Expression of *gag*-CA Gene of Jembrana Disease Virus with Cationic Liposomes and Chitosan Nanoparticle Delivery Systems as DNA Vaccine Candidates

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Highlights

- The *gag-CA* gene from the Jembrana Disease Virus was successfully optimised and constructed in the pEGFP-C1 vector to produce recombinant DNA pEGFP-C1-gag-CA.

- Chitosan successfully encapsulates the recombinant DNA producing nanoparticle using mass ratio of DNA with chitosan 1:2 to 1:4.

- The recombinant DNA vaccine was successfully delivered and expressed in cell line with cationic liposomal and chitosan nanoparticle delivery systems.

Expression of *gag*-CA Gene of Jembrana Disease Virus with Cationic Liposomes and Chitosan Nanoparticle Delivery Systems as DNA Vaccine Candidates

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Abstract: One of the obstacles in increasing the population of Bali cattle is the presence of infectious diseases called Jembrana disease that attacks only Bali cattle. This disease is caused by Jembrana Disease Virus (JDV). A prevention method has been introduced; A DNA vaccine candidate was developed by inserting the *gag*-CA JDV gene in the pEGFP-C1 vector and a delivery system using cationic liposomes and chitosan nanoparticles, which is an important factor in the success of DNA vaccines in inducing immunity. The purpose of this study was to know the expression of the *gag*-CA gene with these delivery systems and the effectiveness of each delivery system. The expression of the gag-CA gene was tested using HeLa cells. The *gag*-CA gene JDV has been optimised using codon and inserted in pEGFP-C1. Then, the recombinant DNA was cloned using the *E. coli* DH5α host. The improvement result was confirmed using polymerase chain reaction (PCR), restriction analysis and sequencing. Recombinant DNA plasmids have been isolated and formulated with cationic liposomes and chitosan nanoparticles. The best characterization of encapsulation with gel inhibition test was chitosan nanoparticles with ratio mass of DNA with chitosan 1:2, diameter size about 178.0 nm and zeta potential +22.5 mV from the measurement using Particle Size Analyzer, while cationic liposome complex was prepared using commercial reagents. The expression results were analysed using fluorescence observation, Two-Step RT-PCR followed by Real-Time PCR analysis and Western Blot. The results showed the design of recombinant DNA plasmids with cationic liposomal and chitosan nanoparticle delivery systems expressing the *gag*-CA gene JDV. This is evidenced by the proliferation of EGFP-Capsid fusion proteins in HeLa cells and Normalised Expression in Real Time PCR, i.e. 5.36029 for cationic liposome conducting system and 0.00533 for

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chitosan nanoparticle delivery system. Also a positive band was also obtained with cationic liposomal delivery system on Western blot.

Keywords: Jembrana Disease Virus, Gen *gag*-CA, Delivery System, Cationic Liposomes, Chitosan Nanoparticles

INTRODUCTION

The fulfillment of people's need for meat protein resources have to be balanced with the increase of livestock population. Bali cattle (*Bos javanicus*), as a local meat source, is expected to be the main beef source instead of imported livestock. However, the effort of increasing Bali cattle population is hampered by complex problems one of which is the presence of Jembrana disease, an infectious disease that only attacks Bali cattle. The disease is caused by virus of family *Retriviridae* genus *Lentiviridae* (Kertayadnya *et al.* 1993; Chadwick *et al.* 1995), known as Jembrana Disease Virus (JDV) that has caused high mortality of Bali cattle across Indonesia for many decades.

A therapy using antibiotics against viral diseases is less effective. Therefore, an alternative prevention strategy is important to mitigate mortality within the animal, such as developing more efficacious vaccine. Recently, vaccine is produced primarily from an attenuated disease agent or an antigen isolated from unknown disease agent. The limited supply of materials due to complicated, time-consuming isolation techniques encourages experts to develop vaccines through recombinant DNA techniques. One of the recombinant DNA technology products is the DNA vaccine. This vaccine is made by inserting a DNA or gene that encodes an immunogenic protein into a eukaryotic expression vector.

One of the structural genes of JDV used as a source of DNA vaccine is the *gag* gene subunit Capsid (*gag*-CA). The gene that encodes the Capsid (CA) protein is targeted by vaccine sources a major protein (Kertayadnya *et al.* 1993) and an immunodominant antigen (Barboni *et al.* 2001). In addition, CA proteins are positive with antibodies from JDV-infected animals (Hartaningsih *et al.* 1994) and these proteins are often used for serologic detection of JDV-infected Bali cattle (Burkala *et al.* 1998, 1999; Zheng *et al.* 2001).

DNA vaccine requires a delivery system to obtain the target action. The demerit of DNA vaccine is its low efficacy in inducing immune system in organisms due to the limitation in its delivery system (Wang *et al.* 2011). In intramuscular injection of DNA vaccines, DNA molecules fail to enter antigen-presenting cells (APCs) and to induce immune responses because the nucleic acids cannot easily penetrate cell membranes (Pachuk *et al*. 2000). On the other hand, the cationic liposomal delivery system is often used as a DNA delivery system in gene therapy; this is because the positive charge of cationic liposomes can be used as DNA condensation reagents (Jufri 2004) and also, chitosan and its derivatives are among the most widely used natural polymers for drug delivery or DNA delivery for gene therapy (Hashimoto *et al*. 2005). Cationic liposomes and chitosan nanoparticles

are known to have strong electrostatic interactions with negative DNA charges (Fang *et al.* 2001). Due to the presence of amino groups, chitosan has a positive charge so that it can stick with negative-charged DNA forming complex and protect it from nuclease degradation. Biocompatible chitosan also helps to facilitate DNA in penetrating cell phospholipid membranes both *in vitro* and *in vivo*, and can be degraded by nuclease in serum (Hashimoto *et al.* 2005). These property causes cationic liposomal and chitosan nanoparticles to be good candidates for DNA delivery systems. The purpose of this study was to know the expression of the *gag*-CA gene with these delivery systems and the effectiveness of each delivery system.

MATERIAL AND METHODS

Gene Design and Preparing for Making DNA-Delivery Systems Complex

The sequence of nucleotide bases optimised codon and designed on the pEGFP-C1 vector was a sequence of nucleotides of the *gag*-CA JDV genes isolated and cloned by Kusumawati *et al.* (2002). The *gag*-CA genes were optimised based on the model of mammalian expression systems using the services of Gene Universal (www.geneuniversal.com). Furthermore, the *gag*-CA gene was constructed by adding *BglII* restriction sites (AGATCT), ATG codons as start codon, as well as TAATAA codons as double-codon stop and *EcoRI* (GAATTC) to form double digestion for confirmation purpose with restriction enzymes. The gene sequence was synthesised using the Custom Gen Synthesis service by Gene Universal (www.geneuniversal.com) and cloned to one of the available standard vectors, namely pEGFP-C1 so the JDV *gag*-CA target gene would bind the *egfp* gene and produced the conjugated EGFP protein in the N-terminus Capsid protein from gen *gag*-CA JDV. In addition, the design of this synthetic gene was made with the intention to insert it in another expression vector in subsequent research. The synthetic gene design can be seen in Supplementary data 1.

Then, plasmid recombinant transformation method used host bacteria (*E. coli* DH5α) and the colony was confirmed by colony PCR using *Primer gag*-CA *gene detection* (Table 1). The primary pair used was a specific primer pair to detect the *gag*-CA genes that were designed using the Online Primer3 Plus Software (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Next step, isolation of recombinant DNA plasmid was followed by electrophoresis. The plasmid recombinant obtained from the isolation method was then confirmed by restriction method using *BglII* and *EcoRI* enzymes and the restriction result was used for electrophoresis confirmation.

Table 1: List of primers used for the PCR amplification.

Code	Forward	Reverse
gag-CA gene detection	TCCGACGGCTCTCCTAG	TGGCTCTCCTTCCACACTCT
gag-CA gene sequencing	CATGGTCCTGCTGGAGTTGGTG	GAAATTTGTGATGCTGTTGC
Combination Primer I	CATGGTCCTGCTGGAGTTCGTG	TGGCTCTCCTTCCACACTCT
gapdh gene	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
Combination Primer II	CATGGTCCTGCTGGAGTTCGTG	AAATCTTCAGCTGCTTGGTCAG

Next, sequencing method used *Primer gag-CA gene sequencing* (Table 1) (references from the BD Bioscience Clontech Manual). Then sequenced data were analysed using the Clustal W program (www.genome.jp/tools-bin/clustalw).

Preparation of Chitosan Solution

A total of 20 mg of medium chain chitosan (Sigma) was dissolved in 100 mL of 1% acetic acid solution (Sigma) while the pH of the solution was adjusted to 5.0 using 1M NaOH (Sigma). A polymer solution with a final concentration of 0.02% was sterilised using a 0.2 µm filter.

Making Complex Reagents Delivering with DNA

Complex chitosan-DNA nanoparticles were made from as much as 1 µg recombinant DNA of 5 tubes (Biologix) and 2.5; 5; 10; 15; 20 µl (1:0.5; 1:1; 1:2; 1:3; 1:4) chitosan ratio of 0.02% heated separately at 50°C for 10 min. Then, the recombinant DNA solution and chitosan solution were mixed at 2500 rpm for 30 sec. Meanwhile, the implementation of cationic-DNA liposomes complex using 8 µg recombinant DNA was according to the existing guidelines.

Inhibition Test on Agarose Gel

The formation of the complex between the positive-charged chitosan and negative plasmid DNA could be observed by agarose gel electrophoresis. Agarose 0.8% with 50 mL TAE 10× solvent (Invitrogen) was boiled and then added with SYBR Safe DNA Gel Stain (Invitrogen) of 5 µl and poured onto gel mold. Electrophoresis was run at 100V for 40 min and then the result was observed under UV lamp. Samples of chitosan/DNA nanoparticles were compared with free DNA samples alone.

Determination of Particle Size, Zeta Potential and Cytotoxicity Test

The particle size and zeta potential were observed under Horiba SZ-100 conducted at the Laboratory of Pharmacy Department, Faculty of Mathematics and Natural

Sciences, Islamic University of Indonesia, Yogyakarta. Meanwhile, cytotoxic test was conducted by Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University with concentration of 1:2 for DNA and chitosan nanoparticles according to maximum complex capacity in inhibition test on Agarose Gel (Supplementary data 2) and cationic liposome-DNA complex concentration according to Lipofectamine™ 3000 *Transfection Reagent, Invitrogen* official guidance for transfection on a 2 µg culture well. This research used cationic liposome commercial (Lipofectamine™ 3000 *Transfection Reagent, Invitrogen*).

Transfection on HeLa Cell Cultures

HeLa cells were used in this experiment as they were used for Mammalia cells model. The purpose of this research was just to know effectiveness of each delivery system according to the resulted expression. The HeLa cell was cultured on 6 petri dishes with diameter 60 mm given cover slip in the middle with diameter 22 mm and initial cell density 0.8 106 cells/petri dish. Then cell culture was incubated at 5% CO_2 and 37°C for 72 h and culture media replacement was carried out every day. The culture medium consisted of Fetal Bovine Serum (Gibco) of 5 mL, 1 mL of Penstrep (Gibco), Fungizone of 250 µL (Gibco) and RPMI (Sigma) to taste up to 50 mL total solution. After preparing confluent HeLa cell culture 70%–80% with a density of 3.2×10^6, transfection was performed on HeLa cell culture in the following treatment groups: (1) without transfection (negative control); (2) transfection using only chitosan (negative control); (3) using only recombinant DNA plasmids alone (negative controls); (4) with cationic-DNA liposomes complex; (5) with chitosan-DNA complexity; and (6) NTC8685-EGFP DNA (Nature Technology Corp.) plasmid as a positive control method. Each culture treatment was transfected with 8-µg recombinant DNA plasmid. After transfection, the cell culture was incubated for 4 h then replaced with new culture medium and incubated for 24 h to further isolate RNA using RNeasy Mini Kit (Qiagen). The isolation procedure is in accordance with the procedure contained in the kit protocol, measurement of RNA concentrations using Nano drop (Maestro).

Electrophoresis of RNA Isolation Results

A 1% agarose solution was prepared with Tris Acetate-EDTA solvent (Invitrogen) 10× as much as 50 mL by heating. After the temperature reached 50°C, 5 µL Gel Stain (Transgen Biotech) was added and then stirred until blended. The agarose solution was then poured onto the gel mold. The solid agarose gel was prepared on agarose electrophoresis gel device by soaking Tris Acetate-EDTA buffer (Invitrogen) 10×. RNA isolation samples were mixed with Gel Pilot DNA loading dye 5× (Qiagen) and inserted in a gel well. Electrophoresis is run with a voltage of 100 V then the result is observed under UV lamp.

Fluorescence Observation of EGFP-Capsid Protein

Fluorescence observations of the EGFP-Capsid protein were carried out using a ZEISS LSM 800 Confocal Microscope with 20× magnification.

RNA Level Expression Test with Two-Step Reverse Transcription PCR followed by Real-Time PCR and Protein Level Expression Test with Western Blot

Testing antigen expression at RNA level was performed using two steps of RT-PCR (Applied Biosystem). In the first stage, a cDNA of RNA isolation was obtained from each transfection treatment of HeLa cell culture, which was then used as a template for the implementation of 1 µL previously diluted 10× using Nuclease-Free Water (Qiagen), the thermal cycler was adjusted for PCR process by using a combination primer I (Gene Universal) for detecting the target gene (Table 1). In addition, a specific primer was used to amplify genes encoding *gapdh* genes (Gene Universal) (Table 1) as an internal control the normality of the RNA cell expression in HeLa cell culture (Siegert & Nitsche 2004). The mixture of PCR mixture consisted of 1 µL cDNA template, Primary Forward of 0.8 µL, Reverse Primer as much as 0.8 µL, Master Mix PCR of 5 µL (Promega) and NFW of 2.4 µL (Qiagen), then PCR process was carried out with Pre-PCR condition at 95°C for 5 min, denaturation at 95°C for 30 sec, annealing at 51°C for 30 sec, elongation 72°C for 1.5 min, post PCR at 72°C for 10 min. All the above processes were done as much as 25 cycles. The PCR results were then electrophoresed using an agarose concentration of 1.5% and an electric current of 100 V. The PCR product obtained was then visualised on agarose gel by electrophoresis technique. The agarose gel used was 1.5% agarose gel and the dye used was SYBR Safe DNA Gel Stain (Invitrogen) which was mixed at the time the gel was made. The agarose gel electrophoresis and preparation procedure were performed in accordance with the procedure of Su *et al.* (2003). In each gel pit, 10 µL of sample or PCR product were added. To know that the size of the product was successfully obtained, one of the gel deposits was included into 6 µL of 100 bp DNA marker (Vivantis). Electrophoresis was carried out on the electrophoresis unit with a voltage of 100 volts for approximately 30 min. Thereafter, the gel was removed from the electrophoresis unit followed by an observation. The observation of electrophoresed gel was performed on top of UV illuminator. The expression analysis was also carried out with real-time PCR with a combination primer II (Gene Universal) (Table 1). The mixture of real-time mix of PCR was cDNA template which previously diluted with 10 µL Nuclease-Free Water (Qiagen) of 1 µL, Primary Forward of 0.4 µL with concentration 0.2 µM, Reverse Primer 0.4 µL with concentration 0.2 µM, Master Mix qRT PCR of 10 µL (Promega) and NFW of 8.2 µL (Qiagen) with final mixture was 20 µL. Real Time PCR implementation was carried out under Pre-PCR conditions at 94°C for 30 sec, denaturation at 94°C for 5 sec, annealing at 60°C for 30 sec. All of the above processes were carried out for 40 cycles followed by melting curve analysis process of the PCR results that were previously carried

out under heating conditions for 5 sec from 60°C to 95°C at intervals of 0.5°C. Plate reading was performed for each stage of heating at that interval. Analysis method used was double delta cq analysis method to *gapdh* gene.

Furthermore, a 20 µg (Image X) and 40 µg (Image Y) of the total protein isolation per treatment from the HeLa cells culture obtained previously were added with 5 µL 5× SDS protein buffer, heated in boiling water for 2 min, and inserted immediately in ice for 5 min. Then, the mixture was electrophoresed in SDS PAGE 12% at an electric current of 100 V. After the sample reached the bottom of the separation gel, the electric current was turned off, and then the gel was removed from the electrophoresis unit. Then, with a blotter and transfer buffer, the protein on the gel was transferred to the PVDF membrane (Image X) and nitrocellulose (Image Y). In the Western Blot analysis, the PVDF membrane (Image X) and nitrocellulose (Image Y) that has been transferred to the protein was blotted with the primary antibody Anti-eGFP (Fine Test) and secondary antibody Goat Anti-Mouse IgG Alkaline Phosphatase Conjugate (Fine Test) and then dyed NBT–BCIP. Proteins that react with antibodies will form the purple coloured bands (Khyseanderson 1984), then, an analysis to determine the molecular weight of the target protein using the Online ExPasy software – ProtParam tool (https://web.expasy.org/protparam/).

RESULTS

Gene Design and Preparing for Making DNA-Delivery Systems Complex

The *gag*-CA genes used in this study are the *gag*-CA gene that was previously obtained by Kusumawati *et al.* (2002). Based on the codon optimisation report by Gene Universal Corp., the increase of Codon Adaptation Index (CAI) was from 0.60 to 0.94. Furthermore, the gene was added to the side of *BglII* restriction, Start Codon (ATG), GCC codon, Stop Codon (TAATAA) and *EcoRI* restriction side in the corresponding sequence in Supplementary data 1. Adding GCC Codon is potential for next research where it is required for inserting this gene synthetic to NTC8685-EGFP Plasmid. Also, as GCC codon which code for Alanine protein is small molecule protein, it was not harmful for the main protein structure. The gene design was synthesised by Gene Universal Corp. then inserted on the restriction side *BglII* and *EcoRI* on the pEGFP-C1 vector, thus forming a recombinant DNA plasmid with a base length of 5407 bp according to the Construct Map in Supplementary data 3.

Two positive colonies had recombinant DNA pEGFP-C1-*gag*-CA with a 245 bp amplification product (Supplementary data 4). This DNA amplification product was obtained using primers of the *gag*-CA gene detection. Furthermore, the positive colony was cultured overnight in a liquid medium containing kanamycin to isolate the plasmid.

To obtain a recombinant DNA plasmid, a confirmed colony of transformation needs to be isolated from the plasmid. The plasmid isolation was performed using Maxi Extraction Kit (Favorgen Biotech Corp) and the implementation of the insulation based on the manual of the given kit procedure. The result of plasmid isolation had a ratio of purity of 1.960. The results of this plasmid isolation were electrophoresed with a 0.8% agarose gel for 40 min at 100 V (Supplementary data 5).

Before it was sent for sequencing, the plasmid must be confirmed in advance with the restriction. This stage was done using the help of enzyme *BglII* and *EcoRI*. Afterwards, the results were visualised, so two plasmid DNA bands and target genes were obtained from the process (Supplementary data 6).

The result of this restriction was two DNA bands measuring 4729 bp and 678 bp which were showed in Supplementary data 6. Size 678 bp is the size of the inserted target gene. This indicates that the target gene has been successfully inserted in the plasmid.

The results of the confirmed plasmid isolation through the above restriction method were then confirmed again by sequencing. Before shipment of sequencing samples to Genetics Science Corp., the samples were previously amplified to the target genes (*gag*-CA), using the Polymerase Chain Reaction (PCR) method. The samples were amplified using a universal primer for sequencing on a pEGFP-C1 vector, an amplification product of 994 bp due to the increase of several base pairs belonging to the *egfp* gene located on the N-terminus portion of the Capsid protein. PCR results can be seen in Supplementary data 7.

Recombinant DNA plasmids inserted by target genes via restriction and PCR methods were then sequenced using forward and reverse sequencing to determine the sequence of nucleotides. The sequencing results were analysed to ensure the absence of mutations in the target gene. The analysis was performed using Clustal W (application by comparing with the *gag*-CA gene sequence that has been optimised with codon. The following are the results of data processing using Clustal W (www.genome.jp/tools-bin/clustalw). The weakness of Sanger Sequencing method from Sequencing Service Provider in this research resulted in the mismatched base pairs because the length of the gene was too long. This length resulted in low-quality sequence which has been confirmed by chromatogram from DNA sequencing gene. To get better quality sequence, forward and reverse sequencing are required in the same colony to confirm which one DNA sequencing should be used according to its chromatogram result.

The data suggested that, the isolated recombinant plasmid did not undergo a gene mutation in order to express the protein of the *gag*-CA gene that would be proven by using fluorescence observation.

Observation of Chitosan-DNA Nanoparticle Complex, Particle Size Characterisation, Potential Zeta and Cytotoxic Test

Chitosan-DNA nanoparticle complex was formulated by Complex Coacervation method. The observation on occurrence of nanoparticle complex was conducted from a ratio of 1:0.5 to 1:4 with 0.8% agarose gel electrophoresis (Supplementary data 2).

After being observed in UV trans illuminator lamps, DNA with a 1:2–1:4 ratio was observed persisting in agarose gel deposits. The complexes occurring in nanoparticles made the DNA unable to migrate through the agarose gel. Measurement of nanoparticles was conducted using Particle Size Analyzer (PSA) with Dynamic Light Scattering Method conducted in Laboratory of Drug, Food and Cosmetic Testing, Islamic University of Indonesia using chitosan-DNA formulation sample with ratio 1:2, particle size obtained 178.0 nm. The charge from the chitosan or Zeta Potential nanoparticles was +22.5 mV.

Based on the results obtained from the cytotoxic liposomes and chitosan nanoparticles tests, it was concluded that the two transfection-conducting reagents were not toxic against the target cell with two times the mass of DNA according to the chitosan nanoparticles and according to standard protocols given for commercial cationic liposomes (Lipofectamine™ 3000). It was detected from the high percentage of living cells where there were 93% of living cells when it was given cationic liposomes and chitosan nanoparticles in which their respective levels are suitable for DNA delivery, 96% of living cells when it was given a mixture of chitosan-DNA, and 95% of living cells when it was mixed with liposomes.

Expression Test with Fluorescence Observation

This study used a pEGFP-C1 vector that was inserted by the *gag*-CA gene on the side of *BglII* and *EcoRI* restriction so that the Capsid protein expressed by the *gag*-CA gene bound to the EGFP protein expressed by the *egfp* gene. The EGFP protein bound the N-terminus portion of the Capsid protein. Cell culture HeLa that was transfected by treatments (1) without transfection (negative control); (2) transfected with chitosan nanoparticles as a negative control; (3) with transfection using only recombinant DNA plasmids alone (negative controls); (4) by transfection of cationic-DNA liposomes; (5) by DNA-nanoparticle transfection; and (6) with NTC8685-EGFP plasmid transfection as a positive control method followed by fluorescence observation with a confocal microscope. Fluorescence observations showing transfection with Treatments 2 and 3 presented a negative result while the Treatments of 4, 5 and 6 showed a positive result (Fig. 1).

The expression of *egfp-gag* gene from each of these transfection treatments was strengthened by RNA level expression results using a Two-Step Reverse Transcriptase PCR previously isolated RNA.
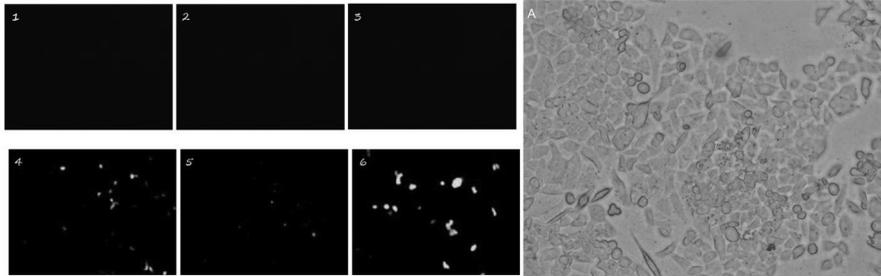
Figure 1. Fluorescence observation result on HeLa cell culture which has been given transfection treatment. HeLa cell cultures (A); without transfection as negative control (1); transfection with chitosan nanoparticles alone as a negative control (2); transfection with only recombinant DNA plasmids alone (3); transfection with cationic-DNA liposome (4); transfection with chitosan-DNA nanoparticles (5); and transfection with plasmid NTC8685-EGFP as a positive control method (6).

Electrophoresis Result of RNA Isolation from HeLa Cells with Treatment

The visualisation results of agarose gel electrophoresis conducted in this study showed 3 bands (Supplementary data 8). Based on the results, RNA isolation has good integrity. In addition to examining the integrity of RNA isolates through agarose gel electrophoresis, a Nano drop quantity check was also carried out. Total RNA isolate concentrations ranged from 11.89–266.15 ng/μL.

Expression Test with Two-Step Reverse Transcriptase PCR followed by Real-Time PCR

The result of electroporation of Two-Step Reverse Transcriptase PCR from treatment with cationic-DNA liposomal transfection agent (F) and chitosan-DNA (G) nanoparticles in Supplementary data 10 showed positive results with single-sized bands in accordance with the target product of 374 bp. The band was derived from amplification using a specific combination primer I to detect the *egfp-gag* target genes in recombinant DNA plasmids.

The results were confirmed expression using Real-Time PCR in the combination primer II. Based on the Real-Time PCR results, we found the expression of the *gag*-CA gene with catonic liposomes delivery system in the 14th cycle (Orang line) and on the 24th cycle for the chitosan nanoparticles (Pink line) in Fig. 2. Furthermore, to confirm the amplification product according to the target product, recombinant DNA plasmid stock was used as a positive control for amplication product analysis with melting curve. The results obtained were positive and had the same melting curve temperature of 85°C (Fig. 3).
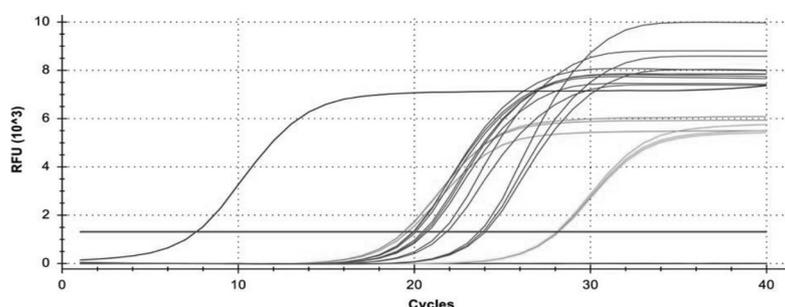
Figure 2. Real-time PCR results. Recombinant DNA plasmid control stock (blue); *gapdh* gene as internal control (green); DNA without delivery system as negative control (red); Nanoparticles of chitosan without DNA as negative control (red); the *gag*-CA genes with cationic liposome delivery (orange); *gag*-CA gene with delivery of nanoparticles (pink).
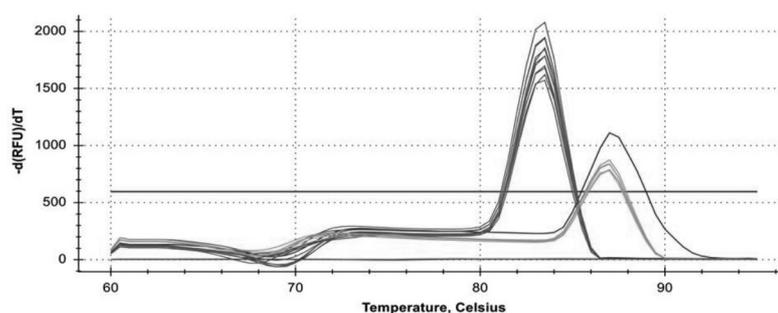

Figure 3. Melting curve result. Recombinant DNA plasmid control stock (blue); *gapdh* gene as internal control (green); DNA without delivery system as negative control (red); Nanoparticles of chitosan without DNA as negative control (red); the *gag*-CA genes with cationic liposome delivery (orange); *gag*-CA with delivery of nanoparticles (pink).

Based on quantification result of Normalized Expression with double delta cq analysis method toward *gapdh* (housekeeping gene) genes in Real Time PCR the value obtained was 5.36029 for cationic liposome delivery system and 0.00533 for cationic nanoparticle delivery system. In addition, according to the result of quantification of expression, the value of delivery effectiveness for cationic liposome delivery system was 1000 times higher than chitosan nanoparticles.

Protein Level Expression Test with Western Blot

The result of Western Blot electrophoresis from treatment with cationic liposome-DNA transfection agent (E for Image X) and (2 for Image Y) in Fig. 4 showed positive results with the band size of target protein of 52.2 kDa. The band was derived from the fusion of EGFP and Capsid proteins, each measuring

25

26.9 kDa and 25.3 kDa. This was confirmed by transfection treatment using cationic liposomes – NTC8685-EGFP (D) which only expressed EGFP protein alone with a size of 26.9 kDa (Cui *et al.* 2012). However, no band detected for the chitosan nanoparticle delivery system.
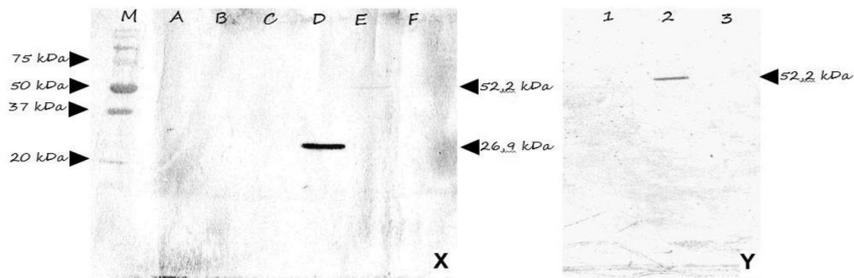

Figure 4. Western blot results with concentration per sample 20 µg and using membrane PVDF (Image X) and Western Blot results with concentration per sample 40 µg and using nitrocellulose membrane (Image Y). Result of protein isolation in HeLa by transfection treatment: without transfection treatment as negative control (A); by transfection treatment using chitosan alone as a negative control (B); with transfection treatment using only DNA [(C for Image X) and (1 for Image Y)]; by transfection treatment using cationic liposomes – NTC8685-EGFP (D); by treatment of cationic liposome-pEGFP-C1-*gag*-CA [(E for Image X) and (2 for Image Y)]; with transfection treatment of chitosan nanoparticles – pEGFP-C1-*gag*-CA [(F for Image X) and (3 for Image Y)].

DISCUSSION

The Codon Adaptation Index (CAI) is a calculation of the frequency used in each codon and calculates the geometric mean of used frequency in each protein in the Expression System target (Sharp & Li 1987). The CAI report from the Gene Universal (Supplementary data 9) guaranteed expression from this synthetic gene would be improving and also this standard method is potential for making a DNA vaccine. Therefore, the increasing CAI could be an indicator to optimise the expression of the *gag* gene encoding the JDV Capsid protein, whereas, the *gag* gene encoding the Capsid protein was used because this in *Lentivirus* has a Pathogen-Associated Molecular Pattern (PAMP) that can trigger the innate immune system (Gottlinger 2001).The optimisation of this gene was suggested to improve the expression of *Lentivirus* gene in eukaryotic host (Haas *et al.* 1996).

Supplementary data 5 showed that DNA has three forms, super-helix circular (I), circular-opened (II) and linear (III). These three forms of DNA have different migration velocities, in which form I migrates faster than forms II and III, respectively. Other factors affecting the speed of migrating DNA are the size of the DNA molecule, the agarose concentration, the electric voltage, the percentage, as well as the electrophoretic buffer composition (Vennison 2009).

The result of purity ratio measurement using Nano drop (260 nm and 280 nm) was obtained at 1.960 with concentration of 1595 ng/μL. DNA purity is expressed as a comparison between absorbance A 260/280. If the ratio is greater than 2.0, the probability of the sample is mixed with RNA and if the ratio is less than 1.8 there may be other proteins or contaminants such as phenols or other organic substances (Clark 2005).

The ionic bonds in this nanoparticle complex were the result of electrostatic interactions between positively charged chitosan and negatively charged DNA. Large molecules have difficulty in passing through the pores of the gel so they migrate more slowly through the gel than smaller DNA (Vennison 2009). Furthermore, DNA condensation of electrostatic interaction results between negatively charged DNA and chitosan are positively charged in the buffer, which is a colloid occurrence mechanism. Colloids are a mixture of heterogeneous substances (two phases) between two or more substances in which the colloidal particles of matter (dispersed/dispersed phases) are spread evenly in another substance (dispersing medium). Chitosan and DNA, each of which was originally dissolved in the buffer solution by the complex method of polyelectrolyte, can form suspended solids in the buffer solution. The ability of chitosan to envelope DNA is because the electrostatic interaction provides DNA protection against external factors. The repulsive force between similar particles prevents these colloidal particles from joining into one.

Dustgani *et al.* (2008) obtained chitosan nanoparticles of approximately 256–350 nm measured using Dynamic Light Scattering Method. Accordingly, the hydrodynamic diameter of the particles measured using the Dynamic Light Scattering Method is greater than the estimated size using a microscope. The size varies primarily due to the high developmental capacity of the chitosan nanoparticles. The size of chitosan complexes in the solution varies from 100 to 1,000 nm, but the population has the mean size of 178.0 nm. The size of the chitosan-DNA complex using coarsevation complex techniques ranges from 100 to 250 nm (Gregory *et al*. 2013) so in this study the formed complexes have a standard size (Ye *et al*. 2013). Other chitosan-DNA complexes were also produced with similar methods resulted in particles with sizes of 227.1 nm (Ye *et al*. 2013). Furthermore, the positive charge on the chitosan nanoparticle complex is critical for the efficiency of transfection in cells monolayer cultures in *in vitro*; the higher the positive charge on the chitosan nanoparticle complex, the higher the efficiency of transfection of chitosan nanoparticle complex as a conductor agent in in vitro monolayer culture cells (Bowman & Leong 2006).

Prior transfection assay, cytotoxicity assay was performed to assess the toxicity potential from chitosan complexes during exposure time with the cells. Chitosan solution and chitosan-DNA complex showed relative viability with the value higher than 80%. The use of similar chitosan complexes that give relative viability ranging from 80% to 90% was still assessed to have no cellular toxicity potential (Martien *et al*. 2007). The acid pH factor (pH 5) of the nanoparticle-carrying agent solution has little effect on the culture medium because the medial culture has a bicarbonate buffer system which maintains a pH of about 7.4. This

test indicates that chitosan complex does not exhibit a high toxicity potential for the cell during 4 h of exposure.

The visualisation results of agarose gel electrophoresis conducted in this study showed three bands (Supplementary data 10). According to Rapley and Heptinstall (1998), two bands of the three bands may show ribosomal RNA of 18S rRNA and 28S rRNA, which are the largest components in total RNA. The thickness (intensity) and sharpness of the tape fluorescence could show the quality of the total RNA isolate well enough. The total RNA isolate obtained in the study had a fairly good quantity because it exhibited a thick fluorescence of 18S rRNA and 28S rRNA bands.

Thin smears were seen in total RNA isolates obtained in the study. According to Farrell (2005), smears can show RNA components such as mRNA and tRNA. The mRNA component appeared as a smear because of the small amount of mRNA (1%–4% of total RNA) and high mRNA size heterogeneity. TRNA and rRNA components include low molecular weight RNAs that were seen as thin smears below the 5.8S rRNA band near the end of the gel. Based on these results, the RNA isolation had good integrity.

Based on RNA level expression test with Two-Step Reverse Transcriptase PCR followed by Real-Time PCR, it can be concluded that cationic liposome conducting system and chitosan nanoparticles can deliver recombinant plasmid of pEGFP-C1-*gag*-CA DNA. Cationic liposomes and chitosan nanoparticles successfully survived and protected DNA from nucleus damage. Cationic liposomes and chitosan nanoparticles manage to remain stable in extracellular fluids because extracellular fluids can interfere with the interaction of polyelectrolytes. After cationic liposomes and chitosan nanoparticles insert into target cells, they bound with a negatively charged cell membrane (Corwin 2009) and then trigger the occurrence of endocytosis.

In Supplementary data 10 and Fig. 2 on the red line, it can be seen that in the absence of cationic liposomes and chitosan nanoparticles as delivery systems, they protect recombinant DNA plasmids. The recombinant DNA plasmid is unable to cross the barrier to deal with nuclease degradation to reach the cell nucleus.

This is reinforced by the fluorescence observations in Fig. 1 which demonstrate the ability of the cationic liposome conducting system and the chitosan nanoparticles to deliver recombinant DNA plasmids into the cells, characterised by the visible green luminescing of EGFP-Capsid protein expression. The cationic properties of liposomes and chitosan nanoparticles with anionic recombinant DNA plasmids reacted with the Complex Coacervation method, and they had the ability to bind the negatively charged plasma membrane. Subsequently, it experienced the process of endocytosis and entered the nucleus to express its proteins.

Cationic-DNA liposomes and compact chitosan-DNA nanoparticles can protect recombinant DNA plasmids from enzymatic degradation. Ultimately, recombinant DNA plasmids can enter the cells and are able to express known EGFP-Capsid proteins from observations with fluorescence microscopy and expression test at RNA level with two-step reverse transcriptase PCR.

Based on the result of expression quantification at RNA level with real-time PCR obtained, the value of effectiveness of delivery in cationic liposome delivery system was 1,000 times higher than chitosan nanoparticles, and then this comparison was confirmed with band of protein expression test result with Western Blot (Fig. 4). Expression at the protein level was compared by visual qualitative. The high differences in expression between cationic liposomal carrier and chitosan nanoparticles were probably due to imperfect formulations of chitosan nanoparticles. Cationic liposomes also have advantages over chitosan nanoparticles, i.e. cationic lipids in liposomes can interact very well with anionic lipids in cell membranes (Shim *et al.* 2013).

The transfection assay indicates that delivery using the chitosan-DNA complex succeeded in bringing plasmid DNA into the cells and expressed to mRNAs which can be detected using RT-PCR and real-time PCR. However, there was a considerable difference when compared to commercial transfecting agent complex (Cationic Liposomes). This phenomenon is thought to be due to the slower release of plasmids from chitosan complex so only relatively fewer expression values in chitosan-treated cells can be detected. The DNA plasmid encapsulated by chitosan will be released in a controlled manner by polymer hydration process and polymer opening over time (Masotti *et al*. 2008). The plasmid DNA on the surface of the chitosan nanoparticles will emerge rapidly from the nanoparticles in the first 3 h at a physiological pH, while the rest will be released constantly up to 72 h with a DNA release of 70% of the initial quantity (Masotti *et al*. 2008). Longer-term expression observations are likely to show higher gene expression as compared with 24 h observation. Chitosan-DNA complexes with a higher chitosan mass ratio are likely to produce better complexes. Using higher chitosan mass ratio, particles with smaller diameter can be produced. Increasing the ratio of chitosan/DNA mass from 2:1 to 200:1 resulted in decreased particle diameter from 181.9 nm to 94.1 nm (Ye *et al*. 2013). The decrease in the particle diameter is expected to facilitate the uptake of particles by cells. In addition, increasing the mass ratio of chitosan/DNA can increase the positive value of the zeta potential of particles. A more positive charge will make it easier to attach the particles to the cell membrane that is negatively charged. Increasing the ratio of chitosan/DNA mass from 2:1 to 200:1 also decreased the zeta potential from +35.4 nm to +41.2 nm (Ye *et al*. 2013).

CONCLUSION

The cationic liposomal and the chitosan nanoparticle delivery systems showed an enhancement in delivering the *gag*-CA gene JDV. Based on the results, the *gag*-CA gene JDV could be expressed on HeLa cells that had transfected with both systems with Normalised Expression values against the *gapdh* gene in real-time PCR of 5.36029 for the cationic liposomes delivery system and 0.00533 for the chitosan nanoparticle delivery system. In addition, the effectiveness of the delivery

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system was found to be 1000 times higher by using a cationic liposomal delivery system that had been confirmed by protein level expression test with Western Blot.

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APPENDIX

<u>AGATCT</u> <u>ATG</u> <u>GCC</u> – *Gene of Interest* – <u>**TAATAA**</u> **GAATTC**
*Bgl*II   Start   Stop   *Eco*RI

Supplementary data 1. Synthetic gene of *gag*-CA from JDV.
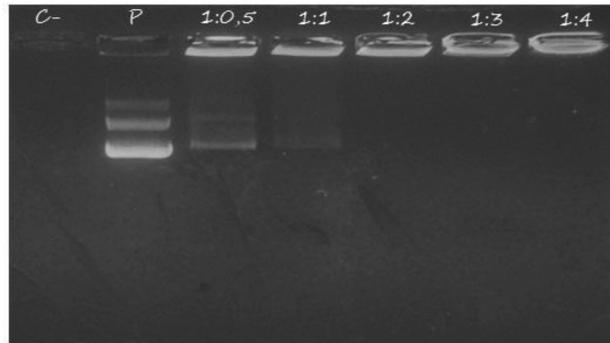
Supplementary data 2. Visualisation of chitosan-DNA complex with Ratio shows the ratio of mass between DNA and chitosan. One part of plasmid DNA is equivalent to 1000 ng of DNA, while one part chitosan is equivalent to 1000 ng of chitosan. All chitosan DNA nanoparticles manufacture using the same total volume, i.e. 30 µL (10 µL DNA solution mixed with 20 µL chitosan solution); Chitosan without DNA (C-); Plasmid without Chitosan (P).

Supplementary data 3. Construct Map of Plasmid Recombinant pEGFP-C1-*gag*-CA
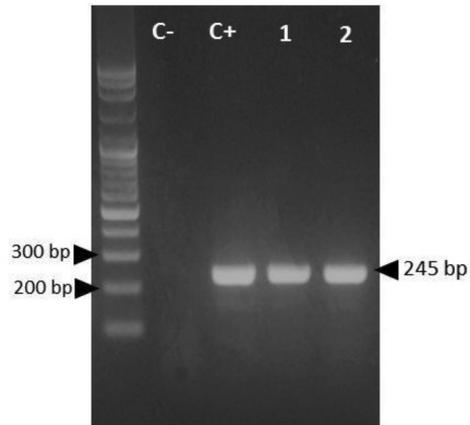
Supplementary data 4. Colony PCR of Transformant Bacteria. Colonies of *E. coli* DH5α Competents not transformed as negative control (C-), recombinant DNA of pEGFP-C1-*gag*-CA from stock as positive control (C +), Colonies 1 (1) and Colonies 2 (2).
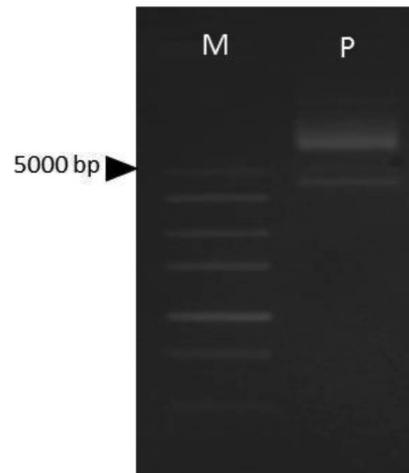
Supplementary data 5. Electrophoresis of Result of Plasmid Isolation on Agarose Gel. Marker 1000 bp (M); Recombinant DNA plasmid (P).
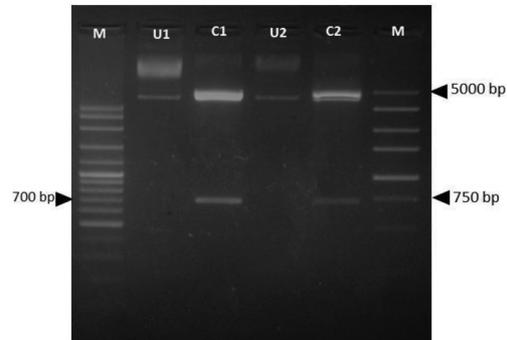
Supplementary data 6. Recombinant Plasmid Restriction with BglII and EcoRI enzymes. The plasmid is isolated from nonrestrictive transformant colonies (U), while the isolated plasmid from the transformant colonies is restricted during overnight (C). The number 1 shows the source of the transformant colony 1, while the number 2 denotes the source of the transformation colony 2; marker 100 bp (M).
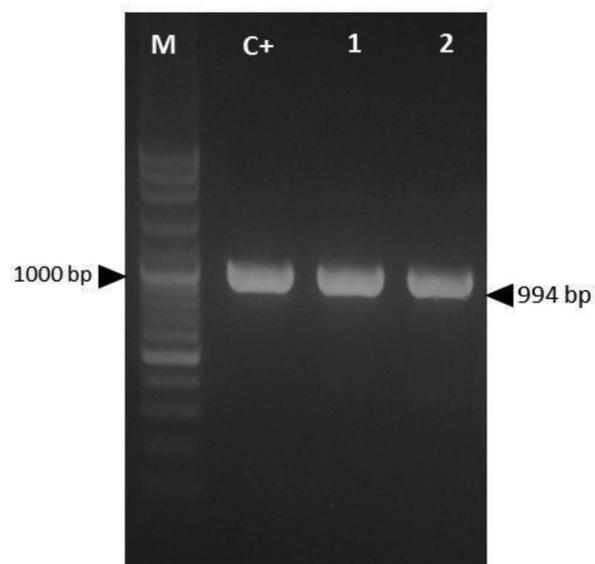
Supplementary data 7. Results of Plasmid DNA Amplification Recombinant with EGFPC and SV40pA sequencing primers. Recombinant plasmid DNA (C +) was used as a positive control, while the isolated plasmid from transformant colonies (1 and 2) was tested for Sanger sequencing process, 100 bp (M) marker.

Supplementary data 8. Electrophoresis results of RNA isolation from HeLa cells with treatment. Without transfection as a negative control (a); by transfection of chitosan nanoparticles alone as a negative control (b); by transfection using only recombinant DNA plasmids alone (c); with NTC8685-EGFP DNA plasmid transfection as a positive control method (described in the appendix) (d); with transfection of cationic-DNA liposomes (e); and by transfection of chitosan-DNA nanoparticles (f); marker 1000 bp (M).

The best value is 1 for sequence optimisation.

CAI: 0.60
Before Codon Adjustment

CAI: 0.94
After Codon Adjustment

Supplementary data 9. Codon Optimisation Report

Supplementary data 10. Results of Two-Step RT PCR Electrophoresis from HeLa cell with treatment. Without transfection as a negative control (A); transfection with chitosan nanoparticles alone as a negative control (B); transfection with only recombinant DNA plasmids alone (C); transfection with plasmid NTC8685-EGFP as a positive control method (described in appendix) (D); recombinant DNA plasmid stock as positive control (E); transfection with cationic-DNA liposome (F); transfection with chitosan-DNA nanoparticles (G); and *gapdh* gene amplification as internal control (H); and marker 100 bp (M).