
2. Cloning of chicken anemia virus VP3 gene in a mammalian expression vector and analysis of its apoptic activity- Dimple Thakuria, Anant Rai, Ashwin Ashok Raut, Sudarshan Kumar, Ankur Saxena 154-179


Biotechnology International, Vol 1, No. 4, 2008

Development of replicase based DNA vaccine containing fowl adenovirus 4 hexon gene

Maninder Sandey, Anant Rai*, Ankur Saxena

Division of Animal Biotechnology, Indian Veterinary Research Institute, Izatnagar, Bareilly-243122, UP, India

*raia48@gmail.com

Abstract

A replicase gene based DNA vaccine against FAV4 by cloning hexon gene in replicase based vector pSinCMV containing replicase gene of alphavirus was developed. The recombinant plasmid designated as pSinCMVfav4hex expressed the target protein in vitro when analyzed by SDS-PAGE, western blot, IFAT and IPT. It was used for immunization of chicks and/or embryonated eggs at various doses with or without booster which rendered high level of immune response (humoral as well as CMI) and protection among chicken challenged with virulent strain of FAV4. Level of immune response and protection was found to be higher among groups vaccinated with replicase based vaccine even at very low doses (1µg) than those injected with live attenuated or conventional DNA vaccines.

Key words: Replicase gene, FAV4, hexon poultry, DNA vaccine, RNA vaccine

Introduction

Fowl adenovirus infections are widespread in most of the poultry raising countries of the world. Domestic avian species of all ages are susceptible to infection. The adenoviruses constitute the Adenoviridae family of viruses, which is divided into two genera, Mastadenovirus and Aviadenovirus (Norrby et al., 1976). The Aviadenovirus genus is limited to viruses of birds, whereas the Mastadenovirus genus includes human, simian,
bovine, equine, porcine, ovine, canine, and possum viruses. Fowl adenoviruses are non-enveloped icosahedral particles that range in size from 70-100 nm in diameter. It has 252 capsomers of which 240 are hexons and 12 pentons (Vertex capsomers).

On the basis of immunological properties, three groups of Aviadenovirus are recognized, with group I containing 12 serotypes designated FAV 1 to 12 (McFerran and Adair, 1993). The fowl adenoviruses belonging to group I have been associated with a number of clinical conditions like effect on egg production (Cook, 1972), respiratory disease (Kawamura et al., 1963; Verma et al., 1971) and inclusion body hepatitis (Aghakhan and Pattison, 1974). Group II Aviadenoviruses include the pathogens responsible for haemorrhagic enteritis in turkeys (Pomeroy, 1972), marble spleen disease in pheasants (Domeruth et al., 1972) and splenomegaly virus of chickens. Egg drop syndrome 1976 is the principal pathogen in Group III and is serologically similar to isolates from water fowl, which are considered to be progenitors of EDS 76 virus (Van Eck et al., 1976). The FAV genome consists of a linear double stranded DNA molecule of 43-45 kb in size, which is significantly larger than its human equivalent (Zsak and Kisary, 1984).

Sheppard et al. (1995) identified the gene for the major capsid protein (hexon) of fowl adenovirus serotype 10. The gene has been found to be located from 46.85 to 52.81 map units.

The hexon protein is the major protein of the adenovirus capsid (Philipson, 1979; Toogood et al., 1989). Each hexon protein is composed of three identical polypeptide chains and the coding region for the hexon occupies nearly 10% of the human adenovirus genome (Philipson, 1979). Since the hexon is the major component of the virion and has been shown
to carry type, group and subgroup antigenic determinants (Norrby, 1969), it is of interest to locate and characterize the FAV hexon gene so as to enable comparison of this major structural protein with other known hexons. The crystallographic investigations of the protein led to the establishment of its three dimensional structure (Roberts et al., 1986). According to this model, hexons are homotrimer structures that give rise to a pseudohexagonal shape with a triangular top superimposed on the base. The pseudohexagonal base has been shown to be composed of three copies each of the two more conserved pedestal regions P1 and P2 (Toogood et al. 1989). The major amino acid changes, whether they are deletions, insertions or mutations are concentrated more frequently in the regions forming the three triangular projections that form the exposed surface of the capsid (Roberts et al., 1986). The triangular projections or towerscomplex non-enveloped icosahedral capsid have been studied. The virus is resistant to lipid solvents, acidic pH and is heat stable at 56°C (Yates and Fry, 1957). The virus is also resistant to trypsin, 2% phenol and 50% ethyl alcohol (Kawamura et al., 1964).

The major structural proteins are the hexon and the fibre, which is non-covalently linked to the penton base, a structure named as penton (Valentine and Pereira, 1965). The hexons consist of a trimer of polypeptide II with a central core; VI, VIII and IX are minor polypeptides also associated with the hexon and thought to be involved in stabilization and/or assembly of the particle. The pentons are more complex; the base consists of a pentamer of peptide III, besides five molecules of IIIa are also associated with the penton base. The pentons have a toxin like activity. Purified pentons cause CPE in the absence of any other virus components. A trimeric fibre protein extends from each of the 12 vertices and is responsible for recognition and binding to the cellular receptors. A globular domain at the end of the adenovirus fibre is responsible for recognition of the cellular receptors. Whereas all mammalian adenoviruses have only a single fibre associated with the penton base, the FAV reflect a morphological peculiarity of two fibres per penton base (Laver et al., 1971). The fibre head is the distal C-terminal part of the protein and carries the type-specific antigen, which is responsible for the haemagglutinating activities (Norrby, 1969). The hexon protein is the major capsid protein of the non-enveloped icosahedral virion on which type, group and subgroup-specific determinants are located (Norrby, 1969).

There have been very few reports on cloning of fowl adenoviruses. Sheppard et al. (1995) identified the gene for the major capsid protein (hexon) of FAV10 by the use of the
expression vector pGEX. They mapped, cloned and sequenced the hexon gene for FAV10 from a Sau3A genomic library of FAV10.

The cloning of hexon gene of FAV4 of Indian origin has been reported by Barua and Rai (2003) who cloned the 2916 bp hexon gene amplified by self designed primers in a mammalian expression vector pcDNA3.1/V5-his-Topo vector (5523 bp, Invitrogen) in India. The recombinant plasmid (8.05kb) was found to express hexon protein in transfected vero cells as detected by immunoperoxidase test. When used as DNA vaccine in 3 days old chicken with 50 µg DNA given intramuscularly the vaccinated birds withstood challenge with 10,000ID50 of virulent liver passaged FAV4 after 21 days of vaccination while the unvaccinated control birds either died or developed the disease (Rai et al., 2005) The field trial of the vaccine showed that it was safe and potent and sera collected from 25 vaccinated birds revealed SN titre of 128-256 against FAV4.

Alpha viruses are enveloped, positive strand RNA viruses that include Sindbis virus, Semiliki Forest virus and the human pathogens Eastern and Western Equine Encephalitis viruses (EEV & WEV). Over the past 15 years, the alpha virus replication and packaging machinery has been adopted for overexpression of heterologous gene products in animal cells (Berglund et al., 1996). After alpha virus enters a cell, the genomic RNA is translated to produce nonstructural (NS) proteins. The NS proteins function to produce negative sense genomic molecules from which large amounts of new genomic and subgenomic RNA are transcribed.

The subgenomic RNA which may reach 1,00,000 copies per cell, codes for viral structural proteins needed for the production of progeny virions. The entire life cycle of alpha viruses takes place in the cytoplasm.

Several characteristics of alpha viruses make them attractive candidates for overexpression of genes in mammalian system for example, size of positive sense ssalpha viral genome (encoding its own replicon) is 11.7 kb which facilitates the construction and manipulation of cDNA clones. Thus productive replication can be initiated either by infection of the cell or by transfection of the genomic RNA into the cytoplasm of the cell. In both cases, vigorous replication is associated with high level production of new virus particles, which may be as many as $10^5$ per cell. Theoretically up to 200000 copies of RNA can be produced in
a single cell within 4 hours and expression of the encoded antigen can be as much as 25% of total cell protein (Rolls et al., 1994). The structural proteins of the virus are expressed from a subgenomic RNA which is colinear with the last third of the genomic sequences. This has allowed the manipulation of the subgenomic sequences without affecting the replication capacity of the system. Additionally, expression is under the control of the virus (not the host), and the lack of complex non-enveloped icosahedral capsid have been studied.

An RNA polymerase II expression cassette is introduced to drive the transcription of a self amplifying RNA (replicon) vector, which allows direct use of plasmid DNA for transfection and expression studies (Berglund et al., 1996; Dubensky et al., 1996).

The benefits of this strategy are lower costs, a more stable molecule and no risk of producing replication proficient virus. Preliminary results from cell culture experiments have shown that this DNA system gives at least 10 fold higher expression levels as compared to conventional naked DNA delivery methods (using pCMV plasmids) (Herweijer, 1995; Dubensky et al., 1996).

**Materials and Methods**

**Vector-pSinCMV**

The vector was obtained from the lab of Dr Suryanarayana, IVRI, Bangalore. This vector was developed as part of Ph.D thesis work by Dr. Nagarajan (Nagarajan, 2005). Replicase gene from pSinHisA was used to amplify the foreign gene at very high copy number. CMV promoter and polyadenylation signal sequence were cloned from the pcDNA3.1+ vector. The final vector was named pSinCMV.
**Birds and fertile eggs**

One-day-old broiler chicks and 11-day old fertile hen eggs were procured from the hatchery section of Central Avian Research Institute (CARI), Bareilly.

**Isolation of plasmid DNA**

Small scale plasmid DNA isolation was done following the alkali lysis method (Birnboim and Dolly, 1979).

**Restriction endonuclease digestion and analysis of plasmid DNA**

The plasmid DNA (5-10 µg) was incubated overnight with appropriate restriction endonuclease (MBI Fermentas, USA/Promega, USA) at 37°C in a water bath. The digested DNA was mixed with 6X loading dye and run on 0.8%-2% agarose (SRL, Mumbai) gel containing ethidium bromide using tris-acetate EDTA running buffer system. The stained gel was visualized under UV-transilluminator and photographed.

**Blunting**

Ends (termini) of gel purified digested products (Insert) was made blunt using T4 DNA Polymerase (Promega, USA). The reaction mix consistef insert 4 µg, 10 X reaction buffer 5 µl, 10mM dNTPs 1 µl, T4 DNA Polymerase (7.9units/µl)1 µl,nuclease free water to 50
µl. The sample was briefly spun and incubated at 37°C for 5 min. Thereafter, it was kept at 70°C for 10 min to inactivate the enzyme. After this sample was subjected to phenol: chloroform precipitation.

**Dephosphorylation of Blunt ended pSinCMV vector**

The dephosphorylation of the StuI digested and blunt ended plasmid vector was carried out using calf intestinal alkaline phosphatase.

**DNA extraction from agarose gel**

The gel extraction of DNA fragments was done using MiniElute Gel extraction kit (Qiagen, Germany) following manufacturer’s instruction.

**Ligation reaction**

The linear plasmid vector (approximately 25 to 50 ng) was ligated with insert in 1:4-6 ratio using 1U of T4 DNA ligase (Promega, USA) in a 10 µl reaction volume containing 1 µl of 10 mM ATP (pH 7.5). The reaction mixture was incubated at 16°C in a water bath for 16-18 hrs. The linear plasmid without insert was ligated as ligation control.

**Transformation**

The 5µl ligated plasmid was added to 195µl of TCM solution and kept in ice for 10 minutes. This mixture was added to 200 µl of E.coli competent cells along with controls having circular plasmid, ligation control plasmid and no plasmid (Sambrook and Russel, 2001). The bacterial cells were mixed gently with the DNA and kept on ice for 1 hour. After incubation, heat shock was given at 45°C for 2 minutes and the cells were again incubated on ice for 5 to 10 mintues. 600 µl of SOC was added to each tube and the tubes were kept in orbital shaking incubator at 37°C for 16–24 hrs. When bacterial colonies were visible on plates, the recombinant clones were amplified in LB broth and plasmids were isolated.

**Agarose gel electrophoresis**

Agarose, Type 1 gelling temperature (40-42°C) from SRL (Mumbai) was used to perform majority of gel electrophoresis in concentrations of 0.8% to 2.0% for the separation of DNA fragments ranging from 0.4 Kb to 14Kb using 1X TAE buffer.

**Sub-cloning of FAV4 hexon gene**
The recombinant pcDNA3.1+hisTopoFav4hex plasmid was digested with BamHI and the insert released was purified by gel extraction method. The eluted product was blunted with T4 DNA polymerase. pSinCMV vector was digested with StuI and purified by phenol-chloroform extraction. Vector was then dephosphorylated with CIAP (Calf intestinal alkaline phosphatase) and reaction was stopped by incubating at 70°C for 10 min. The ligation was done using purified linear pSinCMV vector and hexon gene fragment. Briefly, the ligation reaction mixture contained linear pSinCMV vector 50ng (1µl), hexon gene insert 250ng (6 µl) in 10X ligation buffer (1µl) was incubated along with T4 DNA ligase (1 µl) at 16°C for 16-18 hours. After ligation was over, the product was transformed in E.coli DH5a. The recombinant pSinCMVFav4Hex plasmid containing colonies were amplified in LB broth containing 100 µg/ml ampicillin overnight and miniprep was done to isolate the plasmid. The recombinant plasmid was characterized by restriction endonuclease digestion. The recombinant plasmid was digested with ApaI and XhoI to confirm the right orientation of fav4hexon gene. The digested products of plasmid were analysed on agarose gel.

**Polyfect mediated transfection**

Transfection in CEF cells was done by using Polyfect® Reagent (Qiagen) following manufacturer’s protocol. The cells were stained properly for IFAT or IPT.

**SDS-PAGE and western blot**

Transfected CEF cell monolayer in 25cm² flask was lysed with 1ml of 1X SDS-PAGE sample buffer and SDS-PAGE and Western blot analysis was performed according to the method described by Sambrook and Russel, (2001). Briefly, lysed cells in sample buffer were boiled for 5 minutes and 20 µl of this sample was run on 10% acrylamide gel along with protein molecular weight marker. Mock transfected CEF cells were kept as control. CEF cells transfected with vector alone were taken as vector control. After the run, the gel was stained with Commassie brilliant blue stain followed by destaining. For Western blot analysis, the unstained identical set of samples along with prestained protein molecular weight marker were resolved and the proteins were transferred to a nitrocellulose membrane using Semi-dried System (SDS) transfer apparatus (Atto) at 2mA/ square cm 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 washed
thrice with PBS-tween 20. Primary antibody at a dilution of 1:25 in PBS-T was added. Then membrane was again washed thrice with PBS-T. Secondary antibody was added at dilution of 1:200 in PBS-T. Membrane was again washed with PBS-T followed by addition of substrate.

**Bulk preparation of plasmid DNA**

The plasmids were isolated using Qiagen HiSpeed Plasmid Purification kit following the manufacturer’s instructions.

**Immunoperoxidase test**

The transfected cells were incubated at 37°C for 48 hrs under 5% CO2 tension. The cells were washed with PBS and fixed with 80% chilled acetone. The PBS washed cells were treated with 10% H₂O₂ for 10 min and again washed with PBS. The cells were first incubated with chicken hyper-immune antisera against FAV4 for 2 h at room temperature, washed thrice with PBS and then incubated with goat anti chicken HRPO conjugate (1:400 diluted) at room temperature for 1h. The cells were again washed thrice with PBS and incubated with substrate (Genei) at room temperature for 5 mins for colour development.

**Indirect fluorescent antibody test**

While the procedure was same as for IPT, FITC-conjugated second antibody was used in place of HRP labeled antibody.

**Immunization of birds with pSinCMVfav4hex**

**Inovo vaccination**

Standard size fertile broiler eggs (n=60) were collected from the Experimental Broiler Farm, CARI, Izatnagar and stored in the cold room at 18°C. Prior to setting in the incubator, the eggs were candled and fumigated (1x strength) with formaldehyde gas. On 18th day of incubation, eggs were taken; candled again and fertile live eggs were taken for vaccination. 60 eggs were divided into 2 groups and injected with recombinant plasmid:

30 eggs were vaccinated with pSinCMVfav4hex and 22 hatched, needle length and vaccine delivery site: 25mm, 24 gauge vertical, narrow end, Dose and route: 10µg/egg; Amniotic cavity route
A hypodermic needle with appropriate length was inserted into the egg through a hole punched in the appropriate location of eggshell after the area had been smeared with rectified spirit. A 0.1 ml inoculum of recombinant plasmid DNA was injected into each egg. All procedures were carried out under laminar flow to avoid contamination. Immediately after the injection, site was sealed with sterile paraffin wax and eggs were returned to the incubator. On 19th day the eggs were shifted to the hatcher and kept in the respective pedigree boxes.

After hatching, the chicks (43) were transferred to animal shed, division of Veterinary Biotechnology. Feed and water was given ad-libitum. Booster (Secondary) immunization was done in the hatched healthy chicks with 10µg recombinant plasmid DNA intramuscularly in the quadriceps muscles of hind leg.

Blood and serum samples were collected at regular interval for detection of humoral and cell mediated immune response.

**Immunization of birds**

The 282 healthy chicks (10 days old) were immunized with plasmid DNA intramuscularly in the quadriceps muscles of hind leg. The chicks were grouped as follows:

**Vaccination Schedule for different groups injected with pSinCMVfav4hex rplasmid**

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasmid</th>
<th>Dose</th>
<th>No. of birds</th>
<th>Day of vaccination</th>
<th>Booster</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>pSinCMVfav4hex</td>
<td>1µg</td>
<td>10</td>
<td>7 d</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>pSinCMVfav4hex</td>
<td>1µg</td>
<td>10</td>
<td>7 d</td>
<td>28 d</td>
</tr>
<tr>
<td>III</td>
<td>pSinCMVfav4hex</td>
<td>10µg</td>
<td>10</td>
<td>7 d</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>pSinCMVfav4hex</td>
<td>10µg</td>
<td>10</td>
<td>7 d</td>
<td>28 d</td>
</tr>
<tr>
<td>V</td>
<td>pcDNA3.1/V5-His-Topofav4hex</td>
<td>50µg</td>
<td>10</td>
<td>7 d</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>pcDNA3.1/V5-His-Topofav4hex</td>
<td>50µg</td>
<td>10</td>
<td>7 d</td>
<td>28 d</td>
</tr>
<tr>
<td>VII</td>
<td>In-ovo pSinCMVfav4hex</td>
<td>10µg</td>
<td>10</td>
<td>18 d</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>of incubation</td>
<td>post hatching</td>
</tr>
<tr>
<td>VIII</td>
<td>In-ovo pSinCMVfav4hex</td>
<td>10µg</td>
<td>10</td>
<td>18 d</td>
<td>10 d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>of incubation</td>
<td>post hatching</td>
</tr>
</tbody>
</table>
Detection of FAV4 hexon specific antibody

ELISA and SNT were performed as per the OIE manual (2004). Briefly, 96 well ELISA plates were coated with CEF grown Georgia strain of IBDV and FAV4 diluted 1/3 in 0.05 M carbonate-bicarbonate buffer (pH-9.6), incubated at 4°C overnight. After washing once with PBS-T, plates were blocked with 2% bovine serum albumin in PBS-T for 1 h at 37°C followed by washing with PBS-T thrice. Various dilution of serum in 100 µl volume were added in duplicate wells, incubated at 37°C for 1 h, washed thrice with PBS-T and 100 µl of 1:10000 dilution of rabbit anti-chicken HRPO conjugate (Promega) was added to each well. The plates were incubated for 1 h at 37°C, washed thrice with PBS-T and colour developed with OPD substrate. After 20-30 min of incubation at room temperature, enzyme activity was stopped by 2M H_2SO_4 and plates were read at 490 nm. The SNT was done in 96 well plate using 100 TCID_{50} CEL culture adapted FAV4 and two fold dilutions of sera (1/4 to 1/ 256) in 50 µl volume, incubated at 37°C for 1 h and then 100 µl CEF cells were added per well. Observations were read 72 h later.

Peripheral blood lymphocyte proliferation assay

Whole blood was aseptically collected from the chickens with a syringe containing acid citrate dextrose (25mM citric acid, 51.7mM sodium acetate, 81.6mM d-glucose). Blood was centrifuged at 250 g for 10 min, and buffy coats were taken and resuspended with 0.15M calcium magnesium free-PBS (pH 7.2).These cell suspensions were overlaid onto Histopaque 1.077 density gradient medium (Sigma) and centrifuged at 250 g for 30 min. Lymphocytes at the interface were collected, washed two times in PBS. Cells were again washed once with RPMI-1640 colourless media and resuspended in RPMI- 1640 colourless media supplemented with 10% FCS medium (Boyum, 1976). For proliferation assays, cells were placed into each well (2 x10^7 cells/well) of 96-well flat-bottomed tissue culture plates and Concanavalin A (ConA, Sigma) having stock of 5 mg/ml was added to each well (50 µl / well) except the negative control well and virus control well. Plates were incubated at 37°C in a humidified incubator for 72 hrs. Lymphocyte proliferation assay was done following the method of Bounous et al. (1992). Briefly, 20 µl MTT (from 5 mg/ml stock) was added to each well after 72 hrs of incubation. The plates were further incubated for 4 hrs at 37°C under 5% CO_2 and then 150 µl DMSO was added to each well. After mixing, the dark precipitate
was mixed by pipetting. The plate was read at 550 nm with reference reduction at 655 nm using a microtiter plate reader (Biorad, USA). The stimulation Index (SI) was calculated by the following formula:

\[
\text{Stimulation Index} = \frac{\text{Mean OD of ConA or virus stimulated cells}}{\text{Mean OD of unstimulated cells}}
\]

**Protection test**

All the vaccinated birds along with control were challenged with 10⁴ TCID₅₀ virulent virus 14 days post secondary immunization. The birds were observed for 10 days post challenge for FAV4 specific symptoms and mortality.

**Histopathology**

Small representative pieces (5 mm thickness) of liver and heart from respective groups were collected in 10% formalin. After 3-4 days of fixation, the tissues were trimmed to 2 mm thickness by a sharp blade and subjected to further processing (Luna, 1968), viz., washing in running water for 5-6 hrs, dehydrating in ascending grades of ethanol, clearing in benzene, embedding with melted paraffin (melting point 58°C and sectioning to obtained 4-5μ thick sections). The sectiona were then stained with haematoxylin and eosin stain for histopathological examination.

**Results and Discussion**

**Construction of a replicase based vector encoding FAV4 hexon gene**

The recombinant pcDNA3.1/V5-Topofav4hex plasmid was digested with *BamHI* (Fig. 6) and FAV4 hexon gene released (2940bp) was eluted by gel extraction method. pSinCMV vector was digested with *StuI* and dephosphorylated with CIAP (Fig. 3). A linear band of 10779 bp of pSinCMV was obtained. After that, the hexon gene insert of 2940 bp was successfully ligated to linear pSinCMV vector and the constructed plasmid was then transformed into *E.coli* DH5α competent cells and plated on ampicillin containing agar plate. After 16h of incubation at 37°C, discrete colonies were observed on plate whereas no colonies were observed on control plates. Seventy discrete colonies were selected randomly and grown in LB broth containing ampicillin. The recombinant pSINCMVfav4hex plasmid
DNA was isolated by alkaline lysis method and characterized by restriction enzyme analysis. Digestion with XhoI and ApaI confirmed the right orientation of the hexon gene (Fig. 7 & 8). All of the above restriction sites were predicted in pSinCMVfav4Hex and pSinCMV by using DNASTAR software (Lasergene).

**Expression analysis of recombinant replicase based DNA plasmids**

Immunofluorescence analysis of pSinCMVfav4hex transfected CEF cells revealed diffuse cytoplasmic fluorescence while no fluorescence was seen in mock transfected cells (Fig. 9 &10). Immunoperoxidase test of pSinCMVfav4hex transfected CEF cells showed distinct colour development while mock transfected cells did not show any reaction (Fig.11& 12). In SDS-PAGE, the cell lysate from pSinCMVfav4hex transfected CEF cells revealed major bands of approximate molecular weight 42 kDa and 100 kDa while these bands were absent in control cells and cells transfected with vector alone (Fig.13). Sample resolved in SDS-PAGE and transferred to PVDF membrane revealed ~100 kDa with anti- FAV4 polyclonal sera respectively (Fig.14).

**Immune responses against FAV4 in immunized chicks**

The plasmid DNA prepared from 500 ml LB culture yielded DNA as shown in (Table-1).

Sera collected from birds vaccinated with pSinCMVfav4hex were analysed for FAV4 specific antibody by indirect ELISA. Groups vaccinated at different dose of plasmid showed a variation in ELISA titre from 50-200 (Table 3 and Fig,26). The groups vaccinated with pSinCMVfav4hex @ 1 µg and pcDNA3.1/V5-his-/Topofav4hex showed titre of 50 while titre increased to 100 when booster was given after 21 days of first vaccination in pSinCMVfav4hex @1 µg vaccinated group. The highest titre was shown by group vaccinated with pSinCMVfav4hex @ 10 µg and In-ovo pSinCMVfav4hex @ 10µg and boostered 21 days later. The sera collected 12 days post challenge from survived birds did not show significant difference in the titres.

Lymphocyte proliferation assay using MTT dye showed marked stimulation of lymphocytes collected after second booster from DNA immunized chicks (Table 3). There was a marked increase in stimulation index of virus stimulated cells as compared to ConA stimulated cells (Table 3 and Fig. 27).
On postmortem examination, unvaccinated birds revealed straw coloured fluid in pericardial sac, pale and enlarged liver (Fig.18 & 19). However no gross changes were observed in birds vaccinated with pSinCMVfav4hex plasmid (Fig.17). Microscopically, in liver degeneration of hepatocytes with indistinct cellular membrane and occasional cytoplasmic vacuolation were observed (Fig.21). In heart mild degeneration of myocardial fibres was seen (Fig.22).

Highest (90% & 80%) protection was observed in birds vaccinated with In-ovo pSinCMVfav4hex @10µg and In-ovo pSinCMVibdVP @ 10µg boostered respectively.

In present study, we cloned hexon gene in replicase based DNA vector to elicit protective immune response against FAV4. Transfection of chicken embryo fibroblast cells with recombinant plasmid DNA expressed the hexon protein after 56-72h which was evident from indirect immunofluorescence and immunoperoxidase test. Cell lysates from transfected cells showed expected bands of ~100 kDa in SDS-PAGE which was found immunogenic on western blot. This indicated that hexon is major immunogenic protein of FAV4 and a possible vaccine candidate against HPS.

The birds receiving pSinCMVfav4hex at a dose rate of 10 µg showed higher (90%) protection as compared to group receiving pcDNA3.1/V5-His-Topofav4hex (70%), pointing towards the fact that replicase based vaccines have better protection efficacy than conventional DNA vaccines. Humoral immune response as well as CMI response as could be depicted from ELISA and LTT assay also points towards the superiority of replicase based vaccine over conventional DNA vaccine. The highest ELISA antibody titre (200) was observed with pSinCMVfav4hex @ 10 µg vaccinated groups with or without booster, however, birds receiving booster showed higher immune response than birds vaccinated with single dose ofpSinCMVfav4hex. A lower ELISA antibody titer and SN titre observed in groups vaccinated with pSinCMVfav4hex @ 1 µg indicates a dose dependent immune response but still it was better than the group receiving pcDNA3.1/V5-His-Topofav4hex given at about 50 times higher dose, which supported the previous findings that replicase based vaccines are better even at very low doses compared with conventional DNA vaccines (Wolfgang et al., 2003). Consistent with this observation, we found 80% protection in groups
vaccinated with pSinCMVfav4hex @ 1 µg. Recently, a new concept, which consist of the in ovo injection of a virus-antibody complex vaccine has emerged (Haddad et al., 1997). This novel technology utilises specific hyperimmune antiserum with a vaccine virus under conditions which are not sufficient to neutralise the vaccine virus but which are sufficient for delaying the pathological effects of the vaccine alone. This allows young chicks to be vaccinated more effectively in the presence of passive immunity even with a strain that would be too virulent to use in ovo or at hatching. In-ovo vaccinated birds with pSinCMVfav4hex @ 10 µg (18th day of incubation) showed 70% protection and 90% protection was observed when one booster was given 10 days post hatch while 60% protection was observed with 1 µg dose.

Since IBDV and FAV4 infect 3-6 week age group of broiler bird, we kept a group of birds which received both pSinCMVibdVP\textsubscript{2} and pSinCMVfav4hex plasmid. A dose dependent response was observed in the vaccinated birds with 90% protection was found in group vaccinated @ 10 µg followed by a booster dose at 21 day after primary vaccination while 70% protection was observed in birds vaccinated with recombinant plasmids @ 1µg which is considered satisfactory. MTT and ELISA assay revealed high degree of T cell proliferation and high antibody titre, respectively, against IBDV and FAV4 which marked that replicase based DNA vaccine are able to stimulate both segments of immune response. It becomes amply evident that replicase based DNA vaccines are immunogenic and provided satisfactory protection at 1µg dose level. However 10 µg dose level elicited better immune response and protection.

Table 1: DNA concentration in different plasmid vaccine used for vaccination study

<table>
<thead>
<tr>
<th>Plasmid Vaccine</th>
<th>Total DNA yield (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSinCMVfav4hex</td>
<td>470.77</td>
</tr>
<tr>
<td>pcDNA3.1/V5-His-Topofav4hex</td>
<td>490.12</td>
</tr>
<tr>
<td>Group</td>
<td>Plasmidbirds</td>
</tr>
<tr>
<td>-------</td>
<td>--------------</td>
</tr>
<tr>
<td>I</td>
<td>pSinCMVfav4hex</td>
</tr>
<tr>
<td>II</td>
<td>pSinCMVfav4hex</td>
</tr>
<tr>
<td>III</td>
<td>pSinCMVfav4hex</td>
</tr>
<tr>
<td>IV</td>
<td>pSinCMVfav4hex</td>
</tr>
<tr>
<td>V</td>
<td>pcDNA3.1+/V5-His-Topofav4hex</td>
</tr>
<tr>
<td>VI</td>
<td><em>In-ovo</em> pSinCMVfav4hex</td>
</tr>
<tr>
<td>VII</td>
<td><em>In-ovo</em> pSinCMVfav4hex</td>
</tr>
<tr>
<td>VIII</td>
<td>pSinCMV (Vector control)</td>
</tr>
<tr>
<td>IX</td>
<td>Healthy Control</td>
</tr>
</tbody>
</table>

NS- Non significant
Fig. 6. Digestion of pCDNA3.1/V5 HisTopo.fav4hex rplasmid with BamHI
M: 1 Kb ladder, 1 & 2: Release of FAV4 hex gene from pCDNA3.1/V5HisTopo.fav4hex rplasmid

Fig. 7. Characterization of pSinCMV.fav4hex rplasmid by restriction endonuclease analysis
M: 1 kb ladder, 1-3: Digestion of pSinCMV.fav4hex rplasmid with XhoI yielding fragments of 1234 bp and 12,463 bp, 4: Uncut pSinCMV.fav4hex rplasmid
Fig. 8: Characterization of pSinCMV.fav4hex rplasmid by restriction endonuclease analysis
M 1 kb ladder, 1, 2 & 3: Digestion of pSinCMV.fav4hex rplasmid with MluI yielding band of 934, 4,202 and 8,500 bp, 4: Uncut pSinCMV.fav4hex rplasmid

M 1 2 3 4
1.5 kbp
8.0 kbp
3.0 kbp
1.5 kbp

Fig. 9: Characterization of pSinCMV.fav4hex rplasmid by restriction endonuclease analysis
M: 1 kb DNA ladder, 1-3: Digestion of pSinCMV.fav4hex rplasmid with ApaI yielding fragments of 2797 and 10,922 bp, 4: Uncut pSinCMV.fav4hex rplasmid

M 1 2 3 4
10 kb
3 kb
SAGE-LAGE analysis of CEF cells transfected with pLkneo.

Lane 1: Cell lysates from control CEF cells
Lane 2: Cell lysates from untransfected CEF cells
Lane 3: Cell lysates from pSiCMVrevVP2 transfected CEF cells
Lane 4: Cell lysates from pSiCMVIver4Hes transfected CEF cells

Western blot analysis of CEF cells transfected with pLkneo.

Lane 1: Cell lysates from control CEF cells
Lane 2: Cell lysates from pSiCMVrevVP2 transfected CEF cells
Lane 3: Cell lysates from pSiCMVIver4Hes transfected CEF cells
Fig.: FAV4 specific antibody response in ELISA in birds after first and second vaccination with pSinCMVfav4hex

Fig.: Protection level in challenged birds in terms of mortality, liver and heart lesion
Fig. Indirect immunofluorescence analysis of CEF cells transfected with pSinCMVfav4hex plasmid; a) Mock transfected CEF cells, b) pSinCMVfav4hex transfected CEF cells showing positive fluorescence.

Fig. 12 Indirect immunoperoxidase analysis of CEF cells transfected with recombinant and pSinCMVfav4hex plasmid, a) Mock transfected CEF cells, b) pSinCMVfav4hex transfected CEF cells showing positive reaction.
Fig. 13  a. pSinCMVfav4hex vaccinated bird showing normal heart and liver

b. Unvaccinated bird showing pale liver and fluid filled pericardial sac

Fig. 14  Straw coloured fluid in pericardial sac
References


Anonymous (1996). Report of the study group on poultry diseases appointed by technology mission on dairy development on the advice of National Advisory Committee on Control of Animal Diseases, New Delhi, India.


alpha v beta 5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. 

respiratory syncytial virus (RSV) elicited in mice by plasmid DNA immunisation 


Anonymous (1996). Report of the study group on poultry diseases appointed by technology 
mission on dairy development on the advice of National Advisory Committee on 
Control of Animal Diseases, New Delhi, India.

Hydropericardium hepatopathy syndrome in Asian poultry. *Vet. Rec.*, 141: 271-
273.

amplification and cloning of both genome segments of an Indian infectious bursal 

Ph.D thesis I.V.R.I (India)

24: 85-87.


Sequencing and comparative analysis of hexon gene of fowl adenovirus4 of 

Bayliss, C.D., Peters, R.W., Cook, J.K.A., Reece, R.L., Howes, K., Binns, M.M., 
antigen of infectious bursal disease virus induces protection against mortality 
caused by the virus. *Archives of Virology*, 120, 193– 205.

Bayliss, C.D., Spies, U., Shaw, K., Peters, R.W., Papageorgiou, A., Müller, H., Boursnell, 


Kumar, S., Cloning of Infectious bursal disease virus VP2 genes in a bicistronic mammalian expression vector for use as DNA vaccine. M.V.Sc thesis submitted at I.V.R.I (India).


