 2004). Kai kurių kitų virusų baltymai taip pat sėkmingai gali būti sintetinami mielėse – kiaulytės viruso nukleokapsidės baltymo buvo išgryninta 2,1 mg iš 1 gramo *P. pastoris* mielių (Slibinskas ir kt., 2003), o įvairių žmogaus poliomos virusų VP1 baltymų išeiga siekia 0,44 – 1,05 mg iš gramo *S. cerevisiae* mielių (Norkiene ir kt., 2015). Šiuo požiūriu SBV N baltymas buvo išgrynintas sėkmingai, tačiau chimerinio N_G2 baltymo išeiga yra maža. SBV N_G2ΔH-NHis išgryninta 0,15 mg iš 1 g mielių, o SBV N_G2-CHis – 0,3 mg iš 1 gramo mielių. Įtakos chimerinių baltymų sintezei galėjo turėti ir His uodega. C gale žymėto N_G2ΔH baltymo ir N gale žymėto N_G2 baltymo sintezė visai nebuvo stebima mielių lizatų analizės NDS-PAGE metu. Kitų darbų metu buvo parodyta, jog His uodegos pozicija baltyme gali lemti rekombinantinio baltymo stabilumą. C gale žymėtas *Agrocybe aegerita* ubikvitiną konjuguojantis E2 baltymas buvo mažiau stabilus, nei analogiškas N gale žymėtas baltymas (Li ir kt., 2013).

Kiti mokslininkai yra nustatę, kad hantavirusų N baltymai, išgryninti denatūruojančiomis sąlygomis, sukėlė didesnį imuninį atsaką ruduosiuose pelėnuose, nei natyviomis sąlygomis gryninti N baltymai (Dargeviciute ir kt., 2002). Šio darbo metu pelių imunizacijai naudoti SBV N-His baltymai, kurie gryninti denatūruojančiomis sąlygomis, o vėliau renatūruoti acetatiniame buferiniame tirpale. Denatūracijos–renatūracijos ciklas galėjo paveikti baltymo

erdvinę struktūrą – yra žinoma, jog N baltymas turi tris cisteino aminorūgšties liekanas savo grandinėje, kurios gali formuoti disulfidinius tiltelius. Gautais monokloniniais antikūnais parodyta, jog jie atpažįsta natyviu virusu užkrėstas žinduolių ląsteles. Pagal tai galima spręsti, jog SBV N-His baltymas yra panašus į gamtoje aptinkamo viruso nukleokapsidės baltymą ir turi panašius antikūnų atpažįstamus epitopus. VU BTI Imunologijos skyriaus darbuotojų buvo parodyta, jog monokloniniai antikūnai reaguoja su SBV N-His baltymu imunoblotingo analizės metu, todėl galima numanyti, jog šie MAk atpažįsta linijinius epitopus. Stebint SBV N-His ir SBV N baltymų pavyzdžius elektroniniu mikroskopu nebuvo stebimos į nukleokapsides panašios struktūros. Kiti mokslininkai yra pastebėję, jog rekombinantiniai SBV N baltymai gali formuoti heksamerus ir tetramerus *in vitro* (Dong ir kt., 2013).

Aukšti SBV N-His IFA sistemos jautrumo ir specifiškumo įvertinimai leidžia kelti klausimą, ar ji yra tinkama SBV specifiškiems antikūnams serumuose aptikti. Reikėtų atsižvelgti į tai, jog komercinis testas, su kuriuo buvo lyginta naujai sukurta SBV IFA, gali aptikti ir gimininių Simbu grupės Aino, Akabane, Douglas, Peaton ar kitų virusų infekcijos metu susidariusius antikūnus, todėl teigti, kad nauja SBV IFA atpažįsta antikūnus tik prieš SBV būtų neteisinga.

Neatitikimai tarp komercinio ir SBV N-His IFA testų reiškia, kad šią IFA dar būtų galima tobulinti. Maža priklausomybė tarp naujai sukurtos IFA optinių tankių ir komercinio testo rezultatų gali būti paaiškinta skirtingų N baltymo epitopų atpažinimu šiose sistemose – komercinis testas naudoja *E. coli* susintetintus SBV N baltymus (Bréard ir kt., 2013). Kita vertus, komercinis testas taip pat atpažįsta ne visus SBV antikūnų turinčius mėginius. Pavyzdžiui, PRNT (apvalių plokščių plotelių susidarymo sumažinimo bandymas, angl. *plaque reduction neutralization assay*) yra jautresnis metodas nei IFA, nes geba atpažinti ir antikūnus, specifiškus kitiems struktūriniams SBV baltymams (Mansfield ir kt., 2013). Deja, šio tyrimo metu nebuvo galimybės atlikti PRNT ar kitą virusų neutralizacijos testą, kuris padėtų tiksliau įvertinti sukurto testo

jautrumą ir specifiškumą. SBV N-His IFA būtų galima patobulinti naudojant SBV paviršiaus glikoproteinus, kaip buvo parodyta kitų autorių, kurie panaudojo visą virusą kaip antigeną detekcijos sistemoje (Näslund ir kt., 2014). Disertacijos metu vykdytų tyrimų metu išgrynintas SBV N_G2-CHis baltymas netiko IFA sistemų kūrimui. Su šiuo baltymu vienodai stipriai reagavo tiek SBV specifiškų antikūnų turinčios, tiek šių antikūnų neturinčio karvės serumų mėginiai. SBV N_G2ΔH-NHis baltymo išeiga yra per maža, kad pateisintų šio baltymo panaudojimą antigenu, todėl šio baltymo, kaip antigeno, savybės tirtos nebuvo. Tolesniuose tyrimuose chimeriniai SBV baltymai nebuvo naudoti. Kitų mokslininkų tyrimai atskleidė, jog monokloniniai antikūnai, sukurti pelėse jas inokuliavius SBV užkrėstomis BHK ląstelėmis, buvo specifiški N arba G1 baltymams, o G2 baltymą atpažindavo tik jam esant komplekse su G1 (Wernike ir kt., 2015), todėl chimerinio baltymo panaudojimas tolimesniuose tyrimuose yra abejotinas.

SBV N IFA pasirodė esanti jautresnė nei SBV N-His IFA. Taip galėjo nutikti dėl skirtingų SBV N ir SBV N-His gryninimo metodikų. SBV N baltymas buvo grynintas natyviomis sąlygomis, todėl galimai išsaugojo natūralias konformacinių epitopų antigenines sritis. Maža koreliacija tarp SBV IFA ir komercinės IFA gali reikšti, jog šiose sistemose serumo antikūnai atpažįsta skirtingus SBV N baltymų epitopus.

2014 metais tirtuose Lietuvos galvijuose buvo pastebėti statistiškai patikimi SBV/Simbu grupės viruso užsikrėtimo dažnio skirtumai priklausomai nuo amžiaus grupės. Vyresnėse karvėse buvo dažniau stebimi SBV/Simbu grupei specifiški antikūnai. Taip galėjo nutikti, nes vyresni galvijai dažniau ganosi laukuose, tuo tarpu jaunikliai laikomi tvartuose, kur mažesnė tikimybė kraujasiurbiams perduoti SBV/Simbu grupės virusą. Panašius skirtumus tarp amžiaus grupių rado mokslininkai Etiopijoje (Sibhat ir kt., 2018). Kita vertus, Olandijoje tokių skirtumų nerasta (Elbers ir kt., 2012). Verta paminėti, jog Elbers ir kolegos galvijus tyrė 2011–2012 metais SBV epidemijos įkarštyje, todėl jaunuose galvijuose galimai aptiko iš motinos gautus SBV specifiškus

antikūnus. Buvo parodyta, jog šie ar kitiems Simbu virusams specifiški antikūnai išsilaiko jaunikliuose iki 6 mėnesių (Elbers ir kt., 2014). Didelis SBV paplitimas buvo stebimas 2012 metais Vokietijoje, Olandijoje, Belgijoje ir Prancūzijoje. Iki 80 % tirtų galvijų Europoje tais metais turėjo antikūnus prieš SBV (Garigliany ir kt., 2012; Zanella ir kt., 2015; Wernike ir kt., 2014; Elbers ir kt., 2012), tačiau SBV infekcijos sumažėjimas Vakarų Europoje buvo stebimas jau 2013–2014 metais (Wernike ir kt., 2014). 2013 metais vakarų Europoje buvo stebimas ir SBV nešiojančių kraujasiurbių *Culicoides* vabzdžių sumažėjimas (Elbers ir kt., 2013). Duomenų apie šių vabzdžių kiekius Lietuvoje nepavyko aptikti. Lietuvos karvėse aukštas antikūnų lygis prieš SBV/Simbu grupės virusą buvo stebimas 2013 metais surinktuose mėginiuose, mažesnis – 2014 metais surinktuose mėginiuose. Nėra duomenų apie 2011–2012 metų SBV/Simbu grupės viruso paplitimą galvijuose Lietuvoje. Gali būti, kad SBV/Simbu grupės viruso infekcijos pikas ir infekcijos sumažėjimas Lietuvoje ir Ukrainoje nesutapo su infekcijos piku ir sumažėjimu Vakarų Europoje.

Po SBV infekcijos SBV specifiški antikūnai gali būti aptinkami ne tik kraujyje, bet ir seilėse, piene. Gimininго viruso antigeno naudojimas pieno IgG IFA sistemos riboms nustatyti buvo neišvengiamas, nes tirtų mėginių imtyje nebuvo pakankamai neužsikrėtusių karvių pieno mėginių. Tos pačios serogrupės virusų, pvz. Schamnoda ar Satuperi virusų, nukleokapsidės baltymai būtų suteikę daugiau informacijos apie SBV IFA sistemos specifiškumą, tačiau dėl galimų anti-SBV antikūnų kryžminių reakcijų su šiais antigenais kyla pavojus sukurti mažai jautrią IFA sistemą. SBV pieno IgG IFA sistemos riboms tarp teigiamo ir neigiamo mėginio nustatyti buvo naudotas Andes hantaviruso nukleokapsidės baltymas. Andes virusas yra gimininگas SBV, jo aminorūgščių sekų panašumas siekia 47 %. Literatūroje yra aprašyti metodai, kurie naudoja kitus nei tiriamasis antigenus testo riboms nustatyti (Starkey ir kt., 1995; Lin ir kt., 2008). Pieno antikūnų analizė yra patogi tuo, jog mėginių rinkimas yra neinvazinis, tačiau reikia atsižvelgti į tai, jog mėginių rinkimas nėra sterilus ir antikūnai juose yra linkę degraduoti. Ryšys tarp SBV specifiškų serumo ir pieno

antikūnų galvijuose buvo parodytas jau anksčiau (Daly ir kt., 2015). Šio darbo metu naudojant komercinį ir naujai sukurtą SBV/Simbu grupės virusų antikūnų detekcijos testą taip pat pavyko parodyti, jog galvijai, turintys SBV specifiškų antikūnų savo serume, dažniausiai jų turi ir piene.

Seilės yra patogus mėginys tirti SBV infekcijos paplitimą, nes gali būti renkamas neinvaziniu būdu. Sunkumų dirbant su seilių mėginiais dažnai kyla dėl to, kad seilėse antikūnų yra mažiau nei piene ar kraujyje, taip pat seilių sudėtis kinta priklausomai nuo jų surinkimo metodo, seilių gausos. Paprastai seilių imunoglobulinų testai turi būti labai jautrūs, norint kompensuoti anksčiau paminėtus trūkumus (Parry ir kt., 1987; McKie ir kt., 2002). Dėl savo savybių gerai aptikti SBV/Simbu grupės virusų antikūnus karvių serumuose, karvių seilių IgG ir IgA antikūnų aptikimui buvo pasirinktas SBV N baltymas. Kaip ir pieno IFA atveju, ribai tarp teigiamo ir neigiamo rezultato nustatyti buvo naudotas Andes viruso nukleokapsidės baltymas. Dėl mažo IgG kiekio galvijų seilėse reikėjo jautrios detekcijos sistemos. Tam buvo panaudotos Maxisorp 96 šulinėlių plokštelės. Anot gamintojo, jos sugeba prisijungti 600-650 ng/cm^2 baltymų, tuo tarpu kitiems testams pakako Nerbe plus 96 šulinėlių plokštelės, kurios prisijungia apie 250 ng/cm^2 baltymo. Taip pat į seilių bei antrinių antikūnų buferius pridėjus 5 % Roti-block ir 1 % viščiuko serumo pavyko sumažinti nespecifinių sąveikų lygį. Su viščiuko serumo baltymais greičiausiai reagavo seilėse esantys nespecifiniai antikūnai, kurie vėlesniuose etapuose būdavo išplaunami. Roti-block yra polimerinis blokavimo reagentas, turintis savo sudėtyje Tween-20. Gamintojas neatskleidžia tikslaus veikimo mechanizmo, tačiau gali būti, jog Tween-20 padeda sumažinti nespecifinių reakcijų lygį.

Didelis seilių IgA IFA testo jautrumas leidžia teigti, jog seilių mėginiai gali būti naudojami kaip alternatyvūs, norint aptikti SBV/Simbu grupės virusų antikūnus karvėse. Ši sistema atpažino 98 % mėginių, gautų iš galvijų, kurių serumuose komerciniu testu pavyko aptikti antikūnų prieš SBV/Simbu grupės virusus. Karvėms panaši sistema buvo panaudota jų seilėse ieškant snukio ir nagų ligos virusui specifiškų antikūnų (Archetti ir kt., 1995; Prickett ir kt., 2010).

Tai yra pirmas kartas, kai parodoma galimybė naudoti seilių mėginius SBV diagnostikoje. Karvių seilių IgG IFA pasirodė mažiau tinkama antikūnų aptikimui, greičiausiai dėl mažesnės IgG antikūnų koncentracijos seilėse (Duncan ir kt., 1972).

IŠVADOS

1. Schmallenberg viruso nukleokapsidės baltymas ir chimerinis glikoproteinas 2, sulietas su nukleokapsidės baltymu, gali būti sintetinami mielėse.
2. Monokloniniai antikūnai prieš rekombinantinį Schmallenberg viruso nukleokapsidės baltymą atpažįsta SBV užkrėstas žinduolių ląsteles.
3. Mielėse susintetinti rekombinantiniai SBV N baltymai yra tinkami SBV ar jam artimo Simbu serogrupės viruso specifiškų antikūnų aptikimui galvijų serume, piene ir seilėse.
4. Schmallenberg viruso nukleokapsidės baltymu pagrįsta imunofermentinė analizė savo galimybėmis aptikti SBV ar jam artimo Simbu serogrupės viruso antikūnus kraujo serume ir piene nenusileidžia komerciniams testams.
5. Schmallenberg ar jam artimo Simbu serogrupės viruso paplitimui nustatyti gali būti naudojami karvių seilėse esantys IgA antikūnai.
6. Schmallenberg ar jam artimas Simbu serogrupės virusas buvo paplitęs Lietuvoje ir Ukrainoje 2013–2014 metais.

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APPENDIX: LIST OF PUBLICATIONS (I,II)

The results of this thesis were presented in following publications and conferences:

Publications:

I. J. Lazutka, A. Zvirbliene, I. Dalgediene, R. Petraityte-Burneikiene, A. Spakova, V. Sereika, R. Lelesius, K. Wernike, M. Beer and K. Sasnauskas, 2014. Generation of Recombinant Schmallenberg Virus Nucleocapsid Protein in Yeast and Development of Virus-Specific Monoclonal Antibodies. Journal of Immunology Research, 2014, pp. 1-8. DOI 10.1155/2014/160316.

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Conferences:

- Justas Lazutka, Rasa Petraityte-Burneikiene, Aliona Spakova, Kestutis Sasnauskas; Generation of Recombinant Schmallenberg Virus Nucleocapsid Protein in Yeast; Global Virus Network conference of virologists for the Scandinavia-Baltic-Ukraine region; Laulasmaa, Estonia, 2014.06.11-2014.06.13.

- Justas Lazutka, Kestutis Sasnauskas, Kerstin Wernike, Aiste Bulavaite, Aliona Spakova; Development of The Detection System to Identify Antibodies Against Schmallenberg Virus in Ruminant Sera; International Union of Microbiological Societies Congresses, XVI-th International Congress of Virology; Montreal, Canada; 2014.07.27-2014.08.01.

- Justas Lazutka, Aliona Spakova, Vilimas Sereika, Raimundas Lelesius, Kestutis Sasnauskas, Rasa Petraityte-Burneikiene; Yeast-Derived Schmallenberg Virus Nucleocapsid Protein as an Antigen to Detect Anti-Viral Antibodies in Bovine Saliva; 27° International Conference on Yeast Genetics and Molecular Biology; Levico Terme, Italy; 2015.08.06-2015.08.12.

I

Generation of recombinant Schmallenberg virus nucleocapsid protein in yeast and development of virus-specific monoclonal antibodies

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Research Article

Generation of Recombinant Schmallenberg Virus Nucleocapsid Protein in Yeast and Development of Virus-Specific Monoclonal Antibodies

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Schmallenberg virus (SBV), discovered in continental Europe in late 2011, causes mild clinical signs in adult ruminants, including diarrhoea and reduced milk yield. However, fetal infection can lead to severe malformation in newborn offspring. To develop improved reagents for SBV serology, a high-level yeast expression system was employed to produce recombinant SBV nucleocapsid (N) protein. Recombinant SBV N protein was investigated as an antigen in SBV-specific IgG enzyme immunoassay and used for generation of monoclonal antibodies (MAbs). Yeast-expressed SBV N protein was reactive with anti-SBV IgG-positive cow serum specimens collected from different farms of Lithuania. After immunization of mice with recombinant SBV N protein, four MAbs were generated. The MAbs raised against recombinant SBV N protein reacted with native viral nucleocapsids in SBV-infected BHK cells by immunofluorescence assay. The reactivity of recombinant N protein with SBV-positive cow serum specimens and the ability of the MAbs to recognize virus-infected cells confirm the antigenic similarity between yeast-expressed SBV N protein and native viral nucleocapsids. Our study demonstrates that yeast expression system is suitable for high-level production of recombinant SBV N protein and provides the first evidence on the presence of SBV-specific antibodies in cow serum specimens collected in Lithuania.

1. Introduction

In 2011, an unidentified disease in cattle was first reported in Germany in a farm near the town of Schmallenberg [1]. Metagenomic analysis identified a novel *Orthobunyavirus*, which subsequently was isolated from blood specimens of infected animals. This new virus was called Schmallenberg virus (SBV) after the place of origin of the collected samples. Clinical symptoms of diseased cows include fever, reduced milk yield, and diarrhoea. Also, SBV infection has been implicated in many cases of severely malformed bovine and ovine offspring [2–7]. The inoculation of 9-month-old calves with blood of cattle that were RT-qPCR positive for SBV caused fever and mucous diarrhoea, providing experimental evidence that SBV might be responsible for the clinical signs observed [1]. Analysis of viral genomic sequences has led to the classification of SBV in the Bunyaviridae family and the *Orthobunyavirus* genus. Recent analysis revealed that SBV is most related to Douglas and Sathuperi virus belonging to the Simbu serogroup of *Orthobunyavirus* genus [8]. The majority of bunyaviruses are transmitted by arthropod vectors. Epidemiological data existing so far are in accordance with the hypothesis that SBV is transmitted by biting midges (*Culicoides* spp.). Recently, some studies have

reported the presence of the SBV genome in different species of *Culicoides* collected in different countries of Europe. It has been reported that some *Culicoides* species are present inside farm buildings during the winter and are able to complete their life cycle in animal enclosures. It is possible that SBV is able to persist from year to year in the vector population despite winter temperatures as described in reviews [6, 7]. The qRT-PCR is the primary diagnostic assay used by laboratories in affected countries [1]. This assay has limitations in detecting infected individuals based on blood samples, as it only detects viral RNA when the animal is viraemic [9]. Furthermore, the virus can be isolated on insect and hamster cell lines. For the detection of SBV-specific antibodies, indirect immunofluorescence tests, microneutralization tests, and commercial SBV-based indirect ELISA have been used [9–12].

The genetic structure of SBV is typical for Bunyaviridae, containing a tripartite RNA genome of negative polarity. The genome of SBV contains three segments of single-stranded negative-sense RNA called the large (L), medium (M), and small (S) segments. The L segment encodes the RNA-dependent RNA polymerase; M segment encodes surface glycoproteins Gn and Gc and nonstructural protein NSm. The S segment encodes nucleocapsid protein N and nonstructural protein NSs [13]. The S segment of SBV was shown to share 96.7% nucleotide sequence similarity with S segment of Shamonda virus. Comparably, the similarity between SBV and Sathuperi virus S segment nucleotide sequence is 94% [14].

The N protein of bunyaviruses is the most abundant viral antigen present in the virion and in the infected cells, thus making it an excellent target for serology [15–17]. Recombinant N proteins of different hantaviruses, generated in *Escherichia coli,* insect cells, or yeast have been widely used for serological diagnosis of hantavirus-specific antibodies in human sera and oral fluid [18–22].

The synthesis of N proteins of different European, Asian, and American hantaviruses in yeast expression system has been shown to result in large yields. The proteins were highly pure after nickel chelation purification and during stable long-term storage. Moreover, the recombinant hantavirus N proteins were strongly immunogenic in rabbits and mice. The yeast-expressed N proteins of different hantaviruses have been employed to develop highly sensitive and specific ELISAs and immunoblot tests [17–22]. Initial studies based on *E. coli* expression systems for hantavirus diagnostics have demonstrated lower specificities of these tests due to *E. coli* contaminants remaining in recombinant protein preparation [23, 24]. These problems were eliminated using yeast expression system [17–22].

Epidemiologic situation in regard to SBV infection may differ greatly from country to country and warrants further study. Indeed, to determine the true occurrence and prevalence of the SBV infection, fast, convenient, and cheap diagnostic tests are needed. In the current study, we have generated the N protein of SBV in yeast expression system, demonstrated its antigenic similarity with viral N protein, and developed N protein-specific MAbs reactive with SBV in infected cells.

2. Materials and Methods

2.1. Strains, Media, Yeast Transformation, and Cultivation. Recombinant construct containing SBV N gene sequence was amplified in *E. coli* DH5αF$'$ cells. *Saccharomyces cerevisiae* AH22-214 *MATa* (*leu2 his4 pep4*) was used for expression of SBV N protein. Selection of yeast transformants resistant to formaldehyde was carried out on the YEPD (1% yeast extract, 2% peptone, and 2% dextrose) agar supplemented with 5 mM formaldehyde. *S. cerevisiae* transformants were grown in YEPD medium supplemented with 5 mM formaldehyde or in YEPG induction medium (1% yeast extract, 2% peptone, and 2,5% galactose) as described previously [25].

2.2. Cloning of SBV N Protein-Encoding Sequences into Yeast Vectors and Purification of Recombinant N Protein from Transformed Yeast. All DNA manipulations were performed according to standard procedures [26]. Enzymes, molecular mass standards, and kits for DNA manipulations were purchased from Thermo Fisher Scientific Baltics (Vilnius, Lithuania). SBV N gene was chemically synthesized by GenScript USA Inc. (Piscataway, NJ, USA) according to the published sequence GenBank accession number HE649914.1 [1]. The XmaJI sites compatible with XbaI site for cloning into yeast expression vectors were inserted into the ends of SBV N gene during gene synthesis. For the generation of N-terminally hexahistidine-tagged SBV N protein, the gene was cloned into XbaI site of the *S. cerevisiae* expression vector FX7-6-His under control of galactose inducible *S. cerevisiae GAL10* promoter described previously [17]. The resulting plasmid pFX7-SBV-6-HisN was used for transformation of yeast *S. cerevisiae* AH22-214, as described previously [25]. The primary structure of the cloned gene was confirmed by sequencing.

Cultivation of transformed yeast cells as well as the expression and purification of recombinant proteins was performed as previously described [17, 25]. Briefly, 100 mL of YEPD growth medium (yeast extract 1%, peptone 2%, and glucose 2%) supplemented with 5 mM formaldehyde was inoculated with the transformed yeast cells and grown with shaking at 30°C for 24 h. After addition of 100 mL of YEPG induction medium (yeast extract 1%, peptone 2%, and galactose 5% supplemented with 5 mM formaldehyde), the yeast cells were grown for additional 17 h. The cells were harvested, washed with distilled water, and frozen at −20°C until further use. Thawed cells were resuspended in 8 mL of disruption buffer (6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 0.5% glycerol, 1% Tween-20, 10 mM imidazole, 2 mM PMSF, and pH 8.0) and 8 g of glass beads (0.5 mm diameter, Sigma-Aldrich Co., St. Louis, MO, USA) was added. Cells were disrupted by vortexing at 4°C for 5 min. The cell debris was sedimented by centrifuging the obtained yeast lysates at 3000 ×g for 5 min. Insoluble proteins were spun down by centrifugation at 10.000 ×g for 10 min at 4°C. The supernatant was mixed with 2 mL of Ni-NTA resin equilibrated in disruption buffer and binding was performed by shaking for 1 h at room temperature (RT). N protein purification was performed on a polypropylene column, according to the manufacturer's recommendations for denaturing purification

of insoluble proteins (Qiagen, Hilden, Germany). The main portion of the protein was eluted in buffer E (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, and pH 4.5). To ensure the purity of the eluted recombinant N protein SDS-PAGE, Coomassie Brilliant Blue staining and western blot were performed. Eluted protein was dialysed against sodium acetate buffer (50 mM sodium acetate, 100 mM sodium chloride, and pH 5.0) and stored at −20°C with 40% of glycerol. Yeast transformant containing the plasmid pFX7-6-His without any insert was used as a negative control.

2.3. SDS-PAGE and Western Blot Analysis. Protein samples were boiled in a reducing sample buffer and separated in a SDS-Tris-glycine buffer through polyacrylamide gel electrophoresis (PAGE). Proteins were visualized by staining with Coomassie Brilliant Blue (Sigma-Aldrich Co.). For western blot, purified proteins were electrotransferred to Roti-PVDF membrane (Carl Roth GmbH & Co., Karlsruhe, Germany). The membrane was blocked with RotiBlock (Carl-Roth GmbH & Co.) blocking solution for 2 h at RT and rinsed in PBS with 0.1% Tween-20 (PBST). The membrane was then incubated for 1 h at RT with primary antibodies at working dilution in PBST with 10% RotiBlock and subsequently incubated with goat anti-mouse IgG (H+L)-HRP conjugate (Bio-Rad, Hercules, CA, USA) 1 : 4000 diluted in PBST with 10% RotiBlock. The enzymatic reaction was developed using 4-chloro-1-naphthol and H_2O_2 (Fluka, Milwaukee, WI, USA). For the analysis of MAb specificity, undiluted hybridoma supernatants were used. To check the purity of recombinant N protein, MAb against 6-His-tag epitope (Thermo Scientific, Rockford, IL, USA) was used as a primary antibody.

2.4. Indirect Enzyme-Linked Immunosorbent Assay (ELISA) for Investigation of SBV N Protein-Specific Mouse Antibodies. Micro test plates (Nerbe Plus GmbH, Winsen/Luhe, Germany) were coated with 100 μL/well of SBV N protein dissolved in the coating buffer (0.05 M sodium carbonate, pH 9.5) to a concentration of 5 μg/mL. The plates were incubated overnight at 4°C. The coated plates were blocked with 250 μL/well of PBS with 2% BSA for 1 h at RT. Then plates were rinsed twice with PBST. Mouse antiserum samples, hybridoma growth medium, or polyclonal antibodies were diluted in PBST, added to the wells (100 μL/well), and incubated for 1 h at RT. The plates were then incubated for 1 h with goat anti-mouse IgG (H+L)-HRP conjugate (Bio-Rad) 1 : 5000 diluted in PBST. The enzymatic reaction was visualized by the addition of 100 μL of "NeA-Blue" TMB solution (Clinical Science Products Inc., Mansfield, MA, USA) to each well. The reaction was stopped by adding 50 μL/well of 1 M H_2SO_4 solution. The optical density (OD) was measured at 450 nm (reference filter 620 nm) in a microplate reader (Sunrise Tecan, Männedorf, Switzerland). The apparent dissociation constants (K_d) of MAbs were determined by an indirect ELISA. The K_d values were calculated from 4 parallel ELISA titration curves fitting logistic model obtained by incubating plate-coated SBV N protein with increasing amounts of MAbs ranging from 1.9×10^{-13} M to 3.3×10^{-8} M. The SD was determined from these 4 calculated K_d values. The K_d for each MAb was defined as the concentration (M) of the MAb that gives one-half of the maximum OD_{450} value.

2.5. Indirect IgG ELISA for Detection of SBV N Protein-Specific Antibodies in Cow Serum. Cow blood samples were collected in May-June 2013 from cow farms located in different places of Lithuania. The sera were tested for antibodies against SBV using a commercially available ELISA kit (ID Screen Schmallenberg virus Indirect, IDvet, Grabels, France) [11] before testing them with recombinant SBV N protein. In the current study, 102 serum samples were used from this collection for the evaluation of yeast-derived recombinant SBV N protein as an antigen for ELISA. Micro test plates (Nerbe Plus GmbH) were coated with 400 ng per well of recombinant SBV N protein in 100 μL of 0.05 M carbonate-bicarbonate coating buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed three times with PBST and then blocked by the addition of 150 μL of blocking reagent per well (1x Roti-Block, Carl Roth GmbH & Co.). The plates were incubated at RT for 1 hour. After blocking, the plates were washed three times with PBST and 100 μL aliquots of serum specimens, 1 : 200 diluted in PBST buffer with 10% RotiBlock (Carl Roth GmbH & Co.), was added to the wells. Plates were incubated for 1 h at 37°C and washed five times with PBST. 100 μL aliquots of rabbit antibovine IgG (Sigma-Aldrich Co.) conjugated to HRP, 1 : 10.000 (v/v) diluted in PBS with 5% RotiBlock, was added to each well and the plates were incubated for 1 h at 37°C. After washing five times with PBST, 100 μL of TMB substrate (Clinical Science Products) was added to each well and the enzyme reaction was stopped with an equal volume of 1 M H_2SO_4 solution. The optical density at 450 nm was determined for each sample using an ELISA plate reader (Sunrise Tecan).

2.6. Mass Spectrometric Analysis of Recombinant Proteins. Mass spectrometric (MS) analysis of recombinant SBV N protein was carried out according to Hellman et al. [27]. Proteins were identified by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry using a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems/MDS SCIEX 4800 MALDI TOF/TOF, Framingham, MA, USA). Peptide mass spectra were acquired in reflector positive ion mode with a m/z range of 800–4000 Da. Four hundred laser shots were summarized for each sample with a mass accuracy of ±50 ppm. MS/MS spectra for dominating peptides were acquired in positive mode with the ion collision energy set to 1 keV. Five hundred laser shots were accumulated for each spectrum with a mass accuracy of ±0.1 Da. The proteins were identified in the TrEMBL database (3-23-10 release) using the Mascot algorithm.

2.7. Production of Monoclonal Antibodies. MAbs to recombinant SBV N protein were produced essentially as described by Kohler and Milstein [28]. Eight-week-old female BALB/c mice (obtained from a breeding colony at the Center for Innovative Medicine, Vilnius, Lithuania) were immunized at days

0, 28, and 56 by a subcutaneous injection of 50 μg of recombinant SBV N protein. For a primary immunization, the antigen was emulsified in complete Freund's adjuvant (Sigma-Aldrich Co.). The second and the third immunisations were performed with the antigen dissolved in PBS. Antiserum samples were collected on the 14th day after the first, second, and third immunizations and tested by an indirect ELISA for the presence of IgG antibodies specific to SBV N protein. Three days after the final injection, mouse spleen cells were fused with Sp2/0-Ag 14 mouse myeloma cells using polyethylene glycol 1500 (PEG/DMSO solution, HybriMax, Sigma-Aldrich Co.). Hybrid cells were selected in growth medium supplemented with hypoxanthine, aminopterin, and thymidine (50x HAT media supplement, Sigma-Aldrich Co.). Samples of supernatant from wells with viable clones were screened by an indirect ELISA as described above. Hybridomas secreting specific antibodies to SBV N protein were subcloned twice by a limiting dilution assay. Hybridoma cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM, Biochrom, Berlin, Germany) containing 15% fetal calf serum (Biochrom) and antibiotics. Antibodies in hybridoma culture supernatants were isotyped using the BD Pharmingen Mouse Immunoglobulin Isotyping ELISA Kit (BD Bioscience, San Diego, CA, USA) in accordance with the manufacturer's protocol. All procedures involving experimental mice were performed under controlled laboratory conditions in strict accordance with the Lithuanian and European legislation.

2.8. Immunofluorescence Assay. The immunofluorescence test was performed using SBV-infected BHK cells, clone BRS5 (L194, Collection of Cell Lines in Veterinary Medicine, Greifswald-Insel Riems) as antigen matrix in accordance with the procedure described previously [11]. Briefly, a cell suspension was seeded, incubated for 24 h at 37°C, and subsequently infected with SBV strain BH80/11. Forty-eight hours after infection the medium was removed and the cells were fixed using heat treatment (2 h at 80°C). Both, infected and uninfected cells were incubated with each MAb (1 : 10 diluted hybridoma supernatants) for 1 h at RT. After washing with Tris-buffered saline with 0.1% Tween-20 (TBST), a fluorescein isothiocyanate (FITC-) conjugated goat anti-mouse IgG (Sigma-Aldrich Co.) was added and incubated for 1 h at RT. Thereafter, the cells were washed, embedded with Dabco fluorescence conservation buffer (Sigma-Aldrich Co.), and analyzed using an inverted fluorescence microscope (Nikon Eclipse Ti-U, Nikon Instruments Inc., Melville, NY, USA).

3. Results and Discussion

3.1. Synthesis of SBV N Protein in Yeast Saccharomyces cerevisiae. To express the N protein of SBV virus, we exploited yeast vector system previously used for the high-level expression of hantaviruses N proteins [17]. Expression efficiency of recombinant SBV N protein in yeast was proven both by electrophoresis and immunoblotting. SDS-PAGE analysis of crude lysates of *S. cerevisiae* harbouring pFX7-SBV– expression vector revealed the presence of an additional protein band after induction with galactose. This band of approximately 26 kDa was present in the lysates of yeast transformed with pFX7-SBV-6-His-N (Figure 1(a), lane 3). Meanwhile, no additional band of the corresponding molecular size was observed in crude lysates of *S. cerevisiae* harbouring pFX7-6-His vector used as a negative control (Figure 1(a), lane 1). As evaluated by SDS-PAGE, the expression level of SBV N protein was approximately 2% of the total cellular protein. After cell lysis, the main quantity of SBV N protein was found in the insoluble fraction (data not shown). The yield of the His-tagged N protein after nickel-chelate chromatography was about 3.0–3.5 mg/g wet weight of yeast. Recombinant SBV N protein was soluble in 50 mM sodium acetate buffer (pH 5.0) containing 100 mM sodium chloride. The eluted N protein was visible as a single protein band in Coomassie Brilliant Blue-Stained SDS-PAGE gels (Figure 1(a)). In order to confirm the sequence identity of the full-length recombinant SBV N protein and determine its molecular weight, enzymatic digests were performed using trypsin, chymotrypsin, and endopeptidase-AspN to generate internal peptides for detailed mass spectrometry (MS) analysis. Peptide analysis confirmed the primary structure of recombinant SBV N protein predicted from DNA sequence (data not shown). Also, MS analysis revealed that the molecular weight of SBV N protein is 26 kDa, which is in line with theoretical calculated molecular weight of the protein. Western blot analysis of purified protein with the MAb against 6-His-tag epitope confirmed its identity (Figure 1(b), lane 3).

These data confirmed that we have constructed an efficient recombinant yeast expression system and obtained high-level expression of SBV N protein in yeast *S. cerevisiae*.

3.2. The Reactivity of Yeast-Derived SBV N Protein with SBV IgG-Positive Cow Sera. The reactivity of yeast-expressed SBV N protein with cow serum IgG induced by a natural SBV infection was analysed by an indirect IgG SBV ELISA using a panel of 102 cow serum specimens found to be either positive or negative for SBV-specific IgG antibodies using a commercial diagnostic kit (ID Screen Schmallenberg virus Indirect, IDvet). In the commercial kit, SBV N protein expressed in *E. coli* is used [11]. To define the positive/negative threshold of the newly developed indirect IgG SBV ELISA, 11 serum samples previously determined as SBV IgG-negative by commercial ID Screen test were used. The OD values of ELISA were corrected for nonspecific reactivity and reported as sample-to-positive (S/P) values (S/P = (OD sample/OD positive control (from the commercial test kit))$*$100) according to Breard et al. [11]. To calculate the cut-off value, the reactivities of positive and negative reference serum samples from the ID screen test kit with the recombinant SBV N protein were analysed. The cut-off value was calculated as the mean of S/P values of negative samples plus 2 SD (22 + 10) with 95% confidence. Serum sample was considered positive when its S/P value was greater than 32. The tests were run in duplicate. The correlation coefficient and the standard error were 0.92 and 0.1, respectively, between separate runs. To prove the specificity of the newly developed indirect IgG SBV ELISA, yeast-expressed hantavirus Andes N protein as

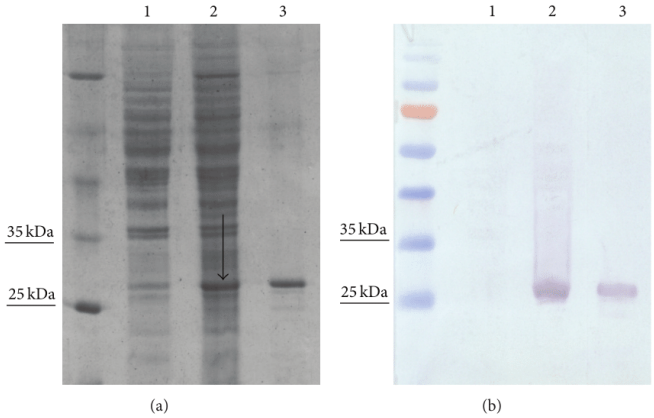

Figure 1: Analysis of yeast cell lysates and purified SBV N protein by SDS-PAGE (a) and western blot (b). Purified SBV N protein (lane 3) or 20 μg of yeast lysates (lanes 1 and 2) was separated in a 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue. PageRuler Unstained Protein Ladder (Thermo Fisher Scientific Baltics) was used. Lane 1, lysate of mock-transformed *S. cerevisiae* [pFX7-6-His]; lane 2, lysate of *S. cerevisiae* transformed with a plasmid [pFX7-6-His-N] encoding SBV N protein (the arrow indicates SBV N protein band); lane 3, Ni-chelate resin-purified SBV N protein. Western blotting was performed using the MAb against 6-His-tag epitope (b) (Thermo Scientific). PageRuler Prestained Protein Ladder (Thermo Fisher Scientific Baltics) was used.

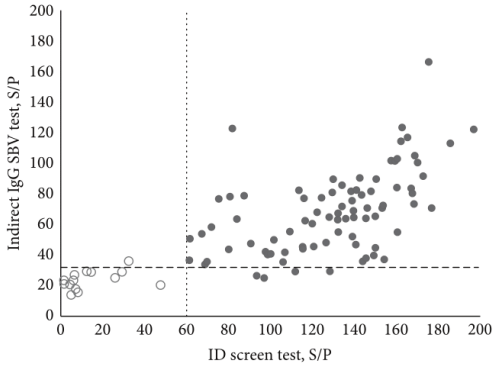

Figure 2: Antibody responses of individual cow serum specimens defined by the newly developed indirect IgG SBV ELISA based on yeast-expressed SBV N protein in comparison to the commercial ID screen test. The S/P ratios of reactivity were plotted. Grey markers represent positive serum samples and white represent negative serum samples obtained by commercial ID screen test. The dashed line represents the cut-off value of the newly developed indirect IgG SBV ELISA. The dotted line represents the cut-off value of the commercial ID screen test.

a negative control antigen was used [29]. No reactivity of cow serum specimens (OD at 450 nm was 0.15 or S/P = 11) with the recombinant hantavirus N protein was detected (data not shown).

Seventy-eight serum specimens out of 82 samples positive by the commercial test were positive and 4 were negative by the newly developed indirect IgG SBV ELISA (Figure 2). Therefore, the sensitivity of the indirect IgG SBV ELISA was calculated to be 95% (78/82 × 100). Fourteen serum samples out of 15 negative specimens by the commercial test were negative and one was positive by the indirect IgG SBV ELISA (Figure 2). Thus, the specificity of the newly developed indirect IgG SBV ELISA was 93% (14/15 × 100). Five serum specimens gave doubtful results by the commercial test. The doubtful serum specimens obtained by the commercial test were excluded from the calculation, as some authors exclude undefined or grey zone sera from the analysis [30, 31].

The high number of sera from SBV-infected cows that were found to be positive in the newly developed indirect IgG SBV ELISA indicated that yeast-expressed SBV N protein may be a suitable antigen for serological diagnosis of SBV infection in cows. The observed discrepancies with the commercial test suggest that IgG SBV ELISA should be further evaluated and optimized using more cow serum specimens collected at variable intervals of the course of the disease. Mansfield and colleagues [12] have shown that the commercial ELISA test could not recognize all SBV-positive serum samples. They conducted focus reduction neutralization assay (PRNT) that appeared to be more sensitive than the commercial ELISA. The essential difference between the PRNT assay and SBV N protein-based ELISA is that the PRNT assay allows detection of antibodies against all structural viral proteins. These results suggest that serologic ELISA tests might be improved by incorporating other viral structural proteins in the test.

This is the first report on SBV antigen expression in yeast *S. cerevisiae* and on the development of an indirect IgG ELISA test based on yeast-expressed SBV N protein. The results showed comparable agreement with commercially available test based on *E. coli*-expressed SBV N protein [11]

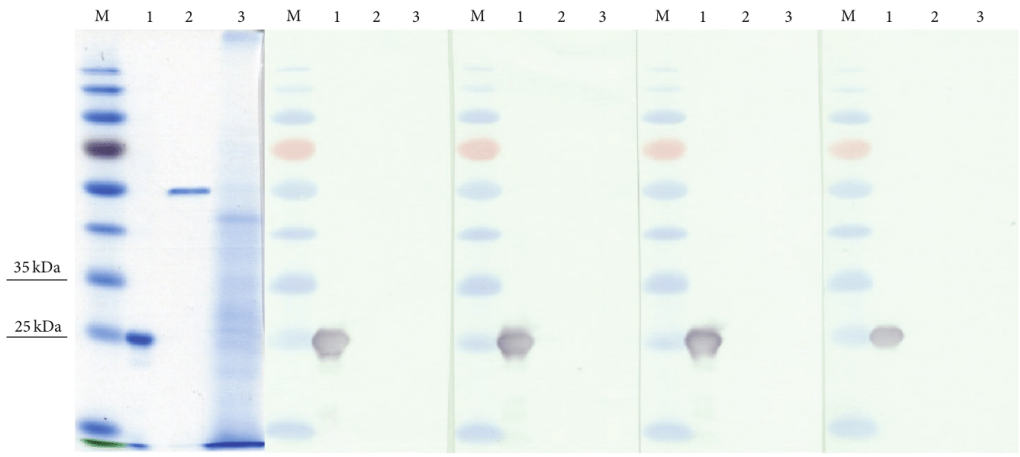

Figure 3: SDS-PAGE (a) and western blot analysis of recombinant SBV N protein with SBV N-specific MAbs ((b)–(e)). Lane M, PageRuler Prestained Protein Ladder (Thermo Fisher Scientific Baltics); lane 1, SBV N protein; lane 2, Puumala N 6 His-tagged protein; lane 3, yeast cell lysate. Undiluted hybridoma culture supernatants were used. MAbs codes are indicated at the bottom of each picture.

indicating that yeast-expressed SBV N protein could provide an alternative for analyzing SBV-specific antibodies in blood sera of SBV-infected cows.

The reactivity of yeast-expressed SBV N protein with SBV IgG-positive cow sera is in line with previous studies that demonstrated the usefulness of recombinant viral N proteins expressed in different heterologous systems for serological diagnosis of bunyaviruses infection both by western blot and ELISA [17–21]. The N protein of bunyaviruses is the most abundant viral antigen present in the virion and in the infected cells, thus making it an excellent target for serology as well as epidemiological studies of viral infections [16–18]. Recombinant N protein of hantaviruses, generated in *E. coli*, insect cells, or yeast, has been widely used for detection of hantavirus-specific antibodies in human sera and oral fluid [17–21]. SBV N protein was expressed recently in *E. coli* [11, 32] and used for the development of a commercial diagnostic kit for serologic diagnosis of SBV infection [11].

Taken together, the reactivity of SBV N protein with cow sera suggests that yeast-derived SBV N protein represents a suitable antigen for serologic detection of SBV infection and generation of virus-specific MAbs.

3.3. Generation of Monoclonal Antibodies against Recombinant SBV N Protein. Purified recombinant SBV N protein was used to immunize mice and generate SBV N protein-specific MAbs. After screening and cloning of positive hybridoma clones, four stable hybridoma cell lines producing IgG antibodies were derived: 4F3, 7B6, 8G10, and 11C10. Hybridoma clones 7B6, 8G10, and 11C10 produced MAbs of IgG1 subtype, whereas the MAb produced by clone 4F3 was of IgG2b subtype (Table 1). The apparent K_d of the MAbs ranged between $2,47 \times 10^{-9}$ and $1,39 \times 10^{-10}$ M, which indicates high-affinity binding (Table 1). All MAbs reacted specifically with recombinant SBV N protein and did not react with yeast-expressed N proteins of Puumala, Hantaan, or Dobrava-Belgrade viruses [17] used to investigate their cross-reactivity (data not shown). To characterize the nature of the epitopes recognized by the MAbs, their reactivity in western blot was analyzed. All four MAbs recognized SDS-denatured SBV N protein in western blot assay (Figures 3(b)–3(e)). This result indicates that the epitopes of the MAbs raised against yeast-derived SBV N protein are not sensitive to denaturation. Recently, it was reported that the MAb 2C8 generated against recombinant SBV N protein expressed in *E. coli* is reactive with virus-derived N protein in the lysates of SBV-infected Vero and BHK cells by western blot [32].

Table 1: Characterization of the MAbs raised against yeast-derived SBV N protein.

Clone	Subtype	K_d, M
4F3	IgG2b	$1,39 \times 10^{-10} \pm 2,9 \times 10^{-11}$
7B6	IgG1	$3,77 \times 10^{-10} \pm 8,9 \times 10^{-11}$
8G10	IgG1	$3,58 \times 10^{-10} \pm 9,3 \times 10^{-11}$
11C10	IgG1	$2,47 \times 10^{-9} \pm 2,4 \times 10^{-10}$

3.4. MAb Reactivities with SBV-Infected Cells. To prove whether the MAbs raised against recombinant SBV N protein recognize viral N protein, the reactivities of the MAbs were tested by an immunofluorescence analysis using BHK cells infected with SBV BH80/11 initially used for the isolation of SBV N protein gene. All MAbs were reactive with SBV-infected BHK cells, although the intensity of the

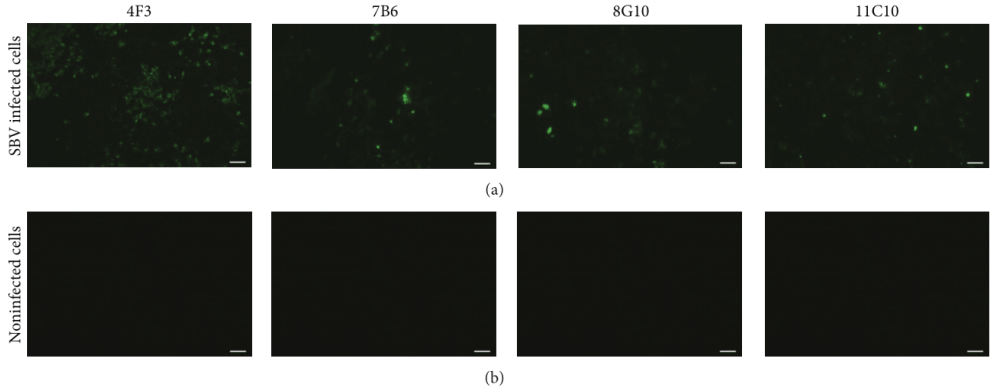

Figure 4: Fluorescence microphotographs showing the reactivity of the MAbs with BHK cells infected with SBV BH80/11 strain (a). Noninfected BHK cells were used as a negative control (b). Hybridoma culture supernatants were used at a dilution of 1 : 10. MAbs codes are indicated on the top of each picture. Scale bar: 100 μm.

immunostaining was differently dependent on MAb clone (Figure 4(a)). No immunoreactivity of the MAbs with noninfected BHK cells used as a negative control was observed (Figure 4(b)).

The immunofluorescence assay data confirm that the MAbs raised against yeast-expressed SBV N protein recognize viral nucleocapsids, which is an additional evidence on the antigenic similarity between yeast-expressed N protein and virus-derived N protein. The MAbs against SBV are of special interest, as they could be used for the development of simple and rapid laboratory diagnostic assays for direct virus detection in biological specimens.

4. Conclusions

Yeast expression system was successfully used to produce recombinant SBV N protein. Purified recombinant SBV N protein was reactive with SBV IgG-positive cow serum specimens collected in Lithuania. For the first time, the circulation of SBV virus in Lithuania was demonstrated. Immunization of mice with SBV N protein resulted in four MAbs that were reactive with SBV-infected cells. The reactivity of recombinant N protein with SBV-specific IgG in cow sera as well as the ability of the MAbs raised against recombinant SBV N protein to recognize native viral nucleocapsids confirms that yeast-expressed SBV N protein resembles native virus in regard of antigenicity and morphology. In summary, yeast-expressed SBV N protein and newly developed SBV-reactive MAbs may provide useful reagents for diagnostics and seroprevalence studies of SBV infection.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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II

Saliva as an alternative specimen for detection of Schmallenberg virus-specific antibodies in bovines

Justas Lazutka, Aliona Spakova, Vilimas Sereika, Raimundas Lelesius, Kestutis Sasnauskas, Rasa Petraityte-Burneikiene

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RESEARCH ARTICLE Open Access

Saliva as an alternative specimen for detection of Schmallenberg virus-specific antibodies in bovines

Justas Lazutka[1], Aliona Spakova[1], Vilimas Sereika[2], Raimundas Lelesius[2], Kestutis Sasnauskas[1] and Rasa Petraityte-Burneikiene[1*]

Abstract

Background: Schmallenberg virus (SBV), discovered in continental Europe in late 2011, causes mild clinical signs in adult ruminants, including diarrhoea and reduced milk yield. However, fetal infection can lead to severe malformation in newborn offspring. Enzyme-linked immunosorbent assays (ELISA) are commercially available for detection of SBV-specific antibodies in bovine sera and milk. Here we describe the development and evaluation of an indirect ELISA based on a yeast derived recombinant SBV nucleocapsid protein (N) for the detection of SBV-specific antibodies in bovine saliva. Development of a non-invasive test to detect antibodies in individual bovine saliva samples could potentially provide a test suitable for calves and adult cattle. The aim of this study was to investigate the agreement between the levels of antibodies (IgG) measured in milk and sera, and the level of antibodies (IgG and IgA) in saliva, in comparison with the antibody levels detected in sera and milk with commercially available test.

Results: Serum, milk and saliva samples from 58 cows were collected from three dairy herds in Lithuania and tested for the presence of SBV-specific antibodies. The presence of IgG antibodies was tested in parallel serum and milk samples, while the presence of IgA and IgG antibodies was tested in saliva samples. The presence of SBV-specific IgG and IgA in saliva was tested using an indirect ELISA based on a yeast-derived recombinant N protein. The presence of SBV-specific IgG in milk and sera was tested in parallel using a commercial recombinant protein based test. The sensitivities of the newly developed tests were as follows: 96 % for the IgG serum assay and 94 % for the IgG milk assay and 85 % and 98 % for IgG and IgA in saliva tests, when compared with data generated by a commercial IgG assay.

Conclusions: Data from testing the saliva IgG and IgA and also the milk and serum IgG with indirect SBV-specific ELISAs showed close agreement with the commercial serum and milk IgG assay data. The level of IgG in saliva was notably lower in comparison to IgA. The newly developed method exhibits the potential to serve as an easily transferable tool for epidemiological studies.

Keywords: Schmallenberg virus, Indirect ELISA, Antibody detection, Cattle, Serum, Milk, Saliva

Background

Schmallenberg virus (SBV), which emerged recently in Europe, was first reported in Germany in a farm near the town of Schmallenberg in late 2011 [1]. Metagenomic analysis identified a novel *Bunyaviridae* family *Orthobunyavirus*, which subsequently was isolated from blood specimens of infected animals. Recent analysis revealed that SBV is most closely related to the Douglas and Sathuperi viruses belonging to the Simbu serogroup in *Orthobunyavirus* [2]. In cattle, clinical symptoms include fever, loss of appetite, reduced milk yield and diarrhoea. Also, SBV infection has been implicated in many cases of severely malformed bovine and ovine offspring [3–8]. Quantitative reverse transcriptase PCR (q-PCR) is the primary diagnostic assay developed by laboratories in affected countries [1]. This assay has limitations in detecting infected individuals based on blood samples, as it only detects viral RNA when the bovine is viraemic [9].

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The first indirect enzyme-linked immunosorbent assay (ELISA) to detect SBV-specific antibodies in serum and milk samples became commercially available shortly after the emergence of SBV (SBV indirect ELISA, IDvet, France) [10, 11]. Testing of bulk tank milk samples by ELISA has been advocated as a convenient way to determine herd-level exposure to SBV [12, 13].

With the availability of vaccines against SBV, it has become important to test animals and apply the test results for herd management. For example a positive bulk tank milk sample indicates that herd-level vaccination is not necessary as natural immunity is present. This test is suitable for dairy farms, but not for males or young cattle. Saliva has a number of advantages over serum for diagnosis. Saliva collection is cheap, non-invasive, is easy to store and transport. Currently a commercial saliva based test for SBV-sero-testing is not available [14, 15].

The aim of this study was to compare the antibody levels in sera, milk and saliva and to develop a method for the detection of SBV-specific antibodies in the saliva of cattle.

Methods

Recombinant SBV N antigen

The cloning of the SBV N gene and purification under denaturing conditions of the recombinant SBV N antigen was recently described [16]. Our recent findings indicated that the SBV N protein purified under native conditions exhibited better antigenicity than the SBV N protein purified under denaturing conditions (See Additional file 1: Figure S1 and Additional file 2: Figure S2). Higher OD readings with positive sera could be obtained using SBV N protein purified under native conditions (See Additional file 2: Figure S2). Thus, for the development of new diagnostic kits, the SBV N protein purified under native conditions seems to have better antigenic characteristics and sero-diagnostic potential. *Saccharomyces cerevisiae* AH22-214 yeast transformation with the plasmid vector was described previously [16, 17]. In this study the pFGG-SBV-N plasmid vector, containing non-tagged full length SBV N protein coding sequence, was used for transformation. After the transformation yeast cells were inoculated in 500 ml of YEPD growth medium supplemented with 5 mM formaldehyde and grown with shaking at 30 °C for 24 h. 500 ml of YEPG induction medium (yeast extract 1 %, peptone 2 %, galactose 5 % supplemented with 5 mM formaldehyde) was added and the yeast cells were grown for an additional 17 h. The cells were harvested, washed with distilled water and frozen at -20 °C until further use. Thawed cells were suspended in 34 ml of PBS at pH 7.4 and 34 g of glass beads (0.5 mm diameter, Sigma-Aldrich Co., St. Louis, MO, USA) were added. Cells were disrupted mechanically by vortexing at 4 °C for 7 min. The lysates were cleared from debris by centrifugation at 2000 × g for 3 min. The insoluble protein fraction was separated by centrifugation at 10,000 × g for 30 min at 4 °C. The supernatant was collected and placed on top of a 70 %/60 %/50 %/40 % sucrose gradient in PBS in an ultracentrifuge tube. The proteins were centrifuged at 110,396 g for 15 h. The 70 % and 60 % gradients, containing the SBV N protein, were collected and concentrated by centrifugation for 2 h at 37,000 rpm through 30 % sucrose solution. The protein pellet, containing the SBV N protein was suspended in 2 ml of PBS. The purity of the resultant N protein was more than 90 % as suggested by SDS-PAGE (See Additional file 1: Figure S1). Two ml of glycerol were added and the protein was kept at -20 °C until further use.

Sera, milk and saliva collection

Bovine blood, milk and saliva samples were collected in September 2014 from dairy farms in different regions in Lithuania. All bovines were clinically healthy at the time of sampling. Samples were stored at -20 °C until tested. Saliva specimens were collected using the Copan Flocked Swabs (Copan, Brescia, Italy) device according to the manufacturer's instructions. The study was conducted according to the Law on the Care, Keeping and Use of Animals, No. 8-500 of the Republic of Lithuania. This research does not need to be approved by an appropriate ethics committee. This is not field studies or experimental research on animals and it complies with institutional, national, or international guidelines.

The sera were tested for antibodies against SBV using a commercially available ELISA kit (ID Screen Schmallenberg virus Indirect, IDvet, Grabels, France) [10] before testing with indirect ELISA with the recombinant SBV N protein.

Reference sera, certified negative or positive for SBV-specific antibodies, were kindly provided by Dr. H. Schirrmeier (Friedrich Loeffter Institut, Germany; [18]).

Indirect IgG ELISA for detection of the SBV N protein-specific antibodies in bovine serum

The ELISA was carried out as described by Lazutka et al. [16].

Indirect IgG ELISA for the detection of SBV N protein-specific antibodies in cow's milk and saliva, and indirect IgA ELISA for saliva

The following protocol was adopted after optimization of the assays. Microtiter plates (Nerbe Plus GmbH, Winsen/Luhe, Germany) were coated with 2 μg/ml of recombinant SBV N protein in 100 μl of 0.05 M carbonate-bicarbonate coating buffer (pH9.6) and incubated overnight at 4 °C. Plates were washed three times with PBST (PBS and 1 % Tween-20) and then blocked by the addition of 150 μl of blocking buffer per well (1x Roti-Block, Carl Roth GmbH

& Co, Karlsruhe, Germany). The plates were incubated at room temperature for 1 h. After blocking, the plates were washed three times with PBST and 100 μl aliquots of milk specimens, diluted 1:10 in PBST, and were added to the wells. Plates were incubated for 1 h at 37 °C and washed five times with PBST. One hundred μl aliquots of rabbit anti-bovine IgG (Sigma-Aldrich Co., St. Louis, MO, USA) conjugated to HRP, diluted 1:20,000 (v/v) in PBST, were added to each well and the plates were incubated for 1 h at 37 °C. After washing five times with PBST, 100 μl of TMB substrate (Invitrogen, Frederick, USA) was added to each well and the enzyme reaction was stopped with an equal volume of 1 M H_2SO_4 solution, after 10 min. of incubation. The optical density at 450 nm was determined for each sample using an ELISA plate reader (Sunrise Tecan, Mannedorf, Switzerland).

The protocol was similar for saliva anti-SBV IgA ELISA, except saliva samples were diluted 1:3 in PBST and sheep anti-bovine IgA, (AbD Serotec, Biorad, Kidlington, UK) conjugated to HRP, were diluted 1:20,000 in PBST with 5 % RotiBlock and 1 % chicken serum (Gibco/Invitrogen, Paisley, UK). For the saliva anti-SBV IgG ELISA 4 μg/ml of the SBV N protein was used per well. Nunc Maxisorp microtiter plates (Thermo Scientific, Roskilde, Denmark) were blocked with 150 μl of 5 % chicken serum in PBS per well. Saliva samples were diluted 1:3 and rabbit anti-bovine Fab'2 IgG (LifeSpan BioSciences, Inc., Seattle WA, USA) conjugated to HRP were diluted 1:30,000 in PBST with 5 % Roti-Block and 1 % chicken serum.

Determining the cut-off values of the different ELISAs

The cut-off value of our serum anti-SBV IgG system was calculated as follows: 27 serum samples that were negative according to the commercial indirect SBV ELISA test were tested against the SBV N protein. The optical density of a sample was divided by the optical density of a reference serum [18] sample and a sample-to-positive (S/P) value in percent was obtained for each sample. The calculation of S/P values was performed according to Breard et al. [10]. The cut-off value for the serum IgG system was then determined using the following formula:

$$\text{Cut off} = (x_1 + x_2 + \ldots + x_n)/n + 3 * \text{SD}(x_1, x_2, \ldots, x_n)$$

where x is an S/P value of the individual sample, n is the number of samples and SD is the standard deviation of the set of samples. The samples that showed reactivity within the average plus 2*SD and average plus 3*SD range were considered doubtful.

We did not have enough negative milk or saliva samples available for us to determine the cut-off values for our milk and saliva assays. Instead, S/P values were calculated after incubating milk or saliva samples with the closely related hantavirus Andes nucleocapsid protein [19]. Other authors also used different antigens for determination of cut-off values. Starkey and co-authors [20] report the use of glutathione *S*-transferase (GST) tagged proteins in enzyme immunoassays (EIAs). They have used GST as a control antigen to permit estimation of background OD in EIAs. Lin and co-authors [21] used a bovine bocavirus protein as a control antigen to define the cut-off in ELISAs established to detect human bocavirus specific antibodies.

The S/P cut-off values for milk IgG, serum IgG, saliva IgA and saliva IgG were 30 %, 28 %, 15 % and 10.5 % accordingly. The cut-off values for these systems were determined as described above.

Evaluation of diagnostic assays in different specimen types

As a first step towards the development of an SBV N ELISA, checkerboard titrations were performed to determine the optimal concentration of the SBV N antigen. To optimize the plate coating, the recombinant SBV N protein was immobilized on ELISA plates at four different concentrations: 4 μg/ml; 2 μg/ml; 1 μg/ml; 0.5 μg/ml. To determine the optimal milk sample dilution, milk samples were serially diluted ranging from 1:5 to 1:50. Saliva samples were tested at dilutions ranging from 1:2 to 1:128. The anti-bovine IgG conjugate was diluted from 1:10,000 to 1:80,000 and the anti-bovine IgA-HRP conjugate was diluted from 1:5000 to 1:40,000. The three milk and saliva samples that were used for the evaluation of the assay had matching serum samples that were characterized by the commercial test as a strong positive, weak positive and negative. After the optimization the tests were used to analyze bovine sera, milk and saliva samples.

Statistical analysis

Statistical significance between separate tests was calculated using MedCalc and Microsoft Excel 2007 software.

Results

Optimization of the assays

The optimal antigen concentration for plate coating as determined by a checkerboard titration was 2 μg/ml for milk IgG and saliva IgA assays and 4 μg/ml for saliva IgG assay. Positive samples revealed significantly higher OD values (0.6 – 2.8) with SBV N protein as compared to the control hantavirus Andes N antigen (0.03–0.15). It was decided to use a dilution of 1:10 for the milk specimens and a saliva dilution of 1:3 as these dilutions gave good discrimination between positive and negative reactions and were more economical in the use of the sample. Nerbe plus plates were used for milk IgG and saliva IgA assays, but for saliva IgG assay we have chosen Nunc Maxisorp plate, in order to increase the

sensitivity of the assays. The maximum reactivity was achieved using weak positive samples, when Nunc Maxisorp plates were used for antigen coating. Nerbe plus plates were not sufficient to detect specific IgG in saliva with low antibody titers, therefore Nunc Maxisorp plate was used to compensate low IgG levels in saliva. A 1:30,000 dilution of anti-bovine IgG -HRP conjugate, and 1:20,000 dilution of anti-bovine IgA-HRP conjugate gave the greatest discrimination between reactivity of positive samples with SBV N antigen and the control antigen - hantavirus Andes N protein.

The saliva sample and anti-bovine IgG/IgA conjugates were diluted in PBS with 5 % Roti-block and 1 % chicken serum solution. The solution was found to be more effective in blocking non-specific binding, leading to lower OD values for the negative control and therefore higher S/P ratios.

Comparison of SBV specific antibody response in milk and saliva samples

Using the newly developed indirect IgG and IgA ELISAs we analyzed sera and their matched milk and saliva samples from 58 randomly selected dairy cows that had been tested before with commercial serum IgG and milk IgG assays. 54 serum samples were positive, one sample was negative and three samples were doubtful in commercial assay. The doubtful serum samples were excluded from further examination. Fifty-two out of 54 positive serum samples were also positive in our developed assay, while two samples were doubtful. One sample was negative in both assays. The agreement between the two tests for the serum IgG assays was 96.30 % (95 % Confidence Interval: 87.25 % - 99.55 %). A small correlation of 0.14 ($p = 0.00515$) between the optical densities of the same serum samples in indirect IgG SBV ELISA and in commercial ELISA was observed.

Further, 58 milk samples were screened with commercial IDvet IgG ELISA test. Fifty-one positive and one negative milk sample was determined by commercial assay. 6 milk samples were determined as doubtful and were excluded from further examination. The remaining 52 samples were tested with newly developed indirect IgG ELISA test. Forty-eight samples were described as positive and one sample was negative. 3 samples, which were determined as positive in commercial test, were doubtful in our test. All 48 positive milk samples in our assay were also positive in commercial assay and the negative sample in our assay was also negative in commercial assay. Our indirect milk IgG assay achieved a sensitivity of 94.12 % (95 % CI: 83.76 % - 98.77 %) (Table 1-A). Based on these values 98 % cows were SBV seropositive. The reproducibility of independently performed ELISAs was high ($R^2 = 0.93$). The correlation between the OD of milk samples in commercial and in our developed assay was low ($R^2 = 0.15$, $p = 0.00597$).

Table 1 Comparison of bovine serum, milk and saliva samples analyzed with newly developed indirect SBV N ELISA tests with commercial IDvet serum or milk indirect IgG ELISAs

(A)

		IDvet milk indirect ELISA		
		Positive	Negative	Total
Indirect milk IgG ELISA	Positive	48	0	48
	Negative	3	1	4
	Total	51	1	52

(B)

		IDvet serum indirect ELISA		
		Positive	Negative	Total
Indirect saliva IgG ELISA	Positive	46	0	46
	Negative	8	1	9
	Total	54	1	55

(C)

		IDvet serum indirect ELISA		
		Positive	Negative	Total
Indirect saliva IgA ELISA	Positive	53	0	53
	Negative	1	1	2
	Total	54	1	55

(D)

		Indirect saliva IgA ELISA		
		Positive	Negative	Total
Indirect saliva IgG ELISA	Positive	45	1	46
	Negative	8	1	9
	Total	53	2	55

Comparison of commercial milk ELISA with our developed indirect milk IgG ELISA (A), commercial serum ELISA with our indirect saliva IgG (B) and saliva IgA (C) ELISA, between saliva IgG and IgA ELISAs (D)

We then tested if the 55 animals tested for anti-SBV IgG in their serum contain SBV-specific IgG and IgA antibodies in their matched saliva samples. Forty-six individuals were positive for SBV IgG antibodies in saliva. Eight cows with no antibodies in saliva had mid-level antibody titers in their sera (Table 1-B). The IgG antibody titer was lower in the saliva than in serum in all cows tested. The only sample that was negative in serum assay was also negative in saliva assay. Thus the sensitivity and specificity was 85.19 % (95 % CI: 72.88 % - 93.38 %) and 100 % (95 % CI: 2.5 % -100 %), respectively, compared to the commercial IgG assay. A comparison of saliva IgG and serum IgG pairs is shown in Fig. 1. Fifty-three cows tested positive in saliva IgA test compared to 54 positive cows in IDvet IgG test. One cow tested negative in both serum IgG and saliva IgA assays and one saliva sample was described as false-negative (Table 1-C). The sensitivity of saliva IgA ELISA was

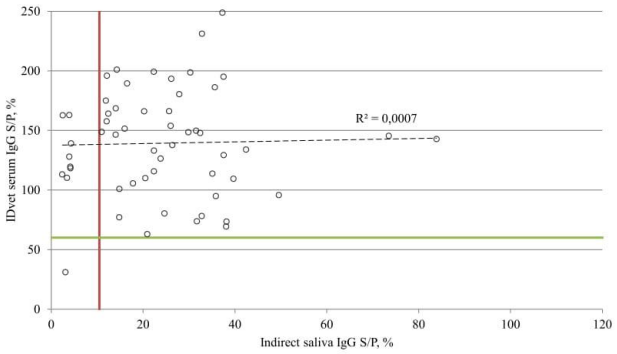

Fig. 1 Relationship between S/P ratios of individual saliva and serum samples. Saliva samples from dairy cows were tested with indirect IgG ELISA using recombinant SBV N protein and serum samples were tested with commercial IgG IDvet ELISA. The horizontal line represent the cut-off value of serum IgG IDvet ELISA, while the vertical line represent the cut-off value of saliva IgG test. Dashed line marks linear regression, *R* squared is the coefficient of determination

98.15 % (95 % CI: 90.11 % - 99.95 %). Forty-five saliva samples were positive for the presence of SBV-specific IgG's out of 53 saliva IgA positives. Eight saliva samples displayed reactivity below the cut-off value in IgG assay while showing a positive reaction in an anti-SBV IgA ELISA (Fig. 2). One saliva IgG positive sample was negative in saliva IgA assay, while one sample was negative in both assays (Table 1-D, Fig. 2). The sensitivity of the saliva IgG test compared to saliva IgA test is 84.91 % (95 % CI: 72.41 % - 93.25 %). There was no linear correlation ($R^2 = 0.0007$) for saliva/serum IgG pairs (Fig. 1). Linear regression analysis estimated the coefficient of determination (R^2) between antibody concentration in saliva IgG and saliva IgA at 0.44 with p value of $2.24 * 10^{-8}$ (Fig. 2).

Discussion

A commercially available antibody ELISA was recently evaluated [11, 13] to detect SBV-specific antibodies in serum and milk samples. Our study aimed to evaluate antibody screening assays for use on both individual milk and saliva samples. We did this by comparing the results of individual milk and saliva antibody testing to individual serum antibody testing. Milk and saliva sample collection is cheap, noninvasive and animal welfare-friendly. However milk and saliva samples are not sterile and are subject to bacterial degradation over time. Furthermore, salivary composition is influenced by the method of collection and the degree of stimulation of salivary flow. Stimulation of salivation before sample collection may lower antibodies concentration in saliva. Dilution effect of fluids from the salivary glands

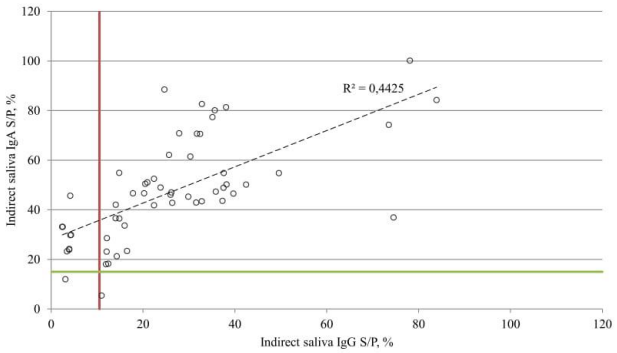

Fig. 2 Relationship between S/P ratios of indirect saliva IgG and IgA assays. Saliva samples from dairy cows were tested with indirect IgG and IgA ELISAs using recombinant SBV N protein. The horizontal line represent the cut-off value of saliva IgA test, while the vertical line represent the cut-off value of saliva IgG test. Dashed line marks linear regression, *R* squared is the coefficient of determination

requires extremely sensitive tests that are able to detect small quantities of antibody. Saliva contains antibodies in concentrations that are 1000-fold less than those in blood. Sensitive detection systems are thus needed to reveal the utility of saliva as a diagnostic medium [14, 22].

Development of an assay for detection of antibodies in milk and saliva required optimization of numerous parameters to maximize sensitivity and specificity. Initial experiments optimized the concentration of SBV N protein used as coating antigen, selection of the microtest plate, the dilution of the test milk and saliva, and the dilution of the HRP conjugated secondary antibody to provide the best discrimination between known positive and negative specimens as determined by commercial test with adequate serum sample.

Three serum samples and six milk samples were excluded from the study for being doubtful. Some authors suggest repeating the tests with longer incubation times to define the antibody status of doubtful samples, or they exclude these samples from their investigation [23, 24]. As the several samples taken over period of time from the same bovine, were not available for the study, therefore the doubtful samples were excluded from further study.

There was close agreement between matched serum and milk IgG assays. The sensitivity of 96 % for serum IgG assay compared to commercial serum IgG ELISA was achieved. This shows a slight improvement over our recently developed indirect serum anti-SBV IgG ELISA [16], where the sensitivity of the assay was 95 %. A low degree of correlation between optical densities of the same serum samples in commercial and our tests was observed and may suggest that different epitopes on the nucleocapsid protein are recognized in these assays.

These results promoted the idea that our newly purified recombinant SBV N protein could also be used to detect antibodies against SBV in milk or saliva samples. All our developed assays agreed with the commercial ones. The agreement of our newly developed milk IgG assay with the commercial test was 94 %. Detection of SBV-specific IgA in saliva yielded similar results, with 98 % sensitivity. However the saliva IgG assay is less sensitive than the saliva IgA assay, probably because of a lower IgG concentration in saliva [25].

After SBV infection antibodies against SBV can be found in bovine serum, milk and saliva. The relationship between individual serum and milk antibodies for SBV was established by others [12]. We suggest using non-invasive sample extraction methods when possible, as antibodies against SBV are present in milk and saliva.

We show that IgA is a reliable marker for SBV diagnosis using bovine saliva samples. There are only few studies demonstrating the production of detectable levels of viral-specific antibodies in bovine saliva samples. This is the first description of a diagnostic assay to SBV based on saliva samples. To date most research on cattle saliva as a diagnostic sample has been carried out with regard to foot and mouth disease virus (FMDV) diagnostics [26, 27].

Conclusions

We have shown that milk and saliva samples are suitable substitutes for serum with minimal loss of sensitivity. Therefore, milk and saliva specimens have the potential to replace serum based screening in large-scale seroprevalence studies.

Additional files

Additional file 1: Figure S1. Analysis of yeast cell lysates and purified SBV N proteins by Western Blot (A) and SDS-PAGE (B). M – Spectra Broad Range Prestained Protein Ladder (Thermo Fisher Scientific Baltics, Vilnius, Lithuania), 1 – lysate of *S. cerevisiae* yeast transformed with mock pFGG plasmid, 2 – 6His- SBV N protein after nickel-affinity chromatography purification under denaturing conditions, 3– SBV N protein after ultracentrifugation in sucrose gradient. Western blotting was performed using mouse monoclonal antibodies raised against SBV N, Mab code 8G10 [16]. (PDF 1256 kb)

Additional file 2: Figure S2. Comparison of the reference sera reactivity with various antigens using IgG ELISA. Positive, weak positive and negative bovine reference sera [18] were tested. Columns represent antibody response against SBV N antigen purified under native conditions (black colums), 6His-SBV N purified under denaturing conditions [16] (grey columns) and control hantavirus Andes N antigen [19] (white columns). The OD values are expressed as obtained in arbitrary units. Bars indicate average values plus standard deviation. (PDF 187 kb)

Abbreviations

ELISA: Enzyme linked immunosorbent assay; SBV: Schmallenberg virus; SBV N: Schmallenberg virus nucleocapsid; HRP: Horseradish peroxidase; S/P: Sample-to-positive value in percent..

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JL developed an indirect ELISA test for serum and milk analysis, was involved in data analysis and drafted the manuscript. AS has cloned the SBV N gene and has purified the recombinant antigen. VS and RL participated in sample collection and data analysis. KS raised the funding for the study, coordinated it and drafted the manuscript. RPB carried out the indirect ELISA for saliva samples, was involved in data analysis and interpretation, and drafted the manuscript. All authors read and approved the final manuscript.

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