Cloning of canine distemper virus H gene in replicase based eukaryotic vector and analysis of its expression

Sudarshan Kumar, Anant Rai, Praveen K Gupta, Soni Gangwar
Division of Animal Biotechnology, Indian Veterinary Research Institute, Izatnagar, Bareilly-243122, UP

Summary: The CDV H gene has been cloned in replicase based mammalian expression vector pSinCMV with the purpose of reducing the dose of vaccination as well as utilizing the special properties of vector to induce innate antiviral immune pathway. The plasmid pSinCMV.cdhv was tested for its expression in vitro by indirect fluorescent antibody test, immunoperoxidase test and western blotting.

Key words: Canine distemper virus, replicase, mammalian expression vector, cloning

Introduction

Canine distemper is one among the infectious diseases of dogs caused by virus canine distemper (CDV) which affects pups of age group mostly between 2 to 6 months old. The mortality is between 30 to 80% and the recovery from the disease depends on the immune status of the animal (Fenner et al., 1987). Many killed and live modified vaccines against CDV are in use (Bolt et al., 1997). Where on one side killed vaccines have been associated with low or no humoral immunity at all (Montali et al., 1993; Williams et al., 1996; van Heerden et al., 2002). Maternal antibodies usually interfere (Pare et al., 1999) with live modified vaccines and reduce its efficacy. Maternal antibodies against canine distemper virus have a half life of around 11 days (Pare et al., 1999). But these antibodies have been reported to interfere with vaccination with modified live virus vaccines till 18 weeks of age (Pare et al., 1999). Modified live virus vaccines have been suspected to retain residual virulence (Appel and Harris, 1988; Durchfeld et al., 1990; Carpenter et al., 1976, 1998).

Materials and Methods

Vector: The replicase based pSinCMV vector obtained from Dr. Suryanarayana, IVRI Bangalore campus, (Nagarajan, 2005), was used.

CDV H gene: Already cloned in pGEMT vector was used as the candidate gene, the sequence accession No. is AM903376. The primers are as given Forward primer: 5'-AAC AAT GCT CTC CTA CCA GGA C-3' and Reverse primer: 5'-TGT CAG GGA TTT GAA CGG TTA-3'

Real time PCR primers:

1. Canine IL-2 RT forward 461 bp
   CTC ACA GTA ACC TCA ACT CCT GC
2. Canine IL-2 RT reverse 24 TTC TGT AAT GGT TGC TGT CTC GTC
3. Canine IFN gamma RT forward 281 bp
   TCG GAC GGT GGG TCT CTT TTC G
4. Canine IFN gamma RT reverse 25 CAC TTT GAT GAG TTC ATT TAT CGC C
5. Canine IL-4 RT forward 317 bp 23 TAA AGG GTC TCA CCT CCC AAC TG
6. Canine IL-4 RT reverse 24 TAG AAC AGG TCT TGT TTG CCA TGC
7. Beta actin gene forward 540 bp 21 GTGGCCGCTCTAGCCACCAA
8. Beta actin gene reverse 27 TCATTGATGCACGCAGCATTTCT

**Conjugates and hyperimmune serum:** Direct polyclonal antibody conjugate for CDV was purchased from VMRD, Pullman, USA. Hyperimmune sera against H gene were raised in mice using recombinant plasmid. The recombinant plasmid (50µg/mouse) was administered four times at interval of one week, to six mice. The blood was collected from retro-orbital plexus of eye of the mouse and serum was prepared.

**Experimental Animals:** Four weeks old Swiss albino mice and one month old pups were used.

**Revival of pGEM-T Easy clone:** pGEM-T easy clone containing CDV H gene insert was revived in LB broth and plasmid DNA was isolated. Restriction endonuclease digestion of rplasmid pGEMT.cdvh with NotI enzyme.

**Blunting of NotI generated CDV H gene staggered ends:** For blunting of staggered ends generated by NotI, T4 DNA polymerase was used in 50µl reaction mix, Insert 20µl, 10X T4 DNA polymerase buffer 5 µl, T4 DNA polymerase 2µl, dNTPS mix, (10mM each)1µl, Nuclease free water 22 µl. The reaction mixture was incubated at 37°C for 10 min and the reaction was stopped by heating at 75°C for 10 min in thermal cycler. The blunted CDV H insert was purified following protocol of (Sambrook and Russell, 2001).

**pSinCMV vector preparation:** StuI was used to create blunt end using 50µl reaction mix. The linearised plasmid was checked and quantitated on 1% agarose gel electrophoresis.

**Dephosphorylation of 5' ends:** CIAP (calf intestinal alkaline phosphatase) was used to remove 5’ phosphate group from both the ends of the linearized plasmid. Reaction mix was: linearized vector pSinCMV (100ng/µl) 11µl, CIAP enzyme 1µl, buffer (10X) 5 µl, nuclease free water 33 µl. Fermentas protocol was followed. Purification of dephosphorylated linearized vector was done as used in purification of H gene insert.

**Blunt end ligation of PSinCMV vector and CDVH gene:** A 10 µl reaction mixture was standardized with 30% PEG8000 for blunt end ligation containing T4DNA ligase (Fermentas) 2 µl, PSinCMV Vector (100 ng/µl) 0.5 µl, CDV H gene (150 ng/µl) 4 µl, ligation buffer (10X) 1µl, 30% PEG8000 (Amersco) 1.5 µl, nuclease free water 1 µl. The reaction mixture was incubated overnight at 16°C.

**Transformation of E.Coli DH5α cells with the ligated product** was done as per Sambrook and Russell (2001).

**Screening of recombinant clones:** A large number of colonies (approximately 100) were picked from the overnight grown transformants. The individual colonies were inoculated in fresh ampicillin (50 µg/ml) containing LB broth and allowed to grow for 18 to 24 hours. Plasmid was isolated from these colonies by alkali lysis method as well as using Promega SV miniprep kit. All plasmids were checked on 1% agarose gel electrophoresis.
agarose gel electrophoresis for any detectable change in its size due to transformation.

**Digestion of plasmid with *EcoRV* and *KpnI* to check the presence of CDVH insert:** Isolated plasmids were checked for the presence of insert by digestion with enzyme *EcoRV* and *KpnI*. The digestion mixture was prepared by mixing the components in amounts as mentioned below.

<table>
<thead>
<tr>
<th>Component</th>
<th>Digestion with <em>EcoRV</em></th>
<th>Digestion with <em>KpnI</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid</td>
<td>2.0 µl</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Enzyme (Fermentas) (10 U/µl)</td>
<td>1.0 µl (<em>EcoRV</em>)</td>
<td>1.0 µl (<em>KpnI</em>)</td>
</tr>
<tr>
<td>Buffer (10X)</td>
<td>1.5 µl</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>10.5 µl</td>
<td>11.5 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>15.0 µl</strong></td>
<td><strong>15.0 µl</strong></td>
</tr>
</tbody>
</table>

The reaction mixture was vortexed and spun and incubated in a waterbath at 37°C overnight. The digested mixture was then electrophoresed in 1% agarose (Low EEO, Bangalore Genei).

**Digestion of plasmids with enzyme *XbaI* to identify the clones with insert in correct orientation**

- Plasmid DNA (300 ng/µl) 1.0 µl
- *XbaI* enzyme (Fermentas, 10U/µl) 1.0 µl
- Tango buffer (10X) 1.5 µl
- Nuclease free water 11.5 µl
- **Total** 15.0 µl

**Sequencing of cloned canine distemper virus H gene:** Once the recombinant plasmids were identified containing CDV H gene in correct orientation, a part of the same plasmid lot was sequenced with BGH reverse primer. The obtained sequence was analysed by BLAST (NCBI). The sequence was submitted to public domain of NCBI GeneBank and accession number obtained.

**Transfection of CrFK/MDCK cells:** Cell lines were obtained from NCCS, Pune. For IPT and IFAT, cell was subcultured in 24-well plate and for Western blot analysis 25 cm² flask was used. At 60 to 80% confluency, cells were transfected with ultra pure plasmid DNA using Polyfect Transfecting Reagent (Qiagen, 2 mg/ml) as per the protocol of Qiagen transfection kit.

**Raising primary antibody against pSinCMV.cdvh in mice:** Primary polyclonal antibody against the CDV H gene was raised in mouse by hyper immunization with pSinCMV.

**Indirect fluorescent antibody test (IFAT):** After transfection, Cells (CrFK) were washed twice with 1X PBS and fixed with 4% paraformaldehyde. Mouse primary anti CDVH hyperimmune sera (1:50 dilution) was added in vaccine, vector and healthy wells in duplicate incubated at room temperature for two hours. After the
incubation, wells were washed with 1X PBS and incubated with 1:40 dilution of FITC conjugated Secondary antibodies goat anti-mouse (Banglore Genei). Separately vector and healthy control wells were also given similar treatment. After two hour of incubation at room temperature wells were washed with 1X PBS, mounted with 50% glycerol in PBS. The plate was examined under fluorescent microscope (Nikon) and photographed.

**Immunoperoxidase test (IPT):** In a 24 well plate MDCK cells, transfected with rpl amrid and vector each in duplicate along with healthy controls were incubated for 48 hours at 37°C. Cells were washed with 1X PBS twice and fixed with 4% paraformaldehyde. After washing the fixed cells with PBS, cells were treated with 2% H₂O₂ in PBS for 10 minutes. Cells were incubated with mouse anti CDVH hyperimmune sera for 2 hours and washed thrice with PBS, 5 minutes each. HRPO conjugated rabbit anti-mouse antibody was added to wells and incubated for 1 hour at room temperature. The cells were again washed with PBS thrice and incubated with 3, 3'-diaminobenzidine (DAB 1mg/ml in PBS with 1 µl/ml H₂O₂) for 5 minutes at room temperature. After the development of color, cells were washed with PBS, dried in air and observed under microscope and photographed.

**Western blot analysis:** MDCK cells were transfected in 25 cm² flasks with pSinCMV.cdvh vaccine (5 µg) and pSinCMV vector (5 µg). After 48 hours of transfection, cells were processed following the method of Sambrook and Russell, (2001). Briefly, cell were trypsinized with 0.5 ml of trypsin (0.2%) and pelleted in 1ml PBS by centrifugation at 8000 rpm for 4 minutes. The cells were resuspended in extraction buffer; freeze- thawed and supernatant was aliquoted in 40 µl tubes. Laemali buffer was added to each aliquot to a final concentration of 1X, boiled in hot water bath for 5 minutes and loaded on 10% polyacrylamide gel in parallel with molecular weight marker. After completion of electrophoresis, the protein bands from unstained gel were blotted on to nitrocellulose membrane using Semi-dried system apparatus (Atto) at 2mA/cm² for 90 minutes. The nitrocellulose membrane was incubated in 5% skimmed milk in PBS-T (PBS containing 0.05% Tween –20) solution overnight at 4°C for blocking. Blocked membrane was probed with mouse anti-CDVH hyperimmune serum diluted 1:100 in 2.5% skimmed milk in PBS-T for one hour at 37°C with constant shaking. Washing was performed four times with PBS containing 0.05%Tween-20, allowing ten minutes per wash. HRPO conjugated rabbit anti-mouse antibody was added and incubated for one hour at 37°C with constant shaking. After washing thrice for 10 minutes each, TMB H₂O₂ (Bangalore Genei) was added as substrate to develop colour. The reaction was thereafter stopped by washing the membrane in running water and bands were photographed.

**Results:**

**Subcloning of cdvh gene in pSinCMV.cdvh**

The CDV H gene cloned in pGEM®-T Easy vector was released by digesting with NotI enzyme and the 1870 base pairs including some sequences from the vector’s MCS and remainder vector backbone of 3015 bp (Fig. 11). The CDV H gene was converted into blunt end fragment by treatment with T4 DNA polymerase enzyme (Fig. 12) and further cleaned of its other constituents by phenol chloroform precipitation method. The pSinCMV vector was linearized by digesting with StuI enzyme (Fig. 12), gel eluted, purified by phenol chloroform precipitation method
and dephosphorylated with calf intestinal alkaline phosphatase (CIAP) enzyme and further purified.

Blunt end ligation was done in the presence of PEG 8000 and ligated vector was transformed in E.Coli (DH5α) cells. The colonies were screened by PCR amplification of isolated plasmids with CDVH gene specific primers. It resulted in amplification of H gene giving a product of length of 1830 bp on 1% agarose gel electrophoresis perfectly matching with the fragment size amplified with gene specific primer on positive recombinant clone pGEM-T Easy (Fig. 13).

Such plasmids giving amplification of desired product length were again checked for the presence of insert with restriction enzymes. Vector sequence along with the cloned insert was analysed in mapdraw to find restriction enzymes which could release the product of desired length. The enzymes KpnI and EcoRV were chosen which had two sites for each enzyme. Digestion with KpnI enzyme released a smaller product of 4767 bp and a larger fragment of 7882 bp, while digestion with enzyme EcoRV released a smaller fragment of 4128 bp and a larger fragment of 8521 bp (Fig. 14). The fragments size were in agreement with the prediction from mapdraw analysis.

Once the presence of CDVH insert was confirmed by PCR and restriction digestion analysis, the orientation of insert in these plasmids were detected by specific restriction enzyme XbaI which has two sites in the recombinant plasmid, one in the insert and the other in the vector. The fragment length resulting from this digestion in both the possibilities of wrong and right orientation was predicted from sequence analysis on mapdraw (DNASTAR). The digestion with XbaI enzyme released two fragment of size 739 and 11909 bp on clone 14 while 1386 and 11262 bp fragments were obtained from digestion on clone 15 and 18. The respective length of fragments size confirmed that clone 14 contained insert in wrong orientation while plasmid clones 15 and 18 contained insert in right orientation (Fig. 15).

The rplasmid clone with insert in right orientation (pSinCMV.cdvh) (Fig. 16) was sent for sequencing at Chromous Biotech Ltd. The sequence data was blasted (NCBI BLAST) and found to be 96-98% homologous to other reported H gene of canine distemper virus.

Expression of recombinant plasmid

The expression of rplasmid was detected in transfected cells. Good amount of fluorescence was observed in cells transfected with rplasmid pSinCMV.cdvh (Fig.17A) while vector transfected cells (Fig.17B) and healthy mock cells which were not transfected with DNA (Fig. 17C) failed to show any immunofluorescence. The recombinant plasmid was found to express the protein in MDCK cell line as detected by IPT (Fig.18A). The vector alone (Fig.18B) and untransfected healthy cells (Fig.18C) failed to show any colouration indicating that the CDVH gene was expressed in cells due to the presence of rpSinCMV.cdvh plasmid. The western blot analysis (Fig. 19) of expressed protein indicated a specific isolated band approximately in the range of 72 to 80 kDa protein marker band which was very close to the molecular weight of CDV glycosylated H protein (78 KDa). The vector transfected cells and mock transfected cells failed to show any band. In a separate flask transfection with pSinCMV.cdvh in MDCK cell line was compared with other flask of same cell line transfected with vector and mock cells (Fig. 20). An apoptosis type of appearance was observed in flask transfected with
pSinCMV.cdvh, indicating that the plasmid is expressing the insert along with dsRNA.

![Fig. 11. Restriction digestion of pGEM-T.cdvh plasmid with NotI enzyme]

**Lane M:** Marker 1Kb ladder.
**Lane 1:** pGEM-T.cdvh r- plasmid undigested
**Lane 2:** pGEM-T.cdvh r- plasmid digested with NotI releasing a product of 1870 bp and 3015 bp

![Fig. 12. Preparation of Vector & Insert]

**Lane M:** 1Kb DNA marker
**Lane 1:** pSinCMV vector
**Lane 2:** pSinCMV vector digested with Stul enzyme
**Lane 3:** Blunted CDV/H gene
**Lane 4:** pSinCMV.cdvh vaccine
Fig. 13 PCR amplification of pSinCMV.cdvh plasmids with CDV H gene specific primers along with confirmed pGEM-Tcdvh plasmid

**Lane M:** Marker 1Kb ladder.
**Lane 1&2:** Sample no 14 & 18 (pSinCMV.cdvh) plasmid with product size 1330 bp.
**Lane 3:** A confirmed pGEM-Tcdvh plasmid with CDVH insert.

Fig. 14 CDV H gene confirmation on 1% Agarose gel

**Lane M:** 1 Kb Marker
**Lane 1-3:** EcoRV digested fragments of 4128 bp & 8521 bp
**Lane 4-6:** KpnI digested fragments of 4767 bp & 7832 bp
Fig. 15 Confirmation of plasmid in right orientation by digestion with XhoI enzyme
Lane M: 1 Kb ladder
Lane 1, 3 & 5: pSinCMV-cdvh undigested
Lane 2: Sample no 14 releasing product of 739 & 11909 bp indicating wrong orientation of the insert.
Lane 4 & 5: Sample no 16 & 18 releasing the product of 1326 & 11262 bp indicating the right orientation of the insert.

Fig. 16 Indirect Fluorescent Antibody Test (IFAT)

Fig. 17 Immunoperoxidase test (IPT)
Fig. 18. Western Blot pSinCMV cdvh in vitro expression
Lane M: Kd protein marker
Lane 1: Mock transfected
Lane 2: Vector control
Lane 3 & 4: pSinCMV cdvh

Fig. 19A MDCK Cell line transfected with pSinCMV cdvh
Fig. 19B MDCK Cell line transfected with pSinCMV (Vector Control)
Fig. 19C MDCK Cell line (Healthy Control)

Fig. 19 pSinCMV cdvh induced apoptosis

Discussion

Canine distemper is one among the infectious diseases of dogs caused by virus canine distemper (CDV) which affects pups of age group mostly between 2 to 6 months old. The mortality is between 30 to 80% and the
recovery from the disease depends on the immune status of the animal (Fenner et al., 1987). Many killed and live modified vaccines against CDV are in use (Bolt et al., 1997). Where on one side killed vaccines have been associated with low or no humoral immunity at all (Montali et al., 1993; Williams et al., 1996; van Heerden et al., 2002). Maternal antibodies usually interfere (Pare et al., 1999) with live modified vaccines and reduce its efficacy. Maternal antibodies against canine distemper virus have a half life of around 11 days (Pare et al., 1999). But these antibodies have been reported to interfere with vaccination with modified live virus vaccines till 18 weeks of age (Pare et al., 1999). Modified live virus vaccines have been suspected to retain residual virulence (Appel and Harris, 1988; Durchfeld et al., 1990; Carpenter et al., 1976, 1998). DNA vaccines were attempted to achieve a balance between strength of immunity conferred and the extent of safety (Cirone et al., 2004; Dahl et al., 2004; Polack et al., 2000). But a serious study on DNA vaccination on dogs against canine distemper is still lagging.

Among the six proteins of canine distemper namely H, N, P, M.L and F, the highest antigenic variation is found in the H protein, whereas F and P proteins are affected to a much lower extent (Blixenkrone-Moller et al., 1993). As the H protein’s primary function is cell receptor binding, its characteristic is therefore most closely related to the effective infectivity and host specificity of the virus (Gassen et al., 1986). The protective efficacy of anti H monoclonal antibodies is higher than that of anti F mAbs (Hirayama et al., 1991). So in our study we have used CDVH gene of Lederle strain to induce immunity against canine distemper. Although, there exists highest variation in H gene among all the proteins of CDV of different strains (Hashimoto and Mochizuki, 2001; von Messling et al., 2001) we have used Lederle strain in our DNA vaccine. CDV Lederle strain was first isolated in 1951 from dog with encephalitis (information provided by ATCC) (Lednicky et al., 2004). Since the Lederle strain has stabilized in the local animals and is still actively circulating (Lednicky et al., 2004), we assume that the H protein of this strain will be a good immunogenic to be used as vaccine. On BLAST the sequence was found to bear 96-98% homology with other genomic and CDVH gene complete CDS.

DNA vaccines have been criticized for its lower potency in terms of antibody titers and the cost of production. We assume a direct correlation between dose of DNA vaccine and the cost of production. The reported dose of DNA vaccines by intramuscular dose is 100 to 300 µg per animal. We have tried to clone the H gene in replicase based mammalian expression vector namely pSinCMV, a fourth generation DNA vaccine and see its immunological response in dogs. Replicase based vectors are superior over other conventional vectors in terms of its lower requirement of dose of immunization (Xiong et al., 1989; Hariharan et al., 1998, Berglund et al., 1998, Leitner et al., 2000), the mechanism of breaking tolerance against self antigen, the power of inducing apoptosis (Leitner et al., 2003) so that transient but robust expression of antigen is achieved in short period without taking risk of integration into host chromosomes (Jolly, 1994; Miller et al., 1993, Samulski et al., 1989), a broad host range, and infection of non dividing cells (Strauss et al., 1994). pSinCMV is an alpha virus (Sindbis virus) based plasmid vector which serves as negative strand once it enters into the host cell. There after entering into the nucleus it is transcribed by host RNA polymerase enzymes from CMV promoter into a full length RNA transcript. This full length transcript then acts as positive sense alpha virus which in turn is translated in cytoplasm to form
replicase protein. This protein serves as RNA dependent RNA polymerase enzyme and forms negative sense RNA from positive sense transcript. From this negative sense strand full length as well as smaller fragments from subgenomic promoters are transcribed which in-turn is translated into proteins. Since the cloned insert is downstream to the subgenomic promoter, the translated proteins represents our target proteins. The subgenomic promoter of alpha virus is very strong so that it makes large number of target mRNA from the sequence downstream to it.

In comparative studies of conventional (nonreplicating) plasmid DNA vectors and alphavirus DNA-based replicon vectors, the latter generally induces stronger immune responses and at a significantly lower DNA concentrations than does conventional vectors (Berglund et al., 1998 Hariharan et al., 1998) It has been shown that innate antiviral pathways implicated in the molecular mechanisms of innate antiviral immunity (double stranded RNA recognition and interferon action) are also one of the mechanism underlying the superior efficacy of replicate based DNA vaccines (Leitner et al., 2003). By various experiments it is now beyond doubt that apoptosis has direct relation with immunostimululatory action. Genes for proapoptotic molecules have been codelivered with DNA vaccines to specifically induce apoptosis in transfected cells. Apoptosis inducing alpha virus replicase based RNA and DNA constructs have been used to deliver antigens of interest. These naked nucleic acid vaccines owe their enhanced immunogenicity not to increased antigen production, but to the requisite production of double stranded RNA species, which results in the quantitative induction of apoptosis and innate immunity (Leitner et al., 2000; Ying et al., 1999). Induced apoptosis is one of the mechanisms for the enhanced immunogenicity of replicate based DNA vaccines apart from the large amount of antigen production under its subgenomic promoter (Leitner et al., 2004).

*In vitro* expression analysis showed that plasmid transfected MDCK and CrFK cells expressed the CDVH protein. Western blot analysis revealed a single band of molecular weight around 78 KDa which is in the range of CDVH glycoprotein reported by others (Orvell, 1980; Rima et al., 1991; Iwatsuki et al., 1997). Japanese field isolates and the Onderstepoort strain have 607 and 604 amino acids respectively and have molecular mass of 68.4 and 67.3 respectively (Iwatsuki et al., 1997). The size of cloned insert in our experiment is 1830 bp with an open reading frame of 1824bp which by prediction after translation should be of 607 amino acids. Although, the ideal approach to demonstrate vaccine efficacy is to perform challenge studies in vivo. However it is well known that experimentally reproducible clinical canine distemper is hard to achieve. Only one authorized challenge system has been adopted world wide: an intracranial challenge exposure of the dog passaged virulent Snyder Hill strain that belongs phylogenetically to the old CDVs (Mochizuki et al., 2002). To assess the efficacy of recombinant pSinCMV.cdh, *in-vivo* studies of immunological responses has been done by serum neutralization test, lymphocyte stimulation test, measurement of CD4+ and CD8+ cells by FACS and measurement of different types of cytokines by Real time PCR.

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