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Detection of mRNA and Anibody in Mice Injected with Plasmid pCDNA3.1-SRM Carrying African Swine Fever Virus Gen Able to Produce Self-Replicating RNA

(PELACAKAN mRNA DAN ANTIBODI MENCIT YANG DIINJEKSI PLASMID pCDNA3.1-SRM PEMBAWA GEN VIRUS *AFRICAN SWINE FEVER* MAMPU MENGHASILKAN *SELF-REPLICATING* RNA)

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ABSTRACT

Vaccine technology and gene therapy have been well developed, in which DNA and mRNA is used as a base. This technology has a drawback as high quantities of DNA/RNA are needed, and therefore it is not feasible economically, especially for animals. Self-replicating/amplifying RNA (saRNA) is a promising technology to cope with the drawback of DNA and mRNA vaccines. A pCDNA3.1-SRM plasmid with a gene of picornavirus encoding polymerase enzyme and 5'-and 3'-untranslated region (UTR) might produce saRNA to be used as a vaccine. In this research, the pCDNA3.1-SRM plasmid was inserted with DNA "ASF-276R-224L" encoding antigens for the African swine fever (ASF) virus. The main aim of this research was to compare the amount of mRNA and antibody of the pCDNA3.1-SRM vaccine with the pCDNA3.1 without polymerase gene as a control vaccine in mice. A total of 46 mice were divided into four groups according to the amount of plasmid per vaccine and type of plasmid. The mRNA quantity was obtained from CT-Values in the qRT-PCR analysis of mice thigh muscles that were sampled at day 3, 6, and 9 post-injections. African swine fever antibodies were measured using ELISA applying synthetic peptides and the optical density (OD) were statistically analysed using T-test method. The results of both mRNA quantity and antibody level of pCDNA3.1-SRM were found to be higher when compared to the control vaccine, but they are not significantly different statistically ($p>0.05$). For future research, it is recommended to improve the construction of pCDNA3.1-SRM plasmid.

Keywords: mice, mRNA, plasmid, vaccine

ABSTRAK

Teknologi vaksin dan gen terapi dalam bentuk DNA atau mRNA sudah berkembang pesat saat ini. Kekurangan teknologi itu adalah DNA atau mRNA diperlukan dalam jumlah besar untuk penggunaannya sehingga teknologinya tidak ekonomis, khususnya untuk penggunaannya pada hewan. Plasmid yang mampu menghasilkan *self-replicating/amplifying* RNA (sa-RNA) adalah salah satu cara untuk menghadapi kekurangan vaksin DNA atau mRNA. Plasmid pCDNA3.1-SRM dengan gen penyandi RNA-polimerase virus picornavirus dapat berfungsi seperti saRNA ketika digunakan sebagai vaksin. Pada penelitian ini, plasmid tersebut disisipi dengan DNA "ASF-276R-224L" penyandi antigen virus *African Swine Fever* (ASF). Tujuan dari penelitian ini adalah untuk membandingkan mRNA yang dihasilkan dan titer antibodi dari vaksin plasmid pCDNA3.1-SRM

dengan vaksin kontrol pada mencit. Sebanyak 46 mencit dibagi menjadi empat kelompok, Masing-masing mendapatkan dosis vaksin dan jenis plasmid yang berbeda. Kuantitas mRNA diperoleh dari hasil CT-*Value* qRT-PCR otot mencit yang diambil pada hari ke-3, ke-6 dan ke-9 pascainjeksi. Antibodi terhadap ASF diukur dengan ELISA dan dianalisis dengan metode *T-test*. Hasil penelitian menunjukkan bahwa kuantitas mRNA vaksin dan kuantitas antibodi berdasarkan nilai OD dengan pCDNA3.1-SRM lebih tinggi jika dibandingkan dengan vaksin kontrol walaupun secara statistika tidak berbeda nyata. Rekomendasi dari penelitian ini adalah konstruksi plasmid pCDNA-SRM perlu disempurnakan.

Kata-kata kunci: mencit; mRNA; plasmid; vaksin

INTRODUCTION

Vaccine technology has developed in recent years, from using attenuated organisms such as viruses or bacteria, to only using nucleotides. These nucleotide vaccines consist of DNA or RNA encoding antigen sequences for certain pathogens, with RNA vaccines having the advantage over DNA vaccines in term of activation rate, creating a faster immunity reaction (Verbeke *et al.*, 2019), but RNA is very unstable and easily degraded in the host's cells (Liu *et al.*, 2021). Injected RNA will not last long, so to trigger immunity inside the host's body, high level of RNA is required (Bloom *et al.*, 2021). To tackle this problem, a self-amplifying RNA (saRNA) with ability to replicate or amplify its own RNA inside the host's body was designed, resulting in the production of more antigens and stronger immunity response, without using large doses of mRNA (Bloom *et al.*, 2021). The saRNA usually uses the RNA sequences from a single-stranded self-replicating virus such as alphavirus (Lundstrom, 2018) that can encode its own RNA polymerase (RNA-dependant-RNA-polymerase or RdRP), in which the sequences that encode for the virus structural protein being replaced with desired antigen encoding sequences (Bloom *et al.*, 2021). The use of saRNA is still under development, due to its larger size compared to regular mRNA or subunit proteins and difficulty in inserting the desired antigen sequences (Pardi *et al.*, 2018).

The Animal Biomedical and Molecular Biology Laboratory, Faculty of Veterinary Medicine, Udayana University has developed a new synthetic plasmid that can function like a saRNA vaccine once it reaches the cell, as it contains sequences encoding the RNA polymerase (RdRP) of picornavirus, while also contains 5' and 3' In-Fusion end sequences and unique single cleavage sites of EcoRV. Therefore, it can be used as antigen sequence insertion sites using seamless cloning methods or any PCR products using blunt-end cloning. This plasmid is used as an alternative to saRNA as it is more flexible to be used for different types of antigen sequences. Besides, it also shows very high stability. The main goal of this research was to compare the capabilities of this plasmid (pCDNA3.1-SRM) used as a vaccine control plasmid (pCDNA3.1+) with no polymerase encoding sequences in it. DNA encoding antigen sequence of the African Swine Fever (ASF) virus (ASF-276R-22L) was used as insert DNA for both plasmids. Both vaccines were injected to a number of mice (*Mus musculus*) and the antibody and RNA quantity were measured and analyzed.

RESEARCH METHODS

Ethical Clearance

This research was done in accordance with the standard ethical clearance issued by the Animal Ethics Committees of Veterinary Medicine Faculty of Udayana University, Number: B/7/UN.14.2.9/PT.01.04/2023

Plasmid pSRM-ASF and pCDNA-ASF

Plasmid pSRM-ASF is a plasmid pCDNA3.1-SRM that has been inserted with the ASF gene, while pCDNA-ASF is a plasmid control (pCDNA3.1+) that has been inserted with the same gene. Both plasmids were obtained from The Animal Biomedical and Molecular Biology Laboratory of the Faculty of Veterinary of Udayana University (Head Laboratory). Both plasmids have been transformed into *Escherichia coli* strain stellar HST08 (636763, Takara Bio©, Takara Bio Inc, San Jose, USA). The bacteria were grown into 150 mL Terrific Broth (Thermo-Fisher©, Thermo Fisher Scientific, Waltham, USA) media and were incubated for 24 hours at 37°C. Both plasmids were collected from the bacteria using Plasmid isolation kit midiprep (Zymo Research©, Irvine, USA). Plasmid concentration was quantified

using Qubit fluorometer 3 (Invitrogen©, *Thermo Fisher Scientific,* Waltham, USA).

Vaccine Preparation of pSRM-ASF and pCDNA-ASF

Both vaccines were formulated into two types of doses. High dose contained 10 µg of plasmid in 200 µL, while the low dose contained 1 µg of plasmid in 200 µL. Vaccines were formulated for 15 doses. The two mixtures were prepared separately. High dose vaccines formulated as such: 1.125 mL of Gibco Opti-mem media (Thermo-Fisher©, Waltham, USA) were mixed with 225 µL lipofectamine 3000 (Thermo-Fisher©, Waltham, USA) as the mixture 1. The mixture 2 composed of 2.250 mL of Gibco Opti-mem media mixed with 300 µL p3000 reagent (Thermo-Fisher©, Waltham, USA) and 150 µg of each plasmid for each vaccine. Both mixes were then combined. Low dose vaccines formulated as such: 1.125 mL of Gibco Opti-mem media (Thermo-Fisher©, Waltham, USA) were mixed with 22.5 µL lipofectamine 3000 (Thermo-Fisher©, Waltham, USA) as mixture 1. Mixture 2 composed of 2.250 mL of Gibco Opti-mem media was mixed with 30 µL p3000 reagent (Thermo-Fisher©, Waltham, USA) and 15 µg of each plasmid for each vaccine, as mix number 2. At the end, both mixtures were combined at ambient temperature. This protocol was based on the manufacturer's manual.

Plasmid Vaccination and Sample Collection

A total of 46 mice (*Mus musculus)* were divided into four groups, namely "HDT" (received high dose pSRM-ASF vaccine), "HDC" (received high dose pCDNA-ASF vaccine), "LDT" (received low dose pSRM-ASF vaccine), and "LDC" (received low dose pCDNA-ASF vaccine). HDT and HDC composed of 14 mice, while LDT and LDC only nine. Each mouse was injected with 200 µL vaccines according to their respective groups. Vaccines were injected intramuscularly at the back right hind leg thigh muscle. Three mice from each group were sacrificed on the three, six, and nine-days post-injection. The mice were euthanized using xylazine overdose. RNAs were extracted from the right hind leg of those mice. Serums from HDT and HDC mice were collected at weeks 2, 4, and 6 after injection. Serums were collected by puncturing the sinus orbital site of the mice.

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Measurement of Antibody Titter of the Mice Serums Using ELISA

Synthetic peptides of p276R consisting of 76RC (KELVFNCSVLLEMV) and 76RD (IECAQHCFKLQSYVVD), and p224L peptides consisting of 24LD (LANAFIPPYRKYIHKI) and, 24LE(FKFAAHLLSFHKV), presented by The Animal Biomedical and Molecular Biology Laboratory, Faculty of Veterinary Medicine, Udayana University, were added into an 8x12 ELISA plate combined with ELISA coating buffer. Each well was deposited with 0.5 µg, as well as 1 µg/µL of Bovine Serum Albumin (BSA, Sigma©, Merck, *Darmstadt, Germany*). The plate was incubated for 24 hours at ambient temperature. Five percent skim milk was added into each well, and incubated for 1 hour at ambient temperature. Each collected serum was diluted and added into different wells, and incubated for 1 hour at ambient temperature. Those wells were added with "goat anti-mouse IgG AP" (Invitrogen ©, Thermo-Fisher, Waltham, USA) antibody diluted in PBS with 1:500 concentration. Lastly, Alkaline Phosphatase was added as the suitable substrate. In between those steps, the plate was washed three times using PBS-Tween before proceeding into adding the next reagent. As the well started to experience color change, the plate was analyzed using an ELISA reader at the wavelength of 492 nm. Absorbance values of antibody level as optical density were recorded and further analyzed using Independent and paired T-Test.

The mRNA Isolation from Mice Muscle Tissue

The obtained mice thigh muscles were grinded using pastels until it is completely disintegrated inside a 1.5 mL microtube. One millilitre of physiological NaCL (0.9%) were added to each tube and was homogenised. The microtubes then centrifuged at 10000 rpm for five minutes. As many as 250 µL supernatants were collected and mixed with 750 µL Trizol LS reagent (Invitrogen ©, Thermo-Fisher, Waltham USA). The mix was then vortexed for one minute and incubated for five minutes at room temperature. The mix was added with 200 µL chloroform and vortexed for 15 seconds. The mix then incubated for 15 minutes, then proceeded to be centrifuged at 12000 rpm for 15 minutes. The mix was added by 500 µL isopropyl alcohol, incubated for 10 minutes, and then centrifuged at 12000 rpm for 10 minutes. The resulting supernatant was discarded. The

pellet was diluted with 1000 µL 70% alcohol. The diluted pellet was then centrifuged at 7500 rpm for five minutes, and the supernatant was discarded. The tube was then air-dried at room temperature. After completely dried, 20 µL of sterile aquabidest was added. One microliter of DNase (Thermo-Fisher©, Waltham, USA) enzyme was added into each sample. Samples were incubated for 10 minutes at 37°C before stored in -20°C until used for quantitative real-time PCR (qRT-PCR).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) pSRM-ASF and pCDNA-ASF

The reaction volume of this qRT-PCR analysis was 25 µL. Each sample was added to different microtubes, with each received 2 µL pure RNA from the previous process, 12.5 µL RealQ Plus 2X Master Mix (Thermo-Fisher©, Waltham, USA), 0.5 µL 10 µM "BGH" primer, 0.5 µL 10 µM, "T7" primer, 0.5 µL superscript-taq polymerase (Thermo-fisher©, Waltham, USA) and 9 µL sterile aquabidest. The thermal cycler (Thermal Cycler RT-PCR 48E. Xi'an Tianlong Science and Technology Co/Tianlong Ltd, Xi'an-Shaanxi, China) used for this process was. " The PCR conditions were as follows: 50ºC for 1 hour, 94ºC for 5 minutes, then 40 cycles of 94ºC for 1 minute, 57ºC for 45 seconds, and 72ºC for 3 minutes with the final sequence at 72ºC for 5 minutes. PCR conditions were obtained from previous experience with the same primer at The Animal Biology and Molecular Biology of Veterinary Medicine Faculty of Udayana University. The results were visualized in a sigmoid graph with the cycle threshold (CT) value noted.

RESULTS AND DISCUSSION

The mRNA Quantification of Mice

The mRNA quantification is according to the cycle threshold value (CT Value) result of qRT-PCR. The data are presented in Table 1. The mRNA concentration and CT Values have an inverse correlation, meaning the lower the CT values of a sample, the higher the mRNA concentration in said sample, or vice versa. All mRNAs were detectable from samples collected at the third-day post-injection, while only a few of them were detected from samples collected on the sixth- and ninth-day post-injection. The HDT has the lowest average of CT-Value in all samples collected three days post-injection, meaning that the group has the highest concentration of mRNA among the four groups from that day's samples. LDT has the highest CT-Value average from samples collected on the same day, meaning the group had the lowest mRNA concentration.

Antibody Detection using 276R and 224L Antigens from Serum

Antibody detection using 276R and 224L are presented in Table 2. The Table present the optical density (OD) results of ELISA process. The OD readings and antibodies level in mice serums have a direct correlation. The higher the OD readings, the higher the level of antibodies in the serum samples. In both treatments (mice injected with high dose pSRM-ASF) and control (mice injected with high dose pCDNA-ASF) the OD values increased from two weeks to four weeks post-injection, then decreased in six weeks post-injection. The OD values in treatments were overall higher than control. Both independent and paired T-test showed no significant difference between control and treatment as well as the time of sampling ($p>0.05$).

Plasmid pCDNA3.1-SRM were developed as a solution for the problems in saRNA technology. This plasmid has many gene components that are vital for its function. This plasmid has cloning sites on pCDNA3.1+, having 5' and 3' Untranslated Region (UTR) for recognition by the translated picornavirus polymerase enzyme (Ferrer-Orta *et al.*, 2015; Kwon *et al.*, 2022), sites that facilitate protein cleavage in cells (Tse *et al.*, 2014), tag sequences for easy protection detection (Olins *et al.*, 1988), 5' and 3'-In-Fusion sequences for gene insertion using In-Fusion-cloning method (Park *et al.*, 2015), EcoRV restriction enzyme site for plasmid linearization and gene insertion cloning.

mRNA Quantification of Mice Samples

The qRT-PCR was done using SYBR™ Green PCR Master Mix (Invitrogen®, Waltham, USA) reagent, which is cheaper and not as complex as using probes. This is due to no requirement of extra processes to design the probe, as long as primers used for specific PCR reactions are available (Wei *et al.*, 2009). The fluorescence intensity goes higher as more double-stranded DNA is formed (Ramos-Payán *et al.*, 2003), which has an inverted correlation with the resulting CT-Value.

Table 1. CT-Value mRNA of mice samples

Sample	CT Value	Sample	CT Value
HDT 1.1	27.30	HDC 1.1	28.40
HDT 1.2	28.68	HDC 1.2	27.92
HDT 1.3	20.04	HDC 1.3	27.60
Average	25.34	Average	27.97
HDT 2.1	0.00	HDC 2.1	0.00
HDT 2.2	0.00	HDC 2.2	0.00
HDT 2.3	25.33	HDC 2.3	0.00
Average	0.00	Average	0.00
HDT 3.1	0.00	HDC 3.1	0.00
HDT .3.2	0.00	HDC 3.2	28.98
HDT 3.3	0.00	HDC 3.3	0.00
Average	0.00	Average	0.00
LDT 1.1	28.15	LDC 1.1	28.42
LDT 1.2	27.70	LDC 1.2	28.13
LDT 1.3	29.88	LDC 1.3	27.29
Average	28.58	Average	27.95
LDT 2.1	0.00	LDC 2.1	0.00
LDT 2.2	0.00	LDC 2.2	0.00
LDT 2.3	0.00	LDC 2.3	0.00
Average	0.00	Average	0.00
LDT 3.1	0.00	LDC 3.1	0.00
LDT 3.2	0.00	LDC 3.2	0.00
LDT 3.3	0.00	HDC 3.3	0.00
Average	0.00	Average	0.00

Legends:

"HDT"= Mice that received high dose pSRM-ASF injection; "LDT"= mice that received low dose pSRM-ASF injection; "HDC"= Mice that received high dose pCDNA-ASF injection; "LDC"=Mice that received low dose pCDNA-ASF injection;
Numbers "1.x", "2x" and "3x" indicates samples collected from three-, six-, and nine-days post-injection respectively; numbers "x.1" to "x.3" indicates samples number 1 to three.

Table 2. Optical density (OD) values of serums towards 276R-224L antigens

Sample number	Treatment			Control		
	Second weeks	Four weeks	Six weeks	Second week	Four weeks	Six weeks
1	0.203	0.399	0.286	0.155	0.298	0.122
2	0.197	0.255	0.302	0.056	0.322	0.213
3	0.098	0.276	0.143	0.102	0.424	0.193
4	0.322	0.452	0.221	0.155	0.233	0.255
5	0.244	0.177	0.266	0.188	0.166	0.098
Mean*	0.2128	0.3118	0.2436	0.1312	0.2886	0.1762
Standard Deviation	0.08	0.11	0.06	0.05	0.1	0.06

*All means in the same row are not statistically significant ($p>0.05$)

Legends

"Treatment" indicates mice that received high dose pSRM-ASF injection

"Control" indicates mice that received high dose pCDNA -ASF injection

The mRNA quantification results show that all samples were able to be detected on the three days post-injection (Table 1), meaning vaccines have been received successfully by the mice's muscle cells and phagocytized by macrophages (Teijaro and Farber, 2021). The detected mRNAs were products of both plasmids' expression, as the primer being used only attach to the gene segment of the mRNA from the plasmid's transcription. The addition of DNase enzyme prevents any remaining plasmid to interfere with the analysis. The result in Table 1 showed that high dose injection of pSRM-ASF (HDT group) had the highest concentration of mRNA caused by the polymerase expressed from the plasmid, amplifying the numbers of mRNA present in the cell (Bloom *et al.*, 2021).

There are two samples that were able to be detected on six- and nine-days post-injection (Table 1), however both of these data could not be averaged. This could happen is due to the fact of using different mice for different days of sampling. Each individual may receive different level of plasmid or different level of plasmid expression rate. Furthermore, we found that LDT (low dose pSRM-ASF mice) has lower mRNA concentration than control (Table 1).

The reduction in mRNA could be due to the natural existence of ribonuclease in animal cells (Goldspink and Pennington, 1971), reduction in RNA polymerase functionality, or due to the unstable nature of RNA itself (Lee *et al*., 2010). Other factors that could affect these results are improper plasmid encapsulation. Lipofectamine 3000 cannot transport plasmids to the nucleus completely, where it needs to function (Bai *et al.*, 2017; Rao *et al.*, 2015). Some plasmids might not reach the nucleus and were degraded/cleaved by nuclease enzymes. Lower dose vaccine would suffer more, as much less plasmid entering the nucleus compared to high dose with higher level of plasmid.

The same result was found by Nakamura *et al.* (2022), with mRNA that functions like saRNA having higher copy numbers than normal mRNA sampled from transfected BHK21 cells. Their saRNA's copy numbers decreased at 72 hours post-transfection. The decrease in mRNA concentration post-injection has also been reported by Maruggi *et al.* (2022). Multiple organs and blood samples of mice injected intramuscularly with the spike of the Covid-19 vaccine were analysed. Their results show that the highest mRNA concentration was found in injection location muscle and lymph nodes, while most mRNA concentration has reduced starting from eight days post-injection to 60 days post-injection except on lymph node. All of these results showed reduction in mRNA concentration starting from three days on this research (Table 1) and research by Nakamura *et al.* (2022) and eight days post-injection found in Maruggi *et al*. (2022) post-injection, but there might be mRNA left in the mice lymph node. For future research, further analysis in pCDNA3.1-SRM RNA polymerase function duration and capability are necessary.

Antibody Forming of pSRM-ASF Vaccine

The OD values have a direct correlation with antibody quantity (Table 2). Higher OD values means higher antibody level. Both vaccines can induce detectable antibodies with 10 µg of plasmid. African Swine Fever virus only infects domestic and wild pigs (Hyeon *et al.*, 2023). The mice used for this experiment have never been exposed to ASF virus previously, so it is highly possible that the obtained ELISA result in Table 2 resulted from the plasmid injection. The pSRM-ASF (treatment) antibody values are higher than the pCDNA-ASF (control) according to Table 2. The reduction in antibody value at six weeks post-injectionpost-injection could be caused by the reduction of antigen numbers, and these led to lower antibody values (Guirakhoo *et al.*, 2022).

Both T-test shows no significant difference statistically ($p>0.05$). Despite that, the higher antibody value of treatment is caused by the polymerase expressed from the plasmid, causing more RNA and directly increasing the antibody values (Ballesteros-Briones *et al.*, 2020; Pearson *et al.*, 2014). The result is not statistically significant, might be due to improper plasmid encapsulation as previously mentioned. Lower plasmid uptake by the cell's nucleus could result in lower antigen proteins expression, which also means weaker immunity.

The research of Vogel *et al.* (2017) reported similar results, with 1.25 µg saRNA influenza vaccine triggering equivalent immunity strength as 80 µg regular mRNA influenza vaccine. Similar results were also found by Geall *et al.* (2012), as 0.1 µg lipid nanoparticle (LNP) encapsulated saRNA can emulate the same immunity level as 1×10^6 IU viral replicon particle. They also found that 20 µg plasmid DNA encoding saRNA delivered using electroporation has higher immune response than that of a naked saRNA (Geall *et*

al., 2012). The use of electroporation and LNP can help the uptakes of plasmid into nucleus (Sokołowska and Błachnio-Zabielska, 2019). Other solutions are by increasing the amount of plasmid used for each vaccine dose, so that there would be more plasmid received by the nucleus. *In vitro* tests need to be done in further studies in addition to increase the accuracy of the mRNA analysis.

CONCLUSION

Injection of pSRM-ASF vaccine at high dose resulted in higher mRNA concentration and antibody induction than pCDNA-ASF.

SUGGESTIONS

Future research needs to be conducted using cell culture for *in-vitro* observation regarding mRNA concentration, to properly monitor mRNA fluctuation. Multiple, higher doses of vaccine need to be tested as well to find the optimal dose, while also maintaining cost. The antibody biological activity should also be tested using serum neutralization test and/or challenge test.

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