

## An oral Sindbis virus replicon-based DNA vaccine containing VP2 gene of canine parvovirus delivered by *Escherichia coli* elicits immune responses in dogs

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Received March 29, 2011; accepted September 16, 2011

**Summary.** – A Sindbis virus replicon-based DNA vaccine containing VP2 gene of canine parvovirus (CPV) was delivered by *Escherichia coli* to elicit immune responses. The orally immunized dogs developed CPV-specific serum IgG and virus neutralizing antibody responses. The cellular immune responses analyzed using lymphocyte proliferation test and flow cytometry indicated CPV-specific sensitization of both CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> lymphocytes. This study demonstrated that the oral CPV DNA vaccine delivered by *E. coli* can be considered as a promising approach for vaccination of dogs against CPV.

**Keywords:** canine parvovirus; DNA vaccine; VP2; Sindbis virus replicon; *E. coli*

### Introduction

Canine parvovirus (CPV, the genus *Parvovirus*, the family *Parvoviridae*), an extremely virulent and contagious virus affecting dogs, wolves, foxes and other canines, causes most threatening disease to puppies between the time of weaning and six months of age. It causes severe acute leukopenia and enteritis in young and adult dogs, leading to death by dehydration and shock. Conventional vaccines against CPV include inactivated and modified live virus vaccines (Smith-Carr *et al.*, 1997; Martella *et al.*, 2005). Inactivated vaccine requires high dose of antigen per immunization and adjuvant and on the other hand, modified live virus is usually excreted after vaccination and not recommended during pregnancy.

To overcome these problems, attempts were made to develop other CPV vaccines including a recombinant vaccine utilizing baculovirus expression system, a synthetic peptide vaccine and CPV DNA vaccine (Turiso *et al.*, 1992; Casal *et al.*, 1995; Gupta *et al.*, 2005; Patial *et al.*, 2007).

DNA vaccination has been proven as a major breakthrough in the development of novel immunization strategy to elicit immunity against a series of infectious agents (Dhamma *et al.*, 2008, for review). Although, DNA vaccination induces robust immune responses in mice, less successful in large animals mainly due to high doses of plasmid DNA required to induce sufficient immunity and improper delivery of plasmid DNA to immune cells (MacGregor *et al.*, 1998; Babiuk *et al.*, 2003). To overcome these limitations, the alphavirus replicon-based self-amplifying and self-limiting expression system has been developed and used to enhance antigen expression and improve DNA vaccination (Xiong *et al.*, 1989; Herweijer *et al.*, 1995; Hariharan *et al.*, 1998; Berglund *et al.*, 1998; Miller *et al.*, 2008; Saxena *et al.*, 2008; Anraku *et al.*, 2002, 2008). For delivery of plasmid DNA directly to cytoplasm of antigen-presenting cells, a variety of Gram-positive and Gram-negative bacteria, including, *Salmonella*, *Shigella* and *E. coli* have been used successfully as carriers for

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**Abbreviations:** CFU(s) = colony forming unit(s); CMI = cell mediated immune/immunity; Con A = concavalin A; CPV = canine parvovirus; PBMCs = peripheral blood mononuclear cells; PHA = phytohemagglutinin; VN = virus neutralization/virus neutralizing

efficient delivery of either DNA vaccine constructs or vaccine antigens (Liljeqvist and Stahl, 1999; Gentschev *et al.*, 2001; Schoen *et al.*, 2004 for review) and for vaccination of model animals against a variety of infectious diseases of both bacterial and viral origin, including human immunodeficiency virus (HIV, Karpenko *et al.*, 2004), hepatitis B virus (HBV, Patrick *et al.*, 2001), Newcastle disease virus (NDV, Fang *et al.*, 2003), and infectious bursal disease virus (IBDV, Li *et al.*, 2006; Mahmood *et al.*, 2007). The enteropathogenic bacteria evaluated as DNA vaccine carriers are either extracellular pathogens, such as *E. coli* (Giacalone *et al.*, 2007; Mahmood *et al.*, 2007) and *Yersinia* spp. or intraphagosomal pathogens like *Salmonella* spp. (Li *et al.*, 2006; Qu *et al.*, 2008; Yang *et al.*, 2009, 2010) or intracytosolic pathogens, like, *Listeria monocytogenes* and *Shigella* spp. (Schoen *et al.*, 2004).

In the present study, the potential of Sindbis virus replicon-based DNA vaccine has been investigated by delivering the vaccine orally by non-pathogenic *E. coli* DH5a to induce immune responses against CPV in dogs. The induced CPV-specific humoral and cellular immune responses were compared with responses developed with commercial vaccine.

## Materials and Methods

**Virus and cells.** CPV type 2b adapted to Madin Darby canine kidney (MDCK) cells was used in virus neutralization (VN) test and in preparation of inactivated purified antigen. MDCK cells were procured from National Centre for Cell Science (NCCS, Pune, India) and grown at 37°C under 5% CO<sub>2</sub> in Dulbecco's modified minimum essential medium (DMEM, Hyclone), supplemented with 10% fetal calf serum (FCS, Hyclone) and 50 µg/ml gentamicin.

**DNA vaccine.** To construct Sindbis virus replicon-based CPV DNA vaccine (pAlpha-CPV-VP2), the DNA fragment containing full length VP2 gene was obtained by digesting pTarget-CPV-VP2 (Gupta *et al.*, 2005) with *NheI* and *SmaI* restriction endonucleases and ligated into *XbaI* and *StuI* sites in Sindbis virus replicon-based DNA vaccine vector, pAlpha (Saxena *et al.*, 2008).

**Commercial vaccine.** Inactivated vaccine, Megavac-P Inact (Indian Immunologicals, India) was used as commercial vaccine.

***E. coli* transformed with DNA vaccine.** The *E. coli* DH5a transformed with pAlpha-CPV-VP2 was grown on LB agar plates containing kanamycin for 10 passages. The stability of the plasmids was evaluated by plasmid isolation and restriction enzyme analysis. Safety of transformed *E. coli* DH5a as vaccine vehicle was analyzed by inoculating orally 10 times higher dose (10<sup>10</sup> CFU per dog) of bacterial culture to a group of three dogs. The dogs were observed for any symptoms of reaction or abnormality during the period of experimentation. For large scale preparation of *E. coli* carrying vaccine plasmid, bacterial culture carrying vaccine plasmid was grown overnight with shaking. Bacterial cells were recovered by centrifugation at 5,000 rpm for 10 mins and the pellet was resuspended in PBS (pH 7.2). The bacterial colony forming units (CFUs)

per ml were determined by plating serial dilutions of resuspended culture on LB agar plates. The concentration was adjusted to 10<sup>9</sup> CFU per ml and used as vaccine.

**Vaccination of dogs.** Two groups of seronegative (with VN titer <1:10) apparently healthy dogs (each n = 3) aged between four and eight weeks, were orally inoculated by 1 ml of *E. coli* containing pAlpha-CPV-VP2 in concentration 10<sup>9</sup> CFU or by empty vector. One seronegative group (n = 3) of dogs was immunized intramuscularly with 1 ml (one dose) of commercial vaccine. One group (n = 3) of dogs received PBS and kept as negative control group. All groups of dogs received booster on day 21 post-immunization. The serum samples were collected from immunized dogs on day 0, 21, 30, and 40 for determination of CPV-specific IgG and VN titer.

**ELISA.** To evaluate IgG antibody response against CPV in immunized dogs, sera from all dogs were analyzed in ELISA following the method described earlier using inactivated CPV as ELISA coating antigen (Patil *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.

**Virus neutralization test.** To determine the protective status among the vaccinated groups, sera from all dogs were analyzed in VN test following the method described earlier (Gupta *et al.*, 2005). The VN titer was calculated as the reciprocal of the highest serum dilution of sera that neutralized 100 TCID<sub>50</sub> of CPV and presented as GMT±SEM. A VN titer 1:20 and above was considered as protective as described earlier (Smith-Carr *et al.*, 1997; Pollock and Carmichael, 1982a,b; www.veterinarypartner.com).

**Lymphocyte proliferation test.** The CPV-specific cell mediated immune (CMI) response in immunized dogs was determined by lymphocyte proliferation test and immunophenotyping of effector cells in peripheral blood mononuclear cells (PBMCs) from all the immunized dogs on day 40 after immunization. The lymphocyte proliferation test was performed using MTT dye in PBMCs isolated from each dog following the method described earlier (Saxena *et al.*, 2008) after stimulation with inactivated CPV antigen along with positive controls, concavalin A (Con A) and phytohemagglutinin (PHA). Stimulation indices (SI) were calculated as ratio of absorbance of stimulated cells to absorbance of unstimulated cells.

**Immunophenotyping.** For immunophenotyping of effector (CD4<sup>+</sup> and CD8<sup>+</sup>) cells, the PBMCs were stimulated *in vitro* with inactivated CPV antigen for 48 hrs. The cells were stained with FITC, RPE and ALEXA 647-conjugated cocktail of monoclonal antibodies specific for cell surface antigens CD3, CD4, and CD8 (Serotec), respectively following the method described earlier (Saxena *et al.*, 2008). The numbers of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> cells from duplicate samples collected from all dogs were acquired per 10,000 cells per sample using BD FACS Calibur flowcytometer (BD Biosciences) and acquired data were analyzed using BD CellQuest program (BD Biosciences). Fold increase in effector cell

population on stimulation with inactivated purified CPV antigen over unstimulated cells was calculated.

**Statistical analysis.** The significance for induction of CPV-specific responses induced in lymphocyte proliferation test, IgG ELISA, and VN titers was analyzed using two-way ANOVA using GraphPad Prism version 4.03 software (GraphPad). All data were presented as the GMT±SEM.

**Results and Discussion**

*Antibody responses to DNA vaccine*

The aim of this study was to assess the efficacy of oral vaccination in dogs with non-pathogenic *E. coli* DH5α delivering a Sindbis virus replicon-based CPV DNA vaccine. The oral vaccination was selected with advantages of bacterial delivery of DNA encoding antigens to both mucosal and systemic immune systems, cost efficiency and acceptability of oral and other forms of mucosal delivery. To assess the immunogenicity of DNA vaccine, CPV-specific humoral IgG immune response in immunized dogs was measured at different time intervals and compared with dogs immunized with commercial vaccine. CPV-specific seroconversion was observed in all vaccinated dogs. The DNA vaccine demonstrated significantly high IgG ELISA titer on day 21 post-immunization which increased after booster dose (Fig. 1). The commercial vaccine also showed seroconversion, however, it was not significant on day 21 post-immunization but increased to significant level after booster dose. There was no or non-significant seroconversion in dogs in control groups.

To assess the protective efficacy of CPV vaccines, the sera collected from all immunized and control dogs were analyzed

for presence of VN antibody. The DNA vaccine demonstrated VN titer (>32) on day 21 post-immunization which crossed the protective status (VN titer ≥20). As described earlier, a VN titer >1:20 indicates protective status against CPV (Pollock and Carmichael, 1982a,b; Smith-Carr *et al.*, 1997; www.veterinarypartner.com). Further, in this study, an anamnestic response was seen in both CPV vaccine immunized groups after the booster dose with VN titer above the protective threshold (Fig. 2). There was no or non-significant seroconversion (VN titer <4) seen in dogs immunized with either empty vector or healthy control groups.

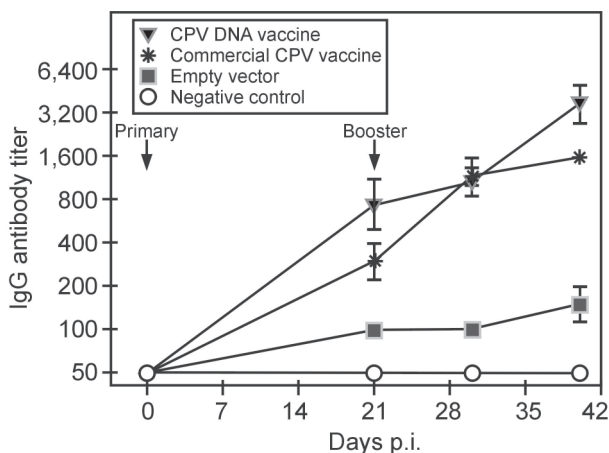


Fig. 1

IgG antibody response to CPV vaccines

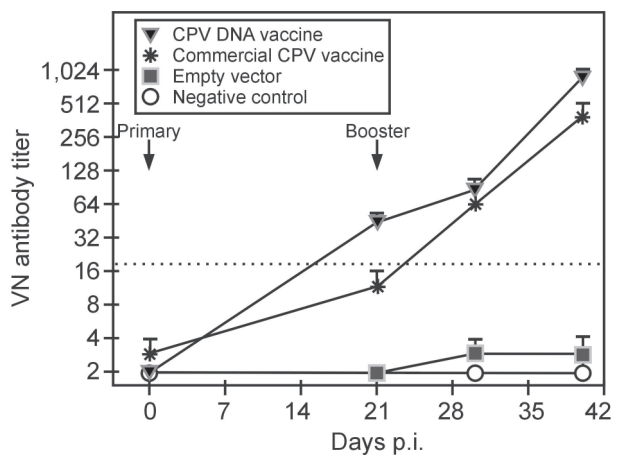


Fig. 2

VN antibody response to CPV vaccines

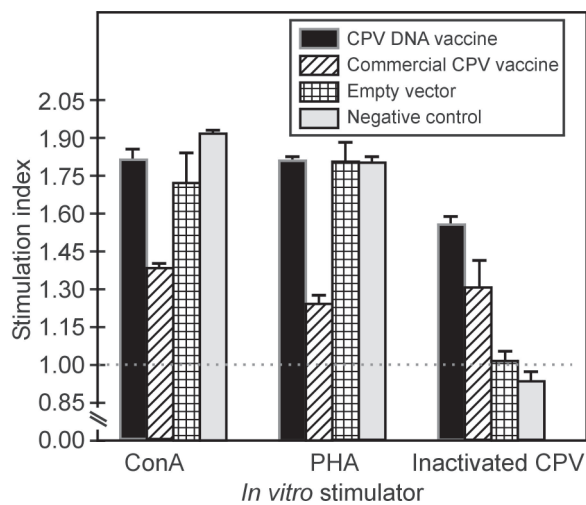


Fig. 3

Lymphocyte proliferation responses to CPV vaccines

Results of the lymphocyte proliferation test after stimulation with Con A, PHA, and inactivated CPV, respectively.

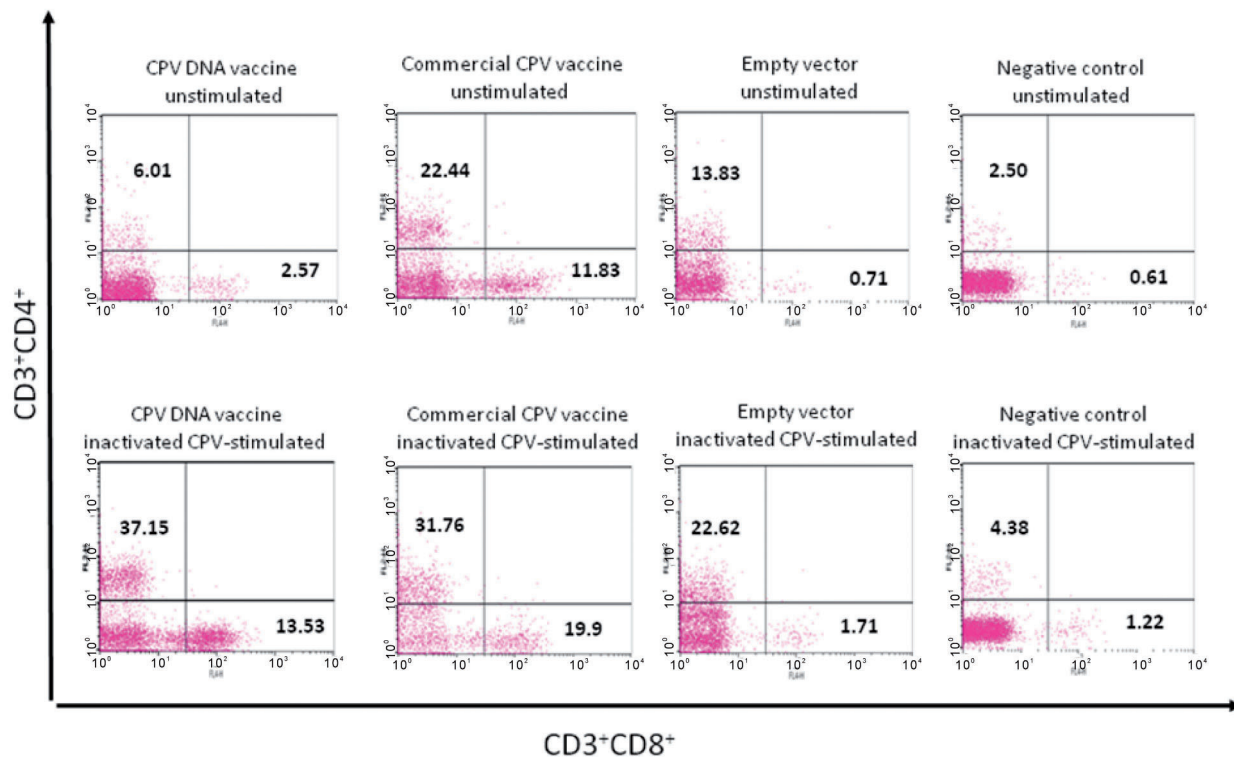


Fig. 4

**Flow cytometry of lymphocyte proliferation responses to CPV vaccines**

Stimulation with inactivated CPV or none.

*CMI responses to DNA vaccine*

The CMI response elicited by DNA vaccine was analyzed in isolated PBMCs after *in vitro* stimulation with inactivated CPV antigen and compared with commercial vaccine. As shown in Fig. 3, the CPV-specific lymphocyte proliferative response with DNA vaccine or commercial vaccine was significantly high. There were no CPV-specific proliferative responses detected in dogs receiving empty vector or PBS (negative control). The proliferative responses of PBMCs from all dogs (immunized and control) with non-specific stimulator (Con A or PHA) confirmed that the cells were healthy and competent to proliferate.

On phenotypic characterization of lymphocytes it was found that both (CD4<sup>+</sup> and CD8<sup>+</sup>) effector cells were stimulated with DNA vaccine and commercial vaccine (Fig. 4). On *in vitro* stimulation with inactivated CPV antigen, there was more than 2-fold increase in both (CD4<sup>+</sup> and CD8<sup>+</sup>) lymphocytes in dogs vaccinated with DNA vaccine (Fig. 5) indicating presence of sensitized lymphocytes in DNA vaccine immunized dogs. It has been reported in earlier studies

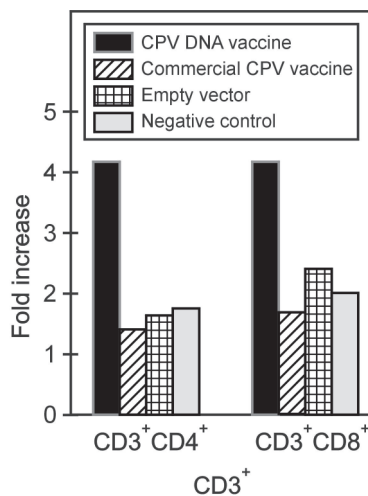


Fig. 5

**Increase in CD3+CD4+ and CD3+CD8+ lymphocytes in response to CPV vaccines**

that replicon-based DNA vaccine against other diseases induced CMI response through induction of apoptosis (Albert *et al.*, 1998; Kohno *et al.*, 1998; Leitner *et al.*, 2000; Saxena *et al.*, 2008) and generation of dsRNA intermediates during the alphavirus-replicon replication which served as T-cell adjuvant to encoded antigen (Leitner *et al.*, 1999; Saxena *et al.*, 2008). In this study, the significantly high CPV-specific CMI response compared to commercial vaccine group may be attributed to the apoptosis and/or the dsRNA adjuvant effects.

This study reports the successful oral vaccination by *E. coli* DH5 delivering replicon-based CPV DNA vaccine for eliciting sufficient levels of antibody and cellular immune responses specific to CPV. This method of immunization is convenient and cost-effective. Taken together, oral DNA vaccination may be an effective alternative strategy for vaccination of the dogs and can be considered as a promising approach for mass vaccination of dogs.

**Acknowledgements.** This work was (in part) supported by the grant No. BT/PR6442/AAQ/01/239/2005 from the Department of Biotechnology, Government of India and by the Indian Council of Agricultural Research, Government of India.

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