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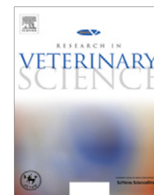

Immunogenicity of a DNA-launched replicon-based canine parvovirus DNA vaccine expressing VP2 antigen in dogs

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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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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.

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