PALACKÝ UNIVERSITY OLOMOUC

Faculty of Science

Department of Biochemistry

SUMMARY OF THE DOCTORAL THESIS

Production of recombinant proteins for veterinary use

P1416-Biochemistry

Carlos Díaz

Supervisor: Prof. RNDr. Ivo Frébort, CSc. Ph.D.

Olomouc 2022

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SOUHRN

Virus afrického moru prasat (ASFV z anglického African swine fever virus) je nejsmrtelnějším členem DNA virů infikujících prasata, a proto masivní epidemie způsobená ASFV musí být zastavena. Mnoho vědců v průběhu let pracovalo na vývoji potenciální vakcíny, ale úspěšnou vakcínu, která by dokázala chránit prasata před virulentním virem po delší dobu se zatím nepodařilo připravit. Tato práce se zaměřuje na přípravu a testování imunogenicity potenciálních kandidátů na podjednotkovou vakcínu sestávajících z virových proteinů CD2v, p22 a termálně labilní B podjednotky enterotoxinu (LTB). Byly navrženy čtyři genové konstrukty (CD2vCt, LTB-CD2vCt, p22Ct, LTB-p22Ct). Pro jejich konstrukci byly vybrány pouze části genů kódující vnější globulární domény virových proteinů, CD2v a p22, samostatně nebo sloučené s genem kódujícím LTB. Hlavní funkcí LTB bylo nabudit imunitní odpověď hostitele. Všechny konstrukty byly použity pro expresi pomocí bakteriálního expresního kmene BL21 STAR a kmene Rosetta 2.

Rekombinantní proteiny p22Ct a LTB-p22Ct byly úspěšně nadprodukovány v buňkách BL21 STAR. Imunoanalýzou pak byla potvrzena jejich schopnost reagovat s různými séry od prasat infikovaných ASFV, což indikovalo správnou konformaci proteinu. Rekombinantní proteiny byly dále použity pro imunizaci myší pro další analýzu. Fúzní protein LTB-p22Ct nevykazoval očekávané adjuvantní vlastnosti a neukázal se jako bezpečný, protože po imunizaci myší byla pozorována tvorba abscesu. Na druhé straně, imunizace samotným proteinem p22Ct ukázala vyšší titr protilátek a nezpůsobila u myší žádné nežádoucí projevy. Tyto výsledky ukazují vysoký potenciál antigenu p22 jako imunogenního proteinu, který je možné použít buď pro budoucí vývoj vakcín proti ASFV, nebo pro účely jeho specifické detekce.

Tato práce také ukazuje potenciál nové metody, nazývané lineární epitopové pole (LAE z anglického linear epitope array), při přípravě epitopových antigenů pro detekční nebo vakcinační účely.

SUMMARY

ASFV is the deadliest member of the DNA viruses infecting pigs and a massive disaster for pigs caused by the African swine fever virus (ASFV) needs to be stopped. Many researchers have been trying to develop new potential vaccines throughout the years, but there has been no success in preparing a successful vaccine that could protect pigs against the virulent virus for a prolonged time. This work focuses on the design and immunogenicity testing of potential subunit vaccine candidates consisting of ASFV viral proteins CD2v, p22, and heat-labile B subunit of enterotoxin (LTB). Four gene constructs (CD2vCt, LTB-CD2vCt, p22Ct, LTB-p22Ct) were designed. Only the gene parts coding for the outer globular domains of viral proteins, CD2v and p22, alone or fused with a gene coding for LTB, were selected. The LTB's main function is to induce the host immune response. All constructs were used for the expression using bacterial expression strain BL21 STAR and Rosetta 2 strain.

Recombinant proteins p22Ct and LTB-p22Ct were successfully overproduced in BL21 STAR cells. The ability to react with different ASFV positive sera confirmed by immunoanalysis indicated the right protein conformation. Recombinant proteins were further used for mice immunisation to analyse their safety and immunogenicity. The protein fused with LTB did not show expected adjuvant properties and did not prove safe since an abscess formation was observed after mice immunisation. On the other hand, immunisation with p22Ct protein alone showed higher antibody titre and conferred safety in mice. These results show the high potential of the p22 antigen as an immunogenic protein, either used for future ASFV vaccine development or detection purposes.

Furthermore, this work shows the potential of a novel method, called linear array epitope (LAE), in producing epitope antigens for detection or vaccine purposes.

AIMS OF THE WORK

1. Collecting all information about the African swine fever virus and its antigens, and to show how these antigens could be used as diagnostic tools or as potential vaccine candidates for combating the African swine fever.
2. Expression and purification of constructs containing the outer part of selected ASFV antigens from *Escherichia coli* expression strain BL21 (STAR) and Rosetta™ 2(DE3), respectively.
3. Overproduce the selected proteins and use them for two following analyses; the immunoassay against positive pig sera for confirmation of proper conformation and mice immunisation for safety and immunogenicity evaluation for respective proteins.
4. Determination of the level of produced antibodies in mice blood samples collected after the immunisation by ELISA analysis.

AFRICAN SWINE FEVER VIRUS

The theoretical part focuses on the properties of African swine fever virus, such as virus structure, genetic classification, viral infection and vaccine development.

VIRUS STRUCTURE

The viral particle of the ASFV has icosahedral symmetry, as shown in Figure 1. As mentioned above, ASFV consists of three envelopes: outer, capsid, and inner envelope. The primary role of the envelopes is to protect the core of the viral particle, where linear dsDNA is localized.

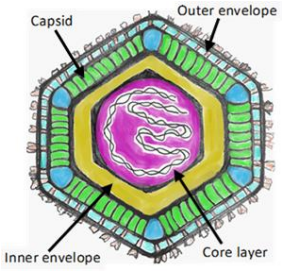

Figure 1: The structural characteristic of the African swine fever virus; adapted from Díaz *et al.*, 2021.

The outer layer is composed of the structural proteins p12 (*pO61R*) and CD2v (*EP402R*) (Alcamí *et al.*, 1992; Rodríguez *et al.*, 2004). The p12 protein (*pO61R*) is a late structural protein known as an attachment protein during the entry of the viral particle to the host cell (Alcamí *et al.*, 1992). However, p12 protein was also found to be a part of an inner envelope (Salas and Andrés, 2013). CD2v is a more complex protein which plays different roles during ASFV infection. It is a transmembrane protein containing 402 amino acids showing a high degree of similarity to CD2, an adhesion receptor of T lymphocytes (Rodríguez *et al.*, 1993).

The major capsid p72 protein (encoded by the viral *B646L* gene) is known for its assembly in the area of the inner core matrix and outer capsid layer of the viral particle (Cobbold and Wileman, 1998). Another crucial structural protein is p49 (*B438L*), which forms the icosahedral shape of the viral particles by localising in the vertices of the capsid (Dixon *et al.*, 2013).

The inner envelope contains five structural proteins: the abundant transmembrane p17 (*D117L*); the late structural pE248R (*E248R*), j5R (*H108R*) and j18L (*E199L*); p54

(j13L, *E183L*) and p22 (KP177R) (Brookes *et al.*, 1998; Rodriguez *et al.*, 1994; Rodríguez *et al.*, 2009; Suárez *et al.*, 2010; Sun *et al.*, 1996; Alejo *et al.*, 2018).

The core layer is composed of structural proteins, which originate from polyproteins pp62 (*CP530R*) and pp220 (*CP2475L*) (Simón-Mateo *et al.*, 1993; Simón-Mateo *et al.*, 1997). Both polyproteins are processed by SUMO-like protease (*S273R*), yielding different structural proteins, which in the case of pp62 are p15 and p35 and in the case of pp220 are p14, p34, p37 and p150 (Simón-Mateo *et al.*, 1993; Simón-Mateo *et al.*, 1997).

ASFV genome is around 170 kbp long and contains 151 open reading frames (ORFs) (Chapman *et al.*, 2008, Yáñez *et al.*, 1995). The genome of the virulent strain Georgia 2007/1 contains genes involved in many processes, such as DNA replication, transcription and processing, the assembly of viral particles, host defences, and last but not least, there are also multigene families, which take part in 30 % of the genome (Dixon *et al.*, 2013).

GENETIC CLASSIFICATION

Distinct ASFV genotypes were identified based on the p72 structural protein. Phylogenetic analysis of the C-terminal end of the p72 gene showed the presence of 22 different genotypes (I–XXII) (Boshoff *et al.*, 2007). Recently two new genotypes were added, XXIII and XXIV (Achenbach *et al.*, 2017; Quembo *et al.*, 2018).

VIRAL INFECTION

ASFV's primary target host cells of ASFV include macrophages and monocytes (Gómez-Villamandos *et al.*, 2013; Galindo *et al.*, 2015). ASFV has evolved a complex of interactions with the host cells in order to infect and evade the host immune system. All interactions and processes are divided into five phases: virus entry, endosomal pathway, replication and transcription of the ASFV genes near the nucleus, virion assembly, and viral release.

The first step in viral infection is a viral entry. Early studies on ASFV entry focused on Vero cells and showed that ASFV enters the cells by receptor-mediated endocytosis, a temperature, pH, and energy-dependent process (Alcamí *et al.*, 1989; Valdeira *et al.*, 1998). However, a later study by Hernáez and Alonso (2010) shows that the virus enters through clathrin-mediated endocytosis. Another way ASFV may enter the host cell is macropinocytosis (Sánchez *et al.*, 2012).

Early infection depends on the production of PI-3P, involved in early endosome (EE) maturation and multivesicular body (MVB) formation, and on the transition between PI-3P and PI-3,5-biP (Cuesta-Geijo *et al.*, 2012). After the virions move to multivesicular endosomes, they lose their outer and capsid envelopes, which depend on the low acidic pH inside the endosome (Hernáez *et al.*, 2016). Thus, the inner envelope

is exposed and subsequently fused with the late endosomal (LE) membrane to release the viral genome into the cytosol (Andrés, 2017).

The further transport of the genome is mediated by p54 protein, which interacts with the light chain of dynein until it reaches the perinuclear spot near the microtubular organizing centre (MTOC), where DNA replication and transcription take place (Alonso *et al.*, 2001). However, based on the study by Brookes *et al.* (1996), genomic DNA is localized not only on the site of viral factories and in the virions outside the cells but also inside the cell nucleus. Later, it was found that nuclear interactions are essential for the early stage of ASFV infection, where the genome is localized at the beginning of infection (Ballester *et al.*, 2011).

The ASFV viral replication occurs at the virus factories, which resemble aggresomes, a place of protein aggregates that enable rearrangements of cellular membranes and cytoskeleton reorganization (Wileman, 2006). For this reason, microtubules' integrity is necessary for forming viral factories (Galindo and Alonso, 2017). Viral factories are localized at the MTOC near the nucleus, where viral proteins and genomic DNA are assembled to form new viral particles (Galindo and Alonso, 2017).

The last step of the ASFV intracellular infection is the release of complete assembled viral particles outside the infected cell. Completed viral particles are subsequently recognized by kinesin, a plus-end microtubule, which transfers them from the virus factories to the cell plasma membrane (CPM) (Jouvenet *et al.*, 2004). The final virus particle release happens through the budding process, where it obtains a third outer envelope from the CPM (Breese and DeBoer, 1966).

VACCINE DEVELOPMENT

The development of vaccines for combating ASFV began in the 1960s (Arias *et al.*, 2017). Multiple vaccines were developed during those early years, but none proved successful enough for commercial purposes. Three main types of vaccines were designed against ASFV: inactivated vaccines with a killed virus, live attenuated vaccines and subunit vaccines (Fig. 2).

Inactivated vaccine approaches were unsuccessful since such vaccines could not enhance the immune response in pigs, even with the addition of different types of adjuvants (Blome *et al.*, 2014).

Live attenuated vaccines contain weakened viruses with deleted or mutated genes responsible for host invasion, virus infectivity, and immune system inhibitors. They were found to enhance cellular and humoral immunity and further protected pigs against the virulent virus type (Sánchez *et al.*, 2019). There are three successful LAVs based on three ASFV isolates: the OURT88/3, NH/P68 and BA71ΔCD2v (Leitão *et al.*, 2001; Monteagudo *et al.*, 2017; Mulumba-Mfumu *et al.*, 2015).

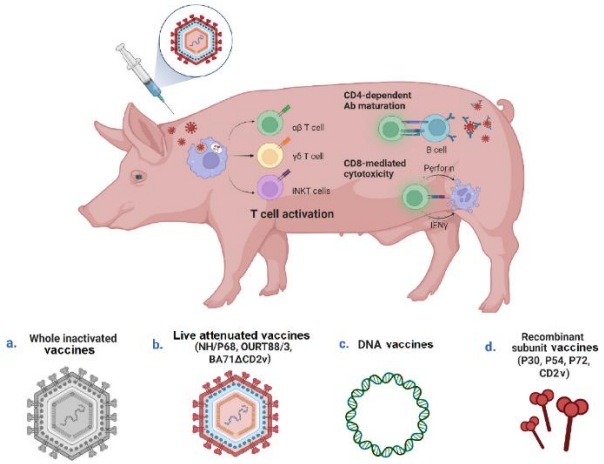

Figure 2. Main vaccine strategies for combating African swine fever virus, including the immune response inside the host body; edited from Urbano and Ferreira, 2022

DNA vaccines have one main disadvantage: their reduced immunogenicity in large animals. This fact was confirmed by failed immunisation of the ASFV genes-containing DNA vaccine (Argilaguet *et al*., 2011).

Subunit vaccines use purified antigenic parts of the pathogen necessary to induce the immune response in the form of antibodies. An example of ASFV subunit vaccine immunisation includes the immunisation with baculovirus-expressed p30, p54, p72 and p22 viral proteins showing only a temporal delay within the onset of disease and reduced viremia (Neilan *et al.*, 2004). The study by Gómez-Puertas *et al.* (1996) shows that the immunisation with two viral proteins, p54 and p72, raised neutralising antibodies against them, which could even inhibit virus attachment to the host cell membrane. A study regarding the CD2v viral protein conducted in 1996 by Ruiz-Gonzalvo *et al.* showed that immunisation with recombinant CD2v inhibited the haemagglutination, restricted the infection temporally and, in some cases, also conferred protection against lethal disease.

EXAMINATION OF IMMUNOGENIC PROPERTIES OF RECOMBINANT ANTIGENS BASED ON CD2v AND p22 PROTEINS FROM AFRICAN SWINE FEVER VIRUS

INTRODUCTION

An enormous economic loss in the pig industry caused by the African swine fever virus (ASFV) needs to be stopped. Many researchers have been trying to develop new potential vaccines throughout the years. Despite the work done in developing new vaccines, there was no success in preparing one successful vaccine that could protect pigs against the virulent virus for a prolonged time. This work focuses on developing potential proteins based on ASFV antigens (CD2v and p22) and their epitopes and uses them for immunogenicity and safety testing in mice.

MATERIAL AND METHODS

Prediction of protein tertiary structure, gene cloning, expression and purification of recombinant proteins, conformation analysis using ASFV-positive sera, mice immunisation, ELISA test and antibody titre measurement with consequent statistical analysis are described in work by Díaz *et al*., *J Vet Res* 2022; 66.

RESULTS AND DISCUSSION

Bioinformatic analysis and construct design

CD2v and p22 proteins are transmembrane proteins containing an N-terminal domain (large outer domain in CD2v; short inner domain in p22), a single membrane-spanning helix and an outer C-terminal domain (large inner domain in both proteins). In this work, only the outer globular part of the proteins was selected in order to facilitate the extraction and purification step. Two constructs were established from each protein, one contained the outer globular part of the protein alone, and the second one had the outer globular part fused with the heat-labile enterotoxin B-subunit (LTB).

Prior to the expression of the selected proteins, bioinformatic analysis was performed to estimate the tertiary structure of the target proteins of interest. Using the Phyre2 protein fold recognition server (Kelley *et al*., 2015), a putative structure (Figure 3) was modelled by comparing the amino acid sequence of the N-terminal domain of CD2v and the C-terminal domain of p22, respectively, with other known proteins from the database. Following the initial tertiary structure prediction, further bioinformatic analysis was performed using the online ProtParam tool from the ExPASy server (https://web.expasy.org/protparam/; Gasteiger *et al*., 2005) to calculate the physio-chemical parameters of the target proteins. Results show that these proteins are suitable for production in *Escherichia coli* expression strains.

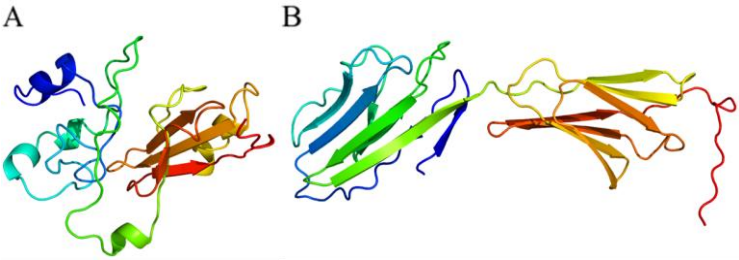

Figure 3: Predicted protein structure by the Phyre2 protein fold recognition server (Kelley *et al.*, 2015).

Four DNA constructs (named *CD2vCt*, *p22Ct*, *LTB-CD2vCt*, and *LTB-p22Ct*; Figure 4) were codon-optimized for the expression in *E. coli*, the first two coding for the above C-terminal globular fragment of the p22 protein and CD2v protein, and the other two for N-terminal fusion of that fragment with the heat-labile B-subunit of enterotoxin (LTB). All constructs were cloned into the pET28b(+) vector prior to the expression experiments.

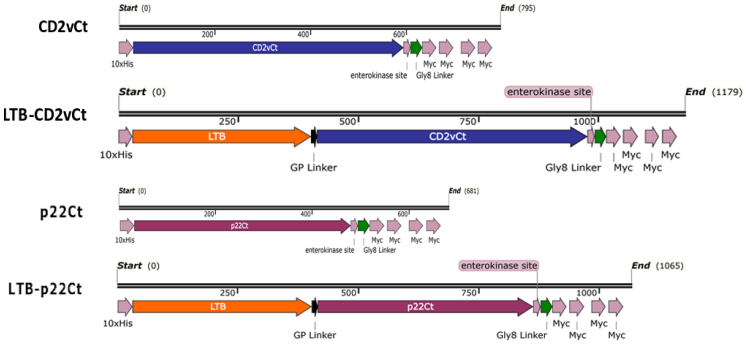

Figure 4: All DNA construct design, CD2vCt, LTB-CD2vCt, p22Ct and LTB-p22Ct.

Expression of CD2vCt and LTB-CD2vCt

CD2v protein shares homology with CD2, a T cell receptor containing disulphide bonds inside the immunoglobulin part of the protein (Bromberg, 1993). The BL21 strain might be inappropriate for expression since it maintains a stable, reducing environment inside the cells and thus disables the expression of disulphide-bond proteins (Kong and Guo, 2014). Therefore, several strains, such as Rosetta or Origami, have been developed

with silenced *trxB* and *gor* genes and are widely used for the expression of disulphide bond proteins (Bessette *et al.*, 1999).

Small-scale expression experiments were performed using Rosetta™ 2(DE3) strain. Target proteins were not clearly recognized on SDS-PAGE, indicating minimal expression level. Purification was done using Ni-NTA agarose as a matrix, and the elution fractions were pooled and concentrated using 10 kDa cut-off Amicon centrifugation filters. SDS-PAGE analysis shows that elution fractions are not clean and contain impurities in both cases. On the other hand, Western blot analysis shows no positive signal.

According to obtained results, two possible reasons for protein detection inability were identified. One of them can probably be due to the experimental instability of both proteins, CD2vCt and LTB-CD2vCt, and their degradation before or during the extraction. Another reason includes the potential issue of the expression strain while considering them toxic and degrading them during the expression. Due to the inability to produce CD2vCt and LTB-CD2vCt, both proteins were abandoned and not produced anymore. The following reasons for protein detection inability are only hypothetical since there are no studies concerning the immunoglobular CD2v protein segment production in *E. coli* expression strains, which could be used for comparison. Therefore these two proteins were abandoned and not continued to work with them.

LAE-based proteins

Linear array epitope (LAE) is a method developed by Taiwanese scientists (Lai *et al.*, 2014). This method is mainly used to produce antibodies against the less immunogenic proteins and use them for diagnostic and therapeutic applications.

The principle of the method is to select the potential antigen epitopes, which will be replicated using this method and subsequently expressed and used for either vaccination or detection purposes. Since the previous preparation of the C-terminal part of CD2v protein, as described above, was not successful, its possible immunogenic regions were further examined by the LAE method. Two selected epitopes, F3 and A6, were confirmed to be the most suitable epitopes of CD2v protein (Argilaguet *et al.*, 2012).

Four clones have been selected from A6 epitope repetitions and other three from F3 epitope repetitions. Selected clones were transformed into chemically-competent BL21 STAR expression cells and used for an expression experiment.

A small-scale expression of A6 and F3 LAE proteins in the *E. coli* BL21 STAR cell cultures was performed using the pGEX-5x-1 vector. Samples were taken before and four hours after the induction, centrifuged and used for the SDS-PAGE analysis using 12 % polyacrylamide separating gel and Western blot analysis using an anti-GST antibody. SDS-PAGE (Figure 15A, 15C) and Western blot analysis (Figure 15B, 15D)

showed that all LAE proteins were successfully overproduced in the *E. coli* BL21 STAR strain.

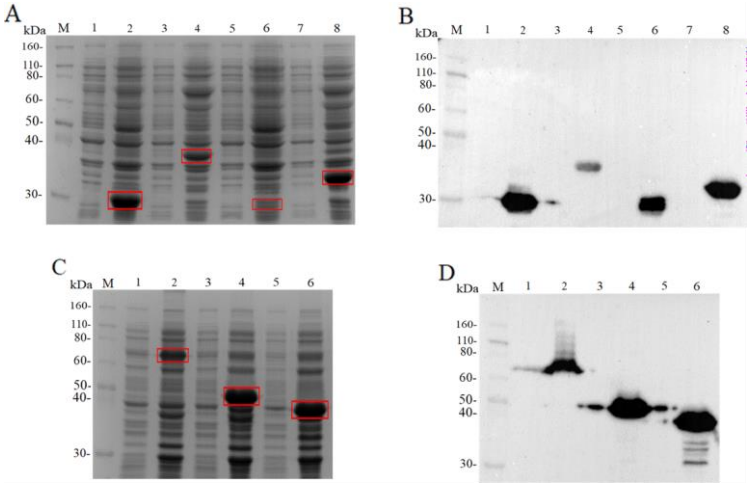

Figure 5: Analysis of expression of four clones containing a different number of repetitions of LAE epitopes made by SDS-PAGE (A and C) and Western blot analysis (B and D)

Presented ASFV LAE antigens were planned to be used for polyclonal antibody production in rabbits during the expected internship at the National Chung Cheng University in Taiwan. These antibodies were supposed to be used for the subsequent development of the diagnostic technique against ASFV.

Expression of p22Ct and LTB-p22Ct

Preliminary expression experiments regarding *p22Ct* and *LTB-p22Ct* constructs were the first successful experiments among all selected constructs. The p22Ct and LTB-p22Ct proteins were expressed in a larger amount to examine the purification efficiency using the Ni-NTA purification matrix. Proteins were concentrated and analysed by Western blot analysis confirming their identity.

However, the mobility on the SDS-PAGE corresponded to higher than the calculated molecular weight. Two additional experiments were performed to prove the protein identity: removal of the Myc-tag by enterokinase and protein identification using Matrix-Assisted Laser Desorption/Ionisation – Time of Flight (MALDI-TOF). After removing the 4xMyc-tag, the mobility of the protein band corresponding to the protein

with a cleaved Myc-tag was again analysed by SDS-PAGE. However, the protein mobility did not change as expected. On the other hand, the MALDI-TOF analysis confirmed the presence of target proteins. Results corresponding to the p22Ct protein correlate with p22 protein.

Following the successful pilot expression experiments, the 4xMyc-tag was no longer required for subsequent experiments; therefore, it was removed from the *p22Ct* and *LTB-p22Ct* gene constructs by site-directed mutagenesis. A small-scale expression of p22Ct and LTB-p22Ct proteins was performed to confirm the protein expression levels after the site-directed mutagenesis (Figure 6).

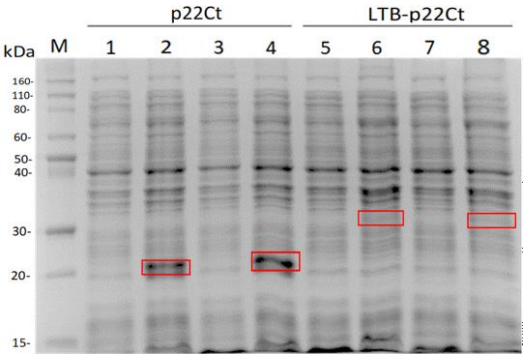

Figure 6: SDS-PAGE analysis of crude extracts from E. coli BL21 STAR cells expressing p22Ct and LTB-p22Ct.

The large-scale expression was performed under the same conditions in 1 L cultures to produce higher protein quantities for purification and further experiments. The cells were harvested by centrifugation and lysed by sonication. The soluble protein fractions were then used to purify the His-tagged recombinant proteins p22Ct and LTB-p22Ct using the Ni-NTA agarose matrix.

The purified recombinant proteins p22Ct and LTB-p22Ct were then tested by immunoblot analysis for their ability to interact with serum antibodies (IgG) from ASFV-infected pigs. Both proteins showed positive reactions with the four sera used as primary antibodies (Figure 7).

The positive reactions of both proteins to four different positive sera indicate the correct conformation of both proteins, thus showing the possibility of induction of the immune response in the host and the production of specific antibodies. As implied by the article by Scheiblhofer *et al.* (2017), conformational stability of the target proteins is essential for the immunogenicity of recombinant vaccines.

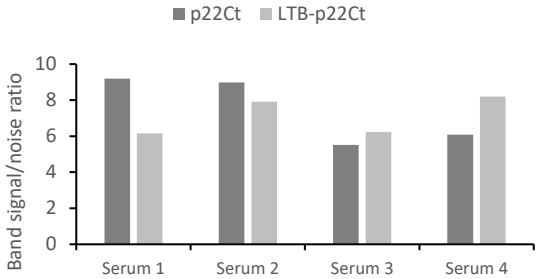

Figure 7: Immunoblot assay of the purified recombinant proteins p22Ct and LTB-p22Ct with four different sera from ASFV infected pigs; the binding intensity was calculated as the intensity of the bands compared to the background over the whole lane of the immunoblot assay.

The general immunogenicity of the prepared recombinant proteins was assessed by mice immunization. For three mice in each group, the protein samples mixed with an adjuvant were injected subcutaneously into the back behind the head with a two-week interval between doses. The control mice group was vaccinated with the adjuvant alone. With the p22Ct protein, the mice were fully immunized with three doses. However, in the case of the LTB-p22Ct group, an abscess occurred after the first dose. Due to this fact, only one dose was administrated. The blood of all mice was collected on day 42, and serological activity against the recombinant proteins was analysed by ELISA. Antibody titre was high for the mice group immunized with p22Ct, while the samples from the LTB-p22Ct group did not react at all (Figure 8).

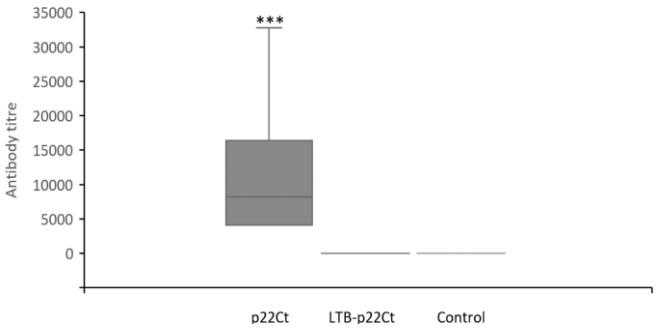

Figure 8: ELISA test results of sera samples taken from mice groups immunised with p22Ct and LTB-p22Ct; the measurement was done in three technical replicates; the

statistical significance of the results was verified by a one-way ANOVA analysis, $P < 0.001$.

Our results show that p22Ct, the C-terminal globular part of the p22 protein, but not the fusion protein LTB-p22Ct, can induce an immune response in mice. The exact cause of the observed difference is unclear. The most probable reason is that mice immunised with LTB-p22Ct were injected with only one dose rather than the three doses of p22Ct administered. This is probably also the reason why the mice immunised with LTB-p22Ct could not produce specific antibodies against the protein.

Our results also show that p22Ct can produce a high antibody titre in mice and is thereby indicated to be a highly potent and immunogenic region. It can be considered a candidate primarily in serological diagnostics for the development of specific antigen-based detection techniques such as fluorescent antibody tests, ELISA and immunoblotting (Qiu *et al.*, 2021). However, further experiments should be conducted to investigate its possible link to other immunostimulatory domains.

CONCLUSION

African swine fever virus was and still is the reason behind an economic crisis causing the death of millions of pigs. ASFV infects mainly macrophages and uses them for replication of new viral particles. Multiple vaccines were developed to combat the disease caused by this virus, but no vaccine was 100 % effective. Recent studies show the development of new attenuated vaccines in eradicating the ASF virus (Borca *et al.*, 2020; Gladue *et al.*, 2021).

In this work, we have prepared potential recombinant proteins containing a globular hydrophilic fragment of the ASFV antigens, CD2v and p22. From four initial constructs, two (*CD2vCt*, *LTB-CD2vCt*) were unable to express using the bacterial expression system, while the other two (*p22Ct*, *LTB-p22Ct*) were expressed successfully and used for further experiments.

After successful expression, the protein identity of p22Ct and LTB-p22Ct were analysed by MALDI-TOF since the protein mobility on SDS-PAGE was slower than expected. The most probable reason behind this phenomenon could be the high protein hydrophilicity (Shirai *et al.*, 2008). After the deletion of the 4xMyc-tag, the correct protein conformation was confirmed by the immunoblot analysis with positive ASFV sera, which is essential for the immunogenicity of recombinant proteins (Scheiblhofer *et al.*, 2017). Following mice immunisation with p22Ct and LTB-p22Ct showed high contrast between both proteins in the inductions of antibodies. No specific antibody induction by the LTB-p22Ct was probably due to only one antigen dose injected since the abscess formation was observed. In contrast, the high specific antibody level

induced by p22Ct shows its potential as a serological marker for the development of new diagnostic techniques for combating the ASFV spread.

In this work, the LAE technique was used for the first time in the ASFV research. CD2v epitopes were chosen based on the Argilaguet *et al.* (2012) study, showing the epitopes' potential. Proteins were successfully overexpressed using a bacterial expression system. However, future experiments are needed to measure the immunogenicity and show LAE proteins' potential in the development of diagnostic tools.

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PALACKÝ UNIVERSITY OLOMOUC

Faculty of Science

Department of Biochemistry

Production of recombinant proteins for veterinary use

Ph.D. Thesis

Author:	**Mgr. Carlos Díaz**
Study program:	P1416 Biochemistry
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Supervisor:	**Prof. RNDr. Ivo Frébort, CSc., Ph.D.**
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Abstrakt

Virus afrického moru prasat (ASFV z anglického African swine fever virus) je nejsmrtelnějším členem DNA virů infikujících prasata, a proto masivní epidemie způsobená ASFV musí být zastavena. Mnoho vědců v průběhu let pracovalo na vývoji potenciální vakcíny, ale úspěšnou vakcínu, která by dokázala chránit prasata před virulentním virem po delší dobu se zatím nepodařilo připravit. Tato práce se zaměřuje na přípravu a testování imunogenicity potenciálních kandidátů na podjednotkovou vakcínu sestávajících z virových proteinů CD2v, p22 a termálně labilní B podjednotky enterotoxinu (LTB). Byly navrženy čtyři genové konstrukty (CD2vCt, LTB-CD2vCt, p22Ct, LTB-p22Ct). Pro jejich konstrukci byly vybrány pouze části genů kódující vnější globulární domény virových proteinů, CD2v a p22, samostatně nebo sloučené s genem kódujícím LTB. Hlavní funkcí LTB bylo nabudit imunitní odpověď hostitele. Všechny konstrukty byly použity pro expresi pomocí bakteriálního expresního kmene BL21 STAR a kmene Rosetta 2.

Rekombinantní proteiny p22Ct a LTB-p22Ct byly úspěšně nadprodukovány v buňkách BL21 STAR. Imunoanalýzou pak byla potvrzena jejich schopnost reagovat s různými séry od prasat infikovaných ASFV, což indikovalo správnou konformaci proteinu. Rekombinantní proteiny byly dále použity pro imunizaci myší pro další analýzu. Fúzní protein LTB-p22Ct nevykazoval očekávané adjuvantní vlastnosti a neukázal se jako bezpečný, protože po imunizaci myší byla pozorována tvorba abscesu. Na druhé straně, imunizace samotným proteinem p22Ct ukázala vyšší titr protilátek a nezpůsobila u myší žádné nežádoucí projevy. Tyto výsledky ukazují vysoký potenciál

antigenu p22 jako imunogenního proteinu, který je možné použít buď pro budoucí vývoj vakcín proti ASFV, nebo pro účely jeho specifické detekce.

Tato práce také ukazuje potenciál nové metody, nazývané lineární epitopové pole (LAE z anglického linear epitope array), při přípravě epitopových antigenů pro detekční nebo vakcinační účely.

Klíčová slova	Africký mor prasat, tepelně-citlivá enterotoxinová B podjednotka, imunitní odpověď, p22 protein, CD2v protein
Počet stran	104
Počet příloh	2
Jazyk	Anglický

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Abstract

ASFV is the deadliest member of the DNA viruses infecting pigs and a massive disaster for pigs caused by the African swine fever virus (ASFV) needs to be stopped. Many researchers have been trying to develop new potential vaccines throughout the years, but there has been no success in preparing a successful vaccine that could protect pigs against the virulent virus for a prolonged time. This work focuses on the design and immunogenicity testing of potential subunit vaccine candidates consisting of ASFV viral proteins CD2v, p22, and heat-labile B subunit of enterotoxin (LTB). Four gene constructs (*CD2vCt*, *LTB-CD2vCt*, *p22Ct*, *LTB-p22Ct*) were designed. Only the gene parts coding for the outer globular domains of viral proteins, CD2v and p22, alone or fused with a gene coding for LTB, were selected. The LTB's main function is to induce the host immune response. All constructs were used for the expression using bacterial expression strain BL21 STAR and Rosetta 2 strain.

Recombinant proteins p22Ct and LTB-p22Ct were successfully overproduced in BL21 STAR cells. The ability to react with different ASFV positive sera confirmed by immunoanalysis indicated the right protein conformation. Recombinant proteins were further used for mice immunisation to analyse their safety and immunogenicity. The protein fused with LTB did not show expected adjuvant properties and did not prove safe since an abscess formation was observed after mice immunisation. On the other hand, immunisation with p22Ct protein alone showed higher antibody titre and conferred safety in mice. These results show the high potential of the p22 antigen as

an immunogenic protein, either used for future ASFV vaccine development or detection purposes.

Furthermore, this work shows the potential of a novel method, called linear array epitope (LAE), in producing epitope antigens for detection or vaccine purposes.

Keywords	African swine fever, heat-labile enterotoxin B subunit, immune response, p22 protein, CD2v protein
Number of pages	104
Number of appendices	2
Language	English

I hereby declare that this Ph.D. thesis has been written solely by me. All the sources quoted in this work are listed in the "References "section. Co-authors approve all published results included in this work.

September……..… …….

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Table of Contents

Aims of the work

1. Collecting all information about the African swine fever virus and its antigens, and to show how these antigens could be used as diagnostic tools or as potential vaccine candidates for combating the African swine fever.

2. Expression and purification of constructs containing the outer part of selected ASFV antigens from Escherichia coli expression strain BL21 (STAR) and Rosetta™ 2(DE3), respectively.

3. Overproduce the selected proteins and use them for two following analyses; the immunoassay against positive pig sera for confirmation of proper conformation and mice immunisation for safety and immunogenicity evaluation for respective proteins.

4. Determination of the level of produced antibodies in mice blood samples collected after the immunisation by ELISA analysis.

THEORETICAL PART

Introduction

An enormous economic loss in the pig industry caused by the African swine fever virus (ASFV) needs to be stopped. Many researchers have been trying to develop new potential vaccines throughout the years. Despite the work done in developing new vaccines, there was no success in preparing one successful vaccine that could protect pigs against the virulent virus for a prolonged time. This work focuses on developing potential proteins based on ASFV antigens (CD2v and p22) and their epitopes and uses them for immunogenicity and safety testing in mice.

African swine fever virus

African swine fever virus (ASFV) is a large virus with linear 170 kbp long double-stranded DNA protected by three envelopes consisting of multiple structural proteins (Chapman *et al.*, 2008, Yáñez *et al.*, 1995). ASFV, as a sole member of the genus *Asfivirus* within the family *Asfarviridae* (MacLachlan *et al.*, 2011), is causing a highly infectious disease called African swine fever, which targets all species of the family *Suidae*. The vector of the virus is ticks from the genus *Ornithodoros* (Frant *et al.*, 2017).

The transmission of the virus exists not only between ticks and swine but also between swine individuals (Guinat *et al.*, 2016). There are four possible known transmission cycles of ASFV: **sylvatic cycle**, primarily present in eastern Africa and between the soft ticks from the genus *Ornithodoros* and warthogs; **tick-pig cycle**, in Africa and the Iberian Peninsula, where *Ornithodoros* spp. ticks infest domestic pigs in their pig pens; **a domestic cycle** that occurs between the domestic pigs, where secretions and excretions of infected pigs transmit the virus; **transmission from sylvatic to domestic cycle** between African wild suids and domestic pigs, which occurs due to shared water supply (Costard *et al.*, 2013). All the possible transmission cycles are shown in Figure 1, divided into two parts, one for Africa and the other for Europe and Asia.

The history of the African swine fever goes back to the beginning of the 20th century when it was first identified in 1921, but its first occurrence was already in 1910 in British East Africa (Kenya Colony; Montgomery, 1921). From there, it spread throughout Africa and gradually to other adjacent continents. The first presence of the virus in Europe goes back to 1957 in Portugal (Cwynar *et al.*, 2013), from where it

was spread to other European counties. Interestingly, its first occurrence in the Czech Republic was only recently, in 2017 (Forth *et al.*, 2020).

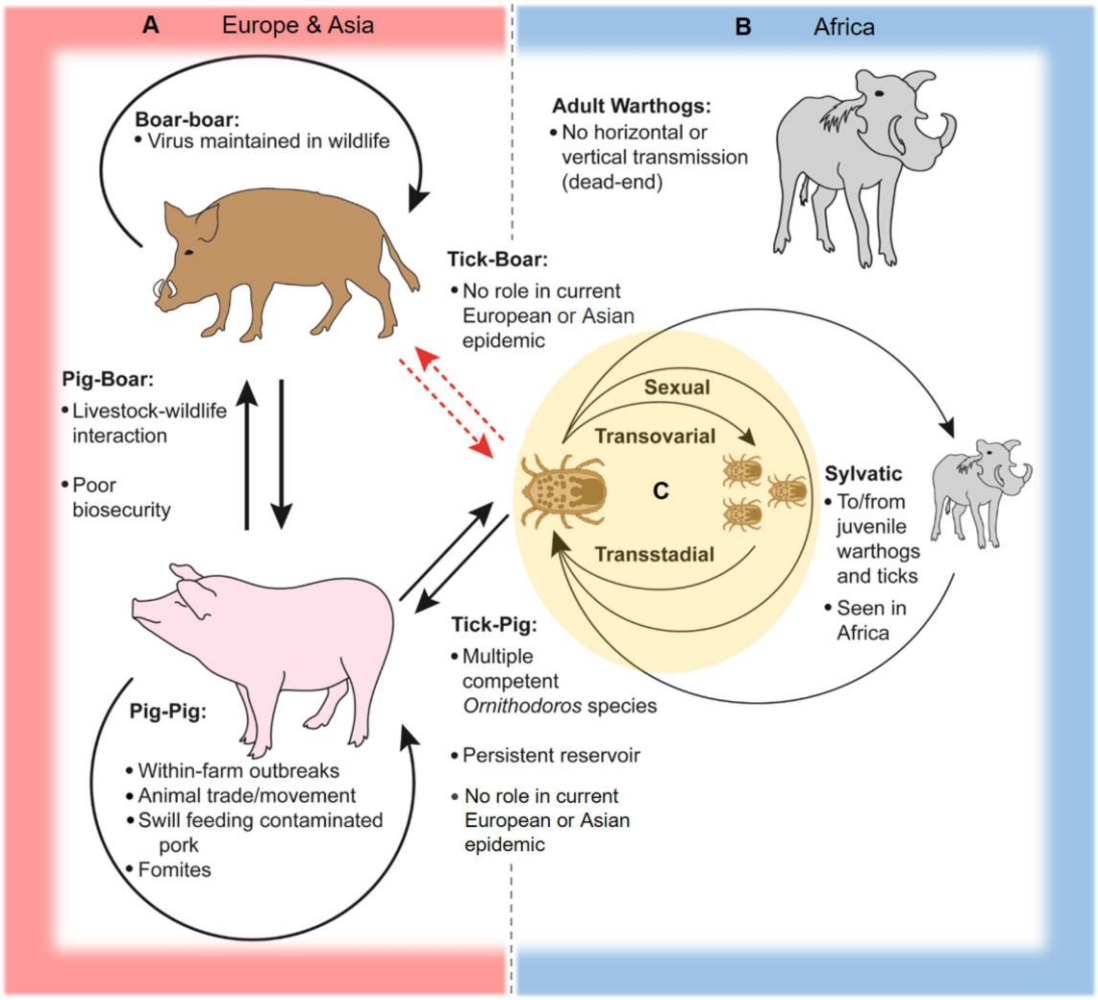

Figure 1: Transmission cycles of the ASFV in Europe, Asia, and Africa
Part A represents the transmission within Europe and Asia, where the transmission is primarily dependent on the contact between wild boars and domestic pigs, individually or separately; Part B represents the transmission within Africa, where the transmission is mostly between ticks and warthogs, called as a sylvatic cycle.; adapted from Gaudreault *et al.*, 2020

This chapter is focused on many features of the ASFV, including the structure, the process of infection inside the host cells, and the development of the vaccines throughout the years and their typology.

Virus structure

The viral particle of the ASFV has icosahedral symmetry, as shown in Figure 2. As mentioned above, ASFV consists of three envelopes: outer, capsid, and inner envelope. The primary role of the envelopes is to protect the core of the viral particle,

where linear dsDNA is localized. A description of each envelope's most important structural proteins, including their function and role, follows.

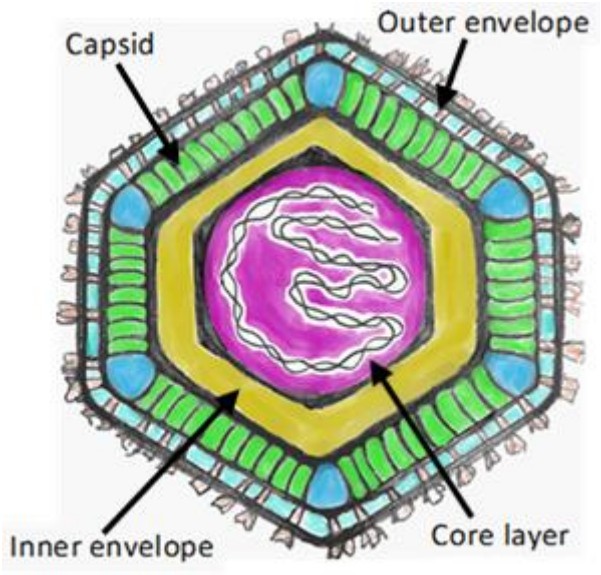

Figure 2: The structural characteristic of the African swine fever virus
Viral particle consists of three envelopes: inner envelope, capsid and outer envelope. The middle part (purple) is called the core layer and contains linear dsDNA of around 170 kbp; adapted from Díaz *et al.*, 2021.

Outer envelope

The outer layer is composed of the structural proteins p12 (*pO61R*) and CD2v (*EP402R*) (Alcamí *et al.*, 1992; Rodríguez *et al.*, 2004). The p12 protein (*pO61R*) is a late structural protein known as an attachment protein during the entry of the viral particle to the host cell (Alcamí *et al.*, 1992). However, p12 protein was also found to be a part of an inner envelope (Salas and Andrés, 2013).

CD2v is a more complex protein which plays different roles during ASFV infection. It is a transmembrane protein containing 402 amino acids showing a high degree of similarity to CD2, an adhesion receptor of T lymphocytes (Rodríguez *et al.*, 1993). This protein functions in the adsorption of red blood cells on the surface of infected host cells (Borca *et al.*, 1998; Rodríguez *et al.*, 1993) and was found to interact with an adaptor protein complex (AP-1) through the diLeu motif in the C-terminal domain (Pérez-Núñez *et al.*, 2015). An AP-1 complex is a group of cytosolic heterotetramers that sort membrane proteins into endosomes by forming clathrin-

coated vesicles using clathrin as a scaffold protein (Nakatsu and Ohno, 2003). In this way, CD2v helps ASFV to enter the host cells.

Capsid envelope

The major capsid p72 protein (encoded by the viral *B646L* gene) is known for its assembly in the area of the inner core matrix and outer capsid layer of the viral particle (Cobbold and Wileman, 1998). This assembly is mediated by a chaperone encoded by *B602L* and takes place on the membrane of the endoplasmic reticulum (ER), where the process of envelopment is localised (Cobbold and Wileman, 1998). Another crucial structural protein is p49 (*B438L*), which forms the icosahedral shape of the viral particles by localising in the vertices of the capsid (Dixon *et al.*, 2013).

Inner envelope

The inner envelope contains five structural proteins: the abundant transmembrane p17 (*D117L*); the late structural pE248R (*E248R*), j5R (*H108R*) and j18L (*E199L*); p54 (j13L, *E183L*) and p22 (KP177R) (Brookes *et al.*, 1998; Rodriguez *et al.*, 1994; Rodríguez *et al.*, 2009; Suárez *et al.*, 2010; Sun *et al.*, 1996; Alejo *et al.*, 2018). Their functions have also been characterised.

j5R and p54 (j13L) are involved in the assembly of viral particles in which p54 (j13L) is accumulated on the ER membrane and involved in recruiting viral membrane precursors (Brookes *et al.*, 1998; Rodríguez *et al.*, 2004).

Protein p17 is also involved in recruiting viral precursors (Suárez *et al.*, 2010). The function of pE248R has been ascertained as an actor in the early phase during virus entry into the host cell (Rodríguez *et al.*, 2009).

The p22 protein, an early structural protein, which was initially believed to be part of the outer membrane (Camacho and Viñuela, 1991), was recently found in the inner membrane (Alejo *et al.*, 2018). An initial experiment using a non-ionic detergent showed that the p22 protein is part of the outer envelope; however, as was learned later, the detergent could disrupt the external and the capsid envelope (Andrés *et al.*, 1998; Camacho and Viñuela, 1991).

Core layer

The first step in forming the viral particle is protecting the genomic DNA with a core layer of proteins. This layer is composed of structural proteins, which originate from polyproteins pp62 (*CP530R*) and pp220 (*CP2475L*) (Simón-Mateo *et al.*, 1993;

Simón-Mateo *et al.*, 1997). Both polyproteins are processed by SUMO-like protease (*S273R*), yielding different structural proteins, which in the case of pp62 are p15 and p35 and in the case of pp220 are p14, p34, p37 and p150 (Simón-Mateo *et al.*, 1993; Simón-Mateo *et al.*, 1997).

Genomic DNA

ASFV genome is around 170 kbp long and contains 151 open reading frames (ORFs) (Chapman *et al.*, 2008, Yáñez *et al.*, 1995). The complete genome of the virulent strain Georgia 2007/1 containing multiple genes with different functions is shown in Figure 3. These genes are involved in many processes, such as DNA replication, transcription and processing, the assembly of viral particles, host defences, and last but not least, there are also multigene families, which take part in 30 % of the genome (Dixon *et al.*, 2013).

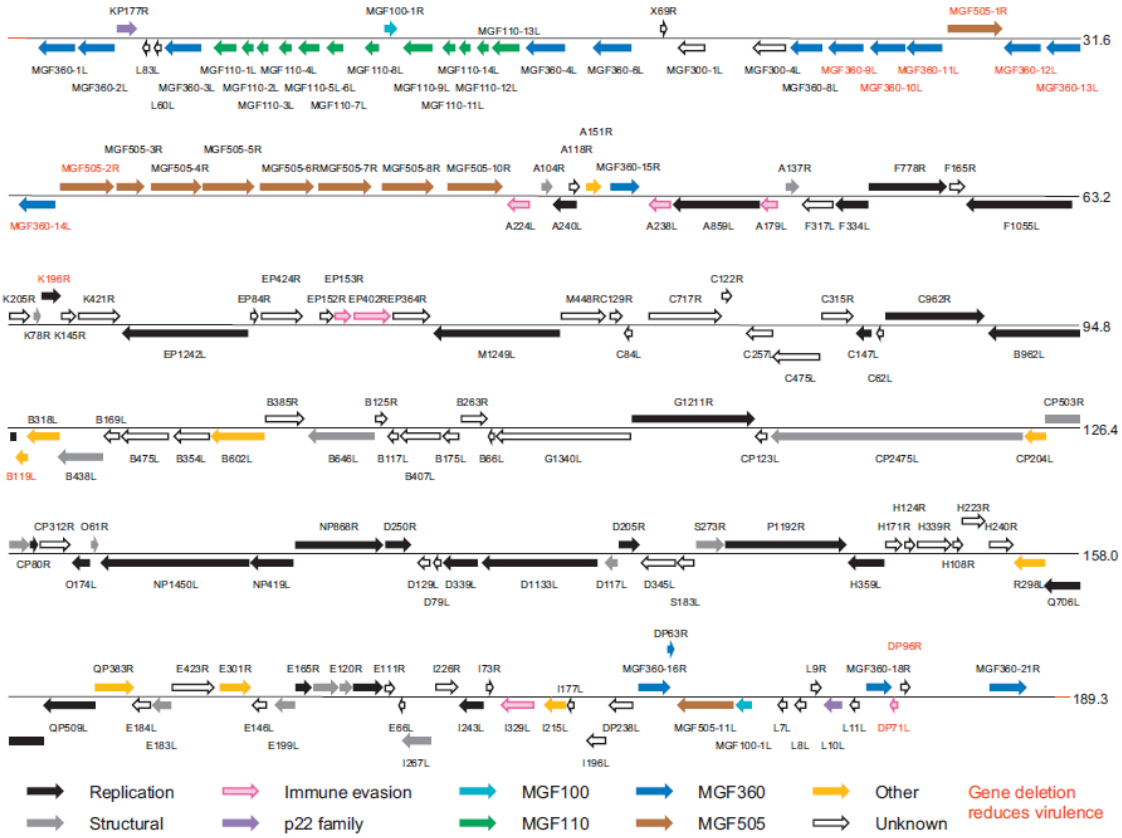

Figure 3: Genome organisation of the ASFV virulent strain Georgia 2007/1.
All ORFs are shown as coloured arrows; different colours show the gene family to which the ORFs belong; adapted from Dixon *et al.*, 2013

Genetic classification

Distinct ASFV genotypes were identified based on the p72 structural protein. Phylogenetic analysis of the C-terminal end of the p72 gene showed the presence of 22 different genotypes (I–XXII) (Boshoff *et al.*, 2007). Recently two new genotypes were added, XXIII and XXIV (Achenbach *et al.*, 2017; Quembo *et al.*, 2018), of which XXIII shares a common ancestor with the genotypes IX and X (Achenbach *et al.*, 2017). In Europe, two types of genotypes caused outbreaks: genotype I in Sardinia and genotype II in Eastern Europe (Bellini *et al.*, 2016). A phylogenetic tree in Figure 4 shows virulent strains divided into the most known ASFV genotypes worldwide, while the red-labelled strains are the most frequent ones.

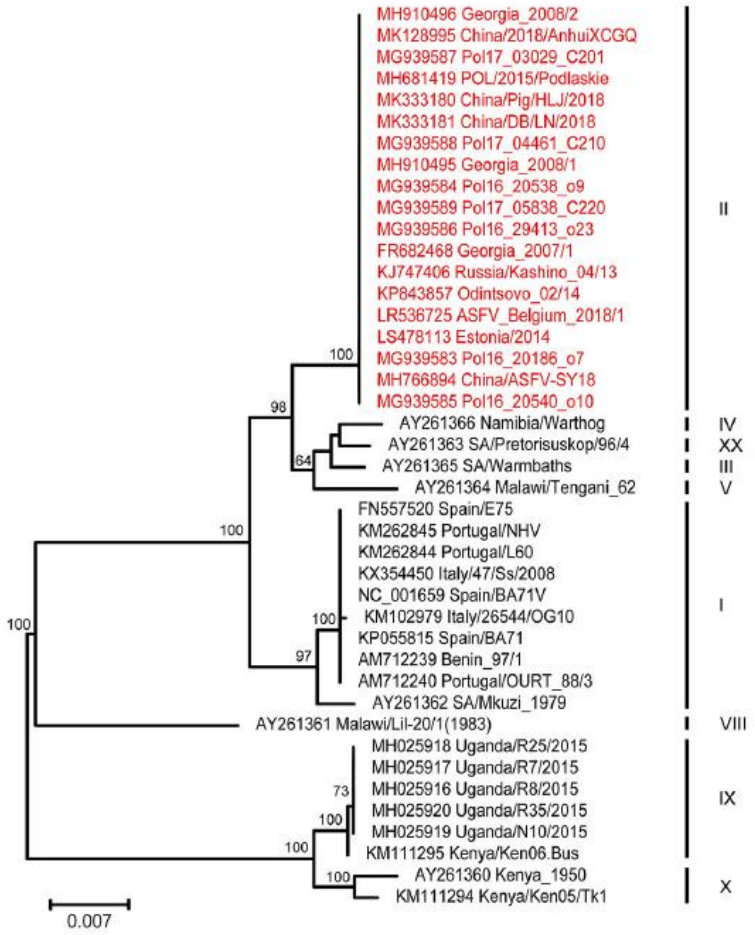

Figure 4: Phylogenetic tree dividing virulent strains into genotypes based on the partial C-terminal part of the p72 gene of ASFV.

The strains shown in red belong to the most currently widespread genotype. Numbers near the individual nodes represent the percentage of each node's support within the tree; adapted from Wang *et al.*, 2020.

Virus infection

ASFV's primary target host cells of ASFV include macrophages and monocytes (Gómez-Villamandos *et al.*, 2013; Galindo *et al.*, 2015). ASFV has evolved a complex of interactions with the host cells in order to infect and evade the host immune system. All interactions and processes are divided into five phases: virus entry, endosomal pathway, replication and transcription of the ASFV genes near the nucleus, virion assembly, and viral release, which will be described in detail within the following subchapters. A scheme of the viral replication cycle is shown and described in Figure 5.

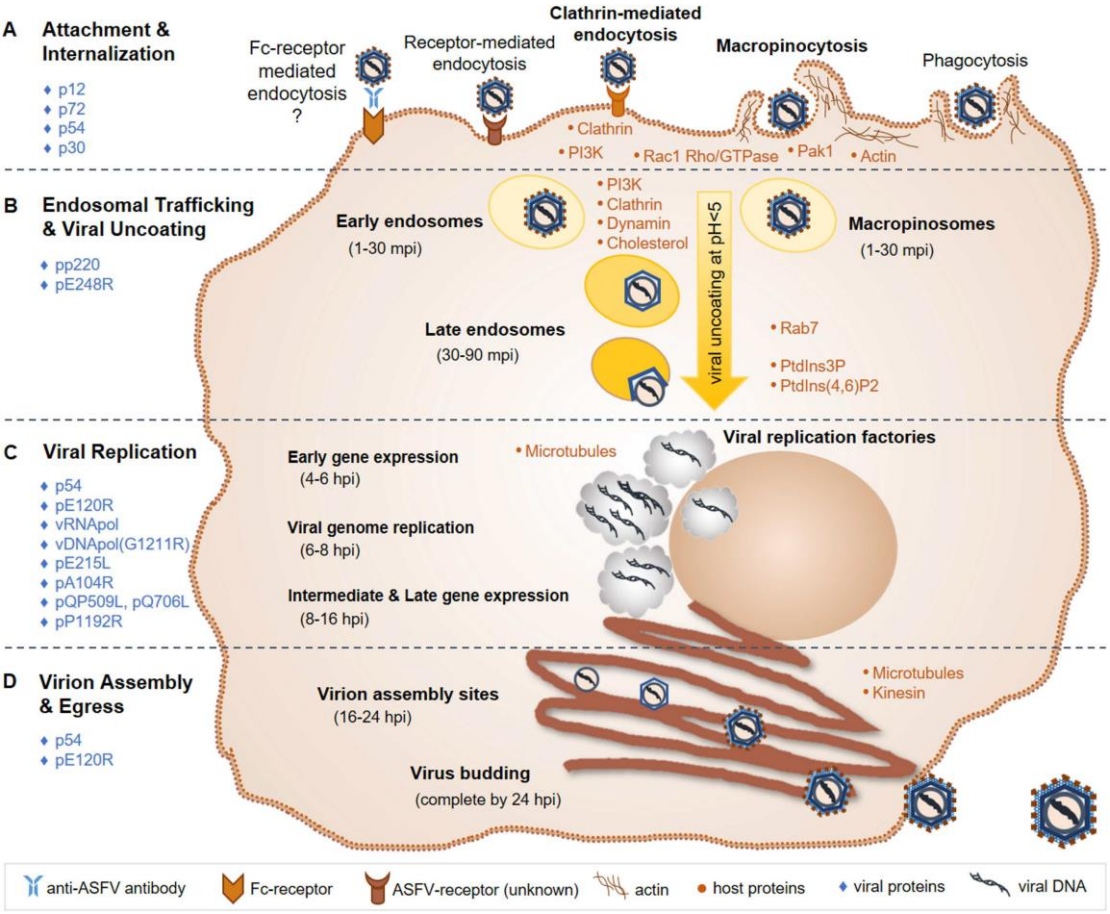

Figure 5: ASFV infection cycle
(A) Virus entry into the cells by multiple mechanisms from which the most frequent are clathrin-mediated endocytosis and macropinocytosis; (B) The endosomal pathway, virion uncoating process and subsequent genome release into the cytosol; (C) Gene expression, including replication and transcription of early, intermediate and late genes; (D) Viral particle assemble and release into the environment of host body; adapted from Gaudreault *et al.*, 2020

Virus entry

The first step in viral infection is a viral entry. Early studies on ASFV entry focused on Vero cells and showed that ASFV enters the cells by receptor-mediated endocytosis, a temperature, pH, and energy-dependent process (Alcamí *et al.*, 1989; Valdeira *et al.*, 1998). However, a later study by Hernáez and Alonso (2010) shows that the virus enters through clathrin-mediated endocytosis. Clathrin-mediated endocytosis is a process composed of a series of reactions resulting in membrane scission (Taylor *et al.*, 2012). These reactions include the recruitment of dynamin, in which GTPase activity recruits actin molecules serving as a dynamic scaffold for dynamin and N-terminal containing BIN/Amphiphysin/RVS domain containing (N-BAR) proteins at sites of membrane scission (Taylor *et al.*, 2012). Bernardes *et al.* (1998) also show that cholesterol is important during the viral entry, while its fluctuation inside infected cells can affect the ASFV infection.

Another way ASFV may enter the host cell is macropinocytosis (Sánchez *et al.*, 2012). Macropinocytosis is an actin-dependent endocytic process associated with the formation of membrane ruffles and blebs (Sánchez *et al.*, 2012). These modulations provide the membrane and energy to form the macropinosomes, endocytic vacuoles (Mercer and Helenius, 2009)

Uncoating of viral particles

Early infection depends on the production of PI-3P, involved in early endosome (EE) maturation and multivesicular body (MVB) formation, and on the transition between PI-3P and PI-3,5-biP (Cuesta-Geijo *et al.*, 2012). After the virions move to multivesicular endosomes, they lose their outer and capsid envelopes, which depend on the low acidic pH inside the endosome (Hernáez *et al.*, 2016). Thus, the inner envelope is exposed and subsequently fused with the late endosomal (LE) membrane to release the viral genome into the cytosol (Andrés, 2017). This fusion is mediated by the pE248R transmembrane protein of the inner envelope (Andrés, 2017). The ASFV is also known to modulate the Rho family GTPase activity within the host cell, which is critical for virus morphogenesis and viral transport during early infection by stabilizing microtubules (Quetglas *et al.*, 2012). Rac1 protein from the Rho family modulates the ASFV transport within the cell by inducing microtubule acetylation (Quetglas *et al.*, 2012). On the other hand, GTPase Rab7 activity and processes related

to LE compartment physiology are also crucial during early infection (Cuesta-Geijo *et al.*, 2012).

Cholesterol also plays a vital role during an endocytic pathway, where its efflux from endosomes helps release the ASFV genome to the cytosol (Cuesta-Geijo *et al.*, 2016). ASFV can also affect cholesterol uptake into the infected cells, concretely to the replication sites to ensure lipid flow to produce more virions (Cuesta-Geijo *et al.*, 2016).

The further transport of the genome is mediated by p54 protein, which interacts with the light chain of dynein until it reaches the perinuclear spot near the microtubular organizing centre (MTOC), where DNA replication and transcription take place (Alonso *et al.*, 2001).

Genome replication

As mentioned above, the genome is transported to the place of viral replication with the help of p54 (j13L) viral protein after its release from LE. However, based on the study by Brookes *et al.* (1996), genomic DNA is localized not only on the site of viral factories and in the virions outside the cells but also inside the cell nucleus. Later, it was found that nuclear interactions are essential for the early stage of ASFV infection, where the genome is localized at the beginning of infection (Ballester *et al.*, 2011).

During the early stage of infection, the genome should be transferred to the proximity of the nucleus. Two ASFV proteins, p37 and p14, have been found to have a transport activity between the nucleus and cytoplasm (Eulálio *et al.*, 2004). A later study by Eulálio *et al.* (2007) showed that protein p37 was found in the proximity to the viral DNA during the early stages of infection, indicating that p37 protein may transport the viral DNA to the nucleus and then from the nucleus to the viral factories.

After the ASFV genome is transferred to the nucleus, it can replicate independently on the host cell (Dixon *et al.*, 2013). ASFV gene expression is divided into four phases: immediate, early, intermediate and late. Early genes are coding for proteins and enzymes for genome replication necessary for late gene expression and multigene family (Gaudreault *et al.*, 2020; Wang *et al.*, 2021). Mid and late genes are coding for structural proteins required for the virion assembly and early transcription factors (Gaudreault *et al.*, 2020; Wang *et al.*, 2021).

Particle assembly

The ASFV viral replication occurs at the virus factories, which resemble aggresomes, a place of protein aggregates that enable rearrangements of cellular membranes and cytoskeleton reorganization (Wileman, 2016). For this reason, microtubules' integrity is necessary for forming viral factories (Galindo and Alonso, 2017). Viral factories are localized at the MTOC near the nucleus, where viral proteins and genomic DNA are assembled to form new viral particles (Galindo and Alonso, 2017). This replication place also causes the collapse of vimentin, which enables the formation of characteristic cages around the viral factories (Figure 6) (Heath *et al.*, 2001). Vimentin is the major protein of intermediate filaments of vertebral mesenchymal cells (Franke *et al.*, 1979). For vimentin to work correctly and form the cage need to be phosphorylated by calcium calmodulin-dependent protein (CaM) kinase II (Stefanovic *et al.*, 2005). At the same time, ASFV DNA replication is responsible for activating CaM kinase II and thus vimentin phosphorylation on Ser82 (Stefanovic *et al.*, 2005). Furthermore, vimentins' cage formation and phosphorylation are essential for DNA replication and late gene expression (Stefanovic *et al.*, 2005).

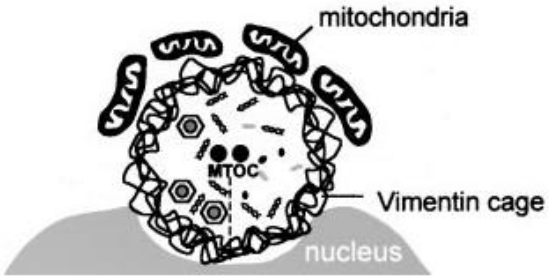

Figure 6: Structure of the viral factory
The viral factory is localized on the MTOC, surrounded by the vimentin cage and mitochondria. Its localization is near the nucleus, essential for an early stage of replication; edited from Heath *et al.*, 2001

Other than that, viral factories also cause the recruitment of the mitochondria and cellular chaperones, as shown in Figure 6 (Heath *et al.*, 2001). Mitochondria, in this case, is used as a source of energy for protein folding and degradation, while chaperons are used for viral protein folding.

In the early stage of virion assembly, structural protein p54 (E183L) targets the ER membrane and thus recruits it into the precursors (pp220 and pp62), forming the core-shell of the viral particle (Rodríguez *et al.*, 2004). Later, non-completed empty

virions are formed within the viral factory, while simultaneously formed nucleoprotein is inserted inside the empty particles once formed (Brookes *et al.*, 1996). The subsequent delivery process of the viral membranes to the viral factory is still unclear, but p54 (j13L), j5L and pXP124L proteins could play an essential role in the delivery (Brookes *et al.*, 1998; Rouiller *et al.*, 1998; Windsor *et al.*, 2012). The origin of lipid membranes needed for building the inner and capsid envelopes for new viral particles has been found. The origin of the membranes comes from two-membrane collapsed ER cisterna wrapped around the core-shell (Rouiller *et al.*, 1998; Andrés *et al.*, 1998), while ASF viral protein pXP124L may play a role in this process (Rouiller *et al.*, 1998). In addition, protein p54 (j13L) is responsible for the collapse of the ER cisterna, while disulphide bond dimerization of p54 (j13L) is essential in this process (Windsor *et al.*, 2012). The process of wrapping and obtaining inner and capsid envelopes is demonstrated in Figure 7.

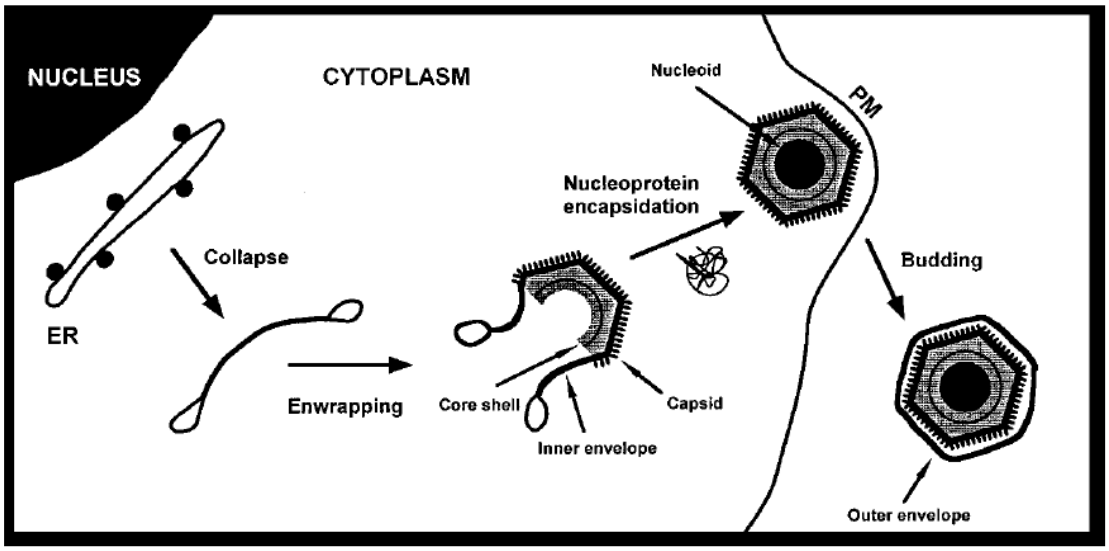

Figure 7: Origin of inner and capsid envelope of ASFV
ER collapsed cisterna is wrapping the core-shell containing genome. This process requires viral proteins to be incorporated into the membrane of the cisterna; Adapted from Andrés *et al.*, 1998

Viral release

The last step of the ASFV intracellular infection is the release of complete assembled viral particles outside the infected cell. Completed viral particles are subsequently recognized by kinesin, a plus-end microtubule, which transfers them from the virus factories to the cell plasma membrane (CPM) (Jouvenet *et al.*, 2004). The pE120R virus protein helps in the microtubule-mediated transfer of viral particles from the viral factory to the CPM (Andrés *et al.*, 2001). The protein is attached to the surface of intracellular virions by binding to the p72 major capsid protein, which helps

to incorporate pE120R into the viral particle (Andrés *et al.*, 2001). Once the ASFV viral particles are at the CPM, ASFV cause actin polymerization and form the so-called actin tail on the cytosolic part of the CPM (Jouvenet *et al.*, 2006). This local actin polymerization is caused by the host and/or viral proteins' interaction at the CPM, but these proteins are still not well known (Jouvenet *et al.*, 2006). The final virus particle release happens through the budding process, where it obtains a third outer envelope from the CPM (Breese and DeBoer, 1966).

Evasion from the host immune system

The first step in protecting the organism against the ASFV viral infection is to kill the infected cells by the host immune system or apoptosis. However, to survive and reproduce, ASFV has developed proteins that evade programmed cell death (PCD) of the infected host cells, including apoptosis and autophagy. ASFV contains multiple genes coding for viral proteins that inhibit the function of interferon type I (IFN I), resulting in inhibition of the antiviral state in infected host cells (Dixon *et al.*, 2019). One study suggests that the MGF 360 and 505 multigene families are involved in evasion from the antiviral state due to the sensitivity of the virus to IFN I when MGFs were deleted (Golding *et al.*, 2016).

Inhibition of apoptosis

The critical part of escaping the host immune system includes inhibiting cell death by apoptosis. Proteins, which disable the apoptosis mechanism of the host cell, are called inhibition of apoptosis proteins (IAPs) (Figure 8). The well-known IAPs from ASFV include A238L, A224L DP71L and EP153R (Nogal *et al.*, 2001; Hurtado *et al.*, 2004; Silk *et al.*, 2007; Zhang *et al.*, 2010).

Viral protein A238L inhibits the tumour necrosis factor-alpha (TNFα) and nuclear factor NFκB (Granja *et al.*, 2006; Silk *et al.*, 2007), resulting in apoptosis inhibition. Protein A238L also binds to the calcineurin and is thus stabilized; however, this binding inhibits calcineurin phosphatase activity, which is essential for activating the nuclear factor of activated T cells (NFAT) (Miskin *et al.*, 2000). NFAT is a transcription factor playing a role in controlling the immunomodulatory processes (Miskin *et al.*, 2000).

Another IAP protein which can inactivate apoptosis is A224L. This protein is recognised by the BIR motif and uses TNF-α as a stimulus for inhibition of apoptosis

(Dixon *et al.*, 2019). A224L is binding and thus inhibiting caspase-3 (Nogal *et al.*, 2001). It also activates the NFκB, which activates the expression of an inactivated caspase-8 homologue, cFLIP, which subsequently blocks caspase-8 activity (Dixon *et al.*, 2019). However, this protein is not essential for growth or viral virulence (Neilan *et al.*, 1997), suggesting that inhibition of apoptosis by TNF-α is unnecessary for ASFV replication.

Viral protein pEP153R, a C-type lectin homologue, also inhibits apoptosis in the infected cells by reducing the activity of cellular protein p53 (Hurtado *et al.*, 2004).

The last but not the least of the IAPs is protein DP71L. DP71L dephosphorylates the translation initiation factor eIF2α, which later inhibits ATF4 and its target CHOP, resulting in the inhibition of apoptosis (Zhang *et al.*, 2010).

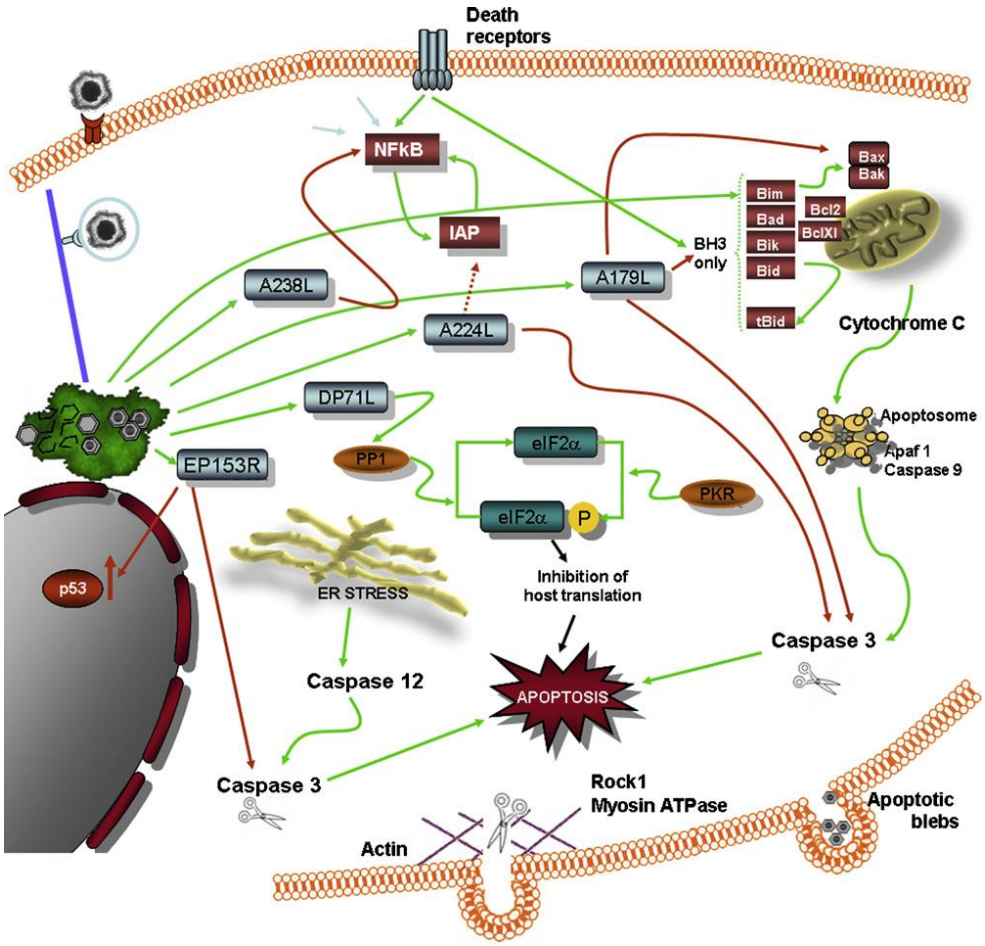

Figure 8: Inhibitions of apoptosis affected by multiple ASFV proteins and their pathways Red arrows represent the inhibition of corresponding proteins, while green arrows show the activation of shown proteins. ; adapted from Alonso *et al.*, 2013.

The A179L protein belongs to the B-cell lymphoma Bcl2 family (Banjara *et al.*, 2017), which is characterised by an anti- or pro-apoptotic function depending on the

type of homology region (BH1–BH4) and the protein interactions (Kvansakul *et al.*, 2017; Youle and Strasser, 2008). This protein is known for interacting with proteins containing the BH3 domain (such as Bak and Bax) and their resultant inactivation (Banjara *et al.*, 2017). Bak and Bax are primary gatekeepers, which upon activation by apoptosis inducers, cause disruption of mitochondrial membranes, and the following release of cytochrome *c* activates the caspase cascade resulting in apoptosis (Kvansakul *et al.*, 2017; Youle and Strasser, 2008).

All described proteins are incorporated in Figure 8, showing their corresponding role in the anti-apoptotic mechanisms.

Activation of apoptosis

ASFV at later stages of infection can induce apoptosis, facilitating virus spread and uptake by host macrophages in apoptotic bodies, which help in evasion from inflammatory responses by necrotic cell death (Dixon *et al.*, 2019). A recent study by Li *et al.* (2021) shows that ASFV viral protein pE199L plays an essential role in inducing mitochondrial-dependent apoptosis in ASFV-infected cells. The pE199L was found to interact with Bcl2 family members and compete with Bak for BCL-X_L, which promotes activation of Bak and Bax (Li *et al.*, 2021). A more detailed picture of pE199L activation of apoptosis is shown in Figure 9.

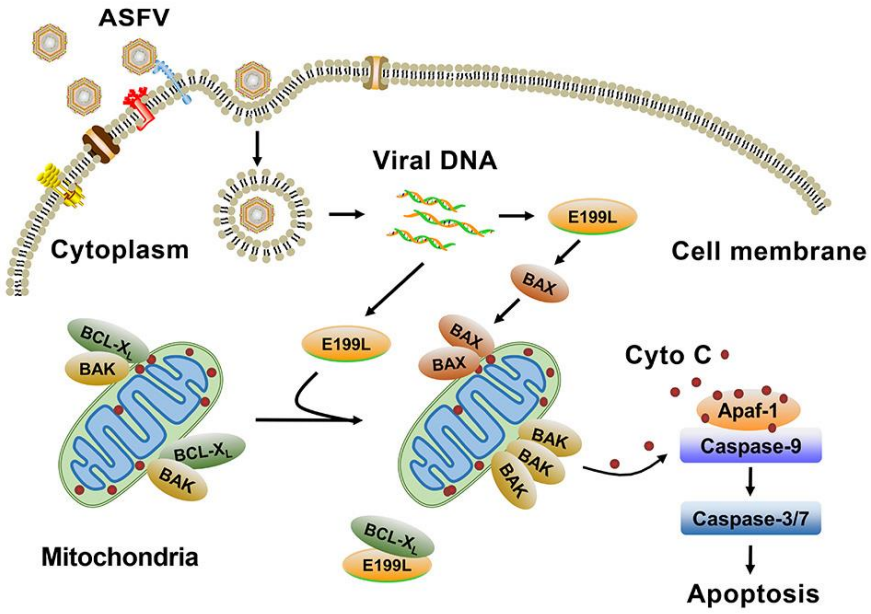

Figure 9: Apoptosis activation by pE199L protein
ASFV protein pE199L binds to and so activates Bax, which sticks to the surface of mitochondria and thus induces cytochrome C release, which subsequently begins the caspase cascade with resulting apoptosis; adapted from Li *et al.*, 2021

ASFV infection is also associated with lymphocyte apoptosis during the subacute and acute stages of the infection (Ramiro-Ibáñez *et al.,* 1996; Gómez-Vallamandos *et al.*, 2013; Dixon *et al.*, 2019). The acute infection causes the activation and thus increases levels of pro-inflammatory cytokines, such as IFNβ, IFNγ, TNFα, and IL-1β, which could be the possible cause of lymphocyte apoptosis (Gómez-Vallamandos *et al.*, 2013; Salguero *et al.*, 2002). Another reason for lymphocyte apoptosis could be their close contact with infected macrophages, which could present cell surface receptors inducing further apoptosis (Dixon *et al.*, 2019).

Autophagy regulation

Autophagy is a cellular homeostatic process involved in various pathological and physiological processes, including defences against viruses and lack of nutrition (Ravanan *et al.*, 2017). ASFV has developed complex processes for how to affect and regulate autophagy. These processes include proteins, which could either inhibit or induce autophagy.

The viral protein A179L, the ASFV Bcl2 homolog, is binding to the BH3 motif of autophagy regulatory factor Beclin1, thus inhibiting the formation of autophagosome and so autophagy (Hernaez *et al.*, 2013; Banjara *et al.*, 2019). In addition, a ligand-binding groove inside the A179L is essential for this interaction (Banjara *et al.*, 2019). ASFV can also inhibit autophagy independently on A179L protein through its complex interactions with the host cell, including Akt/mTORC1 and PI3K/Akt signalling pathways (Shimmon *et al.*, 2021).

On the other hand, viral protein E199L was found to interact with pyrrolline-5-carboxylate reductase (PYCR) 2, which, expressed at low levels, induces autophagy (Chen *et al.*, 2021). This interaction downregulates PYCR2 expression levels resulting in autophagy activation (Chen *et al.*, 2021). Another protein activating autophagy is K205R, which indirectly activates autophagy via kinase 1 (Wang *et al.*, 2022). Moreover, the K205R protein activates NFκB and induces ER stress (Wang *et al.*, 2022).

Vaccine development

The development of vaccines for combating ASFV began in the 1960s (Arias *et al.*, 2017). Multiple vaccines were developed during those early years, but none

proved successful enough for commercial purposes. Three main types of vaccines were designed against ASFV: inactivated vaccines with a killed virus, live attenuated vaccines and subunit vaccines (Figure 10). Inactivated vaccine approaches were unsuccessful since such vaccines could not enhance the immune response in pigs, even with the addition of different types of adjuvants (Blome *et al.*, 2014).

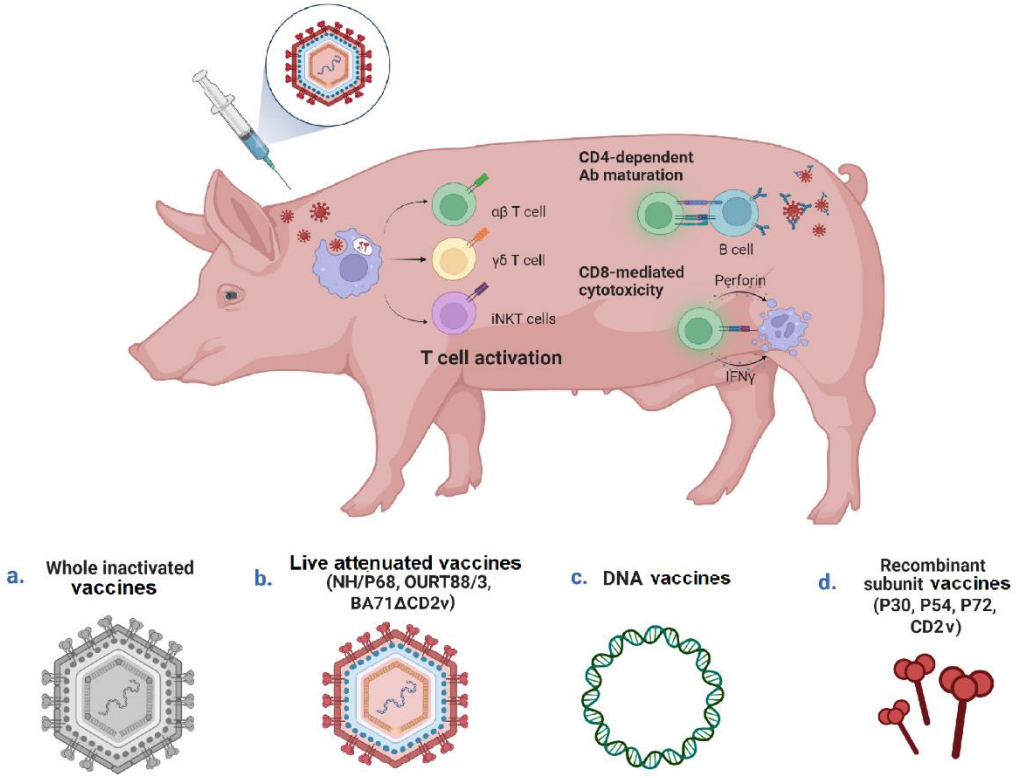

Figure 10: Main vaccine strategies for combating African swine fever virus, including the immune response inside the host body

T cell activation plays a crucial role in host survival. CD4+ T helper cells support the interaction with B cells and induce antibody production and maturation. CD8+ T cells induce the production of perforin and IFNγ; Vaccine strategies include a. whole inactivated vaccines containing killed virus (non-effective); b. live attenuated vaccines containing viruses with deleted or mutated genes (such as NH/P68, OURT88 and BA71ΔCD2v); c. DNA vaccines contain ASF genes coding for viral antigens; d. recombinant subunit vaccines containing selected recombinant ASFV antigens (such as p30, p54, p72 and CD2v); edited from Urbano and Ferreira, 2022

Live attenuated vaccines (LAVs)

Live attenuated vaccines contain weakened viruses with deleted or mutated genes responsible for host invasion, virus infectivity, and immune system inhibitors. They were found to enhance cellular and humoral immunity and further protected pigs against the virulent virus type (Sánchez *et al.*, 2019). There are three successful LAVs

based on three ASFV isolates: the OURT88/3, NH/P68 and BA71ΔCD2v (Leitão *et al.*, 2001; Monteagudo *et al.*, 2017; Mulumba-Mfumu *et al.*, 2015).

The OURT88/3 strain has been observed to enhance the production of CDβ8+ lymphocytes, the part of CD8+ lymphocytes confirming the importance of cellular immunity in the resistance to ASF (Oura *et al.*, 2005). Interestingly, using the OURT88/3 isolate, it has been found that deletion of genes involved in virulence such as *DP71L*, *DP96R* and the IFN I interferon modulators MGF 360 and MGF530/505 weakened the infectivity of and conferred subsequent protection against the OURT88/1 virulent strain (Abrams *et al.*, 2013; Reis *et al.*, 2016). However, MGF360/505 and 9GL deletion in the ASFV Georgia 2007 isolate also reduced the virulence of the isolate but without affording protection against the parental virus (O'Donnell *et al.*, 2016). A similar result was observed using the Georgia isolate with the deletion of the thymidine kinase gene involved in the virulence of ASFV (Sanford *et al.*, 2016). It has also been noted that cross-protection provided by the non-virulent OURT88/3 isolate and virulent OURT88/1 isolate used in combination induced protection against two isolates, Benin 97/1 and genotype X Uganda 1965 (King *et al.*, 2011).

Interestingly, the mutant virus BA71ΔCD2v conferred protection to two strains, parental strain BA71 and heterologous E75 virulent strain, both belonging to genotype I (Monteagudo *et al.*, 2017). Furthermore, pigs also survived a lethal challenge with the virulent Georgia 2007/1 genotype II strain (Monteagudo *et al.*, 2017). Sánchez-Cordón and his co-workers (2017) pointed out that the way of vaccination is crucial for the protection against ASFV; since the intranasal route was much more effective compared to the intramuscular route (Sánchez-Cordón *et al.*, 2017).

DNA vaccines

DNA vaccines have one main disadvantage: their reduced immunogenicity in large animals. This fact was confirmed by failed immunisation of the ASFV genes-containing DNA vaccine (Argilaguet *et al.*, 2011). The study of Argilaguet *et al.* (2011) attempted the construction of a new DNA clone encoding ASFV genes fused with a fragment of an antibody specific to a swine leukocyte antigen II and yielded the observation that targeting antigens to the antigen-presenting cells induced an immune response in pigs. Unfortunately, protection against lethal challenge was not achieved. In contrast, in the more recent study by Lacasta *et al.* (2014), partial

protection was observed while immunizing with a DNA vaccine containing ubiquitin-fused viral genes encoding p54, p30 and the hemagglutinin extracellular domain. The protection was observed against challenge with the virulent E75 strain.

Subunit vaccines

Subunit vaccines use purified antigenic parts of the pathogen necessary to induce the immune response in the form of antibodies. Such antigenic parts are viral proteins, peptides, or only their small fragments, including epitopes. In contrast to DNA vaccines, protein antigens are more effective, even though they do not confer high protection in all cases.

An example of ASFV subunit vaccine immunisation includes the immunisation with baculovirus-expressed p30, p54, p72 and p22 viral proteins showing only a temporal delay within the onset of disease and reduced viremia (Neilan *et al.*, 2004). The study by Gómez-Puertas *et al.* (1996) shows that the immunisation with two viral proteins, p54 and p72, raised neutralising antibodies against them, which could even inhibit virus attachment to the host cell membrane.

Neutralising antibodies specific to the p30 antigen, the most immunogenic among ASFV antigens, were also found to inhibit virus internalisation (Gómez-Puertas *et al.*, 1996; Petrovan *et al.*, 2019). New p30-specific monoclonal antibodies were recently prepared, and their binding epitopes were mapped (Petrovan *et al.*, 2019).

Gómez-Puertas and his co-workers (1998) found that immunisation with either p30 or p54 recombinant antigens did not protect pigs, and they died after some time. However, interestingly, immunisation was successful when both antigens were mixed as a cocktail, and pigs raised neutralising antibodies delaying the disease and stopping the infection (Gómez-Puertas *et al.*, 1998).

A study regarding the CD2v viral protein conducted in 1996 by Ruiz-Gonzalvo *et al.* showed that immunisation with recombinant CD2v inhibited the haemagglutination, restricted the infection temporally and, in some cases, also conferred protection against lethal disease. A more recent study from 2016 reports a similar result, which was that serotype-specific CD2v or C-type lectin induced haemadsorption-inhibition serotype-specific protective immunity. This data shows that CD2v could be used for future vaccine development (Burmakina *et al.*, 2016).

Proteins of interest

This chapter is focused on a more detailed description of two ASFV viral proteins, p22 and CD2v, and one protein involved in the increased induction of immune response, called heat-labile enterotoxin subunit B or LTB. This work is focused on all three proteins, which were produced by heterologous expression using the bacterial expression system.

Viral protein p22

The p22 protein is a structural protein, which was initially believed to be part of the outer membrane (Camacho *et al.*, 1991), but recently it has been found to be taking part of the inner membrane (Alejo *et al.*, 2018). An initial experiment showing that p22 protein is part of the outer envelope used non-ionic detergent, which could disrupt not only the outer but also the capsid and inner envelope, which was found later (Camacho *et al.*, 1991; Carrascosa *et al.*, 1991; Andres *et al.*, 1998). Recently, it has been found that the p22 protein interacts with host proteins related to biological processes, including virus binding, cell structure, signal transduction, and cell adhesion (Zhu *et al.*, 2021). Some of these proteins participate in pathways like the ribosome, spliceosome, actin filament organization and movement, and DNA replication or can affect phagocytosis and endocytosis (Zhu *et al.*, 2021). Even though p22 protein can interact with many host proteins, as stated before, a research group of Vuono *et al.* (2021) showed that p22 protein is not essential for ASFV replication or virulence in swine.

The gene coding p22 protein was initially discovered on the left end of the BA71 genome and was named based on the ORF K'177 (Gónzalez, 1990; Camacho *et al.*, 1991). Later it was renamed to KP177R. The KP177R was also found on the right end of the Malawi LIL120/1 genome (Vydelingum *et al.*, 1993). In the study of Vydelingum *et al.* (1993), authors suspect that it could be a consequence of evolution by duplication, deletions or sequence transposition from one end of the genome to another. KP177R is also known as one of the most conserved genes of the African swine fever virus, among other genes coding structural antigens (Luka *et al.*, 2016). It was also found that the open reading frame I10L is coding the protein similar to p22 (Dixon *et al.*, 1994). The nucleotide sequence has only 40 % homology compared to the p22 protein from BA71V (Dixon *et al.*, 1994). The ORF-I10L have 100 % homology in Benin97/1 and ORT88/3 isolates, which shows its conservation

throughout isolates (Chapman *et al.*, 2008). However, it is still not sure whether protein expressed from this gene is taking part in the viral structure as p22 protein (Chapman *et al.*, 2008).

Viral protein CD2v

CD2v protein (coded by ORF *EP402R*), a T lymphocyte cell adhesion receptor CD2 homologue, is an outer-membrane viral protein produced during the late phase of viral infection (Rodríguez *et al.*, 1993). CD2v play a role in the haemadsorption phenomenon typical for ASFV, while it was found that it can also induce the haemadsorption *in vitro* by itself without adjacent viral particles (Rodríguez *et al.*, 1993; Ruíz-Gonzalvo and Coll, 1993). Open reading frame EP402R coding for CD2v has high similarity to the ORF 8DR from Malawi Lil-20/1 strain, while both are coding the same CD2v protein (Borca *et al.*, 1998).

CD2v protein is a transmembrane protein composed of the extracellular N-terminal, transmembrane and intracellular C-terminal domains. The study by Mima *et al.* (2015) showed that the N-terminal domain of CD2v protein contains 28-30 highly glycosylated sites and provides the immunoglobulin Ig domain, which is localized on the outer part of the cell membrane. On the other hand, C-terminal domain interacts with AP-1 protein complex (Pérez-Núñez *et al.*, 2015). Moreover, three different forms of CD2v have been found in infected host cells: first glycosylated having a full-length of 89 kDa, second short 26 kDa non-glycosylated part of C-terminus and third 63 kDa glycosylated part of N-terminus (Goatly and Dixon, 2011). However, the processing is not defined yet (Goatly and Dixon, 2011).

Many studies show CD2v interacting with cellular and viral proteins, including SH3P7, CD58, EP153R, and AP-1, while these interactions play an essential role in ASFV infection (Kay-Jackson *et al.*, 2004; Chaulagain *et al.*, 2021; Galindo *et al.*, 2000; Pérez-Núñez *et al.*, 2015). One of the proteins CD2v binds to is the cytoplasmic adaptor protein SH3P7, involved in signal transduction and actin transport. This interaction occurs in the surroundings of the virus factories (Kay-Jackson *et al.*, 2004). Interaction with cell receptor CD58 activates NFκB-dependent IFNβ resulting in apoptosis of lymphocytes and macrophages (Chaulagain *et al.*, 2021), while for this interaction, C-type lectin (EP153R) may be essential (Galindo *et al.*, 2000). Specifically, the diLeu motif of the CD2v C-terminal sequence is also targeted by an adaptor protein complex AP-1 around the virus factories near trans-GA (Pérez-Núñez

et al., 2015), playing a role in arranging the membrane proteins to endosomes by forming clathrin-coated vesicles (Nakatsu *et al.*, 2003).

Recently, researchers have been conducting studies on the identification of the CD2v epitopes for improving the design of future potential vaccines. A study by Yang *et al.* (2021) identified the CD2v epitope RAAS located on the C-terminal part of the protein, which can play a role in entering the cells through endocytosis and macropinocytosis. The recent work by Ren *et al.* (2022) identified the linear epitope localized on the AA28-AA51 fragment of the extracellular domain. They also developed a highly immunogenic CD2v nanoparticle containing the fusion of CD2v protein and Norovirus P particle (Ren *et al.*, 2022). Three B cell epitopes of CD2v have been identified for the first time using monoclonal antibodies raised against CD2v protein (Jia *et al.*, 2022).

CD2v is also an immunogenic protein, and studies by Burmakina *et al.* (2016) and Sereda *et al.* (2018) show its potential for future vaccine development. It was also found that its deletion caused the delay in replication and spread and reduced virus titer in the lymphoid tissue and bone marrow (Borca *et al.*, 1998), indicating its importance for ASFV infection.

Heat-labile enterotoxin from Escherichia coli

Heat-labile enterotoxin (LT) from *Escherichia coli* is a heterohexameric protein consisting of one A subunit and five B subunits (Spangler, 1992). The LT promotes cell adherence of *E. coli* to the intestinal epithelial cells mainly due to ADP-ribosylation activity (Johnson *et al.*, 2009).

The A subunit (LTA) possesses strong ADP-ribosyl transferase activity (Gill *et al.*, 1981). The LTA consists of two parts: the N-terminal part A1 playing a role in catalytic activity and C-terminal part A2 inserted inside the pore of the pentameric structure of B subunits (Gill *et al.*, 1981).

The B subunit (LTB) has been found to have immunomodulatory properties as it binds ganglioside GM1 on the surface of the immune cells (Truitt *et al.*, 1998). LTB also induces the apoptosis preferentially of CD8+ T-cells via rapid loss of mitochondrial membrane potential and cell viability (Salmond *et al.*, 2004). LTBs ability to induce apoptosis is not dependent on its immunomodulatory properties and is also caspase-independent (Salmond *et al.*, 2004; Truitt *et al.*, 1998). Even though caspases are essential for apoptosis, cell death is caspase-independent (Salmond *et al.*,

2004). LTB has been used multiple times in different studies as a potent and robust adjuvant either individually or fused to other proteins (Ji *et al*., 2015; El-kassas *et al*., 2015).

Recent development in combating the ASFV spreading

Recently, many studies have focused on developing attenuated vaccines to eradicate the ASF virus. Among them, the studies by Borca *et al.* (2020) and Gladue *et al.* (2021) are the most advanced. Borca *et al.* (2020) show that the attenuated vaccine ASFV-G-ΔI177L induced virus-specific antibody production, and the pigs were protected against the parental strain ASFV Georgia2007 (Borca *et al.*, 2020). In work by Gladue *et al.* (2021), another ASFV-G-ΔA137R attenuated vaccine was shown to induce protection against the virulent strain ASFV Georgia2010 causing the current Eurasian pandemic (Gladue *et al.*, 2021). The article of Gladue and Borca (2022) reviews all the past and present attenuated vaccines, either effective or ineffective, in protecting the host against highly virulent ASFV strains.

Another way of combating the virus spread could be the development of a highly efficient detection system, which would be supposed to detect the infection in the early stage. Such a system could incorporate the techniques like ELISA or immunoblotting (Qiu *et al.*, 2021).

EXPERIMENTAL PART

No efficient vaccine for combating the deadly disease caused by the African swine fever virus has been developed so far. However, some antigens were shown to be potential subunit vaccine candidates since they could partially inhibit the infection of the virus. The experimental part of this work focuses on preparing and testing the potential vaccine candidates from ASFV produced in the bacterial expression strain *Escherichia coli* BL21. Overproduced proteins were used for the analysis against ASFV-positive pig sera and subsequently for mice immunisation. Antibodies raised against potential candidates could help to distinguish better candidates for future vaccine and serological diagnosis development.

Material and methods

Chemicals and solutions

Chemicals were *p.a*. purified provided by the given companies (Table 1).

Table 1: List of used chemicals and their providers

Name of the chemical	**Provider**
2 –mercaptoethanol	Merck
Acetic acid	Penta
Acrylamide	Merck
Agar	Calbiochem
Agarose	IAB
Amidoschwarz 10B	Merck, Germany
Ammonium persulphate (APS)	Fluka
Ampicillin sodium	Merck
Bromophenol blue sodium salt	Fluka
Calcium (II) chloride anhydrous	Penta
Chloramphenicol	Duchefa
Coomassie brilliant blue G-250	Serva
Ethanol	Penta
Ethidium bromide	Merck
Ethylenediaminetetraacetic acid (EDTA)	Penta
Glucose monohydrate	Lach-Ner
Glycerol, 100 %	Lach-Ner
Glycine	Penta
Guanidinium hydrochloride	Applichem
Hydrochloric acid	Penta
Imidazole	Roth
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Duchefa
Isopropyl alcohol	Penta
Kanamycin monosulfate	Duchefa

Lysozyme	Merck
Methanol	Penta
N – butanol	Penta
N, N – methylene bisacrylamide	Merck
N,N,N´,N´ – tetramethylethylenediamine (TEMED)	Merck
Nickel (II) sulphate hexahydrate	Lach-Ner
Phenylmethylsulfonyl fluoride (PMSF)	Merck
Potassium acetate	Penta
Potassium hydroxide	Lach-Ner
RNase	Macherey-Nagel
Sodium acetate anhydrous	Lach-Ner
Sodium chloride	Penta
Sodium hydroxide	Penta
Sodium dodecyl sulphate (SDS)	Penta
Tetracycline hydrochloride	Merck
Tris base	Duchefa
Triton X100	NeoLab
Tryptone	Duchefa
Tween 20	Merck
Yeast extract	Himedia

Immobilon-P polyvinylidene difluoride (PVDF) transfer membrane was provided by Merck Millipore. Restriction endonucleases were from New English Biolabs (NEB), Taq DNA polymerase from Invitrogen and T4 DNA ligase from NEB.

Protein extraction and purification through Ni-NTA

Protein extraction buffer A (for p22Ct, CD2vCt, and LTB-CD2vCt)

0.05 M Tris-HCl, 0.3 M NaCl, 2 % glycerol, 0.1 % Triton X-100, 0.001 M PMSF, 0.015 mM imidazole, pH 8.0

Protein extraction buffer B (for LTB-p22Ct)

0.05 M Tris-HCl, 0.3 M NaCl, 2 % glycerol, 0.1 % Triton X-100, 0.001 M PMSF, 0.015 M imidazole, pH 9.0

Washing buffer A

0.05 M Tris-HCl, 0.3 M NaCl, 2 % glycerol, 0.1 % Triton X-100, 0.04 M imidazole, pH 8.0 (or 9.0, the same as for the used extraction buffer A or B)

Washing buffer B

0.05 M Tris-HCl, 0.3 M NaCl, 2 % glycerol, 0.1 % Triton X-100, 0.05 M imidazole, pH 8.0 (or 9.0, the same as for the extraction buffer A or B)

Elution buffer

0.05 M Tris-HCl, 0.3 M NaCl, 2 % glycerol, 0.1 % Triton X-100, 0.3 M imidazole, pH 8.0 (or 9.0, the same as for the extraction buffer A or B)

Regeneration buffer

0.02 M MES, 0.1 M NaCl, pH 5.0

Storage buffer

20 % ethanol

SDS-PAGE and immunoblot assay

15 % SDS polyacrylamide separating gel

15 % acrylamide – bisacrylamide, 0.382 M Tris-HCl pH 8.8, 0.05 % (w/v) SDS, 0.05 % (w/v) APS, 0.05 % TEMED, in water

4 % stacking gel

4 % acrylamide – bisacrylamide, 0.125 M Tris-HCl pH 6.8, 0.05 % (w/v) SDS, 0.05 % (w/v) APS, 0.05 % TEMED, in water

5x Tris-glycine running buffer, pH 8.8

0.124 M Tris-base, 0.96 M glycine, 0.05 % SDS, pH 8.8 (adjusted with HCl)

2x Sample buffer for SDS-PAGE

0.0625 M Tris-HCl, 10 % glycerol, 2 % SDS, 5 % 2-mercaptoethanol, 0.025 % bromophenol blue, pH 8.0

Coomassie Brilliant Blue staining solution

0.1 % CBB G250, 50 % methanol, 10 % acetic acid

Destaining solution

40 % methanol, 10 % acetic acid

10x Transfer buffer

0.25 mM Tris-HCl, 1.92 M glycine, 10 % methanol (no pH adjusted)

Amidoblack solution

0.5 % Amidoblack 10B, 50 % methanol, 7 % acetic acid

TBS buffer

0.02 M Tris-HCl, pH 7.5, 0.5 M NaCl

TBST buffer

0.02 M Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05 % Tween 20

PBS buffer

0.01 M phosphate buffer, pH 7.3, 0.15 M NaCl

PBST buffer

0.01 M phosphate buffer, pH 7.3, 0.15 M NaCl, 0.1 % Tween 20

Enterokinase reaction buffer

0.02 M Tris-HCl, pH 8.0, 0.05 M NaCl, 0.002 M $CaCl_2$

Adjuvant solution for mice immunisation

10 % aluminium hydrogel

Bacterial strains

Escherichia coli One Shot® TOP 10 strain was used for gene cloning, including plasmid isolation, and *E. coli* BL21 STAR strain was used for protein overproduction (both strains were from Thermo Fisher Scientific, Brno, Czech Republic). Strains Rosetta gami B and Rosetta™ 2(DE3) (Merck, Darmstadt, Germany) were used to overproduce disulphide bond proteins CD2vCt and LTB-CD2vCt. All strains were grown under vigorous shaking (180 rpm) at 28 °C and 37 °C, respectively, in Luria-Bertani (LB) medium. For cloning and expression of the genes, pMA-RQ (Thermo Fisher Scientific), pET28b(+) (Merck, Darmstadt, Germany), and pGEX-5x-1 (GE Healthcare, Czech Republic) were used, respectively.

Gene cloning

Constructs containing the part of the gene KP177R (GenBank: MK333183) and EP402R (GenBank: U18466), respectively, coding for the C-terminal globular segment (amino acids 17-204 of the CD2v protein; amino acids 42-189 of the p22 protein) and the same gene parts fused at the 5' end with the gene coding for the B-subunit of enterotoxin (LTB, GenBank: M17873) were designed. In addition, a

10xHis-tag coding sequence was attached to the 5' end of each construct for the encoded protein purification and detection purposes. In comparison, 4xMyc-tag was attached to the 3' end of each construct only for detection purposes. Designed gene sequences, named *p22Ct*, *LTB-p22Ct*, *CD2vCt*, and *LTB-CD2vCt*, were codon-optimised for the production in *E. coli*, synthetically prepared using a commercial service (Thermo Fisher Scientific) and cloned into the pET28b(+) vector at the *Nco*I restriction enzyme site. For colony screening, primers His-tag forward (occasionally T7 promoter forward) and T7 terminator reverse (shown in Table 1) were used. The constructs within the pET28b(+) vector were verified using a commercial sequencing service (SEQme, Dobříš, Czech Republic). Confirmed clones, pET28b(+)-*p22Ct* and pET28b(+)-*LTB-p22Ct*, were used for the recombinant protein production.

Table 2: List of general primers

Primer name	**Sequence**	**Function**
His-tag forward	CCATCACCATCACCATCACC	Colony screening of p22Ct and LTB-p22Ct constructs in pET28b(+) and pMA-RQ
T7 promoter forward	GTAATACGACTCACTATAGGGCG A	Colony screening of p22Ct and LTB-p22Ct constructs in pET28b(+)
T7 terminator reverse	AGCCAACTCAGCTTCCTTTC	Colony screening of p22Ct and LTB-p22Ct constructs in pET28b(+)
ColEI origin reverse	TTTTTGTGATGCTCGTCAGG	Colony screening of p22Ct and LTB-p22Ct constructs in pMA-RQ
pGEX 5 forward	AGGCGCACTCCCGTTCTGGATAA TG	Colony screening of epitope repetitions in pGEX-5x-1
pGEX 3 reverse	CCGGGAGCTGCATGTGTCAGAGG	Colony screening of epitope repetitions in pGEX-5x-1
p22Ct SmaI StopMut forward	AAACATGCGGACCCGGGCTGATG ATGATAAAGGC	Stop-mutation insertion into p22Ct and LTB-p22Ct constructs in pMA-RQ
p22Ct SmaI StopMut reverse	GCCTTTATCATCATCAGCCCGGG TCCGCATGTTT	Stop-mutation insertion into p22Ct and LTB-p22Ct constructs in pMA-RQ

Site-directed mutagenesis

Following the initial successful small-scale expression, Myc-tag was deleted by site-directed mutagenesis prior to the large-scale expression. For this reason, special primers (p22 SmaI StopMut forward and p22 SmaI StopMut reverse; shown in Table 2) were designed to insert stop mutation inside the enterokinase cleavage site. The total volume of individual PCR reactions was 20 µL. The master mix contained a 1xQ5 reaction buffer containing $MgCl_2$, 0.5 µM primers (forward and reverse), 200 µM dNTP, and 30 ng of template DNA. Finally, 0.05 unit of Q5 DNA polymerase was added per one reaction. The temperature program of the PCR is stated in Table 2. PCR products were purified using Nucleo-Spin™ Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), then cleaved by SmaI restriction enzyme and ligated into expression vector pET28b(+) using T4 DNA ligase. Ligated products were transformed into the chemically competent *E. coli* TOP 10 cells and screened by PCR using His-tag forward and ColEI origin reverse (Table 2).

Table 3: PCR temperature program using Q5 DNA polymerase

Step		**Temperature**	**Time**
Initial denaturation		98 °C	30 s
28 cycles	denaturation	98 °C	10 s
	annealing	50-56 °C	20 s
	polymerization	72 °C	30 s/1kb
Final polymerisation		72 °C	2 min
Cooling		4 °C	∞

LAE method

LAE (Linear Array Epitopes) method is a PCR-based method for producing multiple repetitions of selected epitopes from chosen immunogenic proteins. Two epitopes (9-10 amino acid long) from selected CD2v protein from ASFV were chosen based on published information (Argilaguet *et al.*, 2012). Preparation of epitope repetitions is a two-set PCR method incorporating TR-PCR (Tandem-Repeat PCR) and Ad-PCR (Adapter PCR), as described in the study of Lai *et al.* (2014). Two primers were used as templates for the TR-PCR step, and Q5 DNA polymerase (NEB) was used to amplify epitope repetitions. The temperature program was similar to the one described above (Table 3) but with minor changes: 30 cycles of amplification were run instead of 28, and the final polymerization step was prolonged from 2 to 10 min. The next step was to attach adapters containing restriction sites to both ends of

all repetitions, which was done by Ad-PCR, where another two primers were designed for this purpose. The slight change also applied to Ad-PCR compared to TR-PCR: 20 cycles of amplification instead of 28. All designed primers for TR-PCR and Ad-PCR are shown in Table 4. Following adapter attachment, a set of variable repetitions was cloned into the expression vector pGEX-5x-1 using EcoRI and XhoI restriction enzymes. After the transformation, clones were screened, and positive clones were sequenced and subsequently used for the expression experiment in *Escherichia coli* BL21 expression strain. Samples from expression were analysed using 4 % stacking and 12 % separating polyacrylamide gel electrophoresis and subsequently by western blot analysis.

Table 4: List of primers for TR-PCR and Ad-PCR

Primer name	**Sequence**	**Function**
F3 Fw	AGCGTGGACAGCCCGACCATTACCTAT	For TR-PCR of F3 epitope.
F3 Rev	GGCTGTCCACGCTATAGGTAATGGTCG	For TR-PCR of F3 epitope.
Adapter EcoRI F3 Fw	GATCGAATTCAGCGTGGACAGCCCGA	To attach adapter in Ad-PCR of F3 epitope.
Adapter XhoI F3 Rev	GATCCTCGAGGCTGTCCACGCTATA	To attach adapter in Ad-PCR of F3 epitope.
A3 Fw	ACCAACGGCGACATCCTGAACTACTAC	For TR-PCR of A6 epitope.
A3 Rev	GTCGCCGTTGGTGTAGTAGTTCAGGAT	For TR-PCR of A6 epitope.
Adapter EcoRI A6 Fw	CATGAATTCACCAACGGCGACATC	To attach adapter in Ad-PCR of A6 epitope.
Adapter XhoI A6 Rev	GATCCTCGAGGTCGCCGTTGGTGTA	To attach adapter in Ad-PCR of A6 epitope.

Expression of recombinant proteins

All prepared clones (pET28b(+)-*p22Ct*, pET28b(+)-*LTB-p22Ct*, pGEX-5x-1-*LAE-F3*, pGEX-5x-1-*LAE-A6)* were transformed into chemically competent *E. coli* BL21 STAR cells by heat shock. In contrast, pGEX-5x-1-*CD2vCt* and pGEX-5x-1-*LTB-CD2vCt* were transformed into chemically competent *E. coli* Rosetta gami B cells and *E. coli* Rosetta^{TM} 2(DE3) pLysS cells respectively, by heat shock. Positive clones were inoculated into 20 mL of LB medium (5 mL, in case of small-scale expression) and grown overnight at 37 °C. Then, 20 mL of the culture (0.2 mL, small-

scale) was inoculated into 1 L of the LB medium (5 mL, small-scale) and let grow at 37 °C while shaking until the optical density (OD_{600}) reached 0.7-0.8. Recombinant protein production was induced by adding IPTG at a final concentration of 0.2 mM. The culture was grown at 28 °C for 16 h and then collected by centrifugation (8,000 *g*, 10 min, and 20 °C). Bacterial pellets were used immediately for protein purification or stored at -20 °C.

Purification of recombinant proteins on Ni-NTA agarose

Bacterial pellets were resuspended in the extraction buffer A and B (A for p22Ct, CD2vCt, and LTB-CD2Ct; B for LTB-p22Ct). Lysozyme (1 mg/ml final concentration) and PMSF (1 mM final concentration) were added to the cell suspension for better lysis and inhibition of proteases. After lysozyme was added, cells were incubated on ice for 30 min. Cells were then disrupted by ultrasonic homogenisation (3x 10 min, 6s pulse and 9s pause periods) or French press. The insoluble protein fraction was removed by centrifugation at 14,000 *g* for 15 min. The soluble protein fraction was vacuum filtered through a 0.4 µm filter and used for the immobilised metal affinity chromatography (IMAC) purification step.

The Ni-NTA agarose (Qiagen, Hilden, Germany) was pre-equilibrated with an equilibration buffer (same as extraction buffer). The soluble fraction was loaded twice on the matrix to increase the binding of the desired His-tagged proteins of interest. Unbound proteins were washed out with the same buffer, and further elution was performed with the washing buffers A, B, and finally by elution buffer. Eluted protein samples were concentrated and buffer exchanged for the buffer without imidazole using Amicon 10,000 Mw centrifugal filters (Thermo Fisher Scientific). Subsequently, the samples were analysed by SDS-PAGE and Western blot. The purified proteins were stored at -80 °C. Protein concentrations were determined by Bradford reagent (Bio-Rad, Hercules, CA, USA) and band density on SDS-PAGE gels using ImageLab software (Bio-Rad) with bovine serum albumin (BSA) as standard.

The prediction of expression yield of p22Ct and LTB-p22Ct were estimated using BSA as a standard. The procedure was performed by running samples on SDS-PAGE, the gel was stained by Coomassie Brilliant Blue G250 dye and bands were analysed by the ImageLab software. Samples consisted of known BSA concentrations (1.25, 2.5, 5, and 10 µg) and three sample replicates of purified proteins p22Ct or LTB-

p22Ct (5.0, 7.5, and 10 µL of protein sample). Calibration curves designed by ImageLab software were used for the estimation of protein concentrations.

SDS-PAGE and Western blot analysis

Whole-cell extract samples were prepared by centrifuging 1 mL of the cell cultures at 16,000 g; the pellets were then resuspended in 50 µL of extraction buffer A and B, respectively, and mixed with a sample buffer at the volume ratio of 1:2. Alternatively, the purified protein samples were mixed with the sample buffer at the ratio of 1:1. All samples were heated for 5 minutes at 100 °C, spun down, and separated under reducing conditions using the vertical 4 % stacking and 15 % separating SDS PAGE gels. The electrophoresis was run in the running buffer at 80V for 15 minutes and then at 150 V until the sample dye reached the end of the gel. The gels were stained with the staining solution and unstained using 40 % methanol and 10 % acetic acid.

SDS PAGE was also used to assess protein mobility under non-reducing conditions. In that case, the sample buffer did not contain 2-mercaptoethanol, and the samples were not heated.

The electrophoresed proteins were transferred to a polyvinylidene difluoride (PVDF) membrane for Western blot analysis using a wet transfer apparatus (Bio-Rad). Conditions were as followed, for 16 h at a constant voltage of 20 V and the next day for an additional 1 h at 100 V. The membrane was rinsed twice with TBS buffer and stained with Amidoblack solution, followed by blocking with 5 % non-fat milk in TBST buffer for 90 min, and then incubated with a 1:500 diluted anti His-tag primary antibody (MA1-21315, Thermo Fisher Scientific) for 90 min. Immunodetection was performed for 90 min with a 1:4000 diluted m-IgGκ BP-HRP anti-mouse secondary antibody (sc-516102, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and enhanced chemiluminescent substrate (ECL kit, Bio-Rad). As a molecular mass marker, Novex^{TM} Sharp Pre-Stained Protein Standard (Thermo Fisher Scientific) was used.

Matrix-assisted laser desorption/ionisation – time of flight

The p22Ct and LTB-p22Ct were excised from the gel, digested by trypsin and used for Matrix-Assisted Laser Desorption/Ionisation- Time of Flight (MALDI-TOF) mass spectrometry to analyse the protein identity. In-gel digestion by trypsin and

MALDI-TOF analysis was performed by by Professor Marek Šebela in the Department of Biochemistry, Faculty of Science, Palacký University Olomouc. Performed analysis was done based on the work by Šebela *et al.* (2018).

Cleavage by enterokinase

The enterokinase cleavage site was localized between the gene part and the Gly8 linker and was used to cleave out the 4xMyc-tag. Enterokinase light chain (P8070, New English Biolabs) was used. In order to cleave the protein, protein had to be buffer-exchanged for enterokinase reaction buffer. Subsequently, 25 µg of protein in a total reaction volume of 20 µL were mixed by pipetting with 1 µL of enterokinase. After mixing, the reaction was incubated at 25 °C for 16 h. Samples before and after cleaving were analysed by SDS-PAGE and stained by CBB G250.

Immunoassay with ASFV-positive pig sera

Immunoassay with ASFV-positive pig sera was performed by Dagmar Břínek Kolařová at the Department of Infectious Diseases and Microbiology, University of Veterinary Sciences, Brno, Czech Republic. The membrane prepared as above was cut into strips, each containing one protein sample. Individual strips were blocked with 2 % BSA in PBS and incubated for 1 h while shaking with 1:100 diluted ASFV positive pig serum (Serum 1, European Union Reference Laboratory for African Swine Fever, Valdeolmos, Spain; Sera 2-4, positive sera from wild pigs provided by Dr. Grzegorz Woźniakowski, National Veterinary Research Institute, Puławy, Poland). The strips were rinsed with PBST with 2 % BSA and incubated for 1 h with 1:30,000 diluted Anti-Pig IgG (whole molecule)-Peroxidase secondary antibody (A5670, Merck). After washing three times for 5 min with PBST containing 2 % BSA, the signals of bound antibodies were developed using 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate (T0565, Merck) and evaluated using ImageLab software.

Mice immunisation

Mice immunisation was performed by Dr. Jiří Salát at the Veterinary Research Institute, Brno, Czech Republic. Six-week-old female BALB/c mice (Envigo, Indianapolis, IN, USA) were vaccinated subcutaneously dorsally in the neck region every two weeks, with three doses in total. A single vaccination dose (prepared immediately before application) was composed of a mixture of the recombinant

protein p22Ct or LTB-p22Ct (10 µg) and 10 % aluminium hydrogel adjuvant (InvivoGen, Toulouse, France) in 0.15 mL PBS. Mice were bled two weeks after the last vaccination, and the presence of antigen-specific antibodies in the sera was analysed by ELISA.

ELISA analysis and antibody titre measurement

ELISA analysis and antibody titre measurement was performed by Dagmar Břínek Kolařová at the Department of Infectious Diseases and Microbiology, University of Veterinary Sciences, Brno, Czech Republic. Nunc MaxiSorp ELISA plates (Thermo Fisher Scientific) were coated overnight with 4 µg/well of the recombinant p22Ct or LTB-p22Ct protein dissolved in 0.05 M carbonate-bicarbonate buffer pH 9.6. The wells were then rinsed with PBS and blocked with 2 % BSA in PBS at 37 °C for 60 min. The 100 µL aliquots of mice sera diluted 1:50 in PBST with 2 % BSA were added to antigen-coated wells and incubated for 60 min at 37 °C. Subsequently, the plates were washed three times with PBST, and 100 µL of 1:30,000 diluted rabbit Anti-Mouse IgG Peroxidase Conjugate (A9044, Merck) in PBST containing 2 % BSA was added to each well. The plates were then incubated for 60 min at 37 °C, washed with PBST containing 2 % BSA, and 100 µL per well of the TMB-Complete substrate (TestLine Clinical Diagnostics, Brno, Czech Republic) was added. Colour development was stopped with 1 M H_2SO_4, and the optical density was read at 450 nm using a multimode plate reader Infinite M200 PRO (Tecan, Austria). Serum titre was determined as the ratio of the dilution of the test serum with an absorbance value equal to the average absorbance of the negative mouse serum.

Statistical analysis

Different levels of antibody titres between the independent groups (p22Ct and LTB-p22Ct) analysed by ELISA from blood samples were statistically analysed using one-way ANOVA analysis. Results of p22Ct and LTB-p22Ct were compared with the corresponding control group, where the P-value of 0.05 and lower was considered statistically significant. Statistical analysis was conducted using the Microsoft Excel program.

Results and discussion

The ASFV proteins CD2v and p22 were initially thought to be structural proteins of the outer envelope of the ASFV viral particle (Camacho and Viñuela, 1991). However, a few years ago p22 protein was found on the inner envelope (Alejo *et al.*, 2018), while CD2v remained the sole protein of the outer envelope. Regardless, both proteins previously produced by the baculovirus expression system have temporarily induced immune response or inhibited the virus's infection in the host (Neilan *et al.*, 2004; Burmakina *et al.*, 2016).

In this work, only the outer globular part of the proteins was selected in order to facilitate the extraction and purification step. Two constructs were established from each protein, one contained the outer globular part of the protein alone, and the second one had the outer globular part fused with the heat-labile enterotoxin B-subunit (LTB). Our primary aim was to design constructs for expression in bacterial strain BL21, Rosetta gami B and Rosetta^{TM} 2(DE3), respectively, and use expressed recombinant proteins to examine their immunogenicity and safety as potential vaccine candidates.

Bioinformatic analysis and construct design

CD2v and p22 proteins are transmembrane proteins containing an N-terminal domain (large outer domain in CD2v; short inner domain in p22), a single membrane-spanning helix and an outer C-terminal domain (large inner domain in both proteins). As the p22 protein takes part in the virus binding (Zhu *et al.*, 2021), and CD2v takes part in haemadsorption of the erythrocytes to the host cell surface (Borca *et al.*, 1998), they deserve further attention regarding its possible recognition by the host immune system.

Prior to the expression of the selected proteins, bioinformatic analysis was performed to estimate the tertiary structure of the target proteins of interest. Using the Phyre2 protein fold recognition server (Kelley *et al.*, 2015), a putative structure was modelled by comparing the amino acid sequence of the N-terminal domain of CD2v and the C-terminal domain of p22, respectively, with other known proteins from the database. The resulting model shows that the separate domains are prone to fold into a stable tertiary structure (Figure 11A, 11B). Hence, we decided to prepare recombinant proteins, named CD2vCt, based on the amino acids 17-204 of CD2v, and

p22Ct based on the amino acids 42-189 of p22 and use them for the expression using the bacterial expression system.

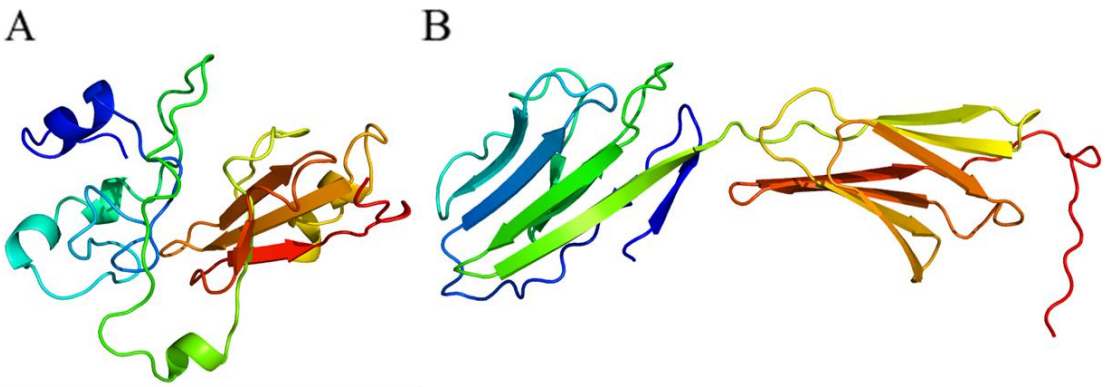

Figure 11: Predicted protein structure by the Phyre2 protein fold recognition server (Kelley *et al.*, 2015).
(**A**) p22Ct protein and (**B**) CD2vCt predicted protein structure; Image is coloured by a rainbow from N- to C-terminal ends.

Following the initial tertiary structure prediction, further bioinformatic analysis was performed using the online ProtParam tool from the ExPASy server (https://web.expasy.org/protparam/; Gasteiger *et al.*, 2005) to calculate the physio-chemical parameters of the target proteins. The parameters included are the molecular weight (Mw; in Da), isoelectric point (pI), number of negative- and positive-charged amino acids, aliphatic and instability index, extinction coefficients (first one when cysteines form disulphide bonds, and second when cysteines are reduced), and Grand average of hydropathicity (GRAVY) score.

From the ProtParam result interpretation, the aliphatic index indicates the protein's thermostability, while the instability index estimates the protein' *in vitro* stability. Extinction coefficients indicate the protein absorption of light at a specific wavelength (280 nm). The grand average of hydropathicity (GRAVY) shows the hydrophobicity (higher than zero) or hydrophilicity (lower than 0) of the proteins (Magdeldin *et al.*, 2012). All the parameters corresponding to the target proteins are stated in Table 5.

Table 5: Physicochemical parameters of the selected target proteins

Physicochemical parameters	Name of target proteins		
	p22Ct	**CD2vCt**	**LTB**
Amino acid (AA) number	149	188	124
Molecular weight (Da)	16 927.99	21 530.04	14 133.37
Isoelectric point (pI)	7.68	5.15	8.97
All negative-charged AA (Asp + Glu)	21	10	12
All positive-charged AA (Arg + Lys)	22	8	16
Aliphatic index	47.72	87.07	85.73
Instability index	48.28	26.65	36.58
Extinction coefficient (+ disulphide bonds)	22055	39350	14565
Extinction coefficient (- disulphide bonds)	21430	38850	14440
Grand average of hydropathicity (GRAVY)	-0.953	-0.256	-0.290

Results from the bioinformatic analysis show that all proteins are more likely to be hydrophilic and globular since their GRAVY level is lower than zero. A high aliphatic index indicates that the target proteins should be thermostable. The instability index of CD2vCt and LTB is below 40, indicating the high probability that proteins will be stable. In contrast, the p22Ct instability index is slightly higher than 40, indicating the possibility of protein being unstable. However, due to the value proximity to 40 and the index being the indication of a theoretical probability, the instability index should be taken with reservation. Therefore, these results show that these proteins are suitable for production in *Escherichia coli* expression strains.

Four DNA constructs (named *CD2vCt*, *p22Ct*, *LTB-CD2vCt*, and *LTB-p22Ct*; Figure 12) were codon-optimized for the expression in *E. coli*, the first two coding for the above C-terminal globular fragment of the p22 protein and CD2v protein, and the other two for N-terminal fusion of that fragment with the heat-labile B-subunit of enterotoxin (LTB). Moreover, all constructs have a coding sequence for a 10xHis-tag attached to the 5' end and a 4xMyc-tag to the 3' end. The enterokinase site and Gly8 linker are between the gene part and the 4xMyc-tag. The 4xMyc-tag was later deleted

using site-directed mutagenesis. All gene constructs *CD2vCt*, *p22Ct*, *LTB-CD2vCt*, and *LTB-p22Ct* were then cloned into pET28b(+) expression vector.

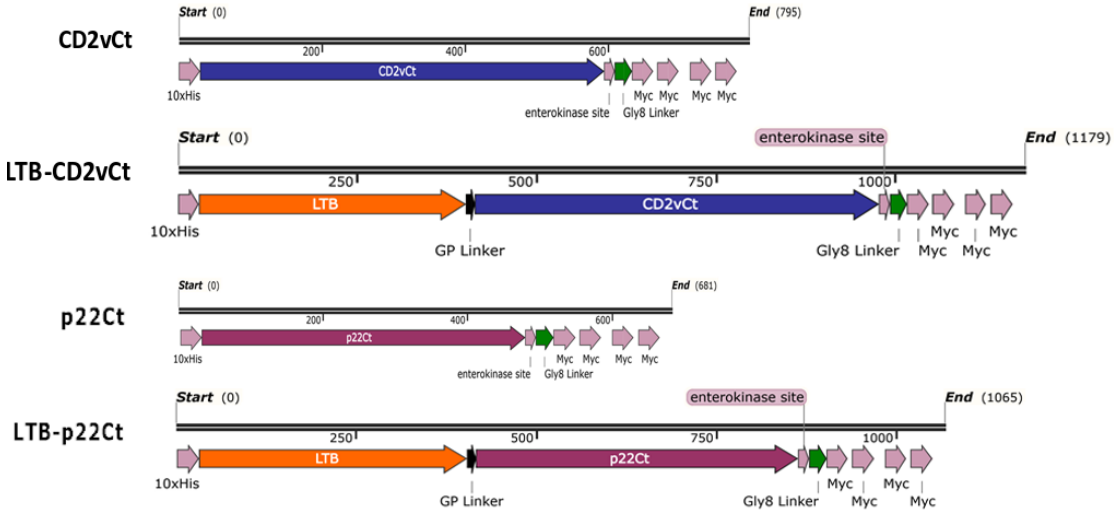

Figure 12: All DNA construct design, *CD2vCt*, *LTB-CD2vCt*, *p22Ct* and *LTB-p22Ct*. The *CD2vCt* gene is coloured in blue, while the *p22Ct* gene is coloured in ruby. The LTB coding sequence is orange, while the pink colour represents the coding sequence for a 10xHis-tag on the 5' end, enterokinase cleavage site and 4xMyc-tag on the 3' end of the DNA. The green-coloured arrow represents the Gly8 linker.

Preliminary expression experiments

In order to preliminarily examine the expression levels of the designed proteins, corresponding coding constructs were expressed in the expression strain *E. coli* BL21 STAR. Prior to the expression, constructs *CD2vCt*, *LTB-CD2vCt*, *p22Ct*, and *LTB-p22Ct*, cloned into pET28b(+) vector, were transformed into the chemically competent cells *E. coli* BL21 STAR. Grown colonies were then used for subsequent expression experiments.

CD2vCt and LTB-CD2vCt proteins

Initial expression experiments regarding constructs containing CD2vCt part cloned into the pET28b(+) vector were performed in BL21 STAR cells. These experiments failed, probably due to the fact that CD2v protein shares homology with CD2, a T cell receptor containing disulphide bonds inside the immunoglobulin part of the protein (Bromberg, 1993). Disulphide bonds are essential during protein folding and key to maintaining the active form of the protein (Bulleid, 2012). Based on these findings, the BL21 strain might be inappropriate for expression since it

maintains a stable, reducing environment inside the cells and thus disables the expression of disulphide-bond proteins (Kong and Guo, 2014). The reason behind this phenomenon lies in the thioredoxin and glutathione/glutaredoxin pathways (Steward *et al.*, 1998). In the strains like BL21, the genes coding the reductase enzymes, namely thioredoxin reductase (*trxB*) and glutathione reductase (*gor*), are responsible for the reduced environment inside the cells. However, several strains, such as Rosetta or Origami, have been developed with silenced *trxB* and *gor* genes and are widely used for the expression of disulphide bond proteins (Bessette *et al.*, 1999).

The first used strain was the Rosetta gami B strain, which is not compatible with kanamycin-resistant vectors, and thus it was necessary to re-clone the constructs into the vector with ampicillin resistance, such as the pGEX-5x-1 vector. The pGEX-5x-1 is a high-copy vector containing the glutathione-S-transferase (GST) tag, which was deleted during the cloning process from the vector. Clones pGEX-5x-1-*CD2vCt* and pGEX-5x-1-*LTB-CD2vCt* were transformed into chemically-competent expression strain Rosetta gami B and later to Rosetta^{TM} 2(DE3) strain. Initial experiments were done using the Rosetta gami B strain, later changed to Rosetta^{TM} 2(DE3) strain due to occurrence of tRNAs for seven rare codons in this strain that enhances the protein expression. RosettaTM 2(DE3) strain was also observed to grow faster than the Rosetta gami B strain.

Small-scale expression experiments were performed as stated in the chapter: *Material and methods*, part: *Expression of recombinant proteins*. All the samples were analysed by SDS-PAGE. Target proteins were not clearly recognized on SDS-PAGE, indicating minimal expression level (Figure 13). For information and better comparison, a table with the predicted isoelectric points and molecular weight of the proteins is shown below (Table 6). Western blot was also performed. However, neither positive signal was observed for the protein CD2vCt or LTB-CD2vCt.

Table 6: Isoelectric point (pI) and molecular weight (Mw) of CD2vCt and LTB-CD2vCt proteins

Protein name	**pI**	**Mw (kDa)**
CD2vCt	4.76	29.36
LTB-CD2vCt	5.35	43.8

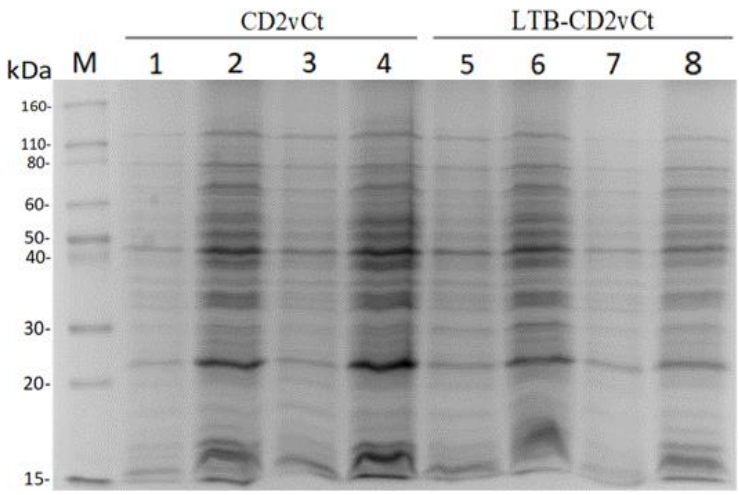

Figure 13: SDS-PAGE analysis of crude extracts from *E. coli* Rosetta™ 2(DE3) cells expressing *CD2vCt* and *LTB-CD2vCt*.

Lanes 1, 3, 5, and 7 show the 20 µL samples from non-induced cells, while lanes 2, 4, 6, and 8 represent the cells after induction with 0.2 mM IPTG. Bands of target proteins were not clearly recognized; therefore, they were not labelled in this figure.

Next, large-scale expression in 100 mL of LB was performed for purification purposes. Purification was done using Ni-NTA agarose as a matrix, and the purified samples were analysed by SDS-PAGE (Figure 14A, 14D). Figures 14A (CD2vCt) and 14D (LTB-CD2vCt) show that even in the elution fractions corresponding to lanes 6 to 9 of both pictures, there is little protein.

For this reason, elution fractions were pooled and concentrated using 10 kDa cut-off Amicon centrifugation filters. Concentrated fractions were analysed by SDS-PAGE and Western blot analysis. SDS-PAGE analysis shows that elution fractions are not clean and contain impurities in both cases (Figure 14B, 14E). On the other hand, Western blot analysis shows no positive signal (Figure 14C, 14F). The intensive band in the first lane of Figure 14A and 14D indicate the expression of the GST tag from an empty pGEX-5X-1 vector.

According to obtained results, two possible reasons for protein detection inability were identified. One of them can probably be due to the experimental instability of both proteins, CD2vCt and LTB-CD2vCt, and their degradation before or during the extraction. Another reason includes the potential issue of the expression strain while considering them toxic and degrading them during the expression. Due to the inability to produce CD2vCt and LTB-CD2vCt, both proteins were abandoned and not produced anymore. The following reasons for protein detection inability are only

hypothetical since there are no studies concerning the immunoglobular CD2v protein segment production in *E. coli* expression strains, which could be used for comparison.

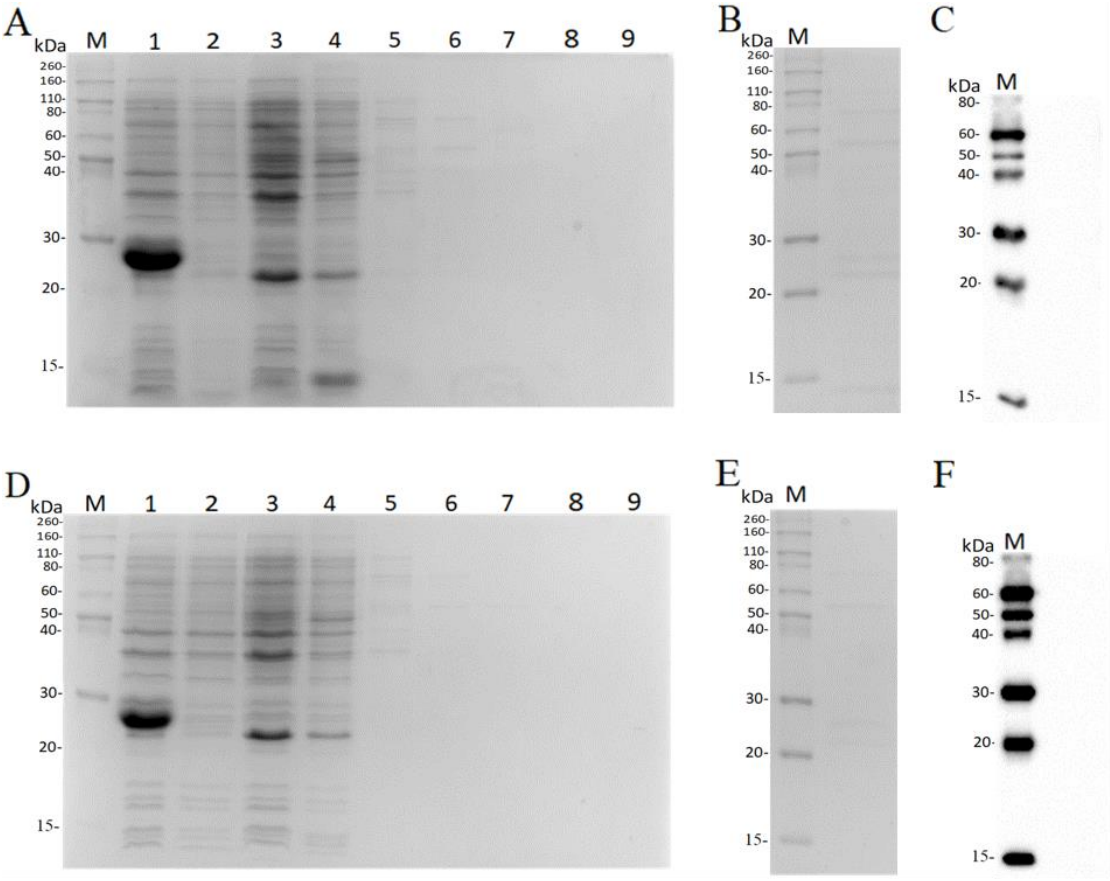

Figure 14: SDS-PAGE and western blot analysis from the expression and purification of recombinant proteins CD2vCt **(A-C)** and LTB-CD2vCt **(D-F)** in *E. coli* Rosetta™ 2(DE3) **(A) 1**, cells harbouring empty pGEX-5x-1 as control (after induction), **2**, cells harbouring pGEXET28b(+)-*CD2vCt* before induction, **3**, the cells after induction, and **4**, soluble protein fraction, **5**, a washout fraction (40 mM imidazole) from Ni-NTA agarose, **6-9**, CD2vCt eluted by 300 mM imidazole, **(B)** purified protein staining and **(C)** Western blot of the purified CD2vCt protein with the anti-His-tag antibody; **(D) 1**, pGEX-5x-1 cells after induction, **2**, pGEX-5x-1-*LTB-CD2vCt* cells before induction, **3**, the cells after induction (each 20 µL), and **4**, soluble protein fraction, **5**, a washout fraction (40 mM imidazole), **6-9**, eluted LTB-CD2vCt protein, **(E)** protein staining and **(F)** Western blot of purified LTB-CD2vCt anti-His-tag antibody; **M** protein molecular mass marker.

These unsuccessful results let us not pursue further investigation related to those proteins. Therefore these two proteins were abandoned and not continued to work with them.

LAE based proteins

Linear array epitope (LAE) is a method developed by Taiwanese scientists (Lai *et al.*, 2014). This method is mainly used to produce antibodies against the less immunogenic proteins and use them for diagnostic and therapeutic applications. It was previously used for RNA viruses, including nervous necrosis virus and dengue virus type 2, but also for the treatment of cancer (Hsu *et al.*, 2000; Lai *et al.*, 2014; Lin *et al.*, 2018).

An LAE method was first introduced to our laboratory by Professor Hau-Ren Chen from the National Chung Cheng University in Taiwan. His research team uses the method primarily for RNA viruses, such as the dengue virus. In our laboratory, LAE was incorporated into the African swine fever virus research as a very innovative method since there is no evidence of the method's application in this research.

The principle of the method is to select the potential antigen epitopes, which will be replicated using this method and subsequently expressed and used for either vaccination or detection purposes. Since the previous preparation of the C-terminal part of CD2v protein, as described above, was not successful, its possible immunogenic regions were further examined by the LAE method. Two selected epitopes, F3 and A6, were confirmed to be the most suitable antigens of CD2v protein (Argilaguet *et al.*, 2012).

Following the LAE method, multiple clone repetitions of both epitopes already cloned into pGEX-5x-1 were screened, and the ones having the number of repetitions above seven were selected. The higher number of repetitions the lower frequency of transformed clones was observed. Four clones have been selected from A6 epitope repetitions and other three from F3 epitope repetitions. The A6 clones were named as A6-1 (7-mer), A6-2 (17-mer), A6-3 (7-mer) and A6-4 (8-mer), while the F3 clones were F3-1 (18-mer), F3-2 (9-mer), and F3-3 (7-mer). Selected clones were transformed into chemically-competent BL21 STAR expression cells and used for an expression experiment.

A small-scale expression of A6 and F3 LAE proteins in the *E. coli* BL21 STAR cell cultures was performed using the pGEX-5x-1 vector. Samples were taken before and four hours after the induction, centrifuged and used for the SDS-PAGE analysis using 12 % polyacrylamide separating gel and Western blot analysis using an anti-GST antibody. SDS-PAGE (Figure 15A, 15C) and Western blot analysis (Figure 15B,

15D) showed that all LAE proteins were successfully overproduced in the *E. coli* BL21 STAR strain.

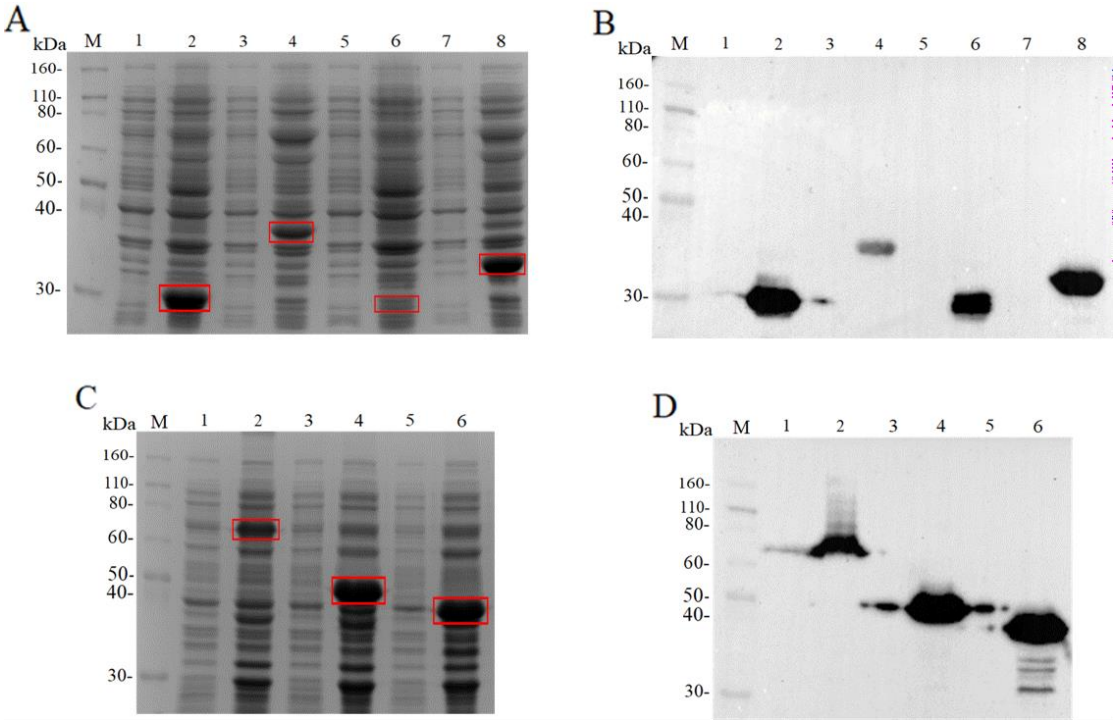

Figure 15: Analysis of expression of four clones containing a different number of repetitions of LAE epitopes made by SDS-PAGE and Western blot analysis

(**A**) SDS-PAGE analysis of crude extracts from *E. coli* BL21 STAR cells expressing a different number of LAE A6 epitopes, Lanes 1, 3, 5, and 7 show the 20 µL samples from non-induced cells, while lanes 2, 4, 6, and 8 represent the cells after induction with 0.2 mM IPTG. Bands corresponding to the recombinant proteins A6 (1-4) and F3 (1-3) are shown in coloured boxes. (**B**) Western blot of expressed clones A6 (1-4) with anti-GST antibody. (**C**) SDS-PAGE analysis of crude extracts from *E. coli* BL21 STAR cells expressing a different number of LAE F3 epitopes . Lanes 1, 3, and 5 show the 20 µL samples from non-induced cells, while lanes 2, 4, and 6 represent the cells after induction with 0.2 mM IPTG. The order of the samples was the same as in the case of LAE A6 clones. (**D**) Immunoblot analysis of expressed clones F3 (1-3) with anti-GST antibody.

Presented ASFV LAE antigens were planned to be used for polyclonal antibody production in rabbits during the expected internship at the National Chung Cheng University in Taiwan. These antibodies were supposed to be used for the subsequent development of the diagnostic technique against ASFV. Unfortunately, the internship was cancelled due to the world pandemic situation causing closed borders during the time of the expected internship. However, a new project proposal presenting the future collaboration with the Taiwanese

laboratory was submitted, and the continuation of these experiments can hopefully happen soon.

p22Ct and LTB-p22Ct proteins

Preliminary expression experiments regarding *p22Ct* and *LTB-p22Ct* constructs were the first successful experiments among all selected constructs. As stated, constructs *p22Ct* and *LTB-p22Ct* were cloned into the pET28b(+) vector and transformed into the BL21 STAR expression strain used for preliminary expression experiments. SDS-PAGE results (Figure 16A) show high expression levels of both proteins. The p22Ct and LTB-p22Ct proteins were expressed in a larger amount to examine the purification efficiency using the Ni-NTA purification matrix. Proteins were concentrated and analysed by Western blot analysis confirming their identity. Table 7 was attached to show both proteins' predicted isoelectric point pI and molecular weight.

Table 7: Predicted isoelectric point (pI) and molecular weight (Mw) of p22Ct and LTB-p22Ct proteins

Protein name	pI	Mw (kDa)
p22Ct	5,58	24,95
LTB-p22Ct	6,03	39,1

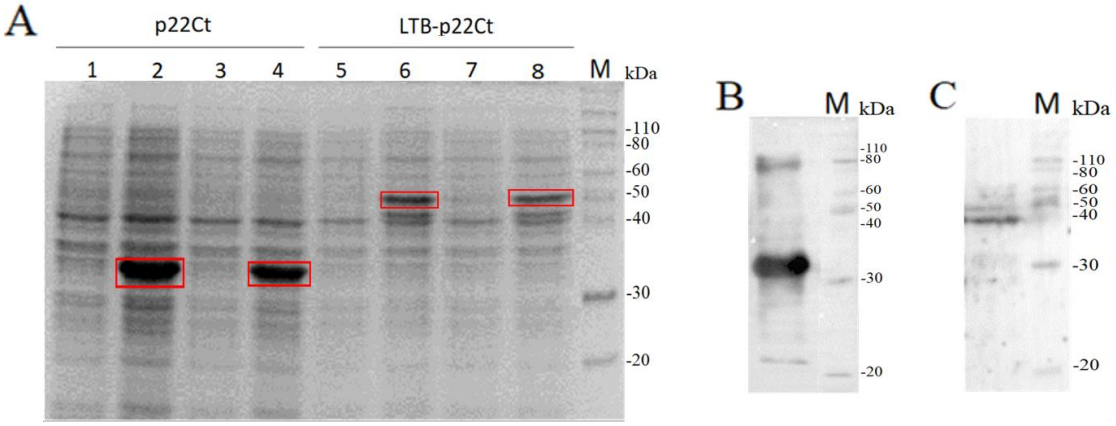

Figure 16: Analysis of expression of p22Ct and LTB-p22Ct recombinant proteins by SDS-PAGE and Western blot analysis

(**A**) SDS-PAGE analysis of crude extracts from *E. coli* BL21 STAR cells expressing *p22Ct* and *LTB-p22Ct*. Lanes 1, 3, 5, and 7 show the 20 µL samples from non-induced cells, while lanes 2, 4, 6, and 8 represent the cells after induction with 0.2 mM IPTG. Bands corresponding to the recombinant proteins p22Ct and LTB-p22Ct are shown in coloured boxes. (**B**)

Immunoblot analysis of purified p22Ct protein, and (**C**) Immunoblot analysis of purified LTB-p22Ct protein with anti-His-tag antibody.

However, the mobility on the SDS-PAGE corresponded to higher than the calculated molecular weight. Two additional experiments were performed to prove the protein identity: removal of the Myc-tag by enterokinase and protein identification using Matrix-Assisted Laser Desorption/Ionisation – Time of Flight (MALDI-TOF).

After removing the 4xMyc-tag, the mobility of the protein band corresponding to the protein with a cleaved Myc-tag was again analysed by SDS-PAGE. However, the protein mobility did not change as expected. The mobility of cleaved p22Ct protein corresponded to approximately 24 kDa, while the calculated Mw is 18.57 kDa. The corresponding protein band is shown in Figure 17 (lanes 2 and 3).

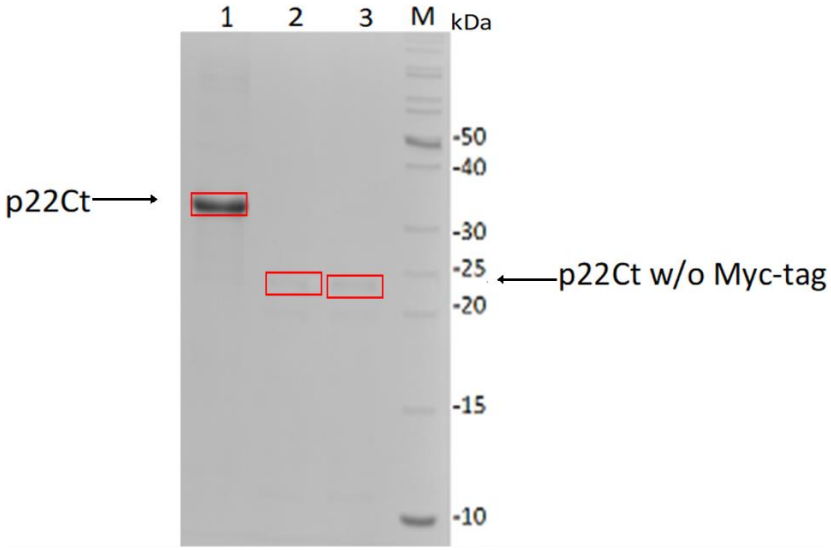

Figure 17: Analysis of purified recombinant p22Ct protein before and after digestion with enterokinase enzyme.
Lane 1 represents purified p22Ct protein, and Lanes 2 and 3 show digested p22Ct protein by enterokinase. Bands corresponding to the recombinant protein p22Ct are shown in coloured boxes.

The reason behind this phenomenon is still unclear. Many factors affect the protein mobility in SDS-PAGE, including the proteins' post-translational modifications and physicochemical properties. However, such post-translational modifications are highly unlikely in the used bacterial expression system. Protein properties, such as the amount of negatively charged amino acid side chains, protein

hydrophilicity and the presence of detergents surrounding the protein, could decrease SDS binding to the proteins and thus lower the electrophoretic mobility (Rath *et al.*, 2009; Shirai *et al.*, 2008; Tiwari *et al.*, 2019). Multiple negative-charged residues result in the decreased binding of SDS to the protein and thus decrease the electrophoretic mobility, but it was observed only in cases when the negative charge/positive charge (NC/PC) ratio was above 1.5 (Tiwari *et al.*, 2019). It was not observed in our case since the NC/PC rate of p22Ct protein was 1, while the one of LTB-p22Ct was 0.89. One of the possible reasons for lowered electrophoretic mobility could be caused by the lower GRAVY score of both proteins (-1.108 for p22Ct; -0.757 for LTB-p22Ct). The low GRAVY value shows high hydrophilicity, which decreases electrophoretic mobility (Shirai *et al.*, 2008). Another reason behind the lower mobility of our proteins could be the presence of Triton X100 since detergents can decrease electrophoretic mobility by competing with SDS (Rath *et al.*, 2009). However, the concentration of Triton in the buffer is very low (0.1 %), which is insignificant in this case. Therefore, the most probable reason behind the low protein motility in the gel is its high hydrophilicity.

However, to confirm our proteins, the protein bands were excised from the gel, and a proteomic analysis was performed by Professor Marek Šebela in the Department of Biochemistry, Faculty of Science, Palacký University in Olomouc. The samples were analysed after proteolysis with trypsin using MALDI-TOF. MALDI-TOF mass spectrometry is primarily used for protein identification by peptide mass fingerprinting (Webster and Oxley, 2011). In this method, proteins separated by SDS-PAGE were excised from the gel, washed, de-stained, reduced, alkylated, followed by trypsin digestion and finally measured by MALDI-TOF mass spectrometry (Webster and Oxley, 2011).

The spectra of the obtained fragments were compared with the database of spectra from known proteins, and the data were extracted. Data corresponding to the p22Ct and LTB-p22Ct protein are shown in Table 8 and 9, respectively. The yellow-marked rows represent the target proteins, while the white rows are considered false or non-significant results.

Table 8: Results of the protein prediction from Mass Spectrometry of p22Ct protein extracted from the SDS-PAGE gel.

The table represents the complete data set from the MS comparison to the database of known spectra

Row	OK	Accession	Protein
1	true	ENV22_ASFB7	Envelope protein p22 OS=African swine fever virus (strain Badajoz 1971 Vero-adapted) OX=10498 GN=BA71V-005 PE=2 SV=1
2	true	ENV22_ASFP4	Envelope protein p22 OS=African swine fever virus (isolate Tick/South Africa/Pretoriuskop Pr4/1996) OX=561443 GN=Pret-004 PE=2 SV=1
3	false	TRYP_PIG	Trypsin OS=*Sus scrofa* OX=9823 PE=1 SV=1
4	false	RRF_NEOSM	Ribosome-recycling factor OS=*Neorickettsia sennetsu* (strain ATCC VR-367 / Miyayama) OX=222891 GN=frr PE=3 SV=1
5	false	ACDA2_METMA	Acetyl-CoA decarbonylase/synthase complex subunit alpha 2 OS=*Methanosarcina mazei* (strain ATCC BAA-159 / DSM 3647 / Goe1 / Go1 / JCM 11833 / OCM 88) OX=192952 GN=cdhA2 PE=1 SV=4

Table 9: Results of the protein prediction from Mass Spectrometry of LTB-p22Ct protein extracted from the SDS-PAGE gel.

The table represents the complete data set from the MS comparison to the database of known spectra.

Row	OK	Accession	Protein
1	true	EFTU2_ECO24	Elongation factor Tu 2 OS=*Escherichia coli* O139:H28 (strain E24377A / ETEC) OX=331111 GN=tuf2 PE=3 SV=1
2	true	ELBP_ECOLX	Heat-labile enterotoxin B chain OS=*Escherichia coli* OX=562 GN=eltB PE=1 SV=2
3	true	EFTU_MARHV	Elongation factor Tu OS=*Marinobacter hydrocarbonoclasticus* (strain ATCC 700491 / DSM 11845 / VT8) OX=351348 GN=tuf PE=3 SV=1
4	true	ALAC_ECOLI	Glutamate-pyruvate aminotransferase AlaC OS=*Escherichia coli* (strain K12) OX=83333 GN=alaC PE=1 SV=1
5	true	ENV22_ASFB7	Envelope protein p22 OS=African swine fever virus (strain Badajoz 1971 Vero-adapted) OX=10498 GN=BA71V-005 PE=2 SV=1
6	true	ENV22_ASFP4	Envelope protein p22 OS=African swine fever virus (isolate Tick/South Africa/Pretoriuskop Pr4/1996) OX=561443 GN=Pret-004 PE=2 SV=1
7	false	TRYP_PIG	Trypsin OS=*Sus scrofa* OX=9823 PE=1 SV=1

The MALDI-TOF analysis confirmed the presence of target proteins. Results corresponding to the p22Ct protein correlate with p22 protein, but some false output was also detected, including trypsin, ribosome-recycling factor and acetyl-CoA decarbonylase/synthase complex subunit α2, which were considered insignificant. On the other hand, data from LTB-p22Ct showed the correlation not only to p22 protein and LTB but also to elongation factor Tu and glutamate-pyruvate aminotransferase AlaC. Elongation factor Tu (EF-Tu) is one of the most abundant proteins in *Escherichia coli* (Furano, 1975). Therefore, there is a possibility that EF-Tu is either binding unspecifically to LTB-p22Ct or being the contamination and thus present in the sample. On the other hand, glutamate-pyruvate aminotransferase AlaC is one of three enzymes playing a role in alanine synthesis with an estimated molecular weight of 46 kDa (Kim *et al.*, 2010) and thus could be excised from the gel together with the target protein having similar molecular weight.

The small-scale expression after mutagenesis

Following the successful pilot expression experiments, the 4xMyc-tag was no longer required for subsequent experiments; therefore, it was removed from the *p22Ct* and *LTB-p22Ct* gene constructs by site-directed mutagenesis. A small-scale expression of p22Ct and LTB-p22Ct proteins in 5 mL of the *E. coli* BL21 STAR cell cultures was performed to confirm the protein expression levels after the site-directed mutagenesis. Expression was done using the T7 expression system and the pET28b(+) vector. The expression was induced by adding IPTG, and the cultures were grown for 4 h at 37 °C. SDS-PAGE showed that the expression level of the LTB-p22Ct fusion protein was much lower (but detectable) than the p22Ct protein (Figure 18), similar to the previous experiments.

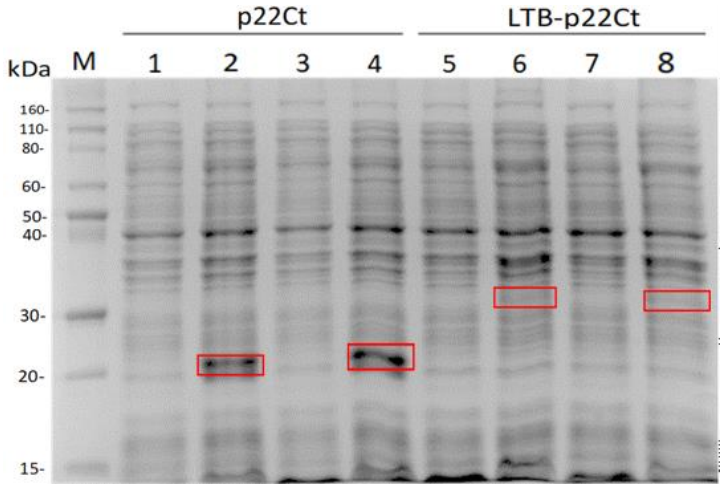

Figure 18: SDS-PAGE analysis of crude extracts from *E. coli* BL21 STAR cells expressing *p22Ct* and *LTB-p22Ct*.
Lanes 1, 3, 5, and 7 show the 20 µL samples from non-induced cells, while lanes 2, 4, 6, and 8 represent the cells after induction with 0.2 mM IPTG. Bands corresponding to the recombinant proteins p22Ct (lanes 2 and 4) and LTB-p22Ct (lanes 6 and 8) are shown in red-coloured boxes.

The results of small-scale cultures indicated that the expression of the LTB-p22Ct protein was much lower than that of p22Ct (lanes 6 and 8 of Figure 18). These results correlate with the study showing that the homologous overexpression of LTB in *E. coli* is often low and prone to forming inclusion bodies (Ma *et al.*, 2010). However, the formation of inclusion bodies was not observed in this work.

Large-scale expression of p22Ct and LTB-p22Ct constructs

The large-scale expression was performed under the same conditions in 1 L cultures to produce higher protein quantities for purification and further experiments. The cells were harvested by centrifugation and lysed by sonication. The soluble protein fractions were then used to purify the His-tagged recombinant proteins p22Ct and LTB-p22Ct using the Ni-NTA agarose matrix. Samples from the individual purification steps were analysed by SDS-PAGE and Western blot with an anti-His-tag antibody (Figure 19). Finally, the fractions containing purified recombinant proteins were pooled, concentrated and stored at -80 °C.

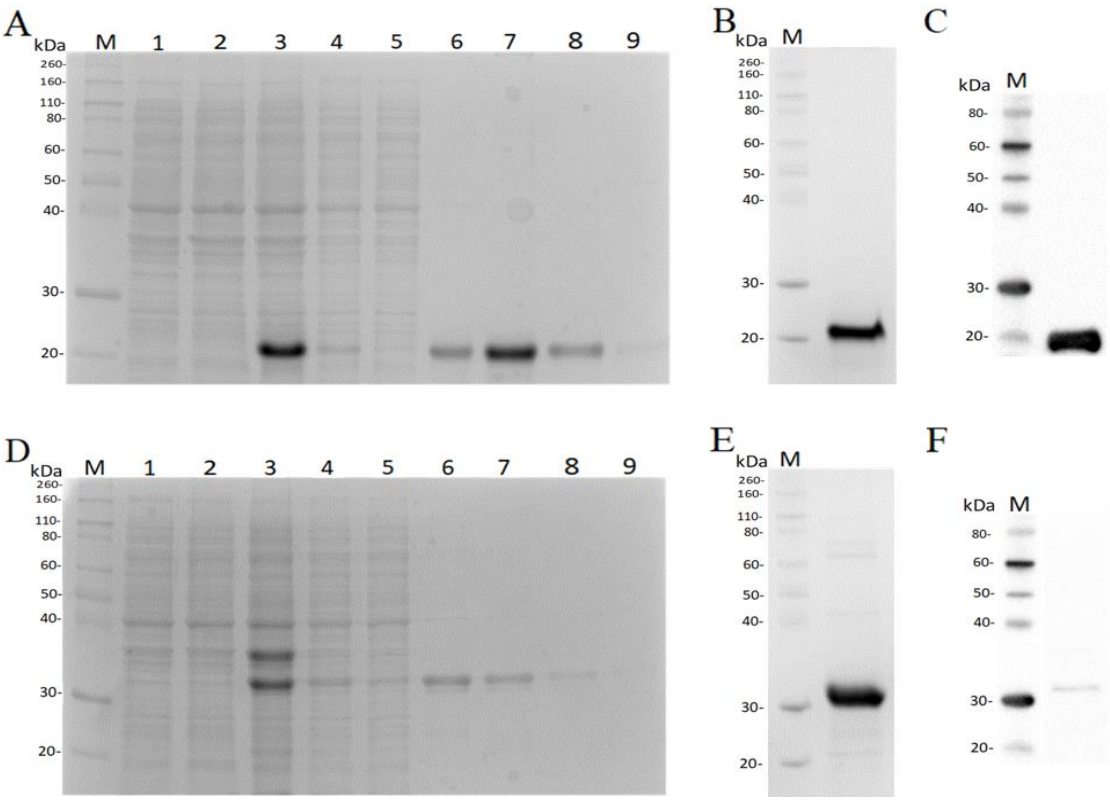

Figure 19: SDS-PAGE and Western blot analysis from the expression and purification of recombinant proteins p22Ct **(A-C)** and LTB-p22Ct **(D-F)** in *E. coli* BL21

(A) 1, cells harbouring empty pET28b(+) as control (after induction), **2**, cells harbouring pET28b(+)-*p22Ct* before induction, **3**, the cells after induction, and **4**, soluble protein fraction, **5**, a washout fraction (40 mM imidazole) from Ni-NTA agarose, **6-9**, p22Ct eluted by 300 mM imidazole (samples of approximately 5, 10, 4, and 0.5 µg, respectively), **(B)** protein staining and **(C)** Western blot of the purified p22Ct protein with the anti-His-tag antibody (11 µg); **(D) 1**, pET28b(+) cells after induction, **2**, pET28b(+)-*LTB-p22Ct* cells before induction, **3**, the cells after induction (each 20 µL), and **4**, soluble protein fraction, **5**, a washout fraction (40 mM imidazole), **6-9**, eluted LTB-p22Ct protein (samples of approximately 4, 3, 2 and 0 µg), **(E)** protein staining and **(F)** Western blot of purified LTB-p22Ct (16 µg); **M** protein molecular mass marker

Western blot analysis showed a low signal of LTB-p22Ct (Figure 19F) compared to its counterpart of the SDS-PAGE result (Figure 19E). This occurrence happened many times in the case of LTB-p22Ct protein, not only during Western blot analysis but also during the purification step, where the protein was washed out from the matrix even with a buffer containing 40 mM imidazole. The possible reason behind this occurrence is probably the native structure of the target protein. Based on the study by Kim *et al.* (2007), the native protein structure may be blocking the histidine tag from the Ni-NTA matrix and thus decreasing the protein yield.

From the calibration curves (Figure 20), it was calculated that the concentration of p22Ct protein was approximately 1.2 µg/µL and that of LTB-p22Ct approximately 1.0 µg/µL. The coefficient of determination (R^2) in both cases is higher than 0.99, stating that calculated protein concentrations are reliable. From calculated concentration, the total yield was estimated. The total yield of p22Ct protein was 4.9 mg, while LTB-p22Ct was 3.8 mg per litre of culture, corresponding roughly to 3 % of the theoretical maximum yield of the protein of interest in *E. coli.*

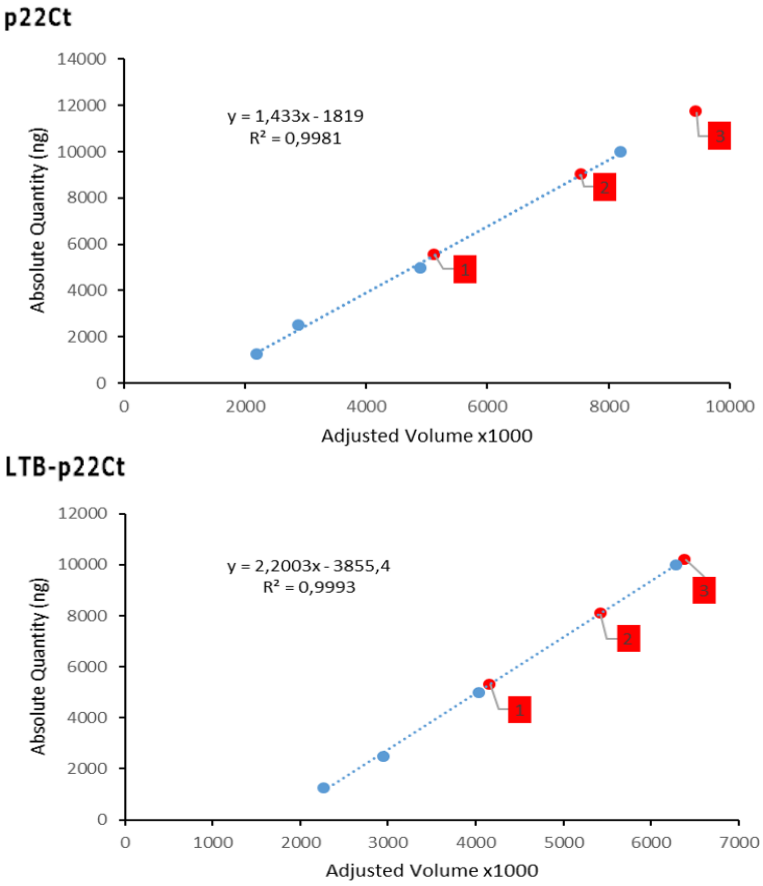

Figure 20: Estimation of protein concentration of p22Ct (upper) and LTB-p22Ct (lower) by comparison with BSA as standard.

The x-axis shows the protein's absolute quantity in nanograms. In contrast, the y-axis shows the adjusted volume of the corresponding band on the SDS-PAGE gel automatically calculated by ImageLab software. Calibration amounts of BSA labelled in blue colour were set to 10 µg (BSA10), 5 µg (BSA5), 2.5 µg (BSA2.5), and 1.25 µg (BSA1.25). Unknown samples are labelled in red colour as p22Ct 1 (10µL), p22Ct 2 (7.5 µL), p22Ct 3 (5 µL), and LTB-p22Ct 1 (10 µL), LTB-p22Ct 2 (7.5 µL), LTB-p22Ct 3 (5 µL).

Analysis of correct conformation and the immunogenicity of proteins

The purified recombinant proteins p22Ct and LTB-p22Ct were then tested by immunoblot analysis for their ability to interact with serum antibodies (IgG) from ASFV-infected pigs. Both proteins showed positive reactions with the four sera used as primary antibodies (Figure 21).

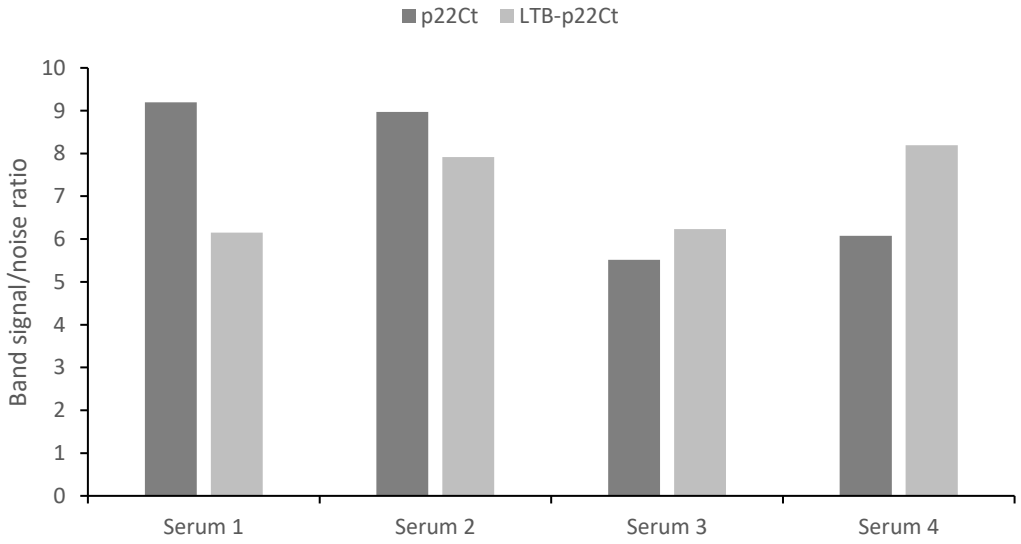

Figure 21: Immunoblot assay of the purified recombinant proteins p22Ct and LTB-p22Ct with four different sera from ASFV infected pigs; the binding intensity was calculated as the intensity of the bands compared to the background over the whole lane of the immunoblot assay.

The positive reactions of both proteins to four different positive sera indicate the correct conformation of both proteins, thus showing the possibility of induction of the immune response in the host and the production of specific antibodies. As implied by the article by Scheiblhofer *et al.* (2017), conformational stability of the target proteins is essential for the immunogenicity of recombinant vaccines.

The general immunogenicity of the prepared recombinant proteins was assessed by mice immunization. For three mice in each group, the protein samples mixed with an adjuvant were injected subcutaneously into the back behind the head with a two-week interval between doses. The control mice group was vaccinated with the adjuvant alone. With the p22Ct protein, the mice were fully immunized with three doses. However, in the case of the LTB-p22Ct group, an abscess occurred after the first dose. Due to this fact, only one dose was administrated. The blood of all mice was collected on day 42, and serological activity against the recombinant proteins was

analysed by ELISA. Antibody titre was high for the mice group immunized with p22Ct, while the samples from the LTB-p22Ct group did not react at all (Figure 22).

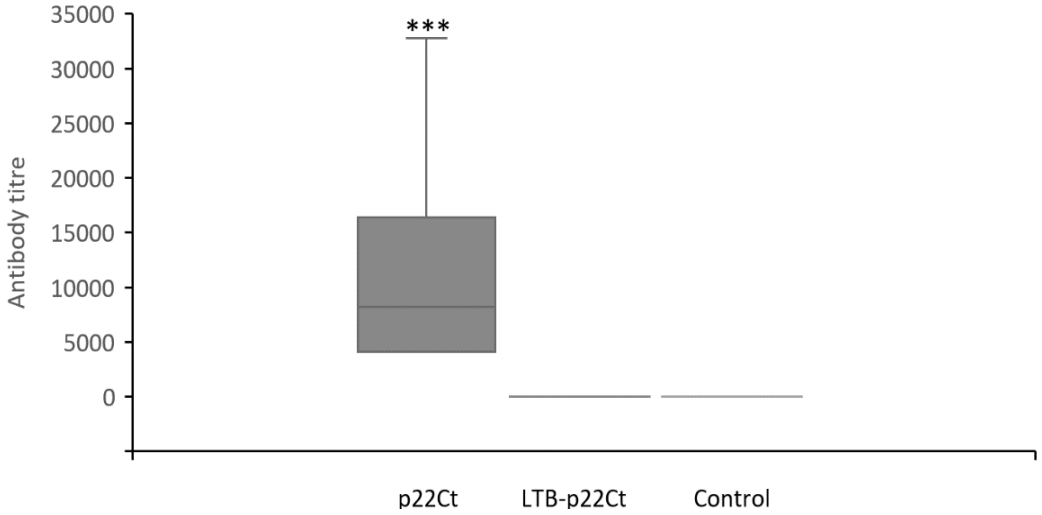

Figure 22: ELISA test results of sera samples taken from mice groups immunised with p22Ct and LTB-p22Ct; the measurement was done in three technical replicates; the statistical significance of the results was verified by a one-way ANOVA analysis, $P < 0.001$.

Our results show that p22Ct, the C-terminal globular part of the p22 protein, but not the fusion protein LTB-p22Ct, can induce an immune response in mice. The exact cause of the observed difference is unclear. The most probable reason is that mice immunised with LTB-p22Ct were injected with only one dose rather than the three doses of p22Ct administered. A significant problem appeared in the form of abscess formation in the mice vaccinated with the fusion protein; therefore, these mice received only one antigen dose. This is probably also the reason why the mice immunised with LTB-p22Ct could not produce specific antibodies against the protein. Another reason may be that LTB could become less active when produced by recombinant technology in *E. coli* (Ma *et al.*, 2010).

Due to the difference in inducing the immune response in mice, the oligomerization of both recombinant proteins was examined by SDS-PAGE under non-reducing conditions (Figure 23). As calculated from comparing the mobility of recombinant proteins with that of protein molecular mass standards, the 33 kDa LTB-p22Ct fusion forms most probably hexameric complexes of 198 kDa. In contrast, the p22Ct protein prevalently retains the monomeric form of 23 kDa.

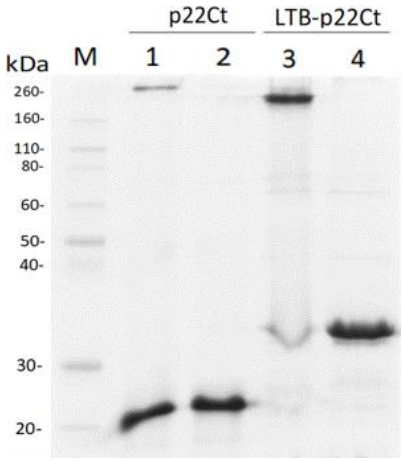

Figure 23: SDS-PAGE of p22Ct (13 µg) and LTB-p22Ct (16 µg) samples under reducing (2, 4) and non-reducing conditions without adding 2-mercaptoethanol and not heated (1, 3); M protein molecular mass marker

As described previously, LTB binds to the GM1 receptor in a pentameric association, which is essential for stimulating the immune response (Kim *et al.*, 2011). However, the SDS-PAGE under non-reducing conditions showed that LTB-p22Ct is more likely to form a hexamer, which may be a reason for its lack of immunogenicity (Figure 23).

Our results also show that p22Ct can produce a high antibody titre in mice and is thereby indicated to be a highly potent and immunogenic region. It can be considered a candidate primarily in serological diagnostics for the development of specific antigen-based detection techniques such as fluorescent antibody tests, ELISA and immunoblotting (Qiu *et al.*, 2021). However, further experiments should be conducted to investigate its possible link to other immunostimulatory domains.

Conclusions

African swine fever virus was and still is the reason behind an economic crisis causing the death of millions of pigs. ASFV infects mainly macrophages and uses them for replication of new viral particles. Multiple vaccines were developed to combat the disease caused by this virus, but no vaccine was fully effective. Recent studies show the development of new attenuated vaccines with promising results (Borca *et al.*, 2020; Gladue *et al.*, 2021).

In this work, we have prepared potential recombinant proteins containing a globular hydrophilic fragment of the ASFV antigens, CD2v and p22. From four initial constructs, two (*CD2vCt*, *LTB-CD2vCt*) were unable to express using the bacterial expression system, while the other two (*p22Ct*, *LTB-p22Ct*) were expressed successfully and used for further experiments.

After successful expression, the protein identity of p22Ct and LTB-p22Ct was analysed by MALDI-TOF since the protein mobility on SDS-PAGE was slower than expected. The most probable reason behind this phenomenon was the high protein hydrophilicity (Shirai *et al.*, 2008). After deletion of the 4xMyc-tag, the correct protein conformation was confirmed by the immunoblot analysis with positive ASFV sera, which is essential for the immunogenicity of recombinant proteins (Scheiblhofer *et al.*, 2017). Following mice immunisation with p22Ct and LTB-p22Ct showed a high difference between both proteins in the inductions of antibodies. No specific antibody induction by the LTB-p22Ct was observed since already a single injected dose of the antigen caused an abscess formation and the experiment had to be interrupted. In contrast, the high specific antibody level induced by p22Ct shows its potential as a serological marker for the development of new diagnostic techniques for combating the ASFV spread.

In this work, the LAE technique was used for the first time in the ASFV research. CD2v epitopes were chosen based on the Argilaguet *et al.* (2012) study, showing the epitopes' high immunogenic potential. Proteins were successfully overexpressed using a bacterial expression system. However, future experiments are needed to measure the immunogenicity and show LAE proteins' potential in the development of diagnostic tools.

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List of Abbreviations

AA	amino acid/-s
AP-1	adaptor protein complex 1
ASF	African swine fever
ASFV	African swine fever virus
Bcl2	B-cell lymphoma 2 protein
BH1-4	Bcl2 family homology regions 1-4
BSA	Bovine serum albumin
CaM kinase II	calmodulin-dependent protein kinase II
CPM	cell plasma membrane
dsDNA	double-stranded deoxyribonucleic acid
EE	early endosomal
eIF2	eukaryotic initiation factor 2
ER	endoplasmic reticulum
GA	Golgi apparatus
GFP	green fluorescent protein
GP	$(Gly\text{-}Pro)_2$ linker
GST	glutathione S-transferase
IAP	inhibitor of apoptosis
IFN	interferon
IL	interleukin
LAV	live attenuated vaccine
LE	late endosome
LTB	heat-labile enterotoxin B subunit
MALDI-TOF	matrix-assisted laser desorption/ionisation – time of flight
MTOC	microtubular organizing centre
MVB	multivesicular body
NFκB	nuclear factor κB
NFAT	nuclear factor of activated T cells
ORF	open reading frame
PBS	phosphate-buffered saline
PI-3P	phosphatidylinositol 3-phosphate
PI-3,5-biP	phosphatidylinositol 3,5-bisphosphate
PYCR2	pyrolline-5-carboxylate reductase

ssDNA	single-stranded deoxyribonucleic acid
TBS	Tris-buffered saline
TNFα	tumour necrosis factor α

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2. Díaz C., Frébort I. Poster:
   Production of recombinant protein for veterinary use in plants. The 14th Asian Congress on Biotechnology, July 1-4, 2019, Taipei, Taiwan.

3. Díaz C., Frébort I. Poster:
   The immunogenicity of a potential vaccine candidate against African swine fever virus based on the recombinant antigen originating from the virus p22 protein. Green for Good VI: Global Challenges, September 12-15, 2022, Olomouc, Czech Republic.

Supplements

Supplement 1

Review article

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REVIEW ARTICLE

The main DNA viruses significantly affecting pig livestock

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Abstract

Swine DNA viruses have developed unique mechanisms for evasion of the host immune system, infection and DNA replication, and finally, construction and release of new viral particles. This article reviews four classes of DNA viruses affecting swine: porcine circoviruses, African swine fever virus, porcine parvoviruses, and pseudorabies virus. Porcine circoviruses belonging to the *Circoviridae* family are small single-stranded DNA viruses causing different diseases in swine including poly-weaning multisystemic wasting syndrome, porcine dermatitis and nephropathy syndrome, and porcine respiratory disease complex. African swine fever virus, the only member of the *Asfivirus* genus in the *Asfarviridae* family, is a large double-stranded DNA virus and for its propensity to cause high mortality, it is currently considered the most dangerous virus in the pig industry. Porcine parvoviruses are small single-stranded DNA viruses belonging to the *Parvoviridae* family that cause reproductive failure in pregnant gilts. Pseudorabies virus, or suid herpesvirus 1, is a large double-stranded DNA virus belonging to the *Herpesviridae* family and *Alphaherpesvirinae* subfamily. Recent findings including general as well as genetic classification, virus structure, clinical syndromes and the host immune system responses and vaccine protection are described for all four swine DNA virus classes.

Keywords: DNA viruses, circoviruses, African swine fever virus, parvoviruses, pseudorabies virus.

Introduction

The genetic material of DNA viruses is either single-stranded (ss) or double-stranded (ds) deoxyribonucleic acid. Virus DNA genomes are variable in size, ranging from small with a size of 1 kilobase pairs (kbp) to large examples of several megabase pairs. DNA viruses use host cells for replication and subsequent infection. The first viral genes to be expressed, which are made by larger viruses, are called early genes. Genes encoding DNA polymerase and proteins incorporated in DNA replication often belong in this group. After DNA replication, viruses change the expression profile to the so-called late genes. Those genes are essential for the production of structural proteins used for coating the replicated DNA genome and forming new viral particles. At the end of the proliferation process, viral particles are released from the cell to infect new sites. In this article, the four main groups of DNA viruses significantly affecting swine are reviewed: porcine circoviruses, African swine fever virus, porcine parvoviruses, and pseudorabies virus. The genetic diversity inside a particular group and family classification, the structures of virus particles, the clinical syndromes, the course of infection, and recent progress in vaccine development as an effective means of protection against infections with swine DNA viruses are described.

Porcine circoviruses (PCVs)

Porcine circoviruses are the smallest autonomously replicating swine viruses containing circular single-stranded DNA (ssDNA) with a size of 1.76 kbp (73, 75). PCVs were first discovered by Tischer *et al.* (113) in 1974 when PCVs were mistaken for picornavirus-like particles in a contaminated PK-15 pig kidney cell line. Those circoviruses were non-pathogenic, and after they

were classified into the *Circoviridae* family, their apparent harmlessness caused them to merit little attention so only a few articles concerning the topic were written until around 20 years later, when a new pathogenic type of porcine circovirus appeared called PCV2 (75). In 2015, PCV3 was discovered and associated with porcine dermatitis and nephropathy syndrome (PDNS) (90), and in 2019, a new type of PCV (type 4) was found in China (124).

Virus structure. PCV virions are small isometric particles with a diameter of 17 nm containing circular ssDNA which only contains three protein-coding genes (114). The virus particle of both PCV1 and PCV2 is composed of a single structural protein called the capsid protein (Cp), with a molecular mass of 30 kDa and which is responsible for spontaneous capsid formation (62) (Fig. 1).

PCV1. This was the first identified porcine circovirus. It was designated PCV PK-15 after its discovery and characterisation as a contaminant in the PK-15 porcine kidney cell line (113). Interestingly, it was also found in lymph nodes from piglets affected by a wasting syndrome in France (4, 60).

PCV2. In 1998, Meehan *et al.* (74) observed that monoclonal antibodies raised to circoviruses causing post-weaning multisystemic wasting syndrome (PMWS) were different from those raised to the PCV PK-15 isolate. They also published the first nucleotide sequences of the circoviruses associated with PMWS, which showed less than 80% identity with the PCV PK-15 isolate, and they thus provided evidence for a new pathogenic type of porcine circovirus, referred to as PCV2 (74). Based on the results of the phylogenetic study using the capsid protein gene region as a marker, PCV2 sequences were divided into two main groups: the first group, which subdivides into three clusters 1A to 1C, and the second group, which branches into five clusters 2A to 2E (40, 85). There is also another grouping method considering the geographic localisation of the virus, dividing PCV2 into PCV2a for the North American-like isolates (which also fall into the first capsid protein gene region-differentiated group of PCV2), and PCV2b for the European-like isolates (also in the second capsid protein group of PCV2) (86).

PCV3. This is a recently discovered type, and yet it has been detected and characterised in many countries throughout the world, including China (55), Italy (31), Brazil (115), and Sweden (121). PCV3 was first identified in 2015 in North Carolina (USA) in isolates from sows showing high mortality, low conception rates and typical signs of PDNS (90). Therefore, PCV3 was associated with PDNS and reproductive failure (90) and it has also been linked to congenital tumours in piglets as well after Chinese PDNS cases were investigated (21). This new type of PCV shares only a small percentage of homology in genomic DNA sequence with those of PCV1 and PCV2 (90). The homology between PCV3 and PCV2 found by sequencing in the *rep* gene sequence is 55% and in the *cp* gene only 37% (90). In China, PCV3 was divided into two groups (a and b) and five subgroups (a1–a3, b1, and b2) by a phylogenetic study using full-length sequences of PCV3 DNA (22). In a phylogenetic study conducted in Germany where only open reading frame (ORF) 2 (coding for the Cp protein) was used for grouping, the number of subgroups differed; group a was not divided but group b was, into three subgroups (22, 39). The difference is caused by the usage of whole-genome sequences in the Chinese study, while ORF2 was considered a critical phylogenetic marker in Germany (85).

PCV4. This type was only discovered in April 2019 (124). Type 4 contains 1.77 kbp long DNA and shares 67% homology with mink circovirus, which is the highest homology across circoviruses, and 43–52% homology with other porcine circoviruses (124). The size of two crucial genes was predicted at 891 nucleotides for the *rep* gene and 687 nucleotides for the *cp* gene (124). For the understanding of porcine circovirus' pathogenicity and infection, further investigations will be necessary.

Clinical syndromes. Postweaning multisystemic wasting syndrome was first described in 1996 and a year later was associated with PCV2 (46). The precise definition of PMWS was proposed by Sorden in 2000 (109). For pigs to be diagnosed with PMWS, they must show all of the following conditions: firstly, clinical signs like wasting, weight loss or failure to thrive; secondly, histological lesions, which are signs of depletion of lymphoid tissues and organs, and inflammation of the lungs and lymphoid tissues in usual cases and less often the liver, kidneys, pancreas or intestine; and thirdly, PCV2 infection inside the lesions. The effect of PMWS on the host immune system is pronounced, causing virus-induced lymphocyte depletion. In the work of Mandrioli *et al.* (69), the presence of activated macrophages was described as an essential factor for the development of the syndrome. Although mainly CD4+ T-lymphocyte counts were decreased during the infection, a dramatic decline in CD8+ and CD4+/CD8+ T-lymphocyte and B-lymphocyte numbers was also observed, associated with the loss of lymphoid follicles (69). The reduced proliferation of lymphocytes thus results in a reduction of cytokines as positive growth factors, which can affect the further expression of major histocompatibility complex I antigens type I and II (MHC I and MHC II) and thus impair the immune response (72). Interestingly, apoptosis was not observed in lymphoid tissues that showed a decreased rate of virus proliferation (69). However, the work of Shibahara *et al.* (106) showed that apoptosis occurred only in B-lymphocytes and not in macrophages (106). This can be explained by the yet-unknown cause of the apoptosis in lymphoid tissues of PMWS in swine (69).

Another disease associated with porcine circoviruses is PDNS. Pigs affected by this syndrome are slightly febrile, depressed, and have ventrocaudal subcutaneous oedema (100). The incubation time of this

disease is very short, and most swine die within three days. There are some similarities between PMWS and PDNS, such as lymphoid depletion and the presence of syncytial cells and others, suggesting that PCV2 may be responsible for this disease. Typically, this disease leads to skin lesions on the hind legs, however PCV2 has not been confirmed as the causative agent of this phenomenon (100).

Porcine respiratory disease complex (PRDC) is a disease that affects mainly 2–8-month-old pigs. PRDC is characterised by poor appetite, weight loss, or weak growth accompanied by clinical signs like anorexia, fever, cough and dyspnoea (19, 52, 86).

Development of vaccines. PCVs are highly resistant to conventional detergents and disinfectants, which makes decontamination problematic (4). To cope with the negative impacts on pig livestock, scientists have developed vaccines for combating these viruses. The first step in producing an efficient vaccine against pathogenic PCV2 is creating and characterising monoclonal antibodies against the pathogen. In 2001, McNeilly *et al.* (72) prepared and characterised monoclonal antibodies against six PCV2 isolates. One year later, Fenaux *et al.* (32) reported the first construction of a DNA clone containing an inserted infectious PCV2 genome and its subsequent use for *in vivo* transfection of pigs. The results from transfection testing showed that the cloned PCV2 genomic DNA could be used for future pathogenesis testing, replacing the virulent virus for greater safety (32). The same research group observed that not only PCV2 genomic DNA could enhance the production of specific monoclonal antibodies, but also that a DNA clone containing a capsid gene from PCV2 inserted into the backbone of PCV1 could achieve the same (34). This DNA clone was further tested as a live attenuated vaccine, which enhanced cell-mediated immune response and thus protected pigs against a pathogenic PCV2 challenge (33).

The first preparation which came onto the market (Circovac®, now produced by Ceva, France) successfully vaccinated sows and piglets older than three weeks (87). Interestingly, the two-dose vaccine was observed to enable the transfer of specific PCV2 antibodies from sow to offspring via colostrum (66). This type of vaccination was named dam vaccination. Another preparation used for immunisation of pregnant sows was a baculovirus-expressed PCV2 vaccine (Ingelvac CircoFLEX®, Boehringer Ingelheim, Germany). Only a single dose of the vaccine could develop neutralising antibodies against PCV2, but 10% of the piglets born to those vaccinated sows contracted *in utero* infection (67, 68). These studies also suggest that the timing of vaccination is crucial, selection of the life stage for administration depending on the desired result. For example, if a farm with sows wants to prevent *in utero* infection in the next generation, they will specify pre-breeding and post-farrowing vaccinations (66, 68). As another example, in the case of protecting piglets in the early stage of growth, the vaccination should be administered pre-farrowing, when colostrum contains more specific antibodies (66). The two vaccines described are currently used frequently for controlling PCV2 infection.

A useful way of combating PCV can also be the application of vaccines or drugs which could block the attachment of viral particles to host cells. Recently two studies have reported two different components which can accomplish that. Li *et al.* (63) found that epigallocatechin gallate from green tea can inhibit the infection of PCV by interfering with the capsid protein and thus inhibiting its binding to the host cells. Another option could be therapeutically neutralising antibodies. In the study of Huang *et al.* (49), a new neutralising monoclonal antibody was prepared capable of blocking the capsid protein attachment to PK15 cells. These findings can provide useful information for the development and synthesis of new vaccines and drugs against porcine circoviruses.

Recent approaches to vaccines mostly target the sole capsid protein (Cp), recognising it as the most important. This protein was either expressed in bacterial strains (*Lactobacillus lactis*) (116) or viruses (adenoviruses) (127) or used to produce PCV2 virus-like particles in insect cells in a baculoviral expression system (18, 70).

African swine fever virus (ASFV)

ASFV is a large DNA virus that is the sole member of the *Asfivirus* genus within the *Asfarviridae* family (64) affecting all species of swine and predominantly vectored by ticks from the *Ornithodoros* genus (37). ASFV causes a highly infectious disease called African swine fever (ASF). Even though ASF was first identified in 1921, its first occurrence had already been observed in 1910 in British East Africa (the Kenya Colony) as an infectious disease affecting domestic pigs (78).

Virus structure. ASFV is a large virus, of which the viral particle has icosahedral symmetry (Fig. 1). The size of ASFV derives from the trilayer viral envelope protecting the core that contains linear dsDNA. Each of the layers is composed of different structural proteins playing not only a protective role but also an infective one. A brief description of each envelope layer and the most important structural proteins follows.

Outer envelope. The outer layer is composed of the structural proteins p12 (*pO61R*), p22 (*KP177R*) and CD2v (*EP402R*) (3, 17, 98). The p12 protein (*pO61R*) is a late structural protein which attaches the viral particle to the host cell (3), and the p22 (*KP177R*) protein is an early structural protein which is localised on the outer envelope of the viral particle (17). CD2v is a more complex protein which plays different roles during ASFV infection. It is a transmembrane protein containing 402 amino acids showing a high degree of similarity to CD2, an adhesion receptor of T lymphocytes, particularly

sharing the immunoglobulin Ig domain with 28–30 highly glycosylated sites (76, 99). This protein functions in the adsorption of red blood cells on the surface of infected host cells (13, 99) and was found to interact with an adaptor protein complex (AP-1) through the diLeu motif in the C-terminal domain (91). Adaptor protein complex 1 is a group of cytosolic heterotetramers which sort membrane proteins to endosomes by the formation of clathrin-coated vesicles using clathrin as a scaffold protein (80). In this way, CD2v helps ASFV to enter into the host cells.

Capsid envelope. The major capsid p72 protein (encoded by the viral *B646L* gene) is knowable by its assembly in the area of the inner core matrix and outer capsid layer of the viral particle (24). This assembly is mediated by a chaperone encoded by *B602L* and takes place on the membrane of the endoplasmic reticulum, where the process of envelopment is localised (24). Another crucial structural protein is p49 (*B438L*), which forms the icosahedral shape of the viral particles by localising in the vertices of the capsid (29).

Inner envelope. The inner envelope contains five structural proteins: the abundant transmembrane p17 (*D117L*); the late structural pE248R (*E248R*), j5R (*H108R*) and j18L (*E199L*); and p54 or j13L (*E183L*) (15, 96, 97, 111, 112). Their functions have also been characterised, and it was learned that j5R and j13L/p54 are involved in the assembly of viral particles in which j13L is accumulated on the endoplasmic reticulum membrane, and involved in recruiting viral membrane precursors (15, 98). Protein p17 is also involved in recruiting viral precursors (111). Although the function of pE248R is not precisely known, it has been ascertained that it is an actor in the early phase during virus entry into the host cell (97).

Core layer. The first step in forming the viral particle is protecting the genomic DNA with a core layer of proteins. This layer is composed of structural proteins, which originate from polyproteins pp62 (*CP530R*) and pp220 (*CP2475L*) (107, 108). Both polyproteins are processed by SUMO-like protease (*S273R*) yielding different structural proteins, which in the case of pp62 are p15 and p35 and in the case of pp220 are p14, p34, p37 and p150 (107, 108).

Genomic DNA. ASFV genome is 170 kbp long and contains 151 ORFs (20, 120). The genome contains multiple genes with different functions. There are genes involved in DNA replication, genes encoding enzymes and factors involved in transcription and processing, genes encoding structural proteins and proteins involved in the assembly of viral particles, genes encoding proteins involved in host defences, and last but not least, multigene families, which correspond to the 30% of the genome (29).

Genetic classification. Distinct ASFV genotypes were identified based on the p72 structural protein. Phylogenetic analysis of the C-terminal end of the p72 gene showed the presence of 22 different genotypes (I–XXII) (14). Recently two new genotypes were added, XXIII and XXIV (2, 94), of which XXIII shares a common ancestor with the genotypes IX and X (2). In Europe, two types of genotypes caused outbreaks: genotype I on Sardinia and genotype II in Eastern Europe (11).

Clinical syndromes. The clinical signs caused by ASFV infection include lesions, high fever, skin haemorrhages and neurological diseases (117). Although these clinical signs may be similar to those of other diseases like classical swine fever virus and porcine reproductive and respiratory syndrome, African swine fever is manifested by additional symptoms including depression, apathy, anorexia, vomiting, and red skin on the ears, abdomen and chest (117).

ASFV and host immune system. The primary target cells of ASFV include macrophages and monocytes (45). ASFV uses macropinocytosis and clathrin-mediated endocytosis as two different mechanisms to enter the host cells (47). When the virus enters the cell, the lower pH inside late endosomes causes the disruption of the outer envelope and capsid (47). Thus, the inner envelope is exposed and subsequently fused with the endosomal membrane to release the viral genome into the cytosol (6). This fusion is mediated by the pE248R transmembrane protein of the inner envelope (6). Cholesterol from the endosome is also essential for the ASFV genome release to the cytosol (26). The further transport of the genome is mediated by p54 protein, which interacts with the light chain of dynein until it reaches the perinuclear spot near the microtubular organizing centre (MTOC), where DNA replication and transcription take place (5). Interestingly, the ASFV genome replicates independently on the host cell (29). The next step of ASFV infection is forming viral factories. These are formed near the nucleus at the MTOC, where virus proteins and DNA are assembled to form new viral particles (41). The integrity of the microtubules is necessary for the formation of viral factories (41). The last step is the release of completed viral particles outside the cells. The pE120R virus protein helps in the microtubule-mediated transfer of viral particles from the viral factory to the plasma membrane (7). The protein is attached to the surface of intracellular virions by binding to the p72 major capsid protein, which helps to incorporate pE120R into the viral particle (7).

Evasion from the host immune system. ASFV contains multiple genes that inhibit the function of interferon type I (IFN I), which results in inhibition of the antiviral state in infected host cells (30). One study suggests that the MGF 360 and 505 multigene families are involved in evasion from the antiviral state, due to the sensitivity of the virus to IFN I when MGFs were deleted (42). The essential part of the escape from the host immune system includes inhibition of cell death by apoptosis. Here, many proteins from ASFV can disable the apoptosis mechanism of the host cell. One of these is a protein encoded by the *A179L* gene, which belongs to the B-cell lymphoma Bcl2 family (10). This family is characterised by an anti- or pro-apoptotic function

depending on the type of homology region (BH1–BH4) and the protein interactions (56, 122). This protein is known for its interaction with proteins containing the BH3 domain (such as Bak and Bax) and resultant inactivation of them (10). Bak and Bax are primary gatekeepers, which upon activation by apoptosis inducers cause disruption of mitochondrial membranes, and the subsequent release of cytochrome *c* activates the caspase cascade resulting in apoptosis (56, 122). However, their inactivation by the *A179L* gene–encoded protein causes the inhibition of apoptosis in infected host cells. Another protein which can inactivate apoptosis is that encoded by the *A224L* gene. This protein belongs to the inhibitors of apoptosis protein family, which is recognised by the BIR motif, and uses tumour necrosis factor alpha (TNF-α) as a stimulus for inhibition of apoptosis (30). That inhibition by this protein is accomplished by inhibition of caspase 3 and activation of the NF-κB nuclear factor (30), which then activates the expression of cFLIP, an inactivated caspase 8 homologue that subsequently blocks caspase 8 activity (30). However, this protein is not essential for growth or viral virulence (81), which suggests that inhibition of apoptosis by TNF-α is not necessary for the replication of ASFV.

Development of vaccines. The development of vaccines for combating ASFV began in the 1960s (9). During those early years, multiple vaccines were developed, but none of them proved effective enough for commercial purposes. There are three main types of vaccines which were designed against ASFV: inactivated vaccines with a killed virus, live attenuated vaccines and subunit vaccines. Inactivated vaccine approaches were not successful at all; such vaccines could not enhance the immune response in pigs, even with the addition of different types of adjuvants (12).

Live attenuated vaccines (LAVs). These vaccines, containing viruses with deleted genes responsible for host invasion, infectivity or immune system inhibitors, were found to enhance cellular and humoral immunity and further protected pigs against the virulent virus type (102). There are three successful LAVs, which derive from the OURT88/3, NH/P68 and BA71ΔCD2v isolates (61, 77, 79). The OURT88/3 strain has been observed to enhance the production of CDβ8+ lymphocytes, the part of CD8+ lymphocytes confirming the importance of cellular immunity in the resistance to ASF (89). Interestingly, using the OURT88/3 isolate, it has been found that deletion of genes involved in virulence such as *DP71L*, *DP96R* and the IFN I interferon modulators MGF 360 and MGF530/505 weakened the infectivity of and conferred subsequent protection against the OURT88/1 virulent strain (1, 95). However, MGF360/505 and 9GL deletion in the ASFV Georgia 2007 isolate also reduced the virulence of the isolate but without affording protection against the parental virus (84). A similar result was observed using the Georgia isolate with the deletion of the thymidine kinase gene involved in the virulence of ASFV (104). It has also been noted that cross-protection provided by the non-virulent OURT88/3 isolate and virulent OURT88/1 isolate used in combination induced protection against two isolates, Benin 97/1 and genotype X Uganda 1965 (53). Interestingly, the mutant virus BA71ΔCD2v conferred protection to both parental BA71 and heterologous E75 virulent strains, which are two genotype I strains (77). Furthermore, pigs also survived a lethal challenge with the virulent Georgia 2007/1 genotype II strain (77). In the study of Sánchez-Córdon *et al.* (103), the immunisation technique was observed to be crucial for protection against ASFV: vaccination through the intranasal route was markedly more effective than the intramuscular route (103).

Subunit vaccines. Subunit vaccines use biomacromolecules for immunisation, such as DNA or protein antigens. DNA vaccines have one main disadvantage, which is their reduced immunogenicity in large animals. This fact was confirmed by failed immunisation with a DNA vaccine containing ASFV genes (8). The study of Argilaguet *et al*. (8) attempted the construction of a new DNA clone encoding ASFV genes fused with a fragment of an antibody specific to a swine leukocyte antigen II and yielded the observation that targeting antigens to the antigen-presenting cells induced an immune response in pigs. Unfortunately, protection against lethal challenge was not achieved (8). There was also protection by a DNA vaccine containing ASFV genes encoding p54, p30 and the HA extracellular domain fused to ubiquitin against challenge with the virulent E75 strain (57). Protein antigens are, however, more effective than DNA vaccines; even if they do not confer protection in all cases. For example, immunisation with baculovirus-expressed p30, p54, p72 and p22 ASFV antigens showed only a temporal delay in the onset of disease and reduced viremia (82). It has been observed that neutralising antibodies were raised to p54 and p72 antigens inhibiting virus attachment to the surface of the host cells (44). Neutralising antibodies specific to the p30 antigen, which is the most immunogenic among ASFV antigens, were found to inhibit virus internalisation (44, 92). Recently, new p30-specific monoclonal antibodies were prepared, and their binding epitopes were mapped (92). It was found that immunisation with either p30 or p54 recombinant antigen was not successful because pigs were not protected and eventually died. However, when the antigens were used together as a cocktail, immunisation was successful and pigs raised neutralising antibodies, which delayed the disease and even stopped the infection (43). The study of Ruiz-Gonzalvo *et al.* (101) conducted in 1996 showed that immunisation with recombinant CD2v antigen inhibited the haemagglutination, restricted the infection temporally and in some cases also conferred protection against lethal disease. A more recent study from 2016 reports a similar result, which was that serotype-specific CD2v or C-type lectin induced haemadsorption-inhibition serotype-specific protective immunity. This shows that these antigens could be used for future vaccine development (16).

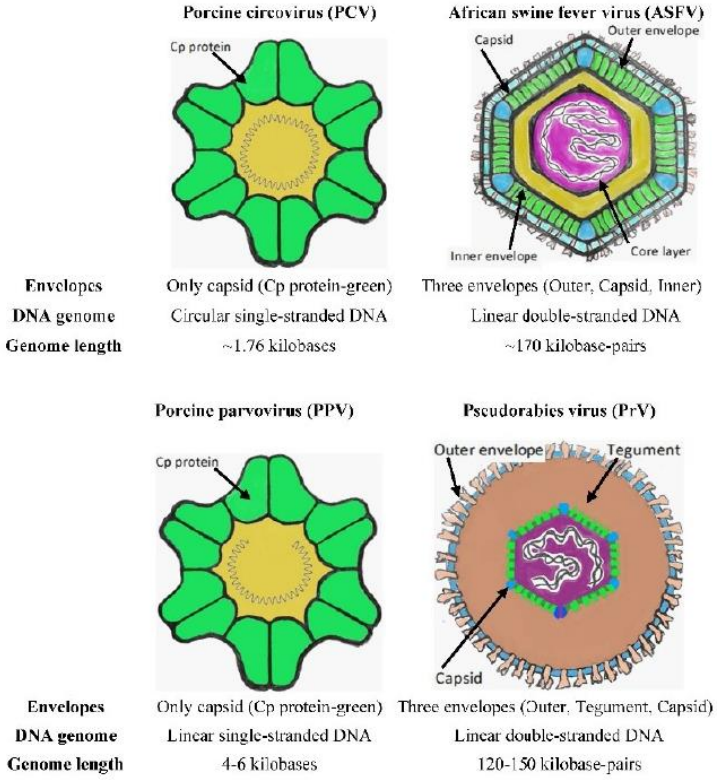

Fig. 1. Structural characteristics of viruses of interest

Porcine parvovirus (PPV)

PPV (58, 65) is a small ssDNA icosahedral nonenveloped virus (Fig. 1) with 5 kbp-long genomic DNA, which belongs to the *Parvoviridae* family, *Parvovirinae* subfamily and *Protoparvovirus* genus. PPV was first isolated in 1965 as a cell-culture contaminant (71) and this first isolate is designated PPV1. From 1965 onwards, different genotypes were identified, and recorded as PPV2 to PPV7, which were further classified based on their different characterisation as a separate genus within the family *Parvoviridae*.

Genetic classification. PPV1 genotype is the first identified genotype that was classified as the *Parvovirus* genus (65). PPV2 and PPV3 were both sorted into the *Tetraparvovirus* genus (27). PPV2 was identified for the first time during a study of the hepatitis E virus in swine sera collected in Myanmar in 2001 (48). The PPV3 genotype is closely related to human parvovirus 4 (PARV4) and porcine hokovirus that was identified for the first time in Hong Kong in 2008 (59). PPV4, PPV5 and PPV6 were classified in the *Copiparvovirus* genus (83, 110). Even though PPV4 belongs to the *Copiparvovirus* genus, it is closely related to the *Bocavirus* genus, containing an additional ORF3 (23) as *Bocavirus* does. The PPV5 and PPV6 genotypes were first identified in 2013 and 2014 in the USA and China, respectively (83, 105, 118). The first occurrence of PPV6 in Europe was observed in Poland in 2017 (28). The last identified genotype was PPV7, which was found in the USA, China and Korea in 2016 and 2017 (88, 119).

Clinical syndromes. The pathogenicity of PPV1 is the best known among the genotypes. PPV1 causes a reproductive failure disease in pregnant sows with clinical signs called SMEDI, an acronym of stillbirth, mummification, embryonic death and infertility (54). The route of infection in gravidity can influence the pathogenesis of the virus. The study by Joo *et al.* (50) shows that the intramuscular route facilitated the transfer of the virus from the dam through placenta and caused infection of foetuses earlier than oral routes of infection. However, the natural PPV entry path is oral, and such infections occur only when dams are exposed in the first part of the middle trimester of gestation (50).

PPV and host immune system. Induction of a cellular immune response to infection with PPV was observed (58). More specifically, CD4+ CD8+ T-cells were found to proliferate, while the activity of cytotoxic T-lymphocytes (CTL) was weak during the infection, indicating the role of humoral activity (58). The invasion by PPV also causes cell death by apoptosis, probably as a result of reactive oxygen species formation, which

activates the Bax apoptosis regulator and translocates it to near the mitochondrial membrane, triggering the subsequent release of cytochrome *c* and a caspase cascade (128). A recent study discovered that the NS1 PPV non-structural protein is responsible for the induction of apoptosis and thus involved in placental tissue damage and reproductive failure (125).

Development of vaccines. Vaccines designed against PPV infection are, in most cases, inactivated virus preparations based on PPV genotype 1 strains. It has been observed that inactivated vaccines can only prevent the disease but not the infection and virus shedding of PPV (35). In 2016, the study by Foerster *et al.* (35) showed that this applies both to homologous heterologous challenges with virulent PPV. Several approaches in vaccine development have been assessed. Vaccines based on genotype 1, including PPV-NADL2, PPV-IDT (MSV) and PPV-143a, and a vaccine based on the Stendal strain (51), are used for combating the disease caused by PPV1 (51, 123). It has been found that these vaccines were able to protect pigs against the disease but not against PPV-27a genotype 2 strain infection (51). PPV-27a was also used to prepare an inactivated vaccine, which likewise was only successful in providing protection from the disease and not from the infection and DNA replication (35). A vaccine against other genotype strains was not designed mainly due to inadequate information on the pathogenicity of these strains.

Pseudorabies Virus (PrV)

PrV is a large enveloped virus with a size of approximately 180 nm containing dsDNA (25). This virus was first described by Aujeszky in Hungary in 1902 as the agent of a disease, and although that disease was not related to rabies, its viral agent was named pseudorabies virus (the disease being termed Aujeszky's disease). The virus symptoms had already been observed previously, however, in the USA in the 1800s (25).

Virus structure. The viral particle appears in diagram form in Fig. 1. It is composed of morphologically different layers including a capsid protecting the dsDNA in the centre of the particle and thus forming a nucleocapsid and a protein matrix known as a tegument coated by the outer envelope, which contains a lipid membrane with distinct glycoproteins (93). A description of all structural proteins and their genes is given in detail in the article by Pomeranz *et al.* (93).

Genetic classification. Originally called suid herpesvirus 1 or Aujeszky's disease virus, PrV is classified into the *Herpesviridae* family and *Alphaherpesvirinae* subfamily containing a single serotype (36). A phylogenetic study based on sequences from the *UL44* gene encoding glycoprotein C (gC) divides PrV into five genotypes (A–E), which are neither country- nor continent-specific, in large part as a consequence of swine imports (36).

Clinical syndromes. Aujeszky's disease is typified by neurological and respiratory disorders resulting in weight loss, decreased growth and high mortality of piglets (93). Recently, it was found that the coinfection with PrV and PCV2 causes severe neurological and respiratory symptoms in pigs while damaging brain and lung tissue in piglets, resulting in higher mortality (126).

Development of vaccines. Two different vaccine types were developed for combating Aujeszky's disease. Inactivated and live attenuated vaccines were explored and live vaccines transpired to show higher efficiency and be more genetically stable than inactivated vaccines (38). Furthermore, live attenuated vaccines were observed to exhibit no or minimal residual virulence, suggesting their safety (38). The development of live attenuated vaccines against PrV is reviewed in the article by Freuling *et al.* (38).

The main DNA viruses significantly affecting swine are divided into four groups: PCVs, ASFV, PPVs, and PrV. Both porcine circoviruses and parvoviruses are small viruses having one capsid protein (Cp) and short genomic ssDNA. Vaccines against both viruses have been developed. However, a new vaccine should be designed, as a response to new genetically different genotypes having been identified which either have demonstrably different or yet unknown pathogenicity. In contrast, the African swine fever virus and pseudorabies virus are large viruses composed of a trilayer envelope and long linear genomic dsDNA. In the case of the African swine fever virus, there are many approaches to vaccine development. However, the effectiveness of every preparation was not sufficient for commercial purposes. In other words, there is no commercial vaccine for combating the viral infection and its disease. Further research is needed in this area to rectify this deficit. In the case of the pseudorabies virus, the majority of developed vaccines are live attenuated vaccines, due to their efficiency.

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Supplement 2

Research article

Examination of immunogenic properties of recombinant antigens based on p22 protein from African swine fever virus

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Examination of immunogenic properties of recombinant antigens based on p22 protein from African swine fever virus

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Abstract

Introduction: The single member of the Asfarviridae family is African swine fever virus (ASFV). This double-stranded DNA virus infects wild and farmed swine and loses the pig industry large sums of money. An inner envelope, capsid, and outer envelope are parts of the ASFV particle containing structural proteins playing different roles in the process of infection or host immune defence evasion. When expressed by the baculovirus system, the p22 protein from the inner envelope was found to induce partial protection against a virulent virus strain. This study aimed to express a part of this protein in a different system and evaluate its immunogenicity. **Material and Methods:** We designed two proteins, the extracellular (C terminal) part of the p22 protein (p22Ct) and its fusion with the heat-labile enterotoxin B subunit from *Escherichia coli* (LTB-p22Ct), which is supposed to be a potent enhancer of the immune response. Both proteins were produced in the *E. coli* expression system and subsequently used for mice immunisation to analyse their safety and immunogenicity. **Results:** The protein fused with LTB did not show the expected adjuvant properties and did not prove safe, because abscess formation was observed after immunisation. In contrast, immunisation with the p22Ct protein alone induced a higher antibody titre but caused no adverse symptoms. **Conclusion:** These results show the high potential of the p22Ct region as an immunogenic protein for ASFV serological detection purposes.

Keywords: African swine fever virus, heat-labile enterotoxin B subunit, immune response, p22 protein.

Introduction

African swine fever virus (ASFV) is a large double-stranded DNA virus belonging to the *Asfarviridae* family that causes enormous economic loss in the pig industry by killing farmed animals with a near-100% fatality rate (19). The vectors of this virus are soft ticks from the genus *Ornithodoros* (10) and wild *Suidae* in Africa. However, the primary route of transmission is contact between domestic pigs and wild boars, especially in regions with a mild climate where *Ornithodoros* ticks are not present. Another route of transmission is porcine products (such as meat and meat products) and slaughter and processing waste. The structure of ASFV particles includes three envelopes protecting the core containing linear double-stranded DNA. These three envelopes are the inner envelope, capsid, and outer envelope. Each envelope consists of different structural proteins playing different roles in the process of viral infection or host immune defence evasion, which is explained in more detail in the previous work (8).

The disease caused by this virus, African swine fever, is associated with many symptoms, including high fever, lesions, and haemorrhagic skin discolouration in the host organism, or rarely is not associated with any signs (29). This peracute infection usually occurs in animals that die even before the immune system can react to the infection (25). For this reason, several vaccines have been developed, but none has been effective enough to protect pigs against a virulent strain of the virus.

Our present study focuses on the ASFV p22 protein, which may become a subunit vaccine candidate. Typically, subunit vaccines contain proteins originating from the virus infecting the host organism. Many structural antigens from ASFV have been tested as subunit vaccines, including p22, p30, p54, p72, CD2v and other proteins (20, 21, 24). Of these proteins, p22, p30 and CD2v were capable of inducing at least a minimal immune response (4, 12, 22). Although none of the antigens successfully protected the host against a virulent virus challenge, they could still be considered potential antigens for future vaccine development.

The p22 protein was initially believed to be part of the outer membrane (5). However, recently it has been found in the inner membrane (1). An initial experiment using a non-ionic detergent showed that the p22 protein is part of the outer envelope; however, as was learned later, the detergent could disrupt not only the outer but also the capsid envelope (2, 5). The structure of the p22 antigen consists of three subparts: a 20-amino-acid-long (1…20) N-terminal domain, a 21-amino-acid (21…41) transmembrane α-helix, and an outer 148-amino-acid (42…189) C-terminal part. Recently, it has been found that the p22 protein interacts with host proteins related to several biological processes, including virus binding, signal transduction, and cell adhesion (30). Some of these proteins participate in ribosome, spliceosome, and actin filament organisation and movement, possibly also in DNA replication, or affect phagocytosis and endocytosis (30). Even though the p22 protein can interact with many host proteins, it was shown recently not to be essential for ASFV replication or virulence (27).

The gene encoding the p22 protein was initially discovered on the left end of the BA71 genome and was named then based on the K′177 open reading frame (ORF) (5, 11), but it was later renamed KP177R and termed the early membrane protein. The KP177R encoding gene was also found on the right end of the Malawi LIL120/1 genome (28). Vydelingum *et al.* (28) suspected that it could be a consequence of evolution by duplication, deletions or sequence transposition from one end of the genome to the other. The KP177R gene is also known among the genes coding for structural antigens as one of the most conserved ASFV genes (17). It was also found that the I10L ORF encodes a protein somewhat similar to p22, of which the nucleotide sequence has 40% homology with the p22 protein from BA71V (7). The I10L ORFs of the Benin97/1 and ORT88/3 isolates have 100% homology, which shows this gene's conservation throughout the isolates identified thus far (6). However, it is still not known whether the protein expressed by this gene is an analogue in the viral structure to the p22 protein (6).

The heat-labile *E. coli* enterotoxin (LT) is a heterohexameric protein that promotes cell adherence of *E. coli* to the intestinal epithelial cells mainly because of the ADP-ribosylation activity of the A subunit (LTA) (14). The B subunit (LTB) has immunomodulatory properties as it binds the GM1 ganglioside on the surface of the immune cells and preferentially induces the apoptosis of CD8+ T-cells *via* the rapid loss of mitochondrial membrane potential and cell viability (26). Multiple uses have been made of LTB as a potent and robust adjuvant, either individually or fused to other proteins (9, 13).

As the p22 protein takes part in virus binding (30), it deserves further attention for its possible recognition by the immune system. We therefore decided to prepare a recombinant protein based on the outer C-terminal part of p22 and examine its immunogenicity in mice.

Material and Methods

Strains, vectors and growth conditions. The *E. coli* One Shot TOP 10 strain was used as the cloning host, and the *E. coli* BL21 Star strain was used for protein overproduction (both strains were from Thermo Fisher Scientific, Brno, Czech Republic). The One Shot TOP 10 and BL21 Star strains were grown under vigorous orbital shaking (4.5 × *g*) at 28°C and 37°C, respectively, in Luria-Bertani (LB) medium. For the cloning and expression of the genes, pMA-RQ (Thermo Fisher Scientific) and pET28b(+) (Merck, Darmstadt, Germany) were used, respectively.

Construct design and cloning. Constructs were designed, for the first protein containing the part of the KP177R gene (GenBank accession number MK333183) coding for the C-terminal globular segment (amino acids 42–189) of the p22 protein (p22Ct) and for the second protein containing the same gene part fused at the 3′ end with the gene coding for the LTB (GenBank accession number M17873) (LTB p22Ct). In addition, a 10× His-tag coding sequence was attached to the 3′ end of each construct for encoded protein purification and detection purposes. The designed gene sequences, named *p22Ct* and *LTB-p22Ct*, were codon-optimised for production in *E. coli*, synthetically prepared using a commercial service (Thermo Fisher Scientific) and cloned into the pET28b(+) vector at the *Nco*I restriction enzyme site. For colony screening, His-tag forward (5′-CCATCA CCATCACCATCACC-3′) and T7 terminator reverse (5′-AGCCAACTCAGCTTCCTTTC-3′) primers were used. After cloning, the constructs within pET28b(+) were verified using a commercial sequencing service (SEQme, Dobříš, Czech Republic). The confirmed clones pET28b(+)-*p22Ct* and pET28b(+)-*LTB-p22Ct* were used for the recombinant protein production.

Expression of recombinant proteins. The clones pET28b(+)-*p22Ct* and pET28b(+)-*LTB-p22Ct* were transformed into chemically competent *E. coli* BL21 Star cells by heat shock. Positive clones were inoculated into LB medium and grown overnight at 37°C. A small-scale expression of LTB-p22Ct and p22Ct proteins in 5 mL of the *E. coli* BL21 Star cell cultures was performed using the pET28b(+) vector. The expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG), and the cultures were grown for 4 h at 37°C.

A large-scale expression was performed to produce higher protein quantities for purification and further experiments. An aliquot of 20 mL of the culture was inoculated into 1 L of the LB medium and allowed to grow at 37°C with shaking until the optical density (OD_{600}) reached 0.7–0.8. Recombinant protein production was induced by adding IPTG at a final concentration of 0.2 mM. The culture was grown at 28°C for 16 h and then collected by centrifugation (8,000 × *g*, 10 min, 20°C). Bacterial pellets were either used immediately for protein purification or stored at −20°C.

Purification of p22Ct and LTB-p22Ct recombinant proteins. Bacterial pellets were resuspended in an extraction buffer (25 mM Tris-HCl pH 9.0, 300 mM NaCl, 2% glycerol, 0.1% Triton X-100, 15 mM imidazole). Lysozyme (1 mg/mL) and phenylmethylsulfonyl fluoride (1 mM final concentration) were added to the cell suspension for better lysis and inhibition of proteases. The resuspended cells were disrupted by ultrasonic homogenisation (3 × 10 min, 6 s pulse and 9 s pause periods) or French press. Cell debris was removed by centrifugation at 14,000 × *g* for 15 min, and then the supernatant was vacuum filtered through a 0.4 µm filter. The final supernatant, representing the soluble fraction, was used further for affinity purification.

Nickel-nitrilotriacetic acid (Ni-NTA) Agarose (Qiagen, Hilden, Germany) was pre-equilibrated with 25 mM Tris-HCl pH 9.0, containing 300 mM NaCl, 2% glycerol, 0.1% Triton X-100, and 15 mM imidazole. The soluble fraction was loaded twice onto the matrix to increase the binding of the desired proteins. Unbound proteins were washed out with the same buffer, and further elution was performed with buffers containing 30, 40, 50 mM, and finally 300 mM imidazole. The eluted protein samples were concentrated and buffer-exchanged for the buffer without imidazole using Amicon 10,000 Mw centrifugal filters (Thermo Fisher Scientific). Subsequently, the samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. The purified proteins were stored at −80°C. Protein concentrations were determined by Bradford reagent (Bio-Rad, Hercules, CA, USA) and band density on SDS-PAGE gels using ImageLab software (Bio-Rad) with bovine serum albumin (BSA) as the standard.

Gel electrophoresis and Western blot analysis. Whole-cell extract samples were prepared by centrifuging 1 mL of the cell cultures at 16,000 × *g*; the pellets were then resuspended in 50 µL of extraction buffer (25 mM Tris-HCl pH 9.0, 300 mM NaCl, 2% glycerol, 0.1% Triton X-100) and mixed with a sample buffer (62.5 mM Tris-HCl pH 8.0, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue) at a volume ratio of 1:2. Alternatively, the purified protein samples were mixed with the sample buffer at a ratio of 1:1. All samples were heated for 5 min at 100°C, spun down, and separated under reducing conditions using vertical 4% stacking and 15% separating SDS-PAGE gels. The electrophoresis was run in 0.25 mM Tris-glycine buffer at pH 8.8 and 80 V for 15 min and then at 150 V until the sample dye reached the end of the gel. The gels were stained with 0.1% Coomassie Brilliant Blue G250 and unstained using 40% methanol and 10% acetic acid. SDS-PAGE was also used to assess protein mobility under non-reducing conditions. In that case, the sample buffer did not contain 2-mercaptoethanol, and the sample was not heated.

For Western blot analysis, the electrophoresed proteins were transferred to a polyvinylidene difluoride membrane using a wet transfer apparatus (Bio-Rad) for 16 h at a constant voltage of 20 V. The membrane was rinsed twice with 25 mM Tris-HCl buffer at pH 8.0 containing 0.9% NaCl (Tris-buffered saline – TBS), blocked with 5% non-fat milk in TBS containing 0.05% Tween 20 for 90 min, and then incubated with a 1:500 diluted His-tag primary antibody (MA1-21315; Thermo Fisher Scientific) for 90 min. Immunodetection was performed for 90 min with a 1:4000 diluted murine immunoglobulin G kappa -anti-mouse secondary antibody conjugated to horseradish peroxidase (sc-516102; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an enhanced chemiluminescent substrate (ECL kit; Bio-Rad). As a molecular mass marker, the Novex Sharp Pre-Stained Protein Standard (Thermo Fisher Scientific) was used.

Immunoblot assay with ASFV-positive pig sera. The membrane prepared as above was cut into strips, each containing one protein sample. Individual strips were blocked with 2% BSA in 10 mM phosphate-buffered saline (PBS) at pH 7.3 and incubated for 1 h while being shaken with 1:100 diluted ASFV-positive pig serum (serum 1 was sourced from the European Union Reference Laboratory for African Swine Fever, Valdeolmos, Spain and sera 2–4 were positive sera from wild boars provided by the National Veterinary Research Institute, Puławy, Poland). The strips were rinsed with PBS containing 0.1% Tween 20 (T-PBS) and 2% BSA and incubated for 1 h with 1:30,000 diluted rabbit Anti-Pig IgG (whole molecule)–Peroxidase secondary antibody (A5670; Merck). After washing three times for 5 min with T-PBS containing 2% BSA, the signals of bound antibodies were developed using 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for Membranes (T0565; Merck) and evaluated using ImageLab software.

Mice immunisation. General immunogenicity of the prepared recombinant proteins was assessed by mice immunisation. Six-week-old female BALB/c mice (Envigo, Indianapolis, IN, USA), three mice in each group, were vaccinated subcutaneously dorsally in the neck region every two weeks, receiving three doses in total. A single vaccination dose (prepared immediately before application) was composed of a mixture of the p22Ct or LTB-p22Ct recombinant protein (10 µg) and 10% aluminium hydrogel adjuvant (InvivoGen, Toulouse, France) in 0.15 mL PBS. The control mice

group was vaccinated with the adjuvant alone. Mice were bled on day 42, two weeks after the last vaccination, and the presence of antigen-specific antibodies in the sera was analysed by ELISA.

Serological test and titre measurement. Nunc MaxiSorp ELISA plates (Thermo Fisher Scientific) were coated overnight with 4 µg/well of the p22Ct or LTB-p22Ct recombinant protein dissolved in 0.05 M carbonate-bicarbonate buffer at pH 9.6. The wells were then rinsed with PBS and blocked with 2% BSA in PBS at 37°C for 60 min. Aliquots of 100 µL of mice sera diluted 1:50 in T-PBS with 2% BSA were added to antigen-coated wells and incubated for 60 min at 37°C. Subsequently, the plates were washed three times with T-PBS, and 100 µL of 1:30,000 diluted rabbit Anti-Mouse IgG (whole molecule)–Peroxidase (A9044; Merck) in T-PBS containing 2% BSA was added to each well. The plates were then incubated for 60 min at 37°C, washed with T-PBS containing 2% BSA, and 100 µL per well of the TMB-Complete substrate (TestLine Clinical Diagnostics, Brno, Czech Republic) was added. Colour development was stopped with 1 M H_2SO_4, and the optical density was read at 450 nm using an Infinite M200 PRO multimode plate reader (Tecan, Männedorf, Switzerland). The serum titre was determined as the dilution ratio of the test serum with an absorbance value equal to the average absorbance of the negative mouse serum from the control group.

Statistical analysis. Antibody titre differences between the two recombinant protein groups (p22Ct and LTB-p22Ct) analysed by ELISA conducted on blood samples were statistically analysed using one-way analysis of variance. The titre results of p22Ct and LTB-p22Ct were compared with those of the corresponding control group, and a P-value of 0.05 or lower was considered statistically significant. Statistical analysis was conducted using Microsoft Excel.

Results

The p22 protein of ASFV contains a short N-terminal domain, a single membrane-spanning helix and an outer large C-terminal domain. Using the Phyre2 protein fold recognition server (15), we predicted by comparing the amino acid sequence of the C-terminal domain of p22 with other known proteins from the database that the separate domain should fold into a stable tertiary structure (Fig. 1).

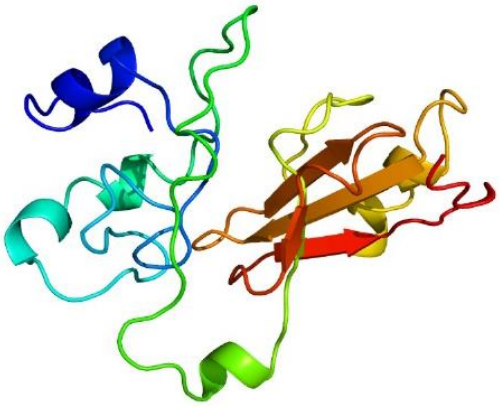

Fig. 1. Predicted protein structure of the p22 C terminal protein (amino acids 42–189) by the Phyre2 protein fold recognition server (15). The colours progress as in a rainbow from the N- to the C-terminus

Two DNA constructs were prepared, *p22Ct* coding for the C-terminal globular fragment of the p22 protein shown in Fig. 1 and *LTB-p22Ct* coding for the N-terminal fusion of that fragment with the heat-labile B-subunit of enterotoxin. We also prepared variants with a 4× Myc tag, but they were not used for further study. The *p22Ct* and *LTB-p22Ct* gene constructs were then cloned into the pET28b(+) expression vector (Fig. 2).

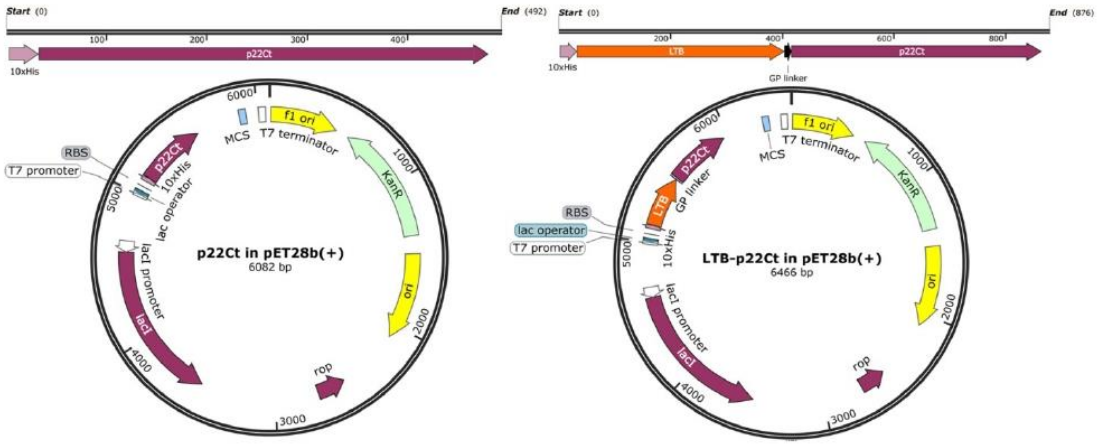

Fig. 2. DNA construct design of p22 C terminal (*p22Ct*) (left) and heat-labile enterotoxin B subunit fused with p22 C terminal (*LTB-p22Ct*) (right) sequences, cloned into the pET28b(+) expression vector. The *p22Ct* gene is coloured ruby red, the LTB coding sequence is orange, the pink colour represents the coding sequence for a 10× His-tag, yellow sections are the origins of replication, pale green the kanamycin resistance gene KanR and blue the multicloning site (MCS)

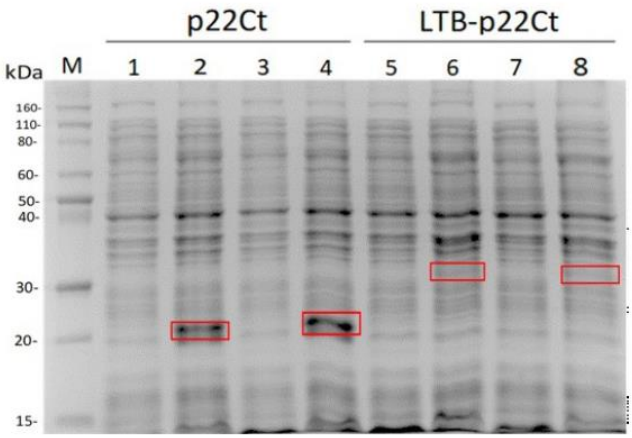

Fig. 3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis of crude extracts from *E. coli* BL21 Star cells expressing p22 C terminal (*p22Ct*) and heat-labile enterotoxin B subunit fused with p22 C terminal (*LTB-p22Ct*). Lanes 1, 3, 5, and 7 show the 20 µL samples from non-induced cells, while lanes 2, 4, 6, and 8 represent the cells after induction with 0.2 mM isopropyl β-D-1-thiogalactopyranoside. Bands corresponding to the p22Ct (lanes 2 and 4) and LTB-p22Ct (lanes 6 and 8) proteins are shown in red boxes

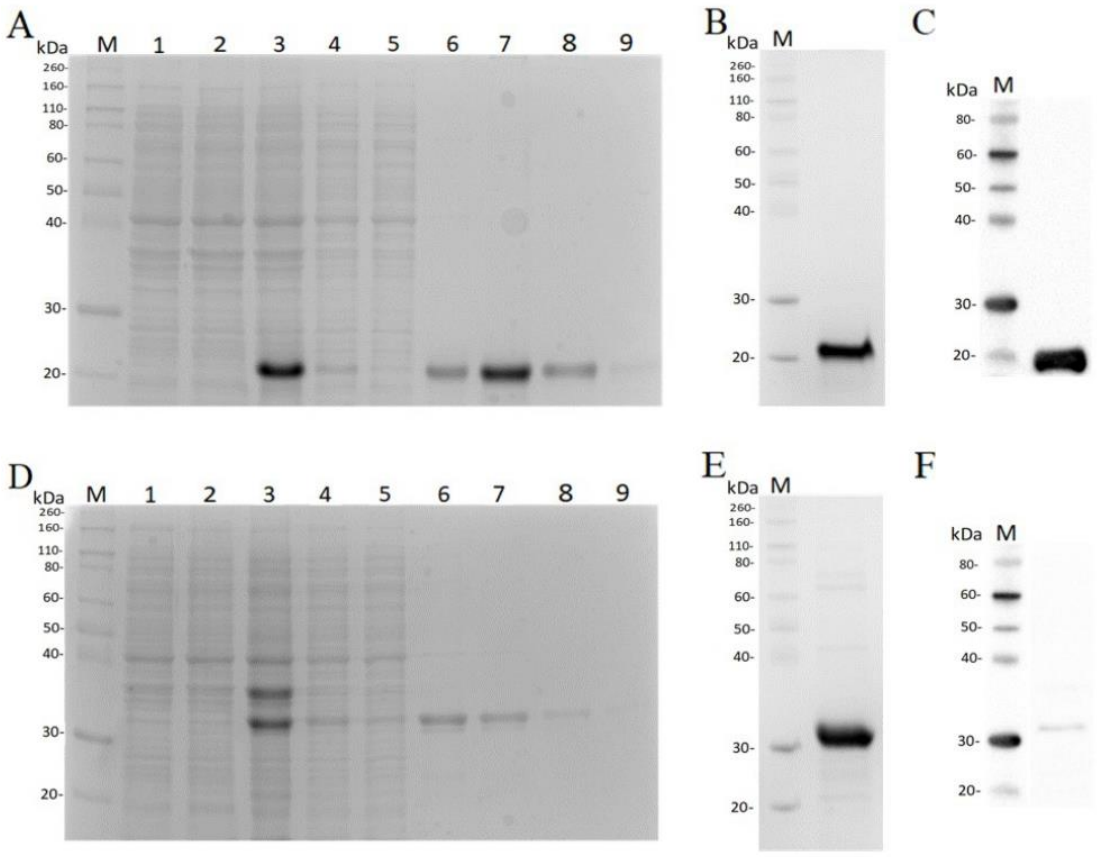

Fig. 4. Expression and purification of p22 C terminal (p22Ct) (A–C) and heat-labile enterotoxin B subunit fused with p22 C terminal (LTB-p22Ct) (D–F) recombinant proteins in *E. coli* BL21 analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blot. A – band 1: cells harbouring empty pET28b(+) as control (after induction); band 2: cells harbouring pET28b(+)-*p22Ct* before induction; band 3: soluble protein fraction; band 4: cells after induction (each sample 20 µL); band 5: washout fraction (50 mM imidazole) from nickel-nitrilotriacetic agarose (20 µL); bands 6–9: p22Ct eluted by 300 mM imidazole (samples of 5, 10, 4, and 0.5 µg, respectively); B – protein staining; C – Western blot with the anti-His-tag antibody of the purified p22Ct protein (11 µg); D – band 1: pET28b(+) cells after induction; band 2: pET28b(+)-*LTB-p22Ct* cells before induction; band 3: soluble protein fraction; band 4: cells after induction (each 20 µL); band 5: washout fraction; bands 6–9: eluted LTB-p22Ct protein (4, 3, 2 and 0 µg); E – protein staining; F – Western blot of purified LTB-p22Ct (16 µg); M – protein molecular mass marker

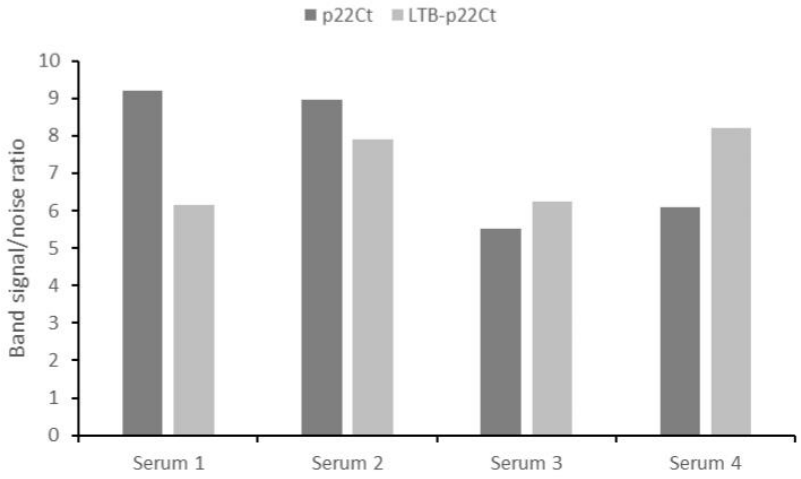

Fig. 5. Immunoblot assay of the purified p22 C terminal and heat-labile enterotoxin B subunit fused with p22 C terminal recombinant proteins with four different sera from African swine fever virus–infected pigs. The binding intensity was calculated as the intensity of the bands compared to the background over the whole lane of the immunoblot assay

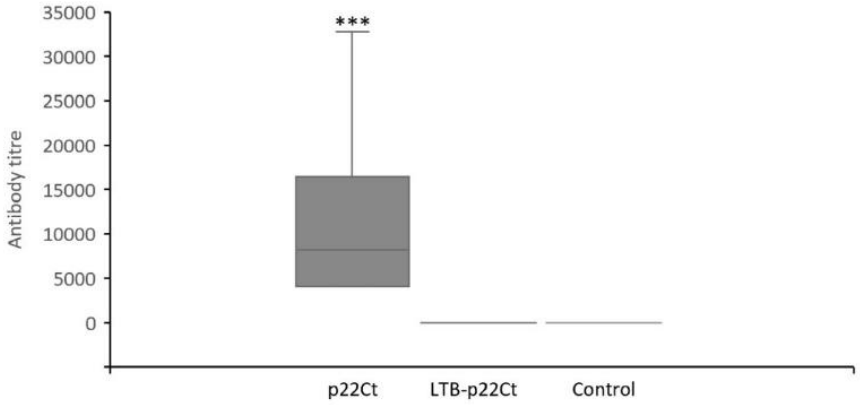

Fig. 6. ELISA test results of sera samples taken from mice groups immunised with p22 C terminal and heat-labile enterotoxin B subunit fused with p22 C terminal

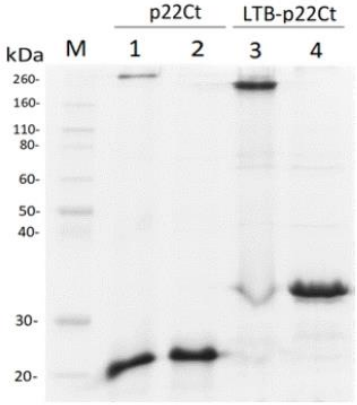

Fig. 7. Gel electrophoresis of p22 C terminal (13 μg) and heat-labile enterotoxin B subunit fused with-p22 C terminal (16 μg) samples under reducing (lanes 2 and 4) and non-reducing conditions without adding 2-mercaptoethanol or heating (lanes 1 and 3); M – protein molecular mass marker

First, a small-scale expression in 5 mL cultures was performed. Gel electrophoresis showed that the expression level of the LTB-p22Ct fusion protein was much lower than that of the p22Ct protein (Fig. 3), while still being detectable.

Secondly, a large-scale expression was performed under the same conditions in 1 L cultures (Fig. 3). The larger-scale expression yields of p22Ct and LTB-p22Ct in 1 L cultures, estimated with BSA as a standard, were 4.9 and 3.8 mg per L of culture.

In the subsequent immunoblot testing of purified p22Ct and LTB-p22Ct recombinant proteins for their ability to interact with serum antibodies (IgG) from ASFV infected pigs, both proteins showed positive reactions with the four sera used as primary antibodies (Fig. 5).

When the serological activity of the two groups' samples against the recombinant proteins was assayed, the antibody titre was high for the mice group immunised with p22Ct, while the samples from the

LTB-p22Ct group did not react at all in ELISA (Fig. 6). The mice allocated to the p22Ct protein group were fully immunised with three doses. However, in the case of the LTB-p22Ct group, abscesses occurred after the first dose. Due to this development, only one dose was administered.

Due to the difference in immune response induction in the mice, the oligomerisation of both recombinant proteins was examined by SDS-PAGE under non-reducing conditions (Fig. 7). As calculated by comparing the mobility of recombinant proteins with those of protein molecular mass standards, the 33 kDa LTB-p22Ct fusion most probably forms hexameric complexes of 198 kDa. In contrast, the p22Ct protein prevalently retains the monomeric form of 23 kDa.

Discussion

The global swine pandemic caused by the African swine fever virus has a negative impact on the economy of many countries throughout the world; therefore, the virus needs to be eliminated. Many researchers worldwide have tried to design a new effective vaccine for combating the disease caused by this virus; however, they have not been successful in protecting the pigs against virulent viral strains. A previous study showed that administration of ASFV proteins from the inner and capsid membranes could somewhat delay the onset of clinical symptoms from the time of viral challenge, but could not stop the further progress of the disease; the pigs administered the proteins eventually died. The four proteins investigated (p22, p30, p54 and p72) were expressed in a baculovirus system. The individual proteins were not purified to homogeneity but used as a mixture to immunise the pigs (20). The p22 protein chosen for our study was previously considered a potential antigen for new vaccine development (4, 22). A study using DNA prime and recombinant vaccinia virus boost (12) showed that p22 (the KP177R early membrane protein) is a potential antigen for inducing a protective immune response or can serve as an infection serological marker. However, the role of p22 in the infection process is still unknown. Recently the focus on developing an effective vaccine turned to live attenuated strains developed using a genetically modified virulent parental virus (3).

In contrast to previous studies, we selected only the large C-terminal globular part of the protein, p22Ct (amino acids 42 to 189). We also prepared its fusion with the heat-labile enterotoxin B-subunit, LTB-p22Ct, which was expected to stimulate the immune response in the host (9, 13).

After cloning the corresponding constructs into the *E. coli* BL21 pET28b(+) expression vector, the results of small-scale cultures indicated that the expression of the LTB-p22Ct protein was much lower than that of p22Ct (Fig. 3). The homologous overexpression of LTB in *E. coli* is often low and prone to forming inclusion bodies (18). However, our experiments in 1 L cultures produced comparable yields of both proteins of about 4 mg per L, corresponding roughly to 3% of the theoretical maximum yield of the protein of interest in *E. coli*. The formation of inclusion bodies was not observed.

Our results show that p22Ct, the C-terminal globular part of the p22 protein, but not the fusion protein LTB-p22Ct, can induce an immune response in mice. The exact cause of the observed difference is unclear. The most probable reason is that mice immunised with LTB-p22Ct were injected with only one dose rather than the three doses of p22Ct administered. A significant problem appeared in the form of abscess formation in the mice vaccinated with the fusion protein; therefore, these mice received only one dose of antigen. This is probably also the reason why the mice immunised with LTB-p22Ct could not produce specific antibodies against the protein. Another reason may be that LTB could become less active when produced by recombinant technology in *E. coli* (18). As described previously, LTB binds to the GM1 receptor in a pentameric association, which is essential for stimulating the immune response (16). However, the SDS-PAGE under non-reducing conditions showed that LTB-p22Ct is more likely to form a hexamer, which may be a reason for its lack of immunogenicity (Fig. 7).

Our results also show that p22Ct can produce a high antibody titre in mice, and is thereby indicated to be a highly potent and immunogenic region. It can be considered a candidate primarily in serological diagnostics for the development of specific antigen-based detection techniques such as fluorescent antibody tests, ELISA and immunoblotting (23). However, further experiments should be conducted to investigate its possible link to other immunostimulatory domains.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The animal experiments were performed in accordance with Czech laws and guidelines for the use of experimental animals. The experiments with mice were approved by the Branch Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic (Approval No. 6929/2019-MZE-17214).

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