Development of recombinant vp1 protein immunised in mice as a model of foot-and-mouth disease vaccine

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Abstract. Foot-and-mouth disease (FMD) is a highly contagious disease that infects cloven-hoofed animals, becoming a serious threat to livestock production and leading to significant economic losses. The re-occurring FMD outbreak in Indonesia was reported back in 2022, causing hundreds of cattle deaths. The immunogenic viral capsid VP1 protein has been extensively researched as a vaccine candidate despite the fact that the existing FMD vaccine uses an inactivated virus. The vp1 gene (648 bps) from FMD virus serotype O was integrated into pET-32b vector and transformed into *Escherichia coli* TOP10F'. The recombinant pET32b-VP1-1D plasmid was expressed in *E. coli* BL21(DE3), followed by N-terminal His tag purification. Protein profiles were determined with SDS-PAGE, showing the target protein at 33KDa. Five 6-week-old BALB/c mice were administered intraperitoneal injections of 50 µg and 100 µg protein, respectively, with two booster shots within two-week intervals. The immune response of polyclonal antibodies was tested using indirect ELISA, resulting in a high absorbance signal compared to non-immunized mice. Thus, the outcomes demonstrate that the VP1 recombinant protein from this study has potential as an immunogen in FMD vaccine development.

1 Introduction

Foot-and-mouth disease (FMD) is a rapidly spreading, highly transmissible viral illness that spreads quickly, affecting cloven-hoofed animals, including cattle, sheep, goats, and pigs. FMD has been endemic in many countries worldwide, except North America, Oceania, Europe and South America, resulting in economic devastation [1–3]. In 2022, Indonesia experienced an FMD outbreak, a re-emerging disease; after three decades, the country was free from the disease [4].

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Foot-and-mouth disease virus (FMDV) exhibits significant variety, resulting in the existence of seven distinct serotypes, including O, A, C, SAT (Southern African territory) 1, 2, 3, and Asian-1. These serotypes frequently show a pattern of recurring within a specific geographic region, following recurrent transmission that might impact numerous nations. FMDV is a positive-sense, single-stranded RNA virus belonging to the genus Aphthovirus, family Picornaviridae. FMDV has one *open reading frame* (ORF) encoding structural proteins (VP1, VP2, VP3, VP4) and a non-structural protein (L, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C, and 3D) [1,5,6].

Currently, commercially accessible FMD vaccines are inactivated whole virus platforms. However, these vaccines have several disadvantages, such as issues with quality control and residual infectivity. As a result, alternative recombinant vaccines have been developed to address these limitations [7]. In this study, we successfully expressed and purified VP1 serotype O in *Escherichia coli*. The proteins efficiently induced humoral response in mice. Hence, comprehending the antigenic and immunogenicity characteristics of every structural protein is crucial in the creation of secure, cost-effective, efficient substitute vaccines. Our findings give valuable insights into the current attempts to develop FMDV vaccines.

2 Materials and methods

2.1 Construction of plasmid vectors and PCR amplification

We used a VP1 synthetic gene construct that was commercially customized (GenScript Biotech, USA) based on Indonesian FMDV type O (accession number AY593813) after codon optimization. Plasmid construction was designed by adding the XhoI and KpnI restriction sites on the 5' and 3' ends of our gene of interest to a pET32b vector utilizing Snapgene software. The plasmid was verified by PCR amplification using Phusion High-Fidelity 2x Master Mix, and PCR amplicon was running at 1% agarose gel electrophoresis.

2.2 Transformation of recombinant plasmid into *E. coli* cells

E. coli TOP10F' cells were inoculated into 5 mL of LB medium and incubated for 16 hours at 37°C with 200 rpm shaking. Re-inoculate the culture into LB medium, followed by incubation for 2 hours until the OD600 reaches 0.5. The cells were harvested by centrifuging for 10 minutes (5000 rpm) and resuspended in CaCl2 solution. The supernatant was discarded then re-added CaCl2 into the tube, and placed on ice for 30 minutes. The bacterial cells were collected and then mixed in 2 mL CaCl2 solution. The competent cells were aliquoted 50 µL into each tube and incubated on ice for 10 mins. Furthermore, recombinant plasmid (5 ng) was added to the cells, mixed well by pipetting, and set aside on ice for 30 mins. Bacterial cells transformed with pET32b empty were designed as a negative control. The tube was then moved to a thermomixer at 42 C for 30 secs for heat shock, and add 950 µL of LB medium was while shaking vigorously for one hour. The competent cells were spread onto LB agar supplemented with ampicillin and incubated overnight.

2.3 Extraction of recombinant plasmid

By following the manufacturer manuals, QIAprep MiniPrep kit was used to isolate the recombinant plasmid. One colony of transformant *E. coli* was cultured into 3 mL of ampicillin-containing LB medium and incubated for 16 hours at 37 ºC while shaken vigorously. Centrifugation was used to extract the cells for three minutes at 10,000 rpm. Pipette the pellets into 250 µL Buffer P1, then add 250 µL Buffer P2 and mix well by

inverting the tube. After flipping the tube 4-6 times, add 350 µL of Buffer N3 and centrifuge at high-speed for 10 minutes. The liquid portion was introduced into the centrifuge tube, subjected to centrifugal force for one minute, and the resulting liquid that passed through was discarded. The spin column was rinsed with 0.75 mL of Buffer PE in order to extract the DNA.

2.4 Expression of VP1 recombinant protein

The recombinant plasmid of pET23b-VP1-1D was subcloned into *E. coli* BL21(DE3), which is the same as the previous procedure. Bacterial cells were cultured in ampicillin-containing LB medium for 2 hours, adding IPTG (Isopropyl-β-D-1-thiogalactopyranoside) to induce protein expression at the final concentration of 1 mM, and later culture was incubated overnight. Approximately 50 mL of culture was harvested and resuspended in 8 ml of native binding buffer (Thermo Scientific, USA). Lysozyme (8 mg) was added, incubated the tube on ice for 30 mins, and disrupted the cells using a high-intensity sonicator with 10 secs on-off interval for 15 mins. Centrifuge cell lysate at 3000 x g for 15 mins to discard cellular debris, followed by transferring supernatant into a fresh tube.

2.5 Protein purification

Protein purification was carried out using Ni-NTA column (Thermo Scientific, USA)), following native conditions from the manual. The lysate was introduced onto a pre-assembled purification column and subjected to incubation at ambient temperature on a rotary shaker, followed by centrifugation at 800 x g to eliminate the liquid portion above the sediment. The column was washed with 8 mL wash buffer by resuspending, centrifuging at low speed, and then carefully aspirating the supernatant. Repeat the washing step three more times and elute protein with 8 mL elution buffer. Inclusion body extraction followed procedures as the manufacturer suggested. using cell pellet ml 5 mL culture, homogenized in B-PER reagent (Thermo Scientific, USA) and centrifuged at 800 x g. Protein refolding followed protocols as described in the previous study [8]. The pellet was submerged in a DOC buffer solution consisting of 50 mM Tris pH 8, 2% sodium deoxycholate, 2 M urea, and 5 mM EDTA, and then subjected to centrifugation. The pellet was thereafter washed in a solution containing 50 millimolar Tris with a pH of 8, and then dissolved in 1 milliliter of 8 molar urea. It was let to rest overnight on a shaker. Later on, centrifuge the sample and dilute the supernatant with TNGA buffer containing 100 mM Tris pH 8, 100 mM NaCl, 1% glycerol and 1 M I-arginine. The mixture was then centrifugated at 18000 g for 30 mins.

2.6 Protein analysis using SDS-PAGE and Western blot

To determine the molecular size of recombinant VP1, we used 10% SDS-PAGE gel with standard protocol and ran at 100 volts for 45 mins. For western blot analysis, protein bands were transferred into the PVDF membrane with a semi-dry transfer buffer. The membrane was blocked with blocking solution, then incubated with anti-6x-His-tagvmonoclonal antibody and anti-VP1 type O polyclonal antibody (Thermo Scientific, USA), respectively, at 1:2000 concentration, followed by 3 hours incubation. The membrane was rinsed three times with 1x PBS before being incubated for 30 minutes with the secondary antibody, IRDye® 800CW goat anti-rabbit IgG for VP1 antibody and anti-mouse IgG for anti-his-tag at a concentration of 1:10.000. Visualization was carried out using an imaging system.

2.7 Ethical statement

All animal experiments are treated in accordance with ethical permission number 013/KE.03/SK/03/2023, granted by the Ethical Clearance Committee of the National Research and Innovation Agency.

2.8 Mice immunization with recombinant VP1

Five female BALB/c mice 6-week-old were administered with 50 μg (Group A) and 100 μg (Group B) protein mixed with aluminum hydroxide adjuvant at one-to-one concentration, respectively, via intraperitoneal route. Immunization was done in two booster shots within two-week intervals. Two days after injection, mice serum was taken to check the humoral immune response using ELISA indirect coated with inactivated FMDV antigen. BSA blocking was done for 4 hours, and the serum was included for overnight incubation. Therefore, the plate was rinsed with 0.1% PBST three times, and a secondary antibody anti-mouse IgG was applied for 30 mins. TMB substrate was added, and the absorbances were measured at 450 nm.

3 Results and discussions

As a viral disease that affects animals, foot-and-mouth diseases are still issued in many nations with advanced animal husbandry. It causes the mortality of young animals and reduces the production of older animals, resulting in significant economic loss and community impact. The illness is a highly contagious transboundary ailment that mostly affects farmed animals, including cattle, pigs, sheep, goats, and buffalo, as well as around 70 other kinds of wild animals with divided hooves [2,9,10].

After the occurrence of an outbreak, authorities will be required to reintroduce vaccines in order to manage and contain the spread of illness effectively. Nevertheless, it has been shown that inactivated foot-and-mouth disease vaccines have certain limitations, such as little or no ability to provide cross-protection against different serotypes and subtypes, requiring regular booster immunisations to sustain adequate levels of neutralizing antibodies, and ineffective stimulators of cytotoxic T-cells that gave temporary antibody responses [10–13]. Hence, researchers have attempted to address these issues by creating substitute immunizations using novel approaches such as recombinant protein, peptide, empty capsid, and genetically engineered subunit vaccines [14–16].

For this investigation, we used *E. coli* expression system as an appropriate approach for bacterial expression due to its simplicity, speed, and affordability [17,18]. One of the problems with synthesized *E. coli* heterologous proteins is the bacterial cytoplasm lacks the necessary stability factors, leading to the formation of insoluble clumps called inclusion bodies[19]. Solubilizing the inclusion usually requires a refolding procedure, which could cause a loss of the function of the protein. In order to prevent the issue, it is necessary to increase the production of proteins. Therefore, the pET-32b(+) vector is engineered explicitly for cloning and facilitating the production of peptide sequences that are coupled with the 109 amino acid Trx-Tag thioredoxin protein. The cloning site also includes both cleavable His-Tag and S-tag. When *E. coli* thioredoxin is over-expressed using plasmid vectors, it may accumulate up to 40% of the total protein. Despite these high expression levels, all of the protein stays in the soluble fraction [17,19].

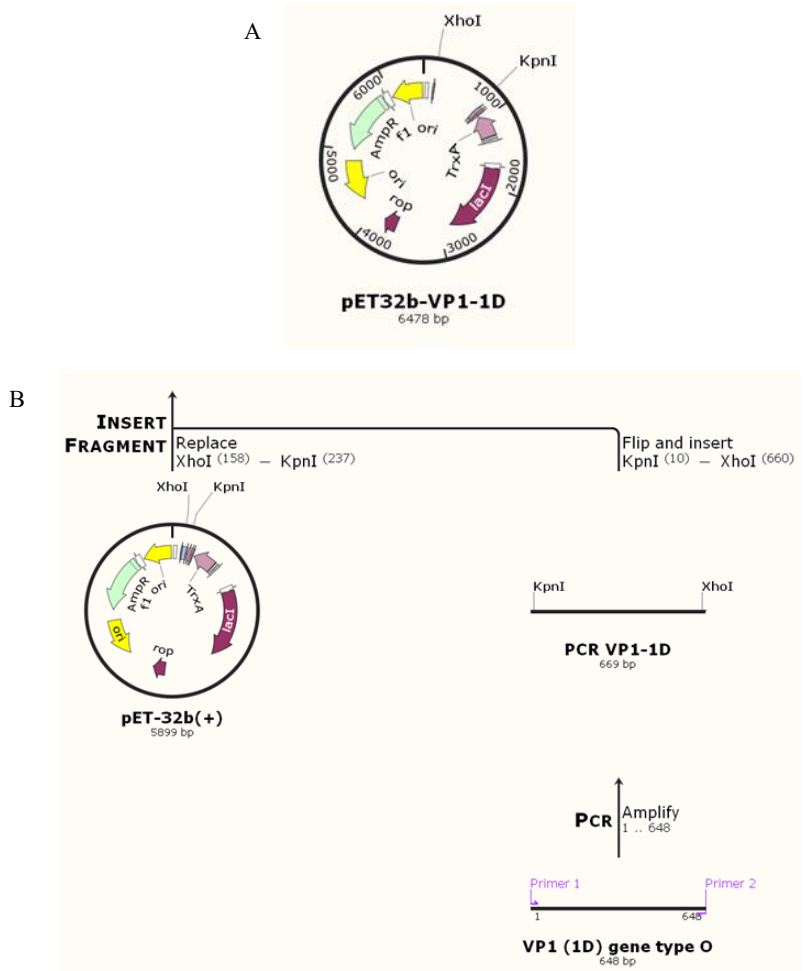

Fig. 1. Plasmid construction of pET32b-VP1-1D (A); the design of vp1 gene type O insert fragment into pET-32b(+) assembling with KpnI and XhoI restriction site

In this study, as depicted in Figure 1, we constructed a full-length VP1 (648bp) into a pET 32b(+) vector. The total size of the vector and our gene of interest is approximately 6478 bp. The PCR result was purified utilizing a Qiagen PCR purifying kit and then analyzed on a 1% agarose gel. The amplification achieved a single band with a length of roughly 669 bp, as can be seen below. Recombinant plasmid pET-32b-VP1-1D and pET-32b(+) were shown as well in Figure 2.

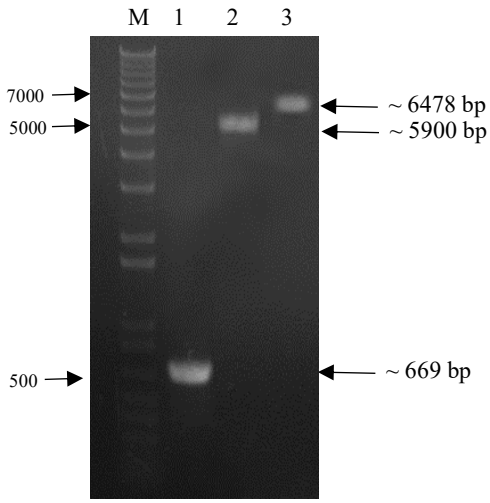

Fig. 2. PCR amplification of VP1 amplicon (Lane 1); pET-32b(+) empty as negative control (Lane 2); and pET-32b-VP1-1D recombinant plasmid (Lane 3) in 1% agarose

The recombinant pET-32b-VP1-1D was successfully introduced into *E. coli* BL21(DE3) for protein expression by IPTG induction. Based on protein profiles, the intended protein was detected at the molecular weight of 64 kDa in supernatant; however, the protein was still in pellet cells in the form of inclusion bodies. In consequence, we performed protein purification both with supernatant and pellet as shown in Figure 3.

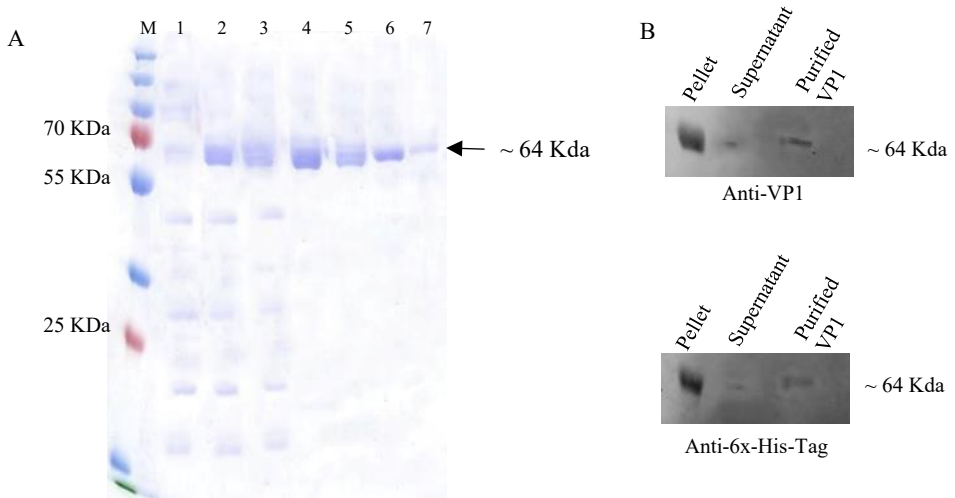

Fig. 3. SDS-PAGE and western blot analysis of VP1 recombinant protein. All samples collected for SDS-PAGE were diluted in 10 folds (A). Lysate of bacterial cells containing pET-32b(+) empty as control (Lane 1); cell pellet of *E. coli* contained pET-32b-VP1 after IPTC induction (Lane 2); Supernatant of *E. coli* transformant carrying recombinant plasmid (Lane 3); Elution fraction 1-4 obtained through protein purification Ni-NTA from inclusion bodies (Lane 4-7). Western blot was done using two antibodies, anti-6x his-tag and anti-VP1 (B), to recognize protein VP1 from the pellet, supernatant, and purified protein, respectively.

Even though the vector used contains thioredoxin fusion, inclusion bodies are still formed. This was also reported by another group that produced SAT2 VP1 protein in the *E. coli* system in pSK and pET28a vector, resulting in VP1 protein at 23 KDa [8]. Interestingly, the molecular weight of VP1 previously reported, which was expressed in pET-32a-His-SUMO-VP1 fusion protein, was 63 KDa [18]. Western blot results show a strong signal from membrane-integrated protein in cell debris. A weak signal was shown on the supernatant, followed by purified VP1 that was collected from inclusion bodies. Our result indicated that recombinant proteins were also expressed into a soluble form, whether the overexpressed protein was aggregated, forming inclusion bodies that bind to the bacterial cell membrane, as mentioned by Xiao et al. [20]. The data is depicted in agreement with similar research from other groups regarding VP1 expression in *E. coli* [8,18,20].

To determine the immunogenicity of protein that we generated, we used inactivated FMDV antigen as a coating and commercial polyclonal anti-VP1 as the positive control. The ELISA assay detected a significant increase in specific antibody (IgG) titer in the serum samples from day 0 serum to the final booster. This finding demonstrates that the structural proteins have the capability to ascertain antigenic specificity.

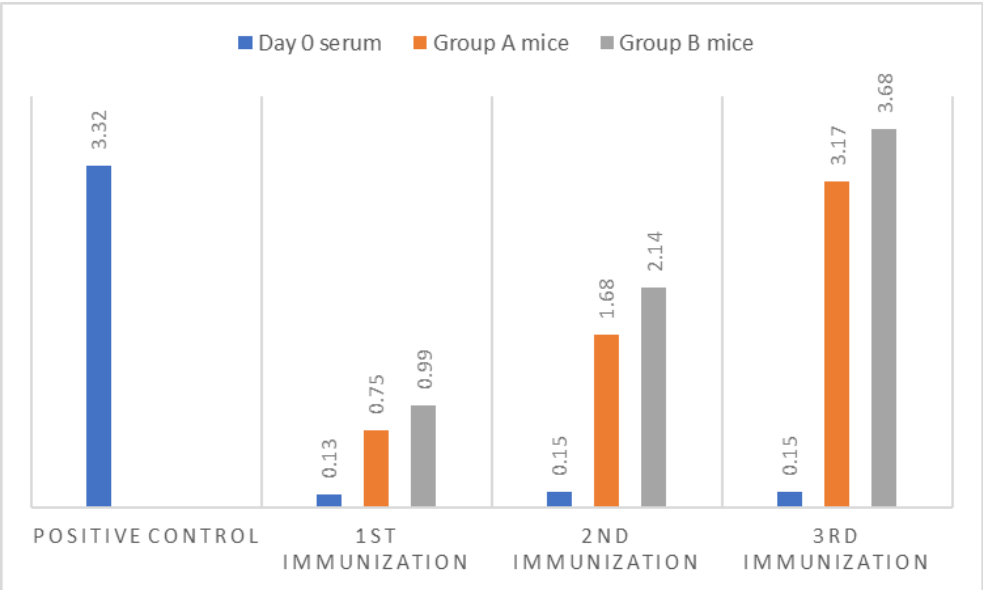

Fig. 4. Humoral immune response was tested by indirect ELISA, indicating that the VP protein succeeded in stimulating antibody generation in mice.

Recently, there have been developing novel techniques in vaccine design aimed at enhancing immunogenicity and antigenicity. VP1 contains crucial epitopes that are responsible for triggering immunological responses against FMDV. Multiple findings have demonstrated that residues 141-160 and 200-213 on the GH-ring of the VP1 protein are recognized as a linear B cell epitope and stimulate the production of neutralizing antibodies. Peptides synthesized from this particular area were also capable of inducing both humoral and cellular T-cell responses and caused cell proliferation [18,21–23].

4 Conclusions

Recombinant VP1 protein expressed in *E. coli* cells from this research has successfully elicited a humoral immune response in mice, promising as a vaccine antigen. Nevertheless, more research should be conducted to determine whether these results can be applied to the development of foot-and-mouth disease vaccines.

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References

1. M. J. Grubman and B. Baxt, Foot-and-mouth disease. Clin Microbiol Rev **17**, 465 (2004)
2. S. M. Jamal and G. J. Belsham, Foot-and-Mouth Disease: Past, Present and Future (2013)
3. M. Mahapatra and S. Parida, Foot-and-mouth disease vaccine strain selection : current approaches and future. Expert Rev Vaccines **17**, 577 (2018)
4. N. Zainuddin, E. B. Susila, H. Wibawa, R. S. D. Daulay, P. E. Wijayanti, D. Fitriani, D. N. Hidayati, S. Idris, J. Wadsworth, N. Polo, H. M. Hicks, V. Mioulet, N. J. Knowles, and D. P. King, Genome Sequence of a Foot-and-Mouth Disease Virus Detected in Indonesia in 2022. Microbiol Resour Announc **12**, (2023)
5. E. E. Fry, · D I Stuart, and D. J. Rowlands, The Structure of Foot-and-Mouth Disease Virus (Springer-Verlag, 2005)
6. N. J. Knowles and A. R. Samuel, Molecular Epidemiology of Foot-and-Mouth Disease Virus. Vir Res **91**, 65 (2003)
7. N. M. T. Le, K. K. So, J. Chun, and D. H. Kim, Expression of virus-like particles (VLPs) of foot-and-mouth disease virus (FMDV) using Saccharomyces cerevisiae. Appl Microbiol Biotechnol **108**, 1 (2024)
8. M. V. Mamabolo, J. Theron, F. Maree, and M. Crampton, Production of foot-and-mouth disease virus SAT2 VP1 protein. AMB Express **10**, (2020)
9. Z. Lu, S. Yu, W. Wang, W. Chen, X. Wang, K. Wu, X. Li, S. Fan, H. Ding, L. Yi, and J. Chen, Development of Foot-and-Mouth Disease Vaccines in Recent Years. Vaccines **10**, (2022)
10. M. S. Bhutta, M. Awais, S. Sadaqat, F. B. Zanchi, N. Shahid, and A. Qayyum Rao, A novel immunoinformatics approach for developing a poly-epitope vaccine targeting foot and mouth disease virus, exploiting structural VP proteins. J Biomol Struct Dyn (2024)
11. L. L. Rodriguez and M. J. Grubman, Foot and mouth disease virus vaccines. Vaccine **27**, (2009)
12. S. F. Li, M. J. Gong, Y. F. Sun, J. J. Shao, Y. G. Zhang, and H. Y. Chang, Molecules **24**, (2019)
13. S. J. Cox, N. Aggarwal, R. J. Statham, and P. V Barnett, *Longevity of Antibody and Cytokine Responses Following Vaccination with High Potency Emergency FMD Vaccines* (2003)
14. J. Shao, W. Liu, S. Gao, H. Chang, and H. Guo, In vitro and in vivo antiviral activity of mizoribine against foot-and-mouth disease virus. Virology **596**, (2024)

15. J. M. Gershoni, A. Roitburd-Berman, D. D. Siman-Tov, N. T. Freund, and Y. Weiss, Epitope mapping: The first step in developing epitope-based vaccines. BioDrugs **21**, 145 (2007)
16. V. Gnazzo, V. Quattrocchi, I. Soria, E. Pereyra, C. Langellotti, A. Pedemonte, V. Lopez, L. Marangunich, and P. Zamorano, Mouse model as an efficacy test for foot-and-mouth disease vaccines. Transbound Emerg Dis **67**, 2507 (2020)
17. S. Wagner, M. M. Klepsch, S. Schlegel, A. Appel, R. Draheim, M. Tarry, M. Hogbom, K. J. van Wijk, D. J. Slotboom, J. O. Persson, and J.-W. de Gier, Tuning Escherichia coli for membrane protein overexpression. Proc Natl Acad Sci U S A **105**, 14371 (2008)
18. G. Li, A. K. Wubshet, Y. Ding, Q. Li, J. Dai, Y. Wang, Q. Hou, J. Chen, B. Ma, A. Szczotka-Bochniarz, S. Szathmary, Y. Zhang, and J. Zhang, Antigenicity and immunogenicity analysis of the e. Coli expressed fmdv structural proteins; vp1, vp0, vp3 of the south african territories type 2 virus. Viruses **13**, (2021)
19. E. R. LaVallie, E. A. DiBlasio, S. Kovacic, K. L. Grant, P. F. Schendel, and J. M. Mc.Coy, A thioredoxin gene fusion expression system that circumvents inclusion body formation in the E. coli cytoplasm. Nat Biotechnol **11**, 187 (1993)
20. Y. Xiao, H. Y. Chen, Y. Wang, B. Yin, C. Lv, X. Mo, H. Yan, Y. Xuan, Y. Huang, W. Pang, X. Li, Y. A. Yuan, and K. Tian, Large-scale production of foot-and-mouth disease virus (serotype Asia1) VLP vaccine in Escherichia coli and protection potency evaluation in cattle. BMC Biotechnol **16**, (2016)
21. H. T. Wong, S. C. S. Cheng, E. W. C. Chan, Z. T. Sheng, W. Y. Yan, Z. X. Zheng, and Y. Xie, Plasmids encoding foot-and-mouth disease virus VP1 epitopes elicited immune responses in mice and swine and protected swine against viral infection. Virology **278**, 27 (2000)
22. M. C. Van Lierop, J. E. P A Wagenaar, J. M. Van Noort, and E. J. Hensen, Sequences Derived from the Highly Antigenic VP1 Region 140 to 160 of Foot-and-Mouth Disease Virus Do Not Prime for a Bovine T-Cell Response against Intact Virus. J Virol **69** 4511 (1995)
23. P. I. Zamorano, A. Wigdorovitz, D. M. P. Filgueira, J. M. Escribano_F, A. M. Sadir, and M. V Borea, Induction of Anti Foot and Mouth Disease Virus T and B Cell Responses in Cattle Immunized with a Peptide Representing Ten Amino Acids of VP1. Vaccine **16** 558 (1998)