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Research article

CLONING OF VERY VIRULENT IBD VP2 GENE IN pALPHA VECTOR FOR USE AS r-DNA VACCINE

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ABSTRACT: The VP2 gene of very virulent infectious bursal disease virus (IBDvv) isolated from Bangalore, Karnataka, India was cloned in pAlpha vector and designated pAlpha.ibdvp2. It was found to induce serum neutralizing (SN) antibody titer of 256, lymphocyte stimulation index 1.8 and gave 90% protection in birds vaccinated with 1µg recombinant plasmid DNA. It is thus a suitable candidate for use as r-DNA vaccine in the field.

Key words: pAlpha vector, replicase vector, IBD, VP2, DNA vaccine, cloning

Introduction

Infectious bursal disease (IBD) is an acute, highly contagious disease of young chickens caused by a Birnavirus. It was first recognized as a specific disease entity in 1962 and designated as "Gumboro disease" after the geographic location of the first recorded outbreaks. In India, the disease was reported in Uttar Pradesh and subsequently from different parts of the country (Kataria *et al.*, 2001). Its most important effect is a severe, prolonged immunosuppression in chickens infected at 3 to 6 weeks of age. There are many strains of the IBD virus, which vary considerably in their pathogenicity and global distribution. In recent years, very virulent strains of IBDV (vvIBDV), causing severe mortality in chickens have emerged in Latin America, South-East Asia, Africa, Middle East and in the Europe and 70% flock mortality was reported in laying pullets due to vvIBDV (Chettle *et al.*, 1989; Van den Berg *et al.*, 1991).

IBDV is a small, hardy Avibirnavirus belonging to the family Birnaviridae and has bisegmented ds-RNA genome. IBDV genome consists of 2 segments, A and B, which are enclosed within a nonenveloped icosahedral capsid.

The genome segment B (2.9kb) encodes VP1, the putative viral RNA polymerase. The larger segment A (3.2kb) encodes viral Proteins VP2, VP3, VP4 and VP5. Among them, VP2 protein contains important neutralizing antigenic sites and elicits protective immune response.

The IBDV has a predilection for lymphoid tissue, especially the Bursa of Fabricius (BF). Between 3 and 6 weeks after hatching, when the BF reaches maximum development, chickens are highly susceptible to the virus.

Although live IBDV vaccines are highly efficacious, the vaccine efficacy decreases in the presence of maternal antibodies, and some of them cause bursal atrophy and immunosuppression (Tsukamoto *et al.,* 1999). Also live attenuated vaccines have the risk associated with the potential for reversion to a virulent phenotype. Inactivated vaccines, although costly, were used successfully until the emergence of the hypervirulent strains (Van den Berg, 2000).

In recent year, VP2 gene has been cloned and used as DNA vaccine (Nitin *et al.,* 2003, Chauhan *et al*., 2005). IBDV VP2 gene expressed by a fowlpox virus recombinant conferred protection against IBD in chicken (Heine and Boyle, 1993).One promising new strategy to improve naked DNA vaccines is to express the target antigen under the control of an alphaviral replicase (Zhou *et al*., 1994) with the premise of using the ability of alphaviruses to produce large amounts of viral mRNA (Schlesinger, 2001) and subsequently protein. Incorporation of alphavirus replicons into plasmid DNA vectors to direct the amplification of RNA expressing the gene of interest has been an exciting approach toward improving DNA vaccination (Dubensky *et al*., 1996). Vectors encoding the alphavirus-derived RNA replicase have been shown to be immunogenic in murine models at doses up to 1,000-fold lower than those used for conventional plasmid vectors (Hariharan *et al*., 1998, Leitner *et al*., 2000b) and are effective when used as vaccines against cancer or viral infections (Andersson *et al*., 2000).

Keeping these facts in view the present work was undertaken to clone vvIBDV VP2 in a replicase based pAlpha vector and analyse its immunogenic potential in chicken.

Materials and Methods

pAlpha vector

The vector was available in Biotechnology Laboratory of IBIT. It has Sindbis virus replicase gene, CMV promoter, BGH and polyadenylation signal sequence .

Birds

One-day-old broiler chicks were procured from the local hatchery.

Bacterial strains

Escherichia coli strain DH5α (Promega, Madison was used for transformation of recombinant plasmids.

Virus

Very virulent infectious bursal disease virus isolated from a field sample from Bangalore, Karnataka, India and its IBD vp2 cDNA were available in IBITCC lab of IBIT Bareilly.

Primers

The IBD VP2 gene forward primer 5’TCGCAGCGATGACAAACCTGCAA GATCA 3’(28 mer) and reverse primer 5’TTCAGACCACCGGCACAGCTATCC TCCT3’ (28 mer) were used for VP2 gene amplification. For nested PCR the primers used were

F 5'CGCTATAGGGCTTGACCCAAAAA3 'and R 5'CTCACCCCAGCGACCGTAACGAC G3'.

Plasmid DNA isolation

Small scale plasmid DNA isolation was done following the TELT method (Ausubel *et al*, 1990).

PCR Amplification of IBD VP2 gene

The PCR reaction mixture in total volume of 50µl contained: 10X PCR buffer (5µl), 25mM MgCl2 (4µl), 10mM dNTPs (1µl), forward primer (1µl), reverse primer (1µl), cDNA (5µl), Pfu polymerase (0.5µl), nuclease free water (ad 50µl). The cyclic conditions were: Initial denaturation (95^0C, 3 min), 30 cycles of denaturation (95^0C, 45 sec), annealing (55^0C, 1min), extension (72^0C, 2 min) and then a final extension (72^0C, 10 min).

Restriction endonuclease digestion and analysis of plasmid DNA

The plasmid DNA (5-10µg) was incubated overnight with selected restriction endonuclease (MBI Fermentas, USA/Promega, USA) at 37^0C in a water bath. The digested DNA was mixed with 6X loading dye and run on 0.7% agarose (SRL, Mumbai) gel containing ethidium bromide using TAE running buffer system. The stained gel was visualized under UV-transilluminator and photographed.

Dephosphorylation of blunt ended vector

To prevent religation of vector, the dephosphorylation of the digested and blunt ended plasmid vector was done. The reaction mix contained: StuI digested gel purified blunt ended pAlpha vector DNA (15 µl),10X reaction buffer (5µl), calf intestinal alkaline phosphatase (CIAP) (1 unit / µl) (1µl), nuclease free water to 50µl. The sample was briefly spun and incubated at 37^0C in a water bath for 30 min and 1µl of CIAP was again added to the reaction and incubated at 37^0 for 30 min and reaction was stopped by heating at 70^0C for 15 min followed by phenol: chloroform extraction.

DNA extraction from agarose gel

The gel extraction of DNA fragments was done using MinElute Gel extraction kit (Qiagen, Germany) following manufacturer's instruction. Elution of DNA from the column was done in 10 µl nuclease free water.

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 10µl reaction volume containing 1 µl of 10 mM ATP (pH 7.5). The reaction mixture was incubated at 16^0C in a water bath for 16-18 hrs. The linear plasmid without insert was ligated as ligation control.

Single step competent cell preparation & transformation

It was done as per method of Chung *et al* (1989).

Cell culture

Chick embryo fibroblast culture (CEF) was prepared from 12 days old embryonated eggs procured from local hatchery and was grown and maintained in DMEM (Gibco, NY) supplemented with 50 µg/ml gentamicin (Hi Media, Bombay) and 10% fetal calf serum (Gibco, NY).

Characterization of recombinant pAlpha.ibdVP2 plasmid

The recombinant pAlpha.ibdvp2 plasmid containing colonies were amplified in LB broth containing 100 µg /ml ampicillin overnight and miniprep was done to isolate

plasmid DNA. The recombinant plasmid was characterized by digestion with restriction endonuclease *ECoRV* and *KpnI* to confirm the right orientation of VP2 gene in vector. Digestion with XbaI and *XhoI* confirmed the release of VP2 gene from the recombinant plasmid. The digested products of plasmid were analysed on agarose gel. Recombinant clone was confirmed by PCR using VP2 gene specific F primer and BGH reverse primer. After amplification, an aliquot of 5µl was subjected for gel electrophoresis along with DNA molecular marker through 0.7 % agarose gel.

Determination of immune response against IBDV in DNA immunized chicken

Bulk preparation of plasmid DNA

pAlpha.ibdvp2, and control plasmid pAlpha were isolated using Silica Gel Technology (Rai *et al*, 2009).

Immunization of birds

The 14 days old healthy chicks were immunized with 1 µg plasmid DNA intramuscularly in the quadriceps muscles of hind leg using 10 chicks in each group. The chicks were grouped as r-DNA group, vector alone group, healthy group, IBD cell culture vaccine group.

Detection of IBDV VP2 specific antibody

SNT was performed as per the OIE manual (2004) in 96 well plate using 100 $TCID_{50}$ CEF culture adapted IBDV Georgia strain and two fold dilutions of sera (1/4 to 1/ 256) in 50 µl volume, incubated at 37^0C for 1 hr and then 100µl CEF cells were added per well. Observations were read 72 hr later.

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, 81.6 mM d-glucose) and 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 colorless media and resuspended in RPMI-1640 colorless media supplemented with 10% FCS medium (Boyum, 1976). The cells were placed into each well (2 $x10^7$ cells/well) of 96-well flat-bottomed tissue culture plates and Con A (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 37^0C in a humidified incubator for 72 hr. Then 20 µl MTT (from 5 mg/ml stock) was added to each well after 72 hr of incubation. The plates were further incubated for 4 hrs at 37^0C under 5% CO2 and then 150 µl DMSO was added to each well. After mixing, the dark precipitate was mixed by pipeting and reading taken at 550 nm with reference reduction at 655 nm. The stimulation Index (SI) was calculated by the following formula: Stimlation Index = Mean OD of ConA or virus stimulated cells / Mean OD of unstimulated cells.

Determination of protective efficacy of pAlpha.ibdVP2

All the vaccinated birds along with control were challenged with 10^5 ID_{50} (50% Infective dose) virulent IBDV 30 days post immunization. The birds were observed for 14 days post challenge for IBDV specific symptoms and mortality.

% Protection =Number of birds showing no symptoms or death X 100/Total number of birds in a group

The level of protection in different group of birds was analyzed according to mortality and liver, heart and bursa lesion found at post mortem. The challenged birds were observed daily for any specific IBDV symptom. The birds showing symptoms like whitish diarrhea, vent pecking, ruffling of feather, closed eyes and recumbency were isolated and examined. The post mortem finding consisted of gross bursal picture and hemorrhages in thigh muscles.

Results and Discussion

Construction of pAlpha vector encoding IBDV VP2 gene

The IBDvv VP2 gene was successfully amplified (Fig 1). The recombinant pAlpha.ibdvp2 plasmid containing colonies were selected randomly and grown in LB broth containing ampicillin. The recombinant plasmid DNA was isolated by TELT method and characterized.The recombinant plasmid was confirmed by PCR amplification using nested primers and an amplified product of 552 bp was obtained (Fig. 2). Digestion with *ECoRV* and *KpnI* confirmed the right orientation of the VP_2 gene (Fig. 3).The recombinant pAlpha.ibdvp2 was digested with XbaI and *XhoI* which released a product of 1667bp along with VP2 gene (Fig. 4). All of these above restriction sites were predicted in pAlpha.ibdvp2 using DNASTAR software (Lasergene).

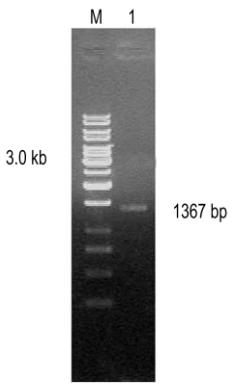

Fig 1. PCR amplification of ibd virus vp2 gene Lane M: 1 kb DNA ladder, 1: 1367bp vp2 gene.

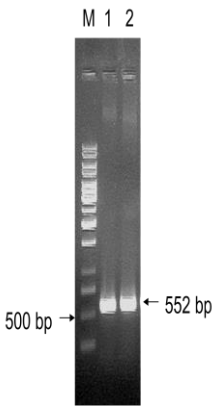

Fig.2. PCR confirmation of pAlpha.ibdvp2 rplasmid
M: 1 kb DNA ladder, 2 : 552 bp PCR product

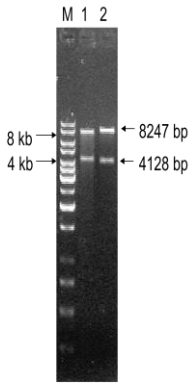

Fig. 3. Characterization of pAlpha.ibdvp2 by RE analysis
M: 1 kb DNA ladder,1: pAlpha.ibdvp2 cut with KpnI yielding 4272 bp and 7882 bp fragments 2 : pAlpha.ibdvp2 cut with EcoRV

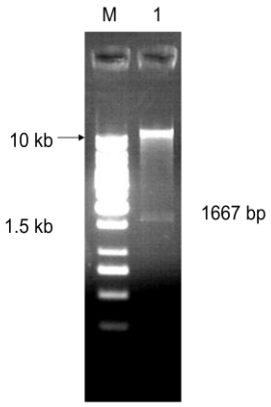

Fig. 4. Characterization of pAlpha.ibdvp2 by REanalysis M: 1 kb DNA ladder,1: pAlpha.ibdvp2 rplasmid cut with XbaI & XhoI

Determination of immune responses against pAlpha.ibdVP$_2$

The plasmid DNA prepared from 500 ml LB culture yielded DNA having concentration 421µg/ml for pAlpha vector and 381 µg/ml for pAlpha.ibdvp2. The SN titre of pAlpha.ibdvp2 was 128, for vector alone 4 and for cell culture vaccine 64. Lymphocyte proliferation assay using MTT dye showed marked stimulation of lymphocytes from DNA immunized chicks with SI 1.8, in vector alone 1.1 and for cell culture vaccine 1.2. There was a marked increase in stimulation index of

virus stimulated cells as compared to ConA stimulated cells.

In situ picture of bursa showed hemorrhages and enlargement in non-vaccinated group which were absent in pAlpha.ibdcp2 vaccinated groups. Grossly bursa revealed postmortem hemorrhages in all the birds died after challenge in vaccinated and non vaccinated groups. Birds sacrificed post challenge from vaccinated groups did not show appreciable gross changes in bursa. Histopathology of bursa of Fabricius of non-vaccinated, challenged birds showed hemorrhages and lymphocytic depletion. Protection of 80% in pAlpha.ibdvp2 and 60% in birds vaccinated with live attenuated vaccine was observed.

IBDV syndrome remains a serious problem for commercial broiler production. Chicken vaccinated with IBD vaccines are not protected against variant or vvIBDV strains (Van den Berg *et al.*, 1999). Inactivated vaccines are safe but more expensive as more antigen is necessary to induce a satisfactory response. However, the use of less attenuated (’hot’) vaccines, even with an acceptable reduction of mortality, is dangerous as these vaccines induce immunosuppression and carry the risk of reversion to virulence (Guittet *et al.*, 1992, Lukert and Saif, 1997). This situation is complicated by the passive transmission of MDA from dams to the offspring via the egg. This passive immunity, although protective, interferes with vaccination. In this context, DNA vaccines could be considered but these have some limitations, especially general low humoral response. Incorporation of alphavirus replicons into plasmid DNA vectors to direct the amplification of RNA expressing the gene of interest has been exciting approaches toward improving DNA vaccination (Dubensky *et al*., 1996). To achieve this goal, VP2 gene was cloned into pAlpha vector which contains replicase gene. Vectors encoding the alphavirus-derived RNA replicase have been shown to be immunogenic in murine models at doses up to 1,000-fold lower than those used for conventional plasmid vectors (Hariharan *et al.*, 1998 and Leitner *et al.*, 2000a) and are effective when used as vaccines against cancer (Leitner *et al.*, 2000a; Cheng *et al*., 2001) or viral infections (Berglund *et al.*, 1998; Hariharan *et al.*, 1998; Andersson *et al.*, 2000). The first product of a plasmid DNA replicon is the RNA replicase, which uses the primary positive-strand RNA transcript as a template to make negative-strand RNA and then makes more copies of full-length positive-strand RNA encoding both replicase and antigen as well as a shorter subgenome length mRNA encoding only the antigen (Leitner *et al.*, 2000b). The enhanced immunogenicity of plasmid DNA replicons cannot be accounted for based only on levels of antigen production but rather through other mechanisms as well. In addition to producing antigen, cells transfected with the plasmid DNA replicon produce double-stranded RNA (dsRNA), which may provide immunostimulatory adjuvant effects (Polo *et al.*, 1998). Furthermore, replicase-based RNA and DNA vaccines have been shown to induce caspase-dependent apoptosis in transfected cells (Ying *et al.*, 1999; Leitner *et al.*, 2000a;) which can increase uptake of transfected cells by dendritic cells. This would likely support dendritic cell maturation (Cella *et al.*, 1999), uptake of apoptotic transfected cells could lead to an

immunogenic signal (Ferguson *et al.*, 2002) and could facilitate antigen processing on presentation to major histocompatibility complex class I (Albert *et al.*, 1998). Thus, alphavirus plasmid replicons may offer both quantitative and qualitative advantages compared with conventional plasmid vectors.

It has previously been shown that replicon based DNA vaccines can be highly immunogenic even at low doses (Wolfgang *et al.*, 2006) compared with conventional DNA vaccines. Consistent with this observation, we found 90% protection in groups vaccinated with 1 µg pAlpha.ibdvp2.

Plasmid construct containing VP2–VP4–VP3 from classical IBDV strain STC induced low or undetectable antibodies in chicken before challenge (Chang *et al*., 2002). DNA vaccines constructed with IBDV strains GP40 and D78 induced antibody production in chickens but some of the immunized chicken, in spite of high antibody response, were not protected from the disease (Fodor *et al*., 1999). However, these two IBDV DNA vaccine studies only examined humoral immune response. In a study using an inactivated IBDV vaccine, T cells were critically involved in protection and antibody alone was inadequate in inducing protection in chickens (Rautenschlein *et al*., 2002b). In our study, direct evidence was seen for involvement of cell mediated immune response in IBDV protection after DNA vaccination. The results obtained from lymphocyte transformation test from lymphocytes collected from pAlpha.ibdvp2 vaccinated group indicated significant degree of proliferation of T lymphocytes. The finding clearly indicates that cell mediated immune response also plays an important role in protection of birds against the challenge of IBDV. Tsukamoto *et al* (1999) suggested that CMI plays important role in providing protection against IBDV in chicken vaccinated with recombinant viral vector based vaccine against IBD. Chicken treated with cyclophosphamide, which caused selective destruction of B cells resulting in severely compromised antibody-producing ability, were protected against virulent IBDV (Yeh *et al*., 2002). Our finding of replicase based DNA vaccine showed marked degree of cell mediated immune responses thus strengthening the findings of above workers. In our study, non vaccinated birds which survived from challenge showed typical symptoms of IBDV like whitish watery diarrhea, anorexia, ruffled feathers, trembling and severe prostration which were further confirmed by postmortem of diseased birds which showed hemorrhagic atrophied bursa of Fabricius.

Replicase based DNA vaccinated chickens which were protected from challenge showed less bursal atrophy and less hemorrhages than the non-vaccinated groups as indicated by gross bursal lesion and histopathology of sacrificed bursa.It becomes amply evident that replicae based DNA vaccine are immunogenic and satisfactory at 1µg dose level.

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