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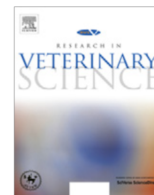
Research in Veterinary Science 93 (2012) 1089–1097

Contents lists available at SciVerse ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc



Immunogenicity of a DNA-launched replicon-based canine parvovirus DNA vaccine expressing VP2 antigen in dogs

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ARTICLE INFO

Article history:
Received 11 October 2011
Accepted 29 January 2012

Keywords:
DNA vaccine
Replicon
Canine parvovirus
Immunization
Dog

ABSTRACT

A replicon-based DNA vaccine encoding VP2 gene of canine parvovirus (CPV) was developed by cloning CPV-VP2 gene into a replicon-based DNA vaccine vector (pAlpha). The characteristics of a replicon-based DNA vaccine like, self-amplification of transcripts and induction of apoptosis were analyzed in transfected mammalian cells. When the pAlpha-CPV-VP2 was injected intradermal as DNA-launched replicon-based DNA vaccine in dogs, it induced CPV-specific humoral and cell mediated immune responses. The virus neutralization antibody and lymphocyte proliferative responses were higher than conventional CPV DNA vaccine and commercial CPV vaccine. These results indicated that DNA-launched replicon-based CPV DNA vaccine was effective in inducing both CPV-specific humoral and cellular immune responses and can be considered as effective alternative to conventional CPV DNA vaccine and commercial CPV vaccine.

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1. Introduction

Canine parvovirus (CPV) is an extremely virulent and contagious non-enveloped single-stranded DNA virus belonging to family *Parvoviridae* and genus *Parvovirus* affecting dogs, wolves, foxes and other canines. CPV, a strain evolved from feline parvovirus occurs as three different mutated forms, namely, CPV-2a, CPV-2b and CPV-2c. The disease caused by this virus is considered as most threatening to puppies between the time of weaning and 6 months of age. In young and adult dogs, it causes a severe acute leukopenia and enteritis leading to death by dehydration and shock in a large proportion of cases (Carmichael, 2005). With severe disease, dogs can die within 48–72 h without treatment. CPV spreads from dog to dog by direct or indirect contact with feces (Parrish, 1990).

Conventional vaccines against CPV include killed and modified live virus (MLV) vaccines (Smith-Carr et al., 1997; Martella et al., 2005). The killed vaccine requires high dose of antigen per immunization and adjuvant while, MLV could be excreted post-vaccination and not recommended during pregnancy. Furthermore, newborns are generally considered unsuitable vaccine recipients due to passive transfer of maternal antibodies leading to antigen clearances and immaturity of their immune system. To overcome these problems, attempts were made to develop new CPV vaccines including, a recombinant vaccine utilizing a baculovirus expression system and a synthetic peptide vaccine (Turiso et al., 1992; Casal et al., 1995). DNA vaccination against CPV has also been investigated with several advantages over conventional CPV vaccines including, eliminating the use of adjuvant and effective in presence of maternal derived antibodies (MDA) in age at which the animal is supposed to be immune (Jiang et al., 1998; Tarpey and Greenwood, 2001; Gupta et al., 2005; Patial et al., 2007; Patel and Heldens, 2009).

Although DNA immunization has several advantages but there are few limitations, namely, DNA vaccination can induce long-term uncontrolled expression of a transgene, possibility of integration into the host genome and possible induction of anti-DNA antibodies (MacGregor et al., 1998; Martin et al., 1999; Beger et al., 2002). Further, enhancing DNA vaccine immunogenicity remains a challenge in large animals (MacGregor et al., 1998; Johnson et al., 2000; Babiuk et al., 2003). To increase antigen production and immunogenicity with DNA vaccines, a new strategy has been developed to express the target heterologous antigen under the control of replicon from positive-strand RNA viruses with the promise of using the ability of these viruses to produce large amounts of viral proteins in infected cells. In addition, exclusive cytoplasmic replication of replicon RNA and inability of the replicon RNA to escape from the transfected cell makes the vector biologically safe (Berglund et al., 1999; Leitner et al., 2000a; Lundstrom, 2000). RNA replicon-based expression vectors have been developed from representatives of most of the positive-strand RNA virus families, namely, *Togaviridae, Flaviviridae* and *Picornaviridae*. Several members of *Alphavirus* genus of *Togaviridae* family, including, Sindbis virus (Xiong et al., 1989; Herweijer et al., 1995; Hariharan et al., 1998; Miller et al.,

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doi:10.1016/j.rvsc.2012.01.017

2008; Saxena et al., 2008; Gupta et al., 2009), Semliki Forest virus (Liljestrom and Garoff, 1991; Berglund et al., 1999; Zhao et al., 2009), Venezuelan equine encephalitis virus (Davis et al., 1989; Lee et al., 2003) and *Flavivirus* genus, including, tickborne encephalitis virus, Kunjin virus (Anraku et al., 2002, 2008), *Pestivirus*-BVDV and *Coronavirus*-HCoV (Curtis et al., 2002) have received considerable attention. These demonstrated that RNA replicon-based DNA vaccines provide higher levels of protective immunity and break immunological tolerance by activating innate dsRNA-mediated anti-viral pathways (Frolov and Schlesinger, 1994; Diebold et al., 2009) and significant dose-sparing advantages compared with conventional DNA vaccines (Miller et al., 2008; Leitner et al., 2003).

In this preliminary study, the potential of a replicon-based CPV DNA vaccine to induce the CPV-specific humoral and cellular immune responses were analyzed in immunized dogs and compared with immune responses induced with conventional CPV DNA vaccine and commercial CPV vaccine.

2. Materials and methods

2.1. Cells, virus and vaccines

Madin Darby Canine Kidney (MDCK) cell line was used to propagate CPV-2b for use in virus neutralization (VN) test and in preparation of CPV antigen. The cell lines like, Baby Hamster Kidney-21 (BHK-21) and Human Embryonic Kidney-293 (HEK-293) were used for *in vitro* transfection. All cell lines were procured from National Center for Cell Science (NCCS), Pune, India and grown at 37 °C under 5% CO_2 in Dulbecco's Modified Minimum Essential Medium (DMEM, Hyclone), supplemented with 10% Fetal Bovine Serum (FBS, Hyclone) and 50 μg/ml gentamicin.

CPV isolate No. NATP/2002/B03, used in this study was isolated from a clinical case from India (Rai et al., 2005) and characterized as CPV type 2b (Gupta et al., 2005). This virus was used in virus neutralization (VN) test and in preparation of inactivated CPV antigen.

The conventional CPV DNA vaccine, pTargeT-CPV-VP2, encoding VP2 gene of CPV-2b was used in this study (Gupta et al., 2005). Megavac-P Inact (Inactivated monovalent CPV vaccine, Indian Immunologicals, India) was used as commercial CPV vaccine.

2.2. Construction of replicon-based CPV DNA vaccine, pAlpha-CPV-VP2

To construct replicon-based CPV DNA vaccine (pAlpha-CPV-VP2), the DNA fragment containing full length VP2 gene was isolated by digesting pTargeT-CPV-VP2 (Gupta et al., 2005) with NheI and SmaI restriction endonucleases and ligated into XbaI and StuI sites of the replicon-based DNA vaccine vector, pAlpha. The VP2 gene insert and ORF in recombinant plasmid was confirmed by restriction digestion and DNA sequencing. The *Escherichia coli* DH5α transformed with recombinant plasmid pAlpha-CPV-VP2 was grown in LB broth containing kanamycin (50 μg/ml). The replicon-based CPV DNA vaccine contained CMV promoter at 5′ end, 5′UTR, non-structural genes (nSP1-4), 26S subgenomic promoter, CPV-VP2 gene, 3'UTR and polyA signal sequence.

2.3. Expression analysis of CPV-VP2 protein in pAlpha-CPV-VP2-transfected cells

The pAlpha-CPV-VP2 plasmid was isolated using EndoFree plasmid column (Qiagen) and transfected into HEK-293 cells using Lipofectamine 2000 transfection reagent (Invitrogen) following manufacturer's instructions. The CPV-VP2 protein in transfected cells were detected in SDS–PAGE and Western blot. At 48 h post-transfection the transfected cells were lysed in SDS–PAGE sample buffer and separated on 10% SDS–PAGE along with protein molecular weight marker (Fermentas). The proteins in SDS–PAGE were stained using Coomassie Brilliant Blue staining. For Western blotting, proteins after SDS–PAGE were transferred onto nitrocellulose membrane and probed with anti-CPV polyclonal sera raised in rabbit (with IgG ELISA titer >6400). The bound antibodies were detected using anti-rabbit secondary antibodies conjugated with alkaline phosphatase (Sigma) and visualized with NBT/BCIP substrate solution (Ameresco).

2.4. Analysis of self-amplification of CPV-VP2 transcripts by pAlpha-CPV-VP2

To analyze the self-amplification of CPV-VP2 transcripts, the BHK-21 cells were transfected with pAlpha-CPV-VP2 plasmid. As non-amplification control, the plasmid pAlpha-CPV-VP2 with deleted 3′UTR (pAlpha-Δ3′UTR-CPV-VP2) was used. The recombinant plasmids pAlpha-CPV-VP2 and pAlpha-Δ3'UTR-CPV-VP2 were transfected into BHK-21 cells using Lipofectamine 2000 reagent (Invitrogen) following manufacturer's instructions. At 48 h post-transfection, the total RNAs were isolated from transfected and control BHK-21 cells using Trizol LS reagent (Invitrogen) and treated with DNase I (Fermentas) following manufacturers' instructions. The DNaseI-treated total RNA samples were analyzed for free of DNA contamination in PCR using total RNA as template and CPV-VP2 gene specific primers. For quantification of CPV-VP2 mRNA transcripts, the total RNAs were reverse transcribed into cDNA using MMLV-reverse transcriptase (Fermentas) and oligo dT primer (Fermentas). In real-time PCR, cDNAs from pAlpha-CPV-VP2 mRNA was kept as test template and cDNA from pAlpha-Δ3′UTR-CPV-VP2 mRNA was kept as calibrator template and GAPDH as internal control. The cDNAs were 1:10 diluted and used for quantitative evaluation of replicase activity using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) kit based on SYBR green dye following the manufacturer's protocol. The primers used were either CPV-VP2 gene-specific primers (CPV-F: 5′-TACCATGGTACAGATCCAG-3′; CPV-R: 5′-CCTCTATATCACCAAAGTTA-3′) or GAPDH primers (GAPDH-F: 5′-CCTGGAGAAACCTGCCAAGT-3′; GAPDH-R: 5′-GCCAAATTCATTGTCGTACCA-3′) (Gupta et al., 2005). The *C*q values for all the reactions were recorded and fold difference in pAlpha-CPV-VP2 gene mRNA transcripts in BHK-21 cells in comparison with cells transfected with pAlpha-Δ3′UTR-CPV-VP2 was determined after normalization with the help of GAPDH internal control as described by Pfaffl (2001).

2.5. Qualitative detection of apoptosis in pAlpha-CPV-VP2-transfected cells

To analyze the induction of apoptosis, the BHK-21 cells were transfected with either pAlpha-CPV-VP2 or pTargeT-CPV-VP2 or empty vector (pAlpha) using Lipofectamine 2000 reagent (Invitrogen). At 48 h post-transfection cells were analyzed for early onset of apoptosis using Annexin V-FITC apoptosis detection kit (Sigma) following manufacturer's instructions. The transfected BHK-21 cells were probed with Annexin V-FITC fluorescent antibody probe which bound to phosphatidylserine translocated outside in apoptotic cells. After counter staining with propidium iodide, the apoptotic cells were seen as green fluorescent cells under fluorescent microscope.

Similarly, the transfected BHK-21 cells were also analyzed for apoptosis specific chromosomal DNA fragmentation using DeadEnd™ Fluorometric TUNEL system (Promega) following manufacturer's instructions. The apoptotic cells with fragmented chromosomal DNA ends were labeled with rTdT which demonstrated bead-like green fluorescence under fluorescent microscope.

2.6. Large scale preparation of plasmid DNA

The DNA vaccines (replicon-based CPV DNA vaccine, conventional CPV DNA vaccine and empty vector) were isolated using EndoFree plasmid Giga kit (Qiagen, Cat#12391). For each plasmid preparation, a single colony was picked and inoculated in 5 ml Luria Bertani (LB) broth with appropriate antibiotic (Kanamycin, at 50 μg/ml or Ampicillin, at 100 μg/ml) and incubated overnight at 37 °C with shaking. Five ml of overnight culture was inoculated into 500 ml of LB broth with appropriate antibiotic and grown overnight at 37 °C with shaking. Bacterial cells were separated by centrifugation and plasmid DNA was isolated using EndoFree plasmid Giga kit following manufacturer's instructions. The purity of plasmid DNA preparations was checked and concentration was estimated spectrophotometrically. The plasmid DNAs used for immunization were ethanol precipitated and resuspended in 150 mM NaCl at concentration of 1 mg/ml. All DNA preparations were stored at −20 °C until used for immunization.

2.7. Vaccination of dogs with different CPV vaccines

A total of 15 healthy mongrel dogs aged between 4 and 8 weeks screened seronegative (with VN titer <1:10) were used in this study. Three groups of seronegative dogs (each $n = 3$) were injected intradermal each with 50 μg of either replicon-based CPV DNA vaccine (pAplha-CPV-VP2) or conventional CPV DNA vaccine (pTargeT-CPV-VP2) or empty vector (pAlpha) in each ear pinna. One group ($n = 3$) of dogs was immunized intramuscularly with 1 ml (one dose) of commercial inactivated CPV vaccine, Megavac-P Inact. One group ($n = 3$) of dogs received PBS injection and kept as negative control group. All groups of dogs received booster on day 21 post-immunization. For the safety assessment of the vaccines, standard health parameters of immunized dogs which included monitoring of food intake, body weight, general behavior, etc. were recorded. Food and water intakes were monitored daily while rectal temperature and physical examination including, body weight, observations of hair coat, salivation, respiration character and rate, eye prominence, and tremors were monitored twice a week. The serum samples were collected from immunized dogs on day 0, 21, 30 and 40 for determination of CPV-specific IgG and VN antibody response.

2.8. Evaluation of CPV-specific antibody response in immunized dogs

To evaluate anti-CPV IgG antibody response in immunized dogs, sera from all immunized dogs were analyzed in ELISA following the method described earlier using purified inactivated CPV as ELISA coating antigen (Patial et al., 2007). For end point titer determination, a positive was scored for any sample with an absorbance more than absorbance from healthy dogs sera with two times the standard deviation. The ELISA titers were defined as the reciprocal of the highest serum dilution positive in ELISA and presented as GMT ± SEM.

To determine the vaccine immunogenicity against CPV in the vaccinated group, VN test was performed following the method described earlier (Gupta et al., 2005) by preparing two fold heat-inactivated serum dilutions starting from 1:2 to 1:1024 in 96 well microtiter plates in triplicate and mixed with 100 $TCID_{50}$ of CPV-2b. The sera from non-vaccinated dogs were also included as control. After neutralization, the MDCK cells were mixed and incubated for three days at 37 °C. The observation was recorded with positive and negative for CPV-specific cytopathic effect and no cytopathic effect, respectively. The VN antibody titer was calculated as the reciprocal of the highest serum dilution of sera that neutralized 100 $TCID_{50}$ of CPV-2b and presented as GMT ± SEM. A VN titer 1:20 and above was considered as protective as described earlier (Pollock and Carmichael, 1982a,b; Smith-Carr et al., 1997; http://www.veterinarypartner.com).

2.9. Evaluation of CPV-specific cell mediated immune (CMI) response in immunized dogs

The CPV-specific CMI response in immunized dogs was determined by lymphocyte proliferation test and immunophenotyping of effectors cells in PBMCs from all immunized dogs on day 40 post-immunization. The PBMCs isolated from each dog were stimulated with inactivated CPV antigen in triplicates in 96 well microtiter plates along with positive stimulator (Con A or PHA) and negative (PBS) control. After 72 h of stimulation at 37 °C, the stimulated cells were mixed with MTT and incubated further for 4 h at 37 °C. The formazon crystals formed were dissolved in 150 μl DMSO and absorbance was recorded at 540 nm with 620 nm as reference wavelength. Stimulation indices (SI) were calculated as ratio of absorbance of stimulated cells to absorbance of unstimulated cells.

For immunophenotyping of effectors ($CD4^+$ and $CD8^+$) cells, the PBMCs were stimulated *in vitro* with inactivated CPV antigen for 48 h. The stimulated cells were stained with FITC, RPE and ALEXA 647-conjugated cocktail monoclonal antibodies (Serotec) specific for cell surface antigens CD3, CD4 and CD8, respectively. Unstimulated cells from each immunized dog were also stained. Briefly, cocktail of conjugated antibodies was mixed with about 1×10^6 cells as per the manufacturer's instructions and incubated at room temperature for 30 min. Stained cells were washed twice with PBS–BSA and resuspended in PBS containing 1% paraformaldehyde. The numbers of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells from duplicate samples collected from all dogs were acquired per 10,000 cells per sample using BD FACS Calibur flowcytometer (BD Biosciences) and data acquired was analyzed using BD CellQuest program (BD Biosciences). Fold increase in effectors cell population on stimulation with inactivated purified CPV antigen over unstimulated cells was calculated.

2.10. Statistical analysis

The induction of CPV-specific IgG ELISA, VN titers and lymphoproliferative responses were compared by statistical analysis using the repeated measures two-way ANOVA followed by Bonferroni post-tests to compare vaccinated and control dogs. All data were presented as the GMT ± SEM. Differences between the groups with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Characterization of replicon-based CPV DNA vaccine, pAlpha-CPV-VP2

The replicon-based CPV DNA vaccine plasmid (pAlpha-CPV-VP2) encoding CPV-VP2 was constructed and evaluated to express CPV-VP2 protein in transfected cells in SDS–PAGE and Western blot (data not shown).

3.2. Analysis of self-amplification of CPV-VP2 mRNA transcripts by pAlpha-CPV-VP2

For quantitative evaluation of CPV-VP2 gene transcripts the BHK-21 cells, pAlpha-CPV-VP2-transfected cells were analyzed using real time PCR and *Cq* values obtained were compared with non-amplifying control (pAlpha-Δ3′UTR-CPV-VP2). The number of CPV-VP2 mRNA transcripts in pAlpha-CPV-VP2-transfected cells was higher ($Cq = 16$) than pAlpha-Δ3′UTR-CPV-VP2-transfected

cells ($Cq = 28$). After normalizing with respective GAPDH internal controls, the Cq values difference was found 14.59. This indicated that the CPV-VP2 transcripts in pAlpha-CPV-VP2-transfected cells were $2^{14.59}$-fold or 24,748 times higher than transcripts from pAlpha-Δ3′UTR-CPV-VP2-transfected cells.

3.3. Qualitative detection of apoptosis in pAlpha-CPV-VP2-transfected cells

In pAlpha-CPV-VP2-transfected and pAlpha-transfected BHK-21 cells, there were large numbers of cells showing apoptosis specific green fluorescence while no in pTargeT-CPV-VP2-transfected and in mock-transfected BHK-21 cells after staining with Annexin V-FITC antibody. This indicated induction of apoptosis specific to replication of the RNA replicon (Fig. 1). When transfected cells were analyzed using DeadEnd™ Fluorometric TUNEL system, bead-like green fluorescence indicative of DNA fragmentation in apoptotic cells or in apoptotic bodies was found in cells transfected with either pAlpha-CPV-VP2 or with pAlpha vector (Fig. 2). There was no bead-like green fluorescence in cells transfected with either pTargeT-CPV-VP2 or mock-transfected BHK-21 cell control. These results indicated that there was induction of apoptosis with chromosomal DNA fragmentation in BHK-21 cells due to replication of the RNA replicon (Fig. 2).

3.4. Evaluation of CPV-specific antibody response in immunized dogs

To assess the immunogenicity of replicon-based CPV DNA vaccine, CPV-specific humoral IgG immune response in immunized dogs was measured at different intervals and compared with conventional CPV DNA vaccine and commercial CPV vaccine immunized dogs. Standard health parameters monitored on vaccinated dogs indicated no adverse reaction in immunized dogs, which recommended the vaccine safe for clinical use. CPV-specific seroconversion was observed in all groups of dogs receiving different CPV vaccine. The dogs immunized with either replicon-based CPV DNA vaccine or conventional CPV DNA vaccine demonstrated IgG ELISA titer on day 21 post-immunization which boosted after booster immunization. The titer was significantly higher in dogs immunized with replicon-based CPV DNA vaccine than conventional CPV DNA vaccine (Fig. 3). The empty vector immunized and healthy controls also showed non-significant seroconversion on day 21, 30 and 40 post-immunization.

To assess the protective efficacy of different CPV vaccines, sera collected from all immunized and control dogs were analyzed for presence of virus neutralizing (VN) antibody. The dogs immunized with replicon-based CPV DNA vaccine and conventional CPV DNA vaccine demonstrated VN antibody response on day 21 post-immunization. However, only two out of three dogs immunized with replicon-based CPV DNA vaccine crossed the protective status (VN titer $\geqslant$1:20). The VN titer in all groups of CPV vaccine immunized dogs boosted after booster immunization and crossed the protective status. However, the VN antibody titer was always maximal with replicon-based CPV DNA vaccine. There was non-significant seroconversion in dogs immunized with empty vector on day 30 and 40 post-immunization (Fig. 4).

3.5. Evaluation of CPV-specific cell mediated immune (CMI) response in immunized dogs

The CPV-specific CMI immune response elicited by replicon-based CPV DNA vaccine was analyzed in PBMCs after *in vitro* stimulation with inactivated CPV antigen and compared with those elicited by the conventional CPV DNA vaccine and commercial CPV vaccine. The CPV-specific lymphocyte proliferative responses, as shown in Fig. 5, was higher in dogs immunized with either replicon-based CPV DNA vaccine or commercial CPV vaccine compared to dogs immunized with conventional CPV DNA vaccine.

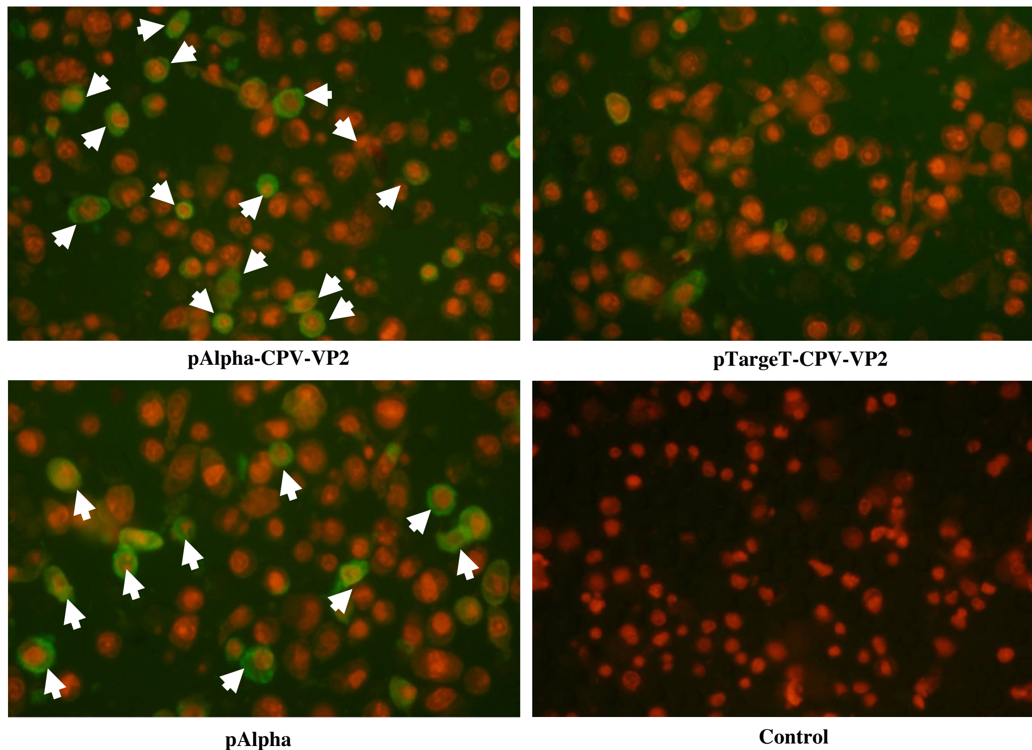

Fig. 1. Qualitative detection of apoptosis in pAlpha-CPV-VP2, pTargeT-CPV-VP2, pAlpha and mock-transfected control BHK-21 cells using Annexin V detection assay. The apoptotic cells were visualized as green cells while cells were visualized as red after counter stain with propidium iodide under fluorescent microscope (200×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

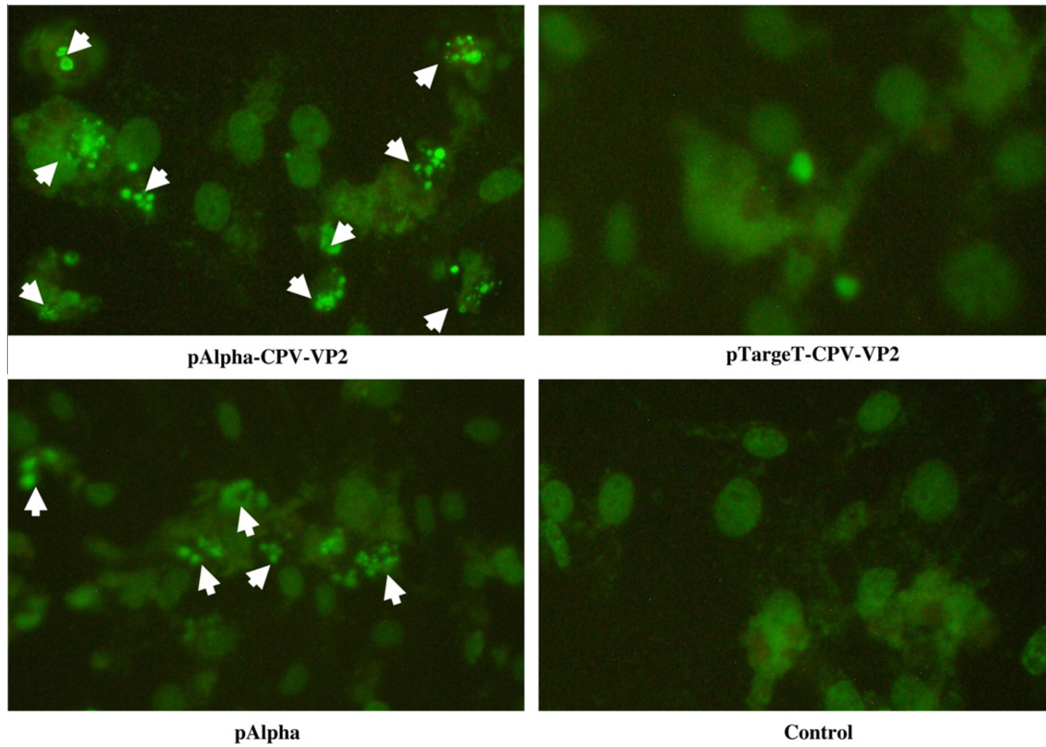

Fig. 2. Qualitative detection of apoptosis in pAlpha-CPV-VP2, pTargeT-CPV-VP2, pAlpha and mock-transfected control BHK-21 cells using DeadEnd Fluorometric TUNEL assay. Fluorescein-12-dUTP labeled DNA in apoptotic cells were visualized as bead-like green fluorescence under fluorescent microscope (200×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

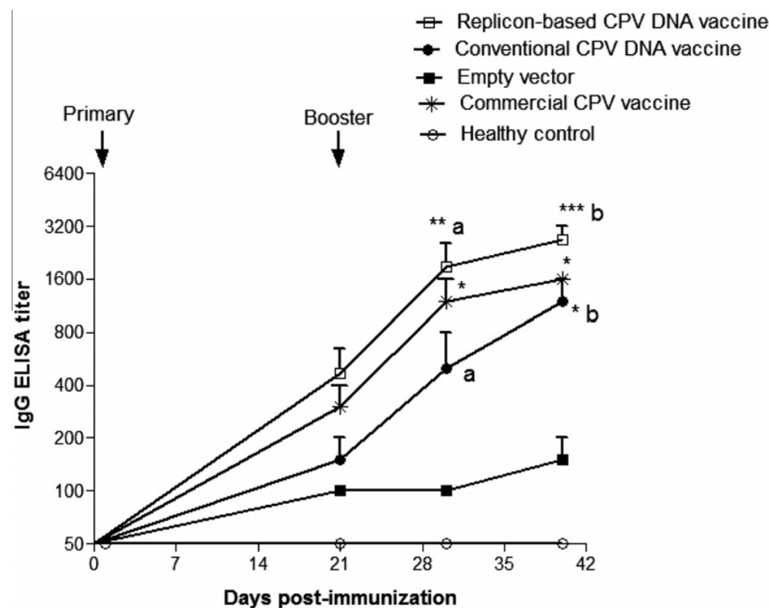

Fig. 3. CPV-specific serum ELISA IgG antibody response in immunized dogs. Sera from different groups of immunized dogs were collected on day 0, 21, 30 and 40 and analyzed for CPV-specific ELISA IgG response. ELISA titers were presented as GMT ± SEM from all immunized dogs in each group. The titers significantly different from controls are marked with *, **, *** at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively. The titers having similar superscript letters differ significantly at $p < 0.05$.

There was non-significant CPV-specific proliferative response detected in dogs receiving the empty vector or dogs receiving PBS. The proliferative response of PBMCs from all immunized and control dogs with non-specific stimulator (Con A or PHA) confirmed that the cells were healthy and competent to proliferate.

To characterize the CPV-specific proliferation of lymphocytes for the most crucial components of antiviral effectors ($CD4^+$ and $CD8^+$) the stimulated lymphocytes were phenotypically analyzed using a panel of different monoclonal antibodies against $CD3^+$, $CD4^+$ and $CD8^+$ markers and compared with their respective unstimulated controls (Fig. 6). The analysis of the increase in effectors cells population after *in vitro* stimulation with inactivated CPV antigen indicated CPV-specific sensitization of $CD4^+$ and $CD8^+$ lymphocytes. There was significant increase in number of $CD4^+$ and

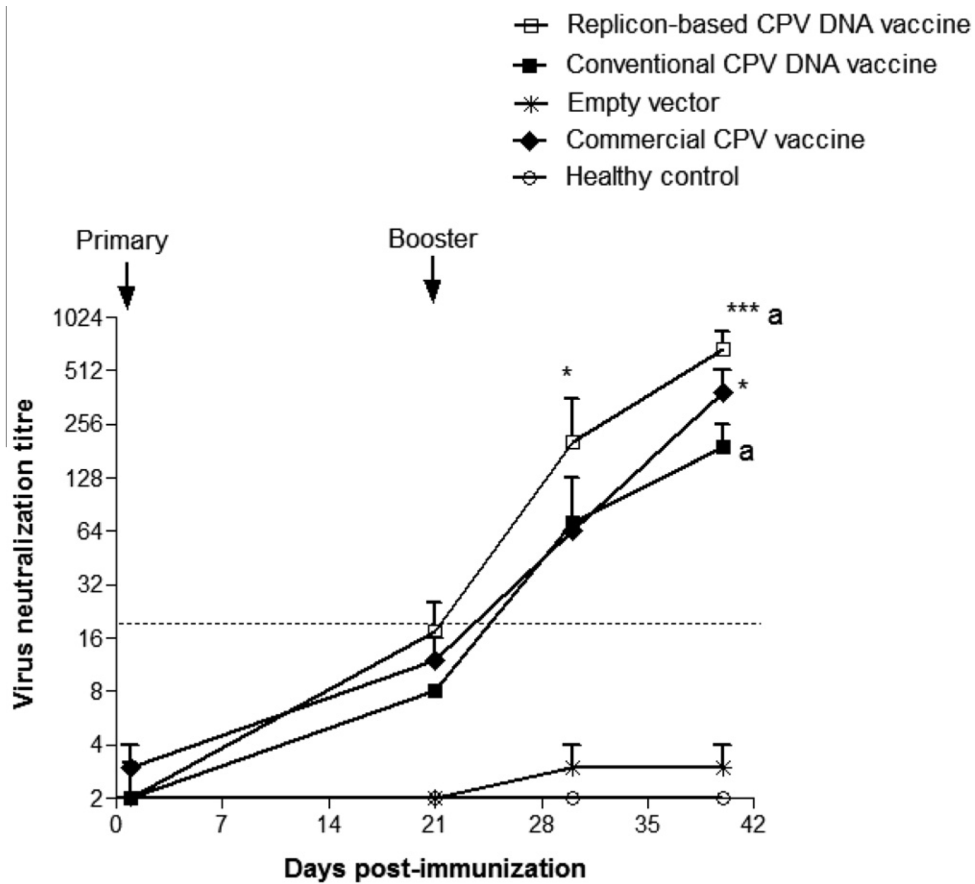

Fig. 4. CPV-specific virus neutralization (VN) antibody response in immunized dogs. Sera from different groups of immunized dogs were collected on day 0, 21, 30 and 40 and analyzed for CPV-specific VN antibody response. VN titers were presented as GMT ± SEM from all immunized dogs in each group. Dotted line represents the protective 1:20 VN titer. The titers significantly different from controls are marked with *, *** at $p < 0.05$, $p < 0.001$, respectively. The titers having similar superscript letter differ significantly at $p < 0.001$.

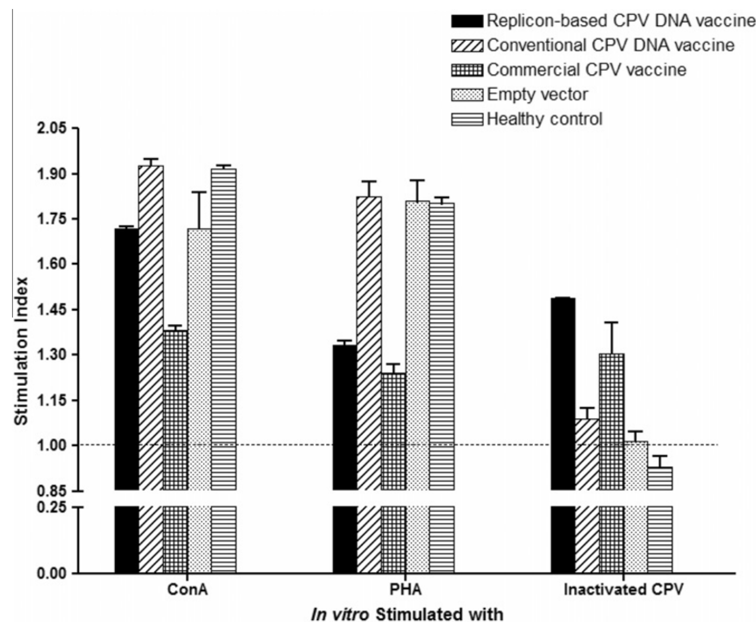

Fig. 5. Proliferative responses of PBMCs from immunized dogs after *in vitro* stimulation with vaccine antigen and inactivated CPV antigen. The PBMCs from different groups of immunized dogs were isolated and stimulated with different non-specific and specific (antigens) stimulators. After 72 h of stimulation, MTT dye assay was done to determine CPV-specific proliferative response in each group. Stimulation index (SI) was calculated as ratio of OD of antigen stimulated cells over OD of unstimulated cells. The ConA and PHA were taken as non-specific stimulator. Data represents mean ± SEM of triplicate wells.

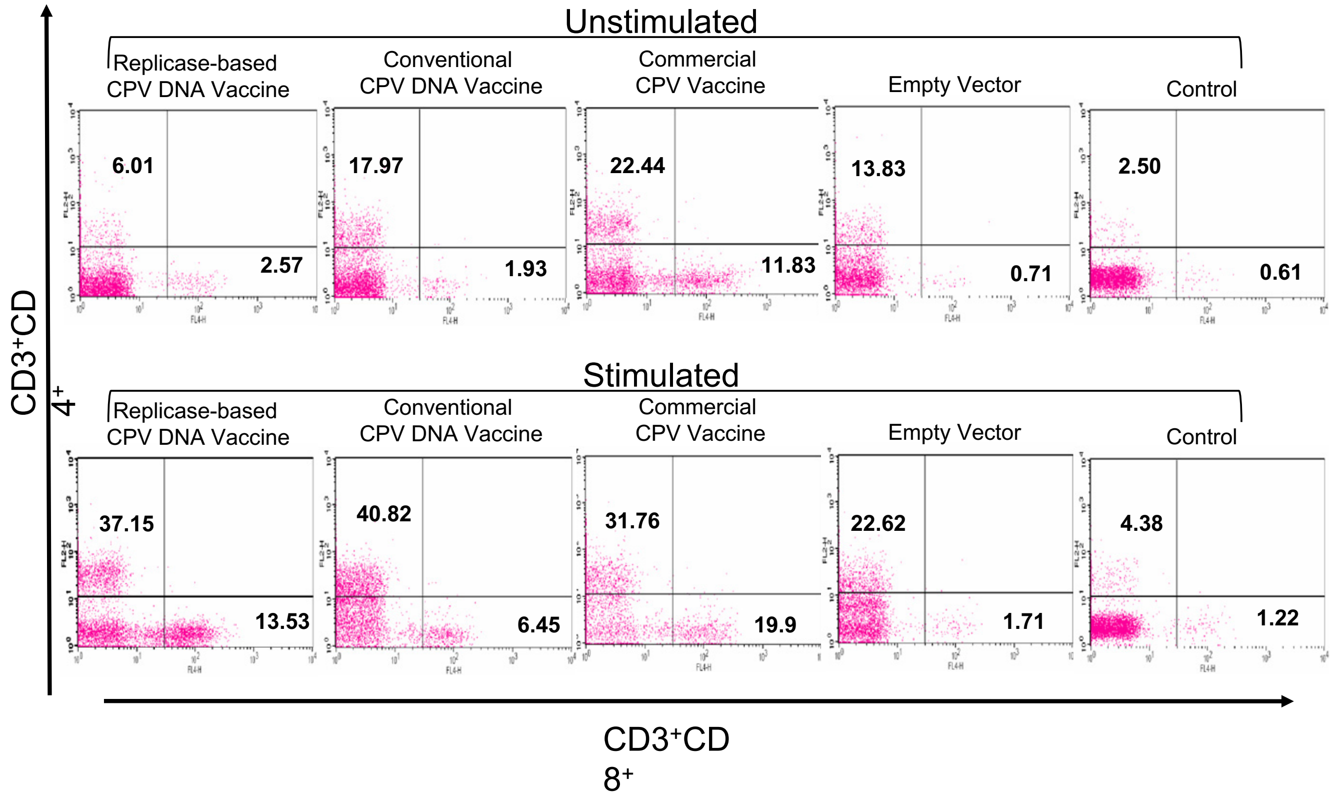

Fig. 6. Immunophenotyping of lymphocyte effectors population after *in vitro* stimulation with inactivated CPV antigen. PBMCs from different vaccinated and control dogs were isolated and cultured *in vitro* with inactivated CPV antigen in duplicate for 48 h. The stimulated and unstimulated PBMCs were stained with anti-dog CD3, CD4 and CD8 antibodies. The numbers of $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ cells were counted by flowcytometry.

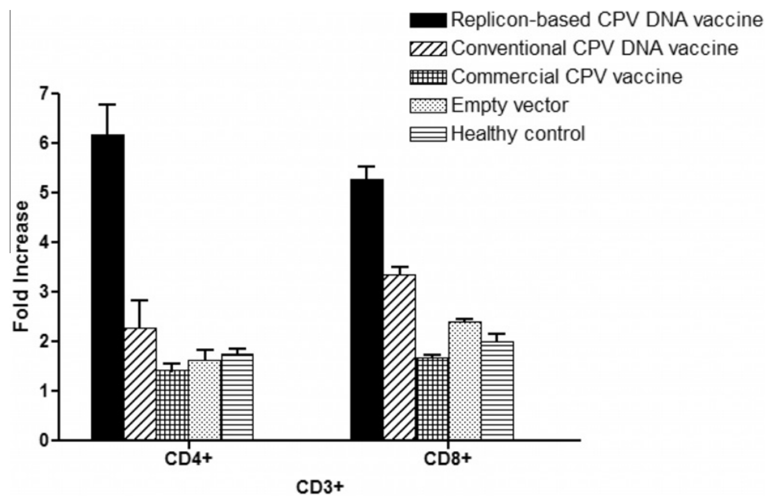

Fig. 7. Fractional increase in effectors CD4 and CD8 population of cells after *in vitro* stimulation with inactivated CPV antigen. PBMCs from different groups of immunized dogs were stimulated in triplicate with inactivated purified CPV antigen for 48 h and stained with anti-CD3, CD4 and CD8 antibodies. The $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ cells were counted by flow cytometry. Fractional increase in effectors cell population on stimulation with inactivated CPV antigen over unstimulated cells was calculated. Data represents mean ± SEM of triplicate samples collected from each group from two independent experiments.

$CD8^{+}$ lymphocytes in dogs immunized with replicon-based CPV DNA vaccine compared to controls (Fig. 7).

4. Discussion

To enhance immunogenicity and to improve biosafety of the conventional CPV DNA vaccine, in this study, we developed a replicon-based CPV DNA vaccine (pAlpha-CPV-VP2). On transfection, the vaccine plasmid transcribed the self-replicating RNA using CMV promoter and host RNA polymerase II inside the nucleus. This transcribed RNA is transported to cytoplasm and translated into replicase protein. The expressed replicase protein catalyzed the synthesis of negative-sense transcripts to act as template for further self-amplification of RNA transcripts and transcription of CPV-VP2 mRNA using 26S subgenomic promoter. SDS–PAGE and Western blot analysis of the pAlpha-CPV-VP2-transfected cell lysate confirmed the translation of CPV-VP2 protein (data not shown). We reasoned that the replicon-based CPV DNA vaccine

would amplify the CPV-VP2 mRNA transcripts utilizing self-replicating ability of replicase and induce apoptosis in transfected cells. To determine the self-replicating ability of replicase-based CPV DNA vaccine, CPV-VP2 mRNA transcripts were quantified using real-time PCR in pAlpha-CPV-VP2-transfected BHK-21 and compared with non-replicating control plasmid (pAlpha-Δ3′UTR-CPV-VP2). The deletion of 3′UTR has made the plasmid non-replicating as the 3′UTR of positive sense RNA virus has been reported as essential element for initiation of anti-sense RNA template and replication (Richard and Charles, 2005; James et al., 2007). There were over 24 thousand times more CPV-VP2 mRNA transcripts in pAlpha-CPV-VP2-transfected BHK-21 cells compared to respective non-replicating control indicating the self-replicating ability of replicon-based CPV-VP2 DNA vaccine.

Another characteristic of a replicon-based DNA vaccine, induction of apoptosis was also analyzed in pAlpha-CPV-VP2-transfected cells. To detect induction of apoptosis with replicon-based CPV DNA vaccine specific to replication of the replicon, apoptotic cell death was analyzed in both pAlpha-CPV-VP2- and empty vector (pAlpha)-transfected cells and compared with pTargeT-CPV-VP2-transfected cells. The induction of apoptosis was detected only in replicon-based CPV DNA vaccine and pAlpha vector as confirmed by Annexin V and TUNEL assays. The induction of apoptosis due to formation of double-stranded RNA intermediates produced by alphaviral replicase during RNA replication (Leitner et al., 2000b; Glasgow et al., 1997) and subsequent activation of dsRNA-dependent pathways (PKR and 2′-5′-A synthetase/RNase L) provided a mechanistic explanation for the observation that every cell transfected *in vitro* with replicon-based DNA or RNA undergoes apoptosis (Leitner et al., 2003). In addition, induction of apoptosis also confers potential immune-potentiating effects and increased safety for the clinical use of DNA vaccines (Ljungberg et al., 2007).

After *in vitro* characterizing replicon-based CPV DNA vaccine, immune responses to vaccine construct was evaluated by injecting intradermally in ear pinna in dogs. CPV-specific virus neutralizing (VN) antibody and IgG ELISA responses were detected in all vaccinated dogs. The VN antibody response was significantly higher in dogs immunized with replicon-based CPV DNA vaccine than conventional CPV DNA vaccine and commercial CPV vaccine. Further, the lymphoproliferative response and CPV-specific priming of $CD4^+$/$CD8^+$ effectors cells were significantly higher with replicon-based CPV DNA vaccine compared to conventional CPV DNA vaccine. Similar enhanced immune responses with replicon-based vaccines over conventional DNA vaccines have been reported earlier for other diseases (Zhou et al., 1994; Dalemans et al., 1995; Hariharan et al., 1998; Vignuzzi et al., 2001; Xiao et al., 2004; Arbele et al., 2005; Saxena et al., 2008).

From this preliminary study, it is premature to compare conventional CPV vaccines with replicon-based CPV DNA vaccine due to the limited number of dogs per group studied. Nonetheless, it is clear from induced antibody and cellular immune responses that some differences are apparent and replicon-based CPV DNA vaccine induced significantly higher responses. The replicon-based CPV DNA vaccine induced dsRNA-mediated apoptotic cell death which makes this vaccine approach effective and biologically safe compared to conventional DNA vaccine. Further, the response of replicon-based CPV DNA vaccine needs to be evaluated in large number of dogs for early immune response, duration of immunity and response in presence of maternally derived antibodies (MDA) before it can be considered as effective alternative to commercial CPV vaccine.

Acknowledgements

We would like to thank the Director, Indian Veterinary Research Institute, Izatnagar for providing facilities for carrying out this study. This work was funded by the Department of Biotechnology (DBT), Government of India under Grant No. BT/PR6442/AAQ/01/239/2005 and additional financial support from the Indian Council of Agricultural Research (ICAR), Government of India.

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