Development of a DNA vaccine construct encoding VP2 gene of Infectious Bursal Disease (IBD) virus and truncated HSP70 gene of *Mycobacterium tuberculosis*

Thesis

Submitted to the

DEEMED UNIVERSITY

Indian Veterinary Research Institute

Izatnagar – 243 122 (U.P.), India

Dr. Hemanta Kumar Maity

Roll No. 5151

FOR THE DEGREE

OF

MASTERS OF VETERINARY SCIENCE

IN

ANIMAL BIOTECHNOLOGY

2014

Development of a DNA vaccine construct encoding VP2 gene of Infectious Bursal Disease (IBD) virus and truncated HSP70 gene of *Mycobacterium tuberculosis*

Thesis

Submitted to the

DEEMED UNIVERSITY

Indian Veterinary Research Institute

Izatnagar – 243 122 (U.P.), India

Dr. Hemanta Kumar Maity
Roll No. 5151

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE

OF

Masters of Veterinary Science

(Animal Biotechnology)

June, 2014

भारतीय पशु चिकित्सा अनुसंधान संस्थान
(सम विश्वविद्यालय)

DIVISION OF VETERINARY BIOTECHNOLOGY
INDIAN VETERINARY RESEARCH INSTITUTE
(Deemed University)
IZATNAGAR - 243 122, U.P., INDIA

Dr. Sohini Dey,
Senior Scientist

Dated: 10.6, 2014

Certificate

*This is to be certified that the research work embodied in this thesis entitled "**Development of a DNA vaccine construct encoding VP2 gene of Infectious Bursal Disease (IBD) virus and truncated HSP70 gene of Mycobacterium tuberculosis**" submitted by Dr. Hemanta Kumar Maity, Roll No. 5151, for the award of **Master of veterinary science** Degree in **Animal Biotechnology** at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

*It is further certified that Dr. Hemanta Kumar Maity, Roll No. 5151 has worked for more than 21 months in the Institute and has put in more than 150 days attendance under me from the date of registration for **Master of Veterinary Science** Degree in this Deemed University, as required under the relevant ordinance.*

Sohini Dey

(SOHINI DEY)
Chairman
Advisory Committee

CERTIFICATE

We the undersigned members of Advisory Committee of **Dr. Hemanta Kumar Maity** Roll No. **5151** a candidate for the degree of **Masters of Veterinary Sciences** with the major discipline in **Animal Biotechnology** agree that the thesis entitled "**Development of a DNA vaccine construct encoding VP2 gene of Infectious Bursal Disease (IBD) virus and truncated HSP70 gene of *Mycobacterium tuberculosis***" may be submitted in partial fulfillment of the requirement for the degree.

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Masters of Veterinary Science** Degree of this Institute.

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Masters of Veterinary Science** Degree of the Deemed University, Indian Veterinary Research Institute, Izatnagar.

.....

(R.S. KATARIA)

External Examiner

Date 28-07-2014.

.....

(Sohini Dey)

Chairman

Advisory Committee

Date 28-07-2014

MEMBERS OF STUDENT'S ADVISORY COMMITTEE

Dr. C. Madhan Mohan, Senior Scientist
Division of Veterinary Biotechnology .....

Dr. Sujoy Kumar Dhara, Senior Scientist
Division of Veterinary Biotecnology .....

Dr. P. Joshi , Principal Scientist
Division of Animal Biochemistry .....

Dr. R. Saravanan, Scientist (SS)
Immunology Section .....

Acknowledgement

It is almost an arduous task to pen down my sincere gratitude and thanks to all who had mentored, supported and helped me during the process of research thesis writing. First of all, I pay my sincere thanks and regards to the chairman of my advisory committee, **Dr. Sohini Dey** (Senior Scientist) for her guidance, enthusiasm, support and trust towards her student. Her prowess in field of Veterinary Biotechnology is unquestionable. I will never forget all the daunting efforts and enthusiasm she had put forth towards successful completion of my MVSc research. I pay my regards and gratitude to my co guide Dr. **C. Madhan Mohan** (Senior Scientist) for painstakingly reviewing my articles with constant encouragements. I am deeply grateful to the members of my advisory committee , **Dr. Sujoy Kumar Dhara** (Senior Scientist) for his immense support and encouragement , **Dr .P. Joshi** (Principal Scientist ,Division of Biochemistry), and **Dr. R. Saravanan** (Scientist (SS), Immunology Section) for their assiduous guidance, constant support , invaluable suggestions on the present investigation and enquiring the progress of the work from time to time.

I sincerely thank to the **Director**, The **Joint Director** (Academic) ,The **Joint Director** (Research) of IVRI, for providing me the necessary facilities to complete this work. I thank ICAR ,New Delhi for providing ICAR Junior Research fellowship throughout my course.

I extend my gratitude to **Dr. Med Ram Verma** (Senior Scientist, Division of LES) for kind assistance in statistical data analysis. **Dr. G. Sai Kumar** (Principal Scientist ,Divison of veterinary Pathology) for their help and kind support.

I pay my regrards to **Dr. Satish Kumar** (Head, Principal Scientist, Division of Veterinary Biotechnology) and other scientists of the Divison of Veterinary Biotechnology for encouraging support elderly guidance and valuable suggestions during the course work. I extend my grateful acknowledgements to the seniors and juniors of Biotechnology division for their friendship and assistance during the course of this study.

I am thankful to seniors in my lab, **Dr(s) Satish Gaikwad, Rajib Deb, Nitin Kamble, Arvind S, Dinesh C Pathak** for their untiring help and immense support throughout the course of research work. I would like to specially mention the enthusiastic support and help provided by **Dr(s) Sagar Ashok Khulape** during research work. I extend my thanks to my MVSc batchmates **Manoj** and **Nighil, Boby, Arun, Ajoy, Himanshu, Arun Saravana kumar, Swati, Senjuti** for their help and support. I also acknowledge the assistance of **Dr(s) Yogesh , Lakshman Santra, Mashidur** during research work and thesis writing. I extend my thanks to **Mr. Niaziji** (Technical officer, Veterinary Biotechnology Division), **Govind Ramji**, **Rajesh** and **Aman** for all the help they provided during research work. I will be grateful to **Krishnenduda**, **Mrintunjoyda**, **Probhakarda**, **Sumanda** for bringing around a cheerful environment to relax and recuperate.

. Above all these, I am indebted to my family members **Maa**, **Baba**, my brother, **Subrata** and sister in law, **Banani** for their sacrifice and relentless perseverance to support me, as well as the unflinching trust they bestowed upon me.

Date: 11/06/2014

Place: IVRI, Izatnagar

(HEMANTA KUMAR MAITY)

List of Abbreviations

bp	:	base pair
BSA	:	bovine serum albumin
cDNA	:	complementary deoxyribonucleic acid
cds	:	coding sequence
DAB	:	3,3′-Diaminobenzidine
DC	:	Dendritic cell
DNA	:	deoxyribonucleic acid
DW	:	Distilled water
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme linked immunesorbent assay
HRP	:	Horse raddish peroxidase
hrs	:	hours
LB	:	Lauria Bertani
LTT	:	Lymphocyte transformation test
MCS	:	Multiple cloning site
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
µM / nM	:	micro molar/ nano molar
OD	:	Optical Density
OPD	:	*o*-Phenylenediamine dihydrochloride
PBS	:	Phosphate buffered saline
PCR	:	Polymerase Chain Reaction
RT-PCR	:	Real time Polymerase Chain Reaction
RE	:	Restriction enzyme
RNA	:	Ribonucleic acid
SDS	:	Sodium dodecyl sulphate
TAE	:	Tris Acetate EDTA
TE	:	Tris EDTA
UTR	:	Untranslated region
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase

List of Figures

List of Tables

List of Plates

Chapter Contents

1 Introduction

Infectious Bursal Disease virus (IBDV) plays an important role in the etiology of economically important disease of young chickens called Infectious Bursal Disease. It was first reported by Cosgrove (Cosgrove *et al*., 1962). Since then the outbreaks has been reported worldwide. Bursa of Fabricius (BF), a major lymphoid organ of chickens is the site of predilection for IBDV. The viral entry into BF is unclear but recent study demonstrated that the α4β1 integrin of BF may act as the receptor for entry of virus into bursa after binding with P domain of VP2 protein (Delgui *et al*., 2009). Infectious bursal disease virus (IBDV) belongs to the *Avibirnavirus* genus of the *Birnaviridae* family, and is having non-enveloped, icosahedral capsid. These are RNA viruses consisting of a double stranded RNA segments namely segment A and B (Muller *et al*., 2012). The larger ORF1 of segment A encodes for a 110 kDa polyprotein which auto-catalytically splices into viral proteins VP2 (48kDa), VP3 (33-35kDa), VP4 (24kDa). VP2 is the major capsid protein and carries the immunogenic determinants and is considered to be the major host protective antigen, able to elicit neutralizing antibodies (Fahey *et al*., 1989). The smaller ORF of segment A encodes VP5 (17kDa), a nonstructural protein considered to be essential for viral release (Brandt *et al*., 2001). Genome segment B (2.8kbp) encodes for VP1 (90kDa) having multiple enzyme activity. IBDV has two serotypes recognized as serotype 1 and serotype 2. Both serotypes of the virus can naturally infect chicken, turkey, duck, guinea fowl, ostriches but pathogenicity is reported only in chicken (Saif *et al*.,1998). Serotype 1 of IBDV is pathogenic whereas serotype 2 is apathogenic in chicken (Macferran *et al*., 1980). Very virulent strain of IBDV (vvIBDV) has emerged in the late 1980(Chettle *et al*., 1989). IBDV is continuously evolving in the field with the changes in antigenicity and virulence. Currently, the disease is controlled by live attenuated or inactivated IBDV but they can revert back to virulence and also may not give full protection against vvIBDV strain (Rautenschlein *et al*., 2005). The inactivated or killed viruses are usually given to birds in pre-laying stage to induce higher level of antibodies for at least 2 weeks. This lead to problem in determining the timing of vaccination. Thus,

there is an urgent need to formulate potentially applicable vaccination strategy to get complete protection against vvIBDV.

Recombinant vaccination strategy like DNA vaccines are popular among new generation of vaccines for their safety, ease of production and stability. Several studies were conducted for the development of a DNA vaccine encoding VP2 gene of IBDV with variable protection (Pradhan *et al*., 2014). However, DNA vaccine encoding VP2 gene alone have limited potency and results in partial protection. Several strategies have been employed to increase the efficacy of DNA vaccine. DNA prime–protein boost vaccination strategy has been reported wherein initial immunization is done with plasmid DNA encoding VP2 gene followed by boosting with recombinant VP2 protein or killed vaccine that showed promising results (Hsieh *et al*., 2007; Gao *et al*., 2013).

Heat shock proteins (HSP) are one of the most conserved proteins present in all prokaryotes as well as eukaryotes. HSPs are expressed both constitutively and under stressful condition (inducible form) to protect the cell from damage as during stress already properly folded protein tends to unfold (Wang *et al*., 2002). HSP may be intracellular or extracellular in origin. Besides the chaperone activity it can interact with cells of immunosystem and shows its adjuvant property. HSP70 is a ligand for TLR2, and TLR4 was shown to enhance the potency of plasmid DNA immunogen in mice (Asea *et al*., 2000). A plasmid protein expressing an antigen–HSP70 fusion gene elicit both humoral and cell mediated immunity that were more potent than plasmid containing antigen alone (Planelles *et al*., 2001). Fusing mycobacterial HSP70 to HIV-1 gag p24, Influenza M2e and synthetic malarial antigen (NANP) a synthetic peptide consisting of 40 (Asn-Ala-40, Asn-Pro) repeats, enhanced the immunogenicity of the antigens and obviated the need for adjuvant. It was shown that the C-terminal fragment of mHSP70 acted as a carrier in mice when fused to the malarial antigen EB200 (HSP70-EB200) and considerably induced vaccine humoral and cell mediated responses (Qazi *et al*., 2005). The effects of two truncated HSP70 molecules, N-terminal domain $HSP70_{1-360}$, amino acids 1-360 and C-terminal domain $HSP70_{359-610}$, amino acids 359-610 of mycobacterial HSP70 was evaluated on the potency of antigen-specific immunity generated by a hepatitis B virus (HBV) DNA vaccination. It was shown that only the $HSP70_{359-610}$ fused HBV DNA vaccination resulted in a significant increase in hepatitis B surface antigen (HBsAg)-specific humoral response, while the $HSP70_{1-360}$ fused vaccine did not.

Moreover, HSP70 $_{359\text{-}610}$ fused DNA vaccine did not induce anti-HSP70 antibody (Li *et al*., 2006).

The previous reports encourage to design the fusion of VP2 gene of IBD with C terminal HSP70 gene of *Mycobacterium tuberculosis* and the genetically linked fusion construct can enhance both humoral and cell mediated immunity and could confer better protection against vvIBDV challenge than immunizing with VP2 alone. Considering the economic importance of IBD my present work was envisaged with the following objectives:

1. **To develop a DNA vaccine construct encoding VP2 gene of Infectious Bursal Disease (IBD) virus and truncated HSP70 gene of *Mycobacterium tuberculosis*.**

2. **To characterize the DNA vaccine construct by Immnoflurosence assay/Western Blot.**

2 Review of Literature

2.1 History of infectious bursal disease (IBD)

Infectious bursal disease (IBD) is an acute infectious, immunosuppressive disease of poultry occurring worldwide (Negash *et al*., 2013). IBD first reported by Cosgrove (1962). The disease was first described as "avian nephrosis" due to the damage in kidney. The disease is also recognized as 'Gumboro disease' as first outbreak of the disease reported in Gumboro, Southern Delaware, USA but the most affected organ Bursa of Fabricius led to designation of the disease as Infectious Bursal Disease by Hitchner (1970). Etiological agent of IBD is Infectious bursal disease virus (IBDV) which is isolated from embryonated egg by Winterfield (Winterfield *et al*., 1962). Immunosuppressive nature of disease was first reported by Allan *et al*., 1972. IBDV is a bi-segmented, double stranded RNA virus belonging to the family Birnaviridae, genus Avibirnavirus (Dobos *et al*., 1979). IBDV have two serotype designated as serotype 1 and serotype 2. The later was identified by Mcferran *et al*., 1980. The target organ for IBDV is the Bursa of Fabricius (BF) which at its maximum development, is a specific source for mature B-lymphocytes in avian species. Bursectomy can prevent illness in chicks infected with virulent virus (Hiraga *et al*., 1994). The severity of the disease is directly related to the number of susceptible cells present in the BF; therefore, the highest age susceptibility is between 3 and 6 weeks, when the BF is at its maximum rate of development. This age susceptibility is extended in the case of vvIBDV infection (van den Berg *et al*., 1991). The occurrence of IBD in India was first reported by Mohanty *et al*., 1971. According to OIE annual animal disease status, 177, 93, 127, 169, 381 and 93 outbreaks of IBD were recorded during the years 2007, 2008, 2009, 2010, 2011, 2012 and June 2013 respectively in India (http://www.oie.int/wahid/ public.php).

2.2. IBDV structure and classification

IBDV belongs to family Birnaviridae and genus Avibirnavirus. Genome of IBDV contain bi segmented, double stranded RNA (Leong *et al*., 2000). The virus has non-enveloped,

icosahedral capsid. The diameter of capsid protein is 60nm and capsid subunits are mainly trimer clustered (Bottcher *et al*., 1997). The two segments of the viral genome are designated as segment A and segment B. Genome segment A has two ORFs. Larger ORF 1 encodes for a 110kDa polyprotein which is spliced into viral proteins VP2 (48kDa), VP3(33-35kDa),VP4(24kDa). VP2 is the capsid protein and carries the major immunogenic determinants (Fahey *et al*., 1989). VP4 is the virus protease, responsible for the cleavage of the polyprotein. VP3 is a ribo nucleoprotein and scaffolding protein. A second ORF designated as ORF A2 is partially overlapped with ORF A1 encode a protein VP5 (Mundt *et al*., 1995). VP5 is not essential for viral replication but considered to have a role in viral release and apoptosis (Lombardo *et al*., 2000). Genome segment B (2.8kb) encodes a structural protein VP1 which is linked to both segments of viral genome and has multiple enzyme activity. VP1 protein of Birnavirus forms a distinct subgroup of RNA dependent RNA polymerases that lacks a GDD motif (Shwed *et al*., 2002)

2.3. Serotype

IBDV has two serotypes recognized as serotype 1 and serotype 2. Both serotypes of the virus can naturally infect chicken, turkey, duck, guinea fowl, ostriches but pathogenicity is reported only in chicken. Serotype 1 of IBDV is pathogenic whereas serotype 2 is nonpathogenic in chicken (Macferran *et al*., 1980). A new very virulent strain of IBDV serotype 1 had emerged in Holland, Belgium in 1986, and then spreaded throughout the world rapidly (Chettle *et al*.,1989)

2.4. Pathology

Disease severity and clinical signs and symptoms depend on the age and sensitivity of the infected birds, the virulence of the strain, and the degree of passive immunity transmitted by the parents. Initial infection in a given farm is generally very acute, with very high mortality rates if a very virulent strain is involved. If the virus persists on the farm and is transmitted to successive flocks, the clinical forms of the disease appear earlier and are then gradually replaced by subclinical forms (Ingrao *et al*., 2013). Moreover, a primary infection may also be unapparent if maternal antibodies are present. Acute IBDV infections are characterized by severe clinical signs and high mortality. Indeed, vvIBDVs produce disease signs similar to classical type 1 infections but the acute phase is exacerbated and more generalized in the affected flock. The incubation period is very short for 2–3 days. In acute cases, the animals are exhausted, prostrated, dehydrated,

suffer from aqueous diarrhea, and their feathers are ruffled. Mortality commences on the third day of infection, reaches a peak, then drops rapidly, and the surviving chickens recover a state of apparent health after 5–7 days. The age susceptibility is extended, covering the entire growing period in broilers and the peaks of mortality show sharp death curves followed by rapid recovery (Chettle *et al*., 1989; Tsukamoto *et al*., 2002; van den Berg *et al*., 2000). Compared with classical virulent strains, vvIBDVs induce higher mortality rates in fully susceptible chickens. But the exact cause of clinical disease and death is still unknown, as it is not clearly related to the severity of the lesions and the extent of the bursal damage. Indeed, after infection, some birds with few bursal lesions can be found dead while others can survive despite extensive bursal damage. Moreover, mortality rates are also variable. On post mortem examination of birds that died during the acute phase of IBD, the BF is the principal diagnostic organ that is turgid,oedematous and sometimes hemorrhagic and turns atrophic within 7–10 days. In addition, dehydration and nephrosis with swollen kidneys are common and ecchymotic hemorrhages in the muscle and the mucosa of the proventriculus are observed in many affected birds. The damage is transient and the structure of the BF is restored and repopulated with lymphocytes; the duration of the recovery process depends on the age at infection and the virulence of the strain. Depletion of lymphoid cells is observed not only in the BF but also in the non bursal lymphoid tissues. In particular, atrophy of the thymus has been associated with the acute phase of the disease and might be indicative of the virulence of the isolates.

2.5. Diagnosis

Pathological changes observed at the BF are characteristic, and histopathological investigations combined with the demonstration of viral antigens by immunohistochemistry confirm an IBDV infection. IBDV can be isolated by the inoculation of antibody free embryonated chicken eggs. The virus-neutralization assay is the only serological test, which can reliably differentiate IBDV isolates into antigenic serotypes and subtypes (Jackwood and Saif., 1987). To demonstrate the presence of IBDV-specific antibodies, ELISA systems are commercially available. Viral antigens can be demonstrated by the agar-gel precipitin assay or by the antigen-capture enzyme-linked immunosorbent assay (AC-ELISA). Commercially four types of indirect ELISAs viz, a commercial IDEXX-ELISA kit, VP2 and or VP3 antigen based ELISAs and a whole virus ELISA were

compared for their diagnostic potential. It was concluded that the sensitivity and specificity of four ELISAs viz., IDEXX-ELISA, VP2-ELISA and VP3-ELISA indicated similar performance whereas whole virus antigen based ELISA showed poor performance in comparison to other ELISAs (Singh *et al*., 2010). Recently a yeast expressed recombinant VP2 subviral particles (SVPVP2) antigen- based single serum dilution enzyme linked immunosorbent assay (ELISA) was described to detect IBDV specific antibodies in chickens which is sensitive, specific and accurate as compared to the serum neutralization test and agar gel immunodiffusion test. The SVPVP2 can be used to diagnose IBDV antibody in positive serum within 3-4 minutes (Dey *et al*., 2010). Recently reverse transcription (RT) loop mediated isothermal amplification (RT-LAMP) assay was reported (Lee *et al*., 2011).

2.6. Immune response towards IBDV

The expression levels of genes related to innate immunity increased during primary infection, while those of adaptive immunity were up-regulated during the secondary infection, with a simultaneous decrease in the expression levels of innate immunity genes. Immune response plays a crucial role in the clearance of the virus and protection from vvIBDV infection. The role of bursa of fabricious (BF) was revealed when bursectomized birds survived without showing any symptoms even after the lethal dose of IBDV was inoculated. In early stage of life BF play major role for differentiation of B cell. Following IBDV infection B cell destruction occur rapidly as virus replicate in immature B cell. In addition to replicating in B cell IBDV are also replicates in macrophage, bone marrow have reported. The protection against IBDV does not solely depends on the neutralizing antibodies T cell involvement also critical for the clearance of virus (Rautenschlein *et al*., 2005). Virus particle were detected in BF and other peripherial lymphoid organ such as thymus, caecal tonsil, spleen following IBDV infection. Rapid infiltration of CD4+T cell and CD8+ T cell immediately after IBDV infection highlighted the importance of T cell in the clearance of virus or might be induction of pathogenesis cannot be ignored (Rauf *et al*., 2011). The role of cell mediated immunity becomes clear by the increased level of mRNA transcription of various pro-inflammatory cytokines like IFNγ, IL-1β, IL6 following in vivo infection with vvIBDV (Mahgoub *et al*,2012). In addition, several Th2 based cytokines as IL4, IL6, IL10 are also increased significantly after in vivo infection (Shini *et al*,2010).

2.7. Vaccines against infectious bursal disease (IBD)

2.7.1. Conventional live and inactivated IBDV vaccines

Live viral vaccines mimic the infection in the target host. They can replicate and induce both cellular and humoral immunity. They do not require an adjuvant to be effective and are suitable for mass administration to the chicken, but they may also have undesirable side-effects. These include horizontal and vertical transmission, reversion to virulence and vaccine reactions that may result in disease or production loss. In general, the live IBDV vaccines in use by the poultry industry have been attenuated by serial passage in tissue culture, eggs or embryo-derived tissues, with the aim of maintaining the immune response induced by the parent virus whilst attenuating the ability of the vaccine virus to cause clinical disease or significant immunosuppression (Schijns *et al*., 2008). Most commercially available conventional live IBDV vaccines are based on classical virulent strains. Those classified as ‘‘mild’’ vaccines exhibit only poor efficacy in the presence of certain levels of maternally derived antibodies and against vvIBDV. ‘‘Intermediate’’ and ‘‘intermediate plus’’ or ‘‘hot’’ vaccines have a much better efficacy and may break through higher levels of maternally derived antibodies, but they can induce moderate to severe bursal lesions and, thus, cause corresponding levels of immunosuppression (Mazariegos *et al*., 1990; Tsukamoto *et al*., 2002; Kumar *et al*., 2000; Rautenschlein *et al*., 2005). They may not fully protect chickens against infection by the vvIBDV strains (Rautenschlein *et al*., 2005) or by antigenic variants. Safety and efficacy of this type of vaccine still remains a major concern.

2.7.2. Inactivated IBD vaccines

Inactivated vaccines are mostly formulated as water-in-oil emulsions, usually combining several antigens. It has been observed that inactivated IBD vaccines were also able to induce IBDV specific T-cell and inflammatory responses in chickens (Rautenschlein *et al*., 2002). It has been reported that inactivated IBD vaccines must have either a high or an optimized antigenic content in order to induce in breeders an immunity that helps to protect the progeny from infection by variant IBDV strains (Rosenberger *et al*., 1985; Muller *et al*., 1992). Inactivated vaccines are most efficiently used in boost regimen, using attenuated live IBDV as priming vaccine.

2.7.3. IBDV immune complex vaccines

IBDV immune complex vaccines (Icx) consist of a mixture of a certain amount of IBDV specific antibodies obtained from the sera of hyperimmunized chickens and infectious IBD vaccine virus (Whitfill *et al*., 1995). Their major advantage is that they are suitable for in ovo vaccination at day 18 of incubation with commercial egg-injection machines, which initially had been used for vaccination against Marek's disease virus and fowl pox virus. Alternatively, the Icx vaccines can be delivered by subcutaneous injection at 1 day old in the hatchery (Ivan *et al*., 2005). In addition it has been shown that these vaccines were effective in the presence of maternally derived antibodies (Haddad *et al*., 1997; Giambrone *et al*., 2001). The conventional live vaccines that are usually given via drinking water or by eye-drop method. At challenge, the experimental efficacy of the Icx vaccines was identical to or better than that induced by vaccination with live IBDV vaccines. The working mechanism was investigated by comparing the infectivity of the IBDV-Icx and the virus alone at various time points after in ovo injection . With both vaccines, IBDV was associated with B lymphocytes, macrophages and follicular dendritic cells in the bursa of Fabricius and spleen, although IBDV complexing with specific antibodies caused a delay in virus detection of approximately 5 days (Jeurissen *et al*., 1998). In another study the virus was first detected in the bursa of vaccinated specific pathogen free chickens at day 14 post vaccination and on days 17 to 21 in broilers with maternally derived IBDV-specific antibodies (Ivan *et al*., 2005). Most remarkable was the low level of depletion of bursal and splenic B lymphocytes in chickens vaccinated experimentally with IBDV-Icx (Jeurissen *et al*., 1998). Furthermore, in ovo inoculation with the IBDV-Icx vaccine induced more germinal centres in the spleen, and larger amounts of IBDV were localized on both splenic and bursal follicular dendritic cells. Recently, recombinant neutralizing antibodies have been developed and used in an experimental IBDV-Icx vaccine (Sapats *et al*., 2006; Ignjatovic *et al*., 2006).

2.7.4. Subunit vaccines

VP2, a major structural protein of IBDV, contains antigenic epitopes responsible for induction of neutralizing/protective antibody (Becht *et al*., 1988; Fahey *et al*., 1989). Denatured VP2 does not induce protection in chickens (Fahey *et al*., 1989), and denatured and renatured VP2 also lost the ability to induce neutralizing antibodies in chickens (Opplingn *et al*., 1989). Different expression systems have been used, such as

Escherichia coli (Fahey *et al*., 1991), yeast (Dey *et al*., 2009), fowl pox virus (Bayliss *et al*., 1991; Heine & Boyle, 1993), baculovirus (Vakharia *et al*., 1993), Semliki Forest virus (Phenix *et al*., 2001), and even plant expression systems (Wu *et al*., 2004). Either the VP2 encoding region alone or the polyprotein gene was used for expression, and polyhistidine tags have been used for effective protein purification. It was observed that IBDV empty capsids offered better protection than did the tubular structures that formed after expression in a baculovirus based system (Martinez-Torrecuadrada *et al*., 2003). To date, three vaccines based on the VP2 subunit have been placed in the market in some countries, with VP2 expressed either in the baculovirus system, or in *E. coli* or in the yeast *Pichia pastoris* (Pitcovski *et al*., 2003). As with inactivated vaccines, the requirements of parenteral administration and booster immunizations are limiting factors for the use of such vaccines.

2.7.5. DNA vaccine

DNA inoculation represents a novel approach to vaccination and immune therapeutic development in early 1990. The direct injection of gene expression cassettes into a living host transforms a number of cells into factories for production of the introduced gene products. Expression of these delivered genes has important immunological consequences and may result in the specific immune activation of the host against the novel expressed antigens (Chattergoon *et al*., 1997). DNA vaccine consists of naked plasmid DNA which contain antigenically important gene regulated under strong mammalian promoter. Direct delivery of DNA encoding human growth hormone into the skin of mice through 'gene gun' resulted in antibody response against the delivered antigen. DNA vaccine is safe, non replicating, inexpensive and technically simple method to induce immune response. DNA vaccine can induce humoral as well as cellular immunity.

2.7.5.1. DNA vaccine encoding VP2 full length gene

A number of studies were conducted on the development of a DNA vaccine, with variable success, to induce an efficient immune response in chickens. DNA vaccine expressing VP2 protein has reported in a number of studies as VP2 is the major structural protein of IBDV containing an antigenic epitope and capable of inducing neutralizing antibody (Fahey *et al*.,1989). Though it has the ability to elicit both humoral and cell mediated immune response, the protective efficacy varied from 40 to 70 % bursal lesions

in most of the cases(Kumar *et al*.,2009; Pradhan *et al*.,2014; Negash *et al*., 2013). A potential use of DNA vaccine for priming in ovo or at 1 day old chicken followed by boosting with inactivated vaccine or vectored vaccine has been described (Haygreen *et al*., 2006; Hsieh *et al*., 2007) and induced protection against a virulent challenge infection. The use of DNA vaccination via the in ovo route was also described (Oshop *et al*., 2003; Haygreen *et al*., 2006; Park *et al*., 2009). The results showed that the in ovo delivery without a booster dose was not sufficient to induce protective immunity. Bacteria, including *Lactococcus lactis, Salmonella typhimurium* and *E. coli*, have been used to deliver IBDV cDNA vaccine orally, but with variable success. Here, difficulties in the secretion or translocation of the expressed viral protein across the bacterial cell wall could be the limiting factor (Li *et al*., 2006; Mahmood *et al*., 2007). Co administration of Cytokine IL-18 and VP2 gene could enhance the immune responses and protection efficacy of DNA vaccine against IBDV infection in chickens, highlighting the potential value of chicken IL-18 as an adjuvant in the prevention of vvIBDV (Li *et al*., 2013). Recently prime boost vaccination strategy has been developed against IBD (Gao *et al*., 2013).

2.8. Heat Shock Protein (HSPs)

HSPs are highly conserved molecules, found in prokaryotes, eukaryotes and even in plants. These proteins undertake crucial functions in maintaining cell homeostasis and are essential for life since they behave as chaperons (Smith *et al*., 1998). HSPs are expressed both constitutively (cognate proteins) and under stressful conditions (inducible forms). Constitutively expressed HSPs appear to serve as molecular chaperons, recognizing and binding to nascent polypeptide chains and partially folded intermediates of proteins, preventing their aggregation and misfolding. HSPs also participate in protein synthesis, suitable protein folding, assembly, trafficking and degradation .Moreover, HSPs as molecular adjuvants have been used as attractive immune stimulatory components in the development of vaccines. HSP70 and other HSP family members endogenously bind antigenic peptides in tumor or virus-infected cells. Such HSP70-peptide complexes, formed by fusing antigens to HSP70, are capable of inducing potent antitumor (Suzue *et al*.,1996a) or antiviral immunity (Li *et al*.,2006 ; Ebrahimi *et al*.,2012). Genetically fusing antigens to HSP70 leads to an enhanced vaccine potency. These investigations have made HSP70 an attractive molecular adjuvant in vaccine development.

2.8.1. HSP70 as adjuvant and carrier

The immunological functions of HSPs began to emerge in the 1980s, when it was observed that homogenous preparations of certain HSPs that were isolated from cancer cells elicited immunity to cancers (Srivastava *et al*., 1998). That study was first carried out with the HSP gp96 (Blachere *et al*. 1993), but similar results were later obtained with HSP70 (Udono and Srivastava 1993, Ciupitu *et al*. 2002), HSP90 (Udono and Srivastava 1994), calreticulin (Basu and Srivastava 1999), HSP170 and HSP110 (Wang *et al*. 2001). Among those HSPs, the HSP70 family is well characterized and attracts much attention because of its versatile functions in the immune system. Besides the chaperone activity, HSP70 molecules can function as endogenous as well as exogenous adjuvants (Vabulas *et al*.2002, Asea *et al*. 2002). HSP70s prepared from tumor cells or virus-infected cells are capable of eliciting CD8+ CTL responses in vivo and in vitro against a variety of antigens expressed in the cells from which these immunogenic proteins have been purified (reviewed in Srivastava 2002). Extracellular HSP70 can complex with antigenic peptides and simultaneously activate professional APCs. This interaction triggers a cascade of events, including re-presentation of chaperoned peptides to MHC I restricted CD8+ and MHC II restricted CD4+ T cells, secretion of pro-inflammatory cytokines and phenotypic and functional maturation of DCs (Asea *et al*., 2000, Tobian *et al*. 2004 (a, b)). These properties combine to make HSP70 a potent adjuvant that integrates innate and adaptive immune responses. HSP70 contains strong T-cell epitopes and serves as a carrier of antigens, effectively inducing antigen specific B cells as well as CD4+ and CD8+T -cell responses without requiring an adjuvant (Udono *et al*. 2001).

Fusing mycobacterial HSP70 to HIV-1 gag p24 (Suzue *et al*.,1996a), Influenza M2e (Ebrahimi *et al*.,2012) and synthetic malarial antigen (NANP) a synthetic peptides consisting of 40 (Asn-Ala-40, Asn-Pro) repeats (Barrios *et al*.,1992), enhanced the immunogenicity of the antigens and obviated the need for adjuvant. Mice immunized with a kineto plamid membrane protein-11 (KMP11) covalently fused to HSP70 from *Trypanosoma cruzi* elicited a CTL response against the Jurkat-A2/Kb cells expressing the KMP11 protein (Maranon *et al*.,2001). Moreover, HSP70 has been used as a carrier for group C meningococcal oligosaccharide, inducing antibodies against oligosaccharide in mice (Perraut .,1993). Furthermore, chimeric proteins formed by antigens coupled to the C-terminal fragment of HSP70 from *M. tuberculosis* and N-terminal fragment from

Leishmania infantum (Rico .,1998), induced humoral and cell mediated immune responses to the coupled antigens.

2.8.2. HSP70 receptors and mechanism of adjuvanticity

The existence of receptors on APCs, specifically mediating the cellular internalization of HSPs was postulated in 1994 by Srivastava (Srivastava . 1994). The first HSP70 receptor was identified in 2000, eliciting considerable interest in this area (Binder . 2000). It has been suggested that the signaling and cross-presentation of chaperoned peptides, might be mediated by different sets of receptors. CD9 is a putative receptor for HSP70, which is specifically endocytic, whereas TLR-2 and TLR-4 are implied as the signaling receptors (reviewed in Binder . 2004). The adjuvanticity of HSP70 is based on the specific interaction of HSPs with the receptors present on professional APCs (DCs and macrophages) having two distinct consequences: 1. stimulation of an innate response (regardless of chaperoned peptides) and 2) activation of adaptive immune events through representation of HSP-chaperoned peptides to MHC molecules, therefore integrating innate and adaptive immune events. HSP70 activates DCs through binding to its cognate receptor CD14/TLR-4 or TLR-2 complexes, expressed on those cells. This is a non-antigen specific event and important for efficient priming of T cells. TLR4/2 receptor mediated binding initiates signaling cascades in immature DCs (Asea ,2002) causing them to differentiate and migrate from the periphery to the draining lymph nodes. This leads to several activities, including up-regulation of MHC and costimulatory (CD86/83) molecules, induction of chemokine secretion, production of NO and secretion of inflammatory cytokines such as IL-1b, IL-12, IL-6 and TNF-a (Asea *et al*. 2000, Moroi *et al*. 2000, Kuppner *et al*. 2001). HSP70 can also interact specifically with the CD91 receptor (Basu *et al*. 2001), that mediates endocytosis and results in cross-presentation of HSP70 associated peptides to both CD8+and CD4+T lymphocytes (Udono *et al*. 2001). This alternate MHC I antigen processing and cross-presentation is mediated via cytosolic mechanisms in dendritic cells and vacuolar mechanisms in macrophages (Tobian *et al*. 2004 (b)).Therefore, the remarkable immunogenicity and adjuvanticity of HSP70 may be ascribed to two crucial features: HSP70 as cross-priming adjuvant and as direct activators of professional APCs. TLR2/4 are the major receptors involved in transducing HSP70 mediated signaling through activation of the MyD88/NF-kB (Asea *et al*. 2002, Vabulas *et al*. 2002). In addition to TLR2 and TLR4, other cell surface receptors, such as CD40 have

been found to be potentially involved in transducing activation signals of HSP70 to APCs, (Wang *et al*. 2001, Becker *et al*. 2002). The idea of the involvement of CD40 in the interaction of HSP70 with APCs draws indirect support from different studies.

2.8.3. *Mycobacterium tuberculosis* HSP70 adjuvant property

Microbial HSP70 derived from *Mycobacterium tuberculosis* (mHSP70) is a 70 kD molecular chaperone having three functionally distinct domains: an N-terminal 44 kDa ATPase portion (amino acids 1-358), followed by an 18 kDa peptide-binding domain (amino acids 359-494) and a 10 kDa fragment (amino acids 495-610).The amino-terminus of HSP70, with mixed alpha-helices and beta pleated sheets, binds and hydrolyzes ATP molecules which were driven from folding action of HSP70.The carboxy-terminal domain of mHSP70 (28 kD) is a loose beta barrel which binds 6-amino acid hydrophobic protein motifs, the object of folding activity (Flahety *et al*.,1990; Zhu *et al*.,1996). Besides the chaperone activity, HSP70 molecules can function as adjuvants (Asea *et al*., 2000). HSP70 is a ligand for TLR2 and TLR4 was shown to enhance the potecncy of plasmid DNA immunogen in mice by inducing expression of Th1 based cytokines (Asea *et al*., 2002). A plasmid protein expressing an antigen-HSP70 fusion gene elicit both humoral and cell mediated immunity that were more potent than plasmid containing antigen alone (Planelles *et al*., 2001). HSP70 prepared from tumor cells or virus-infected cells are capable of eliciting CD8+ CTL responses in vivo and in vitro against a variety of antigen expressed in the cells from which these immunogenic proteins have been purified (Srivastava *et al*., 2002). Extracellular mHSP70 can complex with antigenic peptides and simultaneously activate professional APCs. This interaction triggers a cascade of events, including representation of chaperoned peptides to MHC I restricted CD8+ and MHC II restricted CD4+T cells, secretion of pro inflammatory cytokines and phenotypic and functional maturation of DCs (Tobian *et al*., 2004). These properties combine to make mHSP70 a potent adjuvant that integrates innate and adaptive immune responses.

2.9. C-terminal fragment of mycobacterium HSP70 amino acids 359-610

It was studied that the C-terminal fragment of mHSP70 acted as a carrier in mice when fused to the malarial antigen EB200 (HSP70-EB200) and considerably induced vaccine MHC responses (Qazi *et al*. 2005). The effects of two truncated HSP70 molecules, N-terminal domain $HSP70_{1\text{-}360}$, amino acids 1-360 and C-terminal domain $HSP70_{359\text{-}610}$,

amino acids 359-610, of mycobacterial HSP70 was evaluated on the basis of antigen specific immunity generated by a hepatitis B virus (HBV) DNA vaccination. It was shown that only the HSP70 $_{359\text{-}610}$ fused HBV DNA vaccination resulted in a significant increase in hepatitis B surface antigen (HBsAg)-specific humoral response, while the HSP70$_{1\text{-}360}$ fused vaccine did not. Moreover, HSP70 $_{359\text{-}610}$ fused DNA vaccine did not induce anti-HSP70 antibody (Li *et al.*, 2006). Many studies have been reported using C-terminal of *Mycobacterium tuberculosis* heat shock protein HSP70 provide better protection when fused to antigen compared to complete HSP70 molecule (Wang *et al*., 2002; Zhang *et al*., 2008).

In other studies, the effects of HSP70 on foot and mouth virus (FMDV) and Japanese encephalitis virus in mice were evaluated separately and the HSP70 markedly enhanced both the humoral and cell-mediated immune responses (Fei-fei *et al*., 2006). Therefore C-terminal domain of HSP70 (HSP70$_{359\text{-}610}$) seems to be safe enough immunogenic and function with linkage with small low immunogenic and consider them as attractive vaccine adjuvant (Ebrahimi *et al*., 2010). In a recent reports have shown the induction of specific humoral and cellular immune responses against Hantaan virus in a mouse model following gene fusion of c-terminal HSP70 and Hantaan virus Gn and S0.7 in an adenoviral vector (Cheng *et al*, 2014).

3 Materials & Methods

3.1. Virus and cell line

The very virulent IBD virus was available in Recombinant DNA lab and was given in 11 day old specific pathogen free (SPF) embryonated chicken eggs through CAM route and titer was evaluated by Reed and Muench method. Animal experiments were approved by the animal ethics committee of the institute. Vero cell line was grown and maintained in medium 199 (M199, Life Technology) supplemented with 10% (V/V) heat inactivated foetal bovine serum (FBS, Life Technology).

3.2. Chemicals and reagents

All the chemicals, buffers, media, reagents and various kits related to molecular biology and genetic engineering, were obtained from various manufacturers viz., Amresco (USA), Bangalore Genei (India), Bethyl (USA), Biochem (India), Bio-Rad Laboratories (USA), Eurofins India, Life Technologiy ,USA), Kappa Biosystems (USA), Merck (India/ Germany),Qiagen (Germany),Sigma (USA), Thermo Scientific (USA) and SiscoResearch Laboratories/ SRL(India).Detailed composition of various buffers and reagents, their storage conditions have been provided in the appendix attached and also at various occasions.

3.3. Equipment

Liquid handling systems (micropipettes) were from Eppendorf (Germany), microplate reader (Biorad. USA), Gel electrophoresis apparatus (ATTO), Gel documentation system (Biorad, USA),Blotting apparatus (Bio-Rad, wet tank mini trans blot protein transfer system, USA), table top refrigerated centrifuge (Eppendorf, Germany), Refrigerator (LG India Ltd.), -20˚C to -80˚C freezer (Haier, China), Spinix vortexer (Tarsons, India), Spinwin microfuge (Bangalore Genei, India), BioSpectrometer, Thermal cycler Eppendorf (Germany), water bath (Grant, UK), double distillation apparatus (Borosil), deionisation plant (Borosil), weighing balance (Sartorius, Germany), Laminar Flow

(ESCO), Fluorescence microscopes (Leica), real time thermal cycler (Biorad CFX 96, USA) were used.

3.4. Enzymes

Restriction and modifying enzymes were from Promega (USA), Stratagene (Germany), Invitrogen (USA), New England BioLabs (USA), MBI Fermentas (USA),Superscript III MMLV reverse transcriptase was obtained from Invitrogen (USA).

3.5. Media and buffers

Compositions of media, buffers and solutions used in this study are given in the appendix and in respective sections.

3.6. Construction of recombinant vector

3.6.1. Designing of DNA vaccine construct and recombinant VP2 protein

The eukaryotic expression vector pCI mammalian expression vector with CMV promoter (Promega, USA) was used for the in vitro expression studies. The full length VP2 coding gene (1338 bp) of IBDV was amplified from the cloned IBDV VP2 gene (Dey *et al*., 2009) as the template using the upstream (5'-GCGGAATTCGCCACCATGACAAACCTGCAAGAT-3') and downstream (5'GCGGGATCCTGCTCCTGCAATCTTCA-3') primers that harbored the EcoRI and BamHI restriction sites respectively (Table.1). PCR was performed in 50µl reaction mixture containing Taq polymerase, Taq buffer (10X) 5µl, 10pmol dNTP and 1µmol of each of the primers. Reaction conditions were 95˚C for 5 min followed by 30 cycles at 95˚C for 1 min, 58˚C for 30s, 72 ˚C for 1 min and final extension at 72˚C for 10 min. The c-terminal domain of HSP70 (HSP70 $_{359\text{-}610}$) was amplified (773 bp) from the genomic DNA of Mycobacterium tuberculosis using the specific upstream (5'-GCGGGATCCGAGGTGAAAGACGTTC-3') and downstream (5'-GCGGCGGCCGCTCAATCAGCCGAGCCGGG-3') primers harboring EcoRI and BamHI restriction sites respectively (Table. 1). The PCR conditions were similar to the condition carried out for VP2 amplification. The PCR amplicons VP2 and cHSP70 were gel purified then separately digested with BamHI followed by ligated using T4 DNA ligase (NEB, UK) to form a fusion construct namely VP2-cHSP70 (2111bp). Then using VP2-cHSP70 as a template and VP2 upstream and cHSP70 downstream primers, a PCR reaction was carried out at an annealing temperature of 60˚C for 1 minute. The PCR amplicon obtained from the VP2-cHSP70 fusion construct was

then purified from the gel and subjected to double digestion with EcoRI/NotI restriction enzymes and cloned into the EcoRI/NotI multiple cloning site of digested pCI expession vector to form pCIVP2-cHSP70 construct. Thus pCIVP2-cHSP70 plasmid has a single open reading frame (ORF) with ATG start codon at the 5' end of the VP2 and UGA stop codon in the 3' end of cHSP70. Restriction site for BamHI GGATCC is flanked in between the VP2 and cHSP70 construct(Fig.6). The ORF and the fusion construct was confirmed by sequencing.

Table 1. Primers designed for PCR analysis

Gene	Forward	Reverse	Size	Restriction enzyme site
cHSP70	TGCGC*GG-GATCC*GAGGTGAAAGACGTTC	GCG*GCGGCCGC*TCAATCAGCCGAGCCGGG	773 bp	BamHI / NotI
VP2	GCG*GAATTC*GCCAC-CATGACAAACCTGCAAGAT	GCG*GGATCC* TGCTCCTG-CAATCTTCA	1338 bp	EcoRI/BamHI
VP2-cHSP70	GCG*GAATTC*GCCAC-CATGACAAACCTGCAAGAT	GCG*GCGGCCGC*TCAATCAGCCGAGCCGGG	2111 bp	EcoRI/NotI

The full length VP2 gene was cloned in pCI vector namely pCIVP2. The recombinant VP2 protein expressed in Sacchharomyces cerevisiae formed subviral particle (SVPVP2) (Dey *et al*., 2009) available in the laboratory was used as a vaccine candidate in the DNA prime- protein boost approach.

3.6.2. Preparation of competent cells (Calcium chloride method)

E.coli (DH5α) cells were made competent for transformation by the calcium chloride method described by Sambrook (2001) and as followed in the laboratory. Frozen bacterial stock was thawed at 370C and streaked on LB agar plates using sterile platinum loop and incubated at 370C for 16-18 h. Single colony from the plate was inoculated into a 25 ml LB broth and incubated in a shaking incubator at 370C for 16-18 h. At the end of incubation, 2.5 ml of this culture was transferred to a 250 ml LB broth and incubated at 370C in the shaking incubator until the culture attained an O.D. of 0.3-0.4 at A600 nm (3-4 h incubation). The culture was chilled on ice and the cells were harvested by centrifugation at 6000 x g for 5 min at 40C in a refrigerated centrifuge. The cell pellet was resuspended in 25 ml ice-cold 100mM CaCl2 and incubated on ice for 30 min. The cells were recovered by centrifugation as above. The pellet was resuspended in 2.1 ml of

100mM CaCl2 and 1.5% of sterile glycerol. The cell suspension was thoroughly but gently mixed and aliquoted (80µl) in 1.5 ml microcentrifuge tubes and stored at –70˚C until use.

3.6.3. Transformation by heat shock method

The frozen aliquots of competent cells were thawed on ice and mixed with ligated pCI VP2-cHSP70 plasmid an pCIVP2 plasmid and left on ice for 20 min. The cells were given heat shock at 42˚C for 45 sec followed by snap cooling on ice for 2 min immediately. There after one ml of pre-warmed SOC was added to the cells and the suspension was incubated at 37˚C for 1 h in a shaking incubator. Subsequently, the cells were pelleted by centrifugation at 8,000 x g for 5 min at room temperature and the pellet was resuspended in 100 µl of fresh LB broth. The cells were then plated on LB agar plates containing ampicillin (50µg/ml) and incubated at 37˚C for 16 to 18 h. Colonies that appeared were screened for the presence of recombinant clones or plasmids.

3.6.4. Plasmid isolation by alkaline lysis (mini prep) method

Plasmid DNA from E.coli DH5α cells was isolated with miniprep plasmid isolation kit (QIAGEN, Miniprep kit) as described by manufacturer. Briefly, bacterial colonies transformed with plasmid DNA were inoculated into 10 ml of LB broth containing ampicillin (50 µg/ml) and allowed to grow overnight at 37˚C with shaking. About 1.5 to 3 ml of the culture was centrifuged at 6,000 x g at room temperature to pellet the cells. The cell pellet was suspended in 100 µl of cell suspension buffer (P1) containing RNase-A (20 µg/ml). To this suspension, 200 µl of lysis buffer (P2) was added and the contents mixed by gentle inversion several times until a clear lysate was evident and the mixture turns blue. Then, 150 µl of neutralizing solution (P3) was added to the lysate and the contents were mixed once again by gentle inversion several times until the mixture will became colorless and incubated on ice for 10 min. Chromosomal DNA along with other cell de-bris was pelleted out by centrifuging the above mix at 12,000 x g for 10 min at 40C. The supernatant was collected in Qiagen tip and treated with QC followed by QF buffer at 12,000 xg for 2 min at room temperature. Finally eluted with 50 µl of nuclease free water and stored at –20˚C.

3.6.5. Screening of recombinant plasmids

DNA vaccine construct was confirmed by the release of insert of expected molecular size upon RE digestion of the recombinant plasmid DNA. Suspected recombinant plasmid DNA was extracted by miniprep plasmid isolation method as described earlier. These were subjected to RE digestion and the products were analyzed in an analytical agarose gel alongside a DNA molecular weight marker. Release of the expected size DNA fragment confirmed the presence of recombinants. Single colony picked from the plate was inoculated in LB broth and grown over night. 700 µl of culture was mixed with 300 µl of 50 % sterile glycerol in eppendorf tube for cryopreservation and stored at -80˚C. DNA isolated was quantified in BioSpectrometer at 260 nm and 280nm. The purity was assessed as A260/280 ratio.

3.6.6. Sequencing of recombinant plasmids

The recombinant plasmids were sequenced using the ABI 377 Perkin Elmer Automated DNA sequencer at the Sequencing Facility, Eurofins, Bangalore, India. The data obtained was analyzed using NCBI BLAST and DNASTAR package analysis software.

3.7. In vitro characterization of the fusion construct

3.7.1. Cell Culture

Vero cells that were propagated and maintained with M199 media supplemented with 10% FBS (Foetal Bovine Serum) and penicillin and streptomycin at 37˚C in Recombinant DNA laboratory, IVRI were used in the study.

3.7.2 Transfection of Vero cells

Vero cells were grown to 80 % confluence in 6 well tissue culture plates. 5 µl of Lipofectamine (Invitrogen) was mixed in 300 µl of optiMEMmedia and was kept aside, 5 µg of endotoxin free recombinant/control plasmids were mixed in 300 µl of optiMEM media separately and incubated at room temperature for 5 min. The above mixtures were mixed gently and incubated for 20 min at room temperature. Vero cell monolayer was washed with optiMEM media and layered with DNA lipofectamine mix, incubated at 37˚C for 4 h in 5 % CO2. At the end of incubation period, medium was replaced with 2 ml of fresh growth medium.

3.7.3 In vitro expression of recombinant protein

The vero cell lines were transiently transfected with 4µg of pCIVP2, pCIVP2-cHSP70 or empty vector along with IBDV infected positive and mock infected negative control using Lipofectamine 2000 (Invitrogen, USA) reagent according to manufacturer's protocol. Forty eight hours post transfection the transfected cells were washed with Dulbecco modified phosphate buffer (DPBS, LifeTechnology), fixed with 4% paraformaldehyde in PBS (Affymetrics, USA) for 20 min at room temperature followed by washing with PBS and blocked by 3% bovine serum albumin. The expression of the recombinant plasmids was analyzed by Western blot and immunofluorescence (IFAT) using rabbit anti IBDV-VP2 antibody (1:100) dilution. Briefly ,Cells were lysed with cell lysis buffer (Biosource, USA) and freeze thawed for 3-4 times and centrifuged for 5 min. The pellets were boiled for 10 minutes in a water bath in order to lyse and denature the bacterial proteins. Samples were then spin at 12,000 rpm to pellet the cellular debris following which 40µl of the supernatant was analyzed on 12% SDS-PAGE under denaturing conditions at 100V for 6 h. The gel was stained using Coomasie Brilliant Blue (CBB) dye. The compositions of different buffers used for SDS-PAGE are given in the annexure. The specific reactivity of the expressed VP2-cHSP70 fusion protein and VP2 protein alone was checked by Western blotting using rabbit anti IBDV-VP2 antibody. The resolved proteins from SDS PAGE was subsequently transferred to a nitrocellulose membrane using a semi dry blotting apparatus (Bio-Rad, USA) in 50 nm Tris base, 380 mM glycine, 0.1% SDS, 20% methanol at 100 mA constant current for 3 h. Successful transfer of the protein to the membrane was confirmed by staining the membrane with CBB stain and subsequent destaining. The unbound surface of the membrane was blocked overnight with 2% BSA in PBS, at 40 C. Following washing withPBS-Tween-20 (0.05%) 3 times for ten minutes, primary antibodies (1:200) was added to the membrane and incubated at 37˚C for 5 h. It was washed again with PBS-Tween-20 (0.05%) 3 times for ten minutes. Now, the membrane was treated with 1:2000 diluted HRPO conjugated goat anti-rabbit IgG and incubated at 37˚C for 1 h. The membrane was washed 3 times with PBS-Tween20. Then, prepared a solution containing DAB (Diaminobenzidine) substrate (1 mg/ml) mixed with 30µl H2O2. Further the membrane was than soaked in the solution prepared and keep for sometimes till the colour developed. Once the colour

develop remove the solution and semi dry the blot paper, photographed or preserved for future use.

3.8. Immunization of experimental chicken with DNA vaccine

3.8.1. Endotoxin-free plasmid purification

Purification of the constructed pCIVP2 and pCIVP2-cHSP70 plasmid was performed with EndoFree Plasmid Mega Kit (Qiagen). Single colony of E. coli cells carrying recombinant plasmid, was inoculated into a starter culture of 20 ml LB containing ampicillin (50 µg/ml). The inoculum was incubated at 370C in an orbital shaker (250 rpm) overnight. This starter culture was diluted to 1: 500 into 1500 ml fresh selective LB medium and incubated at 370C for 12- 16 h with vigorous shaking (~250 rpm). The bacterial cells were harvested by centrifugation at 6000 x g for 10 min at 40C. Pellet was resuspended in 10 ml of solution P1 until no clumps were visible. Buffer P2 (10 ml) was added and mixed thoroughly by inverting the sealed tube and kept for 5 minutes at room temperature the mixture turn intense blue colour. Chilled buffer P3 (10 ml) was added, mixed thoroughly until solution turn colorless then incubated on ice for 20 minutes. Cells lysate were centrifuged at 20,000 X g for 30 min. In the meantime, Qiagen Tip 500 was equilibrated with 10 ml of QBT solution. The supernatant was passed through column by gravity flow and washed two times with 30 ml of QC solution. After washing, plasmid DNA was eluted with 15 ml of QF solution in 50 ml tube. Isopropanol (10.5 ml) at room temperature was added and centrifuged at 15,000 g for 30 minutes. The DNA pellet was washed with 70% ethanol after centrifugation at 15000 g for 10 min. The pellet was air dried and dissolved in 100 µl of nuclease free water. Concentration of the recombinant plasmids was checked at A260/280 nm.

3.8.2. Specific pathogen free chickens

Specific pathogen free eggs were obtained from SPF unit Venky's India Limited, Pune and hatched at CARI, Izatnagar. All chickens were housed in separate poultry isolation chambers with ad libitum access to feed and water. The animal experiment was carried out under the approved DBT project and the approval of the institute animal ethics committee.

3.8.3. Immunization trial

A total of 60 birds at 14 days of age were distributed into six groups with 10 birds in each group. The birds were randomly divided into the groups namely Vector control, Control (challenged with vvIBDV), Control (unchallenged), pCIVP2 DNA, pCIVP2-cHSP70 and pCIVP2-SVPVP2 (DNA prime-protein boost). The chickens in pCIVP2 DNA and pCIVP2-cHSP70 were immunized twice at 14 days interval with 100µg of VP2 encoding DNA vaccine and c-terminal of HSP70 linked with IBDV-VP2 (pCIVP2-cHSP70) respectively. The birds in the prime- protein boost group were first immunized with pCIVP2 (100µg/bird) followed by boosting with 50µg of SVPVP2 protein. Vector control birds were immunized with pCI empty vector (100 µg) while control challenged group bird received phosphate buffer saline (PBS). All the groups received the injections intramuscularly.

Table 2 : Immunization schedule for SPF birds

Groups	1st Immunization (14th day)	Booster (28th day)
pCIVP2-cHSP70(10)	100 µg plasmid/bird	100 µg plasmid/bird
pCIVP2-SVPVP2(10)	100 µg plasmid/bird	50 µg SVPVP2 VLP/bird
pCIVP2(10)	100 µg plasmid/bird	100 µg plasmid/bird
pCI vector control (10)	100 µg plasmid/bird	100 µg plasmid/bird
Control challenged (10)	100 µl PBS /bird	100 µl PBS /bird
Control unchallenged (10)	100 µl PBS /bird	100 µl PBS /bird

3.8.4. Evaluation of humoral and cellular immune response

3.8.4.1. Enzyme linked immunesorbent assay (ELISA)

Antibody levels in pre-and post-immunization serum samples were quantified by Enzyme Linked Immunosorbent Assay (ELISA). The serum samples from immunized and control group birds (5 birds/group) were collected at 0, 7, 14, 21 and 28 days post immunization (d.p.i) and tested for IBDV VP2 specific antibody. The assay was performed as described by Dey *et al*., 2009. Briefly, the 96 well ELISA plate (flat bottom) were coated with recombinant VP2 antigen diluted in the coating buffer (100mM bicarbonate buffer, pH 9.0) at 4º C overnight and blocked with 2% bovine serum albumin (BSA). The sera samples were diluted at 1:100 were added on a plate and incubated at 37º C for 1 hour. The relative antibody titer was calculated by sample to positive (S/P) ratio and end point

titer were determined as described previously (Dey *et al*, 2009). Serum samples with end point titer more than 500 were considered positive.

3.8.4.2. Real Time PCR based assay for detection of cytokine gene expression

3.8.4.2.1. RNA isolation from peripheral blood mononuclear cells(PBMC)

Total RNA was isolated from PBMC using Trizol reagent (Invitrogen, USA), according to the manufacturer's instructions at 28 day post immunization. Briefly the steps are follows:

1. Washed PBMC samples which were collected from 3ml of blood were mixed with 1 ml Trizol reagent and mixed properly, incubated in room temperature for 10-15 minutes.
2. 200µl chloroform was added with and mixed vigorously for 10-15 seconds and then kept on ice for 15 minutes.
3. Phase separation: Centrifuge the mixed samples at 12000g for 15minute in 4˚C for phase separation.
4. RNA precipitation: The aqueous colourless phase of the solution was collected into a new DEPC treated eppendorf tube and to that 0.5 ml of isopropanol was added to precipitate the RNA from the aqueous phase by centrifugation at12000g for10 minutes.
5. RNA washing: After precipitation, the RNA was washed with 1 ml of 70% ethanol by centrifugation at 12000g for 10 minutes.
6. Drying: After washing precipitated RNA was air dried and then dissolved in 20µl DEPC treated water and incubated at 37˚C for 15 minutes.
7. The quality and quantity of the samples are checked using spectrophotometer (Eppendorf) by taking the reading at OD260 and OD280 against DEPC treated water as blank. The RNA samples that showed the OD260/OD 280 =2 were free of protein contamination.

3.8.4.2.3. cDNA preparation

First strand of DNA was synthesized from RNA using random hexamer primer and reverse transcriptase in a 20µl reaction mixture as follows:

Component	Volume
Total RNA (1µg)	5µl
Random hexamer primer	1µl
10mM dNTP mix	1µl
DEPC treated water	3µl

The above mixture was incubated at 65 ˚C for 5 minutes and then snap cooled on ice and then following reagents were added.

Component	Volume
10X RT Buffer	2µl
RNAse OUT	1µl
$MgCl_2$	4µl
DTT	2µl
Super scriptIII (SSIII)	1µl

Then the tube was incubated at 25˚C for 10 minute followed by 50˚C for 50 minute and subsequently 85˚C for 5 minute. After the reaction was over RNaseH need to add with the mixture and incubated at 37˚C for 20 minute.

3.8.4.2.4. Cytokine response by real time PCR

Peripheral blood mononuclear cells (PBMCs) was isolated for the extraction of total RNA using Trizol method. After estimation of RNA quantity and quality, 1µg of RNA was used for cDNA synthesis using superscript first strand synthesis kit (Invitrogen). Subsequently cDNA was amplified by PCR with chicken specific primers for IL10, IFNγ, IL12 (Table. 3) in CFX 96 Real Time System (Bio-Rad, CA, USA). The amplification reaction was performed in 20 µl mixture consisting of 1µl cDNA, 10 µl of SYBR Green Master Mix (Qiagen, CA, USA), primers 0.5 µl each (10 pm/µl). All the reaction were performed in triplicate and Ct value were normalized to GAPDH. Fold change (ΔΔCt) of vaccinated group was measured with respect to control group.

3.8.4.3. Lymphocyte transformation assay

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation of heparinized peripheral blood samples obtained from chickens at 28 d.p.i. as previously reported (Kaiser *et al*.,2006). PBMC were stimulated with either concanavalin A (5µg/ml) as positive control, or purified SVPVP2 as the specific antigen

and only media as negative control and incubated at 37˚C in CO2 incubator for 72 hours. Proliferative response was measured by adding 10µl of MTT dye (Invitrogen) to each wells and incubated for 4 hours at 37˚C followed by 100µl of DMSO to dissolve the formazon crystal. The optical density (OD) of each well was measured at 590 nm. Stimulation index (SI) was calculated as the mean OD value of stimulated cells divided by the mean OD value of unstimulated control.

Table 3. Primers designed for real time PCR analysis

Gene	Forward	Reverse	Size	Ac. No.
GAPDH	TGCCATCACAGCCACACAGAAG	ACTTTCCCCACAGCCTTAGCAG	123	AF047874.1
IL-12P40	CGAAGTGAAGGAGTTCCCAGAT	GACCGTATCATTTGCCCATTG	123	AY262752.1
IFN-γ	AAGTCAAAGCCGCACATCAAAC	CTGGATTCTCAAGTCGTTCATCG	132	X99774.1
IL-10	GCTGAGGGTGAAGTTTGAGGAA	GAAGCGCAGCATCTCTGACA	142	AF000631.1

3.9. Determination of protection from challenge

Two weeks after the booster dose, birds from all the groups except control (unchallenged) group were intra occularly challenged with very virulent IBDV strain. Chickens were monitored carefully for the next 10 days for clinical signs and mortality was recorded. Ten days post challenge, the birds were sacrificed and collected bursa from respective birds were weighed. Bursa to body weight, bursal lesion score were calculated and presented in Table. 4. Protection against challenge was assessed by studying the occurrence of mortality in susceptible chickens, presence of viral antigen in bursa four days post challenge (determined by AGPT), bursal gross lesion and bursa to body weight ratio (bursa/body weight B/B%) ten days post challenge. Histopathological examination was done to confirm the status of protection. Clinical signs and mortality were observed daily for 10 days post-challenge. The dead and surviving chickens were sacrificed and Bursa of Fabricious was collected for gross and histological examination. After haematoxylin and eosin (H&E) staining, the tissue were examined at Division of Pathology, IVRI. The bursa form all the groups were examined histiologically to determine the severity of bursal infection. The bursal lesion were graded according to a scale of 0 to 4: 0, no lesions ; 1, mild scattered cell depletion in a few follicles; 2,moderate with 1/3 -1/2 of the

follicle atrophied or with depleted cells; 3, diffuse with atrophy in all follicle; and acute inflammation and acute necrosis, typical of IBDV infection.

3.10. Statistical analysis

All data was presented as the mean± standard error (S.E). Tukey test was employed to evaluate the statistical differences among groups with SAS software. A value of $P < 0.05$ was considered as significant. Relations between the groups were determined by Tukey test.

4 Results

4.1. Development of VP2-cHSP70 and VP2 DNA vaccine construct and its characterization

4.1.1.1. PCR amplification of VP2 and c- terminal HSP70 genes

VP2 gene (1338 bp) of very virulent IBDV strain was amplified from cDNA using the primers VP2 F (EcoRI) and VP2 R (BamHI) as mentioned in Table 1 and depicted in Fig. 1. C-terminal heat shock protein HSP70 was amplified from genomic DNA of *Mycobacterium tuberculosis* using primer HSP70 F (BamHI) and HSP70 R (NotI) primer as shown in Table 1.The amplified product was analysed by agarose gel electrophoresis and a single intense band of 778bp was observed (Fig. 2).

4.1.1.2. Development of VP2 and cHSP70 DNA construct

The amplified fragments, VP2 and HSP70 $_{359–610}$, were gel purified and digested with BamHI restriction enzyme separately. Digested products were again gel-purified and ligated by T4 DNA ligase (NEB, UK) to form VP2–HSP70 $_{359–610}$ fusion construct. The resulting construct was designated as VP2-cHSP70 which encoded the VP2–cHSP70 $_{359–610}$ protein in a single ORF. In this approach BamHI restriction site (GAATTC), encoding two extra amino acids of Gly and Ser separated two parts of the fusion construct. The VP2–cHSP70 $_{359–610}$ fusion construct was amplified using VP2 upstream and cHSP70 $_{359–610}$ downstream primers a PCR reaction. The amplified product was analyzed by agarose gel electrophoresis and 2111bp band was observed (Fig. 3).

4.1.1.3. Cloning of VP2 and VP2-cHSP70 gene in a pCI expression vector

Amplified VP2-cHSP70 and VP2 were purified from agarose gel and ligated to pCI cloning vector individually and transformed into DH5α cells. Following digestion with restriction enzyme using EcoRI and NotI of pCIVP2-cHSP70 released 2111 bp fragment (Fig. 4) and with EcoRI and BamHI digestion for pCIVP2 clones released 1338 bp fragment (Fig. 5).

4.1.1.4. Sequencing of DNA constructs

The positive recombinant clones from each group was further confirmed by DNA sequencing. Both strands of the clones were sequenced by primer walking and analysed using NCBI-BLASTN version 2.2.27+ programme. The DNA sequence of VP2 was found to be 100% identical to the designed sequence of VP2 as well as c-terminal HSP70 without any base substitution (Fig. 7b). Dendrogram showed the presence of BamHI restriction site (GAATTC) in between VP2 and cHSP70 fragment (Fig. 7a).

4.1.2. In vitro expression analysis

4.1.2.1. Indirect immunofluorescence assay

Plasmids pCIVP2 and pCIVP2-cHSP70 were constructed and in vitro expression of VP2 and VP2-cHSP70 fusion protein was confirmed by indirect fluorescence test (IFAT) using anti IBDV-VP2 antibody raised in rabbit. The intensive fluorescence seen in the cells transfected with pCIVP2 and pCIVP2-cHSP70 plasmid indicated the successful expression of VP2 and VP2-cHSP70 protein. No fluorescence was found in pCI empty vector transfected cells (Fig. 8).

4.1.2.2. Western Blot analysis

The expression of VP2 protein and VP2-cHSP70 fusion protein was also confirmed by western blot analysis. The expected bands of 44kDa (Fig. 9a) and 72kDa (Fig. 9b) were observed in the lysates of cells transfected with pCIVP2 and pCIVP2-cHSP70 plasmid respectively.

4.2. Immunization studies

4.2.1 Isolation of vaccine grade endotoxin free plasmid DNA and quantification

Plasmid DNA was isolated in bulk using QIAGEN endofree maxi kit. The integrity of plasmid was checked by agarose gel electrophoresis. Further, the plasmid DNA was also checked for its purity and concentration, spectrophotometrically by taking OD at 260/280 and the total yield of plasmid DNA was calculated to be 350 to 400 µg from 250 ml bacterial cultures. The ratio of OD 260/OD 280 was 1.8, indicating the DNA preparation was pure and free from RNA and protein contamination. The endotoxin free DNA was used to immunize SPF chickens as per the protocol shown in Table 2.

4.2.1. Titration of the challenge virus

Very virulent IBDV strain was maintained in Recombinant DNA Laboratory, Indian Veterinary Research Institute, India. The virus was grown in 11 days old specific pathogen free (SPF) embryonated chicken eggs through CAM route and titre was calculated by Reed and Muench method. For challenge studies, 10^5 ELD_{50} virus was used.

4.2.2. Evaluation of humoral immune response

Antibody titre to IBDV was determined in serum samples collected from birds from each group at 0, 7, 14, 21 and 28 day d.p.i (Fig. 10). After 14 d.p.i. birds immunized with pCIVP2-cHSP70 construct showed detectable antibody titre. However, antibody titre of pCIVP2-cHSP70 group birds was highest in 28 d.p.i. The antibody titre of pCIVP2-cHSP70, pCIVP2- SVPVP2 groups showed significantly higher titre as compared to pCIVP2 vaccinated group ($p<0.01$). It also observed that there was no significant antibody titre difference between pCIVP2-cHSP70 and pCIVP2-SVPVP2 groups. The antibody titre of vector control and control groups were undetectable throughout the study period.

4.2.3. Evaluation of cell mediated immune responses

4.2.3.1. Cytokine expression analysis

Relative expression patterns of IL-10, IL-12p40, IFNγ genes in chicken lymphocytes at 14^{th} days post booster for different plasmid constructs are illustrated in (Fig.11) The expression of IL12 and IFNγ was significantly higher in pCIVP2-cHSP70 immunized group compared to pCIVP2- SVPVP2 and pCIVP2 groups. In contrast the expression level of IL10 in pCIVP2- SVPVP2 group was significantly higher than pCIVP2-cHSP70 vaccinated group ($p<0.01$).

4.2.3.2. Lymphocyte proliferation response

Lymphocyte proliferation responses for the determination of cell mediated immune response of the immunized and control birds was analyzed at 28 days d.p.i. and schematically represented at Fig.12a & 12b. Proliferative response are measured by stimulation index (SI). pCIVP2-cHSP70 and pCIVP2- SVPVP2 groups SI was significantly higher than the pCIVP2 and control group ($P<0.05$) as well as there is no significant

differences between pCIVP2-cHSP70 , pCIVP2- SVPVP2 group while the stimulation index of pCIVP2 was significantly different when compared to vector control and control groups.

4.3. Challenge and protection study

After challenge, chickens in the unvaccinated and unchallenged control group remained healthy and exhibited normal sized bursae, whereas the control challenged group showed typical clinical signs with 90% mortality and severe hemorrhagic bursa. Histopathological lesions were examined (Fig.13). Out of 10 chickens challenged with vvIBDV, 10 chickens died in vector control and control challenged groups. In contrast, chickens in pCIVP2-cHSP70, pCIVP2– SVPVP2 vaccinated and control unchallenged groups were completely protected from mortality against vvIBDV challenge. However, 3 birds died in pCIVP2 vaccinated group. The chickens in the pCIVP2-cHSP70 or pCIVP2-SVPVP2 group had higher B/B ratios and lower bursal lesion scores compared to pCIVP2 vaccinated group (Table 4).

Plate- I

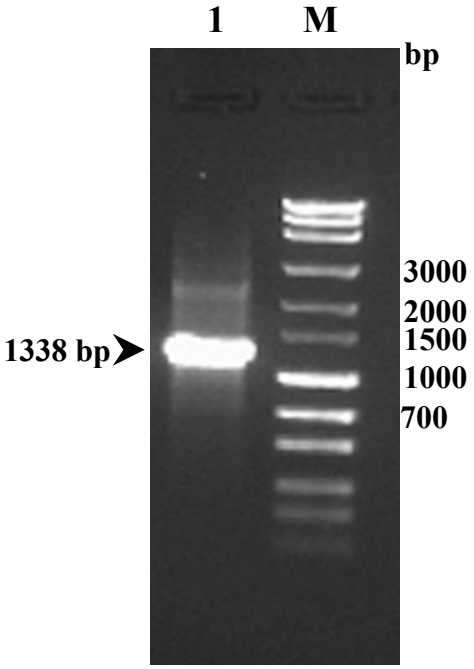

Fig. 1 : PCR amplification of full length VP2 gene of IBDV
Lane 1 : PCR amplicon of VP2 (1338 bp)
Lane M : MassRuler Express Forward DNA marker (MBI Fermentas)

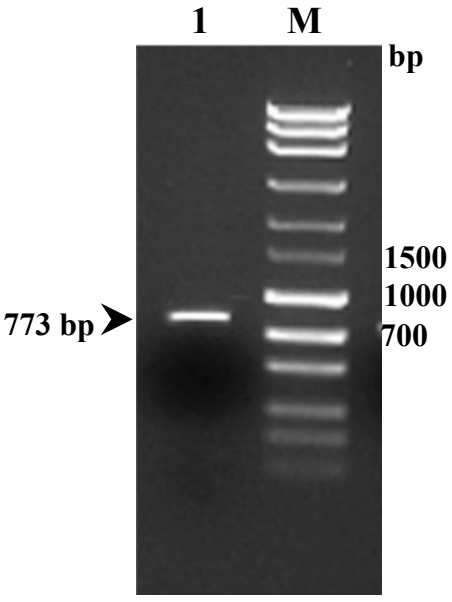

Fig. 2 : PCR amplification of c-terminal HSP70 (cHSP70) gene of *Mycobacterium tuberculosis*
Lane 1 : PCR amplicon of cHSP70 (773 bp)
Lane M : MassRuler Express Forward DNA marker (MBI Fermentas)

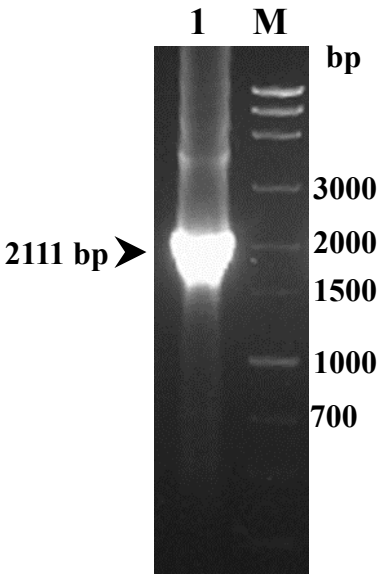

Fig. 3 : PCR amplified fusion VP2-cHSP70 construct
Lane 1 : Fusion construct of VP2-cHSP70 (2111 bp)
Lane M : MassRuler Express Forward DNA marker (MBI Fermentas)

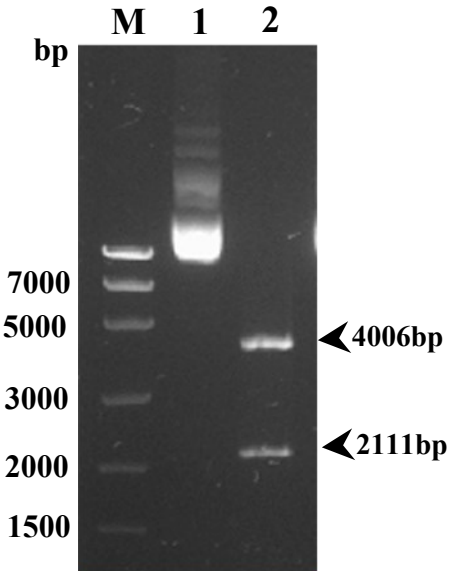

Fig. 4 : Confirmation of VP2-cHSP70 DNA construct
Lane 1 : Uncut pCIVP2-cHSP70 construct
Lane 2 : pCIVP2-cHSP70 construct following digestion with EcoRI and NotI released an insert size 2111 bp and pCI vector back bone (4006 bp)
Lane M : Mass Ruler Express Forward DNA marker (MBI Fermentas)

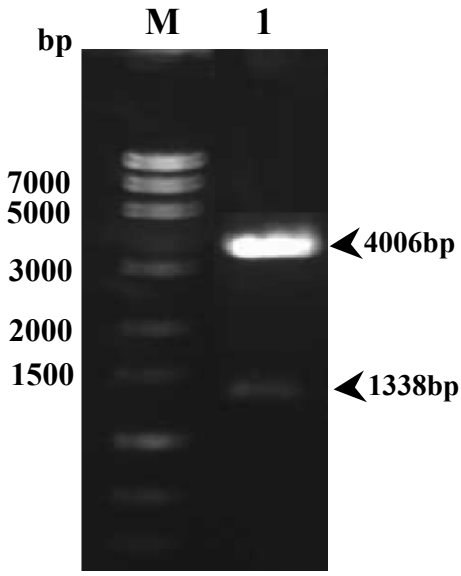

Fig. 5 : Confirmation of pCIVP2 DNA construct by restriction enzyme digestion
Lane 1 : pCIVP2 construct following digestion with EcoRI and BamHI release an insert size 1338 bp and pCI vector backbone (4006 bp)
Lane M : Mass Ruler Express Forward DNA marker (MBI Fermentas)

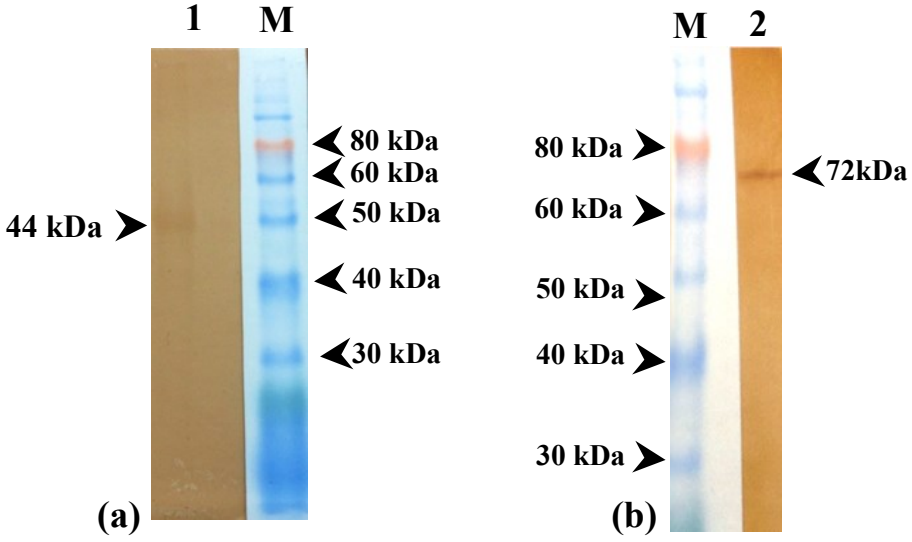

Fig. 9 a & b : Western blot analysis of the expressed (a)full length VP2 protein and (b) VP2-cHSP70 fusion protein from vero cells
Lane 1 : Expressed VP2 protein (44 kDa)
Lane 2 : Expression of fused VP2-cHSP70 protein (72 kDa)
Lane M : Colourplus prestained protein ladder (10-230 kDa,NEB)

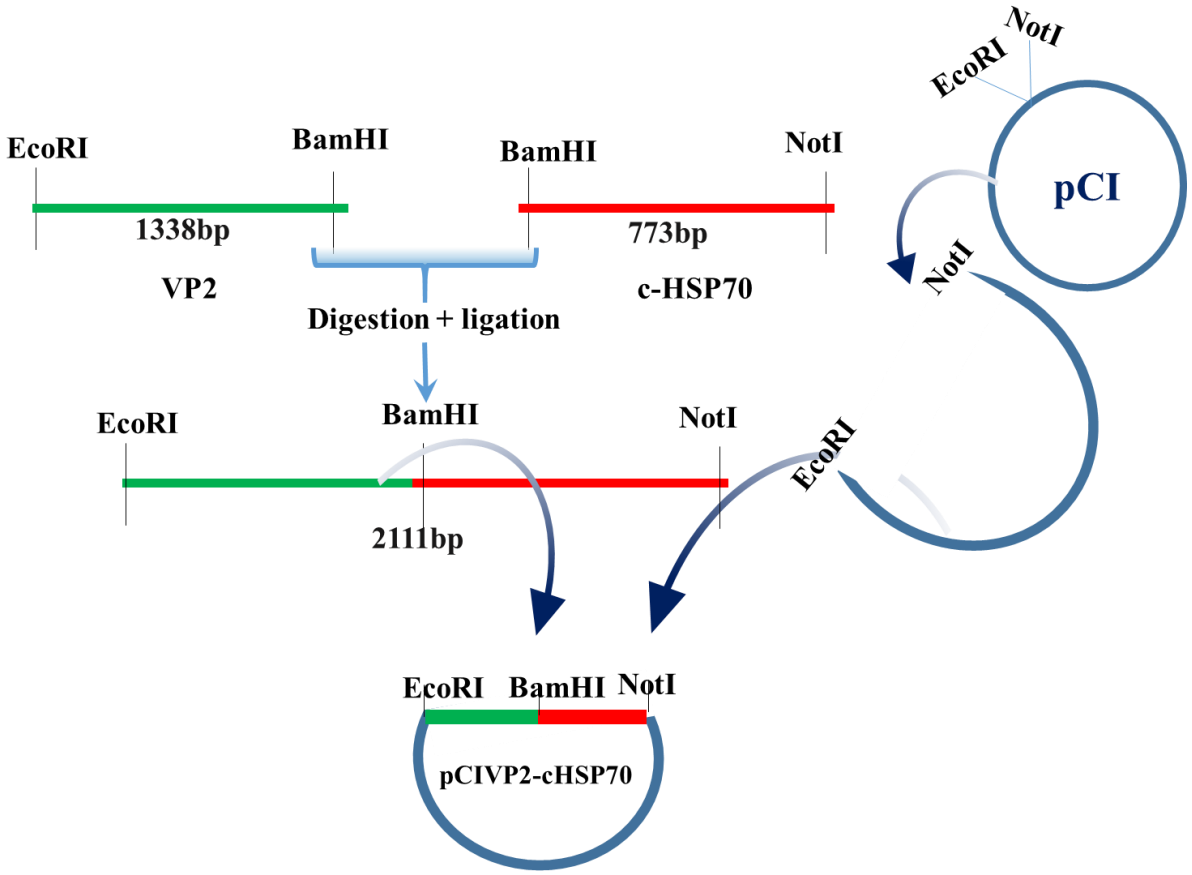

Fig 6: Strategy to construct the genetically linked VP2 and cHSP70 gene in pCI vector

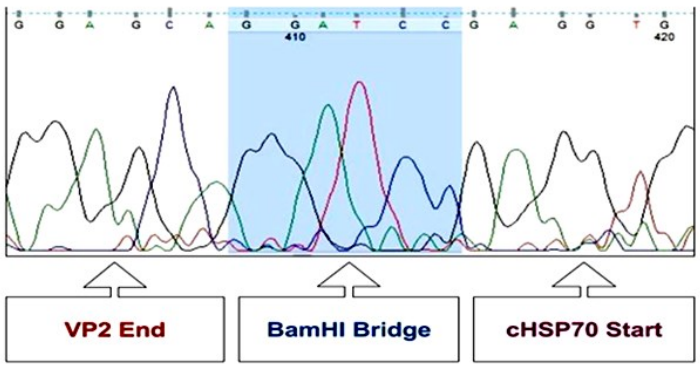

Fig 7a) Confirmation of pCIVP2-cHSP70 fusion gene by sequencing: BamHI (GGATCC) site linking the VP2 and cHSP70 construct.

Plate- V

GCG*GAATTC*GCCACCATGACAAACCTGCAAGATCAAACCCAACAGATTGTTCCGTTCAC-
GGGAGCCTTCTGATGCCAACAACCGGACCGGCGTCCATTCCGGACGACACCCTAGAGAAGCACA
CTCTCAGGTCAGAGACCTCGACCTACAATTTGACTGTGGGGGACACAGGGTCAGGGCTAATTGT
CTTTTTCCCTGGCTTCCCTGGCTCAATTGTGGGTGCTCACTACACACTGCAGAGCAATGGGAACT
ACAAGTTCGATCAGATGCTCCTGACTGCCCAGAACCTACCGGCCAGCTACAACTACTGCAGGCT
AGTGAGTCGGAGTCTCACAGTGAGGTCAAGCACACTCCCTGGTGGCGTTTATGCACTAAATGGC
ACCATAAACGCCGTGACCTTCCAAGGAAGCCTGAGTGAACTGACAGATGTTAGCTACAATGGGT
TGATGTCTGCAACAGCCAACATCAACGACAAAATCGGGAACGTCCTAGTAGGGGAAGGGGTAA
CCGTCCTCAGCTTACCCACATCATATGATCTTGGGTATGTGAGACTCGGTGACCCCATTCCCGCT
ATAGGGCTCGACCCAAAAATGGTAGCAACATGTGACAGCAGTGACAGGCCCAGAGTCTACACC
ATAACTGCAGCCGACGATTACCAATTCTCATCACAGTACCAAGCAGGTGGGGTAACAATCACAC
TGTTCTCAGCTAATATCGATGCCATCACAAGCCTCAGCATCGGGGGAGAACTCGTGTTTCAAACA
AGCGTCCAAGGCCTTATACTGGGTGCTACCATCTACCTTATAGGCTTTGATGGGACTGCGGTAAT
CACCAGAGCTGTGGCCGCAGACAATGGGCTGACGGCCGGCACTGACAACCTTATGCCATTCAAT
ATTGTGATTCCAACCAGCGAGATAACCCAGCCAATCACATCCATCAAACTGGAGATAGTAACCT
CCAAAAGTGGTGGTCAGGCGGGGGATCAGATGTCATGGTCAGCAAGTGGGAGCCTAGCAGTGA
CGATCCACGGTGGCAACTATCCAGGGGCCCTCCGTCCCGTCACACTAGTAGCCTACGAAAGAGT
GGCAACAGGATCTGTCGTTACGGTCGCCGGGGTGAGCAACTTCGAGCTGATCCCAAATCCTGAA
CTAGCAAAGAACCTGGTCACAGAATACGGCCGATTTGACCCAGGAGCCATGAACTACACAAAAT
TGATACTGAGTGAGAGGGACCGTCTTGGCATCAAGACCGTATGGCCAACAAGGGAGTACACTGA
CTTTCGCGAGTACTTCATGGAGGTGGCCGACCTCAACTCTCCCC**TGAAGATTGCAGGAGCA*GGA***
***TCC*GAGGTGAAAGACGTTC**TGCTGCTTGATGTTACCCCGCTGAGCCTGGGTATCGAGACCAAGGG
CGGGGTGATGACCAGGCTCATCGAGCGCAACACCACGATCCCCACCAAGCGGTCGGAGTCTTTC
ACCACCGCCGACGACAACCAACCGTCGGTGCAGATCCAGGTCTATCAGGGGGAGCGTGAGATCG
CCGCGCACAACAAGTTGCTCGGGTCCTTCGAGCTGACCGGCATCCCGCCGGCGCCGCGGGGGAT
TCCGCAGATCGAGGTCACTTTCGACATCGACGCCAACGGCATTGTGCACGTCACCGCCAAGGAC
AAGGGCACCGGCAAGGAGAACACGATCCGAATCCAGGAAGGCTCGGGCCTGTCCAAGGAAGAC
ATTGACCGCATGATCAAGGACGCCGAAGCGCACGCCGAGGAGGATCGCAAGCGTCGCGAGGAG
GCCGATGTTCGTAATCAAGCCGAGACATTGGTCTACCAGACGGAGAAGTTCGTCAAAGAACAGC
GTGAGGCCGAGGGTGGTTCGAAGGTACCTGAAGACACGCTGAACAAGGTTGATGCCGCGGTGG
CGGAAGCGAAGGCGGCACTTGGCGGATCGGATATTTCGGCCATCAAGTCGGCGATGGAGAAGCT
GGGCCAGGAGTCGCAGGCTCTGGGGCAAGCGATCTACGAAGCAGCTCAGGCTGCGTCACAGGC
CACTGGCGCTGCCCACCCCGGCGGCGAGCCGGGCGGTGCCCAC**CCCGGCTCGGCTGATTGA*GCG***
GCCGC

Fig 7b) Sequence of full length VP2 genetically linked with c-terminal of HSP70 gene with BamHI restriction enzyme is linking the two genes

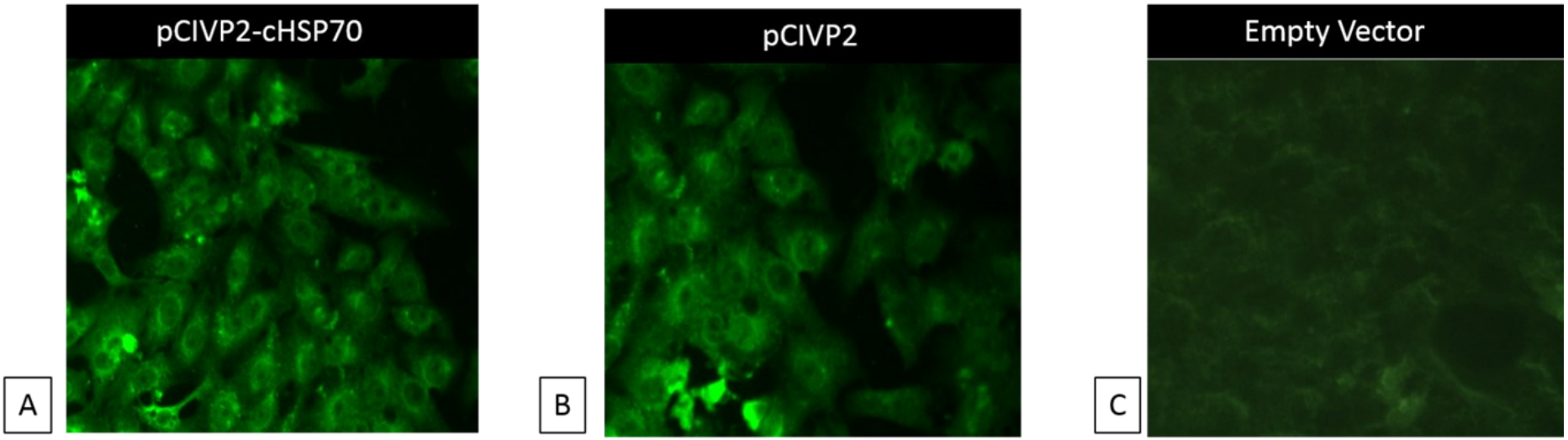

Fig. 8. Indirect immunofluorescence assay of the expressed VP2 protein and fused VP2-cHSP70 protein in transfected vero cells. (A) pCIVP2-cHSP70 transfected vero cells; (B) pCIVP2 transfected vero cells; (C) pCI empty vector transfected vero cells.

Plate- VII

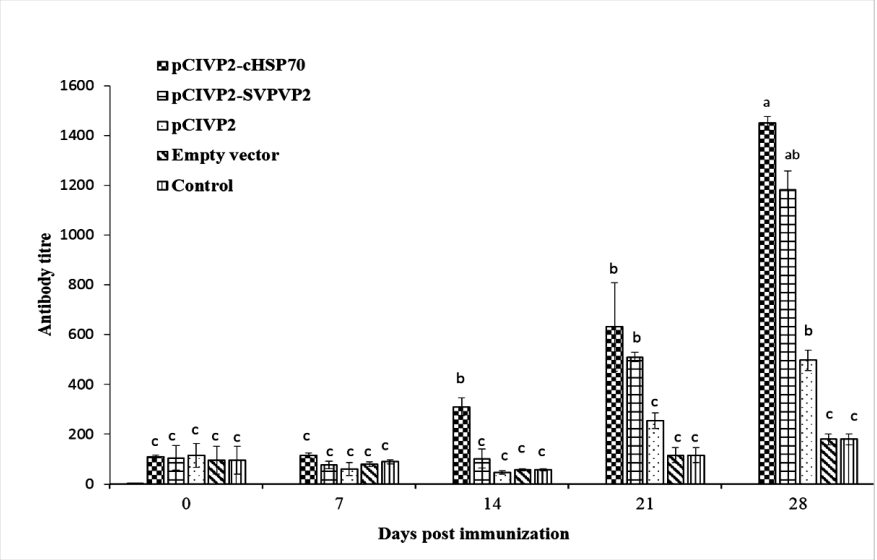

Fig:10. Antibody responses of chickens in different groups measured by ELISA. Chickens were immunized twice at 2-week intervals with pCIVP2-cHSP70, pCIVP2–SVPVP2 and pCIVP2 and pCI empty vector. The serum samples from immunized and control group birds (5 birds/group) were collected at 0, 7, 14, 21 and 28 days post immunization (d.p.i) and tested for IBDV VP2 specific antibody. The antibody titers higher than 500 were considered positive for IBDV antibody. Data represent the means ± standard error. pCIVP2-cHSP70, pCIVP2–SVPVP2 group showed significantly higher ELISA titers than pCIVP2 ($P < 0.01$) after the booster immunization. Level not connected by same letter are significantly different ($p<0.01$).

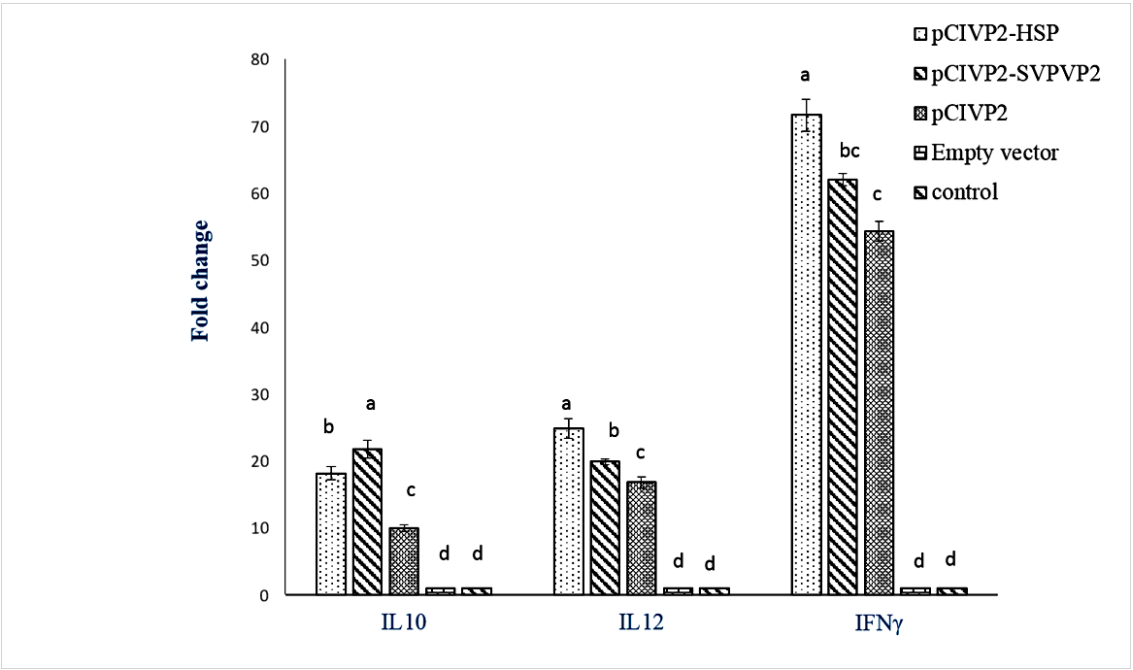

Fig:11. Production of different cytokines by PBMCs from immunized birds groups on 28 days post immunization. Transcription level of each gene was assessed by quantitative real time PCR (RT-PCR) and normalized to that of GAPDH. Relative quantities of mRNA evaluated by using the $2^{-\Delta\Delta Ct}$ method and relative fold change of each cytokine expression between immunized and control challenge group. Data are represented as mean value from three independent experiment in triplicate and error bar represent the standard errors. Levels not connected by same letter are significantly different ($p< 0.01$).

Plate- VIII

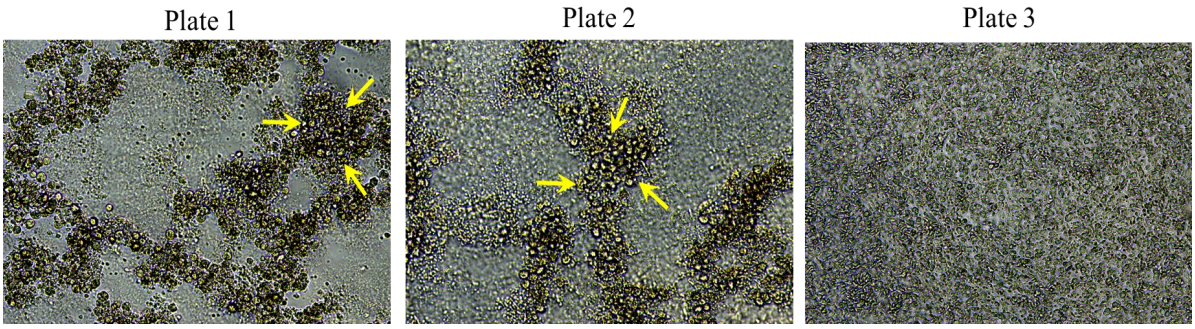

Fig. 12a : Lymphocyte proliferation assay in chicken PBMCs . Plate 1 : ConA stimulated PBMCs , plate 2 : SVPVP2 stimulated PBMCs plate 3 : Unstimulated PBMCs (Control). Arrow indicates the proliferation of lymphocytes.

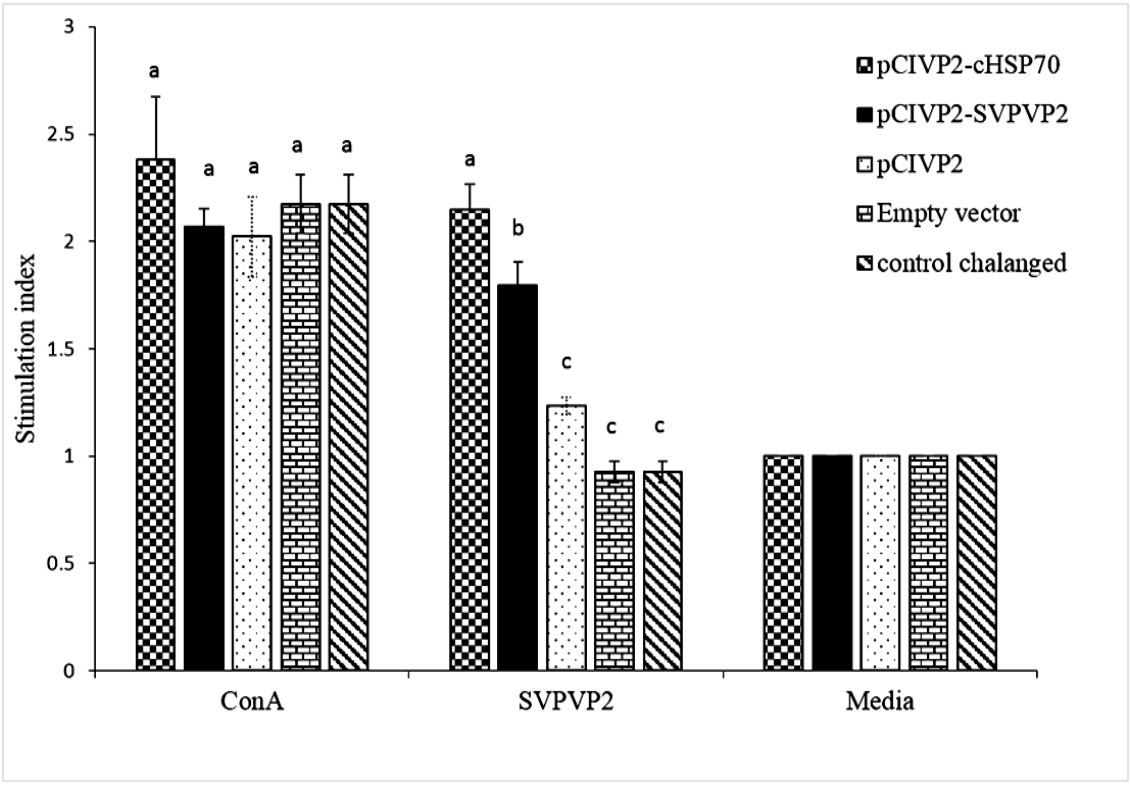

Fig:12 b. Lymphocyte proliferative response in chickens on 28 days post infection. Chicken lymphocytes from immunized and control groups were isolated and stimulated with recombinant VP2 antigen, concanvalin A (conA) as positive control and M199 media as negative control. Lymphocyte proliferative response was measured and expressed as stimulation index. All the data presented as mean value ± standard error. Level not connected by same letter are significantly different ($p<0.05$).

Plate- IX

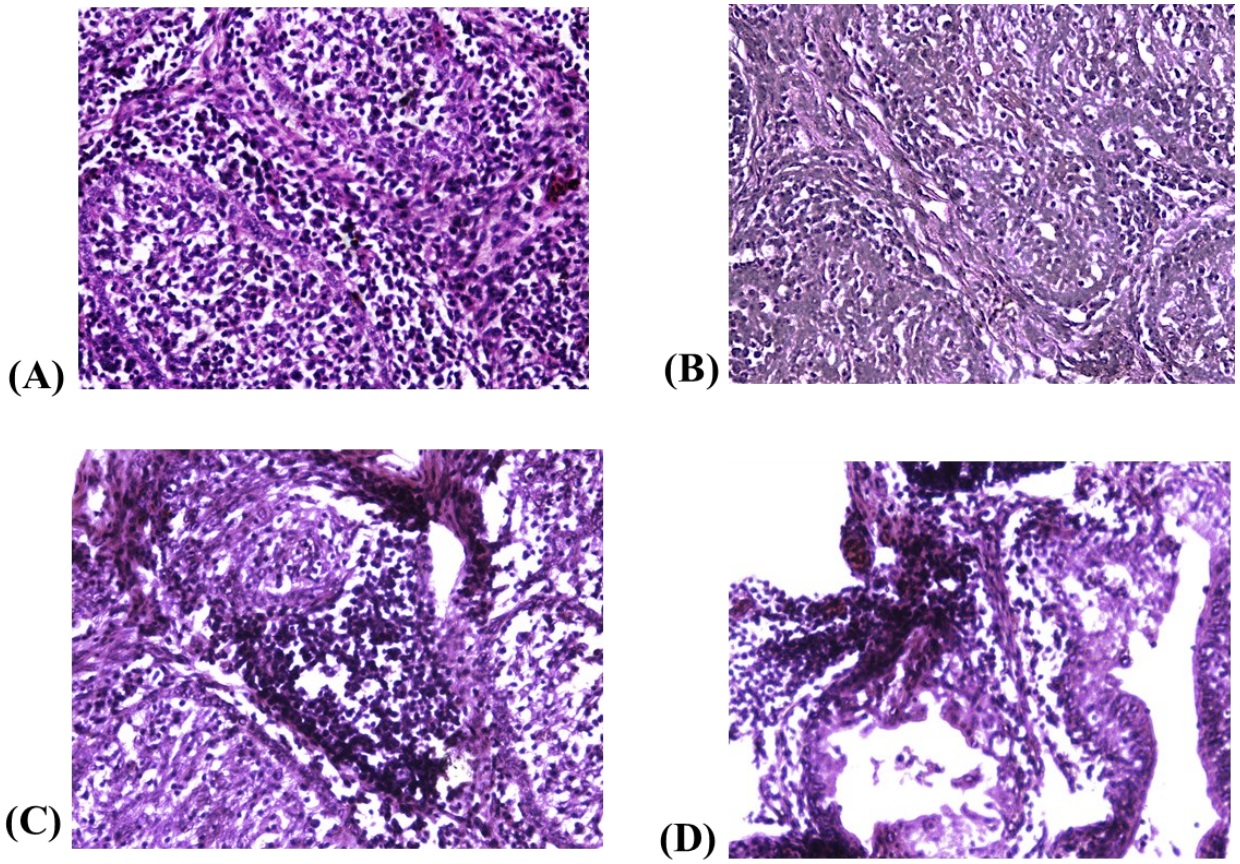

Fig: 13. Histopathology of bursa of fabricius follicle collected from different groups;
A– Normal follicular structure (Control unchallenged)

B– Intact follicles with mild depletion of lymphoid cells in the follicles (pCIVP2-cHSP70)

C– Showing bursal atrophy with moderate depletion of lymphoid cells in the follicles (pCIVP2)

D– Showing necrosis and severe depletion of lymphoid cells in the follicles (pCI empty vector)

Table 4. Protection level in different groups of chickens after vvIBDV challenge

Groups[a]	No. of birds[b]	Mortality[c]	B /B ratio[d]	*BF lesion scored[e]					Protection percentage[f]
				0	1	2	3	4	
Control unchal-lenged	10	0	7.35 ± 0.096 a	10	0	0	0	0	10/10 =100
Control challenged	10	9	1.44 ± 0.156 c	0	0	1	4	4	1/10=10
Empty vector	10	9	1.34 ± 0.173 c	0	0	0	4	5	1/10=10
pCIVP2-cHSP70	10	0	7.96 ± 0.024 a	8	1	1	0	0	0/10=100
pCIVP2-SVPVP2	10	0	7.76 ± 0.042 a	9	0	1	0	0	0/10=100
pCIVP2	10	3	4.99 ± 0.167 b	5	1	1	0	3	7/10=70

[a]Chickens were vaccinated with pCIVP2-cHSP70, pCIVP2-SVPVP2, pCIVP2 and pCI empty vector vaccines. In addition, control challenged, unvaccinated and unchallenged control birds were kept for controls.

[b] Total number of birds taken in each groups.

[c]Mortality was presented as number of dead chickens/total number of chickens in each group.

[d]Bursal/body weight ratios were calculated by bursal weight divided by body weight × 1000 and presented as the mean ± standard deviation from each group. Values followed by different letters within the column are significantly different ($P < 0.05$).

[e]Bursal gross lesions were graded from 0 to 4 based on the severity of bursal involvement at time of euthanasia 0: no lesion, 1: mild scattered cell deletion in a few follicle, 2: scattered or partial bursal damage, 3: 1/2 - 1/3 of the follicle atrophied or depleted cells, 4: Diffuse with atrophy in all the follicle, acute inflammation and necrosis.

[f]Protection percent was determined as the number of protected chickens/total number of chickens in a group.

5 Discussion

Infectious bursal disease (IBD) retards the growth of the poultry industry through its repeated occurrence of outbreaks around the world. The economic impact of IBD is influenced by the virulence of the infecting strain, susceptibility of the flock, interference of primary and secondary pathogens as well as management factors. In classical field outbreaks, the mortality rate may range from 1 to 50% (Muller *et al.,* 2003). Higher mortality rates were observed in layer-type breeds than in broiler breeds following infection with virulent IBDV (van den Berg *et al*., 1991). It is well known that the IBD virus replicates in immature B lymphocytes, particularly in the cells of the BF . However, the studies of Rautenschlein *et al.,*(2002) revealed that functional T cells are needed to control IBDV-antigen load in the acute phase of infection.

5.1. Structure of IBDV

IBDV is a bi-segmented, double stranded, non-enveloped RNA virus. The genome of IBDV consist of two segments designated as segment A and segment B (Mundt *et al.,*1995). Genome segment A has two ORFs. Larger ORF 1 encodes for a 110kDa polyprotein which is later spliced into viral proteins VP2 (48kDa),VP3(33-35kDa),VP4 (24kDa). VP2 and VP3 protein form the outer and inner capsid of the virus respectively (Mahgoub *et al.,* 2012). The capsid protein, VP2 is the major host protective antigen of IBDV carrying the major immunogenic determinants (Fahey *et al.,* 1989). Various studies have been reported using full length VP2 gene for vaccine candidate development (Pradhan *et al.,* 2014; Taghavian *et al.,* 2013). Based on the studies we have chosen the full length VP2 gene for construction of a DNA vaccine candidate.

5.2. Conventional vaccines

IBDV is a highly infectious virus and very resistant to inactivation. Vaccination is the major tool for the prevention and control of IBD. In spite of intensive vaccination programs there are reports of outbreaks in the poultry industries around the world resulting in heavy economic losses. With the emergence of antigenic variant and very

virulent IBDV (vvIBDV) strains (Jackwood and Saif, 1987; Chettle *et al.,* 1989), chickens are not fully protected by traditional inactivated or attenuated vaccines even with high levels of maternal antibodies. In general, live vaccines possess unstable antigenic and pathogenic characters, and induce moderate bursal atrophy, leading to mild disease and immunosuppression. Moreover, intermediate vaccines are insufficient in providing adequate protection against vvIBDV, especially under conditions of high infection pressure. Hence vaccine protocols incorporating the use of intermediate plus vaccines may reduce mortality rates in the field from vvIBDV. Notably, while intermediate plus vaccines are capable of breaking through maternal antibody titers as high as 1:500, they can potentially induce bursal atrophy and immunosuppression (Wu *et al.,* 2012). In addition, conventional live vaccines can be inhibited by maternal antibody making the timing of vaccination difficult (Hsieh *et al.,* 2010). Therefore, it is necessary to develop safer and more efficacious vaccines to prevent IBDV infection.

5.3. DNA vaccines against infectious bursal disease (IBD)

DNA vaccines offer a number of distinctive advantages over conventional vaccines, including chemical and biological stability, simplicity of production, and ease of manipulation (Lewis and Babiuk *et al.,* 1999). DNA vaccines encoding IBDV antigenic protein VP2 has been reported (Negash *et al.,*2013). In this study we have also constructed a DNA vaccine encoding VP2 gene of IBDV to enhance the antigen specific humoral and cell mediated immunity. First we developed a construct encoding full length VP2 gene of IBDV in a mammalian expression vector (pCI) under CMV promoter designated as pCIVP2. Expression of pCIVP2 construct was confirmed by western blotting showing a band of 44 kDa and *in vitro* immunofluorescence assay after transiently transfected in vero cell line. The results confirmed with the other reports (Zhang *et al.,* 2010 and Li *et al,* 2013).

In our study the immunity induced by the DNA vaccines encoding VP2 gene alone has been insufficient to completely protect the chickens from challenge with vvIBDV. Seventy percent of the immunized chickens were protected against challenge with vvIBDV. The results supported the previous experiments which described that DNA vaccine encoding VP2 alone gives protection 50% to 80% against vvIBDV challenge (Fodor *et al.,* 1999; Li *et al.,* 2013). These reports suggest that DNA vaccine encoding VP2 gene alone is not sufficient to provide complete protection against vvIBDV

challenge and new strategies need to be formulated to improve the efficacy of DNA vaccines.

5.4. New strategies for improving DNA vaccine efficacy

A number of strategies have been developed for enhancing the immune response against DNA vaccine encoding VP2 gene of IBDV. DNA vaccine carrying VP234 gene of IBDV and interleukin18 (IL18) enhanced the immune response and protection efficacy (93%) against vvIBDV (Li *et al.,* 2013). In a previous study the IL18 enhanced the protection efficacy, highlighting the potential value of chicken IL18 as an adjuvant in the prevention of vvIBDV infection. Zhang *et al.,*(2010) reported complete protection with DNA vaccine encoding IBDV VP2 gene when fused with defensin (AvBD1) gene and 80% protection was obtained by immunizing with DNA vaccine encoding VP2 gene alone. Thus, several reports have confirmed that partial protection is obtained when chickens are immunized with DNA vaccine encoding IBDV-VP2 gene alone but complete protection could be obtained when immunized together with cytokines or cytosine-phoshphate-guanine(CpG) motif (Hulse & Romero., 2004). This proves the importance of using an adjuvant to enhance the immunogenicity of VP2-DNA vaccine. These reports encouraged us to choose a potent adjuvant which can enhance the antigen specific humoral and cell mediated immunity as well as giving complete protection against vvIBDV challenge.

Previous study also showed that a prime-boost approach, where chickens first primed with DNA vaccine and boosting with killed vaccine or recombinant protein can adequately protect chickens against challenge with IBDV (Hsieh *et al.,* 2007; Gao *et al.,* 2013). Based on the above study, the immune responses and protection efficacy of three different vaccination strategies, namely pCIVP2 DNA vaccine , DNA vaccine containing fusion construct of VP2 gene of IBDV and c-terminal HSP70 gene of *Mycobacterium tuberculosis* (pCIVP2-cHSP70) and 'DNA prime-protein boost' were evaluated for immune response and percentage of protection against vvIBDV.

5.5. HSP70 as potent adjuvant

HSP70 has been described to possess a number of immunomodulatory properties and are being investigated as adjuvant components in vaccine development. HSP70 can be translocated across cell membrane to gain cytoplasmic and nuclear entry. This property

may enable HSP70 to deliver peptide into endoplasmic reticulum and MHC class I pathway. In addition, a CD91 receptor-mediated endocytic pathway was found to allow HSP70-bound peptides or proteins to enter MHC class I and class II processing pathway (Basu *et al.,* 2001; Jiang *et al.,* 2013). However, the ubiquitous presence of HSP70 in eukaryotic and prokaryotic cells, combined with its high degree of sequence homology and intrinsic immunogenicity, have raised concerns about possibility of HSP70s to induce autoimmune responses. Although the previous studies revealed that C-terminal $HSP70_{359–610}$ enhanced HBsAg-specific immunity without producing antibody against the complete HSP70 molecule, while the complete HSP70 or N-terminal $HSP70_{1–360}$ resulted strong anti-HSP70 humoral responses. These results indicated that $HSP70_{359–610}$ might be a superior adjuvant without the potential risk of autoimmunity (Li *et al.,* 2006). Based on these studies we designed a genetically linked VP2 gene with cHSP70 namely pCIVP2-cHSP70 and studied its efficacy as a DNA vaccine candidate against vvIBDV.

5.6. In vitro expression of DNA vaccine constructs

In this study, we constructed a fusion of c-terminal $HSP70_{359–610}$ gene of *Mycobacterium tuberculosis* to VP2 gene of IBDV to enhance the antigen specific humoral and cell mediated immunity. The pCIVP2-cHSP70 construct was expressed under CMV promoter of pCI vector and the expression was confirmed by Western blotting and immunofluorescence assay. The intensive fluorescence seen in the vero cells transfected with pCIVP2-cHSP70 construct indicated that the VP2 DNA vaccine was correctly constructed and expressed. The lysates of cells transfected with pCIVP2-cHSP70 showed the band of 72kDa using in western blotting.

5.7. Evaluation of DNA vaccine constructs in (Specific pathogen free) SPF chickens

We have compared three types of vaccines namely, pCIVP2 DNA vaccine, DNA vaccine containing fusion construct of VP2 gene of IBDV and c-terminal HSP70 gene of *Mycobacterium tuberculosis* (pCIVP2-cHSP70) and 'DNA prime-protein boost' vaccine and the immune responses from these vaccines and their protection efficacy against vvIBDV was studied. Humoral response was measured by ELISA and cell mediated immune response was measured by lymphocyte proliferation assay and cytokine expression assay.

5.7.1. Antibody responses in immunized chickens by ELISA

Neutralizing antibody plays a predominant role in inactivation of virus particles (Fahey *et al.,* 1989). In this study, we measured the antibody titer before challenge continuously from 0 days to 28 days of post vaccination and chickens immunized with pCIVP2-cHSP70 and prime-boost vaccine showed significantly higher levels of antibody titer as compared to pCIVP2 group. The adjuvant like activity of c-terminal HSP70 as reported by Li *et al.,* (2006) might be responsible for enhancing the humoral immune response against IBDV in pCIVP2-cHSP70 vaccinated group. The 'DNA prime-protein boost' vaccination approach in our study has also showed significant humoral immune response compared to pCIVP2 DNA vaccine alone and supports the previous study reported by Gao *et al.,* 2013.

5.7.2. Lymphocyte proliferation response

In IBDV infection, T cells were critically involved in protection and antibody alone is not sufficient in inducing protection in chicken (Sharma *et al.,* 2000). An ideal vaccine should induce both cellular and humoral immune responses to achieve complete protection. In our study, we evaluated the cellular immune response by lymphocyte proliferation response and cytokine assay. The pCIVP2-cHSP70 group induced significantly higher levels of specific lymphocyte proliferative response compared to prime-boost and pCIVP2 groups, when stimulated with SVPVP2 protein. The results confirmed cHSP70 assisted in the augmentation of antigen specific lymphocyte proliferation response and corroborated the results of (Ebrahimi *et al.,* 2012; Fu *et al.,* 2013)

5.7.4. Real time quantification of cytokines mRNA

Since the cytokines also play an important role in the development of cellular immune response and protection of viral infections, the cytokine induction was also detected in the 'DNA prime-protein boost' and pCIVP2-cHSP70 groups. The upregulation of IFNγ and IL12 are indicators for Th1 type response and IL10 indicates Th2 response as reported in (Wang *et al.,* 2002; Rauf *et al.,* 2011; Cheng *et al.,* 2014) and IL10 is also an indicator of Th2 response. The pCIVP2-cHSP70 and DNA prime-protein boost strategy elicited a mixed Th1 and Th2 response against IBDV challenge in chicken with upregulation of IL12, IFNγ and IL10. The chickens in both these groups not only stimulated both the arms of the immune response but also achieved complete protection against vvIBDV challenge

when compared with chicken immunized with DNA vaccine alone.

5.8. Summary

In summary pCIVP2-cHSP70, could be a promising vaccine candidate in eliciting a humoral and cellular immune response against IBDV infection. The c-terminal HSP70 genetically linked to IBDV VP2 gene assisted in augmentation of both the arms of immune response and complete protection in chicken was achieved in chickens when challenged with vvIBDV. This novel strategy of development of a DNA vaccine construct not only improves the efficacy of the vaccine but also provides better immune response when compared to DNA prime-boost vaccination strategy or DNA vaccine alone.

6 Summary & Conclusions

Infectious Bursal Disease virus (IBDV) is a double stranded RNA virus affecting the young chickens. The virus has a selective tropism for bursal B cells causing massive destruction of B cells in lymphoid organs, resulting in lymphopenia (immunosuppression) and secondary infection of the infected bird. There are two distinct types of IBDV, designated as serotypes 1 and 2. While serotype 1 viruses are pathogenic to chickens, serotype 2 viruses are isolated from turkeys and are avirulent for chickens. IBDV serotype I isolate has different levels of virulence and different replicatio efficiency in bursal cells. Beginning in 1990, variant strains of serotype I virus emerged in the Western Europe and subsequently in different parts of Southeast Asia that were more virulent than classical strains and caused mortality rates of over 80 % .The viral genome consists of two segments of linear double-stranded RNA, A and B, 6 kb in length in total. Segment A is 3.2 kb in length and contains two partly overlapping open reading frames (ORF). The largest ORF encodes a polyprotein that is autocatalytically cleaved into two structural proteins, VP2 and VP3, and a serine protease, VP4 . VP2 is considered to be the major host-protective antigen and contains the major antigenic site responsible for eliciting neutralizing antibodies (Abs) (Fahey *et al.,*1989). IBDV is continuously evolving in the field with the changes in antigenicity and virulence. Vaccination is the best method to control the disease. IBDV vaccines include live attenuated and inactivated vaccines. However, there is a growing concern that intensive use of live attenuated vaccines against IBDV could drive this pathogen to revert back virulence. Therefore, these problems have raised increasing interest in the development of subunit vaccines; DNA vaccines offer several advantages for delivering protective antigens. DNA vaccines mimic a natural viral infection in that the antigens they encode are produced in their native structure and are presented in the context of MHC class I and II, evoking a balanced immune response. DNA vaccines encoding IBDV antigenic protein VP2 has been reported in a number of studies (Negash *et al.,* 2013). In this study we have also constructed a DNA vaccine encoding VP2 gene of IBDV to enhance the antigen specific humoral and cell mediated

immunity. But it was able to protect seventy percent of the immunized chicken against challenge with vvIBDV. To increase the efficacy of the DNA vaccine combination of various molecular adjuvant have been studied. A number of strategies have been developed for enhancing the immune response against DNA vaccine encoding VP2 gene of IBDV. DNA vaccine carrying VP234 gene of IBDV and interleukin18 (IL18) enhanced the immune response and protection efficacy (93%) against vvIBD (Li *et al.,* 2013). In a previous study the IL18 enhanced the protection efficacy, highlighting the potential value of chicken IL18 as an adjuvant in the prevention of vvIBDV infection. Zheng *et al.,*(2010) reported complete protection with DNA vaccine encoding IBDV VP2 gene when fused with defensin (AvBD1) gene and 80% protection was obtained by immunizing with DNA vaccine encoding VP2 gene alone. Thus, several reports have confirmed that partial protection is obtained when chickens are immunized with DNA vaccine encoding IBDV-VP2 gene alone but complete protection could be obtained when immunized together with cytokines or cytosine-phoshphate-guanine(CpG) motif (Hulse & Romero *et al.,* 2004). This proves the importance of using an adjuvant to enhance the immunogenicity of VP2-DNA vaccine. These reports encouraged us to choose a potent adjuvant which can enhance the antigen specific humoral and cell mediated immunity as well as giving complete protection against vvIBDV challenge.

Previously truncated c-terminal HSP70 has been described to possess a number of immunomodulatory properties and are being investigated as adjuvant components in vaccine development (Ebrahimi *et al.,* 2012). In the present study we have constructed a fusion of c-terminal $HSP70_{359–610}$ gene of *Mycobacterium tuberculosis* to VP2 gene of IBDV to enhance the antigen specific humoral and cell mediated immunity. The pCIVP2-cHSP70 construct was expressed under CMV promoter of pCI vector and the expression was confirmed by western blotting and immunofluorescence assay. To evaluate the adjuvant property of cHSP70 of *M.tuberculosis* we have compared three types of vaccines namely pCIVP2 DNA vaccine , DNA vaccine containing fusion construct of VP2 gene of IBDV and c-terminal HSP70 gene of *Mycobacterium tuberculosis* (pCIVP2-cHSP70) and 'DNA prime-protein boost' vaccine and the immune responses elucidated by vaccines and their protection efficacy against vvIBDV was studied. Humoral response was determined by ELISA and cell mediated immune response was determined by lymphocyte proliferation assay and cytokine expression assay. Protection level in the vaccinated

groups was assessed by challenging with a very virulent IBDV. The truncated c- terminal HSP70 mediated DNA vaccine genetically fused with VP2 gene construct stimulated both humoral and cell mediated immune responses and conferred complete protection against IBDV. This novel strategy is perhaps a seminal concept in utilizing HSP70 as an adjuvant molecule to elicit an immune response against IBD affecting chicken.

7 Mini Abstract

Infectious bursal disease (IBD) is an acute, infectious, immunosuppressive disease affecting young chicken worldwide. Etiological agent IBD virus (IBDV), is a double stranded RNA virus. The outer capsid protein VP2 of IBDV is the major antigenic determinant capable of inducing neutralizing antibody. New vaccination strategies are in demand to avert the risk with the conventional vaccination approach. DNA vaccines encoding VP2 has been extensively studied achieving partial protection. However, the efficacy of DNA vaccines against IBDV can be augmented by choosing a potential molecular adjuvant. Truncated c-terminal heat shock protein 70 (cHSP70) of *Mycobacterium tuberculosis* is one of the extensively studied molecular adjuvant. In this study, DNA vaccine construct containing fusion of cHSP70 of *Mycobacterium tuberculosis* and full length VP2 gene of IBDV was designed for expression under the CMV promoter of a mammalian expression vector (pCI). The new construct was designated as pCIVP2-cHSP70 and the newly constructed pCIVP2-cHSP70 DNA vaccine has been compared with DNA vaccine encoding only the VP2 gene. Transient expression of the constructs were confirmed by western blot and indirect immunofluorescence assay in Vero cells. The constructs were injected intramascularly into 14 days-old specific pathogen free (SPF) chickens followed by booster in 28^{th} days. Immune responses among three vaccination approaches were studied with ELISA, LTT and cytokine assays. Protection level in the vaccinated groups was assessed by challenging with a very virulent IBDV. The truncated c- terminal HSP70 mediated DNA vaccine genetically fused with VP2 gene construct stimulated both humoral and cell mediated immune responses and conferred complete protection against IBDV. This novel strategy is perhaps a seminal concept in utilizing HSP70 as an adjuvant molecule to elicit an immune response against IBD affecting chicken.

४ लघु सारांष

संक्रामक बसरल रोग (IBD) सम्पूर्ण विश्व में युवा पक्षियों को प्रभावित करने वाला एक तीव्र संक्रामक immunosuppressive रोग है। Etiological agent हैतुक विज्ञानी IBD विषाणू (IBDV), एक (Double stranded) दोहरा असहाय आर एन ए विषाणू है। IBDV का बाहरी capsid प्रोटीन VP2 उदासीन एन्टीबॉडी उतप्रेरण के लिए सक्षम प्रमूख प्रतिजनी निर्धारक है। नई टीकाकरण रणनीतीयां पारम्पारिक दृषटिकोन के साथ जोखिम से बचने के लिए आवश्यक है। VP2 एन्कोडिंग डी. एन. ए. टीके का बडे पेमाने पर रोग से आंशिक संरक्षण प्राप्त करने अध्ययन किया गया है। हालाकीं IBDV के विरुद्व D.N.A. टीके की प्रभाव कारिता एक शक्तीशाली आणविक Adjuvant के चुनाव से बढाई जा सकती है। माइकोबेक्टीरियम क्षय रोग का छोटा सी टर्मीनल प्रोटीन 70 (cHSP 70) बडे पेमाने पर अध्ययन किया हुआ आणविक adjuvant में से एक है। इस अध्ययन मे एम तपेदिक की cHSP 70 का फयुजन और IBDV की पूरी लंबाई VP2 जीन यूक्त D.N.A. टीकाकरण का निर्माण एक स्वनधारी अभिव्यक्ति वेक्टर (पी. सी. आई.) की सी.म.वी. प्रम. ोटर के तहत अभिव्यक्ति के लिए डिजाइन किया गया। नया construct pCIVP2-cHSP 70 के रुप में नामित किया गया और नवर्निमित pCIVP2-cHSP 70 डी. एन. ए. टीके की केवल VP2 जीन वाले डी. एन. ए. टीके से तुलना की गई। Construct की क्षणिक अभिव्यक्ति वेरोकोशिकाऔ Western blot में विधि और अप्रत्यक्ष immunofluorescence पराव विधि पुष्टी द्वारा की गई। इस construct को 14 दिनी विशिष्ट रोग जनक मुक्त मुर्गियों में इन्टरमसकफलर इन्जेक्शन द्वारा दिया गया तथा बाद में 28 दिनी मुर्गियों में बूस्टर डोज दिया गया। ELISA, LTT तथा सायटोकिन्स विधियों द्व ारा तीन टीकाकरण दृष्टीकोण में प्रतिरक्षा प्रतिकिया का अध्ययन किया गया। टीकाकरित समूहों में प्रतिरक्षा के स्तर का मूल्यांकन बहूत अधिक उग्र IBD विषाणु द्वारा चैंलेंज करके किया गया। truncated c–terminal HSP 70 मध्यस्थता वाले डी. एन. ए. टीके आनूवांशिक रुप से जुडे हुए VP2 जीन कन्सट्रक्ट द्वारा humoral और कोशिका की मध्यस्थता वाली प्रतिरक्षा प्रतिकिया को प्रेरित किया और IBDV के विरुद्व पूरी सुरक्षा प्रदान की। यह नई रणनीति संभवतः HSP 70 का एक adjuvant molecule के रुप मे उपयोग से IBDV प्रभावित मुर्गियों में प्रतिरक्षा प्रतिकिया को प्रकाश में लाने हेतु है।

9 References

Allan, W.H., Faragher, J.T. & Cullen, G.A. 1972. Immunosuppression by the infectious bursal agent in chickens immunized against Newcastle disease. Veterinary Record. **90**: 511-512.

Asea,A., Kraeft, S.K., Kurt-Jones, E.A., Stevenson, M.A., Chen, L.B., Finberg, R.W., Koo, G.C., Calderwood, S.K. 2000. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat Med. **6:**435-442.

Asea,A., Rehli, M., Kabingu, E., Boch ,J.A., Bare, O., Auron, P.E., Stevenson, M.A., Calderwood, S.K. 2002. Novel signal transduction pathway utilized by extra-cellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem. **277:**15028-15034.

Barrios, C., Lussow, A.R., Van Embden, J., Van der Zee, R., Rappuoli, P. Costantino, J.A., Louis, P.H. Lambert, and G. Del Giudice. 1992. Mycobacterial heat-shock proteins as carrier molecules. II: The use of the 70-kDa mycobacterial heat-shock protein as carrier for conjugated vaccines can circumvent the need for adjuvants and Bacillus Calmette Guérin priming. Eur. J. Immunol. **22:** 1365-1372.

Basu, S., R.J. Binder, T. Ramalingam and P.K. Srivastava, 2001. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70 and calreticulin. Immunity. **14:** 303-313.

Basu, S., Srivastava, P.K. 1999. Calreticulin, a peptide-binding chaperone of the endoplasmic reticulum, elicits tumor- and peptide-specific immunity. J Exp Med . **189:**797-802.

Bayliss, C.D., Barrios, C., A.R. Lussow., J. Van Embden., R. Van der Zee, R. Rappuoli, P. Peters, R.W., Cook, J.K., Reece, R.L., Howes, K., Binns, M.M. and Boursnell, M.E. 1991. A recombinant fowl pox virus that expresses the VP2 antigen of infectious bursal disease virus induces protection against mortality by the virus. Arch. of Virol. **120**:193-205.

Becht, H., Müller, H. and Müller, H.K. 1988. Antigenic structure of the two serotypes of infectious bursal disease virus. J. Gen. Virol**. 69**: 631–640.

Becker,T., Hartl, F.U., Wieland, F. 2002. CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. J. Cell Biol . **158:**1277-1285.

Binder, R.J., Harris,M.L., Menoret, A., Srivastava, P.K. 2000. Saturation, competition,

and specificity in interaction of heat shock proteins (hsp) gp96, hsp90, and hsp70 with CD11b+ cells. J Immunol . **165:** 2582-2587.

Binder, R.J.,Vatner,R., Srivastava, P.2004 . The heat-shock protein receptors: some answers and more questions. Tissue Antigens. **64:**442-451.

Blachere, N.E., Udono, H., Janetzki, S., Li, Z., Heike, M., Srivastava, P.K.1993. Heat shock protein vaccines against cancer. J .Immunother.**14:** 352-356.

Bottcher, B., Kiselev, N.A., Stel'Mashchuk, V.Y., Perevozchikova, N.A., Borisov, A.V., Crowther, R.A. 1997. Three-dimensional structure of infectious bursal disease virus determined by electron cryomicroscopy. J Virol .**71:** 325–330.

Brandt, M., Yao,K., Liu,M., Heckert, R.A., Vakharia, V.N. 2001. Molecular determinants of virulence, cell tropism, and pathogenic phenotype of infectious bursal disease virus. J Virol. **75:** 11974-11982.

Chattergoon, M., Boyer, J., Weiner, D. B.1997.Genetic immunization: a new era in vaccines and immune therapeutics. FASEBJ. **11:** 753-763.

Cheng, L., Yu, L., Wu, X., Li, K., Wang, F., Zhang, L., Ye, W., Li, P., Zhang, F., Xu, Z. 2014. Induction of specific humoral and cellular immune responses in a mouse model following gene fusion of HSP70C and Hantaan virus Gn and S0.7 in an adenoviral vector. PLoS One. **9:** e88183.

Chettle, N., Stuart, J.C., Wyeth, P.J., 1989. Outbreak of virulent infectious bursal disease in East Anglia. Vet. Rec. **125**: 271–272.

Ciupitu, A.M., Petersson, M., Kono, K., Charo, J., Kiessling, R. 2002. Immunization with heat shock protein 70 from methylcholanthrene-induced sarcomas induces tumor protection correlating with in vitro T cell responses. Cancer Immunol Immunother. **51:**163-170.

Cosgrove, A.S. 1962. An apparently new disease of chickens;avian nephrosis . Avi. Disease. **6:** 385-389.

Delgui, L., Ona, A., et al., 2009. The capsid protein of infectious bursal disease virus contains a functionala4b1 integrin ligand motif. Virology . **386:** 360–372.

Dey, S., Upadhyay, C., Madhan Mohan, C., Kataria, JM., Vakharia VN. 2009. Formation of subviral particles of the capsid protein VP2 of infectious bursal disease virus and its application in serological diagnosis. J Virol Methods. Apr;**157:** 84-89.

Dobos, P., Hill, B.J., Hallet, R., Kells, D.T., Becht, H. & Tenninges, D. 1979. Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. J Virol. **32**: 593-605.

Ebrahimi, S.M. and M. Tebianian, 2010. Heterologous expression, purification and characterization of the influenza A virus M2e gene fused to *Mycobacterium tuberculosis* HSP70359-610 in prokaryotic system as a fusion protein. Mol. Biol. Rep. **37:** 2877-2883.

Ebrahimi, S.M., Dabaghian, M., Tebianian, M., Jazi, M.H. 2012. In contrast to conventional inactivated influenza vaccines, 4xM2e.HSP70c fusion protein fully protected mice against lethal dose of H1, H3 and H9 influenzaA isolates circulating in Iran. Virology. **15:** 63-72.

Fahey, K.J., Chapman, A.J., Macreadie, I.G., Vaughan, P.R., McKern, N.M., Skicko, J.I., Ward, C.W. & Azad, A.A. 1991. A recombinant subunit vaccine that protects progeny chickens from infectious bursal disease. Avian Pathol. **20:** 447-460.

Fahey, K.J., Erny, K. & Crooks, J. 1989. A conformational immuno-gen on VP2 of infectious bursal disease virus that induces virus-neutralizing antibodies that passively protect chickens. J. Gen. Virol .**70 :**1473-1481.

Fei-fei, G., and Y. Qiu., X. Gao., Y. Yang and P. Chen, .2006. Fusion expression of major antigenic segment of JEV E protein-hsp70 and the identification of domain acting as adjuvant in hsp70. Vet Immunol Immunop. **113**: 288-296.

Flaherty, K.M., C. De Luca-Flaherty. and D.B. McKay.1990. Three-dimensional structure of the ATPasefragment of a 70K heat-shock cognate protein. Nature. **346**: 623-628.

Fodor, I., Horva´th, E., Fodor, N., Nagy, E., Rencendorsh, A., Vakharia, V.N. & Dube, S.K. 1999. Induction of protective immunity in chickens immunised with plasmid DNA encoding infectious bursal disease virus antigens. Acta Veterinaria Hungarica. **47:** 481-492.

Fu, F., Tian. H., Li, X., Lang, Y., Tong, G., Liu, S., Li, H.,Wang, W., Li, X., Chen, X. 2013. C-terminal heat shock protein 70 of *Mycobacterium tuberculosis* as a molecular adjuvant for DNA vaccination with the porcine circovirus type 2 ORF2 (capsid) gene in mice. Vet J. **195:**244-247.

Gao, Honglei., Li, Kai., Li, Gao., Xiaole, Qi., Gao, Liting Qin, Yongqiang Wang, Wang, Xiaomei .2013. DNA prime–protein boost vaccination enhances protective immunity against infectious bursal disease virus in chickens. Vet Microbiol . **164:** 9–17.

Giambrone, J.J., Dormitorio, T. & Brown, T. 2001. Safety and efficacy of in ovo administration of infectious bursal disease viral vaccines. Avi. Disease. **45:** 144-148.

Haddad, E.E., Whitfill, C.E., Avakian, A.P., Ricks, C.A., Andrews, P.D., Thoma, J.A. & Wakenell, P.S. 1997. Efficacy of a novel infectious bursal disease virus (IBDV) immune complex vaccine in broiler chickens. Avi. Disease. **41:** 882-889.

Haygreen, E.A., Kaiser, P., Burgess, S.C. & Davison, T.F. 2006. In ovo DNA immunization followed by a recombinant fowl pox boost is fully protective to challenge with virulent IBDV. Vaccine. **24:** 4951-4961.

Heine, H.G. & Boyle, D.B. 1993. Infectious bursal disease virus structural protein VP2 expressed by a fowlpox virus recombinant confers protection against disease in chickens. Arch. of Virol. **131:** 277-292.

Hiraga, M., Nunoya, T., Otaki, Y., Tajima, M., Saito, T., Nakamura, T.1994. Pathogenesis of highly virulent infectious bursal disease virus infection inintact and bursectomized chickens. J.Vet. Med. Sci. **56:** 1057–1063.

Hitchner, S.B. 1970. Infectivity of Infectious bursal disease virus for embryonating eggs. Poult sci. **49:** 511-516.

Hsieh, M.K., Wu, C.C. & Lin, T.L. 2007. Priming with DNA vaccine and boosting with killed vaccine conferring protection of chickens against infectious bursal disease. Vaccine .**25:** 5417-5427.

Hsieh, M.K., Wu, C.C. & Lin, T.L. 2010. DNA-mediated vaccination conferring protection against infectious bursal disease in broiler chickens in the presence of maternal antibody. Vaccine. **28:** 3936-3943.

Hulse, D.J. & Romero, C.H. 2004. Partial protection against infectious bursal disease virus through DNA-mediated vaccination with the VP2 capsid protein and chicken IL-2 genes. Vaccine. **22:**1249-1259.

Ignjatovic, J., Gould, G., Trinidad, L. & Sapats, S. 2006. Chicken recombinant antibodies against infectious bursal disease virus are able to form antibody-virus immune complex. Avian Pathol. **35:** 293-301.

Ingrao, F., Rauw, F., Lambrecht,B., van den Berg, T. 2013 . Infectious Bursal Disease: a complex host-pathogen interaction. Dev Comp Immunol. **41:** 429-438.

Ivan, J., Velhner, M., Ursu, K., German, P., Mato, T., Dren, C.N. & Mesza ros, J. 2005. Delayed vaccine virus replication in chickens vaccinated subcutaneously with an immune complex infectious bursal disease vaccine: quantification of vaccine virus by real-time polymerase chain reaction.Canadian J Vet Res. **69:** 135-142.

Jackwood, D. H., and Y. M. Saif, 1987. Antigenic diversity of infectious bursal disease viruses. Avian Dis. **31**:766–770.

Jeurissen, S.H., Janse, E.M., Lehrbach, P.R., Haddad, E.E. Avakian, A. & Whitfill, C.E. 1998. The working mechanism of an immune complex vaccine that protects chickens against infectious bursal disease. Immunology. **95:** 494-500.

Jiang, J., Xie, D., Zhang, W., Xiao, G., Wen, J. 2013. Fusion of Hsp70 to Mage-a1 enhances the potency of vaccine-specific immune responses. J Transl Med. **11:** 300.

Kaiser, M.G., Cheeseman, J.H., Kaiser, P., Lamont, S.J. 2006. Cytokine expression in chicken peripheral blood mononuclear cells after in vitro exposure to *Salmonella enterica* serovar Enteritidis. Poult Sci. **85:**1907-1911.

Kumar, K., Singh, K.C. & Prasad, C.B. 2000. Immune responses to intermediate strain

IBD vaccine at different levels of maternal antibody in broiler chickens. Tropical Animal Health and Production. **32:** 357-360.

Kumar, S., Ahi, Y.S., Salunkhe, S.S., Koul, M., Tiwari, A.K., Gupta, P. K. and Rai, A. 2009. Effective protection by high efficiency bicistronic DNA vaccine against infectious bursal disease virus expressing VP2 protein and chicken IL-2. Vaccine. **27:** 864-869.

Kuppner, M.C., Gastpar,R., Gelwer, S., Nossner, E., Ochmann, O., Scharner, A., Issels, R.D. 2001. The role of heat shock protein (hsp70) in dendritic cell maturation: hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors. Eur J Immunol . **31:**1602-1609.

Lee, M.S., Lin, Y.C., Lai, G.H., Lai, S.Y., Chen, H.J., Wang, M.Y. 2011. One-step reverse-transcription loop-mediated isothermal amplification for detection of infectious bursal disease virus. Can J Vet Res. **75:**122-127.

Leong, J.C., Brown, D., Dobos, P., Kibenge, F., Ludert, J.E., Müller, H., Mundt, E., Nicholson, B., 2000. Birnaviridae. In: M.H.V. Regenmortel, C.M. Fauquet, D.H.L. Bishop, E.B. Carstens, M.K. Estes, S.M. Lemon, J. Maniloff, M.A. Mayo, D.J. McGeoch, C.R. Pringle, R.B. Wickner (Eds.), Virus Taxonomy Classification and Nomenclature of Viruses, Academic Press, pp. 481–490, ISBN 0-12-370200-3.

Li, Kai., Gao, Honglei.,Gao, Li., Qi, Xiaole., Gao, Yulong., Liting Qin, Yongqiang Wang, Xiaomei Wang.2013.Adjuvant effects of interleukin-18 in DNA vaccination against infectious bursal disease virus in chickens. Vaccine. **31**:1799-1805.

Li, L., Fang, W., Li, J., Huang, Y. & Yu, L. 2006. Oral DNA vaccination with the polyprotein gene of infectious bursal disease virus (IBDV) delivered by the attenuated Salmonella elicits protective immune responses in chickens. Vaccine.**24**: 5919-5927.

Li, X., X. Yang., L. Li., H. Liu and J. Liu. 2006. A truncated C-terminal fragment of *Mycobacterium tuberculosis* HSP70 gene enhanced potency of HBV DNA vaccine. Vaccine. **24**: 3321-3331.

Liu, Y., Wei, Y., Wu, X. & Yu, L. 2005. Preparation of ChIL-2 and VP2 fusion protein by baculovirus expression system. Cell Mol Immunol. **2** :231-235.

Lombardo, E., Maraver, A., Espinosa, I., Fernandez-Arias, A. &Rodriguez, J.F. 2000. VP5, the nonstructural polypeptide of infectious bursal disease virus, accumulates within the host plasma membrane and induces cell lysis. Virology. **277:** 345–357.

Mahgoub, H.A., Bailey, M., Kaiser, P. 2012. An overview of infectious bursal disease. Arch Virol. **157:** 2047-2057.

Mahmood, M.S., Hussain, I., Siddique, M., Akhtar, M. & Ali, S. 2007. DNA vaccination with VP2 gene of very virulent infectious bursal disease virus (vvIBDV) delivered by transgenic E. coli DH5 alpha given orally confers protective immune responses in chickens. Vaccine. **25:**7629-7635.

Maranon, C., Thomas, M.C., Planelles, L. & Lopez, M.C. 2001. The immunization of A2/K(b) transgenic mice with the KMP11-HSP70 fusion protein induces CTL response against human cells expressing the *T. cruzi* KMP11 antigen: identification of A2-restricted epitopes. Mol. Immunol. **38:** 279-87.

Martinez-Torrecuadrada, J.L., Sauri, N., Page`s-Mante´, A., Casto ´n,J.R., Espun ˜a, E. & Casal, J.I. 2003. Structure-dependent efficacy of infectious bursal disease virus (IBDV) recombinant vaccines. Vaccine. **21:** 3342-3350.

Mazariegos, L.A., Lukert, P.D., Brown, J.1990. Pathogenicity and immunosuppressive properties of infectious bursal disease ‘‘inter-mediate’’strains. Avi. Disease. **34:** 203-208.

McFerran, J. B., M. S. McNulty, E. R. McKillop, T. J. Conner, R.M. McCracken, D. S. Collins, and G. M. Allan, 1980.Isolation and serological studies with IBDVs from fowl,turkeys, and ducks: Demonstration of a second serotype. Avian Pathol. **9:** 395 –404.

Mohanty, G.C., Pandey, A. P., and Rajya, B.S. 1971. Infectious bursal disease in chickens. Curr. Sci. **40:** 181-184.

Moroi, Y., Mayhew, M., Trcka, J., Hoe, M.H., Takechi, Y., Hartl, F.U., Rothman, J.E., Houghton, A.N. 2000. Induction of cellular immunity by immunization with novel hybrid peptides complexed to heat shock protein 70. Proc Natl Acad Sci USA. **97:** 3485-3490.

Müller, H., Schnitzler, D., Bernstein, F., Becht, H., Cornelissen, D., Lütticken, D.H. 1992. Infectious bursal disease of poultry: antigenic structure of the virus and control. Vet Microbiol. **33:** 175-83.

Müller, Hermann., Islam, Md. Rafiqul., Rüdiger,Raue.2003. Research on infectious bursal disease the past, the present and the future. Vet Microbiol. **97**: 153–165.

Müller, Hermann., Mundt, Egbert., Eterradossi. Nicolas. & M. Rafiqul Islam. 2012. Current status of vaccines against infectious bursal disease.Avian Pathol. **41:** 133-139.

Mundt, E., Beyer, Jorg., and Muller, Hermann. 1995. Identification of a novel viral protein in infectious bursal disease virus infected cells . J Gen Virol, **76:** 437-443.

Negash, T., Liman, M., Rautenschlein,S. 2013. Mucosal application of cationic poly (d,l-lactide-co-glycolide) microparticles as carriers of DNA vaccine and adjuvants to protect chickens against infectious bursal disease. Vaccine . **31**: 3656 – 3662.

Oppling, V., Muller, H. & Becht, H. 1989. Heterogeneity of the antigenic site responsible for the induction of neutralizing antibodies in infectious bursal disease virus. Arch. of Virol. **119:** 211-223.

Oshop,G.L., Elankumaran, S.,Vakharia, V.N. & Heckert, R.A.2003. In ovo delivery of DNA to the avian embryo. Vaccine. **21:** 1275-1281.

Park, J.H., Sung, H.W., Yoon, B.I. & Kwon, H.M. 2009. Protection of chicken against very virulent IBDV provided by in ovo priming with DNA vaccine and boosting with killed vaccine and the adjuvant effects of plasmid-encoded chicken interleukin -2 and interferon-gamma. J .Vet. Sci. **10:** 131-139.

Perraut, R., Lussow, A.R., Gavoille, S., Garraud, O., Matile, H., Tougne, C., Van Embden, J., Van Der Zee, R., Lambert, P.H., and Gysin, J. 1993. Successful primate immunization with peptides conjugated to purified protein derivative or mycobacterial heat shock proteins in the absence of adjuvants. Clin. Exp. Immunol. **93:** 382-386.

Phenix, K.V., Wark, K., Luke, C.J., Skinner, M.A., Smyth, J.A., Mawhinney, K.A. & Todd, D. 2001. Recombinant Semliki Forest virus vector exhibits potential for avian virus vaccine development. Vaccine. **19:** 3116-3123.

Pitcovski, J., Gutter, B., Gallili, G., Goldway, M., Perelman, B., Gross, G., Krispel, S., Barbakov, M. & Michael, A. 2003. Development and large-scale use of recombinant VP2 vaccine for the prevention of infectious bursal disease of chickens. Vaccine. **21:** 4736-4743.

Planelles, L., Thomas, M.C., Alonso, C., Lopez, M.C. 2001. DNA immunization with Trypanosoma cruzi HSP70 fused to the KMP11 protein elicits a cytotoxic and humoral immune response against the antigen and leads to protection. Infect Immun . **69:**6558-63.

Pradhan, S.N., Prince, P.R., Madhumathi, J., Arunkumar, C., Roy, P., Narayanan, R.B., Antony, U. 2014. DNA vaccination with VP2 gene fragment confers protection against Infectious Bursal Disease Virus in Chickens. Vet Microbiol. **171(1-2):** 13-22.

Qazi, K.R., Wikman, M., Vasconcelos, N.M., Berzins, K., Stahl, S., Fernandez, C.2005. Enhancement of DNA vaccine potency by linkage of plasmodium falciparum malarial antigen gene fused with a fragment of HSP70 gene. Vaccine. **23**:1114–25.

Rauf, A., Khatri, M., Murgia, M.V., Jung, K., Saif, Y.M., 2011a. Differential modulation of cytokine, chemokine and Toll like receptor expression in chickens infected with classical and variant infectious bursal disease virus. Vet. Res. **42 :** 85-92.

Rautenschlein, S., Kraemer, C., Vanmarcke, J. & Montiel, E. 2005. Protective efficacy of intermediate and intermediate plus infectious bursal disease virus (IBDV) vaccines against very virulent IBDV in commercial broilers. Avi. Disease. **49**:

231- 237.

Rautenschlein, S., Yeh, H.Y. & Sharma, J.M. 2002. The role of T cells n protection by an inactivated infectious bursal disease virus vaccine. Vet Immunol Immunopathol. **89:** 159-167.

Rico, A.I., G. Del Real, M. Soto, L. Quijada, A.C. Martinez, C. Alonso and J.M. Requena, 1998. Characterization of the immunestimulatory properties of *Leishmania infantum* HSP70 by fusion to the Escherichia colimaltose-binding protein in normal and nu/nu BALB/c mice. Infect. Immun. **66:** 347-52.

Rosenberger, J.K., Fries, P.A., Cloud, S.S., Wilson, R.A. 1985 . *In vitro* and *in vivo* characterization of avian Escherichia coli. II. Factors associated with pathogenicity. Avian Dis. 1985. **29:**1094-1107.

Saif, Y.M. 1998. Infectious Bursal Disease and Hemorrhagic Enteritis. Poult Sci. **77:** 1186–1189.

Sapats, S.I., Trinidad, L., Gould, G., Heine, H.G., van den Berg, T.P., Eterradossi, N., Jackwood, D., Parede, L., Toquin, D. & Ignjatovic, J. 2006. Chicken recombinant antibodies specific for very virulent infectious bursal disease virus. Arch.Virol. **151:**1551-1566.

Schijns, V.E.J.C., Sharma, J. & Tarpy, I. 2008. Practical aspects of poultry vaccination. In F. Davison, B. Kaspers & K.A. Schat (Eds.).Avian Immunology1st edn (pp. 373-393). London: Academic Press.

Shini, S., Huff, G.R., Shini, A., Kaiser, P. 2010.Understanding stress-induced immuno-suppression: exploration of cytokine and chemokine gene profiles in chicken peripheral leukocytes. Poult Sci. **89:** 841-851.

Shwed, P.S., Dobos, P., Cameron, L.A., Vakharia, V.N., Duncan, R. 2002. Birnavirus VP1 proteins form a distinct subgroup of RNA-dependent RNA polymerases lacking a GDD motif. Virology. **296:** 241-50.

Singh, Niraj K., Dey, Sohini., Madhan Mohan, C., Mohan Kataria, Jag., Vakharia, Vikram N .2010. Evaluation of four enzyme linked immunosorbent assays for the detection of antibodies to infectious bursal disease in chickens. J Virol Methods. **165:** 277-282.

Smith, B.F., Baker, H.J., Curiel, D.T., Jiang, W., Conry, R.M. 1998. Humoral and cellular immune responses of dogs immunized with a nucleic acid vaccine encoding human carcinoembryonic antigen. Gene Ther. **5:** 865-868.

Srivastava, P. 2002. Roles of heat-shock proteins in innate and adaptive immunity. Nat Rev Immunol. **2:** 185-194.

Srivastava, P.K., Menoret, A., Basu, S., Binder, R.J., McQuade, K.L.1998. Heat shock proteins come of age: primitive functions acquire new roles in an adaptive world. Immunity. **8:** 657-665.

Srivastava, P.K., Udono, H., Blachere, N.E., Li, Z. 1994. Heat shock proteins transfer peptides during antigen processing and CTL priming. Immunogenetics . **39:** 93-98.

Suzue, K., and Young, R.A.1996a. Adjuvant-free hsp70 fusion protein system elicits humoral and cellular immune responses to HIV-1 p24. J. Immunol. **156:** 873-879

Taghavian, O., Spiegel, H., Hauck, R., Hafez, H.M., Fischer, R., Schillberg, S. 2013. Protective oral vaccination against infectious bursal disease virus using the major viral antigenic protein VP2 produced in Pichia pastoris. PLoS One. **20:** 8-12.

Tobian, A.A., D.H. Canaday and C.V. Harding, 2004a. Bacterial heat shock proteins enhance class II MHC antigen processing and presentation of chaperoned peptides to CD4+ T cells. J. Immunol. **173:** 5130-5137.

Tobian, A.A., D.H. Canaday, W.H. Boom and C.V. Harding, 2004b. Bacterial heat shock proteins promote CD91- dependent class I MHC cross-presentation of chaperoned peptide to CD8+ T cells by cytosolic mechanisms in dendritic cells versus vacuolar mechanisms in macrophages. J. Immunol.**172:** 5277-86.

Tsukamoto, K., Saito, S., Saeki, S., Sato, T., Tanimura, N., Isobe, T., Mase, M., Imada, T., Yuasa, N., Yamaguchi, S. 2002. Complete, long-lasting protection against lethal infectious bursal disease virus challenge by a single vaccination with an avian herpesvirus vector expressing VP2 antigens. J Virol. **76:** 5637–5645.

Udono, H., Srivastava, P.K. 1994. Comparison of tumor-specific immunogenicities of induced proteins gp96, hsp90, and hsp70. J Immunol. **152:** 5398-403.

Udono, H., Srivastava, P.K.1993. Heat shock protein 70-associated peptides elicit specific cancer immunity. J Exp Med. **178:** 1391-1396.

Udono,H., Yamano,T., Kawabata,Y., Ueda, M., Yui, K. 2001. Generation of cytotoxic T lymphocytes by MHC classI ligands fused to heat shock cognate protein 70. Int Immunol. **13:** 1233-1242.

Vabulas, R.M., Ahmad-Nejad, P., Ghose, S., Kirschning, C.J., Issels, R.D., Wagner, H. 2002. HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal path-way. J Biol Chem. **277:** 15107-15112.

Vakharia, V.N., Snyder, D.B., He, J., Edwards, G.H., Savage, P.K. & Mengel-Whereat, S.A. (1993). Infectious bursal disease virus structural proteins expressed in a baculovirus recombinant confer protection in chickens. J. Gen. Virol.**74:**1201-1206.

Van den Berg, T.P., Gonze, M., Meulemans, G., 1991. Acute infectious bursal disease in poultry: isolation and characterisation of a highly virulent strain. Avian Pathol. **20 :** 133–143.

Van Den Berg, Thierry P. 2000.Acute infectious bursal disease in poultry: A review. Avi Pathol**. 29:** 175– 194.

Wang, X.Y., Kazim, L., Repasky, E.A., Subjeck, J.R. 2001. Characterization of heat shock protein 110 and glucose-regulated protein 170 as cancer vaccines and the

effect of fever-range hyperthermia on vaccine activity. J Immunol . **166:** 490-497.

Wang,Y., Kelly, C.G., Singh, M., McGowan, E.G., Carrara, A.S., Bergmeier, L.A., Lehner, T. 2002. Stimulation of Th1-polarizing cytokines, C-C chemokines, maturation of dendritic cells, and adjuvant function by the peptide binding fragment of heat shock protein 70. J Immunol. **169:** 2422-2429.

Whitfill, C.E., Haddad, E.E., Ricks, C.A., Skeeles, J.K., Newberry, L.A., Beasley, J.N., Andrews, P.D., Thoma, J.A. and Wakenell, P.S. 1995. Determination of optimum formulation of a novel infectious bursal disease virus (IBDV) vaccine constructed by mixing bursal disease antibody with IBDV. Avian Dis. **39:** 687-699.

Winterfield, T. W., Hitchner, S. B., Appleton, G. S., and A. S.Cosgrove, 1962. Avian nephrosis, nephritis and Gumborodisease. L & M News Views **3:** 103.

Wu, H., Singh, N.K., Locy, R.D., Scissum-Gunn, K. & Giambrone, J.J. 2004. Immunization of chickens with VP2 protein of infectious bursal disease virus expressed in *Arabidopsis thaliana.* Avian Dis. **48:** 663-668.

Wu, Y., Peng, C., Xu, L., Zheng, X., Liao, M., Yan, Y., Jin, Y., Zhou, J. 2012. Proteome dynamics in primary target organ of infectious bursal disease virus. Proteomics. **12:** 1844-1859.

www.oie.int/wahis_2/public/wahid. Number of new outbreaks reported in INDIA, 2010-2013.

Zhang, H.H., Yang, X.M., Xie, Q.M., Ma, J.Y., Luo, Y.N., Cao, Y.C., Chen, F., Bi, Y.Z. 2010. The potent adjuvant effects of chicken beta-defensin-1 when genetically fused with infectious bursal disease virus VP2 gene. Vet Immunol Immunopathol. **136:** 92-97.

Zhu, X., Z. Zhao, W.F. Burkholder, A. Gragerov, C.M. Ogata, M.E. Gottesman and W.A. Hendrickson,1996. Structural analysis of substrate binding by the molecular chaperone DnaK. Sci., **272:** 1606-1614.

10 Appendix

I. Solutions for Plasmid extraction

1. Solution -I

Glucose 50 mM

Tris-HCl, (pH8.0) 25 mM

EDTA (pH 8.0) 10 mM

Autoclaved at 15 lb/sq.inch. for 15 min. Stored at 4°C

2. Solution -II (to be made fresh)

NaOH (from a stock of 10 N) 0.2 N

SDS 1.0 %

3. Solution -III

5M Potassium acetate 60.0 ml

Glacial acetic acid 11.5 ml

Distilled water 28.5 ml

The resulting solution is 3M with respect to potassium and 5M with respect to acetate

II. Luria Bertani (LB) Medium (pH 7.0)

Tryptone 1.0%

Yeast extract 0.5%

NaCl 1.0%

For LB plates, add 1.5% agar to the LB broth and autoclave

III. Reagent for Agarose gel electrophoresis

1. TBE (10X)

Tris base 108 g

Boric acid 55 g

EDTA (0.5 M, pH 8.0) 40 ml

Distilled water to make 1L

2. Ethidium Bromide

Ethidium bromide 10 mg

Distilled water 1.0ml

3. Gel loading dye (6X)

Bromophenol Blue 1.0%

Sucrose in water 40%

IV. PCR buffer (10X)

Tris- HCl 100 mM

KCl 500 mM

MgCl2 25 mM

Triton X100 0.1%

V. Miscellaneous Buffers / solutions

Phosphate Buffered Saline, pH 7.4

NaCl 8.0g

Na2HPO4.2H2O 1.44 g

KH2PO4 0.2 g

KCl 0.2g

Distilled water to make 1000 ml

Colony lysis Buffer

1M Tris-Cl, pH 8.0 (100mM) 1ml

0.5M EDTA, pH 8.0 (10mM) 0.2ml

Lysozyme (10mM) 10mg

RNAse (30g/ml) 0.3mg

Distilled water to make 10ml

Phosphate Buffer (10X)

KH2PO4 (0.17M) 23.1g

K2HPO4 (0.72M) 125.4g

Distilled water to make 1000ml

Sterilize by autoclaving

VI. Buffers for cell culture

Maintenance media

DMEM 950ml

TPB(10X) 50ml

Fetal Calf Serum 20ml

Growth media

DMEM 900ml

TPB (10X) 100ml

Fetal Calf Serum 100ml

Phenol red (1% w/v) solution

Phenol red powder 10 g

N/10 NaOH 300 ml

Distilled water 750 ml

The powder was first dissolved in N/10 NaOH solution with the help of a pestle and mortar and then distilled water was added to make the required volume. The solution was stored at 4^0C

Tryptose Phosphate Broth (TPB) (pH 7.3)

Tryptose 20 g

Dextrose 2.0 g

Sodium Chloride 5.0 g

Disodium Phosphate 2.5 g

Suspend 29.5 g in 1000 ml distilled water. Boil to dissolve the medium completely. Sterilized by autoclaving at 15 lb. pressure for 15 minutes

Trypsin Versene Glucose (TVG)

i) Trypsin Stock Solution

Trypsin (Difco; 1:250) 2 g

Distilled water 100 ml

The trypsin was dissolved by agitating the solution at 4^0C for 15 minutes and then filtered through Seitz-KES pads and stored at 4^0C

ii) Versene Stock Solution

Versene (AR) 0.2 g

Distilled water 100 ml

The solution was sterilized by autoclaving at 10 lb pressure for 10 minutes and stored at 4^0C

iii) Glucose Stock Solution

Glucose (AR) 10 g

Distilled water 100 ml

The solution was sterilized by autoclaving at 10 lb pressure for 10 minutes and stored at 4^0C

TVG working solution

1 X PBS 840 ml

2% Trypsin Stock 50 ml

2% Versene Stock 100 ml

10% Glucose Stock 50 ml

1% Phenol red 1 ml

Mix above aseptically in laminar flow, confirm for sterility and store at 4^0C till further use

6. DMEM

4 mM Glutamine

4500 mg glucose/l

1500 mg /l $NaHCO_3$

VII. Reagents for ELISA

Coating Buffer (pH 9.0) -100 ml

Sodium carbonates (Na_2CO_3) – 0.159 g

Sodium bicarbonate ($NaHCO_3$)-0.293 g

Substrate buffer (pH 5.0)- 50 ml

Citric acid -0.26 g

Di-Sodium hydrogen phosphate-0.69 g

VIII. Reagents for SDS PAGE and Western blot

1. Separating gel (12%)

Water-2 ml

Acylamide/bisacrylamide (30%)-2.5 ml

Tris (pH 8.8)-1.4 ml

SDS (10%)-50 µl

APS (10%)-50 µl

TEMED-4 µl

2. Stacking gel (5%)

Water-1.5 ml

Acylamide/bisacrylamide (30%)-310 µl

Tris (pH 6.8)-622 µl

SDS (10%)-25 µl

APS (10%)-25 µl

TEMED-4 µl

3. Lamelli buffer -10 ml

1 M Tris (pH 6.8)-1.87 ml

Glycerol-3 ml

10% SDS-6 ml

4 . Transfer buffer (pH 8.3)

Glycine-2.9 gm

Tris-5.8 gm

SDS-0.37 gm

200 ml Methanol and make upto 1 lit with water

5. Tris Boric acid buffer (TBE) (pH 7.5) -50 ml

10 mM Triscl-0.06 gm

150 mM Nacl-0.435 gm

6. TBST -50 ml

20 mM Triscl -0.12 gm

500 mM Nacl-1.45 gm

0.05% Tween 20- 25 µl

Vitae

Name	:	Dr. Hemanta Kumar Maity
Father's name	:	Sri Haripada Maity
Mother's Name	:	Smt. Maloti Maity
Permanent Addrtess	:	C/o:Haripada Maity, Vill- Janumanu , P.O-Jamualachhimpur, P.S.- Bhupatinagar, Dist- East Medinipur PIN-721422 .West Bengal INDIA
Email	:	dr.hemanta87@gmail.com

Acadamic Details

Degree	University	OGPA	Year
B.V.Sc. & A.H.	West Bengal University of Animal and Fishery Sciences	7.46	2012
M.V.Sc	Indian Veterinary Research Institute	8.41	2014

Honours & Awards:

1. Secured 4th rank (GEN) in ICAR's17th All India Entrance Examination (AIEEA-PG-2012)

2. ICAR Junior Research Fellow (2012-2014)

3. Secured 3rd rank (GEN) in Combined Entrance Examination for Biotechnology, M.V.Sc Biotechnology, conducted by JNU.

4. Recipient of University merit scholarship in the Undergraduate Program (2007-2012).