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OPTIMIZED PRODUCTION OF CD2V EXTRACELLULAR DOMAIN OF AFRICAN SWINE FEVER VIRUS ON ADHERENT CULTURE BACULOVIRUS- SF9 INSECT CELL SYSTEM

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ABSTRACT

The baculovirus expression vector system (BEVS) is one of the strongest tools for producing recombinant proteins such as pharmaceuticals and vaccines. In this study, the multivalent CD2v extracellular domain (CD2v ED) of the African swine fever virus (ASFV) was expressed in Sf9 insect cells using BEVs. Bacmid carrying the gene encoding CD2v ED-Foldon-His was transfected into Sf9 insect cells using the lipid transfer complex Cellfectin II and Opti-MEM I Reduced Serum Medium. The transfection process was observed under microscopy and protein expression would be assessed by western blot. The results indicated the transparent differences in infected cells, including the swelling of cells, the increase of nuclei, granular and vesicular appearance, cessation of cell growth, detachment from the flask bottom, and cell lysis in comparison with normal cells that are round, small, and regular size. The western blot results illustrated the expression of CD2v ED-Foldon-His in trimeric and oligomeric forms with bands of 100 kDa and more than 250 kDa, respectively. Most importantly, the expression of CD2v ED-Foldon-His in adherent culture insect cells was optimized with two main factors, namely multiplicity of infection (MOI) and infection time. The results demonstrated the highest yield of CD2v ED-Foldon-His protein was 4.4 mg/L at a MOI of 5 pfu/cell and 5 days post-infection, which was confirmed by captured pictures throughout the infection time. This is the first study on the expression of the oligomeric CD2v extracellular domain on a baculovirus-insect cell system, aiming to produce subunit vaccines against ASFV. Besides, the study also presented a practical procedure for enhancing the expression of recombinant proteins in this system.

Keywords: Baculovirus expression vector system, insect cell, CD2v, Sf9, multiplicity of infection, time of harvest

INTRODUCTION

African swine fever (ASF) is the most important acute infectious disease in pigs with a high mortality rate of almost 100% within 7 days from the onset of the disease (Li *et al*., 2022; Sánchez-Cordón *et al*.,

2018). First appearing in Kenya in 1920, over the years, African swine fever has been recorded in more than 60 countries around the world. About 75% of the world's pig production is under constant pressure from ASF while prevention and treatment measures are ineffective (Teklue *et al.*, 2020). In Vietnam, (ASF) was discovered in 2019 and has caused significant impacts on the pig farming industry with the death or culling of nearly six million pigs, equivalent to more than 20% of the country's total pig herd (Le *et al*., 2019).

ASF is caused by the African swine fever virus (ASFV) of the Asfarviridae family. ASFV is the only DNA virus that replicates and is transmitted through arthropods (Dixon *et al.*, 2004). They are stable in the environment and have extremely complex genetic structure and composition. ASFV structure consists of five main layers, namely the outermost membrane, outer capsid, inner lipid membrane, inner capsid, and core-shell, from outside to inside (Nguyen *et al*., 2024). The ASFV genome is about 170-190 kb in size, encoding about 160 proteins, many of which participate in inhibiting the host's immune response, which triggers the obstacle in treatment and vaccine development (Dixon *et al.*, 2019; Salas & Andrés, 2012; Teklue *et al.*, 2020). In particular, during ASFV infection, many viral proteins participate in inhibiting the host's immune response, such as A238L-an inhibitor of NFκB activation and calcineurin, IAP, Bcl-2, CD2v...(Dixon *et al.*, 2019) Several candidate vaccines have been developed: inactivated vaccines, live attenuated vaccines, subunit vaccines, DNA vaccines, and viral vector vaccines. However, to date, no vaccine has met the criteria for safety and effectiveness due to incomplete protection from mortality and unwanted side effects (Nguyen *et al.*, 2024).

CD2v is an outer envelope transmembrane glycoprotein encoded by the EP402R gene of ASFV, participating in the pathogenesis of ASFV infection and host immune evasion (Borca *et al.*, 1998; Dixon *et al.*, 2013; Pérez *et al*., 2015). It consists of a signal peptide (SP), a transmembrane region (TM), an extracellular domain (ED), and the cytoplasmic domain (Jia *et al.*, 2017). CD2v has been applied as DNA vaccines or subunit vaccines (Argilaguet *et al.*, 2012, 2013; Ruiz *et al.*, 1996). For example, the Bacman viral vector containing CD2vED-p54-p30 induced T-cell responses but no specific antibodies, providing partial protection in 4 out of 6 pigs (Argilaguet *et al*., 2013). Inoculation of mice with recombinant pseudorabies virus (PRV) expressing CD2v protein (PRV-ΔgE/ΔgI/ΔTK-(CD2v)) induced specific cellular and humoral immune responses and protected 100% of mice against PRV-Fa challenge (Fujian, China) while no mortality was observed after 14 days (Feng *et al*., 2020). More recently, plant-derived oligomeric CD2v extradomain has been proven to induce both humoral and cellular immune responses in mice (Nguyen *et al.*, 2024). These results indicated that CD2v can be used as a safe and potential vaccine candidate for ASFV prevention.

The baculovirus expression vector system (BEV) is one of the best tools available for expressing recombinant proteins with a lot of outstanding advantages, such as high expression yield equal to prokaryotic expression systems, easy scale-up of expression, recombinant proteins are post-translationally modified almost like natural proteins such as glycosylation, phosphorylation, etc. and no infection to

vertebrates. Therefore, BEVs are relatively safe for laboratory manipulation and industrial production of proteins for pharmaceuticals and vaccines (Chambers *et al.*, 2018).

In this study, the expression of the multivalent CD2v extracellular domain on the baculovirus expression system was for the first time investigated towards the development of a protein subunit vaccine against African swine fever. Recombinant bacmid carrying the gene encoding the CD2v ED-Foldon-His antigen constructed in our previous study was used to transfect Sf9 cells to create recombinant baculovirus. The expression of oligomeric CD2v ED was assessed by western blot. Further, the recombinant CD2v expression was optimized under nine cultural conditions with two factors, namely MOI and infection time.

MATERIALS AND METHODS

Monolayer culture of Sf9 insect cells

Sf9 insect cells (Cytion CLS, Germany) preserved in liquid nitrogen were activated on Sf-900 II SFM culture medium (Gibco, USA) supplemented with 5% antibiotic-antimycotic (Gibco, USA) in T-25 flasks (SPL Life Science, Korea) with a density of $4x10^4$ cells/cm^2, at 27°C in an incubator. Cells were harvested when the plate coverage was more than 90%, the survival rate was more than 95%. Cells were counted by Trypan Blue staining method (Aung *et al.*, 2019).

Recombinant bacmid extraction by alkaline lysis method

In the previous study, we successfully designed a recombinant bacmid carrying the gene encoding the extracellular domain of CD2v glycoprotein (CD2v ED) of African swine fever virus circulated in Vietnam VNUA/HY-ASF1 with a Foldon motif for forming a trimer structure and a polyhistidine (His) tail for purification and expression testing with anti-His antibodies (Nguyen *et al*., 2024). In this study, the *E. coli* DH10 Bac strain carrying the bacmid containing the CD2v ED-Foldon-His expression sequence was grown overnight in a liquid medium containing 50 µg/mL kanamycin, 10 µg/mL tetracycline and 10 µg/mL gentamicin at 37°C. The bacmid was isolated by the alkaline lysis method as described in detail by Falak *et al.* (2014).

Transfection of bacmid carrying the gene encoding the CD2v ED-Foldon-His antigen into Sf9 insect cells to create recombinant baculovirus

Recombinant bacmid carrying the gene encoding the CD2v ED-Foldon-His antigen was used to transfect Sf9 cells to create recombinant baculovirus according to the following procedure: 2 mL of Sf-900 II SFM medium (Gibco, USA) containing 10^6 Sf9 cells was grown on a 6-well culture plate (SPL Life Science, Korea) and incubated for 2 hours for the cells to attach onto the plate. At the same time, the transfection mixture of 1 µg of bacmid carrying the gene expressing CD2v ED-Foldon-His with 10 µL of Cellfectin II (Invitrogen, USA) and 250 µL of Opti-MEM I Reduced Serum Medium (Gibco, USA) was prepared and incubated for 15 minutes. The mixture was then added to the cell wells and incubated overnight at 27°C. The virus infection process was observed on a microscope (Olympus CKX41, Japan) and Cellsens Standard capture software. The culture mixture was harvested after 5 days of infection. The virus was

harvested by centrifuging the culture at 1500 rpm for 10 min at 4°C, collecting the clear supernatant. The virus-containing supernatant was supplemented with 2% FBS and stored at 4°C in the dark. The virus content was determined by the plaque assay method as described by Mendoza *et al.* (2020).

The cell pellet was resuspended in 100 µL of 1X Laemmli buffer (50 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% *β*-mercaptoethanol, 0.25% bromophenol blue). The mixture was denatured at 98°C for 10 minutes and the supernatant was recovered by centrifugation at 13,000 rpm at 4°C for 30 minutes. The expression of CD2v ED-Foldon-His was determined by western blotting according to the procedure described by Nguyen *et al.* (2024). The colorimetric signal was captured on an Amersham^{TM} Imager 680 (Cytiva, Sweden).

Optimization of CD2v ED-Foldon-His expression in Sf9 cells

Two factors affecting the protein expression level, including MOI and infection time, were evaluated. 10^8 pfu/ml of virus was used to optimize CD2v ED-Foldon-His protein expression. The experiment was performed on the 12-well plates (SPL Life Science, Korea) according to the following procedure: $5x10^5$ cells in 2 mL of Sf-900 II SFM medium supplemented with 5% FBS containing 'antibiotic-antimycotic' were added to each well. After 3 hours of incubation at 27°C for cell attachment, the virus was added to each well in 5, 25 and 50 µL volumes to achieve MOI = 5 pfu/cell and 10 pfu/cell, respectively. After infection, cells were placed in a 27°C incubator and harvested after 3, 5, and 7 days of culture by centrifugation at 1500 rpm for 10 minutes. The expression of CD2v ED-Foldon-His was determined as described above. The recombinant protein content was calculated based on the standard curve of H5pII-His protein (Phan *et al.*, 2017) at concentrations of 150, 300, and 600 ng in ImageQuant TL 8.0 software (Cytiva, Sweden). The experiment was repeated three times. The effect of two factors on the recombinant protein expression was evaluated by two-factor ANOVA with 3 replications at a significance level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Transfection of bacmid carrying the gene encoding the antigen CD2v ED-Foldon into Sf9 insect cells to create recombinant baculovirus

In a previous report, a bacmid carrying the gene encoding CD2v ED-Foldon-His has been constructed successfully and transformed into *E. coli* DH10 Bac (Nguyen *et al.*, 2024). To generate recombinant baculovirus, bacmid carrying the gene encoding CD2v ED-Foldon-His was transfected into Sf9 insect cells using the lipid transfer complex Cellfectin II and Opti-MEM I Reduced Serum Medium (Gibco, USA). Cellfectin II is a highly efficient transducting lipid substrate, in which the cationic head groups of cellfectin interact with the anionic phosphate backbone of DNA to form a complex that can bind to the cell membrane and probably be taken up by endocytosis (Zitzmann *et al*., 2017). The transfection process was observed daily until the cells were lysed and the virus particles were released (5 days post-transfection). The infection results showed that the cells retained their round, small, regular morphology in the control sample (Figure 1A), whereas in the transfection sample, the

cells swelled, there was a cessation of cell growth, and the cells released from the flask, and the cells appeared lysed (Figure 1B). The results implied that we successfully transfected bacmid and generated recombinant baculovirus carrying the gene expressing the CD2v ED-Foldon-His protein.

To demonstrate that baculovirus induced CD2v ED-Foldon-His expression in Sf9 cells, insect cells obtained by centrifugation of the transfection mixture were suspended in 1X Laemmli buffer, denatured, and examined for the desired gene expression by Western blot. The results showed a clear band corresponding to the size of the H5pII-His protein in the positive control sample as in the previous report (Phan *et al*., 2017), whilst in the negative control well, no band appeared, proving that the western blot experiment worked well and was specific for the His tag-fused proteins. In the transfected cell sample, a band of more than 100 kDa and a band of more than 250 kDa corresponding to the size of CD2v ED-Foldon-His in the trimeric form and oligomeric form were indicated, illustrating that CD2v ED-Foldon-His was successfully expressed in insect cells (Figure 1C).

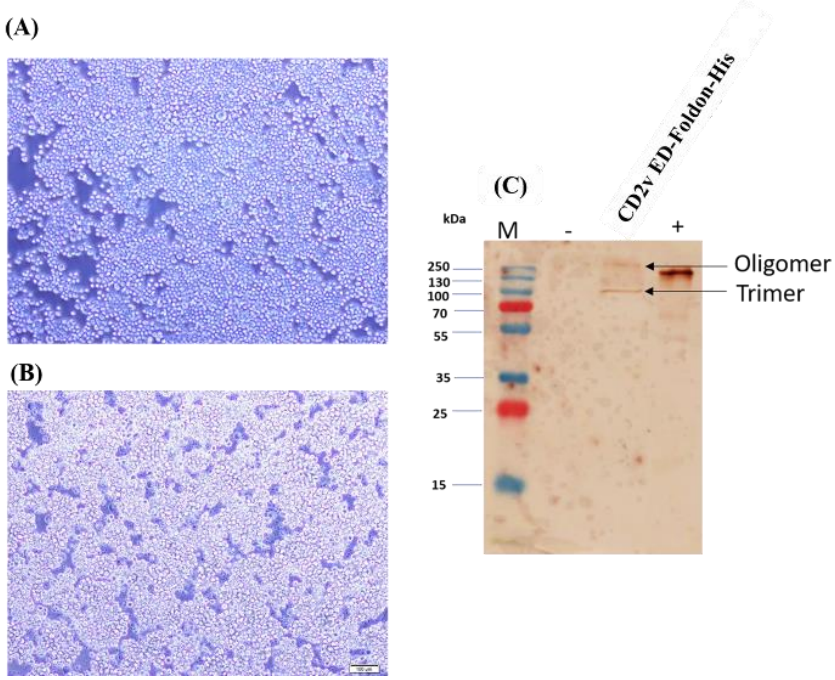

Figure 1. Transfection to create recombinant baculovirus carrying the gene expressing the CD2v ED-Foldon protein. (A): Non-bacmid-transfected Sf9 cells at 10x magnification; (B): Bacmid-transfected Sf9 cells at 10x magnification; (C) The expression of CD2v ED-Foldon-His protein in insect cells was detected by anti-His western blot analysis. Different sizes of recombinant protein were illustrated as oligomers and trimers. M: Marker; -: Negative control is non-infected Sf9 cells; +: Positive control is H5pII-His protein.

Foldon is a small autonomous folding region found in the major coat protein of bacteriophage T4, a virus that infects *E. coli* bacteria. The Foldon motif consists of 27 amino acids and plays an important role in virus assembly. The fusion with Foldon donates protein to form a trimeric state (Tao *et al.*, 1997). Indeed, CD2v ED fused with

Foldon has formed a trimeric structure. Especially at high concentrations, it has formed an oligomer form. Our results are similar to previous studies of Nguyen *et al*. (2024) who expressed successfully trimeric CD2v ED that further formed oligomers at high concentrations after purification, and Harbury *et al.* (1993) who proposed the oligomerization states of trimeric peptides-pII at protein concentrations from 20 to 200 µM.

Optimization of CD2v ED-Foldon-His protein expression in Sf9 cells

The expression of recombinant proteins in the baculovirus-insect cell system can be enhanced by optimizing parameters such as the inoculum cell concentration, MOI, and time of harvest (Zitzmann *et al.*, 2017). Because adherent culture has been used with a constant inoculum cell to ensure the plate coverage of more than 90%, two factors affecting protein levels, including the MOI and infection time, were evaluated. A viral seed containing 10^8 pfu/mL virus was used to optimize CD2v ED-Foldon-His protein expression. Sf9 cells were infected with baculovirus at 3 different concentrations: MOI of 1; MOI of 5 and MOI of 10. Cells were harvested after 3, 5, and 7 days of infection. Protein expression levels were examined by western blot. Colorimetric signals were captured on Amersham™ Imager 680. The results in Figure 2A showed that CD2v ED-Foldon-His protein was expressed highest at a MOI of 5 pfu/cell and after 5 days of infection.

The expression level of CD2v ED-Foldon-His protein in different conditions was quantified based on the standard curve of H5pII-His protein (Phan *et al.*, 2017) at concentrations of 150, 300, and 600 ng in ImageQuant TL 8.0 software (Cytiva, Sweden). The experiment was repeated 3 times. The effect of two factors on the expression level of recombinant protein was evaluated by two-factor ANOVA with 3 replications with a significance level of $\alpha = 0.05$. The results in Figure 2B are in agreement with the western blot result, confirming the highest yield of CD2v ED-Foldon-His at a MOI of 5 pfu/cell and 5 dpi. Particularly, at a MOI of 10 pfu/cell, the highest CD2v ED-Foldon-His protein was obtained at 3 dpi with 3.5 mg/L. However, the amount of protein decreased rapidly on days 5 and 7 after infection with 2.41 mg/L and 1.7 mg/L, respectively. With two virus densities a MOI of 1 pfu/cell and a MOI of 5 pfu/cell, the expression level of CD2v ED-Foldon-His reached its highest level after 5 days of infection at 4 mg/L and 4.4 mg/L, then decreased significantly with only 1.6 mg/L and 1.4 mg/L at day 7 dpi, respectively. This may be the result of the release of protease that degraded the recombinant protein. With higher inoculum virus densities, the whole cell infection rate was faster and the amount of protease produced was high at the early time point.

The results of two-factor ANOVA indicated both MOI and time of harvest effect on the yield of CD2v ED-Foldon-His, in which the latter gave a stronger influence with p of 6.41^{e-15} than the former ($p = 0.0007$) (data not shown).

The infection of baculovirus over time at the optimal virus density was also shown in the infection image on the cells (Figure 3), which confirmed again the above qualification and quantification results. At MOI of 1 and 5, a part of the cells was infected, which increased over the infection time, and by day 7 almost all Sf9 cells were infected, but the cell lysis was not clear. At a MOI of 10, almost all cells were infected

after 3 days of infection, which were lysed at 7 dpi.

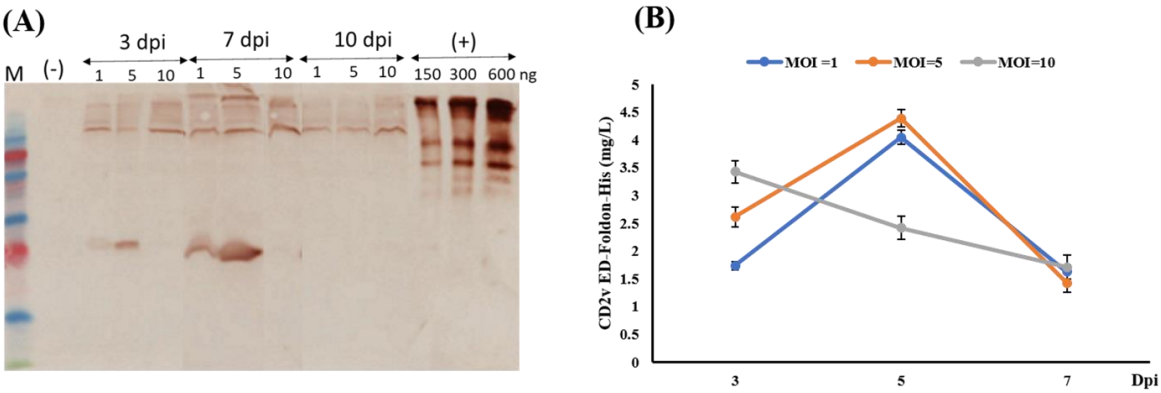

Figure *2.* The optimal expression of CD2v ED-Foldon-His protein in insect cells. (A) Western blot evaluated CD2v ED-Foldon-His expression. The expression of CD2v ED-Foldon-His protein in insect cells was obtained by infection with recombinant baculovirus at densities of 1 pfu/cell, 5 pfu/cell, and 10 pfu/cell at 3, 5 and 7 days post-infection (dpi). M: Marker; -: Negative control is uninfected Sf9 cells; +: 150, 300, and 600 ng of H5pII-His protein was used as positive controls, and a standard curve was constructed to quantify the recombinant protein. (B) Quantitative results of CD2v ED-Foldon-His protein expression levels in Sf9 cells. Data represented as the average value and SD of three replicates.

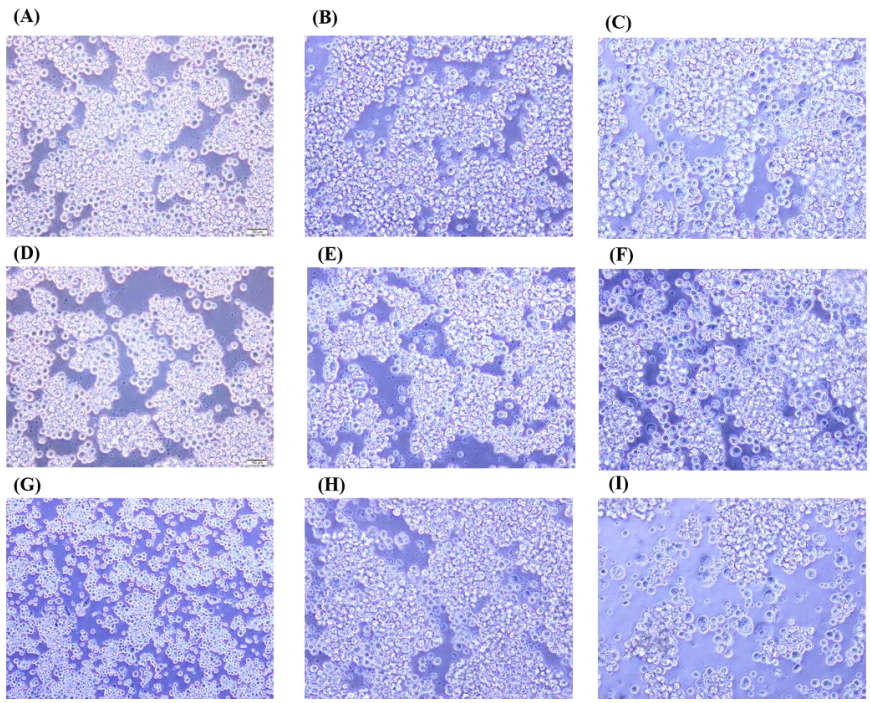

Figure 3. Infection of baculovirus carrying gene encoding the CD2v ED-Foldon-His on Sf9 cells at different conditions. (A, B, C): Infection with a MOI of 3 pfu/cell at 3 dpi, 5 dpi, and 7 dpi respectively.

(D, E, F): Infection with a MOI of 5 pfu/cell at 3, 5, and 7 respectively. (G, H, I): Infection with a MOI of 10 pfu/cell at 3 dpi, 5 dpi, and 7 dpi, respectively. All pictures are captured at 20x magnification.

BEV is one of the most popular systems for expressing a wide range of recombinant proteins. The previous studies documented the yield of protein ranges from 1 to 600 mg/L, depending on the protein, insect cell, bacmid construct, and optimal culture conditions (Druzinec *et al.*, 2013; Käßer *et al.*, 2022; Stolt-Bergner *et al.*, 2018; Zitzmann *et al.*, 2017). In particular, Käßer *et al.* (2022) achieved the maximum yield of SmALDH_312 (aldehyde dehydrogenase of *Schistosoma mansoni*) using BEVS with 47.78 mg/L in Sf-900 II SFM. Stolt-Bergner *et al.* (2018) have expressed intracellular proteins such as Dicer-2 (3 mg/L); Tyrosine-protein kinase ABL1 (4 mg/L), FMRP (fragile X messenger ribonucleoprotein) (25 mg/L) and vNS1-H1 (viral nonstructural protein H1) (18 mg/L). In Vietnam, Do *et al*. (2021) expressed the SARS-CoV-2 spike protein (S protein) using the Sf9 insect cell culture system in culture flasks and bioreactors. The achieved maximum yield of protein was 2.58 mg/L. The expression level of CD2v ED-Foldon-His is comparable to other studies, which is 1.7 times higher than S protein expression (Do *et al*., 2021). In further studies, the immunogenicity and protective ability of CD2v ED-Foldon-His need to be evaluated. In addition, large-scale CD2v ED-Foldon-His expression on bioreactor systems is necessary to increase the scale and expression productivity.

CONCLUSION

This is the first study on the expression of the multivalent CD2v extracellular domain of the African swine fever virus on the baculovirus-insect cell Sf9 expression system, aiming to produce a subunit vaccine for preventing African swine fever. The optimal conditions for CD2v ED-His expression in Sf9 cells cultured in monolayer were determined: 5 days post-infection with a MOI of 5 pfu/cell. The manuscript provides detailed methodology and transparent data that may be a useful guide for the expression of recombinant proteins in the baculovirus-insect cell system.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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