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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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+CD4+ and CD3+CD8+ 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 Paroviridae), 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; Harirahan 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...
efficient delivery of either DNA vaccine constructs or vaccine antigens (Lilleveist 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 Versinia spp. or intraphagosomal pathogens like Salmonella spp. (Li et al., 2006; Qu et al., 2008; Yang et al., 2009, 2010) or intracytосolic 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 DH5α 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% CO2 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 DNA vaccine (pAlpha-CPV-VP2), the DNA fragment containing full length VP2 gene was obtained by digesting pTarget-C-CPV-VP2 (Gupta et al., 2005) with Nhel and SmaI restriction endonucleases and ligated into Xbal and Stul 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 DH5α 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 DH5α as vaccine vehicle was analyzed by inoculating orally 10 times higher dose (10°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°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°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 (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.

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 TCID50 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+ and CD8+) 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+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 acquired data were analyzed using BD CellQuest program (BD Biosciences). Fold increase in effector cell
DAHIYA, S. S. et al.: AN ORAL REPLICON-BASED CPV DNA VACCINE ELICITS IMMUNE RESPONSES IN DOG

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.
DAHIYA, S. S. et al.: AN ORAL REPLICON-BASED CPV DNA VACCINE ELICITS IMMUNE RESPONSES IN DOGS

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+ and CD8+) 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+ and CD8+) 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. 4
Flow cytometry of lymphocyte proliferation responses to CPV vaccines
Stimulation with inactivated CPV or none.

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
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 vac-
cination of the dogs and can be considered as a promising
approach for mass vaccination of dogs.

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canine parvovirus VP2 gene and its use as DNA vaccine

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