Expression of *VP1* gene as a DNA vaccine candidate for foot and mouth disease using phospholipid and poly-lactic acid nanoparticles as a delivery system

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Abstract. The Foot and Mouth Disease outbreaks in Indonesia in 2022 give several disadvantages for livestock. The primary treatment of reproductive pathologies involves a combination of prevention, diagnosis, and treatment. Prevention strategies include vaccination against specific diseases that cause reproductive disorders. Vaccination is the primary preventive measure in managing the spread of the FMD virus. The *Viral Protein 1* (*VP1*) gene encodes the FMD virus capsid protein, which could be a target in developing a DNA vaccine for FMD. Therefore, the development of DNA vaccine in this study is focused on increasing *VP1* gene expression using the pEGFP-N1 vector in HeLa cells as a mammalian cell model, using Lipofectamine as phospholipid nanoparticle and Poly-Lactate Acid (PLA) as polymer nanoparticle to increase delivery efficiency to target cells. The method used in this study is divided into three main stages, namely cloning and transformation of Plasmid DNA (pEGFP-N1-*VP1*) in competent *E. coli* DH5α cells; formulation of recombinant Plasmid DNA complexes of PLA nanoparticles and Lipofectamine Plasmid DNA nanoparticles; and determination of *VP1* gene expression in a mammalian expression system. The *VP1* gene delivered with Lipofectamine was successfully expressed at a level of 516.25-fold, while PLA was expressed at 114.08-fold in HeLa cells as a mammalian model. Both nanoparticles successfully delivered the *VP1* gene into mammalian cells; however, the formulation of PLA nanoparticles requires further optimization to achieve more optimal results.

1 Introduction

Indonesia is one of the Asian countries experiencing the spread of Foot and Mouth Disease (FMD). FMD virus is the cause of FMD, which can infect cloven-hoofed livestock such as cattle, buffalo, pigs, goats, and sheep. According to the World Organization for Animal Health (WOAH), FMD is a critical viral disease in livestock that spreads rapidly and has a considerable economic impact. Data from Statistics Indonesia (BPS) in the

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"Livestock in Numbers 2022" report indicates that the livestock subsector in Indonesia is a key driver for national development, particularly in strengthening the national economy [1]. However, the introduction of the FMD virus into Indonesia has negatively impacted livestock production and hindered the country's ability to export FMD-susceptible livestock products.

The prolonged transmission of the FMD virus can lead to decreased reproductive rates and increased mortality among livestock. A weakened immune system in cows plays a role in disrupting the functioning of reproductive organs, resulting in a longer duration of days open (the interval required for a cow to conceive again after calving), a lower conception rate (the percentage of cows that achieve pregnancy following insemination within a specified timeframe), and a reduced calving rate (the rate of births in animals that may experience fertility decline due to viral infection) [2][3]. Consequently, to revitalize the productivity of cattle, a control program for FMD has been initiated, aiming to achieve FMD-free status for Indonesia, by the Decree of the Minister of Agriculture of the Republic of Indonesia Number 285/KPTS/PK.320/M/06/2023, which outlines the Roadmap for the Eradication of Foot and Mouth Disease from the Territory of the Republic of Indonesia. This initiative is intended to obtain WOAH recognition as an FMD-free country and to restore international trade activities [4][5].

Vaccination is the primary prevention of FMD virus transmission; however, some currently used vaccines still have limitations, such as low effectiveness, limited production capacity, and high production costs. As an alternative, recombinant vaccines have been developed using DNA recombination technology to produce vaccines that are more effective in protecting animals from FMD infection than conventional vaccines [6].

One of the structures of the FMD virus that can be used for the development of recombinant FMD vaccines is Viral Protein 1 (VP1). Among the four structural proteins forming the FMD virus capsid, VP1 is the most immunogenic and effectively induces strong humoral and cellular immune responses in vaccinated animals [7]. VP1 is also the main antigenic determinant and the primary target of neutralizing antibodies, making it the most studied component of the FMD virus and a key candidate for vaccine development [8].

DNA vaccines are small and weak, making them susceptible to degradation by physical and biological agents [9]. Nanoparticle-based delivery systems can enhance vaccine efficacy by directing them to the target site within the host, promoting immune responses through improved antigen absorption and phagocytosis by Antigen-Presenting Cells (APC) [10]. Nanoparticles also serve as adjuvants, replacing traditional adjuvants that are toxic and difficult to degrade. Polylactic acid (PLA) nanoparticles, a type of polymer-based nanomaterial, hold potential for nanomedical applications such as drug, protein, and gene delivery [11][12]. Phospholipid nanoparticles, conversely, act as transfection agents dependent on cationic lipids, which can interact with negatively charged nucleic acids, enabling efficient DNA delivery into cells [13].

Research conducted by Li *et al.* [14] demonstrated that the VP1, VP0, and VP3 proteins from the SAT2 serotype of the FMD virus were successfully expressed in *E. coli* using the pET-32a vector and effectively induced antibody production in mice. Additionally, it was reported that VP1 is the antigen with the highest immunogenicity. However, studies on the expression of the *VP1* gene in HeLa cells using the

Lipofectamine™ 3000 and PLA nanoparticle delivery systems have not yet been conducted. Therefore, in this study, the *VP1* gene from serotype O was expressed in HeLa cells using the pEGFP-N1 vector and the delivery systems of Lipofectamine™ 3000 and PLA nanoparticles.

2 Materials and methods

2.1 Construction of *VP1* gene cassettes

The *VP1* gene sequence was obtained from NCBI (J02185.1) and optimized using codon optimization for system expression of *Bos taurus* by Gene Universal Inc. The cloning and expression cassettes were constructed by adding a start codon, restriction site of *Bgl*II and *EcoR*I restriction enzyme, and double stop codon (Figure 1). The gene was synthesized by Gene Universal Inc. and inserted into a pEGFP-N1 vector to produce pEGFP-N1-*VP1.*

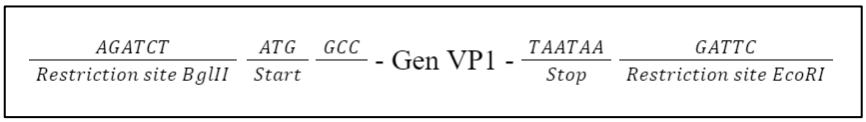

Fig. 1. The *VP1* gene was designed by adding the restriction site of *Bgl*II and *EcoR*I restriction enzyme, codon start, and double codon stop.

2.2 Cloning and transformation

A total of 2 µl of synthetic gene pEGFP-N1-*VP1* (concentration 10 ng/µl) was added to 150 µl of *Escherichia coli* (*E. coli*) as competent cells. Subsequently, the cells were incubated on ice for 30 minutes. Afterward, the heat shock technique was applied to the cells at 42°C for 45 seconds, followed by rapid re-incubation on ice for 2 minutes. Afterward, 1 ml of Luria Bertani broth medium was added following the heat shock treatment. The cells were incubated for 1 hour at 37°C using a shaker at 140 rpm. Subsequently, 25 µl and 100 µl of the cell culture from the LB broth medium were plated onto LB agar medium that contained 50 µg/ml kanamycin. The culture then incubated for 18 hours at 37°C.

2.3 PCR colony

Five colonies that grew on kanamycin selection media (50 µg/ml) were suspended in PCR tubes using a sterile white tip (10 µl). The PCR tubes had been pre-filled with PCR mix consisting of 12,5 µl PowerPol, 0,5 µl Forward Primer *VP1* 10 µM, 0,5 µl Reverse Primer *VP1* 10 µM, and 11,5 µl NFW. The PCR mix had a total volume of 25 µl. Primer sequence for *VP1* (forward 5'AAC CAC CGT GGA GAA CTA CG 3' and reverse 5'GAT CTC CAG GTC GCT GAA GT 3'). The primer pair was a specific primer pair designed to detect the *VP1* gene from the FMDV virus and was intended with Primer3Plus (https://www.primer3plus.com). PCR was carried out following the PowerPol protocol under these conditions: an initial pre-denaturation at 98°C for 45 seconds, followed by 40

cycles of denaturation at 98°C for 10 seconds, annealing at 58,2°C for 30 seconds, and extension at 72°C for 30 seconds, and one cycle of post-extension at 72°C for 7 minutes. The PCR products were then subjected to electrophoresis on a 1% agarose gel run at 100 V. Afterward, the electrophoresis result was visualized through a UV transilluminator.

2.4 Plasmid isolation

A single transformant bacterium from the selection media containing 50 µg/ml kanamycin was cultured in 15 ml of liquid media containing 50 µg/ml kanamycin and incubated at 37°C using a shaker at 180 rpm for 16 hours. The culture was then subcultured into 110 ml of liquid media containing 50 µg/ml kanamycin at 37°C. The culture was incubated with a shaker at 180 rpm for 16 hours, followed by plasmid isolation using the Plasmid DNA Extraction Maxi Kit (Favorgen Biotech Corp). The isolation of recombinant plasmid DNA was carried out according to the instructions in the kit. Subsequently, the concentration of the isolated plasmid was measured using a NanoDrop (MaestroGen).

2.5 Confirmation using restriction enzyme

A restriction enzyme confirmed the isolated Plasmid DNA. Amount of 1 µL isolated Plasmid DNA was mixed with 1 µL of *Bgl*II enzyme (10 U/µL), 1 µL *EcoR*I enzyme (20 U/µL), 2 µL of restriction enzyme buffer, and NFW and then incubated for 15 minutes. The enzyme was inactivated by heating at 85°C for 20 minutes. The plasmid was then subjected to electrophoresis to confirm the success of the restriction using agarose gel 0.8% running at 100 V and then visualized using a UV transilluminator.

2.6 Sequencing

50 µl of unpurified PCR product and the pEGFP-N1 universal forward and reverse primers were sent for sequencing to PT Genetika Science Indonesia. The obtained sequencing data was then aligned with the *VP1* sequence from the database and optimized using the BLAST program (NCBI).

2.7 Plasmid DNA nanoparticle formulation

Formulation of PLA nanoparticle based on a method from Unsunidhal *et al.* [15]. The complex of PLA-pEGFP-N1-*VP1* nanoparticles was carried out using the water-in-oil-in-water method (double emulsion technique). Amount of 0,4 mg PLA nanoparticles were dissolved in 1 mL dichloromethane (DCM) to make a concentration of 0,04% of PLA. PLA was mixed with 1,9 µL plasmid DNA and sonicated (on ice) using a sonicator for 30 seconds. 4 mL Polyvinyl Alcohol (PVA) 2% (dissolved in water) was added to the complex, vortexed for 10 seconds, and sonicated for 1 minute to form w/o/w complex. The complex was stirred for five hours for homogenization and evaporation of DCM. Centrifugation was carried out to obtain the PLA-Plasmid DNA nanoparticle complex in

pellet form. Formulation of phospholipid and plasmid DNA nanoparticles was carried out based on the Lipofectamine™ 3000 reagent procedure using 2,5 µg of DNA.

2.8 Transfection into HeLa cells

The initial stage of transfection in HeLa cell culture began with seeding cells at an initial density of 1,5 x 106 cells/well in 2 mL of complete medium in a 35 mm cell culture dish. The complete medium consisted of basal RPMI supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.5% fungizone. The cells were then incubated in a CO2 incubator at 37°C for 24 hours until they reached 70-80% confluency. The old medium was then removed and replaced with a new medium containing a sample mixture, and the cells were incubated again in a CO2 incubator at 37°C. The transfection of HeLa cells was done with the following treatments: 1) cell control, 2) PLA-based without plasmid DNA, 3) naked plasmid DNA, 4) LipofectamineTM 3000-Plasmid DNA, and 5) PLA-Plasmid DNA complex. All treatment cultures were transfected with 2.5 µg of recombinant plasmid DNA and incubated for 24 hours. Fluorescence observation of the EGFP-VP1 protein was performed on transfected HeLa cells that had reached 70-80% confluence on a cover slip. The fluorescence was observed using a Microscope Confocal ZEISS LSM 800 at 40x magnification.

2.9 Real-time quantitative PCR

RNA isolation was performed using a kit (Zymo Research), resulting in 35 ng of RNA per transfection treatment in HeLa cell cultures. Subsequently, the RNA was used as a template for cDNA synthesis using the SMOBIO RP1400 Reverse Transcription Kit II for RT-PCR. For mix A preparation: the RNA samples were adjusted to a concentration of 2 µg and mixed with one µl of Oligo (dT)/Random Primer mix in a tube, followed by the addition of DEPC-Treated H_2O to make a total volume of 10 µl. The mixture was incubated in a thermocycler at 70°C for 5 minutes, then placed on ice for 1 minute. Mix A in the PCR tube was combined with mix B containing 4 µl of 5x RT Buffer (DTT/dNTPs), 5 µl of DEPC-Treated H_2O, and 1 µl of RTase/RI Enzyme Mix, resulting in a total volume of 20 µl for the combined mixes (mix A and mix B). Reverse transcription was carried out in a PCR tube incubated at 25°C for 10 minutes, followed by incubation at 42°C for 50 minutes, and the reaction was terminated at 85°C for 5 minutes. The thermocycler was then set up to perform PCR using primers to detect the *VP1* gene.

2.10 Analysis statistical

Data analysis was conducted by performing relative gene expression analysis adapted from the method by Livak and Schmittgen [16]. The Ct values of the *VP1* gene obtained from RT-qPCR readings were then used to calculate the relative expression values or ΔΔCt. This analysis was used to determine the relative expression of the *VP1* gene compared to the GAPDH as an internal control gene.

3 Results and discussion

3.1 Construction of pEGFP-N1-VP1

The codon optimization conducted at this stage refers to the codon preferences of *Bos taurus* or water buffalo as the expression system model for mammals, particularly cloven hoof animals. The quality of codon optimization can be measured by the Codon Adaptation Index (CAI), a metric used to evaluate the codon usage of a specific gene by the codon preferences of the target organism. The *VP1* sequence of the Foot-and-Mouth Disease Virus (FMDV) had a CAI value of 0,67 before optimization. After optimization, the CAI value increased by 0,31, reaching 0,98. According to Sharp & Li [17], the CAI value ranges from 0 to 1. A CAI value greater than 0,8 indicates that the sequence is well-optimized and can be effectively expressed in the target organism's expression system. The GC content of the *VP1* sequence was 0,54 before optimization, while the GC content of the *VP1* sequence after optimization was 0,66. A higher GC nucleotide content in the sequence leads to increased protein production.

The restriction enzymes used in this study are *Bgl*II and *EcoR*I. It was found that after codon optimization, there are no sequences encoding restriction sites for these two enzymes. The presence of restriction sites in the middle of the *VP1* sequence after codon optimization was avoided to prevent the sequence from being cleaved by *Bgl*II and *EcoR*I at mid-sequence positions, which would result in an incomplete gene expression or sequence damage.

3.2 Transformation and cloning of pEGFP-N1-*VP1*

Colony PCR with *VP1* gene primers will amplify the target gene, producing an amplicon of 199 bp. Suppose the colony PCR electrophoresis results show a DNA band of 199 bp. In that case, it confirms that the bacterial colonies contain plasmid DNA with the inserted *VP1* gene of the Foot-and-Mouth Disease Virus (FMDV). The negative control for colony PCR is non-transformed *E. coli* DH5α bacterial colonies, while the positive control is the synthetic *VP1* gene that has not been transformed. PCR result is shown in Figure 2.

The absence of DNA bands in the negative control is due to the lack of a *VP1* DNA template that the *VP1* gene primers can amplify. The positive control PCR successfully amplified a DNA fragment of 199 bp, consistent with the expected amplicon size produced by the *VP1* gene primers. Subsequently, the electrophoresis results of the bacterial colony samples also show a DNA band of 199 bp, parallel to the DNA band produced by the positive control. Based on these results, it can be confirmed that the bacterial colonies have plasmid DNA with the inserted *VP1* gene of the Foot-and-Mouth Disease Virus (FMDV).

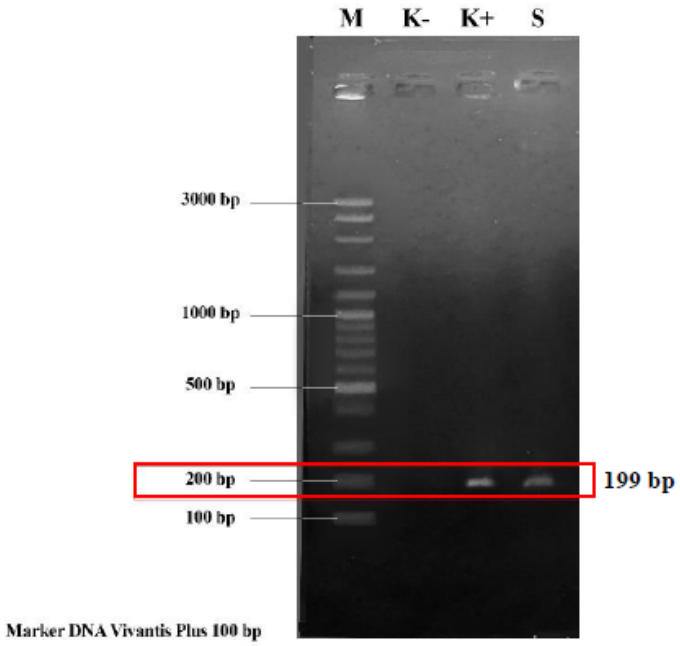

Fig. 2. PCR result to confirm the presence of *VP1* gene in bacteria colonies, PCR result visualization using agarose gel 1% with Vivantis Plus 100 bp as DNA ladder. Marker (M), negative control (K-), positive control (K+), and sample (S).

3.3 Isolation of plasmid DNA pEGFP-N1-*VP1*

The bacterial colonies confirmed to contain plasmid DNA with the inserted *VP1* gene of the Foot-and-Mouth Disease Virus (FMDV) were subsequently isolated to obtain only the plasmid DNA. This purified plasmid DNA will be used with PLA nanoparticles in the subsequent formulation step. The concentration of isolated plasmid DNA obtained was 2106,69 ng/µL with a DNA yield or absorbance value of 1,935.

After electrophoresis, restriction treatment using *Bgl*II and *Eco*RI generated two DNA bands in agarose gel (Figure 3). Sample 1 in Figure 3 is Plasmid DNA cut by a restriction enzyme. Sample 2 is plasmid DNA uncut. There are two DNA bands on sample 1 line; one is approximately 732 bp in size and is estimated to contain the *VP1* gene (711 bp), start codon, stop codon, and restriction sites (21 bp). Another DNA band around 4.707 bp is estimated to be another part of the plasmid DNA. Based on these results, it can be concluded that the isolated plasmid is plasmid DNA with the inserted *VP1* gene.

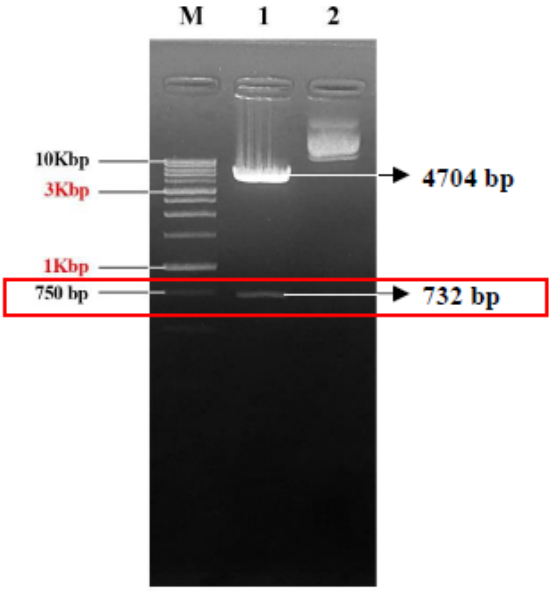

Fig. 3. Result of plasmid DNA restriction using *Bgl*II and *EcoR*I restriction enzyme. PCR result visualization using agarose gel,8% with SMOBIO Plus 1 kbp as DNA ladder. Marker (M), Plasmid DNA cut (1), Plasmid DNA uncut (2).

3.4 Sequencing

PT Genetika Science Indonesia conducted sequencing, and the results were processed using ClustalW software to obtain a consensus sequence. The consensus sequence of the *VP1* gene obtained from sequencing was analyzed using BLAST software to determine its similarity to the *VP1* sequence in the optimized database. The BLAST results showed a 100% identity percentage, indicating a high level of similarity between the recombinant plasmid DNA sequence and the optimized database sequence. The high identity percentage suggests no mutations occurred during the transformation and cloning process.

3.5 Transfection and confocal microscopy

Transfection using Lipofectamine and PLA nanoparticle complexes successfully delivered the plasmid DNA into HeLa cells, as shown in Figure 4. This was confirmed by the presence of green fluorescence, indicating the expression of the EGFP protein. The *VP1* gene was integrated with the N-terminal of pEGFP-N1 and would be expressed before the EGFP protein, so the presence of the EGFP protein signifies that the *VP1* gene was also successfully expressed. No fluorescence was observed in the negative control, which consisted of HeLa cells that were not transfected (1b), indicating no errors in detection or other factors causing fluorescence apart from the presence of the EGFP protein. The fluorescence in cells transfected with the Lipofectamine-Plasmid DNA was brighter

compared to cells transfected with PLA-plasmid DNA. However, cells transfected with PLA-plasmid DNA were more colorful than plasmid DNA without nanoparticles (3b).

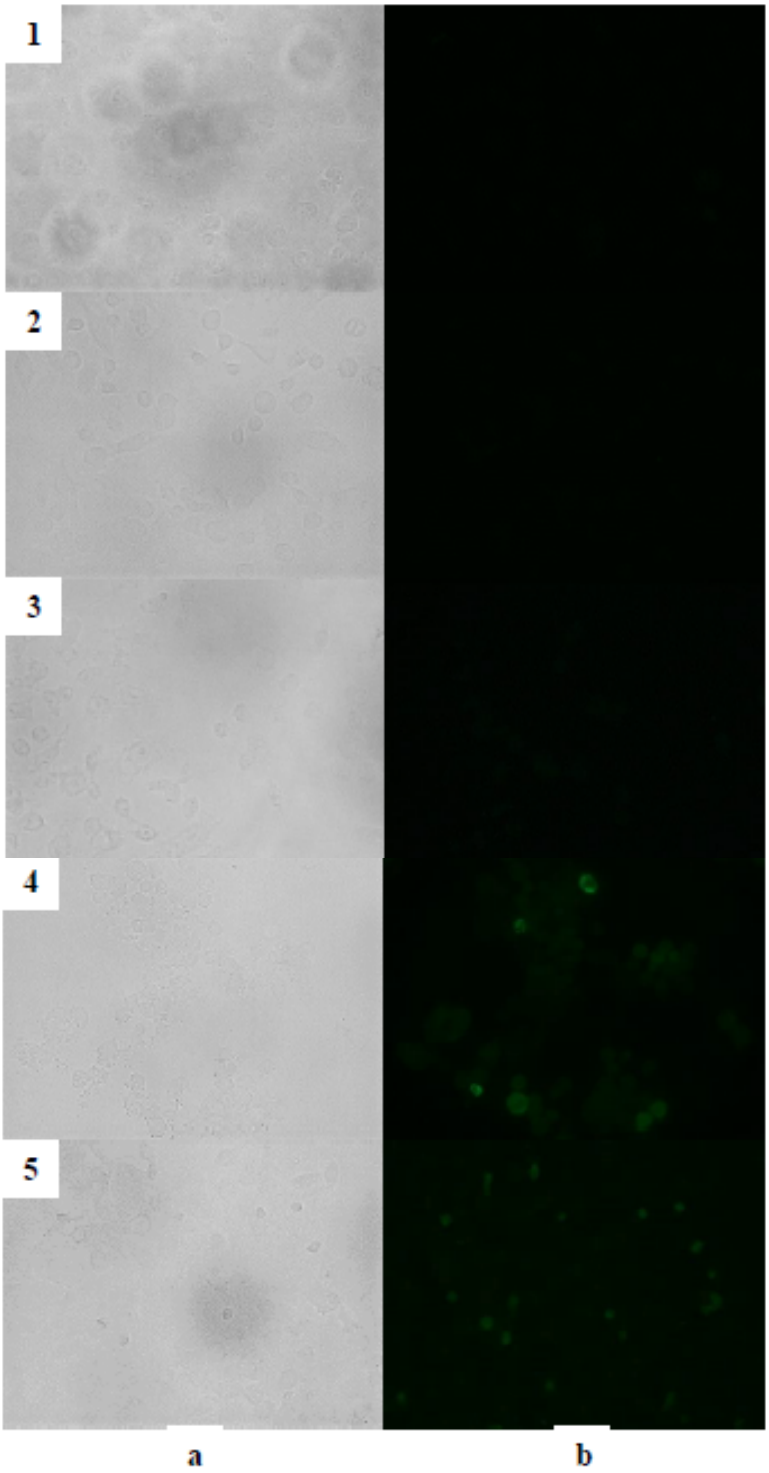

Fig. 4. Confocal image of HeLa cells under a ZEISS 800 confocal microscope at 40x magnification. HeLa cell appearance in bright field mode (a) and green mode (b). HeLa cells without transfection treatment (1), HeLa cells transfected with PLA base (2), HeLa cells transfected with naked plasmid DNA (3), HeLa cells transfected with LipofectamineTM 3000 (4), and HeLa cells transfected with PLA-plasmid DNA nanoparticle complexes (5).

3.6 Expression of *VP1* gene in HeLa cell

The expression level of the *VP1* gene was analyzed using real-time PCR (qPCR) with the generated cDNA. The *VP1* gene expression analysis results for each treatment are depicted in Figure 5. Administration of plasmid DNA delivered with PLA nanoparticles resulted in a 114,08-fold increase in *VP1* gene expression. In contrast, administration of plasmid DNA without nanoparticles led to a 0.01-fold expression or nearly no expression of the *VP1* gene. Based on these results, it can be concluded that delivering plasmid DNA using Lipofectamine and PLA nanoparticles successfully enhances the expression of the *VP1* gene. Additionally, *VP1* gene expression from plasmid delivered by Lipofectamine™ 3000 was higher at 516,25-fold compared to PLA nanoparticles. This finding aligns with qualitative results observed from confocal fluorescence microscopy readings.

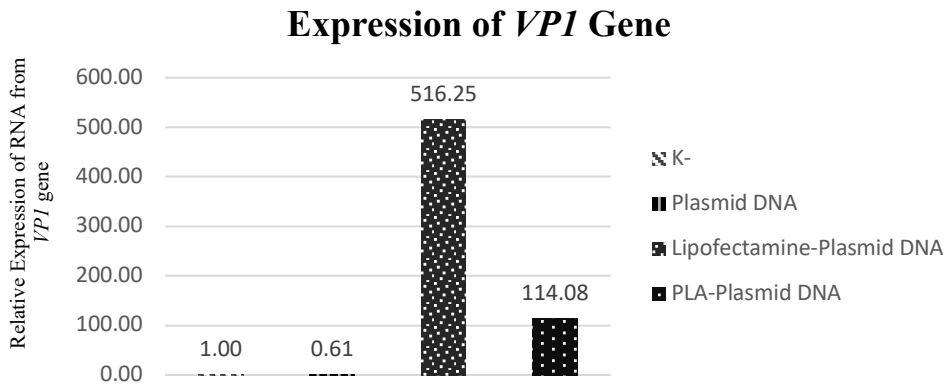

Fig. 5. Expression of *VP1* gene at mRNA level in HeLa cells. Control of HeLa cell without transfection treatment (1,00) as qPCR calibration. The *VP1* gene was expressed 0,01-fold in HeLa cells after being transfected with naked Plasmid DNA. *VP1* gene was expressed 516,25-fold in HeLa Cells after transfecting LipofectamineTM 3000-Plasmid DNA and 114,08-fold after transfected with PLA-Plasmid DNA nanoparticle.

4 Conclusions

The expression level of the *VP1* gene was analyzed using real-time PCR (qPCR) with the generated cDNA. The *VP1* gene expression analysis results for each treatment are depicted in Figure 5. Administration of plasmid DNA delivered with PLA nanoparticles resulted in a 114,08-fold increase in *VP1* gene expression. In contrast, administration of plasmid DNA without nanoparticles led to a 0.01-fold expression or nearly no expression of the *VP1* gene. Based on these results, it can be concluded that delivering plasmid DNA using Lipofectamine and PLA nanoparticles successfully enhances the expression of the *VP1* gene. Additionally, *VP1* gene expression from plasmid delivered by Lipofectamine™ 3000 was higher at 516,25-fold compared to PLA nanoparticles. This finding aligns with qualitative results observed from confocal fluorescence microscopy readings.

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