Title
Improving chicken quality by optimizing in ovo vaccination procedures

Name of University
North Carolina State University

Principal investigator
Isabel M. Gimeno
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Total fund requested: $12,576
Duration of the project: October 1st 2018- September 30th 2019
Entity to which the check will be made payable: North Carolina State University

Keywords
Marek’s disease virus, vaccines, in ovo, immunity
1. PROJECT TITLE:
Improving chicken quality by optimizing in ovo vaccination procedures

2. PROJECT INVESTIGATOR:

**Investigator:** Isabel M. Gimeno
Department of Population Health and Pathobiology
College of Veterinary Medicine
North Carolina State University, Raleigh, NC

3. OBJECTIVES:
Newly-hatched chicks get exposed to numerous antigens within the first few days of life when their immune system is nascent. In commercial chicken flocks, the highest mortality generally occurs during the first week. To avoid early mortality associated with infection, antimicrobials are commonly administered in the hatchery. The use of antibiotics is now becoming restricted and so this might not be an option in the near future. Vaccination in ovo, now commonplace for Marek’s disease (MD) is an alternative means for providing additional protection via innate and adaptive immune responses. Administration of herpesvirus of turkeys (HVT) to 17-19 day old embryos (E17-E19) has been shown to increase protection against MD (6). Our preliminary results show that vaccination with HVT at E18 rendered chicks at hatch more responsive not only to MD but also to an unrelated antigen keyhole limpet hemocyanin (KLH). This interesting experimental finding suggests that immune priming of late stage chick embryos produces chicks that are more immunocompetent at hatch and better able to cope with infection. Our hypothesis is that it is possible to hasten the immune system maturation of the chicken embryos by administering MD vaccines in ovo and that will improve and strengthen the immunocompetency of commercial chicks at hatch. The specific objectives of this project are

1) To optimize the dose of HVT that needs to be administered to achieve the highest benefit on the development of the immune system in chicken embryos

2) To evaluate if recombinant HVTs have the same effect as conventional HVT on the development of the chicken immune system.
4. JUSTIFICATION

The rationale for the proposed experiments rests on the continuing need to improve the health of poultry that are grown for food in ever increasing numbers worldwide. Increasing poultry production has to be accompanied with high standards of animal welfare and food safety and with no risk of human health due to antimicrobials or toxic residues. The only way to achieve such objectives is to improve the immune system of the chickens at hatch. Protective immune responses can be generated in chick embryos by vaccination during late stage development (three days prior to hatch). In ovo vaccination at 18 days of embryonation with MD vaccines is now automated and has become a general practice worldwide. In ovo vaccination against MD confers higher levels of protection against early challenges with Marek’s disease virus than vaccination at day of hatch. In addition to providing protection against MD, we have showed that administration of HVT render chicks more responsive to non-related antigens and therefore that HVT can be used as an adjuvant promoting immune responses to other antigens (12). Furthermore, our preliminary results show that such beneficial effect happens not only in SPF egg type chickens but also in commercial broilers. This finding suggests that with increased understanding of the immune responses to vaccination, immune activation in late stage chick embryos can be further optimized to produce more immunocompetent chicks with little modification of current practices. The results of this study will aid in optimizing in ovo vaccination procedures to improve chick quality.

Preliminary Results.

Experiments showing that in ovo vaccination with HVT hastens maturation of chicken embryo immune system. In preliminary work conducted at our laboratory, we demonstrated that administration of HVT at E18 greatly accelerated appearance of MHC class I (Figure 1), which has been observed repeatedly to appear only slowly following hatch in chicks without HVT vaccination.
Furthermore, lymphocytes of the one-day-old (D1) chickens vaccinated with HVT in ovo were able to proliferative better than the other two groups (D1-sham inoculated and D11) when in presence of concanavalin A (Figure 2).

The adjuvant effect of HVT when administered in ovo was confirmed and extended in a second experiment. In this experiment administration of HVT at E18 was tested for its capacity to enhance antibody responses using a T cell-dependent antigen KLH (Figure 3). The design was similar to the previous experiment but here D1 (inoculated with HVT or vaccine diluent only in ovo at E18), and non-vaccinated D11 chickens were immunized subcutaneously with 1mg of KLH. KLH was administered twice within an interval of 11 days and antibody response was evaluated 11 days after inoculation. After the first exposure, there was no difference in the antibody activity level between 1d-HVT and 1d-sham and both groups had significant less activity than the older chickens (11d).
After the second exposure, the antibody activity level of 1d-HVT groups was higher than 1d-sham although it was lower than older chickens (11d).

**Figure 3. Antibody responses to KLH**

In previous work (12) we had demonstrated that administration of HVT in ovo to SPAFAS chickens hasten immunocompetence. Results of our preliminary studies confirmed that HVT has similar effect in commercial broilers. Since in ovo vaccination of broilers with HVT is a general practice it would be possible to optimize the “adjuvant” effect of HVT with minimum modifications of the standard practices. Because HVT seems to have stronger effect on the cellular immune responses on the broiler chicken embryos than in the humoral immune responses, in the present study we will focus on evaluating cellular immune responses.

5. **PROCEDURES**

Two animal experiments will be conducted using commercial meat type chickens. **Experiment 1 (Aim 1)** will evaluate the effect of HVT dose on the immune system of chicken embryos when administered in ovo. In this experiment four doses of a commercial conventional HVT will be used (2000, 4000, 8000, and 16000 PFU). **Experiment 2 (Aim 2)** will evaluate if two recombinant HVTs have the same effect on the immune responses as a conventional HVT. Immune responses will be evaluated by the ability of splenocytes to proliferate and to express IFN-γ when exposed to Con-A.

**Experimental design.**

Experiment 1: Effect of vaccine dose on “adjuvant” effect of HVT when administered in ovo, 5350 E18 embryonated eggs will be inoculated with HVT at either 2,000 PFU, 4000, 8000, or 16000 or
with 0.1ml of the vaccine diluent (sham-inoculated). At hatch, 70 chickens of each treatment groups will be euthanized and samples of spleen will be collected for flow cytometry and in vitro studies (lymphoproliferation and IFN-γ expression). Spleen of 10 chickens will be pooled to have a total of 7 samples per treatment group. In addition, there will be a control group of 21 naïve chickens that will be 11 days old at the beginning of the experiment.

Experiment 2. Comparison of the adjuvant effect of a conventional HVT and two recombinant HVT vaccines. 280 E18 embryonated eggs will be inoculated with either a conventional HVT or a recombinant HVT (rHVT-A or rHVT-B) at 2,000 PFU or with 0.1ml of the vaccine diluent (sham-inoculated). At hatch, 70 chickens of each treatment groups will be euthanized and samples of spleen will be collected for flow cytometry and in vitro studies (lymphoproliferation and IFN-γ expression). Spleen of 10 chickens will be pooled to have a total of 7 samples per treatment group. In addition, there will be a control group of 21 naïve chickens that will be 11 days old at the beginning of the experiment.

**Marek’s disease vaccines and vaccination.** FC-126 strain at 19 passages in chicken embryo fibroblasts (CEF)(29), and two recombinant HVTs of commercial source will be used. rHVT-A includes inserts of infectious bursal disease and rHVT-B includes inserts of Newcastle disease. Vaccination will be conducted at E18 by intra-amniotic route manually as described (23). Vaccine replication will be monitored by collecting feather pulp samples and evaluating vaccine DNA load by real time PCR as described (8).

**Collection of spleens.** Spleens will be collected aseptically from humanely euthanized newly-hatched chicks. Splenocytes will be released by gently pressing the spleens through sterile gauze. Tissue debris will be separated by decantation and cells will be washed in PBS twice.

**Lymphoid proliferation assays (MTT).** Splenic lymphocytes will be prepared in RPMI 1640 Medium without phenol red (Life Technology, Grand Island, NY) containing 5% fetal calf serum (Life Technology, Grand Island, NY), 5% chicken serum (Sigma Chemical Co., St Louis, MO), 2mM L-glutamine, 100 U penicillin/ml, 100 g streptomycin/ml, 2X10-6 M 2-mercaptoethanol, 5 g 5-fluorocytosine/ml, and 1mM sodium pyruvate (IMDM-10). 3.13 X10⁵ cells in a volume of hundred microliters will be put into each well of a 96-well flat-bottomed tissue culture plate (Sigma Chemical Co., St Louis, MO). One hundred microliters of Con A (Sigma Chemical Co., St Louis, MO) will be added to each well (12.5 units per well). Medium without Con A will be used as negative control. Each assay will be performed in triplicate. Plates will be incubated at 41°C for 48hr in a 5% CO₂ incubator. The MTT assay will be performed using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) following manufacturer’s recommendations.
**IFN-γ ELISA.** A total of 2 X 10⁶ splenocytes per chicken will be incubated for 48h in a 24-well plate in 500 µl of culture medium at 41⁰C in a 5% CO₂ incubator, without stimulation or in the presence of ConA. Afterwards, supernatants will be tested for ChIFN-γ using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, California USA) according to the manufacturer’s instructions.

**Key equipment, reagents and personnel required.** The laboratory of the PI has the facilities and equipment necessary to conduct this study. The PI and the technical support has ample experience in conducting all the necessary assays.

### 6. LITERATURE REVIEW

**Development of immune responses in chicken embryos.** In the absence of vaccination in ovo, the immune system develops rapidly only following hatch. Mast and Goddeeris (18) studied the response of embryos and early post-hatch chickens (E16, E18, D1, D7, and D12) immunized with BSA, a thymus-dependent antigen, and found that only the D12 chickens were capable of mounting significant IgG and IgM responses. Similarly, chickens become fully competent alloimmune responses to MHC-B antigens only 2-3 weeks following hatch (20). Lehtonen and coworkers (16) studied the ontogeny of alloreactivity in chickens and D7 chickens had higher alloreactivity compared with D3 chickens with D1 chickens entirely lacking the capacity for alloreactivity. Seto (22) compared the immune responsiveness of E19 embryos and chickens between D2 and D9 and discovered that D8 chickens could mount higher immune responses in the spleen compared with the younger chickens and embryos. Lowenthal et al. (17) found that chickens display transient poor T cell responsiveness during the 1-2 weeks of life. The lack of T cell responsiveness is not due to lack of cells, but rather to the inability to the T cells in very young animals to perform, under the test conditions, the steps necessary for cell activation including cytokine secretion or receptor expression (17). Abdul-Careem et al. (1) reported that cytokine gene expression was significantly lower in the spleen of chick embryos compared with post-hatch chicks; particularly when compared to D7 chicks. Similarly Karpala et al. (15) found that although interferon types 1 and 3 transcripts were present in the embryo, expression of related receptors that mediate the IFN responses appear to be delayed and develop more gradually. While all these experiments illustrate the immaturity of the chick immune system around hatching, none investigated what would occur if the test embryos were exposed to antigens in ovo prior to hatch.

**Early immune events following HVT vaccination in ovo.** There is evidence to show that both NK and adaptive immune cells respond to vaccine delivered in ovo. Sharma et al. (25) showed that
chickens inoculated with HVT *in ovo* had higher NK activity at hatch than those inoculated at hatch. Abdul-Careem et al (2) found that *in ovo* vaccination with HVT resulted in high replication of HVT in the spleen and a delay in the transcription of IFN-γ and IL-10 when compared to chickens vaccinated at hatch. Our preliminary results (above) show that inoculation of HVT at E18 resulted in high levels of HVT replication in both lung and spleen and an increase in IFN-γ transcripts as early as 24 hours after vaccination. In addition, *in ovo* administration of HVT hastened maturation of the chicken embryo immune system and rendered one-day-old chickens capable of mounting an advanced humoral immune response against an unrelated antigen, KLH.

Industry applications of vaccination *in ovo* against Marek’s disease. Vaccination of chicken embryos at the late stages of embryonation (E17-E19) against MD has become a general practice in the poultry industry. Both innate and adaptive immune responses develop against replicating MD vaccine virus, such as HVT, delivered *in ovo* to late stage embryos (14, 23). Previously, MD vaccines were administered at hatching by the subcutaneous route. Today, *in ovo* vaccination machines are being used in over 90% of U.S. broiler hatcheries, and their use is growing rapidly in Europe and Latin America. *In ovo* vaccination is currently restricted to broiler flocks because eggs of both sexes need to be vaccinated and so this method is cost effective (21). Injection *in ovo* is given at the time eggs are transferred from the incubator to the hatcher, usually around embryonation day E18. Automated, multiple-head injectors deliver a precise quantity of vaccine simultaneously to an entire tray of eggs (19). The automatic injectors deposit the vaccine inoculum into the amniotic fluid of the majority of the eggs. Sharma and Burmester found that vaccination of E18 embryos accelerated development of protective immunity against Marek’s disease by several days (23). Embryo vaccination was advantageous even in the presence of maternal antibodies (23) and with several types of vaccines (24). Pathogenesis of MD vaccines when administered *in ovo* is poorly understood and depends on the vaccine serotype used and the age of the embryo at inoculation. When HVT is administered at or before 14ED, chicken embryos develop immunotolerance and no immune response is elicited (31). In addition strain SB-1 and HVT were shown to induce brain and nerve lesions when inoculated as early as E5 (3). However, when inoculated at E17 or later, vaccination with HVT is safe and protective immune responses are developed (23).

*In ovo* inoculation with Serotype 3 MD vaccines (HVT) have been studied more extensively than with other serotypes. HVT replicate extensively in the embryonic lung during the first 24-72 hours post-inoculation; subsequently, the virus spreads to other tissues (9, 26). During *in ovo* vaccination vaccine is delivered into the amniotic fluid, which enters the embryo by mouth and is then ingested into the intestinal and respiratory track. It moves into the lungs as the result of rhythmic respiratory
movements that begin between E17 and E18 (13). The target cell for HVT infection in the lung has not been identified, but it seems to be an adherent cell type when cultured in vitro (26). The limited antisera available in 1987 provided evidence that these cells were not thymocytes, bursa cells, or MHC class II positive cells. The path of infectivity and pathogenesis of Serotype 1 MDV when inoculated in ovo at E17-E19 days of embryonation is also poorly elucidated. Sharma et al. (27) detected the genome of Serotype 1 in various embryonic tissues, including lung, but no VIA antigen expression was detected. Extensive replication of the virus, however, was detected in embryos inoculated intravenously (28). On the other hand, in ovo inoculation by the amniotic route with CVI988 conferred protection when challenged within the first three days post hatch (30). Therefore, some type of replication of the Serotype 1 MDV is expected to occur in embryo. In our preliminary studies, we demonstrated that inoculation with HVT at E18 hastens the immune system maturation. This suggests that HVT can be used not only as an antigen, but also as an adjuvant to generally enhance immune responses in newly hatched chicks. In addition, we confirmed earlier observations that replication of HVT differs from replication of other serotype MD vaccines (i.e. SB-1 and CVI988). We further found new evidence that differences exist in the immune responses within the late stage embryos elicited by various MD vaccines (See preliminary results, above)

References

7. RESUME OF INVESTIGATOR

<table>
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<th>NAME</th>
<th>POSITION TITLE</th>
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<tbody>
<tr>
<td>Isabel M. Gimeno DVM, MS, PhD, ACPV</td>
<td>Associate Professor</td>
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## EDUCATION/TRAINING

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## Professional Experience

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<td>Research Associate</td>
<td>Centro the Investigacion en Sanidad Animal</td>
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<td>2001 - 2002</td>
<td>Research Associate</td>
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<td>USDA-ARS Avian Disease and Oncology Lab</td>
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<tr>
<td>2006 - present</td>
<td>Assistant Professor</td>
<td>North Carolina State University, CVM</td>
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Selected peer review publications in the last five years (Total number of publication 48)


8. CURRENT RESEARCH ON SUBJECT BY INVESTIGATOR

Dr. Gimeno’s area of research is the development of better methods for controlling MD. We have conducted several studies to better understand protection induced by MD vaccines. We have evaluated early immune responses in the spleen and in the lung elicited by serotype 1 MD vaccines (8). Following that study, we evaluated factors influencing MD vaccine replication in the lung and onset of pulmonary immune responses (7). We are currently further evaluating the immune responses in the lung by characterizing the inflammatory infiltrates and expression of MHCII after vaccination with various serotype 1 MD vaccines in ovo or subcutaneously. In addition, we have standardized a method to reproduce the benefits of double vaccination under laboratory conditions; optimized MD revaccination protocols; and evaluated immune mechanisms associated with the benefits of revaccination (10, 9). Furthermore, we have evaluated the role of diluting MD vaccines on various outcomes of MD infection (11). Recently, we have developed a method to evaluate MDV-induced immunosuppression (MDV-IS) under laboratory conditions and we have evaluated mechanisms behind MDV-IS, factors influencing MDV-IS, and developed control methods for MDV-IS (4-6). We have also demonstrated that inoculation of HVT in SPAFAS chicken embryos hasten the development of the immune system (12) and conducted several preliminary studies (see preliminary information of this grant) on the effect of HVT on the immune responses of the commercial meat type chicken embryos.

9. FACILITIES AND EQUIPMENT REQUIRED AND AVAILABLE FOR THIS PROJECT

These studies will be conducted at the College of Veterinary Medicine, North Carolina State University following the guidelines of the IACUC. Adequate facilities and appropriate equipment are available to conduct the proposed study.

10. RESEARCH TIMETABLE

(a) Date project is scheduled to begin: October 1st, 2018
(b) Date project is scheduled to end: September 30th, 2019

11. PERSONNEL SUPPORT PROVIDED BY THE UNIVERSITY

Dr. Gimeno will devote 3% of her time to this project and Dr. Lucia Cortes will devote 5% of her time to this project
12. FINANCIAL SUPPORT

The research proposal submitted is not currently supported by any agency.

Financial support for other research projects of the principal investigator is listed in table below

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13. INSTITUTIONAL UNITS INVOLVED

Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University. This is the affiliation of the PI and where the proposed research is going to happen.
14. BUDGET

1. BUDGET

Personnel 2,936

5% of technical support (Gimeno’s laboratory) 2208
Fringe benefits for technical support 728

Research supplies 8,000

- KLH and KLH-ELISA kits 3200
- IFN-γ ELISA kits 2500
- Con A 200
- Mitogen stimulation test 1,600
- plastic ware, disposable, others 500

TOTAL $10,936

2. TOTAL FUNDS REQUESTED $12,576 (10,936 + 1,640)

3. INDIRECT COST 15% ($1,640)

4. RECEIPT OF FUNDS NEEDED

Make check payable to: North Carolina State University

5. This study, the investigator and North Carolina State University comply with the provisions of the Institutional Animal Care and Use Committee as specified by the Animal and Plant Health Inspection Service, USDA in 9 CFR part 1 (1-91)
6. The Population Health and Pathobiology Department of North Carolina State University agrees to provide the following to USPOULTRY:

(a) Progress reports on the research project every six months until the project is completed.

(b) Within three months following completion of the research funded, to provide the final project report (using the format for final reports) of the results.

(c) The North Carolina State University understand that USPOULTRY will retain 25% of the approved funds until the final report has been provided to the association.

(d) Give permission to the association to provide the information to the industry. By accepting the funding provided by USPOULTRY and the USPOULTRY Foundation, the researcher grants to USPOULTRY the right and license to share the results of the research with, and provide links or copies of the final research report to, its membership and the research community through USPOULTRY’s website, direct and electronic mail, or any other form of dissemination.

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7. Progress reports will be provided every six months. A final report will be sent within three months following completion of the research project as specified by the research proposal.

8. USPOULTRY makes no claim on discoveries or invention patents made by scientists/institutions utilizing USPOULTRY research funds. USPOULTRY assumes no liability associated with either the conduct of research or the outcome or use of research findings acquired with USPOULTRY funds.

9. Authorized signatures

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Isabel M. Gimeno                Paula Fedorka-Cray  
Project Leader                 Department Head

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University Official/Research Organization President
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9. Authorized signatures

   [Signatures]

   Isabel M. Gimeno  
   Project Leader

   [Signatures]

   Paula Fedorka-Cray  
   Department Head

   [Signatures]

   Stefanie D. Saunders  
   Assistant Director, Operations  
   NCSU Sponsored Programs  
   University Official/Research Organization President