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Development and Evaluation of an *in ovo* Plasmid DNA Vaccine Against Infectious Bursal Disease Virus

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Abstract: Infectious Bursal Disease Virus (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.

Key words: Infectious bursal disease virus, vaccine, *in ovo* vaccination, serology, histopathology, chickens, protection

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 and Johnston, 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 *et al*., 1999; Hassett *et al*., 2000; Siegrist, 2001).

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.

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

Materials and Methods

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.

***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.

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°F 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.

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

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

Spodoptera frugiperda (Sf9) cells and plaque-purifying the recombinant virus. The recombinant virus was grown in large amounts in Sf9 cells.

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 28°C. 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-20°C 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 and 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).

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-20°C.

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.

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×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 analyzed using a panel of strain-specific IBDV monoclonal antibodies (Synbiotics, San Diego, CA).

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

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

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.

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.

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

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 (Fig. 1).

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 (Fig. 2, lane 4).

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.

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

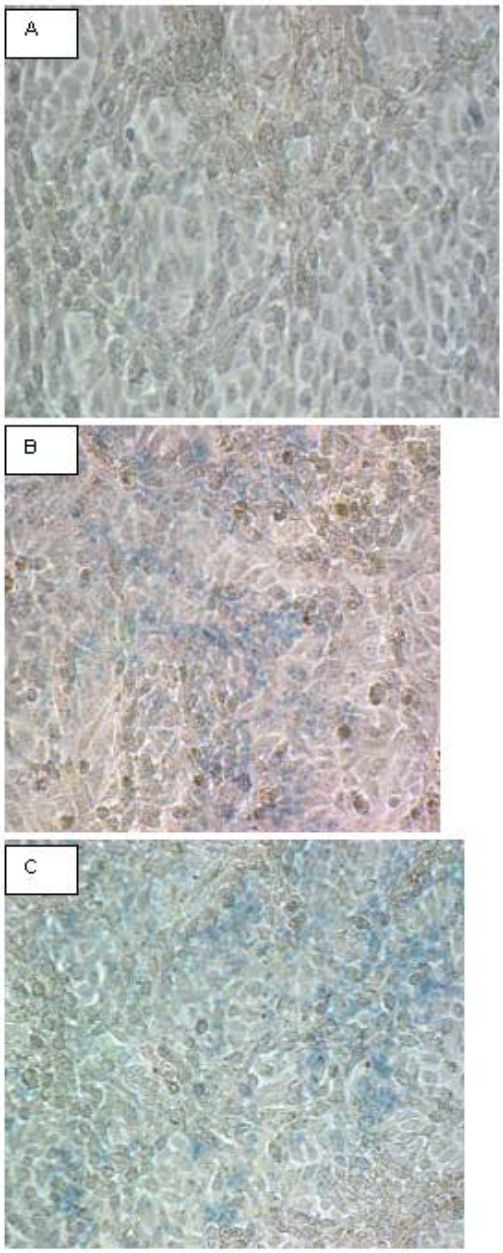

Fig. 1: *In vitro* expression of IBDV proteins in Vero cells after transfection with pVAX1-VP2-VP4-VP3 plasmid DNA. Vero cells were transfected with 5 µg of plasmid using Lipofectin and immunostained 48 h post-transfection. Cells were treated with polyclonal chicken anti-IBDV, labeled with goat anti-chicken peroxidase and stained with Trueblue® Kirkegaard and 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)

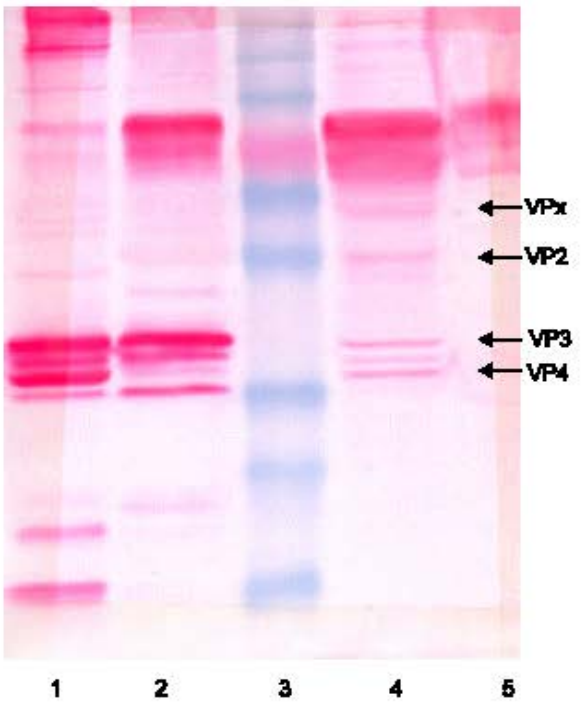

Fig. 2: Western blot results of vIBD-7 proteins expressed in Sf9 infected cells. Sf9 cells were infected with a recombinant baculovirus (vIBD-7) expressing VP2-VP3-VP4 polyprotein of IBDV. Cells were harvested 3 days post-inoculation. The samples were separated by SDS-PAGE on a 12.5% slab gel, blotted 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

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.

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

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 10^3EID_{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

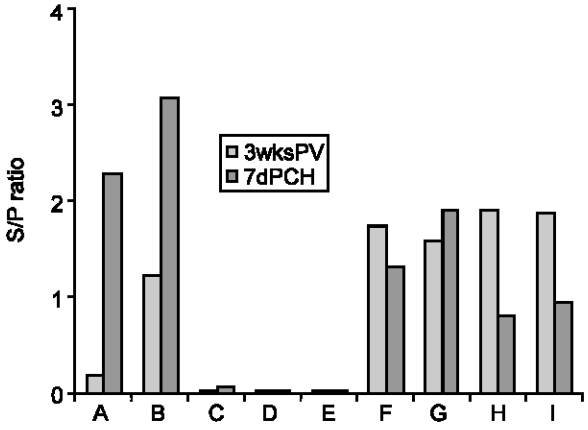

Fig. 3: ELISA results from birds vaccinated with pVAX1-IBDV DNA vaccine three weeks post *in ovo* vaccination and seven days post IBDV-STC challenge. A) SPF embryos vaccinated with plasmid DNA; B) SPF embryos vaccinated with plasmid DNA and boosted at one week of age with baculovirus expressed-IBDV proteins; 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

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.

Serology: Serological results from ELISA test are shown in Fig. 3. 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.

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 (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.*, 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; Seo *et al.*, 1997; Wang *et al.*, 2003).

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