UDC 619:616.98-078:578.842.2:577.2.08:602.6:636.4 **DOI 10.36016/JVMBBS-2021-7-3-4**

DEVELOPMENT OF RECOMBINANT ANTIGEN EXPRESSION AND PURIFICATION FOR AFRICAN SWINE FEVER SEROLOGICAL DIAGNOSTICS

Kit M. Yu.

National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Kharkiv, Ukraine, e-mail: maryna_kit@ukr.net

Summary. The paper reports the purification and its optimization of recombinant proteins p10, p32, p54, p54ΔTM, DNA ligase and DNA ligaseΔDBD of African swine fever virus. The corresponding coding sequences were subcloned into pASG-IBA105 and pASG-IBA103 vectors, multiplied and used for transformation of competent *E. coli* expression strain. Expressed proteins were purified using Strep-Tactin XT purification system under native and denaturing conditions, as well as using detergents according to the optimized protocol for recombinant proteins solubilization from inclusion bodies. Among all expressed and purified proteins p32 and p54 were found to be immunoreactive and specific. Although p54 was unstable during long-term storage, after further storage condition optimization, the protein can be used for indirect ASF ELISA development. Recombinant p32 was shown to be an effective antigen for ASF ELISA providing detection of antibodies against ASFV with low background signal

Keywords: ELISA, p10, p32, p54, DNA ligase

Introduction. African swine fever (ASF) is a viral contagious disease of domestic pigs and wild boars. An acute form of the disease causes up to 100% mortality of infected animals (Beltrán-Alcrudo et al., 2017). The causing agent, African swine fever virus (ASFV), is one of the most complex livestock viruses (Dixon et al., 2012; Oura, 2017; Alonso et al., 2018). For this reason, the virus is highly resistant to a variety of chemical and physical factors and remains stable in the environment for months and even years while being kept at a temperature below 0°C in a suitable reach in proteins environment (Mazur-Panasiuk, Żmudzki and Woźniakowski, 2019). The virus is transmitted from animal to animal via direct contact with blood, saliva, feces, urine, tears, secretions from genital tract and nose (Beltrán-Alcrudo et al., 2017). Soft ticks of the genus *Ornithodoros* (especially *O. moubata* and *O. erraticus*) have been identified as both specific arthropod vectors for ASFV transmission and natural reservoir. Transmission from pig to tick and *vice versa* occurs during feeding (Beltrán-Alcrudo et al., 2017; Dixon et al., 2020). ASF is widely spread in Africa, Eastern and Western Europe, the Caucasus region, Asia and the Pacific (Alonso et al., 2018). The first case of ASF in Ukraine was confirmed in 2012 and more than 550 cases have been registered so far in all regions of the country (SSUFSCP, 2021; OIE, 2021b).

Depending on the situation and the aim of the test, different diagnostic techniques can be used for ASF diagnostics. The laboratory tools used for this purpose are divided into two groups: aimed at causing agent identification (virus isolation, conventional and qPCR, antigen detecting ELISA, fluorescent antibody test) or immune response detection (antibody detecting ELISA, immunoperoxidase test, indirect fluorescent antibody test, immunoblotting). For an outbreak confirmation, using both virus and antibodies detection is recommended (Beltrán-Alcrudo et al., 2017; OIE, 2021a).

In the case of infection with a highly virulent strain of ASFV, animals die before the antibodies formation, whereas in the case of infection with a low virulent virus, animals survive. Antibodies to ASFV start forming at 7–10 dpi and persist in blood for months and sometimes years (Beltrán-Alcrudo et al., 2017; OIE, 2021a). Since there are no commercial vaccines against the disease available, the antibodies in a test sample always indicate the ASFV infection. Mutant attenuated variants of the virus are known to occur in areas where the ASF virus has long been established. Thus, such isolate has been detected during ASF serological surveillance study among wild pigs in Estonia (Zani et al., 2018). In addition to surveillance studies, ASF ELISA is used for eradication strategies improvement, as well as for conformation ASF-free status of populations and territories, which is important for animal movement and international trade (OIE, 2021a).

According to the OIE Terrestrial Manual 2021, the use of the antigen prepared from ASFV infected MS culture is recommended for ELISA (OIE, 2021a). However, since work with live ASFV requires the conditions of BSL-3 laboratory, obtaining such antigens for in-house diagnostics is impossible at laboratories with limited resources.

Therefore, the **aim of the study** was to express recombinant ASFV proteins, which could be used as antigens for ASF serological diagnostics.

Materials and methods. *Sera samples.* ASFV positive and negative sera samples (n = 15) were used for Western blot analysis of recombinant proteins and ASF ELISA development. The samples were kindly provided by PD Dr. S. Blome (Friedrich Loeffler Institute, Institute of Diagnostic Virology, Germany; ASF Serumpanel Charge:11/2018) and Dr. H. von Buttlar (Bundeswehr Institute of Microbiology, Germany, sera described in Al Dahouk et al. (2005) was used as negative sera sample).

Molecular cloning. ORFs coding ASFV proteins p10, p32, p54 and DNA ligase, as well as two truncated proteins with deletions of the transmembrane domain

(p54ΔTM) and DNA binding domain (ligaseΔDBD), have been selected for the study. The PCR primers flanking sequences of interest and including sites for Star Gate combinatorial cloning are shown in Table 1. The PCR products were generated using AmpliTaq Gold DNA Polymerase (Applied Biosystems, USA) according to manufacturer instruction and the data in Table 1. Using *Esp3*I restriction enzyme and T4 DNA ligase, purified and quantified PCR products were subcloned into vectors pASG-IBA103 and pASG-IBA105 (IBA Lifesciences, Germany), which include Twin-Strep-tag coding sequence for target protein purification at C- and N-termini respectively.

Table 1 — Primer sets used for the recombinant proteins development

Primer name	Primer sequence, 5'⟶3'	Encoded gene	Amplicon length, bp	Annealing temperature, °C
p10_F	AGCGCGTCTCCAATGCCTACAAAAGCTGGC	*K78R*	237	59
p10_R	AGCGCGTCTCCTCCCTTTTGACCGTTTAATTTTTTTCTCC			
p32_F	AGCGCGTCTCCAATGGATTTTATTTAAATATATCCATG	*CP204L*	615	52
p32_R	AGCGCGTCTCCTCCCAAACATTAAATGTAGGTGAG			
p54_F	AGCGCGTCTCCAATGGATTCTGAATTTTTTCAACCGG	*E183L*	552	52
p54_R	AGCGCGTCTCCTCCCCAAGGAGTTTTCTAGGTC			
p54ΔT_F1	GCCGCGTCTCGAATGGATTCTGAATTTTTTCAAC	*E183LΔ*	115	50
p54ΔT_R1	CACCACACGTCTCGAACCGCCACCGAAGAAGCTCGG			
p54ΔT_F2	GCCGCGTCTCAGGTTCGATTATTATCATCG		461	
p54ΔTM_R2	CGGCCGTCTCATCCCCAAGGAGTTTTCCAGGTC			
ligase_F	AGCGCGTCTCCAATGCTAAATCAATTTCCTGGG	*NP419L*	1260	52
ligase_R	AGCGCGTCTCCTCCCAATGATTTCTAAAACATTTATCGG			
ligaseΔDBD_F	TAATCGTCTCAAATGAGAGGAATGATCCCCCCTATG	*NP419LΔ*	928	60
ligaseΔDBD_R	CGGCCGTCTCATCCCAATGATTTCTAAAAC			

E. coli NEB Turbo (NEB, USA) competent cells were transformed with the resulting vector. After blue-white screening and carbenicillin selection, amplified plasmids were extracted from bacterial culture using Monarch Plasmid Miniprep Kit (NEB, USA). The plasmids with correct insertion confirmed by sequencing (Eurofins Genomics, Germany) were used for transformation of expressing *E. coli* BL21 Lemo (D3) (NEB, USA) competent cells.

Expression and purification of recombinant proteins. Overnight culture of transformed *E. coli* BL21 Lemo (D3) was diluted 1:50 in fresh LB medium with carbenicillin and grown at 37°C until OD was 0.4–0.6. Protein expression was induced with anhydrotetracycline (200 ng/ml) followed by 3 h incubation at 37°C. The cells were harvested by centrifugation at 6,700 *g* for 10 min and stored at −20°C until use.

In case of native and denaturing protein purification, cells were disrupted by homogenization for 30 s at 6 m/s. Protein purification was performed using Strep-TactinXT 4Flow gravity-flow columns and Strep-TactinXT 4Flow resins (IBA Lifesciences, Germany). Purification under native conditions was performed according to columns manufacturer instruction. Briefly, buffer W (100 mM Tris-HCL, pH 8.0, 150 mM NaCl) was used for the column equilibration and washing, elution was performed using buffer BXT (100mM Tris-HCL, pH 8.0, 150 mM NaCl, 50 mM biotin). Purification under denaturing conditions was also performed according to manufacturer instructions. In this case, both buffers used for purification included 6 M urea. The eluted protein fractions were dialysed against buffer W and refolded using Slide-A-Lyzer G2 Dialysis Cassette 3.5 K MWCO (Thermo Scientific, USA) according to manufacturer instruction.

Non-denaturing purification with protein solubilization from inclusion bodies was performed as followed. The pellet from 100 ml culture was resuspended in 2 ml buffer W additionally containing 150 mM NaCl and 0.1% sodium deoxycholate. 10 μl lysozyme (50 mg/ml), 2 μl benzonase (25–29 U/μl), 2 μl 2 M $MgCl_2$ were added to the suspension and incubated for 20 min at room temperature and for 15 min on ice. After 20 cycles of sonication (30 s on, 20 s off) the suspension was centrifuged for 20 min at 11,000 *g*. The supernatant was removed and the pellet was resuspended in 1 ml of buffer W containing 150 mM NaCl and 0.3% sarcosyl. After 4 cycles of freeze-thawing (−80°C for 15 min, 37°C for 10 min), the suspension was sonicated and centrifuged as mentioned above. The supernatant was incubated overnight at 4°C rotating mixed with 1 ml of equilibrated with buffer W+0.3% sarcosyl Strep-TactinXT 4Flow resins. The pellet was resuspended in 2 ml buffer W+0.3% sarcosyl and incubated overnight at 4°C in a rotator. The suspension was centrifuged again and the supernatant was added to the resins. Protein purification was performed according to the resins manufacturer instructions. Dialysis of proteins from detergent was performed directly during purification at the stage of washing by stepwise reducing the sarcosyl concentration in the buffer W from 3 to 0%. Briefly, 1 ml of buffer W with sarcosyl concentration reduced by 0.5%

was used for each washing step, three last washing steps were performed by buffer W without the detergent. For the long-term storage, glycerol was added to the purified proteins to the final concentration of 5%.

Detection of recombinant proteins. The concentration of recombinant proteins was measured with spectrophotometer DeNovix DS-11 (DeNovix, USA). The purity of the eluted fractions was verified by SDS-PAGE electrophoresis and protein transfer to the nitrocellulose membrane followed by reversible protein staining with Pierce Reversible Protein Stain Kit (Thermo Scientific, USA). Detection of Tween-Strep-tagged proteins was performed by Western blot with Strep-MAB-Classic HRP IgG (IBA Lifesciences, Germany) according to manufacturer instruction.

The recombinant proteins specificity testing was performed by Western blot with ASF positive and negative swine sera samples as primary antibodies. The purified proteins were separated by SDS-PAGE and transferred to Nitrocellulose Membrane (Invitrogen, США). After overnight blocking with 10 ml of PBS containing 5% skimmed milk powder and 0.05% Tween 20, the primary antibodies were added directly to the blocking buffer in dilution 1:500. The membrane was washed 3 times for 10 min with washing buffer (PBS+0.05% Tween 20) and incubated for 1 h with secondary Rabbit-anti-Pig IgG HRP (Thermo Scientific, USA) diluted 1:5,000 in washing buffer. After washing twice with washing buffer and once with PBS the membrane was incubated in dark with TMB SeramunBlau precipitate solution (Seramun Diagnostica, Germany) for 10 min. The enzymatic reaction was stopped by washing the membrane with ultrapure water.

ELISA development. Nunc MaxiSorp 96 well plates (Thermo Scientific, USA) were coated overnight at 4°C with recombinant antigen titrated in carbonate buffer (pH 9.6) from 10 μg/ml to 0.1563 μg/ml from A to G. After discarding coating buffer, the wells were blocked with 10% skimmed milk powder and 1% rabbit serum in PBS for 1 h at 26°C followed by blocking buffer removal. ASF positive and negative swine sera samples were titrated from 1:50 to 1:400 in the dilution buffer (10% skimmed milk powder and 1% rabbit serum in PBS with 0.2% Tween 20). The final volume of the sera dilution was 50 μl per well. After 2 h incubation at 26°C, the buffer was removed and the wells were washed 4 times with 300 μl washing buffer (PBS with 0.2% Tween 20). The secondary Rabbit-anti-Pig IgG HRP-coupled antibodies (Invitrogen, USA) were also titrated in dilution buffer from 1:10,000 to 1:30,000 (50 μl of a dilution per well) and incubated for 1 h at 26°C. After washing as described above, 50 μl of TMB SeramunBlau slow solution substrate (Seramun Diagnostica, Germany) were added to each well followed by 10 min incubation in dark. The reaction was stopped by addition 50 μl of 0.25 M sulfuric acid. The OD was measured at a wavelength of 450 nm with a reference wavelength of 620 nm. When testing the reference sera samples, the OD threshold was established at the level of average OD value for negative sample duplicates (cut-off = average (native sera samples) + (3×SD (average (native sera samples))).

Results and discussion. *Molecular cloning.* Four proteins were selected for this study: p10 — a DNA binding protein (8.3 kDa), p32 — a structural protein responsible for the virus entry into a host cell (22.4 kDa), p54 — an inner envelope protein (20 kDa) and a 48 kDa DNA ligase. These proteins were described among 14 the strongest ASFV antigens (Kollnberger et al., 2002). Truncated proteins with deletions of transmembrane (p54ΔTM, 18.3 kDa) and DNA binding (ligaseΔDBD, 34.6 kDa) domains were developed in order to potentially simplify the purification process.

Although coding sequences of all the proteins were subcloned into two vectors including either C- or N-terminal Tween-Strep-tag, not for all of them the cloning process was successful. Thus, cloning to both types of vectors was successful for truncated and full-length DNA ligase, as well as for p54ΔTM, whereas for other proteins only one construct was obtained: pASG-IBA103_p10, pASG-IBA105_p32, pASG-IBA105_p54. All the plasmids were used for *E. coli* transformation and were shown to successfully express target proteins after induction (data not show).

Protein purification. In the first stage, the purification of recombinant proteins was performed under physiological conditions. However, Western blot of purified proteins with antibodies against Twin-Strep-tag revealed that the proteins were insoluble. As a result, only a small amount of the synthesized protein was present in the lysate (the concentration of pooled eluted fractions for each protein did not exceed 70 μg/ml), and most of the protein remained in the cell debris pellet (Fig. 1A). The only exception was the p10, which was successfully purified under native conditions and the concentration of the pooled purified fractions was 3.5 mg/ml (data not shown).

One reason for the unsuccessful purification could be the formation of inclusion bodies, which are dense spherical aggregates of proteins of the same type that are formed in bacterial cells during the expression of heterologous proteins. Twin-Strep-tag is known not to affect the folding and aggregation of tagged proteins, therefor, most often inclusion bodies are formed when the expression level of heterologous protein exceeds 2% of the total amount of cell proteins (Zhao, Li and Liang, 2013; Singh et al., 2015). About 70% of recombinant proteins overexpressed in *E. coli* form inclusion bodies (Yang et al., 2011). This can be caused by high temperature during protein expression, high concentration of the inducer, expression under the regulation of a 'strong' promoter, a large copy number of the target gene. As a result of a large amount of expressed target protein accumulation, aggregates consisting of misfolded and not completed folding molecules, as well as proteins of native structure, are formed. Proteins can also be inversely disaggregated from inclusion bodies and fold to native conformation (Singh et al., 2015).

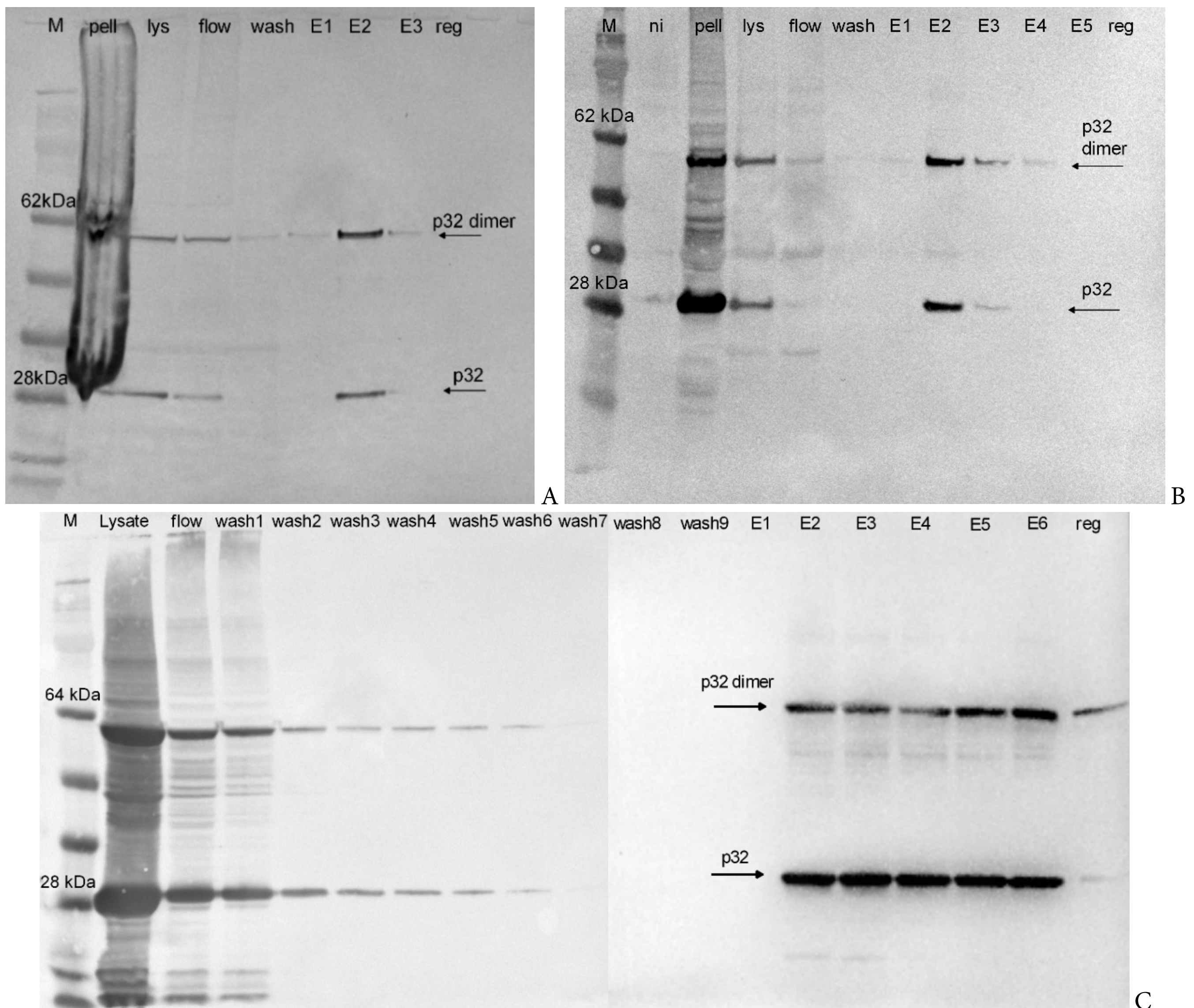

Figure 1. Western blot with Twin-Strep-tag antibodies of the fractions obtained during p32 purification under physiological conditions (A), denaturing conditions (B), non-denaturing purification with inclusion bodies solubilization (C) (M — SeeBlue Plus2 Pre-stained Protein Standard, ni — bacterial culture control before induction, pell — cell debris pellet, lys — lysate before application to the column, flow — flowthrough, wash — column washing fraction, E — eluted protein fractions, reg — column regeneration fraction)

One of the most common ways to solubilize recombinant proteins from inclusion bodies is bacterial culture lysate preparation and further protein purification under denaturing conditions in the presence of urea. By this approach, it was possible to purify a larger amount of soluble protein. In Fig. 1B the purification of p32 under denaturing condition is shown as an example. Similar results have been obtained for the proteins p54, p54ΔTM, DNA ligase and DNA ligaseΔDBD (data not shown). However, the largest part of the synthesized proteins still remained in the pellet, which indicated the need for further improvement of the purification protocol. A general disadvantage of the denaturing protein purification is the need for dialysis of the obtained fractions to free them from urea. Since proteins are in denatured form, dialysis and refolding should be performed gradually and slowly to prevent re-aggregation of proteins after urea removal.

It is known that the use of detergents in low concentrations results in mild protein solubilization, which allows obtaining active recombinant proteins without refolding, and the combination of different detergents can increase the efficiency of solubilization and further purification (Ma, Lee and Park, 2020; Singh et al., 2015; Tao et al., 2010; Burgess, 1996). Thus, based on an in-house method from the Bundeswehr Institute of Microbiology and a protocol for purifying His-tagged proteins (Heimerman et al., 2018) the two anionic detergents: sodium deoxycholate and sodium lauryl sarcosinate (sarcosyl) were used for further purification optimization steps. The sodium deoxycholate is used for lysis of membranes and solubilization of proteins from

inclusion bodies, is particularly effective for the disruption of protein-protein interactions (Burgess, 1996; Lau and Othman, 2019; Johnson, 2013). The use of sarcosyl in the purification of recombinant proteins is well established. Its advantages include the non-denaturing effect, the lack of interference in spectrophotometric measurement of protein concentration, the inhibitory effect on proteases and the low cost (Burgess, 1996; Johnson, 2013). Despite its widespread use in protein studies, sarcosyl has been used for the first time to solubilize Twin-Strep-tagged proteins. Sodium deoxycholate has a mild denaturing effect, while sarcosyl helps protein refolding (Johnson, 2013; Coligan, 1998). Despite the treatment with a denaturing agent, the proteins do not require an additional refolding phase after solubilization due to a 16 h incubation phase in the presence of sarcosyl. It was found that the supernatant contained only a very small amount of target protein after pellet treatment with sodium deoxycholate buffer. However, this step was crucial for purity since this treatment let to the removal of major part of native bacterial proteins from the cell lysate (Burgess, 1996). Subsequently, the treatment with sarcosyl effective enough for further protein solubilization of our target protein. As shown in Fig. 1C, the use of the solubilization protocol allowed to obtain a large amount of purified p32 with a low content of contaminating protein fractions.

Specificity test of the recombinant proteins. After purification, the antigenic properties and specificity of the obtained recombinant proteins were tested by Western blot with ASF positive and negative reference swine sera. The p32 and p54 proteins were proved to have antigenic properties and did not show a background signal in a study with ASF negative serum, while the p10 protein as well as the truncated and full-length DNA ligases did not show any difference between the reaction with ASF positive serum nor negative serum (Fig. 2). This might be due to the lack of necessary posttranslational modifications that *E. coli* expression system could not ensure. Since ASFV native proteins are expressed in eukaryotic cells, immunoreactive recombinant proteins are likely required to be expressed in eukaryotic systems such as HEK-293, insect cell culture, or *Leishmania tarentolae* (Basile and Peticca, 2009; Ikonomou, Schneider and Agathos, 2003; Ma, Lee and Park, 2020; Thomas and Smart, 2005).

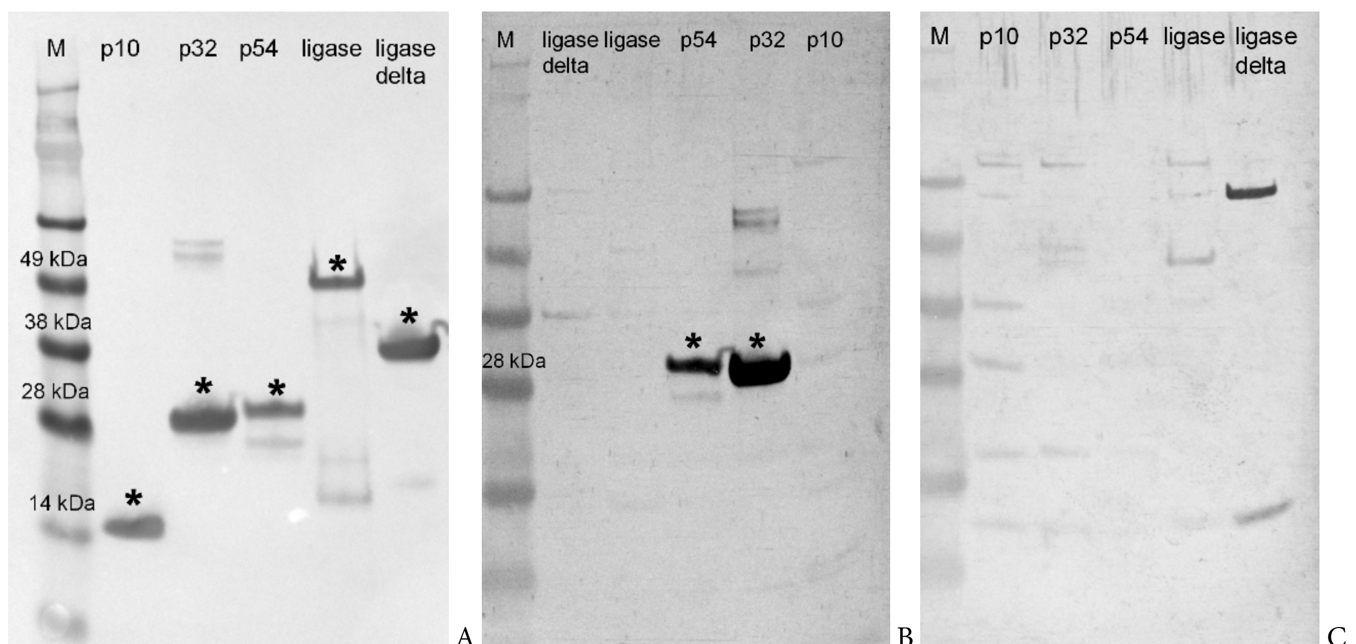

Figure 2. Western blot of purified recombinant proteins with Twin-Strep-tag antibodies (A), ASF positive (B) and negative (C) swine sera (M — SeeBlue Plus2 Pre-stained Protein Standard, p10, p32, p54, ligase, ligase delta — purified recombinant proteins. Recombinant proteins of the target size are marked with asterisks)

The antigenic properties and specificity of the p54ΔTM protein were tested separately. The results of Western blot with both ASF positive and negative sera were positive, indicating nonspecific binding of the antigen to antibodies in swine sera. Therefore, recombinant p54ΔTM protein cannot be used as an antigen for serological diagnosis of ASF. The loss of p54ΔTM protein specificity can be explained by the fact that deletion of the transmembrane domain could affect the folding and tertiary structure of the protein, potentially altering the structure of epitopes which bind to swine antibodies. Thus, p32 and p54 proteins were selected for further ELISA development with the obtained proteins as antigens.

Pilot ELISA development. When choosing the optimal antigens and antibodies concentration, the ratio of OD values of positive and negative samples and the dynamics of signal reduction was considered. Thus, after a sharp

nonlinear decrease of the OD value, the previous antigen concentration was chosen, because it provided the maximum saturation of the well surface with antigen without its redundant use. After selecting the optimal antigen concentration, the optimal serum dilution was chosen. The serum dilution providing high OD values for the positive samples and OD value about 0.2 for negative samples was considered optimal.

After titration of p32 as coating antigen, its optimal concentration was determined to be 5 µg/ml as it was the closest concentration before nonlinear OD decrease (Fig. 3).

The optimal OD values ratio for positive and negative samples was observed with 1:100 sera dilution. In case of establishing optimal dilution of secondary antibodies, a nonlinear OD decrease was observed after a dilution of 1:10,000, so this concentration was selected for further work as optimal (Fig. 4).

A panel of swine ASF reference sera was tested by the developed ELISA based on the use of recombinant p32 protein. The obtained data met the expected criteria. The ASF sera panel included as internal control a negative sample. This sample was #7 which was clearly identified. All other samples have been ASFV positive (Fig. 5).

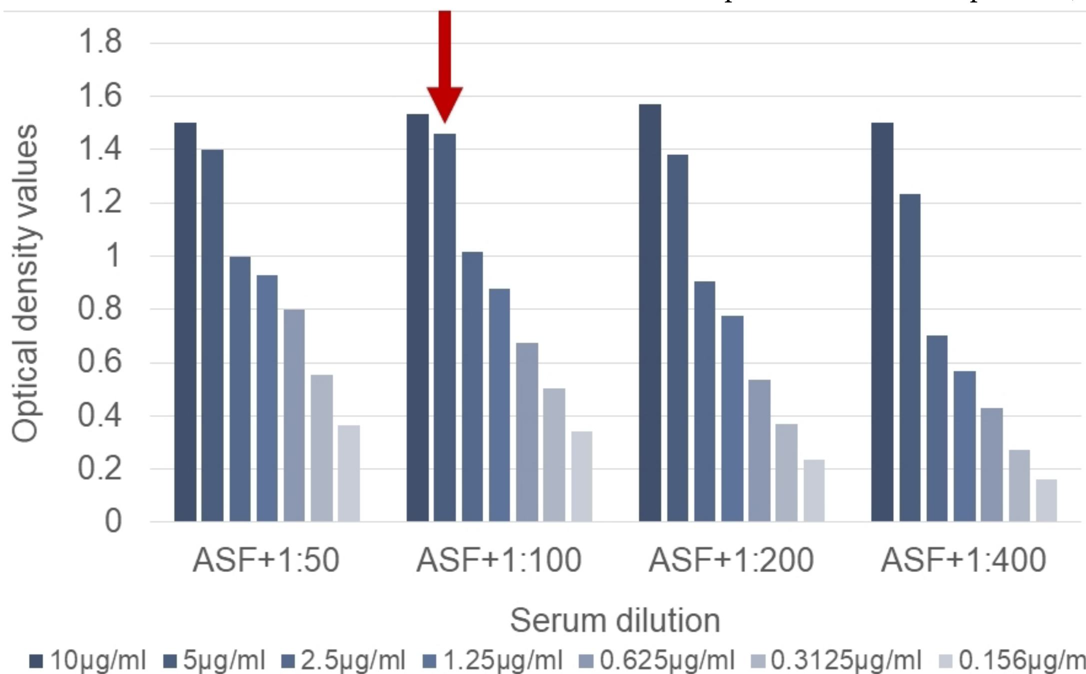

Figure 3. Recombinant p32 protein titration with ASF positive serum (the chosen concentration of the antigen and serum is indicated with the arrow)

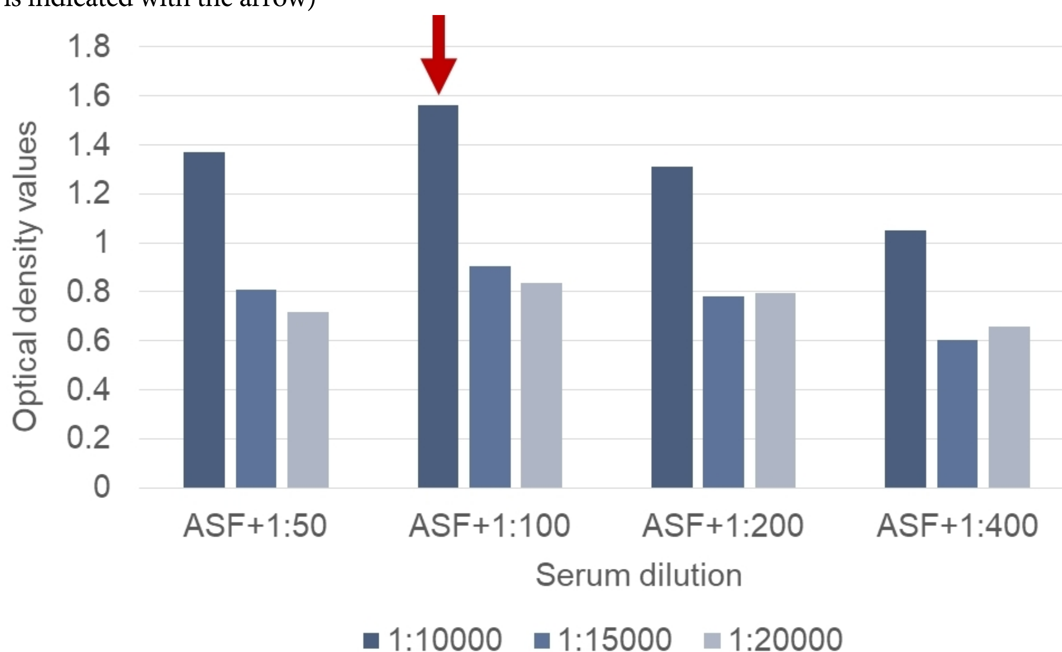

Figure 4. Secondary antibodies titration with ASF positive serum (the chosen concentration of the conjugate and serum is indicated with the arrow)

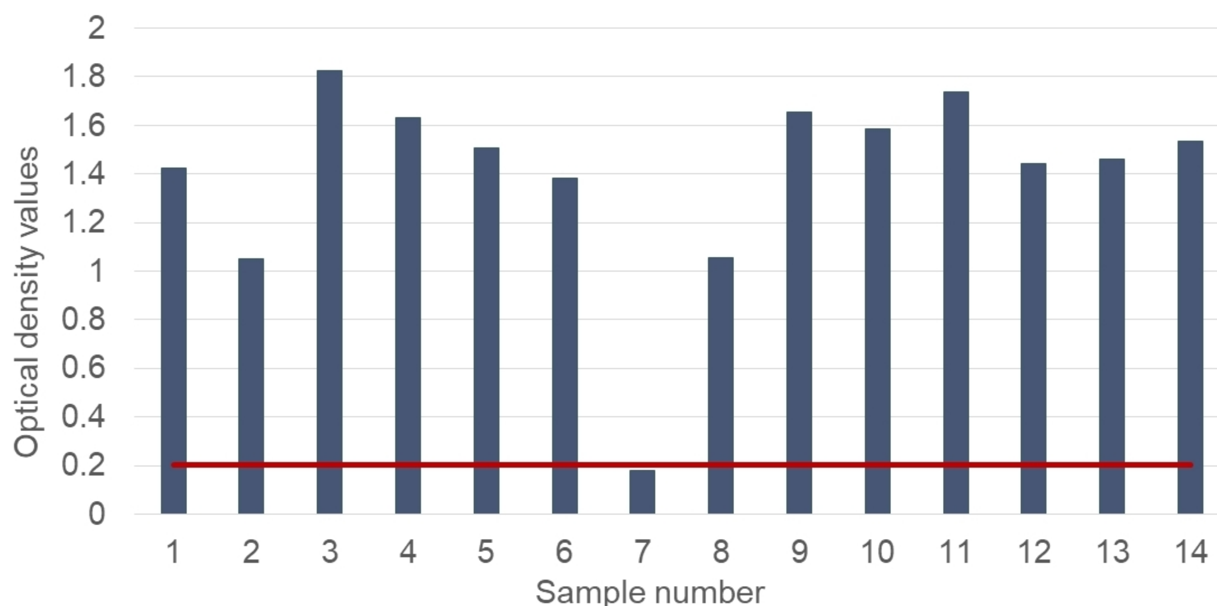

Figure 5. Reference swine sera testing with the developed p32 based ELISA (the red line indicates the average OD threshold for negative results (0.2))

Titration of p54 protein was performed several times, but despite a multiple increase of the antigen introduced into the wells, the maximum saturation of the surface could not be achieved. After storage at –20°C, a sharp drop in the protein concentration was observed and ELISA development with p54 as antigen was suspended for the moment. The concentration decrease can be explained by protein degradation, binding to the storage tubes and re-aggregation of molecules after removal of the solubilizing agent. It is known that proteins in a solution with a concentration less than 1 mg/ml can lose stability (Pierce Biotechnology, 2009; Simpson, 2010) and this could be prevented by concentrating of protein fractions, for example by filtration. Glycerol was added to all aliquots of proteins for long-term storage to a final concentration of 5%, but in the case of p54 samples, it did not lead to any stability. The use of other cryoprotectants (ethylene glycol, polyethylene glycol, Tween 80), protease inhibitors, reducing agents (DTT, 2-mercaptoethanol) could improve the protein stability (Pierce Biotechnology, 2009; Simpson, 2010). Another possible way to solve the problem is to use special protein low bind tubes. Reaggregation of protein molecules can be avoided by optimizing the purification protocol and adding a separate step of dialysis using cassettes. Nevertheless, after identifying the reason for the concentration decrease and optimization of storage conditions, p54 can be used as an antigen for ELISA due to its specificity and low background signal in the study with negative sera.

Conclusions. Immunoreactive recombinant ASFV proteins p32 and p54 were successfully expressed and purified using the optimised protocol for solubilization of Twin-Strep-tagged proteins. After establishing optimal long-term storage conditions, p54 can be used for indirect ASF ELISA development.

For p32 based ELISA, the optimal concentration of antigen, serum and secondary antibodies were determined, which sets the basis for the further development and validation of the assay. Additional studies with weak-positive samples use are necessary to evaluate the sample-to-noise ratio. Testing of the sera samples positive to other swine viral diseases are required for assessment of the assay specificity.

Acknowledgements. This work was funded by the German-Ukrainian biosecurity initiative on zoonosis risk management at the EU external borders. The author expresses her sincere gratitude to Dr. C. Popp, Dr. J. Schwarz, Dr. H. von Buttlar (Bundeswehr Institute of Microbiology, Germany) and Prof. A. Gerilovych (National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Ukraine) for their help and support during the study process and the manuscript writing.

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