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A B S T R A C T

In the present study, we describe the development of a DNA vaccine against chicken anemia virus. The VP1 and VP2 genes of CAV were amplified and cloned into pBudCE4.1 to construct two DNA vaccines, namely, pBudVP1 and pBudVP2-VP1. In vitro and in vivo studies showed that co-expression of VP1 with VP2 are required to induce significant levels of antibody against CAV. Subsequently, the vaccines were tested in 2-week-old SPF chickens. Chickens immunized with the DNA-plasmid pBudVP2-VP1 showed positive neutralizing antibody titer against CAV. Furthermore, VP1-specific proliferation induction of splenocytes and also high serum levels of Th1 cytokines, IL-2 and IFN-γ were detected in the pBudVP2-VP1-vaccinated chickens. These results suggest that the recombinant DNA plasmid co-expressing VP1 and VP2 can be used as a potential DNA vaccine against CAV.

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1. Introduction

Chicken anemia virus (CAV) belongs to Gyrovirus genus of Circoviridae family. It is a small nonenveloped virus resistant to thermal inactivation and treatment with lipid solvents and many of the common disinfectants [1,2]. Infections with CAV are considered to be economically significant because vertical transmission of the virus by eggs from infected breeder flocks can result in increased mortality in young chicks [1–3]. In addition, the virus has the potential to induce immune dysfunction alone or in combination with other pathogens [3]. The genome, a circular single-stranded DNA, codes for three viral proteins: VP1, the 52 kDa structural capsid protein; VP2, a 28 kDa non-structural protein with dual-specificity phosphatase activity; and VP3, the smallest protein of about 13 kDa, known as apoptin which induces apoptosis in erythrocyte precursors and thymocytes, resulting in immunodeficiency [4–6]. Among them, VP1 is the main protective protein inducing neutralizing antibodies [7].

Vielitz and Landgraf [8] have developed a vaccine against CAV, which is based on non-attenuated virulent CAV propagated in chicken embryos. An attenuated live vaccine, developed by Steenhuisen et al. [9] is also commercially available. However, these vaccines cannot be used in chickens in lay and within 21 days of slaughter. Furthermore, live attenuated vaccine may cause clinical disease if not attenuated sufficiently and sometimes spreading of the modified viruses to young chickens may cause the disease.

In a recent development, plasmid DNA-based vaccines have emerged as one of the more promising applications of non-viral gene therapy. DNA vaccines are potentially safer than other traditional vaccines; have the potential for simultaneous immunization against multiple antigens or pathogens; and more importantly, they can stimulate both humoral and cell-mediated immune responses, making them attractive for the development of effective subunit vaccines against viral and bacterial pathogens.
which will likely require both neutralizing antibody and cell-mediated immune responses for protection [10–12]. One such subunit vaccine against infectious chicken anemia was developed by using recombinant baculovirus as a vector for the expression of the CAV proteins [7]. It was shown that co-synthesis of VP1 and VP2 was required for the induction of neutralizing antibodies that protected progeny chicks against CAV challenge [7,13,14]. However, vaccination with plasmid DNAs encoding these proteins of CAV has not been studied. We therefore report the development of a DNA vaccine against CAV using a eukaryotic co-expression vector encoding the VP1 and VP2 proteins of CAV, simultaneously.

2. Materials and methods

2.1. Chickens and CAV genes

Specific pathogen free (SPF) chicken eggs were obtained from Malaysian Vaccines & Pharmaceuticals Sdn Bhd, Selangor, Malaysia. The eggs were hatched and maintained under specific-pathogen free condition with free access to feed and water. All procedures were conducted with the protocols approved by Animal Care and Use Committee of the Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). Recombinant pCR2.1(VP1–VP2) cloning vector containing VP1 and VP2 genes of CAV isolate SMSC-1 (Accession No. AF285882) was obtained from the Faculty of Veterinary Medicine, UPM.

2.2. Construction of DNA vaccines

The pBudCE4.1 co-expression vector (Invitrogen, USA) was used to construct the DNA vaccines, pBudVP1 encoding the CAV VP1 protein and pBudVP2-VP1 encoding the VP1 and VP2 genes of CAV, synchronously. The full length of the VP1 with the exception of the last 12 nucleotides and the full ORF of VP2 were amplified from the pCR2.1(VP1–VP2) plasmid using a pair of VP1-specific primers 5′ GTAAC-GCGGCGCCACCATGCCAACAGAGCTCGC3′ and 5′ CTAATGG-TAACCAGTACATCTGGCTGTG3′ and VP2-specific primers 5′ GTAAGCTCAGACATCGACCAGGACGC3′ and 5′ CATGGATCCACACTATCGTACCAGGCC3′, and then cloned into pBudCE4.1 to construct the DNA plasmids. As shown in Fig. 1, the VP1 gene was inserted into the NotI and KpnI sites of the vector under the control of the EF-1α promoter, while the gene for VP2 was inserted into the Sall and BamHI sites, downstream of the CMV promoter. After transformation into Escherichia coli, cells carrying the recombinant plasmids were selected on LB agar containing Zeocin (25 μg/ml, Invitrogen, USA). Plasmids were extracted using the alkaline lysis method as described by Sambrook and Russell [15]. The correct orientation and nucleotide sequence of the inserted genes were verified by double-stranded sequencing (Medigene, Malaysia). All the plasmid constructs and the control plasmid were purified using a large-scale endotoxin-free plasmid purification kit (Qiagen, USA). The purified plasmids were resuspended in sterile endotoxin-free PBS.

2.3. Transfection of MSB1 cells

Chicken MSB1 cells were transfected with the constructed plasmids using Lipofectamine™ 2000 reagent (Invitrogen, USA). Briefly, a MSB1 cell suspension was prepared in RPMI medium supplemented with 10% (v/v) FBS and 10% (v/v) TPB. The cells were harvested and washed with serum-free medium without antibiotics. The cell suspension (0.5 ml) containing 1 × 10^6 cells was then dispensed into each well of 6 wells culture plate. For each well, 4 μg of plasmid in 250 μl of serum free medium was mixed with 10 μl of Lipofectamine 2000 in 250 μl of serum-free medium and then the mixture was incubated at room temperature for 20 min before adding to the wells. After rocking the plate, the cells were incubated at 39 °C in a CO2 incubator for 5 h. Thereafter, 2 ml RPMI medium containing 10% FBS and 10% TPB was added to each well and then incubated in the above conditions for 48 h prior to testing for expression.

2.4. In vitro expression analysis

In vitro expression of the gene inserts was studies in the transfected MSB1 cells by RT–PCR. After 48 h of transfection, total RNA was extracted using Trizol reagent from the cells and then treated with DNase I (1 μg/μl, Fermentas, Canada) prior to RT–PCR. After verification of DNA removal by PCR using the specific primers used for PCR amplification (see above), DNA-free samples were processed directly for RT-PCR. RNA extracted from normal MSB1 cells was used as
negative control. The RT-PCR reactions were performed in 25 μl volume in a Gradient Thermal Cycler (BioRad, USA). Following an reverse transcription step at 45 °C for 45 min and then denaturation at 94 °C for 3 min, the samples were subjected to 35 cycles of 40 s at 94 °C; 1 min at 55 °C; 2 min at 68 °C; and a final elongation step at 68 °C for 10 min. The reaction products were analyzed by electrophoresis on a 1% agarose gel, and then visualized by staining with ethidium bromide.

The transfected cells were also tested for the presence of the viral proteins by Western blot analysis using mouse anti-His monoclonal antibody (Promega, USA) or chicken anti-CAV hyperimmune serum prepared by Hailemariam et al. [16] as primary antibodies and anti-mouse IgG or anti-chicken IgY conjugated to alkaline phosphatase (Promega, USA) as secondary antibodies.

2.5. Indirect immunofluorescence

The expression of the genes was also evaluated by immunofluorescence test according to Richter and Wick [17]. Transfected cells were washed with 1× PBS (pH 7.4) and fixed with cold acetone–methanol solution (ratio 1:1) for 10 min. The cells were then overlaid with anti-His monoclonal antibody or chicken anti-CAV serum diluted at ratio 1:500 in TBST [0.01% (v/v) Tween-20, 20 mM Tris base, 0.5 M NaCl, pH 7.5] and incubated at RT for 1 h. Cells were washed three times by TBST and incubated with a secondary fluorescein isothiocyanate (FITC) anti-mouse IgG or anti-chicken IgY (Promega, USA) diluted at 1:200 at RT for 1 h. Following three times washing with TBST, the cells were air dried and observed using an inverted phase contrast microscope with fluorescent light.

2.6. DNA vaccination

Two-week-old SPF chicken were divided into four groups (n = 10). Groups 3 and 4 were immunized with 150 μg of plasmid pBudVP1 and pBudVP1-VP2 in PBS, respectively, by intramuscular injection. As control, chickens in Groups 1 and 2 were injected with PBS or the parental plasmid, respectively. Chickens were immunized three times, at 2 weeks intervals.

2.7. In vivo expression analysis

In vivo expression of the viral genes VP1 and VP2 was studied in the skeletal muscles at the site of injection of the vaccinated chickens 10 days post-immunization by RT-PCR and Western blotting. Total RNA was extracted from the skeletal muscle of the vaccinated chickens using Tri-zol. After treatment with DNase I and verification of DNA removal by PCR, DNA-free samples were subjected to RT-PCR. RNA extracted from the control chickens was used as a negative control. The RNA preparation was standardized by RT-PCR for the chicken housekeeping gene, β-actin using the specific primers 5’ATGGTGACAGGCCGTTCGC3’ and 5’TCCTAGGGGCTACTCTCACG3’ which were designed based on the published sequence of the gene (Accession No. EU931581).

For Western blot analysis, the skeletal muscles at the site of injection was harvested; homogenized and their proteins were extracted using RIPA (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 5 μg/ml Aprotinin, 5 μg/ml Leupeptin, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF) and then subjected to Western blotting using anti-His monoclonal antibody.

2.8. Antibody assays

Blood was withdrawn from the wing vein of the chickens before vaccination and 10 days after the last vaccination and serum antibody titers against CAV were determined by IDEXX ELISA kit (IDEXX, Portland, ME, USA). The kit uses a blocking format ELISA for the detection of antibodies to CAV in chicken serum. The assay has correlation to virus neutralizing titer. ELISA was performed according to the procedures recommended by the manufacturer. ELISA titers higher than 1000 were considered positive.

2.9. Virus neutralization assay

Virus neutralization (VN) test is an assay used frequently to determine the ability of antibodies to neutralize the virus infectivity after vaccination. Traditional formats of CAV virus neutralization test use the microscopic observation of cytopathic effect (CPE) in the infected cells. However, the replication of CAV in only MSB1 suspension cells makes microscopic reading of CPE difficult. Therefore, in the present study, neutralizing antibodies were evaluated by the measurement of cell proliferation and viability using cell proliferation reagent WST-1 (Roche, Germany) following the method described by Lehtoranta et al. [18] with some modifications. Briefly, two-fold serial dilutions of heat-inactivated (56 °C for 30 min) sera were prepared in RPMI medium to achieve a dilution range from 1:4 to 1:2048. The diluted sera (50 μl) were transferred into flat-bottom 96-wells microplates and then mixed with equal volume of 10^5 TCID50 of CAV SMSC-1 strain. After 60 min incubation at 39 °C, 200 μl MSB-1 cells were added to each well at a concentration of 1.5 × 10^5 cells/ml followed by further incubation at 39 °C and 5% CO₂ for 5 days. RPMI medium with virus (non-neutralized virus) and or with positive anti-CAV (SMSC-1) serum was served as negative and positive controls, respectively. At 5 days of incubation, 10 μl of WST-1 reagent (Roche, Germany) was added to each well and the plates were further incubated at 37 °C for 4 h in a humidified atmosphere to let the colour reaction develop. Subsequently, the plates were centrifuged at 1000 × g for 10 min to remove the cells. The supernatants were transferred into new microplates and the absorbance of the samples against a background control as blank was measured at 450 nm wavelength. The mean absorbance value from the triplicates was measured for each serum dilution and the controls. Neutralizing activity was calculated as described by Lehtoranta et al. [18].

2.10. Splenocyte proliferation assay

Ten days after the last vaccination, spleens of the immunized and control chickens were harvested, and single
cell suspensions were generated in PBS-EDTA solution (1 × PBS, 2 mg/ml EDTA) supplemented with 2% penicillin/streptomycin. Red blood cells were lysed using red blood cell lysis solution containing 0.84% NH4CL, 0.1% penicillin/streptomycin. Red blood cells were lysed using red blood cell lysis solution containing 0.84% NH4CL, 0.1% penicillin/streptomycin. Viable cells were counted by trypan blue exclusion on a hemocytometer, prior to starting the experiment. Chicken spleen cells were added to 96-well plates in 0.1 ml at 2 × 10^5 cells/well (2 × 10^5 cells/ml) and incubated in triplicate with the purified CAV VP1 antigen (3 μg), and mitogen phytohemagglutinin (PHA 1 mg/ml, Sigma), or medium alone as negative control. For preparation of the VP1 protein, the VP1 and VP2 genes of CAV were cloned into pETDuet-1 vector to construct pETVP2-VP1 co-expressing the VP1 and VP2 genes (data not shown). After transformation into E. coli BL21 (DE3) and induction by IPTG, the VP1 protein was purified on Ni2+ affinity columns (data not shown). The purified VP1 protein and PHA served as experimental and positive stimulator, respectively. The response was evaluated by BrdU cell proliferation assay kit (Exalpha Biologicals, USA). Data is reported as stimulation indices (SI), which is the mean of experimental wells/mean of antigen free wells (negative control). SI greater than two was considered as positive proliferative response.

2.11. Cytokines evaluation

Serum levels of Th1 cytokines, interleukin 2 (IL-2) and interferon γ (IFN-γ) were determined by commercially available ELISA kits (Cusabio Biotech, USA). The assay was performed in 96-wells microplates pre-coated with antibody specific to chicken IL-2, or IFN-γ. The standard curve concentrations were 0.19–12 pg/ml and 31.2–2000 pg/ml for IL-2 and IFN-γ, respectively. The concentration of the cytokines in the samples was determined by comparing the OD of the samples to the standard curves.

For the reason that no commercial ELISA kits were available for the detection of chicken IL-12, IL-4 and IL-6 which play critical role in differentiation of CD4+ T cells into Th1 or Th2 cells, transcriptional expression of these cytokines were evaluated in the spleen of the immunized chickens by RT-PCR using the specific primers 5′ACACATCTGATGAAAGCAGCTGCC3′ and 5′TTGGGATATGTCCAGGTCACAG3′ for IL-12 (Accession No. AY262752), 5′AGCCTCCAGTGCGGCTGAG3′ and 5′TAGCCTTGTGGGAGGGAAGG3′ for IL-4 (Accession No. NM_00107079), and 5′ATGACCTCACGGGGGTCGC3′ and 5′ACGGTCTTCTCCATAACAGAG3′ for IL-6 (Accession No. NM_204628) which were designed based on the published sequence of the genes. RT-PCR of chicken β-actin (see above) was used as a positive control for verification the quality of the RNAs.

2.12. Statistical analysis

The data was analyzed by t-test and statistical significance was set at p < 0.05. The results were expressed as means ± standard error of mean. All the analysis was carried out using GraphPad Prism 5 and Windows Microsoft Excel 2007.

3. Results

3.1. In vitro and in vivo expression of the viral genes

In order to determine the expression of the gene inserts, transient gene expression was carried out in MSB1 cells. RT-PCR analysis showed the expression of the both VP1 and VP2 genes in the cells transfected with pBudVP2-VP1, whereas VP1 expression was observed in the cells transfected with pBudVP1 (Fig. 2A).

In order to determine the translational expression of the DNA vaccines in vitro, transfected MSB1 cells were tested by Western blot using anti-His monoclonal antibody or anti-CAV serum as primary antibody. As shown in Fig. 2B, the cells transfected with the constructs, pBudVP2-VP1 or pBudVP1, successfully expressed the gene inserts. The expression of the VP1 protein (∼56 kDa) was detected in the both pBudVP2-VP1- and pBudVP1-transfected cells using anti-His monoclonal antibody (Fig. 2B, lanes 2 and 3). However, anti-CAV serum only recognized the VP1 protein.
Fig. 3. *In vitro* expression of the viral proteins by indirect immunofluorescence. MSB1 cells were transfected with each of the recombinant plasmids using Lipofectamine 2000 reagent and at 48 h later, the expression was detected by indirect immunofluorescence using anti-His monoclonal antibody (A and B) or anti-CAV serum (C and D) as primary antibody. The images were observed under ×20 objective. A and C: cells transfected with pBudVP1; B and D: Cells transfected with pBudVP2-VP1; E & F: Cells transfected with the parental plasmid.

which was co-expressed with the VP2 protein in the cells transfected with pBudVP2-VP1 (Fig. 2B, lane 4), not in the cells transfected with pBudVP1 (Fig. 2B, lane 5).

Indirect immunofluorescence using anti-His monoclonal antibody or anti-CAV serum also revealed the expression of the proteins. Cells transfected with pBudVP2-VP1 exhibited bright cytoplasmic fluorescence using both anti-His antibody (Fig. 3B) and anti-CAV serum (Fig. 3D) as primary antibodies, while pBudVP1-transfected cells exhibited bright cytoplasmic fluorescence only by using anti-His monoclonal antibody (Fig. 3A). The pBudVP1-transfected cells showed very weak fluorescence using anti-CAV serum (Fig. 3C). Cells transfected with the parental plasmid showed no reaction with the antibodies (Fig. 3E and F).

Expression of the genes in the constructs was also confirmed *in vivo* in the skeletal muscle at the site of injection post-immunization using RT-PCR and Western blotting (Fig. 4).

3.2. Antibody response of the immunized chickens

To evaluate the DNA vaccines, 2-week-old SPF chickens were immunized intramuscularly with pBudVP1 or pBudVP2-VP1 in PBS and twice boosted with the same amount of plasmids as the first injection. Sera antibody titer
against CAV was evaluated pre- and post-immunization by IDEXX ELISA kit. Table 1 summarizes the antibody titers to CAV in the immunized chickens. Although chickens had no detectable antibody titer to CAV before vaccination at 2 weeks old, chickens in Group 4 (injected with pBudVP2-VP1) had average moderate antibody titer (1853 ± 89) compared to Group 3 (injected with pBudVP1), which showed no positive antibody titer post-immunization. There was no detectable response in chickens immunized with the control plasmid or PBS. These results indicated that co-expression of VP1 and VP2 is necessary to efficiently induce immune system against CAV, in agreement with previous studies[7,13].

3.3. Virus neutralizing antibody titers

The serum samples from pBudVP2-VP1 and pBudVP1-immunized chickens were tested for neutralization activity against CAV infection in MSB1 cells. According to the results, summarized in Table 2, the serum samples from pBudVP2-VP1-injected group were found to be positive for neutralizing antibodies against CAV with titer ranging from 1:256 to 1:512, while the sera from the pBudVP1 group showed negative neutralizing activity.

3.4. Cytokines evaluation in the vaccinated chickens

Serum levels of Th1 cytokines IL-2 and IFN-γ were assessed in the pBudVP2VP1-immunized chickens by ELISA. The sera from the vaccinated group showed significantly (p<0.05) high levels of IL-2 and IFN-γ after immunization when compared to the controls (Fig. 5). No significant differences were observed between the vaccinated group and the controls pre-immunization. RT-PCR analysis showed the presence of IL-12 mRNA in the spleen of pBudVP2-VP1-injected chickens, whereas no detectable expression of IL-4 and IL-6 was found in the vaccinated chickens (results not shown).

3.5. Antigen-specific proliferation of splenocytes

In vitro proliferation assay of splenocytes which is primarily used to monitor T-cell responses after immunization was carried out 10 days after the last vaccination. The splenocytes of the chickens from pBudVP2-VP1 and the control group vaccinated with the empty plasmid were prepared and stimulated with the purified CAV VP1 protein as antigen, or PHA as positive stimulator to give a non-specific stimulation. As shown in Fig. 6, there was no significant difference (p>0.05) in SI between the pBudVP2VP1-vaccinated group and the control group, when the splenocytes were stimulated by PHA. However, pBudVP2-VP1-immunized group showed significantly higher level of proliferative responses (p<0.05) to the CAV VP1 protein (mean SI 7.63) in comparison to the control group (mean SI 1.15). These results indicated positive proliferative response of splenocytes against the VP1 antigen in pBudVP2-VP1-immunized chickens (SI>2).

Table 1
ELISA antibody titers results in the vaccinated chickens.

<table>
<thead>
<tr>
<th>Vaccinated groups</th>
<th>Serum antibody assays</th>
<th>After vaccination</th>
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<tr>
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<td>ELISA S/N ratios¹</td>
<td>Antibody titers (mean ± S.D.)</td>
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<td>pBudVP2-VP1</td>
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<tr>
<td>pBudVP1</td>
<td>&gt;0.8</td>
<td>–</td>
</tr>
<tr>
<td>pBudCE4.1 (control)</td>
<td>&gt;0.8</td>
<td>–</td>
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<tr>
<td>PBS (control)</td>
<td>&gt;0.8</td>
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a S/N = sample OD600/negative control OD600.
b ELISA S/N >0.8 negative titers; S/N 0.8–0.2 moderate titers; <0.2 high protective titers.
c Antibody titers higher than 1000 are considered positive.
benthamiana had a marked alteration on the distribution of sequencing analysis prior to expression studies. characterized using restriction enzyme analysis, PCR and were constructed. The DNA plasmid constructs were first pBudVP2-VP1 co-expressing the VP1 and VP2 genes of CAV, plasmids, pBudVP1 expressing the CAV VP1 protein and to develop DNA vaccines against CAV where two DNA co-expression vector pBudCE4.1 was chosen to be used a subunit vaccine against CAV infection. Therefore, the VP1, forming large VP1 aggregates throughout the nucleus [14]. As a result, VP1 and VP2 can be used to generate anti-CAV antibodies, when expressed synchronously with CAV antibodies indicating that VP1 protein recognized by the pBudVP2-VP1-transfected cells was recognized by anti-CAV antibodies. From the ELISA results, chickens immunized with pBudVP2-VP1 had positive antibody titer to CAV, whereas pBudVP1 did not produce significant antibody titer and inoculated chickens should be considered to have a negative immune status, because according to the criteria established in IDEXX CAV ELISA kit, samples with titers to CAV lower than 1000 are considered negative. These results were in agreement with the Western blot and immunofluorescence results where only the expressed VP1 protein in the pBudVP2-VP1-transfected cells was recognized by anti-CAV antibodies indicating that VP1 protein recognized by anti-CAV antibodies, when expressed synchronously with VP2. The results confirmed the previous studies [7,13] that co-synthesis of the VP1 and VP2 proteins is required to produce the essential neutralizing form of VP1 resulting in efficient induction of antibody response against CAV, whereas separate expression of VP1 does not.

4. Discussion

In the present study, two DNA vaccines against CAV have been developed using the VP1 and VP2 genes of CAV isolate SMSC-1 as the target genes. It was showed that the neutralizing epitope on VP1 was formed only when VP1 and VP2 were synthesized synchronously [7,13]. The interaction of the CAV proteins has been also demonstrated by transient expression of the viral proteins in transgenic plants whereby co-expression of VP1 and VP2 in Nicotiana benthamiana had a marked alteration on the distribution of VP1, forming large VP1 aggregates throughout the nucleus [14]. As a result, VP1 and VP2 can be used to generate a subunit vaccine against CAV infection. Therefore, the co-expression vector pBudCE4.1 was chosen to be used to develop DNA vaccines against CAV where two DNA plasmids, pBudVP1 expressing the CAV VP1 protein and pBudVP2-VP1 co-expressing the VP1 and VP2 genes of CAV, were constructed. The DNA plasmid constructs were first characterized using restriction enzyme analysis, PCR and sequencing analysis prior to expression studies.

In vitro expression analysis in chicken MSB1 cells revealed that VP1 protein capable to be recognized by anti-CAV antibodies was produced, when expressed synchronously with VP2. These data were agreement of with the finding of Noteborn et al. [13] that only Sf9 cells infected with recombinant baculovirus co-expressing VP1 and VP2, or co-infected with VP1- and VP2-recombinant baculoviruses, react with the neutralizing antibodies. One potential explanation of the requirement of the VP2 protein might be that it act as a scaffold protein in virion assembly [7,13].

In vivo evaluation of the constructs in SPF chicken showed that only pBudVP2-VP1 co-expressing both VP1 and VP2 can induce the generation of anti-CAV neutralizing antibodies. From the ELISA results, chickens immunized with pBudVP2-VP1 had positive antibody titer to CAV, whereas pBudVP1 did not produce significant antibody titer and inoculated chickens should be considered to have a negative immune status, because according to the criteria established in IDEXX CAV ELISA kit, samples with titers to CAV lower than 1000 are considered negative. These results were in agreement with the Western blot and immunofluorescence results where only the expressed VP1 protein in the pBudVP2-VP1-transfected cells was recognized by anti-CAV antibodies indicating that VP1 protein recognized by anti-CAV antibodies, when expressed synchronously with VP2. The results confirmed the previous studies [7,13] that co-synthesis of the VP1 and VP2 proteins is required to produce the essential neutralizing form of VP1 resulting in efficient induction of antibody response against CAV, whereas separate expression of VP1 does not.

Protective immunity against CAV infection has been shown to have correlation with the presence of antibody titers to CAV and neutralizing antibody can provide complete protection [19–23]. To determine virus neutralization (VN) activity of the antibodies in the vaccinated groups, the sera were further tested for neutralization activity against CAV infection in MSB1 cells. The results indicated positive neutralization activity of the sera from the pBudVP2VP1-vaccinated group with protective titer ranging from 1:256 to 1:512, whereas the sera from the pBudVP1-vaccinated group showed negative neutralizing activity. The neutralizing titer of 1:256 which has been shown to be necessary for the prevention of shedding and vertical transmission of CAV [24] was achieved in all chickens vaccinated with pBudVP2-VP1. Nevertheless, in order to confirm this issue, it is suggested that breeder flocks producing day-old chicks with maternal antibodies be vaccinated for virus challenge.

Induction of cellular immune responses after DNA immunization has been reported for variety of antigens in previous studies [25–30]. Therefore, in this study, cell-mediated immune responses were also evaluated in the vaccinated chickens by in vitro proliferation assay of splenocytes and cytokine evaluation, although neutralizing antibodies can provide complete protection against CAV.

The splenocyte (lymphocyte) proliferation assay is widely used to evaluate cell mediated immune responses. It is suggested that antigen-stimulated proliferation response of splenocytes is directly proportional to cell-mediated immune response [31–33]. In the present study, the proliferative response of the splenocytes to the VP1 antigen was evaluated in the pBudVP2VP1-vaccinated group. Splenocytes from the immunized chickens proliferated in response to the antigen indicating the successful expression of the construct in the systemic immune system of the vaccinated chickens, resulting in activation of antigen-specific immune responses.

Cytokines play a critical role in the development of cell-mediated immune responses and prevention of viral infections [34]. To explore whether the pBudVP2-VP1 DNA vaccine can induce Th1 T-helper cell responses, the serum levels of Th1 cytokines, IL-2 and IFN-γ were evaluated in the pBudVP2VP1-vaccinated chickens by ELISA. According to the results, high levels of IL-2 and IFN-γ were detected in the vaccinated group after vaccination (p < 0.05) compared to the controls, whereas there were no significant differences between the vaccinated group and controls before
Fig. 5. Serum level of IL-2 and IFN-γ in the vaccinated chickens. The serum levels of Th1 cytokines, IL-2 (A) and IFN-γ (B) were determined in pBudVP2VP1- and PBS-injected (as a control) chickens. The results showed that IL-2 and IFN-γ serum levels in the immunized group are significantly higher when compared with the control group.
vaccination. Furthermore, RT-PCR analysis showed the detectable expression of IL-12 which is involved in differentiation of CD4+ T cells into Th1 cells [35,36] in the spleen. Detectable expression of IL-12 which is involved in differentiation of CD4+ T cells into Th1 cells [35,36] in the spleen.

Fig. 6. VP1 stimulation of splenocytes in the pBudVP2-VP1-immunized chickens. Ten days after the last injection, proliferation response was evaluated by BrdU cell proliferation assay kit as described in material and methods. Chickens injected with the parental plasmid were used as a control. Mitogen phytohemagglutinin (PHA) was used as a positive control for cell proliferation. Data is reported as stimulation indices (SI). SI greater than two is considered positive. This showed the positive VP1-stimulated proliferation of splenocytes in the chickens immunized with pBudVP2-VP1.

In conclusion, our study demonstrates that the DNA plasmid pBudVP2-VP1 co-expressing the VP1 and VP2 genes of CAV could result in both humoral and cell-mediated immune responses suggesting that this DNA plasmid could be a potential DNA vaccine against CAV infection. Our future efforts will concentrate on using an in ovo delivery system for administration of the DNA vaccine to study protective immunity against CAV infection in young chicks, because age resistance to CAV develops during the first week of life and becomes complete within 3 weeks [20–23].

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