ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF
POULTRY SCIENCE

ANSI*net*
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

International Journal of Poultry Science 6 (11): 776-783, 2007
ISSN 1682-8356
© Asian Network for Scientific Information, 2007

Evaluation of *in Ovo* Delivery System for Plasmid DNA Vaccination

L. Moura[1], M. Liu[2] and V.N. Vakharia[3]
[1]Department of Pathobiology, School of Veterinary Medicine,
Ross University, PO Box 334, Saint Kitts, West Indies
[2]Department of Microbiology and Immunology, Uniformed Services University of Health Sciences,
4301 Jones Bridge Road, Bethesda, Maryland, USA-20814
[3]Center for Biosystems Research, University of Biotechnology Institute,
College Park, Maryland, USA-20742

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 isotope-ELISA assays.

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

Introduction

In the 1990s, an entirely new type of vaccine (DNA vaccines) was first described (Wolff *et al*., 1990; Robinson *et al*., 1993; Ulmer *et al*., 1993). 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 (Scholz *et al*., 1993; Fynan *et al*., 1993; Sakaguchi *et al*., 1996; Corr *et al*., 1996). DNA vaccines have also been successfully used in poultry to immunize against several pathogens (Fynan *et al*., 1993; Robinson *et al*., 1993; Sakaguchi, 1996). 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*., 2002; 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 dimethyl sulfoxide (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.*, 2002; 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.*, 2002) 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

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'-A C C G C G G T A C T A A C C A G A C C T G GCTTCTCTAAC-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 (Fig. 1). *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).

***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 transect Vero cells using Lipofectin (Invitrogen, Grand Islands, NY). Protein expression was observed by

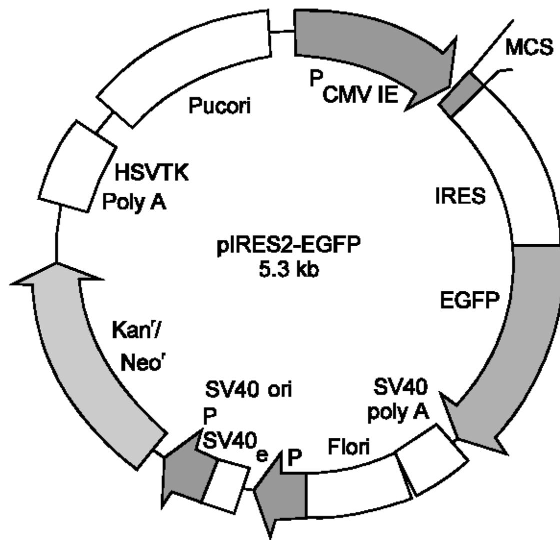

Fig. 1: Schematic diagram of a 5.3Kb eukaryotic expression vector used to express HN protein. NDV-HN gene was inserted in the Multiple Cloning Sites (MCS). Independent EGFP expression was possible due to the presence of Internal Ribosomal Entry Site (IRES) downstream of MCS

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 and 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 4°C 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.

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.

Serology: Humoral immune responses to HN protein were measured by ELISA (Synbiotics, San Diego, CA), isotope ELISA and HI. To verify specific antibody isotope (IgG, IgA and IgM), serum and tear samples were analyzed 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 isotope, 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.

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

***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 (Fig. 2).

HN expression by pIRES-HN-EGFP was confirmed by the transfection of Vero cells and immunostaining technique (Fig. 3).

***In vivo* EGFP expression after *in ovo* injection:** Tissue distribution of EGFP expression is shown in Fig. 4. 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.

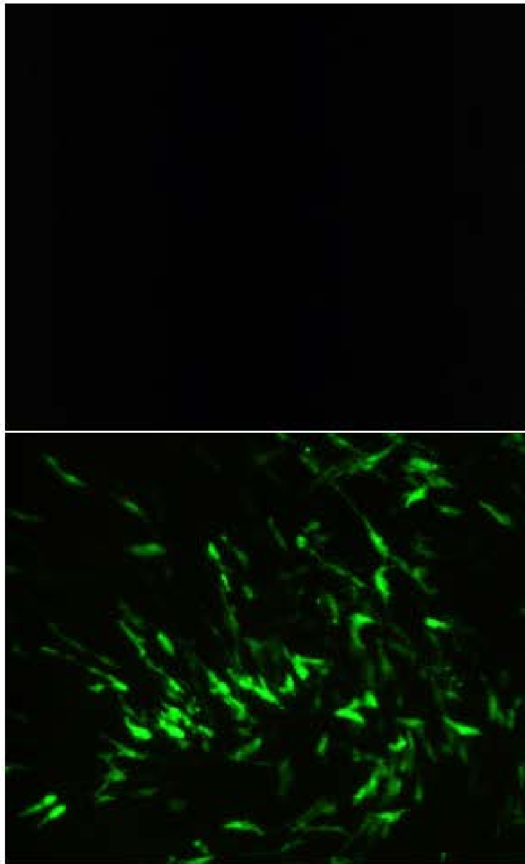

Fig. 2: HD11 avian macrophage cells transfected with plasmid CMV-EGFP-BGH. A) mock-transfected cells; B) cells visualized under UV light 48 h post-transfection with 5 µg of plasmid DNA (magnification 100X)

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.

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 Fig. 5. 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

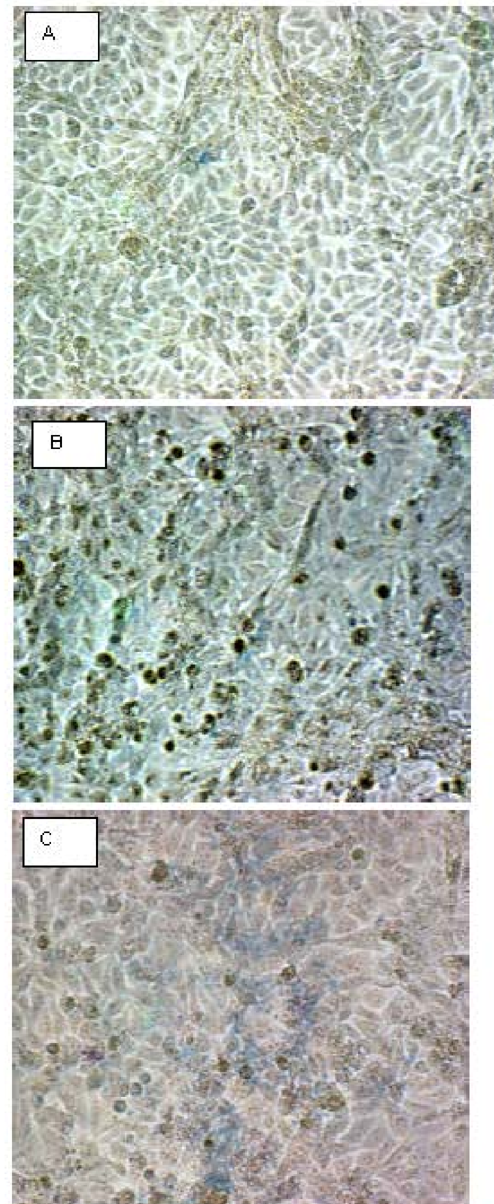

Fig. 3: *In vitro* expression of HN protein in Vero cells after transfection with pIRES-HN-EGFP. Cells were transfected with 5 µg of plasmid using LipofectAmine transfection reagent. Cells were immunostained with polyclonal chicken anti-NDV, labeled with goat anti-chicken peroxidase and stained with Trueblue® (Kirkegaard and 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)

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

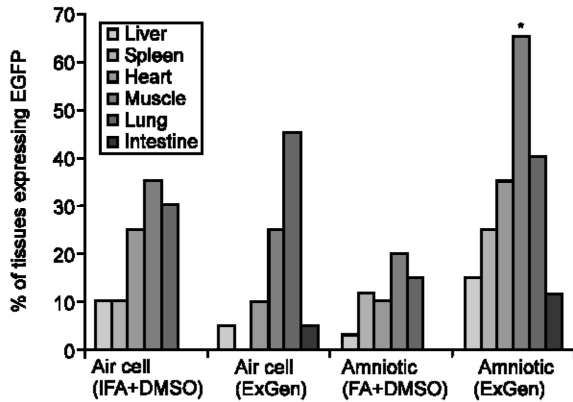

Fig. 4: Tissue distribution of EGFP expression from embryos inoculated with plasmid pCMV-EGFP-BGH DNA (60 µg/egg) through air cell and amniotic cavity using two different formulations (IFA+DMSO and PEI-ExGen®). All embryos were inoculated at 18 days of embryonation. Tissues were collected prior to hatch and examined under UV light. *Significantly different from all other tissues ($p<0.05$)

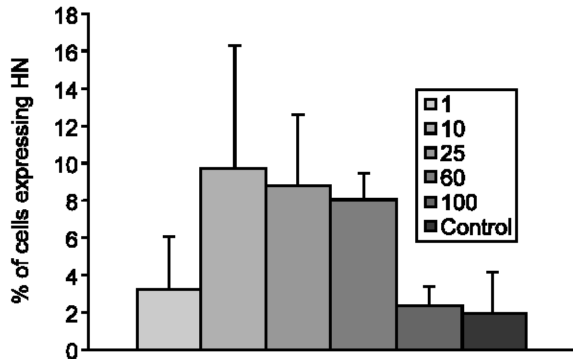

Fig. 5: Percentages of splenocytes expressing HN from embryos inoculated at 18 days of embryonation. Embryos were inoculated through the amniotic cavity with 1, 10, 25, 60 and 100 µg/egg of pIRES-HN-EGFP DNA mixed with PEI ExGen®, whereas, the control group consisted of 60 µg/egg of pIRES-EGFP DNA. The 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

(1 µg/egg) are able to transect 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.

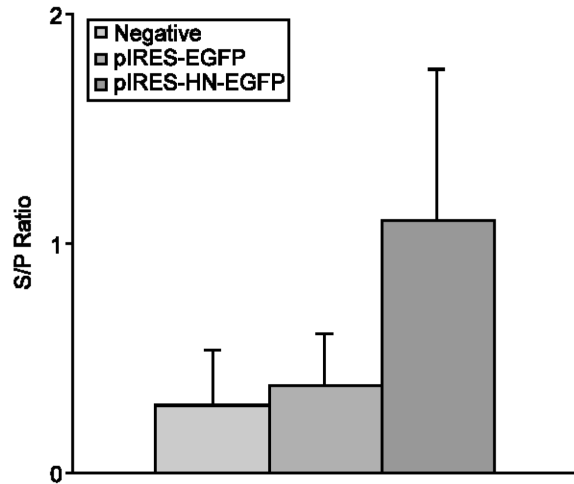

Fig. 6: IgM isotope-ELISA results from serum samples collected at 5 weeks post *in ovo* vaccination with 60 µg/egg of pIRES-HN-EGFP DNA. SPF, 18-day-old embryos were vaccinated through the amniotic cavity using PEI-ExGen®. Graph shows the mean of the ratios between sample and positive control. Negative control embryos were unvaccinated. Plasmid DNA control embryos were inoculated with pIRES-EGFP plasmid DNA lacking the HN gene

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. Fig. 6 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 analyzed by Hemagglutination Inhibition (HI) test were negative at all collection periods.

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 *et al.* (2002, 2003) 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 transect 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, isotope 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 isotope 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.

References

Akiyama, T., B. Whitaker, M. Federspiel, S.H. Hughes, H. Yamamoto, T. Takeuchi and T.D. Brumeanu, 1994. "Tissue-specific expression of mouse tyrosinase gene in cultured chicken cells". Exp. Cell Res., 214: 154-162.

Corr, M.D., D.J. Lee, D.A. Carson and H. Tighe, 1996. "Gene vaccination with naked plasmid DNA: mechanism of CTL priming". J. Exp. Med., 184: 555-1560.

Elankumaran, S., R.A. Heckert and L. Moura, 2002. "Pathogenesis and tissue distribution of a variant strain of infectious bursal disease virus in commercial broiler chickens". Avian Dis., 46: 169-176.

Elankumaran, S., G.D. Raj, A. Thangavelu, A. Koteeswaran, A. Albert and A.T. Venugopalan, 1996. "Estimation of antibodies to Newcastle disease virus in tears". Trop. Anim. Health Prod., 28: 293-297.

Fodor, I., E. Horvath, N. Fodor, E. Nagy, A. Rencendorsh, V.N. Vakharia and S.K. Dube, 1999. "Induction of protective immunity in chickens immunized with plasmid DNA encoding infectious bursal disease virus antigens". Acta Vet. Hungarica, 47: 481-492.

Fynan, E.F., R.G. Webster, D.H. Fuller, J.R. Haynes, J.C. Santoro and H.L. Robinson, 1993. "DNA vaccines: protective immunizations by parenteral mucosal and gene-gun inoculations". Proc. Natl. Acad. Sci. USA, 90: 11478-11482.

Heckert, R.A., S. Elankumaran, G.L. Oshop and V.N. Vakharia, 2002. "A novel transcutaneous plasmid-dimethylsulfoxide delivery technique for avian nucleic acid immunization". Vet. Immuno. Immunopath., 89: 67-81.

Kapczynski, D.R., H.S. Sellers, G.N. Rowland and M.W. Jackwood, 2002. "Detection of *in ovo*-inoculated infectious bronchitis virus by immunohistochemistry and in situ hybridization with a riboprobe in epithelial cells of the lung and cloacal bursa". Avian Dis., 46: 679-685.

Kodihalli, S., D.L. Kobasa and R.G. Webster, 1997. "Strategies for inducing protection against avian influenza A virus subtypes with DNA vaccines". Vaccine, 18: 2592-2599.

Lewis, P.J. and L.A. Babiuk, 1999. DNA vaccines: a review. Adv Virus Res. 54:129-88. Review

Oshop, G.L., S. Elankumaran and R.A. Heckert, 2002. "DNA vaccination in the avian". Vet. Immuno. Immunopath., 89: 1-12.

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

Robinson, H.L., L.A. Hunt and R.G. Webster, 1993. "Protection against a lethal influenza virus challenge by immunization with a hemagglutinin-expressing plasmid DNA". Vaccine, 11: 957-960.

Sakaguchi, M., H. Nakamura, K. Sonoda, F. Hamada and K. Hirai, 1996. "Protection of chickens from Newcastle disease by vaccination with a linear plasmid DNA expressing the F protein of Newcastle disease virus. Vaccine, 14: 747-752.

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. "Molecular cloning". A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, Long Island, NY.

Scholz, E., C. Zhang and P. Guo, 1993. "Transactivation of the early SV40 promoter by avian infectious laryngotracheitis virus in avian hepatoma cells". J. Virol. Methods, 45: 291-301.

Sharma, J.M., 1986. "Embryo vaccination of specific-pathogen-free chickens with infectious bursal disease virus: tissue distribution of the vaccine virus and protection of hatched chickens against disease". Avian Dis., 30: 776-780.

Suarez, D.L. and S. Schultz-Cherry, 2000. "The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chicken using an avian influenza model". Avian Dis., 44: 861-868.

Ulmer, J.B., J.J. Donnelly, S.E. Parker, G.H. Rhodes, P.L. Felgner, V.J. Dwarki, S.H. Gromkowski, R.R. Deck, C.M. DeWitt and A. Friedman, 1993. "Heterologous protection against influenza by injection of DNA encoding a viral protein" Sci., 259: 1745-1749.

Wakenell, P.S., T. Bryan, J. Schaeffer, A. Avakian, C. Williams and C. Whitfill, 2002. "Effect of *in ovo* vaccine delivery route on herpesvirus of turkeys/SB-1 efficacy and viremia". Avian Dis., 44: 536-541.

Wolff, J.A., R.W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani and P.L. Felgner, 1990. "Direct gene transfer into mouse muscle in vivo". Sci., 247: 1465-1468.