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Protective Effect of *In ovo* Vaccination with IBV-Spike-Recombinant DNA and Chicken Interferon as an Adjuvant

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Abstract: Infectious Bronchitis Virus (IBV) can cause high mortality, poor weight gain and low feed efficiency of infected chickens, which leading to considerable economic losses to the poultry industry. In this study, a plasmid DNA pTracer-CVM2-IBVS (*abbr:* pCMV-S) that expresses the immunogenic S glycoprotein genes of IBV serotype Massachusetts 41 was inoculated *in ovo* into 18-days-old embryonating Specific-Pathogen-Free (SPF) chicken eggs as a vaccine. Recombinant chicken interferon type I alpha (rChIFN-α) was added as an adjuvant to enhance the immunogenicity of the vaccine. After hatch, birds were challenged at 4 weeks of age with a homologous pathogenic Massachusetts 41 field type IBV. Birds receiving a single dose of pCMV-S vaccine *in ovo* showed mild clinical signs and were protected at the level of 66%, whereas groups receiving a combination of the pCMV-S with 2000 IU or 500 IU of rChIFN-α showed protection at the level of 83 and 89% respectively. The pCMV-S + 500 IU rChIFN-α vaccinated group, when boosted with a live attenuated commercial IBV vaccine at 2 weeks of age indicated a significant protection (> 92 %) against IBV challenge. At 5 days post challenge, no clinical signs were seen from the birds vaccinated with 5 ug of pCMV-S +500 IU rChIFN and the birds received booster with commercial IBV vaccine. RT-PCR based diagnostic test indicated detecting IBV infection was reduced in birds received pCMV-S with 2000 IU or 500 IU of rCHIFN-α. This study indicates that the application of rChIFN-α as an adjuvant with pTracer-CMV-IBVS vaccine expressing S glycoprotein gene was somewhat effective in controlling IBV infection. Additionally an increase in protection against the homologous IBV challenge was observed in birds boosted with the live attenuated IBV commercial vaccine.

Key words: *In ovo*, recombinant DNA vaccine, chicken interferon alpha, infectious bronchitis virus

INTRODUCTION

Infectious Bronchitis Virus (IBV) is a highly contagious pathogen of poultry causing important economic loss in chicken operations. In laying birds, infection with IBV induces drops in egg production and egg quality and in broilers, condemnation due to airsaculitis at processing plants (Cavanagh and Naqi, 1997). Both live and inactivated virus vaccines are used for protection against IBV infection. However, the use of live vaccines poses the concern of artificially introducing the virus into the flocks and provides the potential of emerging new IBV variant strains (Ignjatovic *et al*., 2003; Moore *et al*., 1998; Wang *et al*., 1993; Wang and Khan, 2000). On the other hand, inactivated vaccines require manual injection of each bird, which is labor intensive and costly. Thus, there is a need for vaccines of higher efficacy and safety. Lately, a new approach has been directed towards the use of recombinant DNA vaccines as safer, more efficient ways of inducing immunity against diseases (Oshop *et al*., 2002; Ulmer *et al*., 1993; Wang *et al*., 2001; Zhou *et al*., 2003). The IBV genome encodes four structural proteins, small membrane protein (E), Membrane protein (M), Nucleocapsid (N) and Spike (S) protein (Boursnell *et al*., 1987; Godeke *et al*., 2000). The protein encoded by the spike gene (S gene) is post-transnationally cleaved yielding two subunits, S1 and S2 (Cavanagh, 1983). The S1 subunit is the bulbous part of the spike protruded outside the membrane (Kant *et al*., 1992; Zhou *et al*., 2003), while the S2 subunit has a small hydrophobic segment and anchors into the viral membrane (Cavanagh, 1983). The S1 glycoprotein induces Virus Neutralizing (VN) antibodies and is considered as the most likely inducer of protection (Johnson *et al*., 2003; Kant *et al*., 1992). S2 may also be important since it carries highly conserved epitopes for induction of cross-reaction antibodies (Cavanagh, 1983; Dhinakar and Jones, 1997). *In ovo* vaccination makes DNA-based vaccines more competitive and effective

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against poultry disease agents, because manual labor is eliminated and hatching chickens have been already vaccinated, thus giving them a better chance to be protected in an early age (Khan and Fabis, 2002; Kapczynski *et al*., 2003; Fabis and Khan, 2004; Stone *et al*., 1997; Wakenell and Sharma, 1996). In recent years, it has been shown that interferon modulates the type and the extent of immune responses after infection or vaccination and has the potential to serve as potent vaccine adjuvant (Asif *et al*., 2004; Le Bon *et al*., 2001; Lowenthal *et al*., 1998; Marcus *et al*., 1998; Rautenschlein *et al*., 2000; Sekellick *et al*., 1994; Schijns *et al*., 2000; Sick *et al*., 1998).

In this study, a plasmid DNA containing the entire S glycoprotein gene of Massachusetts 41 serotype of IBV along with recombinant chicken interferon alpha (rChIFN-α) was inoculated simultaneously *in ovo* and protection against the homologous IBV serotype was examined.

MATERIALS AND METHODS

Viruses and RNA extraction: A reference IBV strain Massachusetts 41, plaque-purified and maintained in laboratory was inoculated into the allantoic sac of 9-11-day-old fertile Specific-Pathogen-Free (SPF) chicken embryos (Gelb and Jackwood, 1998). Allantoic fluid was harvested for IBV virus after 48 h incubation at 37°C and was used for cloning and PCR tests. A virulent Massachusetts 41 IBV (Charles River, SPAFA, CT) was used as a challenge virus. Allantoic fluid was centrifuged at 40,000 x g for 1 h and the pellet was subjected to RNA extraction using the Trizol method according to the manufacturer's protocol (Invitrogen, CA).

Cloning and sequencing of the Spike gene: The following primers, IBV-S-F1: 5'-TTT GAG ATT GAA AGC AAC GCC-3' and IBV-S-F2: 5'-GAA GGA CGT GGG ACT TTG GAT-3' were designed from IBV Mass41 sequence data base and used for amplification of S gene. Briefly, RNA of IBV was reverse transcribed into cDNA in 20 µl mixture containing 2 µl of 10x buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 40 IU RNasin™ (a RNase inhibitor), 10 mM of each dNTP (2 µl each), 200 U of M-MLV reverse transcriptase (Invitrogen, CA). 1 µl (50 pmol) of IBV-S-F2 reverse primer and 5 µl (500 ng) RNA. The reaction was carried at 45°C for 1 h followed 95°C for 5 min. PCR was carried out using a master mix containing 8 µl of 10x buffer, 2 µl of 25 mM $MgCl_2$, 1 µl of 50 pmol IBV-S-F1 forward primer, 1 µl (5 U) of Taq DNA polymerase was added to 20 µl of RT reaction and the volume raised to 100 µl by adding molecular biology grade water. Amplification was conducted at 74°C for 6 min followed by a denaturation step performed at 94°C for 5 min, followed by 35 cycle of 1 min at 94°C, 2 min at 45°C and 5 min at 74°C. A final extension step was performed at 72°C for 10 min. Amplified DNA products were visualized on 1.2% agarose gel using TEA (Tris-Acetate-EDTA) and staining with ethidium bromide. A 3,200-bp amplified DNA product of S gene was excised from gel, then extracted and purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA). This amplified product was cloned into plasmid, pTracer-CMV-2 and transformed into competent *E. coli* according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). This recombinant plasmid construct was abbreviated as pCMV-S. Presence and orientation of the cloned S gene in pTracer-CMV-2 was checked by using S1 and S specific PCR methods (Fig. 3) and DNA sequencing analysis. DNA sequencing was performed at the DNA Biotechnology Facility, UCONN, Storrs, CT.

Immunohistochemistry (IHC) to detect expression of S gene in chicken embryo: Two methods of *in ovo* inoculation were used to confirm the expression in embryo tissues. Five (5) µg each of pCVM-S DNA in 0.2 ml of saline buffer was inoculated into the amniotic cavity of 18-day-old embryonating eggs. The second method of inoculation was performed using 5 µg of DNA in 5% DMSO in 0.2 ml as a vehicle and injected into the air chamber of the 18 day-old embryonating eggs as described previously (Moura *et al*., 2007; Oshop *et al*., 2003). Three days post-inoculation or at hatching, all hatchlings (day-old chicks) were humanly euthanized and their Bursas of Fabricius and thymuses were removed and stored at -80°C before testing. Ten micron sections of all the tissues were immediately examined under a UV microscope (Axiovert 200M, Zeiss Jena, Germany), equipped with filters specific for GFP and images were photographed. Polyvalent chicken antibodies against IBV (Charles River, SPAFAS, CT) and Cy5-labelled donkey anti-chicken (Jackson Immuno Research Lab, PA) were used in the immunoassay. The duplicate tissue sections were placed in staining chambers and immunoassayed for IBV-S protein. Tissues were mounted using FA mounting medium and immediately examined under a microscope (Zeiss Axiovert 200M, Zeiss Jena, Germany) equipped with a UV GFP-specific and a Cy5-specific filters and photographed.

***In ovo* immunization and challenge:** One hundred twenty 18 days-old embronating SPF chicken eggs were randomly divided into twelve groups as described in Table 1. Embryo vaccination (*in ovo*) was performed in each group as specified in Table 1 according to procedure described previously (Wakenell and Sharma, 1996). After hatching, all of the groups were housed in separate High Efficiency Particulate Air (HEPA) filter isolators under negative pressure at the University of Connecticut's animal facility. They were provided food and water *ad libtium*. Two weeks after hatching, the chicks in groups 3, 7 and 10 were vaccinated intranasally with $10^{3.8}$ $TCID_{50}$ live attenuated commercial Mass 41 vaccine (Fort Dodge, Iowa, USA).

Table 1: Protective immunity against IBV after *in ovo* vaccination

Groups	*In ovo* vaccine	Secondary vaccine at 2 weeks of age	Clinical signs 5 days post-challenge	Virus detection by RT-PCR from tracheal swab	Percent of protection (virus recovery test)
1	pCMV-S (5 μg) + 2000 U rChIFNα	None	25%	-	89*
2	pCMV-S (5 μg) + 500 U rChIFNα	None	25%	-	83*
3	pCMV-S (5 μg) + 500 U rChIFNα	LAV	None	-	92*
4	pCMV-S (5 μg)	None	50%	+	61
5	2000 U rChIFNα	None	75%	+	58
6	500 U rChIFNα	None	75%	+	65.71
7	500 U rChIFNα	LAV	25%	-	76.66
8	pTracer-CMV no insert (5 μg)	None	100%	+	48
9	Vehicle (PBS)	None	100%	+	38
10	Vehicle (PBS)	LAV	25%	-	89.74*
11	Control (Challenge)	None	100%	+	25
12	Control (non vaccinated-non-challenge)	None	None	-	100

LAV = live attenuated vaccine

Twenty-eight days (4 weeks of age) after hatching, all of the birds in groups 1-11 were challenged intranasally with 5.0 x $10^{4.7}$ EID_{50} /bird with a virulent field strain of Massachusetts 41 IBV. Birds in all groups were monitored daily for clinical signs and necropsied 5 days after virus challenge. At necropsy clinical signs were recorded and blood samples were collected from each bird for serology. The tracheal swabs were taken from each bird for virus isolation. Treacheal swabs from each bird in each group were placed in 2.0 ml sterile tryptose phosphate broth (GIBCO, MD) and stored in -80°C freezer. In addition sections of tracheas were taken and fixed in 10% formalin for histopathology.

Virus recovery/protection: According to method described for IBV virus recovery (Gelb and Jackwood, 1998), five 9-11 day-old SPF embryonating chicken eggs were inoculated in the allantoic cavity with 0.2 ml of each tracheal swab filter sterilized sample. Eggs were incubated at 37°C. The eggs were candled daily for 5 days and the eggs with dead embryos were kept at 4°C. On the fifth day of post-inoculation, all eggs were kept overnight at 4°C. The allantoic fluid was harvested and the embryos were examined for lesions typical of IBV infection. These lesions included dwarfing, stunting, or curling of embryos, being clubbed down or death during the 2-5 day post-inoculation period.

RT-PCR identification: Viral RNA extraction was performed on allantoic fluids recovered as above using TRizol reagent according to manufacturer protocol (Invitrogen, CA). Amplification of the S1 gene was carried out using the primers forward S1OLOGO5': 5'TGAAACTGAACAAAAGACA3' and the reverse S1OLIGO 3':5'CATAACTAACATAAGGGCAA3' (14). Briefly, Reverse Transcription (RT) reaction was carried out using M-MLV (Invitrogen, CA) and reverse S1 oligo primer. The first strand of cDNA was immediately used for amplification by PCR. A mixture containing 5 μl of viral RNA, 1 μl of 10 mM dNTPs, 1 μl of reverse Primer (100 pmol) and 5 μl of water was made for the RT reaction. The mixture was heated at 65°C for 5 min and then quickly chilled on ice. The microtubes were briefly centrifugated and a mixture of 4 μl of 5x first strand buffer, 2 μl of 0.1 M DTT and 1 μl (40 IU) of RNase inhibitor (Invitrogen, CA) were added to the reaction. The microtube was heated at 37°C for 2 min and then 1 μl of M-MLV was added to each tube and mixed gently. The reaction was carried on at 37°C for 50 min and continued at 70°C for 15 min.

PCR amplification was carried out using 3 μl of cDNA from above RT reaction and added to the pre-mixture (2 μl of 25 mM magnesium solution, 5 μl of 10x buffer, 1 μl of 10 mM dNTPs, 1 μl (100 pmol) of each oligo primers, 2 μl of Taq DNA polymerase 5U/μl and 35 μl of autoclaved distilled water). PCRs were performed with an initial denaturation at 95°C for 1 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 2 min and extension at 72°C for 5 min. A final extension step was conducted at 72°C for 10 min. PCR amplification products were analyzed on a 1.2% agarose gel stained with ethidium bromide.

Serology: To determine if DNA vaccination with the S gene could induce virus-specific antibodies, the humoral immune response to IBV was measured during the course of the experiment. After *in ovo* DNA vaccination blood samples were collected at 14, 28 and 33 days of age, Enzyme-Linked Immunosorbent (ELISA) was performed using infectious bronchitis virus antibody test kit (IDEXX, Portland, ME).

Histopathology: Tracheal tissue samples fixed in 10% formaline were processed and cut into 5 um sections and stained with hematoxylin and eosin for histopathologic examination. The tracheal lesions were scored according previously described scoring method (Kapczynski *et al*., 2003). Statistical analysis of the data was performed by Kruskal-Wallis one-way analysis of variance with the Dunn's post-hoc with SigmaStat Version 3.0.

RESULTS

S gene *in ovo* expression in tissues and Immunohistochemistry (IHC): Inoculation of the pCMV-S using DMSO as a vehicle and injecting into the air

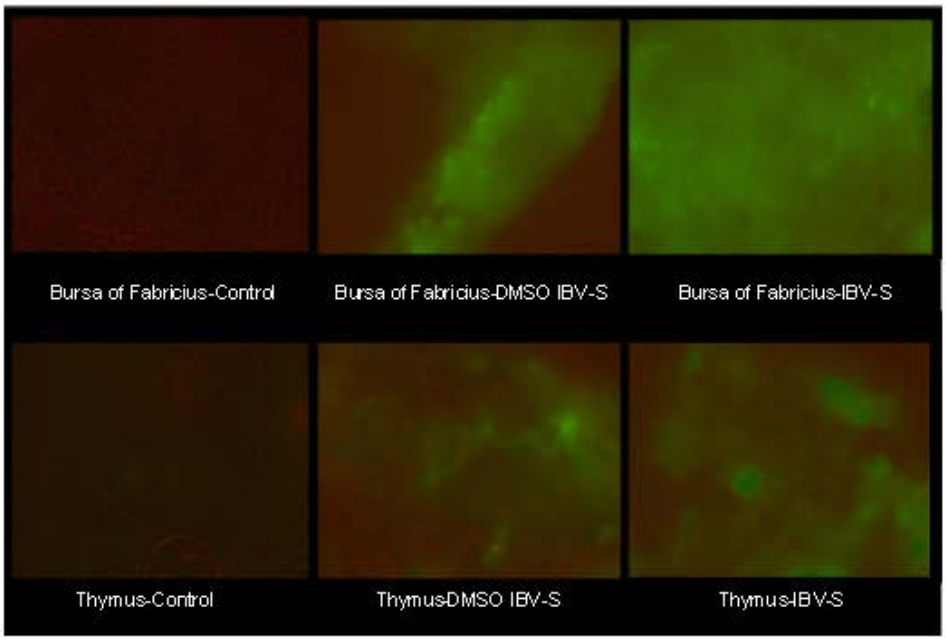

Fig. 1: *In ovo* expression of the IBV-S glycoprotein (IBV-S) in Bursa of Fabricius and thymus of day old chick using UV microscope equipped with GFP specific filters. Control tissues sections from Bursa of Fabricius and Thymus day-old chick without inoculation of IBV-S. Tissue sections of day-old chick from Bursa of Fabricius and Thymus inoculated with IBV-S using DMSO inoculation in air-chamber and *in ovo* injection in amniotic cavity of embryonating eggs. Photographs were taken after 2 sec of light exposure time

chamber or the amniotic cavity have showed no differences in expression of pCMV-S in the tissues. Images photographed from the tissue sections and GFP expressions (Fig. 1) have shown that the plasmid, pCMV-S was absorbed by the developing embryo and expressed in the tissues of Bursa of Fabricius and thymus. Immunoassay results were also complimentary to the tissues images of GFP expression. Detection of expression of S protein using GFP and Cy5 labeled anti-IBV antibodies on tissue sections of Bursa of Fabricius and thymus indicated the absorption and expression of pCMV-S (Fig. 2).

Protection against homologous IBV challenge after *in ovo* DNA vaccination: As expected, the non-challenged non inoculated group (group 12) was negative for clinical signs and histopathological lesions of IBV infection (Table 1; Fig. 4). The groups vaccinated with the pCMV-S and rChIFN-α (group 1, 2) showed minor clinical symptoms and lower histopathological lesion scores, they were protected 89% and 83% respectively (Table 1; Fig. 4). However, when the live attenuated vaccine was administered at two weeks of age in group 3 (pCMV-S + 500 IU rChIFN-α), no clinical signs and histopathological lesions were observed. The birds in this group were 92% protected.

Birds in challenged groups 5 and 6, which did not receive pCMV-S but were inoculated only with rChIFN-α (2000IU, 500IU) were protected between 58% and 66% respectively. Birds in these two groups showed complications and clinical signs associated with IBV infections such as tracheal rales, coughing and ruffled feathers. These groups had relatively higher histopathologic lesion scores (Table 1; Fig. 4).

There was no significant statistical difference between groups that received PBS, plasmid with no insert (group 8 and 9) and control challenge group 11 had clinical symptoms, higher histopathologic lesions scores and higher viral infection load (Table 1; Fig. 4).

Virus detection by RT-PCR for S1 gene: Tracheal swabs were tested for the presence of IBV using RT-PCR. The samples were considered negative if after two blind passages in SPF eggs the result was still negative. The non-challenged group (group 12) was negative for IBV-S1 gene PCR while the challenged group (group 9) which received PBS, was IBV-S1 gene PCR positive. The *in ovo* vaccinated groups showed different protection results corresponding to the vaccination regiment. The groups 1 and 2, which received the pCMV-S and ChIFN-α were IBV S1- PCR negative, while the birds receiving the DNA vaccine alone or rChIFN-α only (groups 4, 5 and 6) were IBV S1 -PCR positive. The group which received the DNA vaccine and rChIFN-α followed by a live attenuated vaccine booster 2 weeks later, (group 3) was IBV S1- PCR negative. The groups that received ChIFN-α

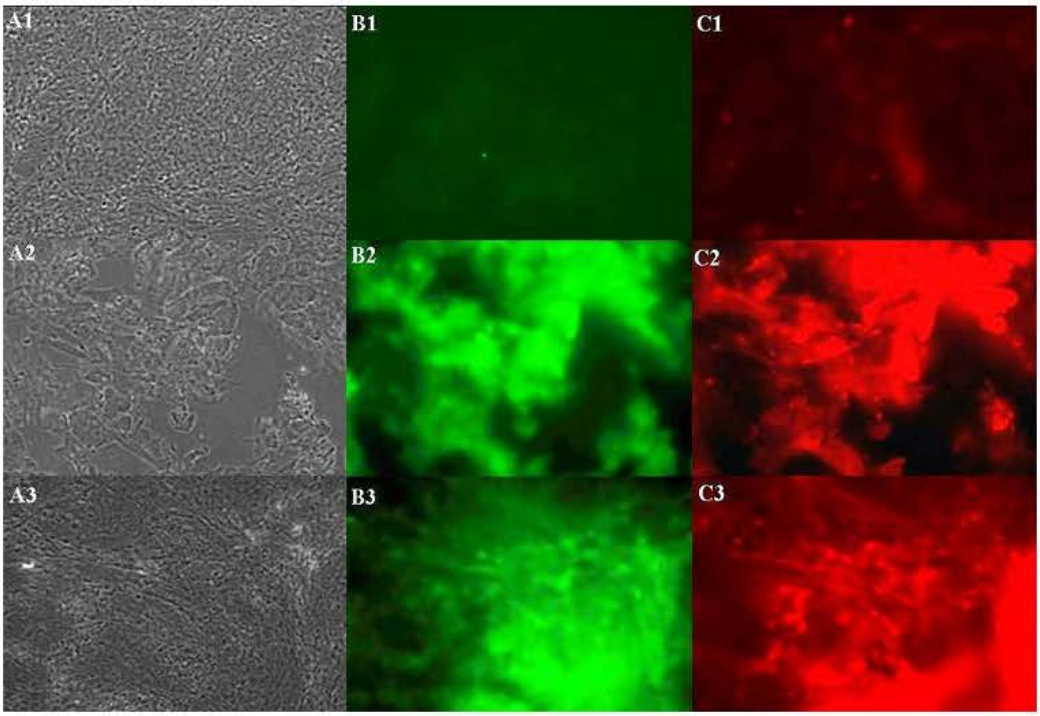

Fig. 2: Indirect Immuno-histochemstry. Bursa of Fabricius tissue sections from a day old chick inoculated *in ovo* with IBV-S at 18 days embyronating SPF eggs were co-expressed using the indirect immunoassay. A 1-3 Bursal tissue sections without staining; B1, direct image with GFP filter without staining; C1 direct image with Cy5 filter without staining; B2 and B3, immunostaining with GFP filter; C2 and C3 immunostaining with Cy5 filter

or PBS only and were boostered with attenuated live commercial vaccine at 2 weeks age (groups 7 and 10) were IBV S1-PCR negative.

Humoral immune response: The birds vaccinated with pCMV-S had no detectable antibody levels using IBV ELISA kit (IDEXX, Portland, ME). The birds who receive *in ovo* DNA vaccine had the same antibody levels as unchallenged buffer-vaccinated birds at day 14, 28 and 33. However, for groups received a commercial live attenuated vaccine as booster (groups 3, 7, 10), the antibody titer was detectable and mean titers at day 28 were 1224, 411 and 745 respectively.

Histopathology: The median score for tracheal lesions of each group (Table 1; Fig. 4) was evaluated. A Kruskal-Wallis one-way analysis of variance with the Dunn's post-hoc test was used to compare the control group to each test group using $p<0.05$ for significance. There were no significant differences between group 1, 2, 3, 7 and 10 vs. control groups (group 12), which indicated efficiency of vaccination regiment. The birds in group 5, 6, 8 and 11 that did not receive the pCMV-S and those who did not receive secondary live attenuated commercial vaccine as booster had median tracheal lesion scores $\geq$1.75 which was significantly higher than negative non-challenge group.

DISCUSSION

The presented results indicate *in ovo* application of rChIFN-α as an adjuvant with a pCMV-S containing the whole S glycoprotein gene produces significant protective immunity. It has been shown that vaccination of chickens with the DNA vaccine containing the S1 glycoprotein gene from IBV can lead to a protective immunity against infectious bronchitis (Johnson *et al*., 2003; Wang *et al*., 2001). Further, immunization of 18-day old embryonating chicken eggs with this combination followed by a commercial live attenuated vaccine significantly protects birds from homologous virus challenge. Antibody responses after *in ovo* vaccination were evaluated using an IBV ELISA test. Birds vaccinated with pCMV-S had non-detectable antibody level. However, birds in group 3, 7 and 10 which received a live attenuated commercial vaccine at 2 wk of age showed high detectable antibody titers against IBV. Therefore the results of this study correspond to the reports from previous studies in that the DNA-vaccinated birds did not show antibody titer before virus challenge (Dhinakar and Jones, 1997; Fynan *et al*., 1993). The reason for undetectable antibody response after

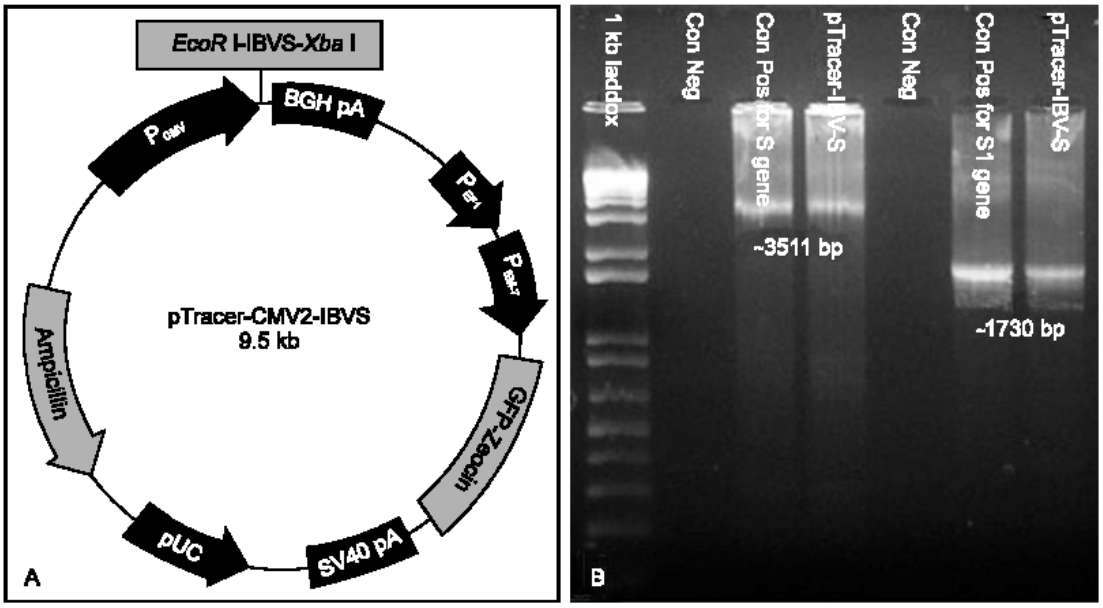

Fig. 3: A pTracer-CMV2-IBVS. CMV promoter = human cytomegalovirus Mediated-early promoter; IBVS = S gene from Mass 41; BGH pA = bovine growth hormone transcription and processing signals; SV40 pA = simian virus 40 polyadenylation signals; ampicillin = ampicillin resistance gene. B. PCR conformation of existence of the S and S1 genes

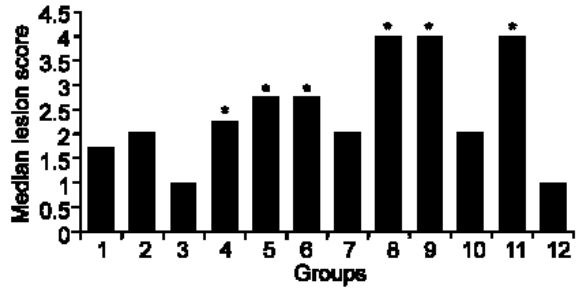

Fig. 4: Median histolpathologic lesion scores for the experimental groups 1-11 challenged with Mass 41-IBV at 28 days after hatch. Group 12 in non-challenge non-vaccinated (Negative control)

application of the DNA-vaccine is still unclear. However, protection against IBV challenge in absence of detectable antibody indicates induction of a cell mediated immune response in the vaccinated birds with a DNA-vaccine. From the expression images and immunohistochemistry results, one may conclude that there should be a population of IBV-sensitized T and B-lymphocytes in the hatchlings involved for protection against IBV challenge.

Adjuvant activity of the IFN-α/β has been shown in the mouse model (LeBon *et al*., 2001). It also has been reported that mammalian interferon involves in innate immunity toward wide range of viral pathogens. It was reported that treatment of chicken with rChIFN-α effects replication of Marek's disease virus strain R2/23 (Jarosinski *et al*., 2001). In present study 500 IU and 2000 IU rChIFN-α was used with the pCMV-S and the result showed some degrees high protection as compare to non-adjuvant group (group 4; Table 1). A significant protection was induced after *in ovo* inoculation of these combinations. An enhanced immune response in chickens and turkeys has been shown when the vaccine contained recombinant chicken IFN-γ (rChIFN-γ) (Marcus *et al*., 1998). It was showed that using a rChIFN II expressed in a Fowl Pox Virus (FPV) vector had superior adjuvant activity over same construct with type I IFN to enhance vaccine response in turkeys (Rautenschlein *et al*., 2000). Current study demonstrated that ChIFN-α can be used as an adjuvant to enhance DNA vaccine response in chickens.

Marcus *et al*. (1998) reported oral administration of rChIFN-α via the drinking water can ameliorate Newcastle disease in treated chickens. Chickens receiving a high amount of rChIFN-α had lesser weight loss and reduced pathology of the tracheal tissue after viral infection.

Results of this study indicate that application of rChIFN-α as an adjuvant may reduce the amount of the DNA vaccine needed for inoculation. In recent study 150 µg of DNA vaccine coding S1 gene of IBV induced IBV protection (Kapczynski *et al*., 2003), however, in this study using 5 µg of the DNA vaccine coding S protein of IBV in combination with rChIFN-α was able to induce similar protection. The results also showed that in order

to achieve higher protection, a booster vaccination with a live attenuated virus maybe required.

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