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Research article

EXPRESSION AND IMMUNOGENIC ANALYSIS OF ALPHAVIRUS REPLICON BASED RECOMBINANT PLASMID CONTAINING FAV4 HEXON GENE

Upasna Sharma[1] and Anant Rai[2*]
[1]IFTM University, Moradabad, Uttar Pradesh, India
[2]Institute of Biotechnology & IT, Bareilly-243122, Uttar Pradesh, India
***Corresponding author: raia48@gmail.com**

ABSTRACT

The fowl adenovirus hexon gene cloned in an alpha virus replicon vector showed high level of gene expression in BGM70 cell line. It produced high level of immunological responses including serum neutralizing antibody and cell mediated immune response. It may be evaluated for a DNA vaccine candidate in a field trial.

Key words: Expression, immunity, alphavirus, replicon, plasmid vector, fav4, hexon gene.

INTRODUCTION

Rai *et al*. (2005) reported cloning of FAV 4 hexon gene and its immunological response in chicken. Several characteristics of alpha viruses make them attractive candidates for expression of genes in mammalian system. Theoretically up to two lakh copies of RNA can be produced in a single cell within 4 h (Rolls *et al*., 1994). An RNA polymerase II expression cassette is introduced to drive the transcription replicon vector, which allows direct use of plasmid DNA for transfection and expression studies (Berglund *et al*., 1996; Dubensky *et al*., 1996).

It was found that this DNA system gave at least 10 fold higher expression levels as compared to conventional naked DNA using pCMV plasmids (Herweijer *et al*., 1995; Dubensky *et al*., 1996; Sandey *et al*., 2008; Kumar *et al*., 2009). Replicase based DNA or RNA induces apoptotic death of the host cell *in-vitro* just as alpha viral infection induces apoptosis in host cells. These apoptotic cells may be picked up by dendritic cells for presentation to the immune system. Transfection with self-replicating genetic vaccines may also cause the production of heat shock proteins in transfected or bystander cells. The activity of the viral replicase may provide a powerful adjuvant effect because of the requisite production of double stranded RNA intermediates. The dsRNA itself is a

potent inducer of the interferon and virus derived dsRNA can function as a strong adjuvant for cellular and humoral immune responses. Interferon-gamma potentiates the apoptotic effects of dsRNA.

Nucleic acid vaccines containing alpha viral replicase gene are now gaining popularity (Sandey *et al.*, 2008) among researchers. It is evident that findings of the present study may be utilised fruitfully to conduct field trials for its immunogenic potential for DNA vaccine in the field.

MATERIALS AND METHODS

Experimental chicks

Broiler chicks 21 days old from apparently healthy parent stock were obtained from the local hatchery unit and were maintained at the Experimental Animal room of the Institute. They were provided proper bedding, sufficient feed and water and comfortable room temperature.

Virus

The Fowl adenovirus serotype 4 adapted to BGM70 cell line at passage 14 (P14) and the purified FAV4 viral DNA was available in the Biotechnology Lab of the Institute.

Recombinant plasmid

The pAlpha-fav4hex plasmid constructed (Sharma and Rai, 2017) was used.

Cell culture

The BGM70 cell line was used for growth of FAV4 and related experiments.

Conjugates

The rabbit anti-chicken Ig^{γ} FITC conjugate and rabbit anti-chicken Ig^{γ} HRP conjugates were procured from Promega (USA).

Propagation of BGM70 cell culture

The BGM70 cell line was grown in DMEM containing 4mM L-glutamine, 4.5g/L glucose, 1.5g/L, NaHCO3/L with 10% fetal calf serum, pH 7.2-7.4 and incubated at 37^{0}C.

Propagation of FAV 4 in BGM70 cell culture

Flask of $25cm^2$ area containing complete monolayer of BGM70 cell culture was washed twice with serum-free DMEM and infected with 0.25 ml of FAV4 virus BGM70 adapted (P14) per flask, the virus was allowed to adsorb onto the cells at 37^{0}C for 60 min and then DMEM containing 2% fetal calf serum was added to the culture and incubated at 37^{0}C. The culture was harvested when the monolayer exhibited more than 80% cytopathic effect.

Preparation of hyperimmune serum against recombinant plasmid

Two healthy, 28 days old chicks were immunized with pAlpha-fav4hex recombinant plasmid DNA intramuscularly in thigh muscle in the leg each with 50 µg DNA. The chicks were kept in IBIT Experimental Animal House with proper bedding, water for drinking and feed. Four injections at weekly interval were given. One week after last injection, they were bled from humeral vein.

Transfection of cells with fav4 hexon gene

It was done as per Rai *et al.* (2014). 100 µl 2.5M $CaCl_2$ was mixed with 20 µl plasmid DNA. Added 80 µl distilled water and kept at room temperature for 1 min and immediately transferred the calcium phosphate DNA suspension using 20µl suspension for each well of the microtiter plate. 100µl BGM70 cell suspension was

added in each well. Suitable controls were kept. Incubated at 37^0C in a humidified incubator with an atmosphere of 5% CO_2 for 72 h.

Indirect fluorescent antibody test (IFAT)

After 48 h transfection, media from the wells was poured off; cells were washed twice with 1X PBS and fixed with 4% paraformaldehyde. Chicken anti-fav4hex sera (1:40) was added in triplicate wells of rplasmid, vector alone and healthy groups, incubated at 37^0 C for 2 h after which the wells were washed with 1X PBS and incubated with rabbit anti-chicken FITC conjugate (1:40) for 2 h. Thereafter the wells were washed with 1X PBS, mounted with 50% glycerol in PBS and examined under fluorescent microscope.

Indirect immunoperoxidase test (IPT)

After 48 h transfection, media from the wells was poured off. Cells were washed twice with 1X PBS and fixed with chilled acetone. The PBS washed cells were treated with 2% H_2O_2 for 10 min and again washed with PBS. The cells were first incubated with chicken anti-fav4hex sera (1:40) for 2 h at 37^0C, washed three times with PBS and then incubated with rabbit anti-chicken HRP conjugate (1:40) at 37^0C for 2 h. The cells were again washed thrice with PBS and incubated with DAB 1mg/ml in PBS with 1 µl/ml H_2O_2 for 5 min at room temperature to develop color. Once the color developed, the cells were washed with PBS, dried in air and were observed under microscope.

Vaccination of chicks

The 4-week old chicks were used. Pre-vaccination blood (heparinised as well as non-heparinised) of chicks of all the groups was collected and then the vaccination was done (Table 1).

Table 1.Vaccination of different groups of chicks with pAlpha-fav4hex rplasmid.

Group	Plasmid injected	Dose	No. of chicks
I	pAlpha-fav4hex	5µg	**5**
II	pAlpha vector alone	5 µg	**5**
III	Healthy control	-	**5**

The chicks were observed for 28 days and given proper feed water and housing conditions. On 28th day, blood (heparinised as well as non-heparinised) of chicks of all the groups was collected for further analysis.

Serum Neutralization Test (SNT)

It was done as per Rai *et al*., (2016). Briefly, sera were inactivated at 56^0C for 30 min and two-fold dilutions were prepared 1:4 to 1/256 in 96 well microtitre plates by mixing 0.05 ml of serial two-fold dilution of sera in PBS with 0.05 ml of FAV4 suspension containing 100 $TCID_{50}$ virus. The serum virus mixture was incubated for 2 h at 37^0C and then 100µl BGM70 cells were added per well. These were incubated at 37^0C for 72 h after which observations were read. The SN antibody titre was calculated as the reciprocal of the highest dilution that neutralized the virus.

Lymphocyte proliferation assay

Whole blood was collected from the chicken without anticoagulant for serum as well as in heparin. Blood was overlaid onto histopaque 1.077 (Sigma) and centrifuged at

250 g for 30 min and lymphocytes at the interface were collected as per Boyum (1976). Cells were washed with RPMI-1640 media and resuspended in RPMI-1640 containing 10% FCS. For assay, $2x10^7$ cells/well in 96-well flat-bottomed tissue culture plates and Con A 50µl/well from stock of 5 mg/ml was added to each well except the negative control well and virus control well. Plates were incubated at 37^0C for 72h. Lymphocyte proliferation assay was done as per Bounous *et al.* (1992). Briefly, 20µl MTT from 5 mg/ml stock was added to each well after 72h of incubation. The plates were further incubated for 4 h at 37^0C 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. The stimulation Index (SI) was calculated by the following formula:

$$\text{Stimulation Index} = \frac{\text{(Mean OD of Con A or virus stimulated cells)}}{\text{(Mean OD of un-stimulated cells)}}$$

RESULTS

FAV4 hexon gene expression

Immunofluorescence analysis of pAlpha-fav4hex transfected BGM70 cells revealed diffuse cytoplasmic fluorescence while no fluorescence was seen in mock transfected cells (Figure 1 & 2). Immunoperoxidase test of pAlpha-fav4hex rplasmid transfected BGM70 cells showed distinct colour development while mock transfected cells did not show any reaction (Figure 3 & 4). These observations demonstrate that hexon gene is in right orientation and showing high level of expression in cells.

Immunogenicity of pAlpha-fav4hex recombinant plasmid

The SN antibody titer in pAlpha-fav4hex vaccinated chicks showed enhanced response with a titer of 128 as compared to vector alone group and healthy control group with titer of 4. The lymphocyte proliferation assay using MTT dye showed marked stimulation of lymphocytes in fav4hex group as compared to vector and control groups which showed that cell mediated immune response was also produced (Table 2).

Table 2. Immune response of chicks vaccinated with pAlpha-fav4hex plasmid DNA.

Group	No. of chicks	DNA	SN ab titer	Stimulation Index	
				virus	Con A
pAlpha-fav4hex	10	5µg	128	2.1	1.33
pAlpha vector	10	5µg	4	1.1	1.07
Healthy control	10	nil	4	1.1	1.05

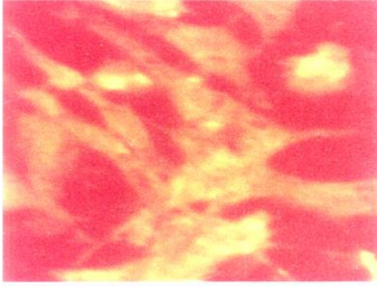

Figure1. Imunofluorescence analysis of mock transfected BGM70 cells showing no fluorescence.

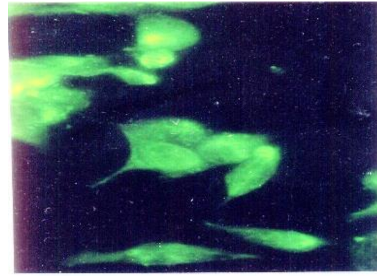

Figure 2. Immunofluorescence analysis of BGM70 cells transfected with pAlpha-fav4hex plasmid showing positive fluorescence.

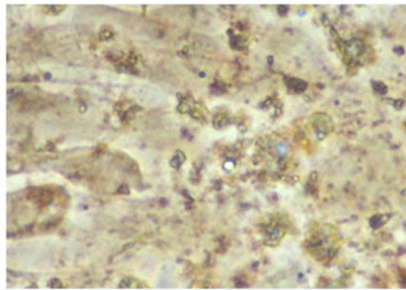

Figure 3. IPT analysis of mock transfected BGM70 cells showing no staining reaction.

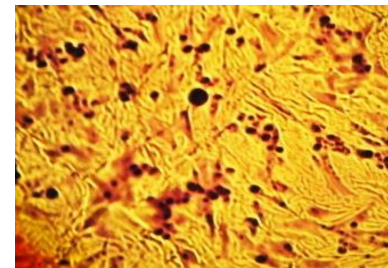

Figure 4. IPT analysis of BGM70 cells transfected with pAlpha-fav4hex plasmid showing positive staining reaction.

DISCUSSION

Sandey *et al*., (2008) reported cloning of hexon gene in a replicase based vector and its immunological response in chicken when given along with an IBDvp2 gene containing recombinant plasmid. 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 field trial of the vaccine showed that it was safe and potent and sera collected from 25 vaccinated birds revealed SN titre of sera 128-256 against FAV4. The field trial of the vaccine showed that it was safe and potent and sera collected from 25 vaccinated birds revealed SN titre of sera 128-256 against FAV4.

The enhanced immunogenicity of plasmid DNA replicons cannot be accounted for only on the levels of antigen production but rather through other mechanisms. In addition to producing antigen, cells transfected with the plasmid DNA replicon produce double-stranded RNA (dsRNA), which may provide immunostimulatory adjuvant effects (Polo and Dubensky, 1998; Leitnet *et al*., 2006; Kim *et al*., 2014). Alphavirus based DNA vaccine have been reported to elicit protective immune

response against disease like IBD, FAV4, rabies, canine distemper, parvoand hepatitis (Kumar *et al.*, 2008; Kumar *et al.*, 2009a; Kumar *et al*, 2009b; Kumar *et al.*, 2009c; Gangwar*et al.*, 2010; Gangwar and Rai, 2013; Schachner *et al.*, 2014; Rai and Tyagi, 2016; Kanojia and Rai, 2016) and other diseases (Lundstrom, 2014). Park *et al.* (2017) carried out molecular analysis of the hexon, penton base, and fiber-2 genes of Korean fowl adenovirus serotype 4 isolates from hydropericardium syndrome-affected chickens. Shah *et al.*, (2016) reported that many characteristics of alpha viral replicase gene were a good candidate for over expression of genes in mammalian system. Theoretically up to 2 lakh copies of RNA can be produced in a single cell within 4 h. (Rolls *et al.*, 1994). Preliminary results from cell culture experiments showed that it gave at least 10 fold higher expression levels as compared to conventional naked DNA vaccine (Herweijer *et al.*, 1995; Dubensky *et al.*, 1996). A major aim of putting antigen coding genes under the control of alpha viral RNA replicase was to enhance antigen expression and presentation. The activity of the viral replicase may provide a powerful adjuvant effect because of the requisite production of double stranded RNA intermediates. The dsRNA itself is a potent inducer of the interferon and virus derived dsRNA can function as a strong adjuvant for cellular and humoral immune responses. Nucleic acid vaccines containing alpha viral replicase gene are now gaining popularity among researchers. It is evident that findings of the present study may be utilised fruitfully to conduct field trials for its immunogenic potential for DNA vaccine in the field.

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