Development of Virus-Like Particles Based Oral Vaccine against Canine Parvovirus

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Abstract

Canine parvovirus (CPV) is a contagious virus that causes enteric disease in all dog populations. The CPV capsid protein, VP2, plays an important role in virus infection and host immune response. To develop a vaccine against CPV, the gene encoding for VP2 protein was cloned and expressed in the baculovirus/insect cell expression system. The recombinant VP2 protein was successfully expressed and detected in the baculovirus-infected insect cells by Western blot analysis, using an antibody specific to CPV VP2 protein. It was found that the recombinant VP2 protein can self-assemble into virus-like particles (VLPs) with the size range from 50-100 nm as revealed by a transmission electron microscope. The VP2-VLPs were also shown to have hemagglutination activity, similar to CPV when incubated with goose erythrocytes. This VP2-VLPs were used for syringe and needle-free, oral vaccine development. Non-viral delivery agents, bacterium like particles (BLPs) derived from non-living *Lactococcus lactis*, known for their safety and efficiency for mucosal immune stimulation, were selected for the oral vaccine formulation. After the ad-mix method, VP2-VLPs bound to BLPs was detected with the immunofluorescent assay. This oral vaccine will be further investigated for its immune stimulation efficiency.

***Keywords*:** Canine parvovirus (CPV), VP2, Virus-Like Particles (VLPs), Bacterium-Like Particles (BLPs), oral vaccine

1. Introduction

Canine parvovirus belongs to Parvoviridae family. CPV virion is a small, icosahedral, non-enveloped particle-containing single-stranded DNA virus with a genome of approximately 5 kb. CPV infection causes severe myocarditis, enteritis and lymphopenia in dogs and is significant economic importance in breeding farms. The most abundant structural protein, namely VP2, plays an important role in virus-host interaction (Feng et al, 2014; Truyen, 2006). Conventional CPV vaccines are attenuated and inactivated the CPV vaccine. The use of the lived virus has raised concerns about the virus mutation and conversion to virulence strain (Mohan Raj, Mukhopadhyay, Thanislass, Antony & Pillai, 2010). Inactivated CPV vaccine also has safety issues regarding insufficient chemical inactivation of virus and required multiple doses. Furthermore, these vaccines are administered via injection. Thus, a safer vaccine should be developed. Virus-like particles (VLPs) which are assembled forms of virus proteins that mimic virus structure and can be conveniently produced by various expression systems, could be a promising alternative CPV vaccine. Parvovirus-like particles had been successfully produced in the baculovirus vector/insect cell expression system and shown to elicit both cellular and humoral immune responses (Jin et al, 2016). Since CPV infection is fatal in young pups, a convenient, painless, needle-free type of vaccine is preferable. The oral vaccine is thus formulated by mixing the CPV-VLP with an immune-stimulating carrier, designated Bacterium like Particle (BLPs), derived from food-grade bacteria *Lactococcus lactis*. A simple pretreatment of bacteria in hot acid destroys all cellular components, including intracellular components such as DNA. Thick layer cell wall made from peptidoglycan (PGN) of *L. lactic* bind with antigen via a binding domain called peptidoglycan hydrolase AcmA (Van Braeckel-Budimir, Haijema & Leenhouts, 2013). Mode of action of BLPs is well-defined and based on activation of the innate receptor, TLR2, which is a membrane surface receptor, specific for numerous bacteria, fungi, and viruses (Ramirez et. al, 2010).

2. Objectives

1. To develop CPV virus-like particles from recombinant VP2 protein (VP2-VLPs) from a baculovirus vector/insect cell expression system.
2. To prepare Bacterium like particles (BLPs) from *L. lactis*.
3. To formulate a VP2-VLPs based oral vaccine against canine parvovirus.

3. Materials and Methods

Materials

Spodoptera frugiperda (Sf9) cell line was purchased from Invitrogen Corp., USA. Canine parvovirus genomic DNA was obtained from the live attenuated vaccine (Primodog™, Merial, France). Mouse-anti VP2 antibody was purchased from Immune Technology, Netherlands).

Methods

3.1 Cloning of gene encoding VP2 protein and construction of recombinant VP2 baculovirus

CPV from the commercial live attenuated vaccine (Primodog™) was pelleted by centrifugation through 40% sucrose cushion at 100,000 g for 3 hours at 4°C. The viral pellet was resuspended in PBS and genomic DNA was extracted using the phenol/chloroform extraction method (Green & Sambrook, 2017). *VP2* was PCR amplified using genomic DNA as a template and cloned into a pFastBacHTb expression plasmid (Invitrogen, USA). The recombinant pFastBac HTb–VP2 was then transformed into *E. coli* DH10Bac competent cells containing baculovirus shuttle genome, bacmid. The transformed *E. coli* DH10Bac were propagated in Luria Bertani broth containing 50 mg/ml kanamycin, 7 mg/ml gentamicin, 10 mg/ml tetracycline, 100 mg/ml X-gal and 40 mg/ml IPTG (Isopropyl b-D-1-thiogalactopyranoside, Sigma). The recombinant bacmid was later extracted and characterized by PCR analysis using *VP2* specific primers. After PCR confirmation of *VP2* integration into the bacmid genome, 1 µg of recombinant bacmid DNA was transfected into of *Spodoptera frugiperda* (Sf9) cells ($1x10^6$ cells/ml) using cellFectin™ reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The culture supernatant of transfected cells containing recombinant baculovirus expressing *VP2*, namely recombinant VP2 baculovirus, was harvested at day 4 post-transfection by centrifugation at 2,500 g for 10 minutes. Titers of the recombinant VP2 baculovirus were determined by end-point dilution method (OReilly, 1992)

3.2 Recombinant VP2 gene expression and protein analysis

Recombinant VP2 baculovirus at MOI 1 was infected into $1x10^6$ cells/ml Sf9 cells cultured in TNM-FH medium supplemented with 10% Fetal Bovine Serum (FBS) in shake flask at 27°C. *VP2* gene expression was confirmed by reverse- transcription PCR analysis (RT-PCR). Total RNA was extracted from infected Sf9 cells using TRIZOL® LS (Invitrogen, USA) and cDNA were prepared using RevertAid Reverse Transcriptase (Thermo Fisher Scientific Inc., USA) as instructed by the manufacturer before PCR amplification using *VP2* specific primers.

Recombinant VP2 protein was also analyzed by Western blot analysis. Infected SF9 cells were harvested and lysed in TN buffer (150 mM NaCl, 50 mM Tris) containing 1X protease inhibitor cocktails (Sigma Aldrich, USA) using 25G needle. The supernatant of infected cell lysate was collected by centrifuge at 14,000 rpm for 10 min. Proteins were separated on 10% SDS/PAGE, transferred onto PVDF membrane (Invitrogen) and blocked with 5% skim milk in PBST (PBS with 0.05% Tween-20) for 30 min. The membrane was washed 3 times in PBST and incubated with 1: 1,000 diluted mouse-anti VP2 antibody (Immune Technology) at 4°C for overnight. The membrane was washed again with PBST and incubated with a 1:5,000 diluted HRP anti-mouse secondary antibody (Abcam, UK) in 5% skim milk in PBST. The specific band on the membrane was visualized using a chemiluminescence substrate and detected by a chemiluminescence scanner (LI-COR®, Bioscience, UK).

3.3 Recombinant VP2 protein purification

The supernatants of cell lysate samples in PBS were loaded onto a 25-70% sucrose gradient and centrifuged at 33,200 rpm for 90 min at 4°C in a TH-660 ultracentrifuge (Beckman). All fractions were collected and subjected to Western blot analysis for recombinant VP2 protein detection as earlier described. Fractions containing recombinant VP2 proteins were pooled and dialyzed in dialysis cassette (Thermo Fisher Scientific) in PBS according to the manufacturer's instructions.

3.4 Virus-like Particles analysis with a transmission electron microscope

Recombinant VP2 protein assembly was confirmed by transmission electron microscope (Hitachi HT7700, Japan). The purified recombinant VP2 protein sample from 3.3 was dropped on a discharged 400 mesh formvar-carbon coated copper grid and stained with 2% phosphotungstic acid. The stained samples on-grid were visualized on the transmission electron microscope at an accelerating voltage of 100 kV.

3.5 Hemagglutination Assay

Goose erythrocytes were prepared by centrifuge goose blood at 1,500 rpm for 5 min at room temperature. Then, the blood cell pellet was washed 3 times with PBS. The cell pellet was prepared as 1% red blood cells in PBS. Sample containing recombinant VP2 protein or VP2-VLPs was diluted in two folds dilution with PBS and 50 µl of each dilution was added into a well in a 96 well plate (V-bottom). Then, 50 µl of 1% red blood cell suspension (described above) was added and gently mixed, incubated for 30 min at room temperature and observe hemagglutination activity.

3.6 Bacterium-like particle preparation

Lactococcus lactis was cultured in MRS broth at 37°C, 200 rpm for overnight. The bacterial cells were harvested by centrifugation at 5,000 rpm for 5 min at room temperature. The cell pellet was collected and boils with 10% trichloroacetic acid (TCA) for 30 min to remove bacterial genomic DNA. Then, the sample was washed with excess PBS to remove the remaining acid.

3.7 VP2-VLP based oral vaccine formulation

The oral vaccine was prepared by using 1 unit of BLPs ($2.5x10^9$ cells/ml) (van Roosmalen et al, 2006) and 100 µg of purified VP2-VLP (from 3.3) mixed by gentle agitation for 30 min at room temperature. Binding of BLP and VP2-VLP was confirmed by Western blot analysis as described in 3.2 and immunofluorescence staining. For immunofluorescence staining, BLPs and VP2-VLP mixture was incubated in a micro-centrifuge tube with primary antibody (1:2,000 diluted mouse anti-VP2 antibody in PBS, Immune Technology) at room temperature for 1 hour. Then, the mixture was centrifuged at 3,000 rpm for 5 mins and the pellet was washed twice with PBS followed by incubation in secondary antibody (1:1,000 diluted Donkey anti-Mouse IgG (H+L) ReadyProbes™ Secondary Antibody (Thermo Fisher Scientific, USA) in PBS at room temperature for 2 hours. After that the pellet was obtained by centrifugation at 3,000 rpm for 5 min and washed with PBS, the mixture was then dropped onto a glass slide that already coated with poly L-Lysine (Sigma, USA), left to dry for 5-10 min, covered with a coverslip and the coverslip sealed with nail polish. The binding of BLPs and VP2-VLP was observed under structure illumination microscope (Zeiss Apotome.2, Germany)

4. Results and Discussion

4.1 Recombinant VP2 protein production by baculovirus/insect cell expression system

At 4 days post-infection with recombinant VP2 baculovirus, infected Sf9 cells were observed under an inverted microscope. Infected cells showed the typical appearance of cytopathic effects such as an increase of cell diameter, irregular shape and cell detachment from the flask as shown in figure 1. *VP2* gene expression in infected insect cells was analyzed by RT-PCR using primers specific to *VP2*. A band at approximately 1.8 kb was shown in figure 2 representing the PCR amplified fragment of *VP2* cDNA which had been converted

Proceedings of RSU International Research Conference (2020)

from VP2 mRNA. This indicates that the Sf9 cells infected with the recombinant VP2 baculovirus were able to express the *VP2*.

The infected cells were harvested and analyzed for the recombinant VP2 protein infection. Intracellular proteins were separated by centrifugation into 2 parts; supernatant containing soluble proteins and pellet containing cell debris and insoluble proteins. Both samples were subjected to SDS/PAGE and Western blot analysis. Figure 3b shows a band around 76 kDa specific to anti-CPV VP2 antibody, found in the cell debris and soluble intracellular protein part (lane 1 and 2) which were neither found in any part in wildtype baculovirus-infected cells (lane 3 and 4) and normal insect cells (lane 5 and 6). Thus, recombinant VP2 protein was successfully synthesized in the recombinant VP2 baculovirus-infected SF9 cells, even though the protein found in cell debris and is mostly found in the intracellular form. Furthermore, this recombinant VP2 protein was able to hemagglutinate goose erythrocytes which is an important characteristic of the CPV virus (figure 4, row A1-2). This can be concluded that the infected Sf9 cells produced functional VP2. Thus, the antibody to be induced by this recombinant protein VP2 protein is expected to be specific to the native CPV VP2 of the infectious CPV.

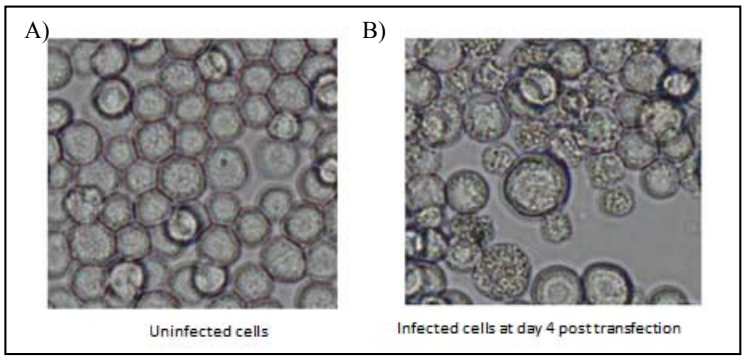

Figure 1 Cytopathic effects of recombinant VP2 baculovirus-infected Sf9 cells (B) compare to normal Sf9 cells (A) 400x magnification

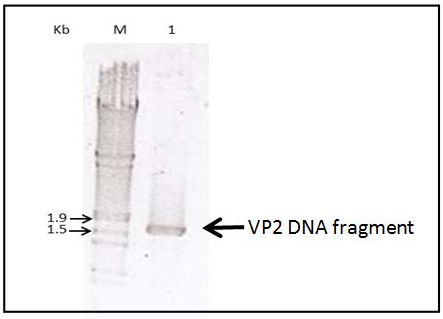

Figure 2 Reverse Transcription PCR analysis of total RNA samples obtained from recombinant VP2 baculovirus (lane 1) infected Sf9 insect cell; lane M: λ-DNA *Eco*RI/*Hind*III marker

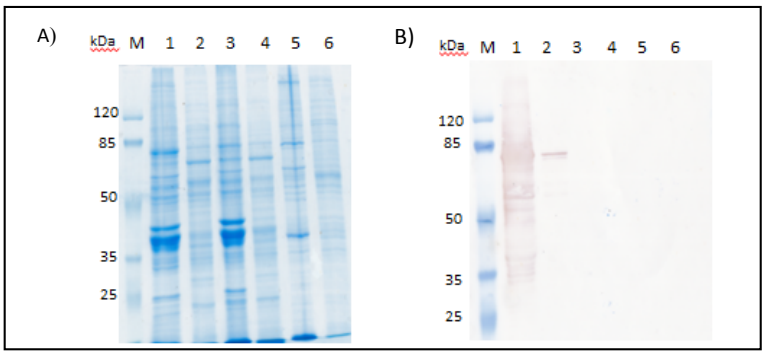

Figure 3 SDS-PAGE with instant blue staining (A) and Western blot analysis using anti-CPV VP2 specific VP2 antibody (B) of pellet and supernatant of cell lysates from; recombinant VP2 infected Sf9 cells (lane 1 and 2)**;** wild-type baculovirus infected Sf9 cells (lane 3 and 4); from non-infected Sf9 cells (lane 5 and 6); lane M **:** pre-stained protein ladder (Thermo scientific)

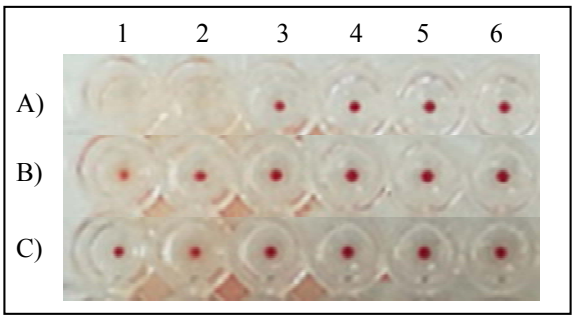

Figure 4 Hemagglutination (HA) assay, using goose erythrocytes, for recombinant VP2 protein analysis. Sample from intracellular of recombinant VP2 baculovirus-infected cells (A) Wild-type baculovirus-infected cells (B) uninfected cells (C).

4.2 Purification of VP2-VLPs

The VP2-VLPs was purified by ultracentrifugation of the soluble proteins from recombinant VP2 baculovirus-infected cells onto 25-70% sucrose gradient at 33,200 rpm for 90 min. Five fractions were collected as shown in figure 5. Western blot analysis revealed that fraction 1, 2 and 3 contain recombinant VP2 protein (data not shown) and Bradford assay was used to determine the amount of protein in each fraction

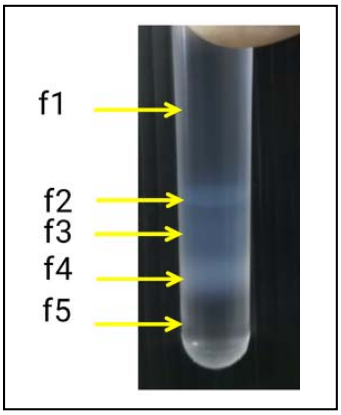

Figure 5 Separation of intracellular proteins obtained from recombinant VP2 baculovirus-infected Sf9 cells in 25-70% sucrose gradients after ultracentrifugation at 33,200 rpm for 90 min at 4°C

4.3 VP2 virus-like particles (VP2-VLP)

The electron micrograph using a transmission electron microscope clearly showed that recombinant VP2 protein self-assembled into parvovirus-like particles (VLPs) with diameters ranging from 50-100 nm (figure 4). VLPs are safe because of their non-replicable nature as they have no genetic materials. They can be easily engulfed by antigen-presenting cells because of their well-defined shape and size resembling a virus particle (Silva et al, 2015) (Zaman et al, 2014). VLPs have repetitive epitopes on their surfaces and therefore constitute a multivalence, which is able to cross-link B cell receptors, results in the maturation of naïve B cells (Chackerian, 2007). In addition, VLPs have also been demonstrated to induce memory cytotoxic T cell responses (McCoy et al, 2013). Thus, the application of VLPs as vaccines offers an excellent solution for safety versus immunogenicity.

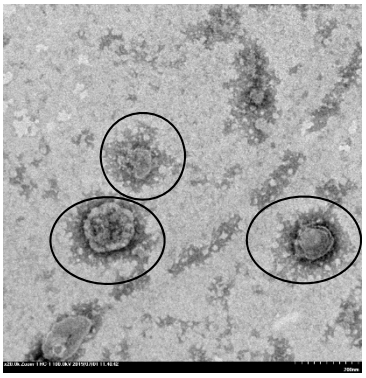

Figure 6 Electron micrograph of VP2 virus-like particles (circles) obtained from the intracellular part of infected Sf9 cells

4.4 Binding of BLP to VP2-VLPs as an oral vaccine

The binding of BLPs to VP2-VLPs was demonstrated by Western blot analysis (Figure 7). After admixed between the two and washing with PBS, the VP2-VLPs was not washed out from BLP. A specific band for VP2 was found with the BLPs on lane 2 while no signal was detected in washed PBS (lane 3). This demonstrated that VP2-VLPs remained binding to BLPs after the washing step.

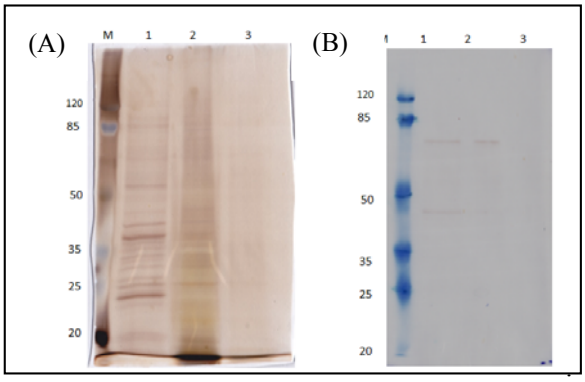

Figure 7 SDS-PAGE (A) and Western blot analysis (B) of BLP binding to VP2 VLP;
Lane M: pre-stained protein ladder (Thermo scientific), lane1: VP2 VLP in PBS (control), lane2: admixed BLP and VP2 VLP, lane3: washed PBS

The binding of BLP to VP2 VLP was also demonstrated by immunofluorescence staining (Figure 8). The *L. lactis* BLPs were seen in the bright field but not when detected with Cy3 filter used for fluorochrome 594 detection. The fluorescent signal was only seen on the BLPs with the presence of VP2-

VLPs bound by the anti-VP2 antibody and detected by the fluorescent, ReadyProbes™ secondary antibody. Thus, VP2 VLPs was successfully linked onto the BLPs and ready to be orally delivered into the animals for the immune stimulation test.

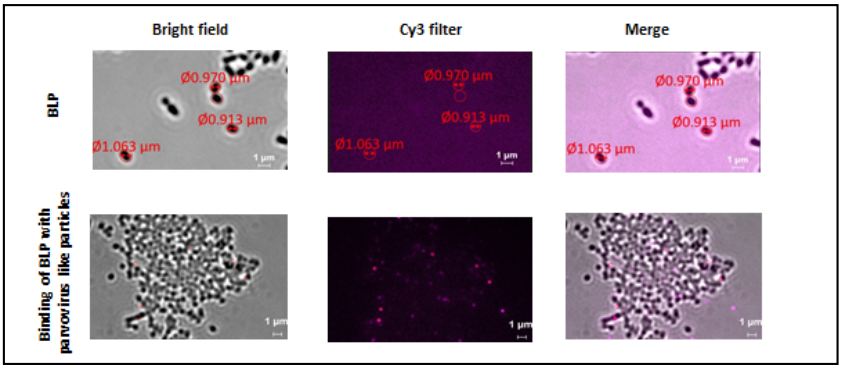

Figure8 Immunofluorescence staining for detection of binding of BLP to VP2 VLPs observed under a structured illumination microscope (ZEISS Apotome.2, Germany).

The thick layer cell wall of BLPs made from peptidoglycan (PGN) binds with VLPs via binding domain called peptidoglycan hydrolase AcmA (Van Braeckel-Budimir, Haijema & Leenhouts, 2013).

L. lactis, tends to elicit minimal immune responses against themselves, instead of inducing high levels of systemic and mucosal antibodies against the expressed foreign antigen following uptake via the mucosal immune system (Wells & Mercenier, 2008). Thus, *L. lactis* BLPs bound with VP2-VLPs, should be a promising antigen for oral vaccine development.

5. Conclusions

Recombinant VP2 protein was successfully produced by the baculovirus vector/insect cell expression system. The recombinant VP2 protein was found to poses hemagglutination function and able to self-assemble into parvovirus-like particles, VP2-VLPs. This VP2-VLPs was also effectively bound to Bacterium like particles (BLPs), a good adjuvant for oral immune stimulation. Thus, this VP2-VLPs-BLPs complex could be a promising antigen for CPV oral vaccine. This vaccine is now being tested for it immune stimulation efficiency.

6. References

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