ABSTRACT

Title of Dissertation: **DELIVERY OF DNA AND RECOMBINANT INFECTIOUS BURSAL DISEASE VIRUS VACCINES *IN OVO***

Lenita Moura, Doctor of Philosophy, 2004

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Infectious bursal disease virus (IBDV) remains a serious problem for commercial broiler producers throughout the world. An *in ovo* delivery system for plasmid DNA vaccines was evaluated by studying parameters, such as the route of delivery (air cell vs amniotic cavity), transfection reagent (IFA+DMSO vs polyethylenimine), dose of plasmid DNA (1 to 100 µg/egg), and the nature of humoral immune responses. An optimal response was detected when embryos were inoculated with 60 µg of plasmid DNA.

This system for *in ovo* delivery was used to determine the efficacy of a plasmid DNA vaccine against IBDV in 18-day-old embryos. The DNA vaccine expresses the polyprotein VP2-VP4-VP3 of IBDV. SPF and fertile broiler eggs with maternal antibodies were vaccinated and challenged against IBDV-STC. Two groups of birds (SPF and broilers) received a booster immunization with baculovirus expressed-proteins of IBDV. The DNA vaccine had no detrimental effect on hatchability or first week post-

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hatch survival. *In ovo* vaccination generated detectable humoral immune responses as measured by ELISA. Antibody response was significantly enhanced two weeks post the IBDV-protein boost. Broilers vaccinated with plasmid DNA or IBDV-protein boost exhibited partial protection against IBDV-STC strain, whereas, vaccinated SPF chicks were not protected and exhibited severe microscopic lesions after challenge.

A second approach in the control of IBDV used a recombinant attenuated vaccine administered *in ovo* to 18-day-old embryos. The vaccine was genetically tailored to protect from challenges in the field against classic and variant strains of IBDV. SPF and fertile broiler eggs were vaccinated and used to evaluate protection against IBDV-STC challenge. A full dose of the vaccine consisting of $5.6x10^3$ pfu was administered to SPF and commercial broiler embryos. In addition, a half dose containing $2.3x10^3$ pfu was injected in SPF embryos. The vaccine generated high antibody titers in chickens vaccinated with either dosage. All vaccinated groups were protected against mortality. The vaccine did not cause bursal damage and fully protected SPF chicks vaccinated *in ovo* with $2.3x10^3$ pfu and broiler embryos that received a full dose of the recombinant vaccine. The vaccine had no effect on hatchability or first week survival in either broilers or SPF birds, even when high doses were administered.

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DELIVERY OF DNA AND RECOMBINANT INFECTIOUS BURSAL DISEASE VIRUS VACCINES *IN OVO*

by

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Dedicated to

my family back in Brazil

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A	alanine
AC-ELISA	antigen capture-enzyme linked immunosorbant assay
AI	avian influenza
bp	basepairs
BF	Bursa of Fabricius
BSA	bovine serum albumin
°C	degrees Celsius
CPE	cytopathic effect
DMSO	dimethylsulfoxide
E	glutamic acid
ECE	embryonated chicken egg
E. coli	*Escherichia coli*
EEV	eukaryotic expression vector
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbant assay
EID_{50}	embryo infectious dose 50
EM	electron microscopy
FPV	fowlpox virus
FBS	fetal bovine serum
G	glycine
g	gram
GFP	green fluorescent protein
h	hour
HVT	turkey herpesvirus
IBD	infectious bursal disease
IBDV	infectious bursal diseases virus
IB	infectious bronchitis
IBV	infectious bronchitis virus

IFA	incomplete Freund’s adjuvant
IHC	immunohistochemistry
kDa	kilodaltons
Kb	kilobases
L	liter
MAbs	monoclonal antibodies
MD	Marek’s disease
MDV	Marek’s disease virus
mg	milligram
µg	microgram
min	minutes
mL	milliliter
NS	non-structural
ND	Newcastle disease
NDV	Newcastle disease virus
OD	optical density
ORF	open reading frame
P	proline
PBST	phosphate buffered saline with Tween-20
PEI	polyethylenimine
pfu	plaque forming unit
Q	glutamine
RdRp	RNA-dependent RNA polymerase
RT-PCR	reverse transcriptase-polymerase chain reaction
RT	room temperature
S	serine
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SD	standard deviation
sec	second

Sf-9	*Spodoptera frugiperda* cell line
SPF	specific-pathogen-free
TBS	tris-buffered saline
$TCID_{50}$	tissue culture infectious dose 50
V	volts
Vero	African green monkey kidney cells
VLPs	virus-like particles
VN	virus neutralization
WB	Western blot
µL	microliter

CHAPTER 1

INTRODUCTION

1.1. *In ovo* vaccination

Today, *in ovo* vaccination against Marek's disease virus (MDV) is a worldwide practice in broiler production. Indeed, *in ovo* injection is practiced in 30 countries around the world, and in more than 85% of all broilers in the U.S.

The embryonated egg is an immobile target that can be easily accessed by high-speed automated equipment, such as the commercial egg injection system used for the vaccination of the embryo. Birds vaccinated *in ovo* for Marek's disease (MD) exhibit better performance in the field than birds vaccinated at hatch due to reduced stress associated with the elimination of manual handling during vaccination. Vaccine delivery is aimed into the amniotic cavity of the embryo. Thus, *in ovo* vaccinated chicks are healthier chicks due to a more uniform, and early immunity. Since the vaccination against MD proved to be successful, attempts have been made to deliver other vaccines, such as infectious bursal disease virus (IBDV) (Haddad et al., 1997), reovirus (Guo et al, 2003), DNA (Oshop et al., 2003), infectious bronchitis virus (IBV) (Wakenell et al, 1995), and Newcastle disease virus (NDV) (Karaca et al., 1998). One important question addressed regarding *in ovo* vaccination research is the ability of the vaccine to overcome maternal

antibodies, mount an immune response, and not interfere with the level of protection conferred by MD vaccine.

1.2. Newcastle disease (ND)

ND is a highly viral contagious, fatal disease of all species of birds. Clinical signs of ND vary from unapparent to highly virulent forms, depending on the virus strain, isolate, and the host species. It's ethiological agent, Newcastle disease virus (NDV) is grouped into three main pathotypes: velogenic, mesogenic, and lentogenic. Velogenic strains cause acute, lethal infections of chickens of all ages, and hemorrhagic lesions of the digestive tract are frequent. This form of disease is termed viscerotropic velogenic Newcastle disease (VVND). Velogenic strains can also cause a second form of disease, characterized by respiratory and neurological clinical signs, denoted neurotropic velogenic (NVND). A less pathogenic form of NVND affecting young birds is caused by mesogenic strains. Mesogenic strains are often used as secondary live vaccines. Lentogenic strains may cause mild or unapparent respiratory infections, and are routinely used as live vaccines (Alexander, 1997).

NDV belongs to the family *Paramamyxoviridae*, genus *Rubulavirus*. Members of this family are enveloped, nonsegmented, single stranded, negative-sense RNA viruses. It's entire genome consists of 15,186 nucleotides, with six structural genes in the order of 3'-NP-P-M-F-HN-L-5', which encodes at least seven proteins (Nakaya et al., 2001; Krishnamurthy and Samal, 2000; Phillips et al., 1998; Steward et al., 1993; Peeples,

1988). The nucleocapsid protein (NP) binds to the genomic RNA forming the nucleocapsid core. The phosphoprotein (P) and the large polymerase protein (L) are associated with the nucleocapsid core. Together, they form a tight functional ribonucleoprotein (RNP) complex. The matrix protein (M) forms the inner layer of the envelope. Hemagglutinin-neuraminidase protein (HN) is a glycoprotein attached to the envelope of the virus and it's responsible for the attachment of the virus to the host cell receptor. HN is recognized by the host immune system, and it can elicit a humoral immune response. NDV envelope also exhibits another glycoprotein called Fusion (F), which mediates fusion of the viral envelope with the host cell membrane. It is considered the most immunogenic protein of NDV.

Mesogenic and lentogenic (La Sota, B1) strains of NDV are widely used as live vaccines against ND to protect birds from severe clinical signs. However, it neither prevents virus infection in vaccinated flocks nor shedding from vaccinated to unvaccinated animals. Live NDV vaccines may cause mild respiratory disease, leading to increased susceptibility to secondary bacterial infections. In addition, these viruses are attenuated and their capacity to revert pathogenicity, spread to susceptible flocks and cause severe disease is always present. Another disadvantage of live vaccines is the need for refrigeration, a major problem in developing countries with intensive poultry production. Inactivated vaccines use β-propiolactone or formalin to kill the virus, and are mixed with aluminum hydroxide or oil-emulsion as an adjuvant. They are expensive to produce and to apply, which creates high costs in labor to administer individual intramuscular or subcutaneous injections. Such vaccines also cause severe local

inflammatory reactions and the site of injection must be removed to improve the appearance of the chicken for consumers.

1.3. Infectious bursal disease (IBD)

IBD, also known as Gumboro disease, is an acute, contagious viral disease of poultry. In chickens, three to six weeks of age infectious bursal disease virus (IBDV) causes severe immunosuppression and mortality (Lukert and Saif, 1997). IBDV targets the lymphoid tissue, specially the B-lymphocytes of the bursa of Fabricius (BF). In younger chickens, less than three weeks of age, IBDV causes subclinical disease with severe bursal damage, which leads to immunosuppression, increased susceptibility to other infections, and vaccination failure (Kibenge et al., 1988).

IBDV belongs to the family *Birnaviridae,* and genus *Avibirnavirus*. It is non-enveloped, and it has two segments of double-stranded RNA. Other members of this family include infectious pancreatic necrosis virus (IPNV), tellina virus, sandgoby virus, oyster virus, blotched snakehead virus, and the Drosophila X virus. IBDV genome consists of a large segment (3.3 Kb) of double stranded RNA, segment A, and a smaller segment (2.9 Kb) of double stranded RNA, segment B (Mundt and Muller, 1995; Azad et al., 1985). Segment A consists of two overlapping open reading frames (ORF). The larger ORF encodes VP2, VP4, and VP3, while the smaller ORF encodes VP5 (Mundt et al., 1995). Segment B encodes VP1, the RNA dependent-RNA polymerase (RdRp) (Hudson et al., 1986; Azad et al., 1985). VP2 is the major structural protein of the virion, and

ranges from 41 to 54 KDa. VP5 is a non-structural protein of unclear function, present only in infected cells, and it is not required for replication (Yao et al., 1998).

Vaccination of broiler breeders with live followed by several inactivated vaccines is a common practice in the poultry industry. This practice provides passive immunity to progeny by the transmission of immunoglobulins (IgY) via yolk (van den Berg et al., 1991). Live attenuated vaccines produced in tissue culture or chicken embryos can be administered via spray, drinking water or eye drop to young chicks. However, it creates a concern regarding the optimal time for vaccination of young chicks, since live attenuated vaccine virus could interfere with maternal immunity. Besides, an incomplete attenuation of IBDV could lead to severe vaccine reaction due to residual pathogenicity. IBDV vaccines also present the risk of reversion from attenuated to a more virulent form of IBDV in the field, as some other live vaccine does. The level of protection increases proportionally to the pathogenicity of the strain used for vaccination. Thus, IBDV strains of intermediate virulence present a higher residual pathogenicity that leads to bursal damage, and resultant immunosuppression.

1.4. DNA vaccines

DNA vaccines, also called nucleic acid vaccines, genetic vaccines, or naked DNA vaccines, are based on recombinant DNA technology, where a DNA sequence encoding the protein or proteins of interest is cloned into a eukaryotic expression vector (EEV). The constructed plasmid is grown in bacteria (*Escherichia coli*), purified, and inoculated

into the host to be vaccinated. Wolff and co-workers were first to describe that a simple inoculation of plasmid DNA could generate protein expression (Wolff et al., 1990). The exact mechanism of how DNA vaccines work is not well understood. One hypothesis is that after intramuscular injection, the plasmid DNA transfects muscle host cells and inside their nuclei starts gene transcription. The resulting mRNA goes to the cytoplasm and initiates protein translation. The resultant protein of interest is processed and presented to the immune system. DNA vaccines are able to induce cytotoxic T lymphocyte (CTL) via the major histocompatibility complex (MHC) class I. Humoral response is generated by free antigens, and by antigen presenting cells (APCs), which activate T-helper cells via the MHC-II pathway (Robinson, 1997). The first report of DNA vaccinations was published in 1992 using mice as a model and gene-gun as the delivery technique (Tang et al., 1992).

Most of the knowledge regarding DNA vaccines was generated using mice as a model in order to study human diseases. In poultry, several DNA vaccines were tested against many pathogens, such as infectious laryngotracheitis (Keeler, 2000; Cheng et al., 2000), avian influenza (Kodihalli et al., 1997; Robinson et al., 1993; Fynan et al. 1993), coccidia (Song et al., 2001), IBDV (Oshop et al., 2003; Wu et al, 2000; Fodor et al., 1999), infectious bronchitis virus (Kapczynski et al., 2003; Seo et al., 1997), and NDV (Oshop et al., 2003; Sakaguchi et al, 1996). The use of DNA technology in poultry to generate specific antibodies for diagnostic purposes was also described successfully using the H5 gene from avian influenza (Lee et al., 2003).

A better delivery system for DNA vaccination has to be developed for its use in poultry, where a large number of birds must be vaccinated without the need to inject individual birds. One approach for large-scale delivery is *in ovo*. The use of DNA vaccines *in ovo* has not been studied in great detail and many aspects that could compromise vaccine efficacy were not addressed.

1.5. Rationale and significance

Commercial poultry comprises the largest segment of food animal production globally. Most of the live vaccines have been developed by manipulation of pathogenic microorganisms, such as virus attenuation by *in vitro* passage or by chemical treatment. These procedures cause changes in their genomes. Although the current vaccines available to control ND and IBD have greatly contributed to the health of commercial poultry, these vaccines are not risk free. It is well documented that IBDV vaccines do cause minor immunosuppression. Live NDV vaccines may cause mild respiratory disease, leading to increased susceptibility to secondary bacterial infections.

IBDV and NDV are viruses of major economic importance to the poultry industry. There are several reports of isolation of very virulent strains in Asia, Europe, and South America as well as antigenic variants of IBDV from vaccinated flocks, in the US. These variant strains of IBDV are able to break maternal immunity and induce disease (Snyder et al., 1992; van den Berg et al., 1991; Chettle et al., 1989).

There is a clear need for a more efficient vaccine in order to control, prevent or even eradicate IBD. It has to be a vaccine capable of protecting young flocks from immunosuppressive as well as classic forms of the disease.

1.6. Research objectives

We propose to develop and evaluate a more effective way to protect poultry flocks against IBDV. In our first objective, we plan to use a well-established practice of *in ovo* vaccination and optimize a delivery system using a DNA vaccine expressing the hamagglutinin-neuraminidase (HN) gene from NDV. In second objective, a DNA vaccine against IBDV will be constructed and delivered following the criteria established in the first objective. Our DNA-based and live attenuated vaccine will be constructed to express epitopes from standard D78 vaccine strain, and the variant GLS strain. We intend to test the IBDV vaccines (DNA and recombinant) in specific-pathogen-free (SPF) and commercial 18-day-old embryos to examine the possibility of maternal antibodies interference.

Initially, we planned to work on DNA vaccines for NDV. However, challenge studies to assess protective immunity would require a biological level three (BL3) facility, unavailable at this moment. So, we decided to switch to a plasmid expressing structural proteins (VP2-VP4-VP3) of IBDV that requires a BL2 facility. All preliminary results obtained from DNA-NDV studies will be used in the following experiments for IBDV. The air cell and the amniotic routes will be compared for *in ovo* DNA vaccination.

In a separate experiment, we will also compare two formulations for DNA vaccine delivery, a transfection reagent (PEI-ExGen®) and dimethylsulfoxide (DMSO) associated with incomplete Freund's adjuvant (IFA). We will use the information regarding route and formulation gathered previously to compare five different dosages of plasmid DNA. In a final *in vivo* experiment, we will access the humoral immune response after hatch of *in ovo* DNA vaccinated chickens.

The plasmid DNA encoding IBDV proteins will be used to address the issue regarding maternal antibody, and vaccine safety and efficacy. Eighteen-day-old, SPF embryonated eggs will be compared to broilers to assess the ability of the virus to break through the maternal antibody barrier and mount a protective immune response against IBDV challenge.

We also propose to verify the possible use of a recombinant live attenuated vaccine by *in ovo* delivery into SPF eggs (lacking maternal antibody), and also in a commercial fertile broiler eggs facing standardized challenge studies against IBDV. This vaccine was developed earlier in our laboratory. Vaccine efficacy will be determined following the standard OIE requirements. All animal studies will be performed accordingly to protocol approved earlier by the Institutional Animal Care and Use Committee, University of Maryland, College Park.

CHAPTER 2

LITERATURE REVIEW

2.1. *In ovo* technologies

The American poultry industry is one of the most prominent in the world. Commercial hatcheries in the 19-state weekly program set 206 million eggs per week in 2003, a 3 percent increase compared to last year. Average hatchability for chicks hatched during this period was approximately 83 percent (WATT poultry publication, Monday December 29, 2003). In order to keep up with this fast pace, this industry is developing new technologies in all sectors of production. In the hatchery, *in ovo* technologies were created to sex, candle, vaccinate, and transfer fertile eggs to increase productivity.

The embryo development takes place outside the hen's body, and it can be easily manipulated to improve poultry production. Considering that a broiler chick reaches marketable weight in 32 to 48 days, the embryonic period composes 30-40% of a broiler's total lifespan, and represents a crucial phase in the avian production life cycle. Minimal changes regarding temperature, setting position, humidity, and contamination levels can have tremendous consequences in broiler performance.

In ovo technology was initially developed for the application of Marek's vaccine in 18-day-old embryos (Sharma and Burmester, 1982). Today, this technology is also

used for antibiotic therapy, delivery of other vaccines besides Marek's, egg-candling to determine viable fertile eggs, and transfer from incubators to hatchers. Also, *in ovo* technologies are being used to provide samples for diagnosis to help disease surveillance and epidemiological studies. The next revolutionary use of this technology is under way to select embryos by sex and even more ambitiously, to change the sex of the embryo by the introduction of avian embryonic stem cells (Ricks et al., 2003). In certain sectors of production, such as egg production, a female sex is more desirable. In broilers, males in general have better feed conversion than females. Additionally, broilers reach marketable weight much faster than females. Under these circumstances, males are highly desirable. This new technology is still in its infancy but it won't be too long before it's available for commercial use.

2.2. *In ovo* vaccination

Embryo vaccination was developed in order to provide adequate time for chicks to respond to MD vaccination before exposure to field virus. MD is a highly contagious malignant T-lymphomatosis of chickens caused by virulent MDV. MD was controlled for years by vaccination. It was the first cancer-causing virus that could be prevented through immunization. Vaccine viruses include apathogenic MDV1, naturally apathogenic MDV serotype 2 (MDV2), and HVT serotype 3 (MDV3). However, during the 1980s, MD control became less successful when more virulence strains of the virus appeared in the field. In 1992, embryo vaccination against MD was introduced commercially to

hatcheries in the USA in order to confer early protection to young chicks exposed to field challenge (Sharma et al. 1982).

Commercial *in ovo* vaccination of embryos between days 18 and 19 of incubation against MD with HVT started in 1992 (Sharma, 1987). At eighteen days of incubation, the eggs are removed from the incubators, vaccinated against MD, and transferred to the hatchers. Not all vaccines currently licensed for MD have been approved for use *in ovo*. However, some of the currently available HVT, SB-1, and serotype 1 vaccines (CVI-988) have been approved for *in ovo* administration (Wakenell, et al., 2002).

HVT vaccines after *in ovo* inoculation resulted in high titers of the virus in non-lymphoid, and non-macrophage cells in the lungs of the embryos indicating the importance of this organ to induce proper immunity (Sharma, 1987).

Many factors may influence the efficacy of vaccines delivered *in ovo*. The embryo position is one of them. The location of the embryo or surrounding fluids is dependent on the percentage of water loss from the embryonated eggs to the environment through incubation, the embryo's age at inoculation, and the size of the eggs (Wakenell et al., 2002). The egg size is influenced by the age of the hen. Older breeders lay bigger eggs. Needles used for automated *in ovo* injection present a standard length. Thus, eggs presenting a larger size may not allow the vaccine to be inoculated into the desired amniotic cavity. Another important factor is the status of the maternal antibodies transferred from the yolk to the embryo after 18-19 days of incubation. Many vaccines

are unable to overcome the maternal antibodies and establish an immune response. Maternal antibodies induced by heavy immunization of breeders could neutralize the *in ovo* administered vaccine. On the other hand, if the vaccination of young chicks is delayed until the maternal immunity wanes, these birds may have an interval where they would be more vulnerable to viral infection since the active protection by immunization may not be available yet.

At eighteen days of embryonation, when most *in ovo* vaccination occurs, fertile chicken eggs consist of four compartments: 1) the air cell, 2) the allantoic sac, a fluid-filled compartment positioned along the shell and below the air cell membrane, 3) the amniotic sac, which surrounds the amniotic fluid, yolk sac, and embryo and 4) the yolk sac, which lies within the amniotic sac. Beginning on day 16-17 of incubation and continuing on day 18, a normal embryo increases in size and pushes the allantoic fluid to the sides and to the small end of the egg. At the same time, the total volume of allantoic fluid is rapidly reduced because of water resorption by the embryo and moisture loss through the eggshell. The yolk sac is being absorbed by the embryo to inside its abdominal cavity (Wakenell et al., 2002).

Commercially, the amniotic cavity is the target for *in ovo* injection. Wakenell and co-workers evaluated one-day-old chicks vaccinated subcutaneously, and four *in ovo* routes (amniotic, allantoic, air cell, and embryonic) using a HVT/SB1 MDV vaccine. Embryos vaccinated via air cell were not protected against MD-RB1B challenge. Challenge protection results of birds vaccinated at hatch were similar to results verified in

embryos vaccinated by the amniotic and embryonic routes when challenged at 5 days post-hatch (Wakenell et al., 2002).

Zhang and Sharma, addressed the concerns regarding inducible immunological tolerance by *in ovo* vaccine administration. HVT was injected *in ovo* in different stages of embryo development. The inability to show HVT antibody response by serologic assays was the criteria used to verify immunotolerance in viremic birds. Embryos exposed to HVT at fourteen days of incubation or earlier presented significant tolerance (Zhang and Sharma, 2003).

Many attempts to use *in ovo* vaccination against an important poultry pathogen, IBDV have been made. Sharma evaluated a low virulence IBDV vaccine (TC-IBDV and BVM-IBDV) by inoculating 18-day-old embryos and challenging them at 3 weeks of age (Sharma, 1985). Protection of the *in ovo* vaccinated group was similar to the group vaccinated at one-day of hatch and hatchability was not affected by the vaccine. However, *in ovo* vaccinated chicks exhibited bursal damage, as verified by histopathology, in a higher degree than birds vaccinated at hatch. Also, the dual vaccination against IBDV and MD was evaluated. Dually vaccinated group showed similar protection against both diseases when compared to chickens vaccinated with single vaccines (Sharma, 1985). In a different experiment addressing the use of *in ovo* vaccine against IBDV, embryos vaccinated with different dosages of a BVM strain of IBDV showed virus replication in several tissues at one day up to 7 days post-vaccination. Lung, thymus, proventriculus, liver, kidney, and spleen were the main sites

for virus replication. Tissue distribution after *in ovo* vaccination showed similar results when compared with birds vaccinated at hatch. In addition, vaccination *in ovo* generated a protective immunity against IBDV challenge at 4, 6, 8 and 10 weeks post-hatch. The lowest dosage tested (6.2 median embryo lethal dose) was also able to protect vaccinated birds against IM-IBDV challenge strain (Sharma, 1986).

Live intermediate IBDV vaccines (containing classic strains of the virus) from three different vaccine companies were administered *in ovo*, and evaluated for safety and efficacy. *In ovo* vaccination with a half dose of vaccine in commercial broilers (with maternal immunity) conferred protection against standard and variant strains of IBDV (87-94%, and 60-74% respectively). Minor bursal damage was observed at one-day post-hatch but not at 3 weeks. Hatchability and post-hatch survival was not affected. The authors indicated the need for a higher protection against variant strains, and proposed to evaluate a new vaccine that includes antigenic variant strains for *in ovo* use (Giambrone et al., 2001).

More recently, a new generation of live attenuated vaccines was created against reovirus and IBDV, where there is a formation of an immune complex of IBDV or reovirus with its specific antibodies. Against IBDV, there are two commercial vaccines, named CEVA Transmune IBDV vaccine, and IBDV-Icx, respectively, produced by Embrex Inc., Research Triangle Park, NC, and CEVA-Phylaxia Ltd., Budapest. Ivan et al. evaluated the transient bursal damage caused by CEVA vaccine. CEVA Transmune IBDV vaccine contains an IBDV strain (identified as 2512), containing $10^{2.0}$ EID50%

per recommended dose. *In ovo* vaccinated birds demonstrated depletion of bursal follicles, detected by immunohistochemistry in SPF and broiler birds. Bursal damage in broilers was detected later in age, and it was less severe than in SPF birds and of less duration (Ivan et al., 2001). The second experiment described below also evaluated the immune response of birds vaccinated with an immune complex against IBDV. IBDV-Icx and IBDV-2512 attenuated strain were injected *in ovo*. Neither vaccine mounted an antibody response by 21 days post-vaccination. Birds vaccinated with IBDV-Icx were challenged at 21 and 35 days after vaccination and showed respectively 83%, and 77% of protection against standard challenge strain of IBDV. The IBDV-2512 vaccinated birds presented bursal atrophy; protection was not evaluated. The lack of humoral responses indicated the presence of cellular immunity since degrees of protection was still observed (Corley et al., 2002). The cell immunity role played in protection was evaluated in a different experiment by Corley et al., 2002. SPF embryos were vaccinated at 18 days of incubation with IBDV-Icx and IBDV-2512. T cell mitogenic responses, CD4+, CD8+ T cell profile, and B cell percentages were examined. The T cell response assay in birds vaccinated with IBDV-2512 induced greater T cell suppression than IBDV-Icx. This suppression was not related to the proportion of T cell since the T cell profile was similar to both vaccines. The detection of B cells by flow cytometry revealed a decreased percentage for both vaccines when compared to unvaccinated group (Corley et al., 2002). Guo, and co-workers described immune complex vaccines against reovirus. Reovirus vaccine strain was compared with vaccines containing the same virus vaccine strain combined with different dilutions of the antibody for the immune complex. All vaccines were administered *in ovo* at 18 days of incubation. Birds vaccinated with immune

complex had delayed antibody responses and virus recovery when compared to birds that received the virus alone. Post-hatch mortality was 13.3% lower in the immune complex vaccinated group (Guo et al., 2003).

Attempts to vaccinate chicken embryos against IBV were also made. IBV is an economically important, highly contagious disease caused by a coronavirus. Wakenell and collaborators compared chickens vaccinated *in ovo* and at hatch using the virulent Holland strain (vIBV), and commercially available Massachusetts type (10^6 EID50%), which was attenuated after 40 passages in tissue culture (p-IBV). High antibody responses were observed between birds vaccinated *in ovo* with either vaccine. The p-IBV caused more lesions in the trachea and lungs when administered *in ovo*, and similar lesions were also observed in one-day-old birds vaccinated with v-IBV. Trachea and lung lesions were almost nonexistent at 17 days post-hatch. No kidney lesions were observed in any vaccinated group (Wakenell et al., 1995). Another study evaluating the use of live attenuated vaccines *in ovo* against IBV was reported by Kapczynski and co-workers. Embryos were vaccinated after 18-days of incubation, in the chorioallantoic sac, using the Arkansas serotype of IBV strain. Tissue distribution was determined by probing bursa, lung, spleen, heart, and thymus with a digoxigenin-labeled antisense S1 riboprobe to detect viral mRNA. Tissues were collected 24, 48, 72 and 120 h post-inoculation. Samples from the lungs were positive for IBV 24, 48 and 72 h after inoculation, and 48 h post-inoculation in the BF. No viral mRNA was detected in heart, thymus, or spleen at any time point. These results suggest that an initial infection by IBV in the lungs of inoculated embryos spread further to the BF (Kapczynski et al., 2002).

In ovo vaccination against NDV using fowlpox virus (FPV) as a vector was also examined (Karaca et al, 1998). Interestingly, this construct not only expresses the HN and F genes of NDV but also the gene encoding interferon type I (IFN). One-day-old SPF chicks and 17-day-old embryonated eggs were inoculated with FPV, FPV-NDV, FPV-IFN, and FPV-NDV-IFN. Two weeks after hatch, the birds were challenged with NDV-GB Texas (standard NDV challenge strain). No significant differences were reported regarding body weight among all groups. Animals from FPV-NDV-IFN and FPV-NDV were fully protected against challenge. Challenge against FPV was also performed and all groups (*in ovo*/one-day old) were protected (90%). All groups exhibited a humoral response to NDV by ELISA and virus neutralization assays (VN). However, the group vaccinated with FPV-NDV-IFN showed significantly lower titers. The authors attributed lower humoral responses to a down regulation played by IFN type I (Karaca et al, 1998). A similar study was performed in turkeys by the *in ovo* route, using FPV as a vector for NDV and interferon type I and II. Hatchability, survival, performance and weight again were not significantly different among vaccinated groups. Higher antibody responses and protection against NDV challenge was detected in turkeys that received FPV-NDV-IFN-II vaccine. Interferon-γ (IFN type II) is a potent macrophage activator, and immunomodulator. The authors speculated its role as a vaccine adjuvant to justify the better results presented by FPV-NDV-IFN-II (Rautenschlein et al., 2000).

The immunity of an avian pneumovirus vaccine administered *in ovo* was also evaluated in turkeys. Turkey embryos after 24 days of incubation without maternal antibodies, received a live attenuated vaccine with a dose 10 times higher than the

manufacturer's recommendation. Hatchability was not affected by the vaccination and 100% protection was observed at both 3 and 5 weeks post-hatch (Worthington et al., 2003).

2.2.2. *In ovo* vaccination against multiple viral agents

Vaccination programs vary among different companies. One-day-old chicks may receive many live vaccines in addition to Marek's. Live attenuated vaccines against IB, ND, and in some cases IBD may be administered in the hatchery via spray or at the poultry farm via drinking water, and spray. Thus, many researchers, intending to take advantage of the *in ovo* technology, have been studying the possibility of administrating multivalent vaccines against several poultry pathogens. This way, vaccination at one-day of age could be avoided, resulting in less labor, handling stress and more uniform performance. The possible drawback of this methodology is the interference among vaccine viruses competing to infect, replicate and stimulate the relatively immature host immune system. This concern is addressed in the following reports.

The use of *in ovo* vaccination against NDV presents another challenge, since all conventional vaccine strains of the virus that are nonpathogenic for hatched birds are lethal for the developing embryos (Ahmad et al., 1992). Therefore, a group of researchers used a recombinant FPV vaccine expressing the HN and F gene of NDV. SPF, 18-day-old embryos were vaccinated with a recombinant cell-associated HVT expressing the NDV gene, and glycoproteins A and B of MDV. The same vaccination program was

applied in one-day old chicks. Control groups consisted of unvaccinated birds, HVT alone, and a NDV-B1 treated with ethylmethane sulfonate. Chicks vaccinated at one-day of age and *in ovo* exhibited similar results. Hatchability and survival were not affected. Birds were considered protected after challenges against NDV (NDV-GB Texas) and against MD (RB1B) at 4 weeks post-hatch. However, the challenge NDV was isolated from the trachea of vaccinated birds, indicating partial protection (Reddy et al., 1996).

Stone and co-workers assessed protection against avian influenza (AI) and NDV using inactivated vaccines *in ovo*. The inactivated vaccine (inactivated by beta-propiolactone treatment) consisted of the Ulster strain of NDV, and H5N9 type of AI propagated in 9-day-old embryos. The inactivated virus mixture was emulsified in an oil-based solution (Drakeol) and inoculated into embryos with three different virus concentrations per egg (1X, 5X, 10X). Seroconversion was observed 2 weeks post-hatch by hemagglutination inhibition assays (HI). Complete protection was observed against NDV and influenza challenge at 53, and 34 days post-hatch, respectively (Stone et al., 1997).

SPF embryos that were vaccinated with a multivalent vaccine against MD, IBDV, FPV, and NDV, were fully protected against challenge. The vaccine mixture consisted of a commercially available recombinant FPV vaccine expressing NDV genes; serotypes 1, 2 and 3 of MD (CVI988, 301/B1, HVT), and of an IBDV strain (2512). The vaccine mixture induced antibody immune response and did not affect survival. Unfortunately, hatchability was decreased 23-26% when rFVP-NDV was present in the mixture (Gagic

et al., 1999). This same mixture was used in commercial embryonated eggs containing maternal antibodies. Protection against IBDV, NDV, and FPV was 100%, 81%, 86%, respectively. Birds vaccinated *in ovo* against MD with HVT alone, at one-day-old against IBV and NDV, and boosted 2 weeks later with IBV and NDV, gave 100%, 19% and 0% protection (Sharma, 2002).

Guo and co-workers determined the association of Marek's vaccine, and immune complex vaccine against reovirus. SPF embryos were vaccinated *in ovo* with an immune complex formed by reovirus vaccine (Synvac, $10^{3.5}$ TCID50%), and serum from hyperimmunized chickens, diluted to 1:8. Commercial embryos with maternal antibodies were vaccinated with a 1:16 serum dilution in the immune complex. The vaccine against MD was a bivalent HVT/SB1 administered alone or associated with reovirus immune complexes. One half of all groups were challenged against MD at 5 days post-hatch, and the other half against reovirus 7 days post-hatch. Neither vaccines affected hatchability or survival. No significant differences in protection were observed in reovirus vaccinated birds with or without MD vaccine. On the other hand, commercial broilers exhibited lower protection levels, indicating that maternal antibodies may interfere with vaccine efficacy. Birds were also protected against MD challenges. However, a lower, statistically insignificant difference was observed in birds challenged against MD when the vaccine was administered in association with reovirus (Guo et al., 2003).

2.3. Newcastle disease

ND is a severe respiratory, neurological, or enteric disease accompanied by high mortality in chickens, which causes significant economic losses to the poultry industry. It can infect all species of birds. The disease exhibits different degrees of severity, depending on the pathotype. The virus is categorized in three main pathotypes: velogenic, mesogenic, and lentogenic. Velogenic strains can produce acute infections with high mortality. Mesogenic strains exhibit intermediate virulence and more moderate clinical signs. Lentogenic strains may cause mild disease in birds and are often used as live vaccines worldwide (Alexander, 1997).

2.3.1. History of ND

Virus isolation techniques were not available when the first ND outbreak occurred. Thus, it is almost impossible to state when the first ND outbreak happened. However, the first official ND outbreaks caused by a Paramyxovirus type 1 (PMV-1) were reported in Java, Indonesia in 1926, and in Newcastle-upon-Tyne, England. There are earlier reports of this disease from Korea and Central Europe. The disease was named “Newcastle disease” by Doyle in 1926.

PMV type 2 was isolated in 1956 by Bankowski from a chicken exhibiting respiratory clinical signs of ND. Epidemiological studies of PMV-2 reported a wider

distribution in turkeys than in chickens. In exotic birds, PMV-2 was primarily identified in passerines and psittacines.

PMV-3 was originally isolated from turkeys in Ontario in 1967, Wisconsin in 1968, and several European countries. Later, PMV-3 was also serologically identified in many other states in the US.

2.3.2. Epidemiology

The severity of NDV varies greatly depending on the host and strains. Also dose, route of administration, host's age, and environmental conditions play major roles in the severity of clinical signs. NDV has been isolated in more than 236 species from 27 of the 50 orders of birds. In commercial poultry, chickens are the most susceptible. In contrast, ducks and geese may be infected and show few or no clinical signs of the disease, even when infected with strains lethal for chickens. The disease is more severe in young chickens. Young flocks infected by virulent strains may cause high mortality, without symptoms. Oral, nasal, and ocular routes are considered natural routes of infection and cause predominantly respiratory signs. Intramuscular, intracerebral, and intravenous infection cause mainly neurological signs (Alexander, 1997).

NDV can be transmitted by aerosol and also by ingestion. Large amounts of virus are excreted in the feces and it is a main source of infection. Oral transmission can also occur by contaminated feed (Alexander, 2001). Transmission via parent to progeny is still

unclear. Experimental studies to address vertical transmission were inconclusive because of mortality during incubation of infected embryos (Lancaster et al., 1975). Another important source of infection is by humans and equipment. Vaccination crews moving from one flock to another may have their conjunctival sacs infected with NDV or may spread the virus by contaminated clothing, and shoes (Alexander, 1997).

The incubation period of ND varies between 2 to 15 days after natural exposure. NDV is sensitive to heat, irradiation, oxidation processes, pH, and chemical compounds and these processes dramatically reduce its infectivity.

2.3.3. Diagnosis of ND

ND has no pathognomonic lesions or hallmark clinical signs. Intestinal contents or cloacal swabs, feces, and trachea swabs or trachea tissues are the preferred material for virus isolation in 9-10 days embryonated chicken SPF eggs. When neurological clinical signs are present, brain should also be included for virus isolation. Vaccination with a live attenuated strain is a common practice and as a consequence it is impossible to distinguish vaccinated from infected birds. Thus, serology has limited diagnostic value. Serology techniques, such as HI, VN in chicken embryos, single radial immunodiffusion, single radial hemolysis, agar gel precipitin, plaque neutralization, and ELISA have significant importance in post-vaccinal monitoring or for diagnosis in non-vaccinated flocks (OIE, 2000).

To measure the true virulence of the isolated virus, laboratory assessment of the pathogenicity of the virus is necessary. Three *in vivo* tests are used: mean death time in eggs (MDT), intracerebral pathogenicity index in one-day-old chicks (ICPI), and intravenous pathogenicity index in 6-week old chickens (IVPI).

2.3.4. Prevention and control

International control policies vary greatly depending on the status of the disease in each specific country. Countries free of the disease do not vaccinate and do not allow any form of NDV to be introduced. Others allow the presence of only live attenuated vaccines made of lentogenic strains. Some countries have very virulent strains of NDV and the control is through vaccination (Alexander, 1997).

In the US, control policy is to prevent the introduction of virus and prevention of spread by strict surveillance, and quarantine. Prevention is reached by biosecurity practices and vaccination in areas of high risk. ND vaccination reduces the appearance of serious clinical signs. However, it does not block infection, replication, and viral spread.

Live vaccines from lentogenic strains, such as Hitchner B1 and La Sota are widely administered to broilers by eye-drop, spray or drinking water. Even though the immune response increases as the pathogenicity of the live vaccine increases, mesogenic strains of NDV are used only in secondary vaccination due to the risk of vaccinal reaction. Inactivated vaccines are produced from allantoic fluid, treated with beta-

propiolactone or formalin to kill the virus, and then mixed with an adjuvant. These vaccines are used in broiler breeders and layers. In broiler breeders, inactivated vaccines are used not only to prevent disease, but also to confer high level of antibodies to the progeny. Maternal antibodies are protective against NDV. Thus, it must be taken into account when timing primary vaccination of broilers (Alexander, 1997).

2.4. Newcastle disease virus

NDV is a member of the genus *Rubulavirus*, family *Paramyxoviridae*, and subfamily *Paramyxovirinae*. Many other important viruses belong to this family, such as simian virus 5 (SV5), mumps virus, and human parainflueza virus type 2, 4A, and 4B. NDV is included in the *Rubulavirus* genus because of their non-conserved intergenic sequences and lack of a C-protein open reading frame (Lamb and Kolakofsky, 2001). This classification is controversial because the organization of the NDV P gene and its mRNA editing profile resembles those of the genera *Morbillivirus* and *Respirovirus*, characteristics not present in the *Rubulavirus* genus (De Leeuw and Peeters, 1999).

2.4.1. Virion structure

Negative contrast electron microscopy of members of the Paramyxovirus genus shows very pleomorphic virus particle, generally round, ranging in size from 100-500 nm in diameter. However, filamentous forms of about 100 nm with variable length can also be seen. NDV virions are composed of a bilayer envelope derived from the host cell and

an internal ribonucleoprotein (RNP) core. The surface of the virus particle is covered with projections about 8 nm in length. The larger projection is formed by HN protein, and it is responsible for hemagglutinin and neuraminidase activities. HN is a 75 KDa protein responsible for attachment of the virus to host cell receptor. The smaller projection, fusion (F) is a 66 KDa protein and it mediates fusion of viral envelope with host cell membrane. The HN and F are the major proteins recognized by the host immune system. Interactions of these two glycoproteins facilitate the virus entry and release, which subsequently play a very important role in viral virulence (Stone-Hulslander and Morrison, 1997).

Matrix protein (M) is located inside the envelope between the inner membrane and the nucleocapsid core. M is implicated in the final assembly of virus particles (Peeples, 1991). The core has helical capsid symmetry and contains the single negative stranded genomic RNA (Nakaya et al., 2001; Krishnamurthy and Samal, 2000; De Leeuw and Peeters, 1999; Phillips et al., 1998). The genomic RNA contains 15,186 nucleotides and is encapsidated by nucleocapsid protein (NP). The NP, P, and L proteins, plus the genomic RNA form a complex, called RNP complex.

2.4.2. Genome structure and organization

The NDV genome is a negative stranded RNA, encoding six genes: 3'-NP-P-M-F-HN-L-5'. These genes are monocistronic, coding for a protein with the equivalent name. The only exception is the P gene, which encodes two additional proteins, V and W,

by RNA editing (Steward et al., 1993). In between these genes there are two consistent regions, 3' extracistronic region containing 55 nucleotides and known as the leader region, and a 5' extracistronic region of 114 nucleotides, known as the trailer (Krishnamurthy and Samal, 2000). These two regions are involved in the control of virus transcription and replication (Lamb and Kolakofsky, 2001). In the beginning and end of each gene, there are conserved transcriptional control sequences known as gene-start and gene-end. The NDV genome also consists of intergenic regions between each gene. These regions consist of 1 to 47 nucleotides (Krishnamurthy and Samal, 2000).

2.4.3. Viral proteins

The viral nucleocapsid formed by NP, P and L proteins, and the genomic RNA, is considered the minimum infectious unit of NDV. All the enzymatic activities required for transcription, replication and translation are associated with the nucleocapsids.

The 53 KDa NP surrounds the viral RNA. It plays a major role in virus replication, including encapsidation of genomic RNA to form RNase-resistant nucleocapsid, association with P-L polymerase during transcription and replication, and, most likely, interaction with M protein during virus assembly. Unassembled NP concentration inside the cell controls the relative rate of viral transcription and replication (Errington and Emmerson, 1997).

P protein of NDV is rich in serine and threonine residues, which are potential sites for phosphorylation (Steward et al., 1993; McGinnes et al., 1988). P is involved in the formation of the viral polymerase complex when associated with L protein. It is also involved in the formation of P-NP complex and acts as a transcription and replication factor. In other paramyxoviruses, phosphorylated P protein is a regulator factor in RNA synthesis (Lamb and Kolakofsky, 2001). P gene is able to produce two additional proteins (V and W), by transcriptional modification of the mRNA by insertion of one or two non-template G residues respectively into the newly synthesized mRNAs (Steward et al., 1993).

The L protein is the largest protein of NDV. However, it is the least abundant one. L is also the major component of the RNA-dependent RNA polymerase in negative-stranded RNA viruses. L is responsible for mRNA capping and it is also involved in the formation of P-NP complexes.

M protein is located in between the inner layer of the envelope and the nucleocapsid core. Function of the M protein is to coordinate virus assembly (Peeples, 1991). It interacts not only with the lipid layer of the envelope and the nucleocapsid, but also with sites of HN and F on the surface of the virion.

HN is the largest glycoprotein of NDV. It is a type-II membrane protein. The host immune system recognizes this glycoprotein and elicits an antibody response. It has hemagglutinin (HA) and neuraminidase (NA) activities. The main function of HN is to

mediate virus attachment to the host cell receptors. The neuraminidase enzymatic activity prevents virus aggregation on the plasma membrane during budding. HN is also involved in cell fusion, indicating that the coexpression of HN and F is required for cell-cell fusion (Hemingway et al., 1995).

F glycoprotein is involved in cell-cell fusion and virus-cell fusion (Morrison et al., 1985). This fusion is pH-independent. The fusion protein is a type-I membrane protein and is synthesized as an inactive form (F0). Trypsinlike protease cleaves F0, which results in two disulfide-linked subunits, F1 and F2. Only after this proteolytic cleavage, fusion can take place between the envelope of the virus and host cell membrane (Gotho et al., 1992; Morrison et al., 1985; Hsu et al., 1983). The cleavage of F0 is the major virulence aspect of NDV (Peeters et al., 2000).

2.4.4. NDV replication, transcription, translation, and assembly

NDV replication follows the same pattern of other non-segmented negative-strand RNA viruses. All stages of NDV replication take place entirely within the cytoplasm. Replication is initiated when HN protein attaches to specific cell surface receptors containing sialic acid (Huang et al, 1980). Fusion protein mediates the fusion of the viral and the host cell membrane. Fusion occurs at neutral pH. The fusion protein precursor (F0) is cleaved by proteases to the active form, a disulfide bond-linked heterodimer composed of F1 and F2 (Morrison et al., 1985; Hsu et al, 1983; Kohama et al., 1981). This cleavage results in an F1 polypeptide with a highly conserved amino acid sequence

at the N-terminus, termed “the fusion sequence”. This sequence is believed to mediate fusion between membranes (Hsu et al., 1983; Richardson and Choppin, 1983). Thus, proteolytic cleavage of the F0 protein is known to be a major determinant of virulence and essential for initiation of infection. In virulent strains of NDV, the F0 protein can be cleaved post-translationally by host protease in a large variety of cell types and tissues, allowing the virus to cause a more severe clinical disease with systemic consequences. In strains of low pathogenicity, cleavage of F0 is accomplished by a more specific cell type, such as endodermal cells of chick embryo. For this reason, these viruses can replicate only in certain type of host cells, which induces a more local infection (Nagai et al., 1976). HN is also involved in the fusion of membranes by triggering a conformational change in the F protein after attachment to cell receptors (Hemingway et al., 1995; Lamb, 1993; Morrison et al., 1991). Following fusion, the nucleocapsid complex enters the cell.

After entering the cell, the nucleocapsid complex is transcribed by the viral RNA-dependent RNA polymerase (RdRp), which results in 5’ capped and 3’ polyadenylated mRNAs (primary transcription). Viral proteins are translated from the primary transcription mRNAs. Newly synthesized viral proteins help in the replication of genomic RNA, and this serves as template for further transcription (Peeples, 1988).

RdRp starts to transcribe the genome at the 3’ end, more precisely in the short leader sequence. After that, the viral polymerase transcribes all the genes in a sequential order by terminating and reinitiating at each gene junction. The junctions consist of gene-end sequence, a short variable non-transcribed intergenic region, and a gene-start

sequence. There is a loss of transcription of downstream genes due to a failure of RdRp to reinitiate transcription. As a result, there is a polar attenuation of gene transcription. When sufficient amounts of unassembled NP protein are present after translation, genome replication starts with the synthesis of a full-length complementary copy, called antigenome (+). RdRp uses the antigenome as a template for the synthesis of new viral genomes. It has been shown that NDV replication follows the 'rule of six' (Peeters et al., 2000). Related to this, is the fact that each NP monomer associates with six nucleotides of the genomic RNA (Calain and Roux, 1993). Thus, when the length of the viral genome is a multiple of six, replication takes place more efficiently. NP mediates encapsidation of the genome and antigenome. The leader and trailer regions of the genome control initiation of encapsidation.

In the first step of viral assembly, there is a formation of a helical RNP structure mediated by the encapsidation of the genomic RNA with unassembled NP protein. Subsequently, there is an association of RNP structure to P-L protein complex forming the nucleocapsid. The envelope is assembled at the cell surface. All the steps involved during viral assembly are still unknown. The viral M protein is thought to be the major driving force that brings the assembled RNP core to the plasma membrane (Peeples, 1991). The virus is released by budding through areas embedded by viral envelope glycoproteins in the host plasma membrane.

2.5. Infectious bursal disease (IBD)

IBD is an acute, contagious viral infection of young chickens. The virus affects the lymphoid tissue, mainly the B cells of BF. Thus, the most prominent lesion is necrosis of the BF, sometimes accompanied by hemorrhages in the thigh and pectoral muscles (Allan et al., 1972). It causes clinical disease and mortality in chickens 3 weeks of age or older. When younger birds are infected, it causes a severe and prolonged immunosuppression, leaving them susceptible to many other diseases, such as *Escherichia coli* (*E. coli*) gangrenous dermatitis, and vaccination failures. Fortunately, IBD is not a zoonotic disease and has no public health importance (Lukert and Saif, 1997).

2.5.1. History of IBD

The first IBD outbreak was reported in Gumboro, Delaware in 1962. In 1957, Albert Cosgrove recognized a syndrome, later termed “avian nephrosis” on a broiler farm near Gumboro, DE. For this reason the disease is also called “Gumboro disease” (Cosgrove, 1962). It was referred to as “avian nephrosis syndrome” due to severe kidney lesions found in birds that succumbed to infection (Lukert and Saif, 1997). The presence of kidney lesions generated some confusion between IBDV and IBV. This syndrome was characterized by 10% morbidity and 1-10% mortality; and it was believed to be a variant strain of IBV, called the “Gray strain”. Winterfield successfully isolated IBDV in embryonated eggs from birds immune to Gray strain, and having signs of IBD

(Winterfield et al., 1962). Several years later, this condition was also detected in other regions of the US, such as Mississippi, Alabama, Georgia, and North Carolina. In 1967, the first vaccine against Gumboro disease was licensed and named “Bursa Vac®”, which was derived from chicken embryos infected with a mild isolate of the virus (Snedeker et al., 1967). In 1970, Hitchner proposed the name “infectious bursal disease” for this disease, which caused 2% mortality, and severe lesions in the BF, and identified this organ as the primary target organ for virus infection (Hitchner et al., 1970).

Allan and collaborators reported that IBDV infection could cause immunosuppression in younger birds, leaving them more susceptible to bacterial infections, other viral infections, and failure to respond to vaccines (Allan et al., 1972).

After the discovery of immunosuppression caused by IBDV, researchers realized the importance of developing a new vaccine to aid in the control of the disease. The first attenuated vaccine for IBDV was made in 1973 from homogenated BF of chickens infected with the field isolate. Later, this vaccine was adapted and attenuated in chicken embryo fibroblasts (CEF) cells by Lukert and co-workers.

The immunosuppressive aspect of IBDV was described by Wyeth in 1975, using 1-day and 3-week-old chickens exposed to IBDV, and challenged with *Salmonella typhimurium* and *E.coli* (Wyeth, 1975).

The existence of a second serotype was described in 1980 (McFerran et al., 1980). Serotype I viruses are pathogenic to chickens. Serotype II viruses, most commonly isolated from turkeys, are apathogenic to chickens. The control of the disease by vaccination was compromised when new variants of IBDV were isolated in the US (Snyder et al., 1992; Jackwood and Saif, 1987). These new variant strains are called Delaware and GLS, which were isolated in Delmarva poultry-producing farms (Snyder et al., 1988; Rosenberger and Cloud, 1986). These variant strains can overcome the maternal immunity barrier and cause disease in the presence of high levels of maternal antibodies, elicited by vaccination with 'classical' strains. These viruses cause rapid bursal atrophy without signs of inflammation. Very virulent strains of IBDV, exhibiting 90% to 100% mortality in infected flocks, were identified in Europe and Asia in the late 1980s (van den Berg et al., 1991; Chettle et al., 1989).

2.5.2. Pathogenesis

IBDV can enter the susceptible host by the respiratory, gastrointestinal or urinary tracts. The virus exhibits tropism for certain organs and infects specific cell types and tissues. During oral infection, IBDV can be detected in macrophages and lymphoid cell populations in the cecum, and later on in the duodenum, jejunum, and liver. It enters the bloodstream, and spreads to other lymphoid tissues, such as the BF. IBDV has tropism for BF and it is the site of a secondary intense viremia (Muller et al., 1979).

The general symptoms include anorexia, soiled vent feathers, whitish or watery diarrhea, trembling, severe prostration, followed by death (Cosgrove, 1962). Chickens 3 to 6 week-old have completely developed BFs, which increases susceptibility to IBDV infections. However, chickens of all ages may become infected by IBDV (Lukert and Saif, 1997). Challenge studies using 3-day-old chicks treated with cyclosphosphamide and 4-week-old bursectomized chickens showed no clinical signs of disease (Fadley, 1976). IBDV targets lymphoid B cells in an active state of division and differentiation, which bear on their surfaces immunoglobulin M (sIgM) (Burkhardt and Muller, 1987). Infection by IBDV results in lysis of these cells, which results in the destruction of the bursa.

IBDV pathogenicity is dependent on the strains used. Classical virulent strains, such as IM strain, cause hemorrhagic lesions, atrophy of the bursa, and about 30% mortality. D78, a classical attenuated strain, causes neither mortality nor gross lesions in the bursa. GLS is a variant strain and causes bursal atrophy and immunosuppression, with very low mortality. With very virulent viruses, mortality can reach up to 70% with very severe bursal damage (Figure 1).

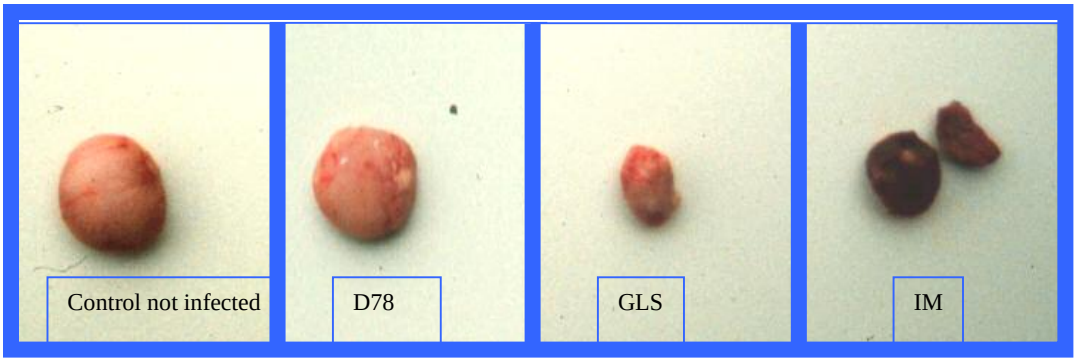


Figure 1 - Gross bursal lesions caused by different strains of IBDV. The control is a normal BF derived from non-infected chicken. Chickens infected with an attenuated strain of IBDV (D78) do not exhibit bursal lesions, whereas variant GLS strain causes bursal atrophy, and the IM strain causes hemorrhagic lesions.

BF plays an important role in the avian immune system. It is the primary site for B cells differentiation, and maturation (Glick et al, 1991). B cells are antibody-producing cells when activated. Therefore, their depletion results in a severe reduction or total abrogation of a humoral immune response. Thus, birds infected with IBDV have their immunity compromised against other pathogens in the field or vaccines. Birds infected with IBDV at day one of age and challenged by NDV virus at one week, two weeks and three weeks of age demonstrated severe, moderate, mild humoral immunesuppression, respectively (Allan et al, 1972). Avian macrophage cell lineage can also be infected by IBDV and it has been implicated in viral dissemination, and disease exacerbation (Inoue et al., 1992; Sharma and Lee, 1983).

2.5.3. Epidemiology

IBDV can infect chickens, turkeys, and ducks. Clinical signs of the disease can be seen in susceptible chickens between 3 to 6 weeks of age. Birds younger than 3 weeks do not exhibit clinical signs of IBD. However, IBDV infection can cause a severe depletion of B cells in the BF, leaving them immunosuppressed and susceptible to other opportunistic infections (Lukert and Saif, 1997).

IBDV can be spread by contact with infected birds and contaminated fomites, such as drinking water, feed, and feces. The virus is stable in poultry houses for a long time due to its resistance to many physical and chemical agents. Thus, it can be easily carried from one flock to a succeeding flock. Benton and co-workers showed that poultry houses with IBDV-infected birds were still infective for other birds 54 and 122 days after removal (Benton et al., 1967). IBDV is resistant to some disinfectants. Iodine and 0.5% formalin reduce IBDV infectivity and are widely used in the poultry industry. There is no evidence to support vertical transmission via eggs or infection via mosquitoes (Lukert and Saif, 1997).

2.5.4. Diagnosis of IBD

Acute outbreaks of IBD with characteristic clinical signs, high morbidity, spiking mortality and rapid recovery can make a preliminary diagnosis. Confirmation of the diagnosis can be made by necropsy examination of the BF, such as enlargement due to

inflammation followed by atrophy. BF, spleen and blood samples can be used for viral isolation, using 9 to 11-day-old embryos. ELISA procedure is the most commonly used serological test for evaluation of IBDV antibodies and its results are comparable to VN (Lukert and Saif, 1997). Reverse transcriptase-polymerase chain reaction (RT-PCR) can also be used to confirm the preliminary diagnosis (Jackwood and Sommer, 1999).

2.5.5. Prevention and control

IBDV can survive for long periods of time in the environment and can easily be spread from one flock to another. Thus, strict biosecurity measures are required. Vaccination of broiler breeders to boost and prolong immunity is a common practice. This vaccination regime can confer maternal immunity to the progeny in the first few weeks of life. In vaccination program breeders are heavily vaccinated with live intermediate, and intermediate plus vaccines, as well as killed vaccines containing classic and antigenic variant viruses. Live attenuated vaccines are produced in tissue culture or chicken embryos and can be administered via drinking water or eye drop to breeder pullets. Inactivated vaccines are effective in producing high levels of antibodies in breeder hens, which can pass along via yolk to their progeny (van den Berg et al., 1991). Unfortunately, oil-emulsion inactivated vaccines have to be administered manually by intramuscular injections. This methodology is not very effective because as the hen ages, the level of immunity passed to the progeny reduces (Lukert and Saif, 1997).

To overcome this problem, the poultry industry routinely vaccinates young broiler chicks, in cases where IBDV is endemic (Lukert and Saif, 1997). One of the most important drawbacks of live attenuated vaccines is determining the optimal time for vaccination of young chicks because maternal antibodies can interfere with vaccine efficacy. Incomplete attenuation of IBDV can leave residual pathogenicity in live vaccines. In addition, there is always a risk of reversion from attenuated to virulent forms of IBDV in the field. IBDV strains of intermediate virulence present a higher residual pathogenicity, even though they are unable to break through the maternal antibodies, and induce strong immune response. Highly attenuated strains used for vaccination may lose virulence and reduce the severity of bursal lesions. However, these vaccines may not be able to overcome the maternal antibody barrier in order to establish immunity.

A new generation of vaccines against IBDV has been developed in conjunction with new advances in molecular biology and recombinant DNA technologies. VP2 is the major immunogenic protein of IBDV and it induces virus-neutralizing antibodies. Several studies demonstrated the protective potential of VP2 protein when expressed in yeast (Macreadie et al., 1990), adenovirus (Francois et al., 2003), FPV (Bayliss et al., 1991), HVT (Tsukamoto et al., 2002; Darteil et al, 1995), and in insect cells by the baculovirus system (Vakharia, 1997; Snyder et al., 1994; Vakharia et al., 1993). The baculovirus-expressed proteins of GLS-IBDV fully protected chickens immunized at 6 weeks and boosted at 8 weeks of age against GLS and Delaware variant strains, and partially protected against standard challenge strain STC (Vakharia et al., 1993). In another study, the baculovirus expressed-proteins from a chimeric GLS/D78 construct also protected

chickens against STC challenge (Snyder et al., 1994). Virus-like-particles (VLPs) produced by the baculovirus system was also used as a vaccine that provided protection against challenge with variant (E/Del and GLS), and classical strains of IBDV (Vakharia, 1997). HVT was used as a vector to express VP2 protein, which conferred immunity against challenge strains (Tsukamoto et al., 2002). More recently, Francois and co-workers expressed VP2 protein in a type 1 fowl adenovirus (chicken embryo lethal orphan virus). This recombinant virus was apathogenic to chickens inoculated by several routes, including *in ovo* with different dosages. No clinical signs or mortality were observed after challenge with a very virulent IBDV (strain 89-163) in vaccinated chickens using subcutaneous or intradermic routes. Birds vaccinated *in ovo* were also protected (Francois et al., 2003).

2.6. Infectious bursal disease virus

2.6.1. Virion

IBDV belongs to *Avibirnavirus* genus of the *Birnaviridae* family and all viruses in this family contain two segments of double-stranded RNA (Dobos et al., 1995; Kibenge et al., 1988; Dobos et al., 1979). There are two other genera in this family, *Aquabirnavirus* and *Entomobirnavirus*. Viruses in the *Entomobirnavirus* genus include Drosophila X virus (DXV) of fruit fly. The *Aquabirnavirus* genus includes IPNV that infects fish, *Tellina* virus (TV), and oyster virus (OV). The virion is non-enveloped and made up of single-shelled icosahedrons of approximately 55-60 nm in diameter, having a

T=13 symmetry as shown in Figure 2 (Bottcher et al, 1997). The virion capsid is constituted of VP2 protein (51%), VP3 (40%), VP4 (6%), and 3% of VP1 (Kibenge et al., 1999; Dobos, 1979).

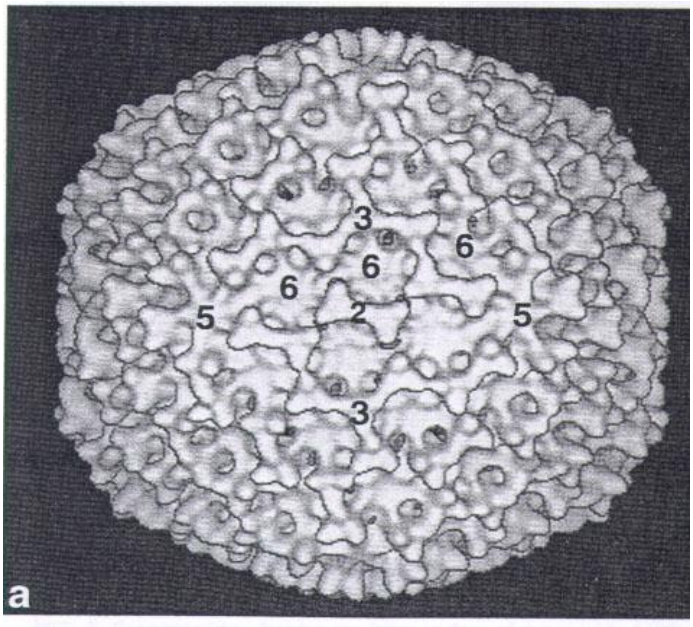


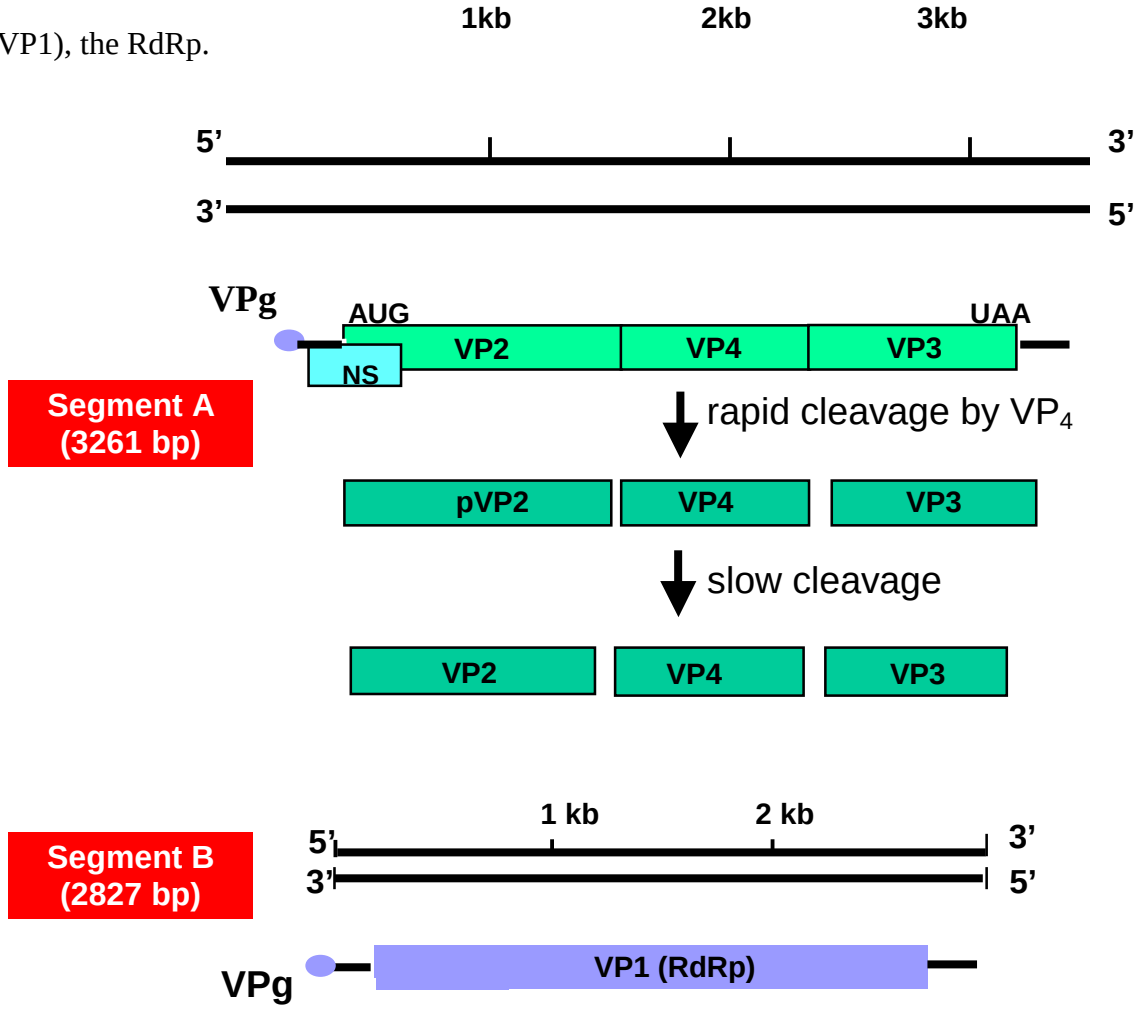
Figure 2 - Three-dimensional map of IBDV virion indicating a T=13 architecture. A close-up view down on the threefold axis of the particle. Graphics adapted from Bottcher et al., 1997.

2.6.2. Genome

IBDV genome contains two double-stranded RNA segments, A and B (Figure 3). Segment A is 3261 nt in length with two overlapping reading frames (ORF). The larger ORF encodes one polyprotein (VP2-VP4-VP3) of 1012 amino acids (Hudson et al., 1986;

Azad et al., 1985). The smaller ORF encodes one 17 KDa NS protein with 145 amino acids (Mundt et al. 1997; Spies et al., 1989). The smaller segment B is 2827 nt long. It contains only one ORF, which initiates at 112 nt from the 5' end and encodes VP1, an 879 amino acid protein with molecular weight of 94 KDa.

Figure 3 – Schematic organization of the IBDV genome. Segment A encodes mature viral proteins VP2, VP4, and VP3, and VP5 (NS protein). Segment B encodes a 94 KDa (VP1), the RdRp.



2.6.3. IBDV proteins

VP4 is a viral protease responsible for the cleavage of the polyprotein VP2-VP4-VP3 (Figure 3). Electron microscopy of the external surface of the capsid demonstrated that VP2 (42 KDa) is the major structural protein of IBDV and it forms trimeric subunits (Bottcher et al., 1997). The VP2 protein has serotype-specific group antigens responsible for antigenic variation between serotypes and strains. It is also the major antigen recognized by the host immune system (Fahey et al., 1989; Becht et al., 1988).

VP3 also forms trimeric subunits, which build up the internal surface of the capsid. This protein is the second largest structural protein of IBD virion (32 KDa) (Bottcher et al., 1997). VP3 also contains group-specific antigen (Becht et al., 1988). VP3 protein appears to bind with dsRNA and may be involved in viral assembly and replication (Tacken et al., 2002).

VP4 is a 28 KDa protein involved in the proteolytic autoprocessing of polyprotein (VP2-VP4-VP3). It has been demonstrated that VP4 is also involved in the formation of tubules (type II) in the cytoplasm of IBDV-infected cells (Granzow et al., 1997).

VP5 is a non-structural protein, which is present in infected cells and not in virion. The function of VP5 is not clearly understood. However, recent studies have shown that VP5 is not essential for virus replication, and the VP5-deficient virus can replicate *in vitro* and *in vivo* (Yao et al., 1998; Mundt et al., 1997). Moreover, this mutant

virus was attenuated and grew to lower titers than wild-type virus. Growth of the mutant in cell culture demonstrated reduced CPE. *In vivo* studies infecting chickens with the VP5 knockout virus did not cause gross microscopic lesions in the BF (Yao et al., 1998).

The RdRp (VP1) is a 90 KDa protein with several functions, such as replicase activity, guanylyltransferase activity, and capping activities (Spies and Muller, 1990; Spies et al., 1987). VP1 is present in the virion as a free polypeptide and as a genomic-linked protein (VPg), bound to the 5' ends of both genomic segments A and B (Dobos et al., 1995; Muller and Nitschke, 1987).

2.6.4. Viral replication

Attachment to the host cell membrane is the first step for viral replication to take place. It requires the interaction of the virion with a cell membrane receptor. The specific cell receptor for IBDV is not known. It seems that VP2 is responsible for interactions with cell receptor and this receptor may be composed of N-glycosylated protein present in sIgM-bearing B-lymphocytes (Ogawa et al., 1998; Nieper and Muller, 1996). After cell entry, the virus particle must be uncoated for further genome release.

After attachment, IBDV enters the cell and may initiate transcription and replication without uncoating (Spies et al., 1987). Replication and assembly take place in the cytoplasm of infected cells. IBDV replication occurs in a semi-conservative way, where the newly synthesized strand remains attached to its template. The positive-strand

RNA is packaged to make new viral particles. Finally, the negative-strand RNA is synthesized within the newly formed viral particle to complete the formation of dsRNA (Patton and Spencer, 2000). The new assembled virus particle is released to the extracellular compartment by host cell lysis and apoptosis (Yao and Vakharia, 2001; Lombardo et al., 2000; Fernandez-Arias et al., 1997).

2.7. DNA vaccines

Wolff and co-workers were first to apply the concept of DNA vaccines, also called the "third vaccine generation". They found that inoculation of chloramphenicol transferase (CAT), luciferase, or beta-galactosidase genes into the muscle of mice generated protein expression in this tissue (Wolff et al., 1990). The host immune system could recognize this expressed protein as foreign and mount an immune response against it. Thus, the idea of a DNA vaccine was generated (Cox et al., 1993; Ulmer et al., 1993; Tang, 1992).

Over the past years, research on DNA vaccination has expanded and many studies have been published. This technique consists of the insertion of DNA sequences encoding the protein (or proteins) of interest into a eukaryotic expression vector (EEV). Bacteria (in most cases, *E. coli*) are transformed by the plasmid DNA, grown in large scale, and the amplified plasmid DNA is purified for further inoculation into the animal to be vaccinated. Host cells then express the plasmid DNA, and the protein produced by the vaccinated animal elicits an immune response (Robinson, 1997; Tang, 1992). DNA

vaccines have been shown to elicit both humoral and cellular immunity, which provide protection from viral challenge (Kowalczyk and Ertl, 1999).

Several different methods and routes can be used to deliver DNA vaccines. Needle-injection into muscle tissue and into the skin is the most commonly used method. Gene-gun delivery of a DNA vaccine is used to transfect skin cells, which requires extremely small amounts of plasmid DNA. The disadvantages of this delivery method are that it requires hair removal in the inoculation site and its costs are prohibitive for large-scale vaccination.

The exact mechanism of how DNA vaccines initiate an immune response is not clearly understood. One hypothesis is that after intramuscular injection, the plasmid DNA enters the host cell and is then transcribed in the nuclei. The mRNA is translated into proteins in the cytoplasm. The new synthesized protein is processed, transported, and presented to the host immune system, initiating both humoral and cell-mediated immunity in a similar mechanism used by viruses. During transport and processing, these peptides are associated to MHC-I molecules and presented on the surface of the cell. T cell receptor (TCR) recognizes peptides associated with MHC-I molecules and these cells become activated (Babiuk et al., 2000).

DNA vaccines have also been shown to initiate humoral responses, leading to protection against viral diseases in animals (Dufour, 2001). In some cases, protection against challenge was achieved even though antibodies were not detected, indicating the

presence of cell-mediated immunity (CMI) (Kowalczyk et al., 1999). Protection using DNA vaccines has been evaluated in many different viruses (Donnelly and Ulmer, 1999), bacteria (Strugnell et al., 1997), and protozoa (Kalinna, 1997).

2.7.1. DNA vaccine advantages

One of the most important advantages of DNA vaccines is their safety. They cannot revert to virulent forms, as seen in live attenuated virus vaccine (Corr et al., 1996). DNA vaccines have a much longer shelf life than vaccines that consist of live viruses. These vaccines do not require the propagation of virulent strains of viruses nor constant refrigeration, and are easy and inexpensive to produce (Beard and Mason, 1998). Their use in animals with circulating maternal antibodies has been investigated with promising results (Babiuk, 1999).

2.7.2. DNA vaccines in poultry

The literature shows several reports of the use of DNA vaccines in poultry. Most of them investigated the route, dosage, promoters, and formulation of the DNA vaccine for optimal delivery and protection.

The first studies that used nucleic acid vaccines in poultry examined the responses to avian influenza virus. One study evaluated the protective response against a lethal virus challenge in chickens that were vaccinated by plasmid DNA containing

hemagglutinin 7 gene (H7). Three-week old chickens were inoculated intramuscularly, intravenously, and intraperitonially with 100 µg of H7 plasmid DNA. After 4 weeks, the birds were boosted with 300 µg of plasmid, using all three-immunization routes. One week post secondary vaccination, birds were challenged with a virulent avian influenza virus of the H7 subtype. Vaccinated birds showed 60% protection. DNA vaccinated birds showed minimal humoral responses after vaccination, which were elevated after challenge (Fynan et al., 1993). A similar study was carried out by Robinson and co-workers, which gave almost the same results (Robinson et al., 1993).

Additional studies were carried, which evaluated four different promoters that express hemagglutinin protein of H5 influenza virus: immediate early cytomegalovirus (CMV), Rous sarcoma virus, chicken actin, and simian virus 40 (SV-40). Only the plasmid DNA containing CMV promoter generated an antibody response in one-day-old chicks vaccinated intramuscularly. Birds in this group were boosted at three weeks of age, which resulted in partial protection. Plasmid containing CMV promoter was also used to examine the beneficial effect of possible vaccine adjuvant, such as 25% sucrose, diethylaminoethyl (DEAE) dextran, calcium phosphate, polybrene (hexadimethrine bromide), and two cationic lipids (lipotaxi and lipofectin) to help increase the uptake of the vaccine. Better results were obtained with the cationic lipids (Suarez and Schultz-Cherry, 2000).

One attempt to control coccidiosis also employed the use of a DNA vaccine, which was prepared by cloning the *Eimeria* gene. Intramuscular and subcutaneous

injections of 5 to 100 µg of plasmid DNA were injected into one-day-old chickens two weeks apart. Results from *Eimeria acervulina* challenge showed that two injections with higher amounts of DNA were more effective than one dose in reducing oocysts. Intramuscular injection resulted in higher levels of serum antibodies when compared to subcutaneous route. Also significant changes in T cell profiles were observed, indicating that this DNA vaccine can initiate local and systemic responses against *Eimeria* (Song et al., 2001).

Potential DNA vaccines for IBDV have also been studied. Fodor and his collaborators evaluated the efficacy of two plasmid DNA vaccines in chickens, one encoding the VP2 gene and the other encoding the polyprotein (VP2-VP4-VP3) genes. DNA vaccine containing the VP2 gene did not induce humoral responses or protection against challenge but groups vaccinated with VP2-VP4-VP3 genes produced antibodies and 36% protection against IBDV (Fodor et al., 1999). Another IBDV study also evaluated the VP2-VP4-VP3 genes for use as a DNA vaccine, which was administered intramuscularly with one or two booster injections. Vaccinated chickens showed no clinical signs or mortality following IBDV challenge (Wu et al., 2000).

One IBV study was conducted with plasmid DNA vaccine encoding the nucleocapsid gene. Vaccinated birds were protected against challenge. However, no antibody response was detected and protection was attributed to cytotoxic T cell responses (CTL) (Seo et al., 1997). Sakaguchi and co-workers evaluated two plasmid DNA vaccines against NDV (linear and circular form). Protection from lethal dose of

NDV was observed in birds that received a mixture of both forms of the plasmid expressing the F gene (Sakaguchi et al., 1996).

The concept of *in ovo* delivery of plasmid DNA vaccines remains novel for most diseases. Only few reports are described. In one study, a DNA vaccine was prepared for IBV, which was delivered *in ovo*. Complete protection (100%) was conferred in birds vaccinated *in ovo* and then boosted at 2-weeks of age with live attenuated vaccines. Birds that were immunized with only the DNA vaccine or live attenuated vaccine showed less than 80% protection. This report is the first to suggest the use of a DNA vaccine to prime the immune system (Kapczynski et al., 2003).

More recently, Oshop and co-workers developed a modified *in ovo* delivery technique, which one can deliver the DNA vaccine to the embryo (Oshop et al., 2003; Oshop et al., 2002).

CHAPTER 3

EVALUATION OF *IN OVO* DELIVERY SYSTEM FOR PLASMID DNA VACCINATION

ABSTRACT

In ovo vaccination against Marek's disease virus (MDV) is a common practice in more than 85% of broilers produced in the US. DNA vaccines represent a new tool to prevent infectious diseases in many species, including poultry. An *in ovo* delivery system for plasmid DNA vaccines is described in which we evaluate the route of delivery (air cell vs amniotic cavity), transfection reagent (IFA+DMSO vs polyethylenimine), dose of plasmid DNA (1 to 100 µg/egg), and the nature of humoral immune responses.

A plasmid DNA (CMV-EGFP-BGH) construct expressing enhanced green fluorescent protein (EGFP) under cytomegalovirus (CMV) immediate early promoter was used to optimize the route of delivery, and formulation for *in ovo* DNA vaccination. A plasmid expressing the hemmagglutinin-neuraminidase (HN) gene of Newcastle disease virus (pIRES-HN-EGFP) was used to evaluate five different dosages of DNA and the humoral immune responses after *in ovo* vaccination.

Higher expression of EGFP and hatchability were obtained when 18-day-old embryos were inoculated through the amniotic cavity using a cationic lipid adjuvant containing polyethylenimine (PEI - ExGen®). Transgene expression was observed even when low amounts of plasmid DNA were used (1 µg/egg). A dose-dependent response was observed with plasmid DNA concentrations of 1, 10, 25, 60, and 100 µg/egg. Better responses were detected when embryos were inoculated with 60 µg of plasmid DNA. Detectable humoral responses were observed as measured by ELISA and isotype-ELISA assays.

INTRODUCTION

In the 1990s, an entirely new type of vaccine (DNA vaccines) was first described (Robinson et al., 1993; Ulmer et al., 1993; Wolff et al., 1990). These new vaccines used naked plasmid DNA to express foreign proteins in the host. DNA vaccines are specially modified bacterial plasmids that usually have an *Escherichia coli* origin of replication, an antibiotic resistant gene, and eukaryotic promoter that drives the expression of the target gene, a target gene, and a polyadenylation signal sequence. The target gene is usually an antigenic protein from a pathogenic infectious organism. The plasmid DNA is commonly delivered either by intramuscular injection or with the use of a gene-gun that forces the DNA into epidermal cells.

Since these initial reports on this novel vaccine technology, DNA vaccines have been successfully used to immunize a number of different animal species against a

multitude of infectious agents (Sakaguchi et al., 1996; Corr et al., 1996; Scholz et al., 1993; Fynan et al., 1993). DNA vaccines have also been successfully used in poultry to immunize against several pathogens (Sakaguchi, 1996; Fynan et al., 1993; Robinson et al., 1993). Many of these experimental procedures used large amounts of plasmid DNA in several applications. These methods employ impractical delivery systems, such as by gene-gun or intramuscular injections, which currently are not suitable for administration to large numbers of birds in a cost effective manner.

In order to be suitable for poultry, DNA vaccines have to be easily administered to large numbers of animals at the same time. The air cell route for *in ovo* delivery of plasmid DNA was examined previously, and protein expression was demonstrated in the embryo using the chloramphenicol acetyl transferease (CAT) reporter gene (Oshop et al., 2003). Several eukaryotic expression vectors with different promoters have been evaluated, and all avian studies reported higher levels of expression when using human cytomegalovirus immediate early promoter/enhancer (CMV) (Oshop et al, 2003; Kapczynski et al., 2003; Suarez et al, 2000; Akiyama et al., 1994; Scholz et al., 1993).

Plasmid DNA can be easily degraded after delivery by host endonucleases (Lewis and Babiuk, 1999). In order to protect and enhance plasmid DNA expression, many attempts to develop an adjuvant have been made. Calcium phosphate, diethylaminoethyl (DEAE) dextran, 25% sucrose, polybrene (hexadimethrine bromide), and two cationic lipids (lipotaxi and lipofectin) were evaluated as adjuvants to an avian influenza DNA vaccine when injected into the muscle of one-day-old chicks. Lipotaxi and lipofectin

induced better antibody responses (Suarez et al, 2000). In addition, two possible adjuvants for *in ovo* DNA vaccination, neutral lipid incomplete Freund's adjuvant (IFA) mixed with dimethylsulfoxide (DMSO), and a cationic lipid (LipofectAmine Plus®), were also evaluated. Better results were obtained by IFA mixed with 50% DMSO (v/v) (Oshop et al., 2003). Another study done *in vitro* suggests the use of PEI (ExGen®) to obtain optimal transgene expression (Heckert et al., 2002).

Another important aspect to be evaluated is the dose of plasmid DNA required to obtain high protein expression. Earlier studies (Oshop et al., 2003; Suarez et al., 2000) observed a dose-dependent response using one-day-old chicks and 18-day-old embryos, respectively. Suarez and co-workers used 10, 50, 100, and 250 µg of pCI-neoHA/bird intramuscularly. A maximum response was observed when 100 µg of plasmid was used (Suarez et al., 2000). *In ovo* inoculation studies by Oshop and collaborators, reported better responses using 60 µg of plasmid (Oshop et al., 2003).

Several studies in recent years addressed the nature of plasmids, such as promoter and polyadenylation signals (Kapczynski et al., 2003; Oshop et al., 2003; Heckert et al., 2002; Suarez et al., 2000). Several routes for optimal plasmid DNA delivery, such as intramuscular (Heckert et al., 2002; Fodor et al., 1999), transcutaneous (Heckert et al., 2002), and *in ovo* (Oshop et al., 2003; Kapczynski et al., 2003) were examined. There are few reports regarding the dose of plasmid DNA vaccine for optimum transgene expression and protection efficacy (Oshop et al., 2003; Heckert et al., 2002; Suarez et al., 2000; Sakaguchi et al., 1996).

There are only a few studies that address the issue of *in ovo* delivery of DNA vaccines in chickens. Therefore, the present study was designed to address several parameters to optimize *in ovo* DNA vaccination in chickens. This study has several objectives. We will compare the air cell route previously examined for plasmid DNA vaccination and the amniotic cavity, the route routinely used by the poultry industry to deliver Marek's vaccine. We also propose to determine the most efficient transfection reagent for *in ovo* DNA vaccines (IFA+DMSO vs PEI-ExGen®). Another important aspect addressed in this chapter is the dose of DNA vaccine for *in ovo* inoculation. We also evaluate the humoral immune responses of DNA vaccine expressing the HN gene of NDV, after *in ovo* delivery.

MATERIALS AND METHODS

3.1. Construction of plasmid DNAs

To evaluate route and formulation for *in ovo* DNA delivery, we used a plasmid CMV-EGFP-CAT BGH, a gift from Dr. Subbiah Elankumaran (Heckert et al., 2002). Cloning procedures were carried out essentially as described (Sambrook et al., 1989). Briefly, the CAT gene was removed by digestion with *Apa*I and *Not*I restriction enzymes. The DNA fragment was excised from a 1% agarose gel and re-ligated. *E.coli* (DH5α) were transformed and recombinants were plated on ampicillin plates. Plasmid DNA was prepared and purified using endotoxin-free silica column kits (Qiagen, Inc., Valencia, CA) as per manufacturer instructions. The plasmid DNA preparation was checked on 1%

agarose gel (w/v) for genomic DNA or RNA contamination. The plasmid concentration was determined by spectrophotometer reading (260 nm). The plasmid DNA was frozen at –20°C to protect it from degradation by endonucleases.

The last two studies utilized the pIRES-EGFP vector in which the HN gene of NDV (Beaudette strain) was inserted to generate the pIRES-HN-EGFP plasmid. Briefly, Vero cells were infected with NDV at a multiplicity of infection (MOI) of 0.01. Three days after infection, viral RNA was extracted and used as template for RT-PCR. The coding sequence of HN consisting of 2265 base pairs was amplified using two specific primers that introduced *Bgl*II and *Sac*II restriction enzyme recognition sites (respectively underlined) at the 5' and 3' end of the NDV HN antigenomic RNA. Primers used were: forward 5'-AAGATCTATGGACCGCGCAGTTAGCCAAGTTG-3' and reverse 5'-ACCGCGGTACTAACCAGACCTGGCTTCTCTAAC 3'. RT-PCR product was excised from 1% agarose gel using a QIAquick gel extraction kit (Qiagen Inc., Valencia, CA). The PCR product was ligated into a pCRII-TOPO vector using the Topo cloning kit (Invitrogen). After ligation, *E.coli* cells were transformed and white colonies bearing the inserted HN gene were selected for plasmid preparation. Plasmid was digested with *Bgl*II and *Sac*II restriction enzymes. HN gene fragment was recovered after gel purification and then ligated between *Bgl*II and *Sac*II sites of pIRES-EGFP vector (Figure 4). *E.coli* cells were transformed and the recombinants selected after plating. The resulting plasmid was designated pIRES-HN-EGFP. The inserted DNA was sequenced to confirm the identity of the HN gene. Large amounts of purified, endotoxin-free plasmid were obtained from Aldevron, Inc. (Fargo, ND).

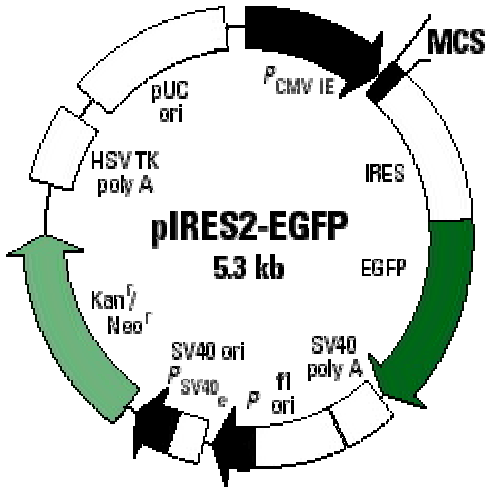


Figure 4 – Schematic diagram of a 5.3Kb eukaryotic expression vector used to express HN protein. NDV-HN gene was inserted in the multiple cloning sites (MCS). Independent EGFP expression was possible due to the presence of internal ribosomal entry site (IRES) downstream of MCS.

3.2. *In vitro* transfection

The plasmid CMV-EGFP-BGH was evaluated *in vitro* using HD11 (avian macrophage cell line) cells to test its transfection capability as described (Heckert et al., 2002). Briefly, HD11 cells were transfected with 5 µg of plasmid DNA using LipofectAmine (Invitrogen). After 24 h incubation, cells were examined under a Nikon Eclipse TE epifluorescent microscope to detect EGFP.

The plasmid pIRES-HN-EGFP (5 µg) was used to transfect Vero cells using Lipofectin (Invitrogen, Grand Islands, NY). Protein expression was observed by

immunostaining techniques. Briefly, after transfection Vero cells were washed three times with phosphate buffered solution (PBS), and fixed with acetone and methanol (v/v) for 20 min. Vero cells were washed twice, and incubated with polyclonal chicken anti-NDV antibody (1:20) for 1.5 h at RT. Cells were then washed three times, and goat anti-chicken IgG peroxidase labeled antibody (1:50) was added, and plates were further incubated at RT for 30 min. Cells were washed three times, and 200 µl/well of Trueblue® (Kirkegaard & Perry Lab., Gaithersburg, MD) peroxidase substrate was added. After 15 min, Trueblue® excess was rinsed and cells were examined under the microscope.

Experiment 1 – In order to determine route and formulation to be used for plasmid DNA delivery, five groups (of at least 10 eggs each), were inoculated into the air cell and amniotic cavity with 60 µg/egg of CMV-EGFP-BGH. A preliminary trial to test the amniotic cavity delivery technique was performed using Indian ink. After dye inoculation, embryos were chilled and opened to inspect the site of inoculation. For air cell inoculations, pre-trials were considered unnecessary. All embryonated eggs used in these experiments were from white leghorn hens, 18 days of embryonation, SPF (SPAFAS Inc, Norwich, Ct., USA) and were incubated at 100ºF with 60-80% humidity, as indicated by the thermometer (dry bulb) and hygrometer (wet bulb), respectively. For either route of inoculation, a small hole was made in the large end of the egg. The air cell inoculation was performed by dropping the plasmid DNA formulation on top of the chorioallantoic membrane, using a 25 gauge, 2.5 cm needle and only half of the needle length was allowed to penetrate the eggshell. Inoculations into the amniotic cavity were

performed using a 23 gauge, 2.5 cm needle, accordingly to preliminary trials described previously.

The ExGen® formulation consisted of 60 µg of plasmid CMV-EGFP-BGH diluted into sterile glucose solution (5%) and six equivalents of the cationic polymer gene delivery reagent (ExGen®500 *in vivo* transfection, MBI Fermentas). The second formulation tested (Oshop et al., 2003), consisted of 60 µg of plasmid DNA combined with IFA, forming a mixture of 50 µl, and mixed vigorously. The same amount of DMSO (Sigma, USA) was added and the mixture was sonicated. One control group consisted of 10 eggs that were un-inoculated.

After inoculation, all eggs were sealed with tape and re-incubated. Right before hatch, eggs were placed at 4ºC to induce death by hypothermia. Tissues such as liver, lung, spleen, muscle, intestine, and heart were collected. Tissues were placed in TissueTek OCT Compound (Sakura, Inc.) and snap frozen in liquid nitrogen. The samples were then stored at –70°C until processing. Frozen tissues sections were cut at 8 µm thickness with Leitz HM-500 Cryostat, air-dried at RT, fixed in acetone for 20 min, and mounted into glass slides with PBS/glycerol (v/v). Tissues samples were then microscopically examined under a UV light (488 nm excitation) at 40, 100, 400X magnification.

Experiment 2 - Having determined the most effective route for *in ovo* plasmid delivery as well as formulation, this experiment was designed to evaluate the dosage of pIRES-

HN-EGFP DNA to obtain higher levels of protein expression. Plasmid DNA was mixed with ExGen® in five different dosages (1, 10, 25, 60, 100 µg/egg) and injected into the amniotic cavity. Fifteen eggs were used per dose tested. One control group was used, consisting of plasmid DNA without HN insert (backbone pIRES-EGFP). Right before hatch, all eggs were placed in the refrigerator to induce death by hypothermia. Spleens were removed aseptically from each embryo.

Experiment 3 - This study was designed to evaluate immune responses elicited by *in ovo* injection of pIRES-HN-EGFP vaccine construct. Eggs were inoculated with 60 µg/egg of plasmid DNA mixed with ExGen® by the amniotic cavity as described in Experiment 1. Group 1 was inoculated with pIRES-HN-EGFP; group 2 received the plasmid DNA backbone control, and a third group was left un-inoculated. Eggs were sealed and incubated until hatch. All feather-dried hatched chicks were transferred directly from the hatcher to biological level 2 (BL2) animal facility in Avrum Gudelsky Veterinary Center and housed in isolation chambers with *ad libitum* access to feed and water. At three weeks of age, all three groups were sampled for serum, and tears. Tears were collected as described (Elankumaran et al., 1996) by applying a pinch of salt to each eye. At five weeks of age all birds were anesthetized using isoflurane, and 3-6 mL of blood was collected by cardiac puncture. The birds were then humanely euthanized.

3.3. Flow cytometry analyses

To determine dosage of plasmid DNA needed for high protein expression, the spleens from birds inoculated with pIRES-EGFP were macerated and filtered through sterile 70 µm nylon cell strainers (Falcon, Becton Dickinson Labware, NJ). Cells were washed three times with 5 mL of sorter buffer (Hanks balanced salt solution w/o phenol red, 3% FBS, 1% sodium azide) for 5 min, 42 x*g* at 4ºC and resuspended in sorter buffer. Viable cells were counted by trypan blue dye exclusion method. A cocktail of NDV monoclonal antibodies (62.5 µl of MAb10D11 and 62.5 µl of MAb 15C4 in 12.37 mL of sorter media) was added (100 µl) to the cells and incubated on ice for 40 min. After two washes, cell pellet was resuspended in 2 mL of sorter buffer and 100 µl of goat anti-mouse IgM+IgG+IgA R-phycoerythrin (PE) labelled (1:500) (SouthernBiotech, Inc., Birmingham, AL) was added. Cells were incubated on ice for 30 min, washed twice and resuspended in 1 mL of sorter buffer. Analyses were performed using an EPICS XL-MCL flow cytometer.

3.4. Serology

Humoral immune responses to HN protein were measured by ELISA (Synbiotics, San Diego, CA), isotype ELISA and HI. To verify specific antibody isotype (IgG, IgA, and IgM), serum and tear samples were analysed by a sandwich ELISA as described (Elankumaran et al., 2002). Briefly, ninety-six well ELISA plates of high adsorption capability (Nunc Maxisorb Immunoplate) were coated with affinity purified goat-anti-

chicken IgG, IgA, or IgM (Bethyl Laboratories, Inc.) antibodies (100 µl/well) diluted 1:200 in carbonate/bicarbonate buffer (0.1M pH 9.6). The plate was then incubated at 37 °C for 1 h. After five washes with PBST (PBS, 0.05% Tween-20) plates were dried, and blocked for 1 h at RT with 1% BSA in PBST. Serum and tear samples were diluted 1:50 in dilution/blocking buffer (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) and 100 µl of each sample was added to the appropriate wells. Each sample was run in duplicate. Anti-NDV chicken polyclonal serum (1:50) was used on each plate to serve as positive control. Normal chicken polyclonal serum (1:50) was diluted and used as a negative control. The plates were then incubated at RT on a plate shaker for 1 h. After incubation, plates were washed five times and incubated with NDV (1:200) at RT for 30 min. Each sample was tested with each antibody isotype, separately. Monoclonal antibodies to NDV (10D11, and 15C4) were diluted (1:200) and added to each well. After 30 min incubation and five washes, goat-anti- mouse peroxidase (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) was added (100 µl). The plate was then incubated for 30 min at RT and washed five times. The substrate TMB (100 µl) (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) was then added to each well and incubated at RT for 10 min. The color reaction was terminated by the addition of 100 µl of sulphuric acid (2M). Absorbances were read at 450 nm (reference wavelength of 550 nm). The S/P ratios were calculated for each sample.

HI was performed using 4 hemagglutination units (HAU). Briefly, serum samples were two-fold diluted in V-botton-96 well plates, and incubated with 4 HAU of the titered antigen (NDV) at RT for 30 min. After incubation, 25 µl of 1% chicken RBC's

were then added to each well. After 40 min incubation, the HI titer was determined as the highest dilution of serum causing inhibition of hemagglutination.

3.5. Statistical analysis

Results obtained from Experiment 1, 2 and 3 were statistically analyzed using the Student *t*-test (Statistix, version 7.0). Experimental group means were considered significantly different from each other if $p<0.05$.

RESULTS

3.6. *In vitro* expression

In order to confirm that CMV-EGFP-BGH plasmid expressed the reporter gene (EGFP), HD11 avian macrophage cells were transfected and observed under UV light. Our transfection experiments demonstrated the expression of EGFP gene by this construct (Figure 5).

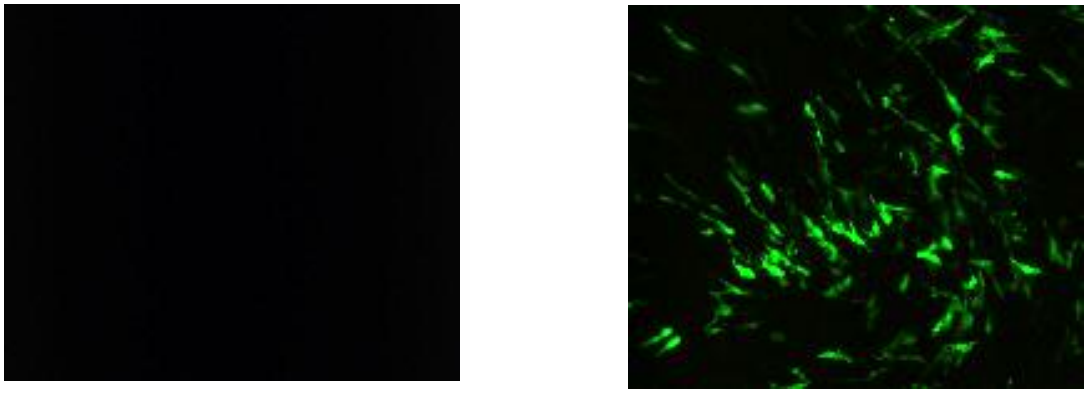


Figure 5 – HD11 avian macrophage cells transfected with plasmid CMV-EGFP-BGH. A) mock-transfected cells; B) cells visualized under UV light 48 h post-transfection with 5 µg of plasmid DNA (magnification 100X).

HN expression by pIRES-HN-EGFP was confirmed by the transfection of Vero cells and immunostaining technique (Figure 6).

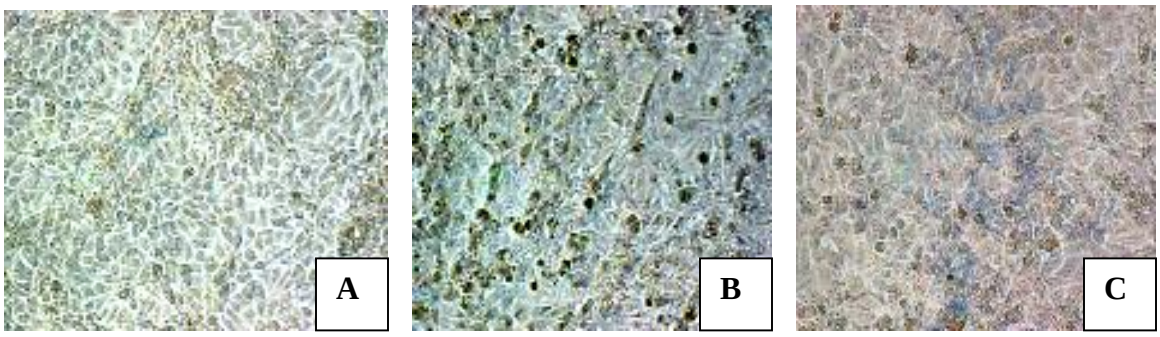


Figure 6 – *In vitro* expression of HN protein in Vero cells after transfection with pIRES-HN-EGFP. Cells were transfected with 5 µg of plasmid using LipofectAmine transfection reagent. Cells were immunostained with polyclonal chicken anti-NDV, labeled with goat anti-chicken peroxidase, and stained with Trueblue® (Kirkegaard & Perry Lab., Gaithersburg, MD). A) mock-transfected Vero cells (negative control); B) cells infected with NDV (positive control); and C) cells transfected with pIRES-HN-EGFP DNA (100X).

3.7. *In vivo* EGFP expression after *in ovo* injection

Tissue distribution of EGFP expression is shown in Figure 7. EGFP expression was detected in all tissues examined. The only two exceptions were the spleens of embryos inoculated by the air cell with ExGen® and intestine of embryos inoculated through either route with IFA+DMSO. Significantly ($p<0.05$) higher expression was observed in the muscle of embryos inoculated by the amniotic cavity using ExGen®. In this group, the percentages were 64, 40, 14, 25, 35, and 12% in the muscle, lungs, liver, spleen, heart, and intestine, respectively. Lower percentages of expression were observed in tissues from embryos inoculated by the air cell using either formulation. Also, lower percentages of expression were observed when plasmid DNA was delivered into the amniotic cavity using IFA + DMSO. Muscle and lung tissues consistently showed higher level of expression irrespective of the route or formulation used.

With IFA+DMSO formulation, 48 and 52% of embryos pipped when inoculated through the amniotic or air cell route, respectively. In contrast, eggs inoculated with ExGen® formulation for DNA delivery, 93% and 97% of the eggshells pipped, when the air cell and amniotic cavity routes were used. These results indicate that either IFA or DMSO may have a detrimental effect on hatchability.

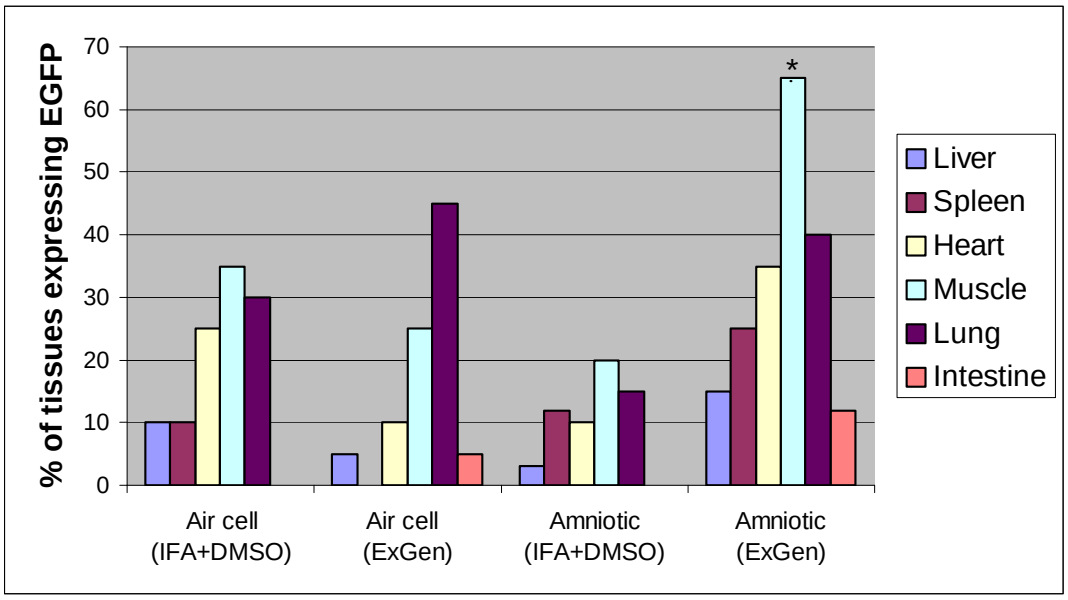


Figure 7 – Tissue distribution of EGFP expression from embryos inoculated with plasmid pCMV-EGFP-BGH DNA (60 µg/egg) through air cell and amniotic cavity using two different formulations (IFA+DMSO and PEI-ExGen®). All embryos were inoculated at 18 days of embryonation. Tissues were collected prior to hatch and examined under UV light. * Significantly different from all other tissues ($p<0.05$).

3.8. Flow cytometry

Five different doses of the plasmid pIRES-HN-EGFP were inoculated *in ovo* into the amniotic cavity of 18-day-old embryos. The spleens were harvested and processed for flow cytometry. Results are shown on Figure 8. Higher percentages of HN labelled cells (9.8%) were determined in the groups inoculated with 10 and 25 µg/egg. However these groups also had a high standard deviation (SD= 6.53 and 3.85, respectively). In contrast, cells of eggs inoculated with 60 µg/egg show slightly lower average, and much lower variability. Embryos inoculated with 20, 25, and 60 µg/egg did not show significant

differences ($p>0.05$) in the percentages of cells expressing HN protein. These results also show that small amounts of plasmid DNA (1 µg/egg) are able to transfect the embryo resulting in protein expression. The backbone plasmid results (1.95%) were considered background. We also observed a dose-dependent response inferring that higher amounts of DNA do not necessarily result in higher expression.

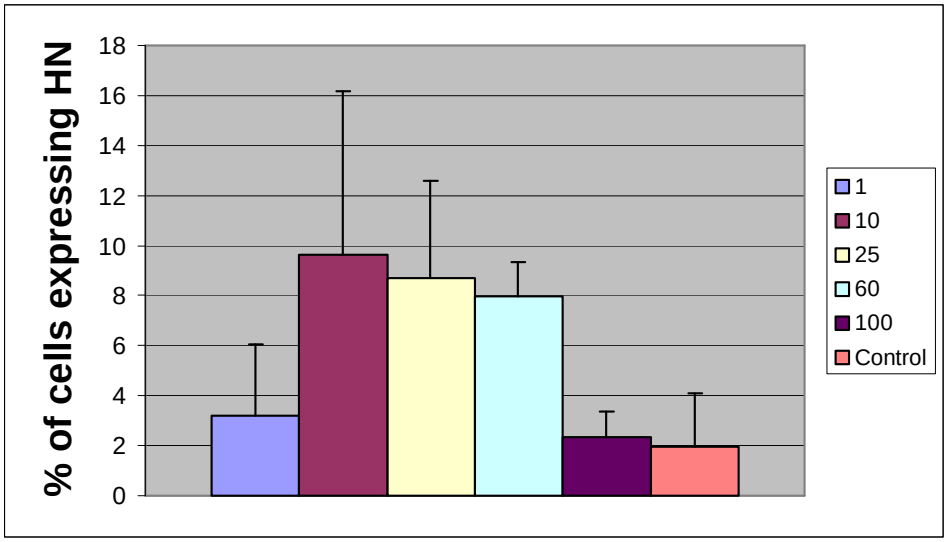


Figure 8 – Percentages of splenocytes expressing HN from embryos inoculated at 18 days of embryonation. Embyos were inoculated through the amniotic cavity with 1, 10, 25, 60 and 100 µg/egg of pIRES-HN-EGFP DNA mixed with PEI ExGen®, whereas, the control group consisted of 60 µg/egg of pIRES-EGFP DNA. The spleens were collected prior to hatch and processed for flow cytometry analyses. Bars represent the standard deviation per group analyzed. No significant differences were observed among embryos inoculated with 10, 25 and 60 µg/egg of plasmid DNA.

3.9. Serology

Tear samples were collected at 3 weeks and serum at 3 and 5 weeks post *in ovo* vaccination. All samples were analyzed for IgA, IgG, and IgM immune responses. All samples were negative for all three immunoglobulins tested at 3 weeks post-vaccination. At 5 weeks of age, three serum samples were positive for IgM. Figure 9 shows the mean of each treatment group.

At 5 weeks post-vaccination, two birds (2/6) that received the pIRES-HN-EGFP plasmid were considered positive as measured by commercial ELISA (data not shown). All tear and serum samples analysed by hemagglutination inhibition (HI) test were negative at all collection periods.

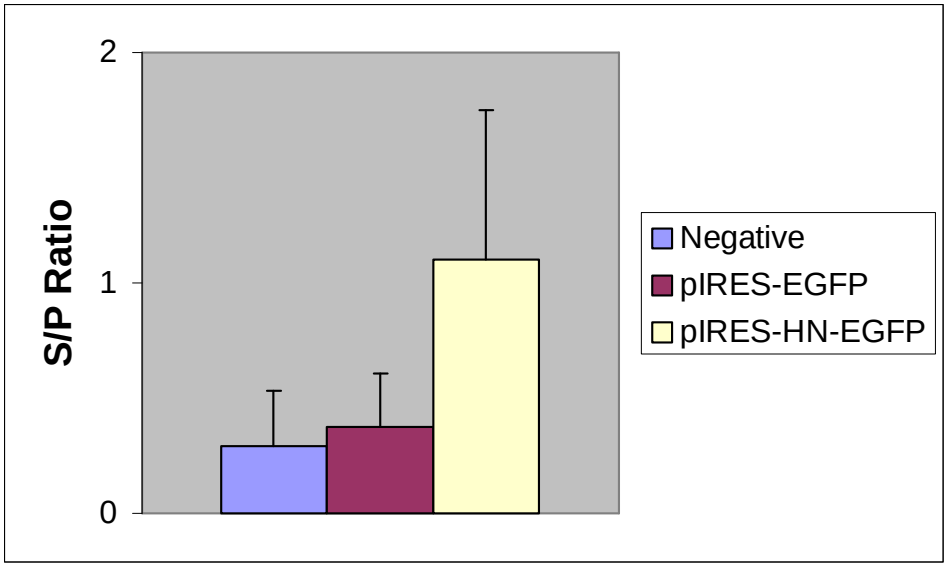


Figure 9 – IgM isotype-ELISA results from serum samples collected at 5 weeks post *in ovo* vaccination with 60 µg/egg of pIRES-HN-EGFP DNA. SPF, 18-day-old embryos were vaccinated through the amniotic cavity using PEI-ExGen®. Graph shows the mean of the ratios between sample and positive control. Negative control embryos were unvaccinated. Plasmid DNA control embryos were inoculated with pIRES-EGFP plasmid DNA lacking the HN gene.

DISCUSSION

Previous research has indicated *in ovo* DNA vaccines can be delivered into the air cell of ECE when encapsulated by neutral lipids such as IFA and DMSO (Oshop et al., 2003). We obtained similar results regarding EGFP expression when using the air cell route. Hatchability rates of 52-57% were also similar. In addition, the air cell was compared with the amniotic cavity route, which is routinely used for Marek's *in ovo*

vaccination of chickens in the poultry industry. When the plasmid DNA was delivered by the amniotic cavity a significantly higher percentage of tissues expressed EGFP.

Wakenell and co-workers evaluated the air cell route for delivery of Marek's vaccine *in ovo*. The authors reported a lack of vaccinal response caused by the inability of the virus to cross the air cell membrane (Wakenell et al., 2002). On the other hand, Oshop and co-workers, suggest that DMSO may increase permeability through membranes, thus enhancing plasmid DNA up take by the embryo (Oshop et al., 2003). Considering that plasmid DNA does not replicate as MDV and thus, its capacity to cross embryos membranes have to be mediated by a carrier such as DMSO. However, we obtained lower level of expression through the air cell. In addition, a dramatic decrease in hatchability of embryos vaccinated with IFA and DMSO formulation (52%) was observed when compared to ExGen® (97%). In our studies, embryos inoculated with DMSO that had died before hatch exhibited evidence of hepatic toxicity (data not shown). Affected livers were larger with severe congestion, and hemorrhages. The average hatchability rate in the poultry industry is 83%; lower rates represent a large loss in productivity. The results obtained in our studies using ExGen® as a vaccine adjuvant for *in ovo* inoculation were excellent. In addition, a consistent expression of the reporter gene in the muscle and lungs of embryos inoculated by either route or formulation was observed. For these reasons, it was decided to use the amniotic route and ExGen® to deliver plasmid DNA in the vaccine experiments. It is also important to point out that the cost of this cationic lipid (ExGen®) is very high to be considered for commercial applications.

High titers of IBDV in the lungs of embryos vaccinated by *in ovo* injection were reported, suggesting that this organ may play a main role in vaccine spread and protection (Sharma, 1986). In this previous experiment, muscle as well as lungs had the highest expression rates of the reporter gene, independent of route or formulation used (64 and 40%, respectively).

Another important aspect of *in ovo* plasmid DNA delivery was addressed in Experiment 2. Five different dosages of plasmid DNA (1, 10, 25, 60, 100 µg/egg) were evaluated for protein expression. A prior report observed a dose-dependent response in 18-day-old embryos and better responses were obtained using 60 µg/egg of plasmid DNA (Oshop et al, 2003). Flow cytometry results also showed a dose-dependent response to plasmid DNA using 1, 10, 25 and 60 µg/egg, but expression efficiency decreased with a dose of 100 µg/egg. The highest percentage of HN labelled cells was seen in the groups inoculated with 10 and 25 µg/egg (9.63% and 8.72%, respectively). However, these groups also had a high standard deviation that suggests inconsistent results. For this reason, we decided to use 60 µg of plasmid DNA/egg in future experiments because there were no significant differences among these three groups (10, 25 and 60 µg/egg). In addition, the 60 µg/egg group presented a much lower standard deviation (1.36). Similar results were observed by Oshop and co-workers when CAT protein expression was measured by AC-ELISA (Oshop et al., 2003). These studies also show that small amounts of plasmid DNA (1 µg/egg) are able to transfect the embryos resulting in protein expression (HN), and that large amounts of DNA may have a detrimental effect on transfection efficiency.

The immune responses induced by *in ovo* inoculation of plasmid DNA pIRES-HN-EGFP was measured by ELISA, isotype ELISA, and HI. A humoral response was not detected until 5 weeks of age. Few birds seroconverted as measured by commercial ELISA. Considerable IgM levels were detected by the isotype ELISA, indicating a primary immune response. All samples were negative for IgA suggesting that DNA vaccines delivered *in ovo* may not stimulate mucosal immunity.

Several important factors for *in ovo* DNA vaccine delivery were considered in this study. Using a well-studied promoter (CMV) (Oshop et al., 2003; Suarez, 2000; Kodihalli et al., 1997) two routes, two formulations, and five dosages for DNA vaccine delivery were evaluated. The humoral immune responses against plasmid DNA that encodes the HN gene from NDV were also evaluated. In the next study, these findings will be applied in the development of a DNA vaccine against IBDV.

CHAPTER 4

DEVELOPMENT AND EVALUATION OF AN *IN OVO* PLASMID DNA VACCINE AGAINST IBDV

ABSTRACT

IBDV is a highly contagious disease of chickens, which is controlled by live and inactivated vaccines. In this study, we evaluated a novel approach to vaccinate chickens against IBDV using DNA vaccinology. Plasmid DNA was administered *in ovo* to 18-day-old embryos. The DNA vaccine expresses the polyprotein VP2-VP4-VP3 of IBDV. The VP2 gene expresses epitopes of D78 strain and variant strain of GLS. VP3 and VP4 genes are from D78. VP2-VP4-VP3 genes were inserted into a plasmid vector (pVAX1) and their expression verified by immunostaining assays. SPF and fertile broiler eggs with maternal antibodies were vaccinated and hatched chicks were challenged against IBDV-STC. Each embryo received 60 µg of the DNA vaccine delivered into the amniotic cavity. In addition, a control group was inoculated with plasmid DNA without insert. Two groups of birds (SPF and broilers) received a booster immunization with baculovirus expressed-proteins of IBDV. The DNA vaccine had no detrimental effect on hatchability or first week post-hatch survival. *In ovo* vaccination generated detectable humoral immune responses as measured by ELISA. Antibody response was significantly enhanced two weeks after the birds received the IBDV-protein boost. However, no significant protection was observed in all vaccinated groups. BF had severe microscopic

lesions. Broilers vaccinated with plasmid DNA or IBDV-protein had partial protection possibly due to maternal antibodies.

INTRODUCTION

Broiler breeders are immunized with live and inactivated vaccines in order to confer passive immunity to the progeny (van den Berg et al., 1991). Delivery of inactivated vaccines in breeders is time consuming, laborious, and inaccurate because each animal is inoculated intramuscularly or subcutaneously (Lukert and Saif, 1997) During the first few weeks of life, broiler chicks are protected against IBDV by maternally acquired passive immunity. However, passive immunity decreases rapidly as the chick ages, leaving it susceptible to IBDV infection. Thus, it is a routine practice in the poultry industry to vaccinate young chicks against IBDV to control the disease. The optimal age for live vaccination in broilers is difficult to predict (Lutticken et al., 1994). If the vaccine is administered too early, neutralization of maternal antibodies may occur. Vaccine administration later in life may leave the birds susceptible to the disease. In addition, live vaccines, when administered *in ovo*, may cause microscopic lesions in the BF because the immune system of the embryo is too immature to respond adequately to the vaccine (Giambrone et al., 2001; Sharma, 1986).

A more recent approach to vaccinate humans and animals against infectious agents was created after the discovery that a simple inoculation of naked plasmid DNA could generate a humoral and cellular immune response (Tang, 1992). Since then, much

advancement in DNA vaccinology has been made. One important advantage of DNA vaccines is their possible use in neonatal animals. Apparently, passive maternal antibodies seem to have no interference with DNA vaccines (Babiuk, 1999; Siegrist, 2001; Hasset et al., 2000).

In order to circumvent the potential disadvantages of live and inactivated vaccines against IBDV and to evaluate the possibility of maternal antibody interference, we developed a plasmid DNA vaccine for SPF and broiler chicks. The specific objective of this study are: 1) evaluate the immunogenicity and protective efficacy of an *in ovo* IBDV vaccine in SPF chickens; 2) in broiler chicks with maternally acquired immunity; 3) examine the efficacy of a prime-boost strategy with IBDV DNA vaccine and baculovirus-expressed subunit vaccine in SPF and broiler chicks.

MATERIALS AND METHODS

4.1. Construction of a DNA vaccine expressing VP2-VP4-VP3 proteins of IBDV

A plasmid DNA that contains VP2 epitopes from variant strain GLS-IBDV (residues A, E, and S), and from standard strain D78 (P, Q, and G) was previously constructed. VP4 and VP3 genes were derived from standard strain D78-IBDV. In addition, the gene that encodes the nonstructural protein (NS) was ablated. This plasmid was denoted pUC19B69GLSVP2ΔNS (Liu, 2003). The insert pUC19B69GLSVP2ΔNS

and pVAX1 vector were digested with *Eco*RI. In order to avoid re-ligation of pVAX1, treatment with alkaline phosphatase was performed. The 3.2 Kb fragment (B69GLSVP2ΔNS) and pVAX1 were purified from a 1% agarose gel and ligated. The resulting DNA vaccine construct was designated pVAX1-B69GLSVP2ΔNS. *E.coli* cells were transformed by the vaccine construct and plated. *E.coli* colonies were selected and plasmid DNA was purified. The correct orientation of the inserted gene was checked by *Bam*HI digestion. The inserted genes were sequenced and analyzed for correctness. Large amount of endotoxin-free plasmid DNA were obtained from Aldevron (Fargo, ND). Plasmid concentration was determined by 260 nm spectrophotometer reading, and RNA/DNA contamination was checked by agarose gel. We decided to use pVAX1 as a vector because it was specifically designed for use in DNA vaccinology. It contains the CMV promoter, kanamycin resistance gene for selection in *E. coli*, and is only 3Kb in size.

4.2. *In vitro* expression of pVAX1-B69GLSVP2ΔNS

Transient transfection of Vero cells was performed to verify protein expression by the vaccine construct. Vero cells were transfected and immunostained as described in section 3.2, except in this study Lipofectin was used as the transfection reagent instead of LipofectAmine. IBDV proteins were detected using a polyclonal chicken anti-IBDV (1:500) and goat anti-chicken IgG (H+L) peroxidase labeled (1:1000), and peroxidase substrate (Trueblue®). A rIBDVNSΔ mutant virus strain was used as positive control. The negative control consisted of cells not transfected by the plasmid DNA.

4.3. Experimental design

Commercial and SPF (Sunrise Farms, Catskill, NY) fertile eggs were incubated at 100°F with 40-60% humidity. All SPF eggs used were from the same source. The commercial eggs were from a 36-week old broiler breeders flock immunized against IBDV from a local company. The vaccination program for these breeders consisted of one live IBDV vaccination at 4 weeks of age, and booster vaccinations at 10 and 18 weeks of age with an inactivated oil-emulsion vaccine containing standard and variant strains of IBDV.

The plasmid pVAX1-IBDV (60 µg/egg) was mixed with 50 µl of 5% sterile glucose solution. Six equivalents of PEI (ExGen®500 *in vivo* transfection, MBI Fermentas) were diluted in 50 µl of 5% sterile glucose solution. ExGen® solution was added to the plasmid DNA, mixed, and incubated at RT for 15 min. *In ovo* inoculation, through the amniotic cavity at 18 days of embryonation, was performed as described in Section 3.2., Experiment 1. Treatment groups and number of eggs per group are shown in Table 1. A larger number of eggs were utilized for treatment groups A, B, C, and F because of the possibility of adverse effect due to vaccination. Sample size was calculated accordingly to protocol previously approved, and available isolators at BL2 facility. After *in ovo* inoculation, all injection sites were sealed with adhesive tape. Eggs were replaced into the hatcher and incubated for three more days at 100 ºF with 60-80% humidity.

All hatched chicks were transferred to BL-2 isolators. Animal care and sample collections were performed as described earlier (Section 3.2., Experiment 3). One-week old birds from groups B and G received of 0.5 mL of IBDV protein lysate (6.8 mg/mL) subcutaneously as a secondary vaccine. At 3 weeks of age, all birds were bled and challenged.

Seven days post-challenge, birds were then anesthetized with isoflurane, and 3-6 mL of blood was collected by cardiac puncture. The birds were then humanely euthanized and spleens and BF were collected. Spleens and half of each BF were fixed in 10% buffered formalin for seven days. After fixation, all tissues were stored in 70% alcohol and submitted to American Histolabs (Gaithersburg, MD) for HE staining. The other half of the BF collected at the necropsy was used for antigen-capture ELISA detection.

Table 1 – Treatment groups to evaluate plasmid DNA vaccine administered *in ovo.*

Group	Egg type	Treatment	# of eggs
A	SPF[a]	Plasmid DNA vaccine[b]	12
B		Plasmid DNA vaccine + protein boost[c]	12
C		Plasmid DNA control[d]	10
D		NV/NCH[e]	8
E		NV/CH[f]	8
F	Broilers[g]	Plasmid DNA vaccine	10
G		Plasmid DNA vaccine + protein boost	9
H		NV/NCH	9
I		NV/CH	9

a - specific-pathogen-free eggs.
b - 18-day-old embryos received 0.2 mL of pVAX1-IBDV DNA vaccine (60 µg/egg).
c - one-week-old birds received 0.5 mL of protein boost expressed in baculovirus subcutaneously.
d - eggs inoculated with 60µg of pVAX1.
e - non-vaccinated, non-challenged control group.
f - non-vaccinated, challenged control group.
g - fertile broiler eggs from a local poultry farm.

4.4. Expression and detection of baculovirus expressed-IBDV proteins

Baculovirus expressing IBDV structural protein genes of GLS strain was a gift from Dr. Raghunath Shivappa. Recombinant virus vIBD-7 was obtained by cotransfecting pGLSBacI and *Autographa california* nuclear polyhedrosis virus (AcNPV) DNA into *Spodoptera frugiperda* (Sf9) cells, and plaque-purifying the recombinant virus. The recombinant virus was grown in large amounts in Sf9 cells.

4.5. Production and detection of baculovirus expressed-proteins of IBDV

Baculovirus expressed-proteins of IBDV used to boost one-week-old chicks were produced and harvested from Sf9 cells infected with baculovirus vIBD-7, as described (Vakharia et al., 1993). Briefly, infected cells were cultured in Grace's insect medium with 1% L-glutamine supplemented with 10% of FBS in 1L spinner flasks at 28ºC. After showing typical cytopathic effect (CPE) the Sf9 cells were centrifuged at 4,000 x*g* for 5 min at 4°C. The cell pellet was washed with cold PBS (pH 6.5) and sonicated for three times 15 sec each, cycle number 5 (Fisher Scientific Sonic Dismembrator, Model 100). Cell lysis was verified by trypan blue exclusion method. Aliquots were collected for western blotting and protein concentration assays. Total protein concentration (6.8 mg/mL) was determined by BCA protein assay (Pierce, Rockford, IL). Clarified lysate was stored at –20ºC for further subcutaneous inoculation into chickens as a booster for primary DNA vaccination.

Detection of baculovirus protein expressed by the vIBD-7 construct was determined by Western blotting. Fifteen microliters of the cell lysate was mixed with 2X loading buffer (15 µl), boiled, and run on a 12.5% SDS-PAGE. The proteins were transferred from the gel by blotting onto nitrocellulose membrane and the membrane was blocked overnight in 2% non-fat dried milk solution. The membrane was incubated for 1 h with rabbit-anti IBDV polyclonal antibodies (1:400). The membrane was washed and a secondary antibody, goat anti-rabbit phosphatase (Kirkegaard & Perry Laboratories) was added (1:1000) and incubated for 1 h, and washed. All washes were made three times, five min each with tris-buffered-saline (TBS) with 0.1% Triton-100X, and one final wash with TBS only. Finally, the protein was detected by naphthol phosphate fast red (Sigma).

4.6. IBDV challenge

The challenge strain was titered using the mean embryo infectious dose (EID_{50}) method since this strain does not grow in tissue culture. Serial dilutions of the IBDV-STC stocks were made and then 100 µl of each dilution was inoculated onto the chorioallantoic membrane (CAM) of 11-day-old ECE. The inoculated eggs were examined by candling for 6 days. IBDV causes embryo mortality from 3-5 days post-inoculation. After seven days, the remaining embryos were chilled for 2 h and examined for IBDV specific lesions such as edematous distention of the abdominal region, cutaneous congestion and petechial hemorrhages, cerebral hemorrages, liver necrosis and hemorrhages, heart and lung congestion, mottled necrosis of kidneys, and small necrotic foci in the spleen. The CAM does not present plaques but may exhibit small hemorraghic

areas (Lukert and Saif, 1997). Then an EID_{50} was determined using the Reed-Muench formula. Aliquots of virus stock were stored at –20ºC.

Each bird received 0.2 mL of 10^3 EID_{50} of IBDV standard challenge strain STC by oculo-nasal route. The birds were observed for clinical signs of IBDV. They were scored on a scale of 1 to 8 (Table 2). Birds that had a total score of 6 or higher from the viral challenge were anesthetized and humanely euthanized. The experiment was terminated on day 7 after viral challenge.

Table 2 - Scoring system to quantify pain, distress, and suffering after IBDV challenge.

A - Physical appearance/unprovoked behavior post-challenge[a]	**Score[b]**
Normal	0
Ruffled feathers;	1
Lack of grooming, reduced mobility;	2
Anorexia, inactive, trembling;	3
Violet comb, nasal and/or ocular discharge, whitish or watery diarrhea	4
B - Behavioral responses to external stimuli post-challenge	**Score**
Normal	0
Depression, tendency for some birds to pick at their own vents	1
Decreased alertness,	2
Severe prostration	3
Comatose	4

a - birds were challenged by ocular/nasal route. Each bird received 0.2 mL of 10^3 EID_{50} of IBDV standard challenge strain STC. They were observed for clinical signs of IBDV three times a day.
b - each bird was scored on a scale of 1 to 8. Birds that had a total score of 6 or higher from the viral challenge were anesthetized and humanely euthanized. The experiment was terminated on day 7 after viral challenge.

4.7. Assessing protection

Vaccine efficacy was determined by bursa/body weight ratios, survival to IBDV challenge, histopathological scoring, detection of viral antigen by AC-ELISA, and humoral immune responses. The bursa/body weight ratio was calculated as bursa

weight/body weight x 1000. The vaccinated group was considered protected if all bursa/body weight ratios were equal or higher than 2SD (standard deviation) of the non-vaccinated, non-challenged control group, indicating absence of bursal atrophy.

To detect the presence of IBDV antigens and assess protection against challenge, a commercial antigen-capture ELISA kit (AC-ELISA) was used. The bursae were homogenized individually according to manufacturer's recommendation and analysed using a panel of strain-specific IBDV monoclonal antibodies (Synbiotics, San Diego, CA).

Protection from IBDV challenge was also determined by evaluating the degree of microscopic bursal damage. BF sections from surviving birds were randomly read and scored using the scoring system shown in Table 3.

Table 3 – Histological scoring system for bursal damage after infection with IBDV.

Damage score[a]	**Histological features**
0	No bursal damage in any follicle, clear demarcation of medulla and cortex
1	Mild necrosis of occasional follicles with overall bursal architecture maintained
2	< 50% of follicles with severe lymphocyte depletion
3	> 50% of follicles with severe lymphocyte depletion
4	Follicular outlines only remaining, increased connective tissue, cysts
5	Loss of all follicular architecture, fibroplasia

a - adapted from Muskett et al., 1979.

4.8. Serology

Commercial ELISA kits (Synbiotics, San Diego, CA) were used to verify immune responses generated by pVAX1-IBDV DNA vaccine. This kit consists of plates coated with bursal derived IBDV antigen.

4.9. Statistical analysis

Data obtained from ELISA were statistically analyzed using the Student *t*-test (Statistix, version 7.0). Experimental group means were considered significantly different from each other if $p<0.05$.

RESULTS

4.10. Cell transfection by pVAX1-VP2 VP4-VP3 plasmid DNA

The complete segment A encoding the polyprotein VP2-VP4-VP3 of IBDV was cloned in to the pVAX1 plasmid vector, as described in Section 4.2. The insert was sequenced to its entirely to confirm identity. In order to confirm that the construct expressed the VP2-VP4-VP3 polyprotein, Vero cells were transiently transfected and tested by immunostaining assay for protein expression. Our *in vitro* transfection experiments demonstrated the expression of VP2-VP4-VP3 (Figure 10).

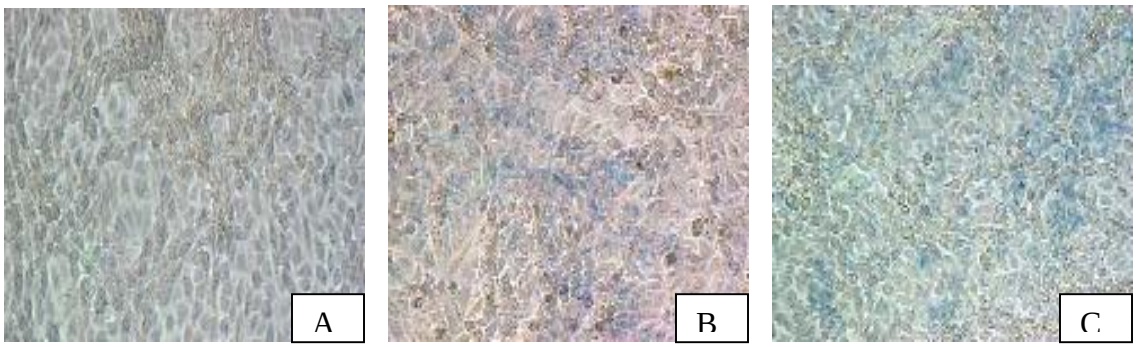


Figure 10 – *In vitro* expression of IBDV proteins in Vero cells after transfection with pVAX1-VP2 VP4-VP3 plasmid DNA. Vero cells were transfected with 5 µg of plasmid using Lipofectin and immunostained 48 h post-transfection. Cells were treated with polyclonal chicken anti-IBDV, labeled with goat anti-chicken peroxidase, and stained with Trueblue® Kirkegaard & Perry Lab., Gaithersburg, MD). A) mock-transfected cells (negative control); B) cells infected with rIBDVΔNS (positive control); C) cells transfected with pVAX1-IBDV plasmid DNA (Magnification 100X).

4.11. Detection of IBDV proteins

In order to determine the expression of VP2, VP4, and VP3 in the baculovirus (vIBD-7) system, the Sf9 cell lysate was analyzed by Western blotting, which gave strong bands indicating IBDV protein expression (Figure 11, lane 4).

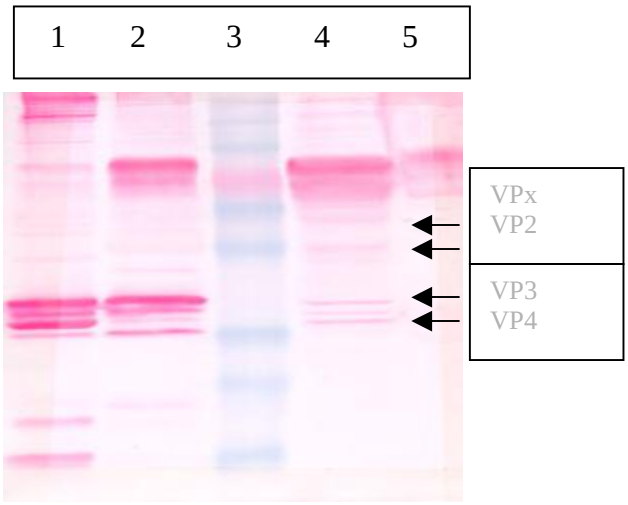


Figure 11 - Western blot results of vIBD-7 proteins expressed in Sf9 infected cells. Sf9 cells were infected with a recombinant baculovirus (vIBD-7) expressing VP2-VP3-VP4 polyprotein of IBDV. Cells were harvested 3 days post-inoculation. The samples were separated by SDS-PAGE on a 12.5% slab gel, blotted onto nitrocellulose, reacted with polyvalent rabbit anti-IBDV serum, and detected with goat anti-rabbit phosphatase labeled and developed by naphthol phosphate fast red. Lane 1, D78; Lane 2, GLS; Lane 3, Marker; Lane 4, vIBD-7; Lane 5, un-infected Sf9 cells.

4.12. Hatchability and first week survival rates post *in ovo* plasmid DNA vaccination

The hatchability and first week survival rates are shown in Table 4. The hatchability percentages from commercial broiler eggs were 70% to 100%. Commercial broiler eggs hatched significantly better than SPF embryos. The plasmid DNA vaccine

may not have been responsible for this low hatchability, since non-vaccinated SPF embryos also had lower hatchability (50%). It is possible that the quality of SPF embryos shipped by our provider was poor.

First week survival rates were 100% in broiler chicks. Survival at first week was compromised in the SPF groups. Hatched chicks in the SPF groups were weak and small, independently of treatment group, again reinforcing the view that egg quality at the time of receipt must have been poor.

Table 4 – Effect of *in ovo* vaccination of IBDV-DNA vaccine on hatchability and survival rates.

Group	Egg type	Chicks hatched (%)	1st week survival (%)
A	SPF[a]	11/12[b] (92)	10 (91)
B		10/12 (83)	10 (100)
C		4/10 (40)	4 (100)
D		4/8 (50)	3 (75)
E		4/8 (50)	3 (75)
F	Broilers[c]	7/10 (70)	7 (100)
G		9/9 (100)	9 (100)
H		9/9 (100)	9 (100)
I		9/9 (100)	9 (100)

a - specific-pathogen-free eggs.
b - number of eggs vaccinated/ number of hatched chicks.
c - fertile broiler eggs from a poultry farm.

4.13. Assessing protection

Table 5 summarizes results from plasmid DNA vaccine efficacy 7 days post IBDV challenge. SPF embryos vaccinated *in ovo* with plasmid DNA (group A) or boost at one-week of age (group B) were not protected against challenge. All vaccinated birds exhibited clinical signs of IBD three days after challenge. Two birds from group B died 7 days post-challenge. Non-vaccinated, challenged control groups (E, and I) became severely ill and were humanely euthanized 3 days after challenge. The severity of clinical signs and mortality rate of group I (broilers) was significantly lower than group E (SPF). No protection was observed in birds from group C that was inoculated with control plasmid DNA. Broilers from group F were not protected either. Broiler embryos that received a boost (group G) were partially protected. In this group, birds did not die after challenge, had the body/bursa weight ratios normal, and no viral antigen was detected by AC-ELISA. However, their BF showed a score of 3.67 in the histopathological examination, indicating severe bursal damage, and 50% of vaccinated birds (3/6) exhibited clinical signs of IBD.

IBDV-specific antigens in the BF were assessed by AC-ELISA and the results are summarized in Table 5. Viral antigen could not be detected by AC-ELISA in the bursae of SPF and broiler non-vaccinated, non-challenged control birds (D, H). Antigen was detected in 100% of samples from non-vaccinated, challenged control group and 66.7% in broilers (groups E, and I). All BFs from plasmid control (group C) had antigen detected at 7 days post-challenge. Three out of seven vaccinated SPF birds had IBDV

antigens detected by AC-ELISA. No antigen was detected in BF of SPF birds that received plasmid DNA and IBDV-protein boost (group B) Antigen was detected in vaccinated, challenged broilers group F (3/6). No viral antigen was detected in broiler birds from group G that received a protein boost at 7 days post-challenge.

Table 5 – Protection rate, bursa/body weight ratio, antigen detection, and histopathological scores from birds vaccinated *in ovo* with pVAX1-IBDV DNA at 7 days post IBDV-STC challenge.

Group	Egg type	Birds with clinical signs/challenged[a]	B/B ratio[b]	AC-ELISA[c]	Lesion Score[d]
A	SPF	9/10[e]	2.18 ± 0.3[f]	3/7	4.62
B		7/10	2.08 ± 0.3	0/6	4.66
C		4/4	4.90 ± 1.7	3/3[g]	5.0
D		NA[h]	7.11 ± 1.4	0/3	0
E		NA	3.63 ± 0.3	3/3	4.6
F	Broilers	5/6	1.96 ± 0.9	3/6	2.0
G		3/6	1.90 ± 0.8	0/6	3.67
H		NA	1.48 ± 0.6	0/6	0
I		NA	2.31 ± 0.5	4/6	4.6

a – number of birds exhibiting clinical signs of IBD post-challenge with 0.2 mL of $10^3 EID_{50}$ of IBDV-STC challenge.
b - (bursa weight/body weight) x 1000.
c - viral antigen detected by antigen capture ELISA.
d - mean of lesion scores.
e - number of birds that died after challenge/number of birds challenged
f - mean of bursa/body weight and standard deviation.
g – number of birds positive for antigen detection as measured by AC-ELISA.
h – not applicable

4.14. Histopathology

Table 5 shows the average of score lesions from microscopic analysis of BF from SPF and broiler chickens at 7 days post-challenge. No microscopic lesions were observed in chickens that were not vaccinated or challenged. In contrast, bursae from non-vaccinated challenged SPF control birds showed lesion score 5.0 and severe lymphocytic necrosis, follicular cell depletion, extensive accumulation of inflammatory cells, and inter and intra-follicular cystic formation. A significant increase of connective tissue was observed, which resulted in the loss of distinction between the cortex and medulla. All vaccinated, and challenged SPF birds had high lesion scores (4.62 and 4.66) and exhibited severe microscopic lesions in the BF at 7 days post-challenge.

Non-vaccinated, challenged control broilers showed bursal lesions. However, they were not as severe as SPF challenged controls. In vaccinated broilers, lesions in the BF as well as spleen were dramatically different from bird to bird. In addition, broilers that received plasmid DNA vaccine presented mild to severe lymphocytic depletion. Embryos vaccinated with plasmid DNA and boosted with baculovirus expressed-IBDV protein had lesion score 2.0 and a high variability in the lesions caused by IBDV challenge.

The spleen was also microscopically analyzed (data not shown). SPF non-vaccinated, challenged birds showed increased numbers of germinal centers when compared to non-challenged control group. The spleen was hemorrhagic and showed infiltration of inflammatory cells as well as white zones characteristic of cell depletion.

Non-vaccinated broilers that were challenged also showed lymphocytic depletion, but more generalized throughout the entire organ. Vaccinated SPF birds exhibited lesions in the spleens similar to the challenged control group. Milder lesions were observed in groups F and G.

4.15. Serology

Serological results from ELISA test are shown in Figure 12. The S/P ratios were calculated for each sample. As expected, all SPF non-vaccinated birds did not show antibody titers after three weeks of vaccination. The maternal antibody detected at three weeks of age in non-vaccinated commercial broilers was not uniform (mean= 1.89; SD=0.95).

At three weeks post-vaccination 3/10 SPF birds vaccinated with plasmid DNA showed antibody responses as measured by ELISA. In addition, SPF birds vaccinated with plasmid DNA and boosted one week later were positive (S/P ratio=1.23). Group F showed ratios of 1.75, and broilers boosted with IBDV protein showed 1.58.

At seven days post-challenge, a significant increase of humoral response was observed in SPF birds that received the plasmid DNA vaccine (groups A, and B). Birds inoculated with the control plasmid DNA remained negative. Commercial broilers vaccinated with plasmid DNA and protein boost had significantly increased ($p<0.05$) antibody responses (1.58 vs 1.90). However, commercial broilers that received plasmid vaccine only did not show an antibody increase 7 days after challenge, as measured by

ELISA. Antibody responses were not detected in SPF non-vaccinated, challenged birds after 7 days of challenge. Non-vaccinated, not challenged control group demonstrated a significant decrease in maternal antibodies at four weeks of age.

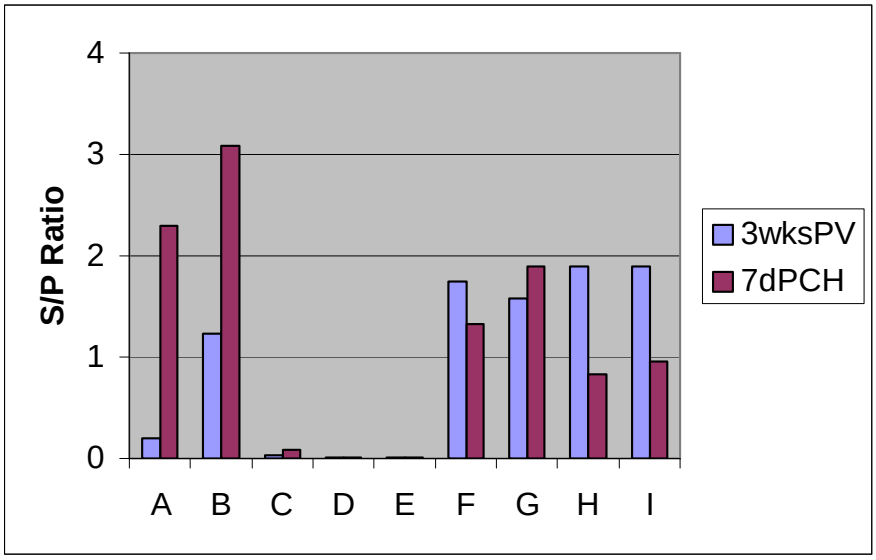


Figure 12 – ELISA results from birds vaccinated with pVAX1-IBDV DNA vaccine three weeks post *in ovo* vaccination and seven days post IBDV-STC challenge. A) SPF embryos vaccinated with plasmid DNA; B) SPF embryos vaccinated with plasmid DNA and boosted at one week of age with baculovirus expressed-IBDV proteins; C) SPF embryos vaccinated with plasmid DNA control (pVAX1); D) SPF unvaccinated, non-challenged control group; E) SPF unvaccinated; challenged at 3 weeks post plasmid DNA vaccination; F) broiler embryos vaccinated with plasmid DNA; G) broiler embryos vaccinated with plasmid DNA and boosted at one week of age with baculovirus expressed-IBDV proteins; H) broiler embryos unvaccinated and non-challenged; I) broiler embryos non-vaccinated, and challenged.

DISCUSSION

IBDV remains a serious problem for commercial broiler producers. Chickens vaccinated with IBD vaccines are not protected against variant or vvIBDV strains. In addition, immunity conferred by live vaccines induced mild to moderate bursal atrophy (Tsukamoto et al., 1995). Previous work has indicated that *in ovo* vaccination against IBDV using live intermediate vaccines, can lead to disease and immunosuppression due to microscopic lesions in the BF (Lukert and Saif, 1997). In addition, these viruses may be able to revert to a virulent state.

Therefore, a safer and more efficacious vaccine to control IBD is necessary. Plasmid DNA vaccination has been used in recent years as a new way to induce host immune responses. Few studies reported the use of a plasmid DNA vaccine against IBDV. Plasmid DNA vaccine expressing the polyprotein VP2-VP4-VP3 induced specific antibodies and partially protected chickens immunized intramuscularly and intraperitonealy (Chang et al., 2001; Fodor et al., 1999). In this study, we demonstrated that a plasmid DNA vaccine expressing the polyprotein (VP2-VP4-VP3) of IBDV conferred partial protection against IBDV challenge in commercial broilers when delivered to 18-day-old embryos. On the other hand, SPF embryos that lack maternal antibodies did not show protection against challenge.

These results were obtained using an expression vector under the control of the CMV promoter containing IBDV genes for VP2-VP3-VP4 polyprotein followed by *in*

ovo inoculation of 18-day-old embryos. In order to enhance the immune responses generated by plasmid DNA vaccination, SPF and broilers were boosted subcutaneously with a subunit vaccine generated in the baculovirus system at one week post-hatch. Our study showed that this secondary vaccination induced a higher level of antibody responses in SPF birds. Antibody responses measured by ELISA two weeks after boost were significantly higher when compared to birds that were vaccinated with plasmid DNA only (S/P ratio = 1.3 vs 0.3). Partial protection was observed in commercial eggs inoculated with plasmid DNA vaccine and boosted one week of age. Previous studies using the same baculovirus construct obtained protection when two-week-old leghorns were inoculated intramuscularly, boosted 4 weeks later and challenged with IBDV-GLS strain (Vakharia et al., 1993). In another study using the same construct but with antigenic mass 4-fold its original resulted in full protection against STC, E/Del, and GLS challenges (Vakharia et al., 1994). It is possible to infer from these results, that better protection could have been provided if boost was performed with higher concentrations of the IBDV-protein later in life. However, the lifetime of a broiler chick is 48-49 days and several inoculations for vaccine boost are not practical.

We also demonstrated that bursal damage and lesions after challenge in non-vaccinated control broilers were highly variable from bird to bird. These findings suggest that the level of protection observed is not exclusively due to the DNA vaccine but as well as to maternal antibodies. As measured by ELISA, maternal antibody titers were not uniform (ranging from 345 to 5455). Also, passive immunity waned significantly after 3 weeks of age to sub-protective levels, indicating that the vaccination program used in the

breeder flock was not adequate to protect young chicks from IBDV challenges in the field during the first few weeks of life.

In this study, we could not demonstrate that DNA vaccines may be able to overcome maternal immunity since broilers that received plasmid vaccine did not show an antibody increase three weeks post *in ovo* vaccination, as measured by ELISA. In addition, only a few SPF birds seroconverted. Low levels of antibody responses after plasmid DNA vaccination have been reported by other investigators (Heckert et al., 2002; Kodihalli et al., 2000; Chang et al., 2001; Kodihalli et al., 1997). Our findings also suggest that cellular immunity may have played a role in partially protecting vaccinated broilers. Other authors obtained similar results, indicating that protection may due to the presence of CTL rather then antibody immune response (Oshop et al., 2003; Wang et al, 2003; Seo et al., 1997).

CHAPTER 5

RECOMBINANT ATTENUATED IBDV VACCINE DELIVERED *IN OVO* CONFERS PROTECTION IN CHICKENS

ABSTRACT

A recombinant attenuated vaccine against IBDV was administered *in ovo* to 18-day-old embryos. The vaccine was genetically tailored to protect from challenges in the field against classic and variant strains of IBDV. The vaccine virus contains neutralizing epitopes from both classic (D78) and variant strain (GLS), and abrogates expression of the nonstructural protein, VP5 of IBDV. SPF and fertile broiler eggs obtained from a local poultry farm were vaccinated and used to evaluate protection against IBDV-STC challenge. A full dose of the vaccine consisting of $5.6x10^3$ pfu was administered to SPF and broiler embryos. In addition, a half dose of the vaccine containing $2.3x10^3$ pfu was injected into SPF embryos. The vaccine had no effect on hatchability or first week survival in either broilers or SPF birds, even when high doses were administered. The vaccine generated high antibody titers in chickens with either dosage. All vaccinated groups were protected against mortality. The vaccine did not cause bursal damage and fully protected SPF chickens vaccinated *in ovo* with half dose of the vaccine and broiler chicks that received a full dose of the recombinant vaccine *in ovo*.

INTRODUCTION

IBD is an acute, contagious disease caused by a double-stranded RNA virus of the *Birnaviridae* family, IBDV. IBDV genome consists of two segments, A and B. The larger segment A contains two overlapping ORF that encodes a polyprotein VP2-VP4-VP3 and a nonstructural protein, VP5. Segment B codes for a 97 Kda protein, designated VP1, which represents the viral RNA-dependent RNA polymerase. The virus targets the lymphoid tissue of chickens mainly the BF, causing severe bursal damage, and consequently immunosuppression. Thus, IBD is of major economic importance to the poultry industry.

A strategy for the control of IBD in chicks involves hyperimmunization of breeders, which allows them to transmit high levels of maternal antibodies to progeny during the critical first few weeks of life. Although maternal antibodies provide protection during this period, continued protection against IBDV must be maintained before the maternal immunity reaches sub-protective levels by the administration of live vaccines. However, maternal antibodies can neutralize vaccine virus and reduce the viral load needed to induce immunity (Sharma et al., 1987). In addition, new antigenic variants of IBDV, which appeared during the 1980's, introduced new problems for poultry production. These new field isolates were able to break through neutralizing maternal antibodies induced by standard IBDV vaccines (Snyder, 1992). Since then, these variant strains have been incorporated into commercial inactivated vaccines for broiler breeders.

Unfortunately, despite these vaccination measures, IBDV continues to be a problem. Very virulent strains of IBDV have caused outbreaks of disease with high mortality in Europe and Asia (Nunoya et al., 1992; van den Berg et al., 1991) despite vaccination programs. In addition, live vaccines that are available for mass vaccination of broilers in the first few weeks of life are not suitable for *in ovo* administration. These vaccines may induce immunosuppression during late stages of incubation, when the embryo is highly susceptible to infection.

Therefore, in an effort to aid in the control of this disease, a recombinant IBD vaccine virus that can protect against both classical and variant strains was created in our laboratory, using reverse genetics system (Liu, 2003). This virus, designated as rD78GLSNSΔ, is deficient in the expression of VP5 nonstructural protein (NS). It grows one log lower than the parental viruses, and exhibits decreased cytotoxic and apoptotic effects in cell culture. This virus fails to induce any pathological lesions in the bursa of infected three-week old chickens. In addition, vaccinated birds challenged with classic (STC) and variant (GLS) strains of IBDV were fully protected.

In this report, we evaluate the potential use of this recombinant attenuated virus *in ovo* to protect SPF as well as commercial chicks from IBDV challenges. Fertile eggs were obtained from a local poultry farm that routinely immunizes broiler breeders with live and inactivated vaccines to confer high levels of maternal antibodies to the progeny. The vaccination program for these breeders consisted of one live IBDV vaccination at 4

weeks of age, and booster vaccinations at 10 and 18 weeks of age with an inactivated oil-emulsion vaccine containing standard and variant strains of IBDV.

Two different doses of this recombinant vaccine were evaluated in SPF eggs in the absence of maternal antibodies. A full dose was used to vaccinate broiler embryos with maternal antibodies to verify its ability to break through antibody barrier and generate a protective immune response against IBDV challenges.

MATERIALS AND METHODS

5.1. Cells and viruses

Vero cells were maintained in medium 199 (M199) supplemented with 5% FBS at 37°C in a humidified 5% CO_2 incubator and used for propagation of the virus. Primary chicken embryo fibroblast (CEF) cells were prepared as described previously (Mundt and Vakharia, 1996). Briefly, ten-day-old embryos from SPF chickens were aseptically removed and cut into pieces. The tissues were rinsed in HBSS and digested with 0.2% trypsin at 37°C for one h to produce a single cell suspension. The suspension was filtered through gauze and washed twice with HBSS. The cells were grown in M199 and F10 (1:1 v/v) with 10% FBS. Secondary CEF cells, used for virus titration, were maintained in growth medium consisting of M199 and F10 with 5% FBS.

5.2. Propagation and purification of IBDV

The recombinant IBD vaccine virus, rD78GLSNSΔ, was prepared as described (Liu, 2003). Large amounts of this virus were grown in Vero cells as stock for *in ovo* inoculations, and stored at –20 °C.

5.3. Plaque assays

Virus stocks propagated in cell culture were titered by plaque assay as described earlier (Mundt and Vakharia, 1996). Briefly, the infected supernatant was diluted in ten-fold increments in MEM without FCS. Confluent monolayers of CEFs were infected with serial dilutions of viruses (10^{-4} to 10^{-7}, 0.1 mL/well). After 1 h adsorption at RT, the media was removed and the monolayer overlaid with 3 mL of 1% SeaPlaque agarose (Difco) containing 10% tryptose phosphate broth, 2% FCS, 0.112% $NaHCO_3$, 100 unit/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL fungizone. On day 3, the agarose overlay was removed and cells were fixed with formalin. After fixing, the cells were stained with crystal violet and plaques were counted and expressed as plaque forming units (pfu/mL).

5.4. Experimental design

The experimental groups and controls are shown in Table 6. A larger number of eggs were utilized for treatment groups A, B, and E (vaccinated) because of a possible

adverse effect due to vaccination. Sample size was calculated accordingly to protocol previously approved. SPF and commercial fertile broiler eggs of 18 days of embryonation were inoculated in a manner similar to that previously described in Chapter 3, using a full dose of rIBDV (group A, and E). Each embryo received $5.6x10^3$ pfu/0.2 mL of rD78GLSNSΔ. Another group of SPF eggs received only half a dose of this virus, which was $2.3x10^3$ pfu/0.2 mL (group B). The negative controls consisted of non-vaccinated, unchallenged SPF and commercial embryos (groups D, and G). The challenge control groups consisted of SPF and commercial eggs, which did not receive the vaccine and were challenged two weeks post-vaccination (groups C, and F). After *in ovo* vaccination, all eggs were sealed with adhesive tape and re-incubated.

Table 6 – Experimental design to evaluate live attenuated rIBDV vaccine.

Group	Egg type	Vaccine dose[a] (pfu)	# of eggs
A	SPF[b]	5.6×10^3	27
B		2.3×10^3	27
C		NV/CH[c]	17
D		NV/NCH[d]	17
E	Broilers[e]	5.6×10^3	24
F		NV/CH	9
G		NV/NCH	9

a - 18-day-old embryos received 0.2 mL of rIBDV vaccine containing either $5.6x10^3$ or $2.3x10^3$ pfu.
b - specific-pathogen-free fertile eggs.
c - non-vaccinated, challenged control group.
d - non-vaccinated, non-challenged control group.
e - fertile broiler eggs from a poultry farm.

The SPF embryos were free of any other immunosuppressive diseases that could compromise our results, such as adenoviruses, and chicken anaemia virus. The commercial eggs were obtained from the same poultry company as described in section 4.3.

After 21 days of incubation, all hatched chicks were housed in BL-2 isolators and cared for under the same conditions described in Chapter 3. Two weeks post-hatch, all birds were bled and challenged with STC strain (0.2 mL by the ocular and nasal route - 10^3 EID_{50}) of IBDV, except group D, and G. At ten days post-challenge, all the remaining birds were anesthetized, bled, and humanely euthanized. Spleen and bursa were collected and bursa/body weight recorded. The bursae were sectioned in half. Spleen and bursa halves were placed in 10% buffered formalin for histology. The other half of the bursa was stored frozen for later testing by AC-ELISA.

The antibody levels in serum samples collected at 2 weeks post-vaccination, and 10 days post-challenge, were determined by ELISA and VN. A commercial ELISA kit was used (Synbiotics, San Diego, CA). The frozen bursae were processed as described earlier and probed for antigen detection by AC-ELISA (Synbiotics, San Diego, CA). Fixed tissues were sectioned at American Histolabs (Gaithersburg, MD) and stained by hematoxylin-eosin (HE). We used the same criteria to determine protection against IBDV challenge as described in Chapter 4.

5.5. Virus neutralization assay (VN)

Serum samples were heat-inactivated at 56°C for 1 h and diluted in serial two-fold dilutions. Each dilution was mixed with 100 $TCID_{50}$ of rIBDV and incubated for 1 h at 37°C. The mixture was added to 85-90% confluent monolayer of Vero cells, grown in 96-well tissue culture plates (Costar, Ithaca, NY). All plates were incubated for 5-6 days until the presence of CPE was detected in the virus control wells. Normal serum from SPF birds was used as a negative control and a polyclonal anti-IBDV (SPAFAS) was used as a positive control. Anti-IBDV titers were determined 5-6 days later, averaged, and expressed as Log2.

RESULTS

5.6. Vaccine safety

Hatchability and first week survival rates are shown in Table 7. The hatchability rates in SPF eggs from group A and B were similar (92.5%), indicating that the vaccine is safe even when higher doses were administered. The percentages of hatched birds and first week survival were not significantly different from vaccinated groups and control non-vaccinated. The hatchability rates in broilers were lower than SPF embryos. However, we do not believe the vaccine caused these changes, since non-vaccinated birds also exhibited lower hatchability (88.8%). We attributed it to stress caused by temperature shock during transportation. Embryonated eggs were removed from

incubators at 37ºC and transported at 22ºC for 2 h. The hatchability in this experiment is much higher than the average observed in the poultry industry (83%) because all unviable eggs were discarded prior to *in ovo* vaccination.

Table 7 – Effect of *in ovo* vaccination of rIBDV on hatchability and survival of hatched chicks.

Group	Egg type	# of chicks hatched (%)	1st week survival rate (%)
A	SPF	25/27 (92.5) [a]	24 (96)
B		25/27 (92.5)	25 (100)
C		16/17 (94.1)	16 (100)
D		17/17 (100)	17 (100)
E	Broilers	18/24 (75)	17 (94.4)
F		7/9 (77.8)	7 (100)
G		8/9 (88.8)	8 (100)

a – percentage of hatched chicks after *in ovo* vaccination with rIBDV.

5.7. Vaccine protection

Results of the IBDV challenge studies are shown in Table 8. All vaccinated birds were fully protected against IBDV-STC. The bursa/body weight ratio was calculated as bursa weight/body weight x 1000. The vaccinated group was considered protected if all bursa/body weight ratios were equal or higher than 2SD (standard deviation) of the non-vaccinated, non-challenged control group. The mean for the SPF control (group D) was 5.35 (2SD=2.48). Thus, all SPF vaccinated birds with either dose were considered protected. Additionally, vaccinated broilers were also considered fully protected.

Table 8 also shows results from antigen detection assessed by AC-ELISA (Synbiotics, San Diego, CA) seven days post-challenge. IBDV antigen was detected in two SPF birds that were vaccinated with full dose of the vaccine. Viral antigens could not be detected in the BF of birds that received half dose of the vaccine or in broilers that received full dose. Antigen was detected in SPF challenge control group (11/12). As expected, no IBDV antigen could be detected in the negative control birds (group D, and G), whereas non-vaccinated, challenged broilers (8/9) were positive by AC-ELISA at 10 days post-challenge.

Table 8 – Protection indices from birds vaccinated with rIBDV-attenuated vaccine and challenged with the classic STC strain of IBDV.

Group	Birds with clinical signs/Challenged[a]	B/B weight[b]	AC-ELISA[c]	VN Log2[d]		Lesion Score
A	0/20[e] (100)[f]	6.04 ± 1.2[g]	2/12	8.76	7.90	2.0
B	0/18 (100)	5.27 ± 1.5	0/11	9.25	5.47	0
C	12/12 (NA)[h]	4.31 ± 1.1	11/12	4.00	2.33	5.0
D	0/12 (NA)	5.35 ± 1.2	0/12	3.16	4.00	0
E	0/17 (100)	2.14 ± 0.8	0/11	9.46	7.58	0
F	10/12 (NA)	1.48 ± 0.6	8/9	5.3	6.8	4.6
G	0/12 (NA)	2.31 ± 0.5	0/9	5.4	6.8	0

a - at two weeks post-vaccination birds received 0.2 mL of 10^3EID50% of IBDV-STC challenge.
b - (bursa weight/body weight) x 1000.
c - number of birds that had antigen detected as measured by antigen capture ELISA.
d - virus neutralization results two weeks post-vaccination and ten days post-challenge.
e - number of birds dead/ number of birds challenged.
f - percentage of protected birds.
g - mean of bursa/body weight rations and standard deviation.
h - not applicable.

5.8. Antibody responses

The antibody responses as measured by commercial ELISA are shown in Figure 13. At two weeks post-vaccination, all vaccinated groups had significantly higher antibody titers ($p<0.05$) than non-vaccinated groups by either test. As expected, all non-vaccinated SPF birds had negative titers for IBDV at 2 weeks post-vaccination. The non-vaccinated, commercial broilers had maternal antibody titers ranging from 455 to 5455 at 2 weeks of age. At 10 days post-challenge, all SPF vaccinated groups (full and half dose) showed significantly ($p<0.05$) higher titers than the same groups at two weeks post-vaccination. However, broilers receiving a full vaccine dose did not show an antibody increase after challenge as measured by ELISA. Antibody responses at 2 weeks post-vaccination in SPF birds that received half dose of the vaccine were relatively higher than SPF birds vaccinated with a full dose. The level of protective maternal antibodies in the non-vaccinated, unchallenged broilers seemed to wane slightly after challenge. Antibody responses were not detected in SPF non-vaccinated, challenged control birds after 10 days of challenge. This result is expected considering that a primary immune response would take at least 2 weeks to be detected.

The results from virus neutralization assay are expressed as log2 in Table 8. All vaccinated groups showed protective levels (between 4-6 log2) of antibodies 2 weeks post-vaccination. Non-vaccinated control broilers showed maternal antibody levels (4.8 log2) lower than normal for the first few weeks of life. All control groups continued to exhibit only low levels of antibodies 10 days post-challenge. As expected, all vaccinated

SPF birds showed higher humoral responses after challenge. Contradicting ELISA results, vaccinated broilers also showed higher antibody response 10 days post-challenge (7.5 to 9.46).

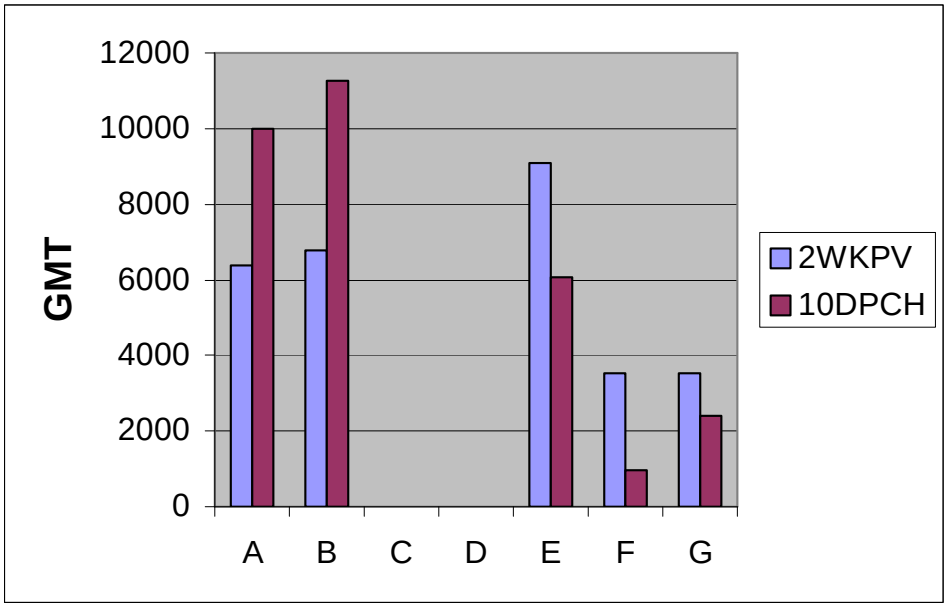


Figure 13 – ELISA geometric mean titer (GMT) from two-week-old chickens post *in ovo* vaccination with rD78GLSNSΔ and 10 days post IBDV challenge. A) SPF embryos vaccinated with 5.6 x 10^3 pfu/egg of the vaccine; B) SPF embryos vaccinated with 2.3 x 10^3 pfu/egg of the vaccine; C) SPF embryos unvaccinated, but challenged at 2 weeks post *in ovo* vaccination; D) SPF embryos were neither vaccinated, nor challenged; E) broiler embryos vaccinated with 5.6 x 10^3 pfu/egg; F) broiler embryos unvaccinated, but challenged at 2 weeks post *in ovo* vaccination; G) broiler embryos unvaccinated, and non-challenged.

5.9. Histopathology

The averages of microscopic lesions are shown in Table 8. At 2 weeks post-vaccination, birds from the control and treatment groups that received either full dose or half dose did not show microscopic lesions in the BF. At 10 days post-challenge, SPF non-vaccinated birds that were challenged showed severe lymphocyte depletion, undulation in the epithelium, intra and interfollicular epithelial cysts, and degeneration of follicular structure (Figure 14 A, Table 8 - C). SPF birds that received full dose of the vaccine (Figure 14 B) showed a mild degree of B-cell depletion, localized in a few follicles. The lesion score for this group was 2.0. SPF birds vaccinated with a half dose of the vaccine did not show histopathological lesions in the bursa (Figure 14 C, Table 8 - B). The control non-vaccinated, challenged broilers showed bursal lesions. However, they were much milder than SPF challenged controls (Figure 14 D, Table 8 - F). In addition, broilers that received a full dose of the vaccine showed normal bursae after 10 days post IBDV challenge (Figure 14 F, Table 8 - E).

The spleens of SPF non-vaccinated, challenged birds showed hemorrhages and lymphocytic depletion. Non-vaccinated broilers that were challenged showed a milder but more generalized reduction of lymphocytes throughout the entire organ. No lesions were observed in the spleens from vaccinated groups (data not shown).

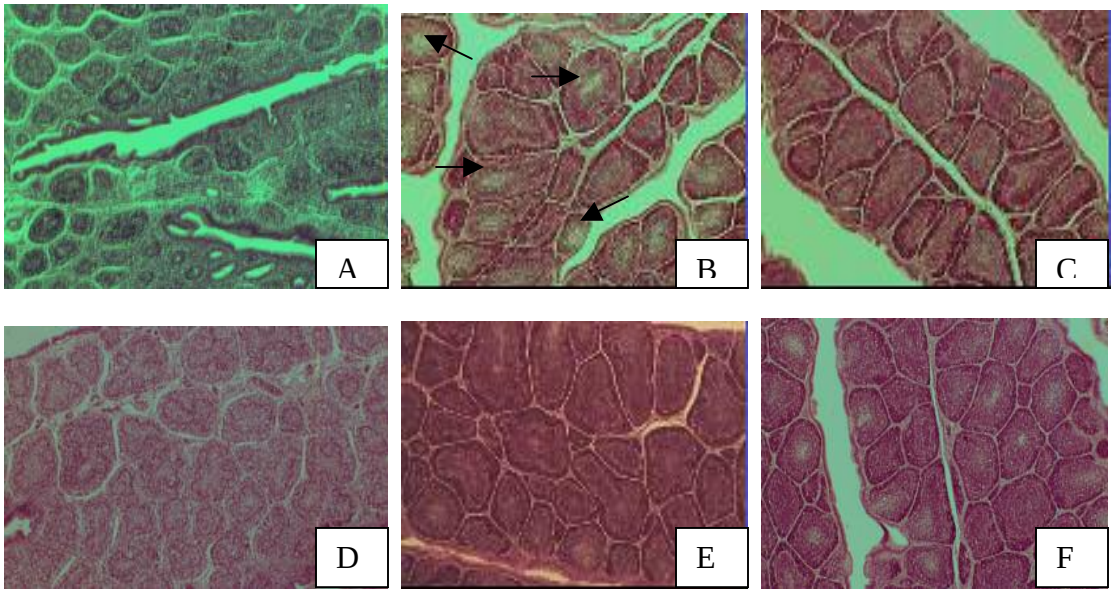


Figure 14 – Sections of the BF stained by hematoxylin-eosin for histopathological examination 10 days post-challenge with IBDV-STC. Eighteen-day-old embryos were inoculated with either 5.6 x 10^3 pfu (full dose) or 2.3 x 10^3 pfu (half dose) of rD78GLSNSΔ. A) non-vaccinated, challenged SPF, shows severe lymphocytic necrosis and heterophilic inflammation; B) vaccinated (full dose) and challenged SPF birds shows lymphocytic depletion (indicated by arrows); C) vaccinated (half dose) and challenged SPF shows no visible microscopic lesions; D) non-vaccinated, challenged broiler shows lymphocytic necrosis and loss of follicular structure; E) unvaccinated, and non-challenged control broiler shows no microscopic lesions; F) vaccinated (full dose) and challenged broiler shows no microscopic lesions.

DISCUSSION

IBD in chickens was first described in 1962 (Cosgrove, 1962). IBDV is a lymphotropic virus able to cause mainly humoral immunosuppresion in chickens infected before three weeks of age (Sharma and Lee, 1983). Efficacy studies with commercial live vaccines *in ovo* against IBDV caused acute clinical signs of the disease when administered in a full dose. Additionally, hatchability was severely decreased (Sharma et al., 2001). Attempts to administer commercial vaccines in a lower dosage induced less mortality, however, microscopic bursal lesions persisted (Lukert and Saif, 1997). In addition, since 1980 new subtypes of serotype I, called 'variant strains' have been isolated in the US (Snyder et al., 1988). Active or passive immunity mediated by vaccination with classic strains do not protect against variant strains (Rosenberger et al., 1985).

In this study, a recombinant live attenuated vaccine that expresses multiple neutralizing epitopes of classical and variant strain of IBDV was evaluated in 18-day-old SPF and commercial embryos. SPF embryos were injected with a full or half dose of the virus through the amniotic cavity. Two weeks post-vaccination, birds were challenged with an IBDV-STC strain. Commercial broilers vaccinated with a full dose and SPF embryos vaccinated with half dose were fully protected. In addition, no significant microscopic bursal lesions were observed in these groups. On the other hand, SPF birds that received a full dose of the vaccine *in ovo* exhibited microscopic lesions similar to unvaccinated, challenged control group.

No detrimental effects on hatchability with either dosage used were observed. However, histopathological results suggested that a higher dose of the vaccine given to birds that lack sufficient maternal antibody may still be virulent even though clinical signs of IBD were not observed. Our findings agree with previous research in SPF and broiler embryos vaccinated with three commercial intermediate vaccines *in ovo*. Microscopic bursal lesions were observed even when half of the recommended dose was used (Giambrone et al., 2001).

In a previous report, microscopic lesions were not observed when a full dose of rIBDV live attenuated virus was used to vaccinate two-week-old chickens ocularly (Liu, 2003). In this study, bursal lesions were observed in SPF birds, lacking maternal antibodies that received a full dose of the vaccine when delivered *in ovo*. At 10 and 15 days of embryonation, prebursal stem cells are migrating via the blood supply from the spleen to the BF (Masteller et al., 1994). Consequently, at eighteen days of incubation, when *in ovo* vaccination occurred, the avian immune system was not fully developed and a viral infection that targets this organ may have caused irreversible damage.

A second aim of this study was to evaluate the efficacy of this chimeric virus as a potential vaccine in the presence of maternal antibodies. Commercial broilers were from breeder flocks vaccinated with classic and variant strains of IBDV. Vaccinated broilers had significant higher antibody titers than non-vaccinated broiler control group at two weeks post vaccination. According to Lutticken et al., 1994, these findings indicate that the vaccine was able to breakthrough maternal antibody barrier and seroconvert. Similar

results were obtained in broilers vaccinated with a chimeric IBDV vaccine 14 days post-vaccination (Mundt et al., 2003). However in their studies, the challenge using classic and variant viruses induced chronic lesions in BF of vaccinated broilers with a chimeric virus expressing classic and variant epitopes of GLS and D78 IBDV strains (Mundt et al., 2003).

On the other hand, despite protection against challenge, vaccinated broilers did not show an increased humoral response after challenge, as expected. One possible explanation is that the sampling period (10 days after challenge) was too early in order to detect an increased antibody response.

Due to the poor quality of SPF eggs, it would be valuable to repeat this experiment in a new set of eggs. However, we had several constraints regarding time, facility, and personnel.

The novelty of this work is its use *in ovo* to vaccinate commercial broilers in the presence of maternal immunity against IBDV. This study suggests the potential use of rD78GLSNSΔ as a vaccine candidate for *in ovo* delivery. This vaccine was shown to be safe, highly immunogenic, and protective against STC-IBDV challenge. More studies regarding properties of the virus in face of variant strain challenges are necessary.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1. CONCLUSIONS

A typical vaccination program for breeders consists of live vaccines, as well as killed vaccines containing classic and antigenic variant viruses. Unfortunately, passive immunity is variable and transient. In addition, vaccination with inactivated vaccines is laboring, time consuming, expensive, and often inaccurate. Live vaccines used in chicks during the first few days of life are not suitable for *in ovo* use. Therefore, to aid IBDV control, we used the well established *in ovo* technology for vaccine delivery to evaluate two new vaccines against IBDV.

Initially we generated a better *in ovo* delivery system to be used for DNA vaccines. We showed that amniotic cavity results in better transfection rates in a safe manner. We also showed that a cationic DNA vaccine adjuvant (PEI - ExGen®) resulted in higher plasmid expression when delivered through the amniotic cavity. Microscopic examination of several tissues revealed the presence of the reporter gene in many different organs, indicating the capacity of the plasmid DNA to transfect several cell types. Our findings also show that plasmid DNA was able to generate a humoral immune response and partially protect 3 week-old chickens against IBDV challenge. Very little is known regarding DNA vaccines for poultry, specially when delivered *in ovo.*

Unfortunately, the cost of PEI-ExGen is prohibitive and more research has to be done to develop a cost-efficient DNA vaccine for the poultry industry. Interesting and promising results are under way using various lipids, and immune estimulators, such as CpGs sequences (Wang et al., 2003). Considering that most viral diseases affecting young chicks have the respiratory tract as the entry site for virus replication and dissemination, it would be interesting to find an adjuvant able to target the plasmid DNA delivery into the mucosa of the upper respiratory tract.

Our IBDV-DNA study showed better protection when followed by an IBDV-protein boost. VP2 is the most immunogenic protein of IBDV and it has been expressed in many different systems. The use of a subunit vaccine consisting of baculovirus expressed VP2-VP4-VP3 proteins to boost immunity mounted primarily by a plasmid DNA vaccine is novel. It elicited a significantly higher immune response than DNA vaccine alone. Thus, protection against challenge was significantly improved. This work also suggests the possible use of plasmid DNA vaccine to prime the host immune system, followed by a live attenuated vaccine.

Throughout our studies regarding plasmid DNA vaccines, several attempts were made to assess cellular immunity after *in ovo* delivery. Two different ELISA tests were used to detect IFN-γ at 7 and 14 days after *in ovo* vaccination. We were unable to detect it by either test, indicating that IFN responses are fast and transient. We also performed T-cell proliferation, and macrophage activation (NO) assays and no responses were observed.

Reverse genetic system allowed us to evaluate a tailored vaccine expressing multiple neutralizing epitopes of classical and variant strain of IBDV. The *in ovo* delivery of this live attenuated vaccine generated a strong and fully protective immune response in chickens challenged at two weeks post-vaccination.

Many other attenuated IBDV vaccines have been tested for *in ovo* use. However, they cause bursal damage and consequently immunosuppression. Our findings indicate that the rIBDV did not affect hatchability or first week survival. In addition, no bursal damage or microscopic lesions were observed in broilers. Regarding the maternal antibody barrier, we observed that our rIBDV vaccines did not overcome it as demonstrated by serology. It is possible that attenuation provided by VP5 deletion did not allow the virus to replicate efficiently in high titers due to antibody neutralization.

6.2. FUTURE WORK

DNA vaccines have shown potential use to protect chickens from different pathogens (Oshop et al., 2003, Suarez et al., 2000; Song et al., 2001). Specifically against IBDV, DNA vaccines containing VP2 gene or the polyprotein (VP2-VP4-VP3) were evaluated (Wu et al., 2002; Fodor et al., 1999). Better results were obtained using VP2-VP4-VP3 proteins. In order to increase the efficiency of plasmid DNA vaccines against IBDV, few approaches were already studied using CpG sequences. Another approach would be the use of cytokines, such as interferon to enhance IBDV plasmid DNA vaccine responses and protection.

In Chapter 5, we discussed the potential use of a chimeric vaccine that expresses epitopes of variant (GLS) and classic (D78) strains of IBDV. Our challenge studies were performed using a USDA standard IBDV challenge strain, STC. We demonstrated that vaccinated birds with or without maternal antibodies were fully protected after challenge. We also demonstrated that the tailor made chimeric vaccine was able to breakthrough maternal immunity against variant and classic strains of IBDV. However, in the future it would be of interest to evaluate the efficiency of this marker live attenuated vaccine in 18-day-old embryos against challenge with a variant strain, such as GLS. In addition, this chimeric vaccine should also be evaluated in conjugation with MD vaccines in order to expand the usefulness of the *in ovo* vaccination technology for poultry. Another control group should also be included, consisting of a vaccinated group with D78, a classical vaccine against IBDV.

We also recommend few extra samplings in the future experiment in order to observe in vivo kinetics of this chimeric construct. For example, we checked for possible bursal damage two weeks post-vaccination. It is possible that after two weeks BF had already recovered in case the vaccine caused any bursal damage. Few more samplings could have been done 1, 3, 7, 10 and 14 days post-vaccination.

It would be useful to perform this experiment for a longer period in the broilers, up to 47 days and include few extra blood samplings to verify antibody responses.

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