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Executive Summary

A major problem in the control of NDV is the cost and effort to vaccinate. *In ovo* vaccination would be ideal, however, to date the various efforts to develop *in ovo* vaccination strategies have resulted in low hatchability and high mortality. We have recently demonstrated that the V4 NDV, when administered as a live *in ovo* vaccination, does not reduce hatchability or increase mortality. Therefore, we propose to determine the vaccine efficacy and protection against NDV challenge. Additionally, we investigated the potential to combine this possible NDV vaccine virus with *in ovo* Marek's Disease Virus vaccination to provide a cost-effective dual *in ovo* vaccination. We showed in two independent studies that the administration of V4 *in ovo* did not decrease hatchability or livability. However, in our third study where we attempted to combine MDV and NDV vaccines the previous two studies were contradicted and hatchability was reduced. This results was unexpected and highlights the continuing need for research to develop a better understanding of the avian immune response to *in ovo* vaccination. At present, there is an ongoing requirement for renewed research in the area of protective immune responses to NDV. A major challenge is to ascertain the molecular mechanisms of immunity associated with protection to infection.

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Introduction

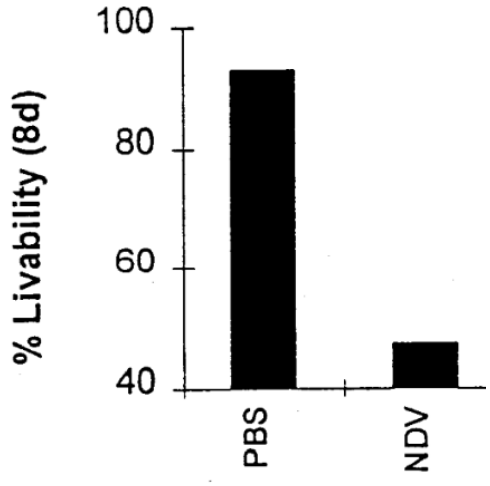
Background

Newcastle disease (ND) is a devastating viral disease of poultry and wild birds that primarily affects animals in developing countries (for example South-East Asia and Africa). There are a number of different strains of ND virus (NDV) with variable clinical presentations. Some highly virulent strains can cause up to 90% mortality in affected flocks, causing significant loss of animal production. From 1998-2002, Australia had multiple outbreaks of NDV leading to the culling of many thousands of birds and a shortage of table eggs to the community until the stocks of birds could be re-established. One of the most effective strategies for control of NDV is vaccination, which since March 2002 and the ratification of the Emergency Animal Disease Response Agreement, had become a mandatory requirement for the poultry industry. Recently however, NDV vaccination of poultry in Australia has become voluntary and there has already been a drop in NDV vaccine sales locally. It is likely that poultry farmers will not vaccinate in order to increase profits, however, this will leave many farms and the Australian poultry industry at a high risk of outbreak.

Furthermore, the route of administration for long lived birds (breeders and layers) is not ideal. It is currently an intramuscular (I.M.) immunisation which is used with commercially available adjuvants (immune stimulators) to enhance the effectiveness of the vaccine. Although effective the adjuvants often lead to off target effects such as wounds at the site of injection. A superior form of vaccination would be *in ovo* (in egg) vaccination as it would be cheaper, reduce stress to the animals and remove the risk of site reactions from the adjuvant. Moreover, if the *in ovo* vaccination could be combined with other important vaccinations, such as Marek's Disease Virus (MDV), the cost may be low enough as to encourage farmers to continue to protect their flocks despite there no longer being a legal requirement to do so.

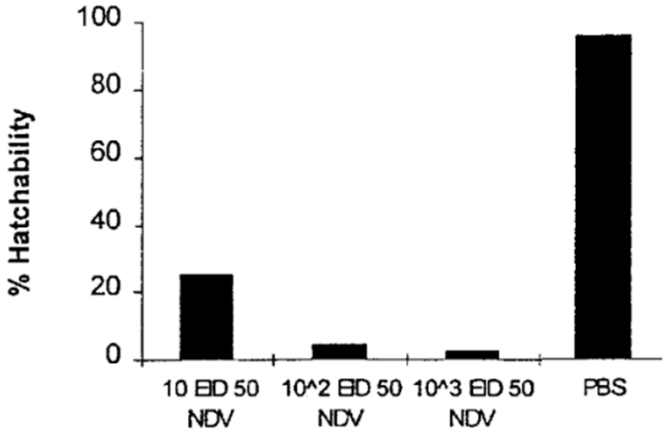
Significant effort has gone into developing an *in ovo* vaccination strategy for NDV. Unfortunately, in studies where vaccine virus strains were used to inoculate day E18 eggs, high doses of virus caused reduced hatchability (Figure 1). Some strains reduced egg hatching to less than 10%. Conversely, when low doses were used, although hatchability and 7 day mortality were unaffected the vaccine did not offer serological protection against NDV.

Figure 1: When live NDV was administered to eggs at a range of doses (10^1,10^2 and 10^3 EID_{50}) there was a dose dependant reduction in hatchability of the eggs.



In addition to issues with low hatchability of eggs, which poses a clear problem commercially, there is also a reduction in the 8 day mortality (livability) of the hatch eggs. When a dose of 1 EID_{50} was administered there was a reduction in livability from ~95% to ~50% (Figure 2). This too would pose a significant issue commercially and ultimately make the potential *in ovo* vaccine non-viable.

Figure 2: When a dose of 1 EID_{50} was administered to day E18 SPF eggs, there was a significant reduction in the 7 day survival of the hatch chicks.



The ideal solution would be a virus strain that is not lethal to the egg but also generates a strong immune response to multiple strains of NDV in the live bird. Recently, our group, in collaboration with Zoetis, have identified a strain of NDV that did not appear to significantly reduce either hatchability or livability when we vaccinated *in ovo*.

This data is extremely encouraging and provides solid evidence that this NDV strain may form part of a new NDV *in ovo* strategy. **The key to this project and a significant priority of our partner, Zoetis, is the**

identification of ideal dose, proof of a protective immune response and generation of a combined NDV/MDV vaccination strategy.

The critical next steps in this project are to repeat the *in ovo* vaccination at a variety of doses to (a) confirm our previous findings and to (b) determine what dose range the eggs will tolerate. Once we have shown conclusively that this strain is well tolerated we will need to assess the protective immune response that is generated by the vaccine through serological (antibody levels) assessment at the different doses. Although serological assessment is a strong, if not conclusive, indicator of protection we will need to determine protection in a challenge model, using both the autologous (strain injected) as well as heterologous (different strain) to show the vaccinated birds can survive an infectious challenge. Ultimately, we would need to test the co-administration of the NDV and MDV vaccines *in ovo* as this will lead to a far cheaper and widely used vaccine.

Objectives

The aims of this project are:

Aim 1: *Determine the optimal dose of novel V4 NDV virus for in ovo vaccination*

Aim 2: *Test the vaccination in a challenge protection model*

Aim 3: *Develop a combined NDV, MDV vaccination strategy*

Methodology

2.1. Ethics

All animal experiments were conducted in accordance with the regulations of the CSIRO-AAHL Animal Ethics Committee under Permit No. 1668.

2.2. Isolation of lymphocytes

Spleens were harvested from 4-week-old specific pathogen free (SPF) chickens and single cell suspensions of spleenocytes were prepared from individual spleens by dispersal through a 70-µm strainer into complete DMEM (10% fetal calf serum (FCS), 2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 µg/mL). Blood was taken via heart bleed from exsanguinated animals using a 25 gauge needle and a 1 mL syringe and then diluted 1:2 in PBS. Both cell suspensions were layered over equal volume of Lymphoprep™ (Stemcell Technologies, Australia) and centrifuged for 20 min at 1000 *g* at room temperature with no brake. The interphase was collected, transferred to a 15 mL Falcon tube and washed in 10 mL complete DMEM

media followed by centrifugation for 5 min at 400 x *g*. The pellet was resuspended in 5 mL complete DMEM media.

2.3. Freezing of cells

Cells were cultured in a T75 flask (Sigma-Aldrich, USA) at 37^{o}C supplied with 5% CO2. Following culture expansion the cells were stored in liquid nitrogen. Briefly, cells were retrieved from tissue culture flasks by adding 2 mL trypsin and incubating for 5 min at 37^{o}C with 5% CO_2 and then 5 mL fresh DMEM was added to stop the trypsin reaction. Media containing cells and trypsin was then transferred to a 10 mL Falcon tube followed by centrifugation for 5 min at 400 x *g*. Supernatant was discarded and 5 mL complete DMEM was added and were diluted with an equal amount of freshly prepared freeze mix (40% DMEM, 40% FCS and 20% dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA)) and cells were aliquoted into 1.5 mL cryotubes (Sarstedt, Germany). The tubes were immediately transferred to a freezing container (Nalgene® Mr. Frosty (Sigma-Aldrich, USA)) containing isopropanol, which was initially stored at -80^{o}C for 24 h and later transferred to liquid nitrogen for long term storage.

2.4. Cell thawing and culture

All cells were retrieved from liquid nitrogen and thawed in a 37^{o}C water bath until the last ice crystal melted and the samples were then transferred to a 15 mL tube containing 10 ml complete DMEM and centrifuged for 5 min at 400 x *g*. The pellet was resuspended in 5 mL DMEM and the cells were counted using a Haemocytometer (Bright-line, Hausser Scientific, USA).

2.5. Measuring gene expression using real time qPCR

2.5.1. Isolation of RNA

Total RNA was harvested from cell and tissue samples using RNeasy® Mini Kit (Qiagen, Netherlands) according to manufacturer's instructions. Briefly, 350uL of RLT-lysis buffer was added to each samples. An additional equal volume of 70% ethanol was added to each sample and the total sample applied to a spin column. Following several washes with different washing buffers the RNA was eluted from column with 25uL RNase free water as elution buffer. RNA concentration and purity was determined by NanoDrop (Thermo Fisher Scientific, USA).

2.5.2. cDNA synthesis

Extracted RNA was reverse transcribed into complementary DNA (cDNA) using the SuperScript® III First-Strand Synthesis SuperMix (Thermo Fisher Scientific, USA). Briefly, 6 µL of extracted RNA, 1 µL annealing buffer and 1 µL primer oligoDT were incubated together at 65^{o}C for 5 min. 2X First strand reaction mix (10

µL) and SuperScript® III enzyme mix (2 µL) were added and incubated in T100 thermal cycler (Bio-Rad, USA) for 50 min at 50°C, 5 min at 85°C and then stored at -20°C.

2.6.3. Real time quantitative PCR

In an MicroAmp® Optical 96-Well Reaction plate (Applied Biosystems®; 403012, USA) we combined 2 µL template cDNA, 10 µL 2x TaqMan PCR master mix (Applied Biosystems, USA), 1 µL primer mix and 7 µL of nuclease free water was added in a final volume of 20 µL. The qPCR protocol consisted of a holding stage of 2 min 50°C followed by denaturation at 95°C for 10 min and a cycling stage of 15 sec. 95°C, 1 min at 60°C. The machine used was an AB Applied Biosystems® Step-one Plus Real Time PCR system and analysed by the software StepOne™ Software v.2.0.

2.6. Vaccine

The live NDV vaccine (NDV V4) was provided by Zoetis (Holland). The vaccine is based on the avirulent V4 strain belonging to genotype I of NDV. The stock vaccine titer was $10^{9.25}$ EID_{50} diluted in phosphate-buffered saline (PBS) to get 10^6, 10^3 and 10^1 EID_{50} dose in 50 µL.

2.7. *In ovo* vaccination and sample collection

Each experimental group consisted of 10 eggs per group for the *in ovo* trial. Vaccine dose was either 10^6, 10^3 or 10^1 EID_{50} dose administered in a total volume of 0.2 mL as well as a PBS alone group. Using a 25 gauge needle the vaccine was administered into the amniotic cavity at 18^{th} embryonic day in eggs. Eggs were then allowed to hatch in incubator and the number of eggs that hatched and survived to day 7 were recorded. Whole blood, serum and spleen samples were collected and stored appropriately. Serum was scored for NDV-specific humoral immunity by haemagglutination inhibition (HI) test.

2.8. Haemagglutination inhibition (HI) assay

Briefly, two fold serial dilution of 25 µL serum was made with PBS in U-bottomed Nunc™ MicroWell™ 96-Well Microplates (Thermo Fisher Scientific, USA) up to the tenth well. Using 25 µL of 4 haemagglutinating (HA) units of NDV were added till the eleventh well. The plates were kept at room temperature for over 30 minutes to facilitate antigen antibody reaction. Then 50µl of 0.5% (v/v) chicken RBC suspension was added to each well. The eleventh well contains antigen and RBCs as the positive control and the twelfth well contains just RBCs as the negative control. After gentle mixing, the RBCs were allowed to settle at 4°C for 40 minutes and agglutination was evaluated by tilting the plates. The samples showing central button shaped settling of RBCs were recorded as positive and maximum dilution of each sample causing haemagglutination inhibition was considered as the end point, which was utilized to evaluate the HI titer. The HI titer of each serum test was evaluated as corresponding of the serum dilution.

2.9. NDV $TCID_{50}$

The virus was tested for its viability in chicken fibroblast cell line (DF1). DF1 at 2 x 10^5 cells/mL were seeded in Nunc™ MicroWell™ 96-Well Microplates (Thermo Fisher Scientific, USA) into quadruplicate for 48 h. NDV virus dilutions were prepared in complete DMEM starting from 1:5, then 1:10 serial dilution were followed till 8^{th} row down the plate. Cells were then infected for 1 h and the virus was replaced with fresh DMEM and the cells were incubated for 96 h. Cytopathic effect (CPE) was observed at each dilution and the $TCID_{50}$ was calculated according to the number of virus particles present at each dilution.

2.10. Detection of NDV in cultured cells

NDV infected DF1 cells were observed in bright-field microscope EVOS® FL (Thermo Fisher Scientific, USA) at 10x and 40x objective. Cell death and syncytia were recorded as the measure for CPE. Further, cells were fixed and fluorescently-labelled antibodies were then used to detect the NDV virus. Fixing buffer comprising of 4% paraformaldehyde (PFA) in PBS was used to fix the cells. For 2 x 10^6 DF1 cells, spent media was replaced by 200 µL of the PBS buffer in the Nunc™ MicroWell™ 96-Well Microplates (Thermo Fisher Scientific, USA). Cell were then incubated in 100 µL 0.1% Triton X 100 (Sigma-Aldrich, USA) for 10 min. To block the non- specific binding the wells were incubated with 100 µL of 0.5% BSA (Sigma-Aldrich, USA) in PBS for 30 min. The cells were then incubated for 1 h with 50 µL/well of primary mouse monoclonal antibody diluted in 0.5% BSA in PBS, followed by three 5 min washes with 100 µL PBS. Species specific secondary antibody conjugated with Alexa fluor® 488 (Life Technologies, USA) was used for detection and was incubated for 1 h in dark. Cells were washed two times with (5 min incubation) 100 µL PBS and followed by two rinses with 100 µL tissue culture (TC) water. Nuclei were labelled with freshly prepared DAPI (Sigma-Aldrich, USA) for 10 min in dark and then rinsed twice with 100 µL TC water. The plate was imaged using the CellInsight Personal Image Cytometer (Thermo Fisher Scientific, USA) at a magnification of 10 x, 49 fields/well representing the entire well for the detection of CPE.

2.11. Antibody labelling for FACS

Approximately 1 x 10^6 PBMC or splenocytes were aliquoted in 96 well Round-bottom microtiter plates (Thermo Fisher Scientific, USA). The plates were centrifuged for 3 min at 400 x *g* and supernatant was discarded. The antibodies were diluted in cold FACS buffer (2% FCS and 0.01% Sodium Azide in PBS) and 50 µL of antibody cocktail was added to the cells. The plate was kept for incubation for 30 min at 4^oC in the dark. Following 30 min incubation, the antibody-cell cocktail was washed using 100 µL FACS buffer and the plate was centrifuged for 3 min at 400 x g. The supernatant was discarded and the cells were resuspended in 150 µL of FACS buffer in preparation for flow cytometric analysis in BD LSR II (BD Biosciences, USA)

2.12. Statistical analysis

Data means and standard error (SE) were calculated using Microsoft Excel (Microsoft Office Excel, 2013). Further statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, USA). ANOVA (one-way analysis of variance) or Kruskal-Wallis were used to determine statistical significance. Unless generally expressed the obtained statistical values were viewed as significant from control values when their probability was under 0.05 ($p<0.05$).

Results:

In this study we aimed to further examine the impact of vaccinating *in ovo* with the V4 strain for NDV as a possible method of providing anti-NDV protection. We previously had shown in a small pilot study that when we vaccinated with either 10^1 or 10^3 EID_{50} *in ovo* that we did not see a significant reduction in hatchability or liveability when compared to PBS controls. These results were contradictory to results previously obtained by Zoetis in their in house testing, where greater than 50% of eggs did not hatch following NDV *in ovo* injection. One of the first steps in determining the source of this disparity was to determine the viability of the NDV vaccine used. To do this we undertook to perform a $TCID_{50}$ in DF1 cells, a continuous fibroblast cell line derived from chickens (figure 1). We showed that the DF1 cells were susceptible to the NDV vaccine and had clear CPE at the 1:5 and 1:50 dilution of virus. We also observed syncytia in the infected wells, which is expected for NDV as it is a paramyovirus. Interestingly, there was little to no CPE in subsequent dilutions (1:500 – 1:50,000) which was unexpected as the stated concentration was $10^{9.25}EID_{50}$ which should have given infection up until a 1:5000 dilution. To address this discrepancy we performed additional infection studies and looked for viral infection through the use of antibody staining and fluorescent microscopy.

We observed a very different result for the fluorescent antibody staining (figure 2) where we had infected cells up until the 1:50,000 dilution of the virus. In the first two dilutions (1:5 and 1:50) we again observed syncytia and CPE, however in this experiment we observed very high levels of virus present in the 1:500 and 1:5000 dilutions and a small amount of virus present in the 1:50,000 dilution. These results indicated that the virus was viable and replicating productively and that our previous findings where there was little to no loss of eggs through *in ovo* vaccination of eggs with NDV were not due to a loss in vaccine viability. We then proceeded to attempt to repeat the pilot study to ensure our findings were not due to experiment to experiment variation.

To validate our earlier findings we aimed to repeat the pilot study whereby we vaccinated *in ovo* with 10^1 and 10^3 EID_{50} of the V4 NDV vaccine. Furthermore, we added an additional group (10^6 EID_{50}) to determine the impact of an extremely high dose of vaccine. Our measures for each of the conditions were hatchability (figure 3), liveability and 7 day mortality (figure 4). The measure of hatchability showed that our PBS injected control group had a hatch rate of 87.5%. From our experience we see between 80-100% hatch, when eggs have been manually injected with PBS so this hatch was considered normal. The 10^1 EID_{50} group had a hatch rate of 77.5% and the 10^3 EID_{50} group had a hatch rate of 80%. These numbers were lower than that seen for our pilot study, however they were not significantly different from the PBS control group and were significantly higher than the hatch rate previously seen by Zoetis with the same dose of vaccine. The 10^6 EID_{50} group had a hatch rate of 67.5%. This was significantly lower than that of the PBS control group.

When we combined the bird losses from both the hatch and 7 day mortality (figure 4) the PBS control group had a total survival of 82.5% compared to 72.5% and 77.5% for the 10^1 and 10^3 EID_{50} groups

respectively. The total survival of the 10^6 EID_{50} group was 60%, which again was significantly lower than that of the PBS group. Although the 10^6 EID_{50} group was significantly lower the total survival of the other two groups was higher than previously seen in in-house experiments with Zoetis. Therefore, we investigated what the possible mechanism of immune protection may have been by studying the immune response in vaccinated chickens.

We first sought to determine the stimulation of the immune response that had occurred in 7 day old chicks vaccinated with the V4 NDV vaccine. One of the earliest responses to viral infection is the interferon response and the triggering of interferon stimulated genes. The triggering of these genes stimulates a cascade of responses that fight the virus and form the basis of long lasting immunity. We investigated the gene Mx, which is a well described interferon stimulated gene in poultry that is known to respond to viral infection. We compared the Mx levels in PBS vaccinated birds to those of the NDV vaccinated birds (figure 5) and interestingly, conversely to what we had expected, the Mx levels dropped dramatically in all groups vaccinated with NDV. The Mx gene expression reached as low at a 20 fold reduction in the 10^3 EID_{50} group. This was a much unexpected result and may indicate an important feature of *in ovo* vaccination survival. Often in viral infection, the signs and outcome of infection are dictated by the immune response and an over stimulation of the immune system is concordant with poorer outcomes. To further investigate this phenomena we analysed the cellular compartment of the immune system.

We analysed several cellular subsets (figure 6) within the blood of vaccinated chickens including CD45+ cells (all leukocytes), CD3+ cells (T cells), CD25+ cells (activated lymphocytes), Bu1+ cells (B cells) and MHCII+ cells (antigen presenting cells). While the major population of cells within the chicken blood is red blood cells we observed no significant changes in the proportions of CD45+ cells indicating no major proliferation event. Interestingly, we did observe a significant increase in CD3+ T cells in the 10^6 EID_{50} group and a significant decrease in the activation marker CD25 in all groups.

A key measure of the efficacy of any vaccination is the production of anti-viral antibodies. Therefore, we measured the protective antibodies produced (figure 7) in response to NDV vaccination in the 10^6 EID_{50} group as compared to PBS controls. As this is a live vaccine we were also interested in the ability for not vaccinated birds to generate a protective response when co-housed with vaccinated birds. We showed that birds vaccinated *in ovo* had an average HI titre of $>2^4$ at 2 weeks post vaccination and $>2^5$ at 8 weeks post vaccination. Interestingly, birds given PBS and co-housed with vaccinated birds had an average HI titre of $<2^1$ at the 2 week bleed but $>2^6$ at the 8 week bleed, suggesting that the live virus vaccine had been productively replicating in the hatched, vaccinated birds. A protective HI titre for a flock of chickens is considered to be 2^3, suggesting that the vaccinated birds had a protective titre at the 2 week time point and the co-housed birds also achieved a protective titre by the 8 week time point.

If NDV were to become a commercially successful *in ovo* vaccine it is likely that it would be co-administered with the *in ovo* MDV vaccine. Therefore, we sought to determine the impact of vaccinating with both MDV and 10^3 EID_{50} (as this dose caused no significant loss of eggs). Furthermore, we aimed to

perform a challenge study by administering a low virulent strain of NDV and determine the immunological memory. In this study we observed drastically different results to that which we had observed in our previous studies. With a 100% hatch rate in our PBS injected group we had a 0% hatch of the NDV vaccinated eggs and a 30% hatch rate of NDV + MDV vaccinated eggs (figure 8). This was highly unexpected as we had observed very high hatch rates in our previous experiments. There are many factors to take into consideration when analysing this result, the most important of which is the SPF stock from which we had obtained these eggs. For a number of months they had experience low hatch rates, preventing us from performing this last experiment. One of the solutions to this problem was to import new breeding stock, an event which may have had a significant impact on our outcomes.

Figure 1: Cytopathic effects of live V4 strain of NDV. DF1 cells were infected with the V4 vaccine strain of NDV to assess its viability. The brightfield images show the control well (a) had no CPE or syncitia formation, however, the 1:5 dilution (b) showed significant loss of cells and cell death. This too was true of the 1:50 dilution (data not shown). Neither the 1:500 (c) or 1:5000 dilution of NDV showed cells death.

Figure 2: Immunofluorescent detection of viral infection. Using fluorescent imaging we examined at what dilution the virus was able to infect the DF1 cells. Antibody staining (green) was detected in all dilutions (a-e) to variying levels. Again CPE was observed in the 1:5 and 1:50 dilutions, however high levels of virus was also detected in 1:500 and 1:5000 dilutions with a small amount in the 1:50000 dilution. The control well (f) showed no antibody staining.

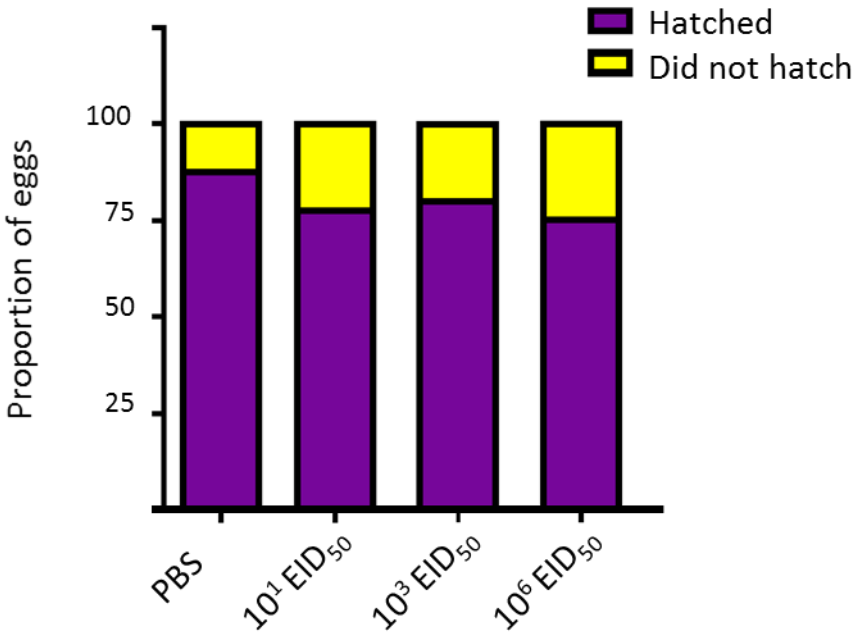


Figure 3: Effect of NDV vaccine on hatchability of SPF chickens. NDV V4 vaccine was administered at 10^1, 10^3 and 10^6 EID_{50} concentration at 18th embryonic day. The bar graph shows the number of eggs hatched and didn't hatched. Negative control group was PBS alone. The values are expressed as mean ± SE.

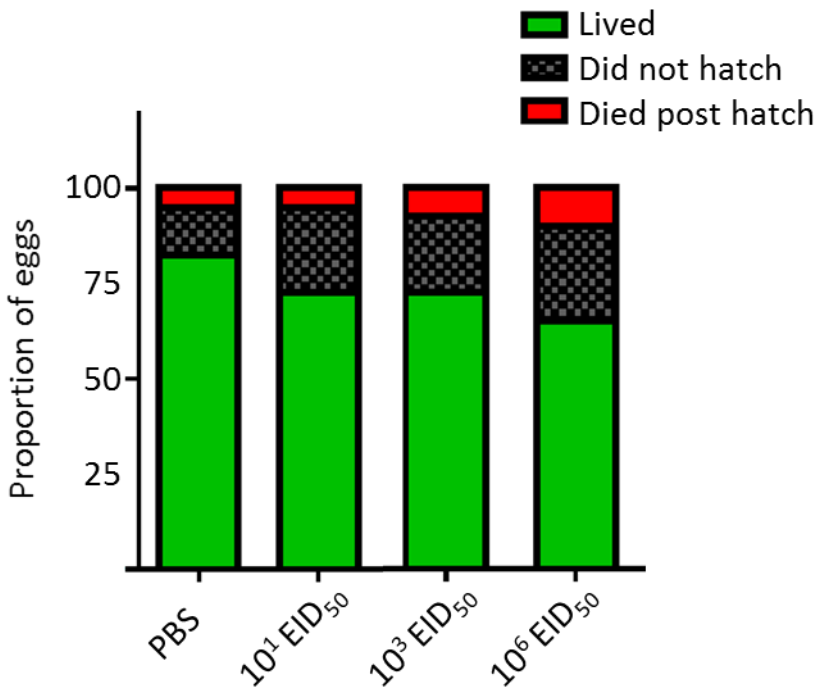


Figure 4: Effect of NDV V4 vaccine on liveability in SPF chickens. NDV vaccine was administered at 10^1, 10^3 and 10^6 EID_{50} concentration at 18th embryonic day. The bar graph shows the number of chicks that lived, died before hatch and died within 7 days of hatch. Negative control group was PBS alone. The values are expressed as mean ± SE.

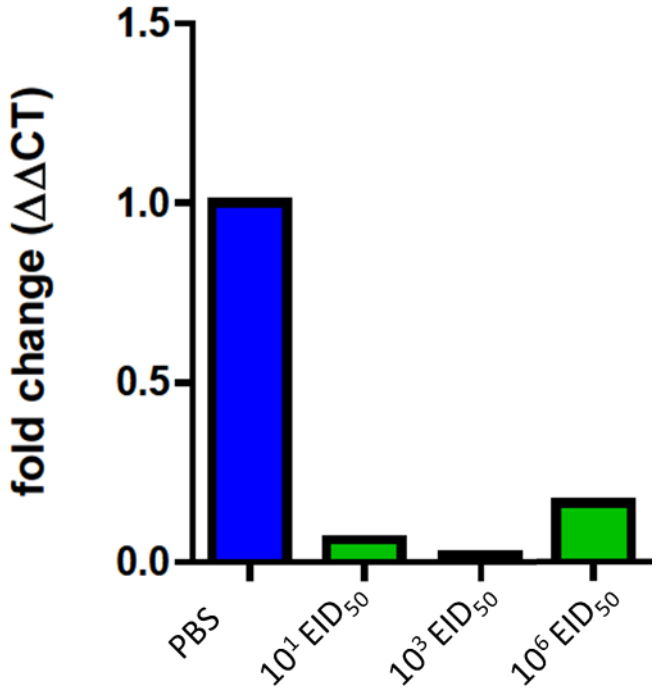


Figure 5: Mx gene expression following *in ovo* NDV vaccination. The bar graph shows the Mx mRNA transcripts following administration of NDV vaccine *in ovo.* The expression is shown relative to PBS as control. qRT-PCR was performed and GAPDH was used as a housekeeping gene to standardize results. The values are expressed as mean.

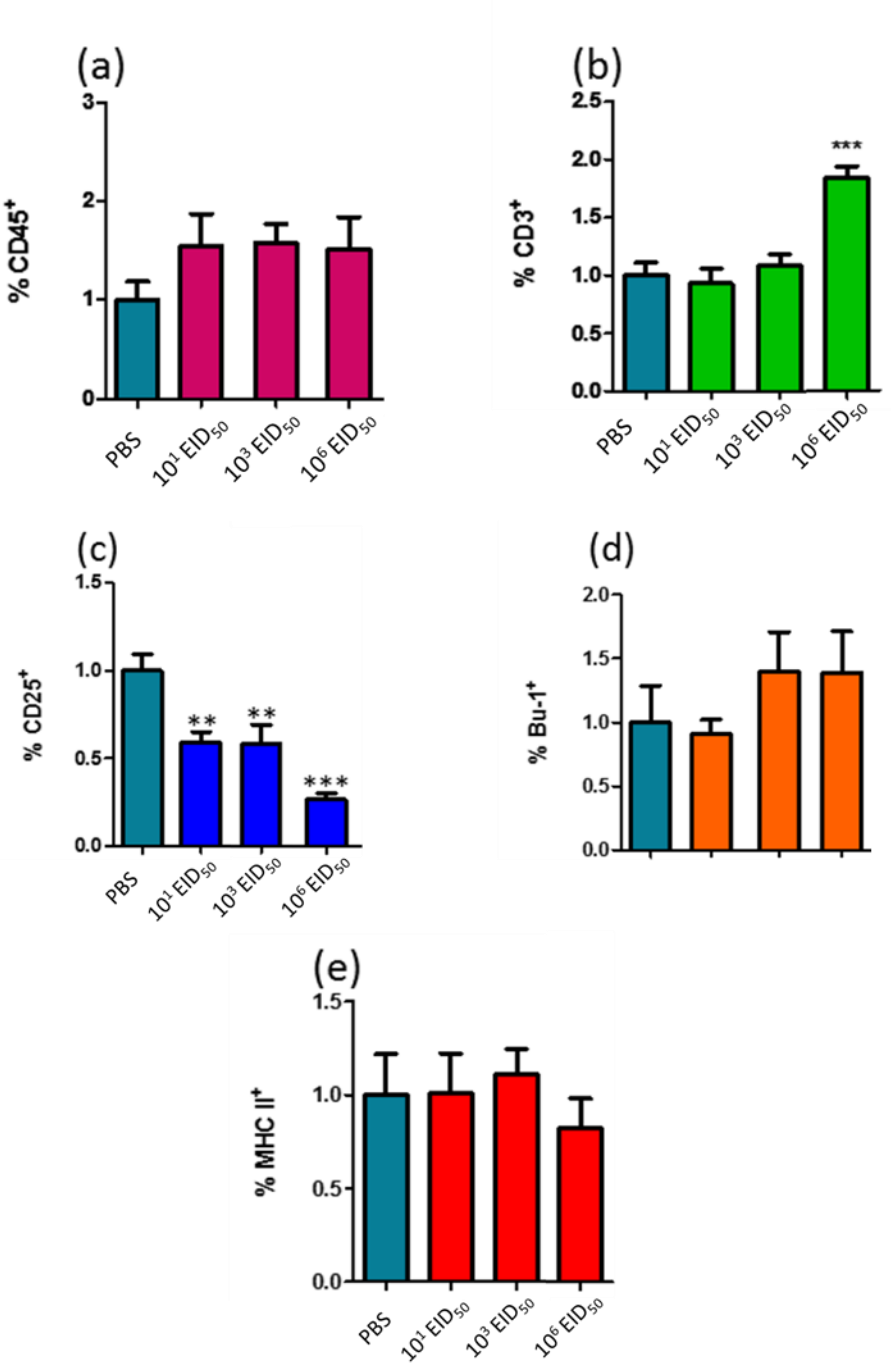


Figure 6: Effect of NDV *in ovo* vaccination on immune cell subsets. FACS was performed to detect the changes in sub-population of cells in the whole blood of chickens after NDV vaccination. The bars graph shows the percentage of subsets of cells as shown in as (a) CD45+, (b) CD3+, (c) CD25+, (d) Bu-1+ and (e) MHC II+. The values are expressed as mean ± SE; $p<0.05$.

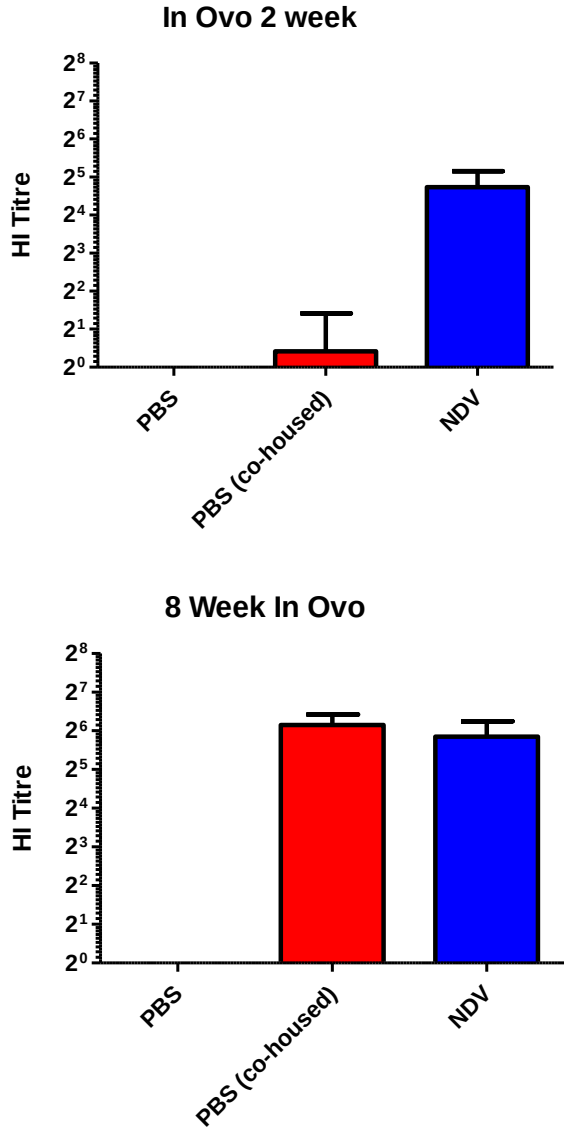


Figure 7: Serum titre of birds vaccinated with 10^6 EID_{50} NDV vaccine. The HI titre of bird vaccinated *in ovo* with the V4 NDV strain was determined at the 2 weeks post hatch and 8 weeks post hatch. Birds that were injected with PBS alone and house separately were used as a control for birds vaccinated with NDV as well as birds co-housed with vaccinated birds.

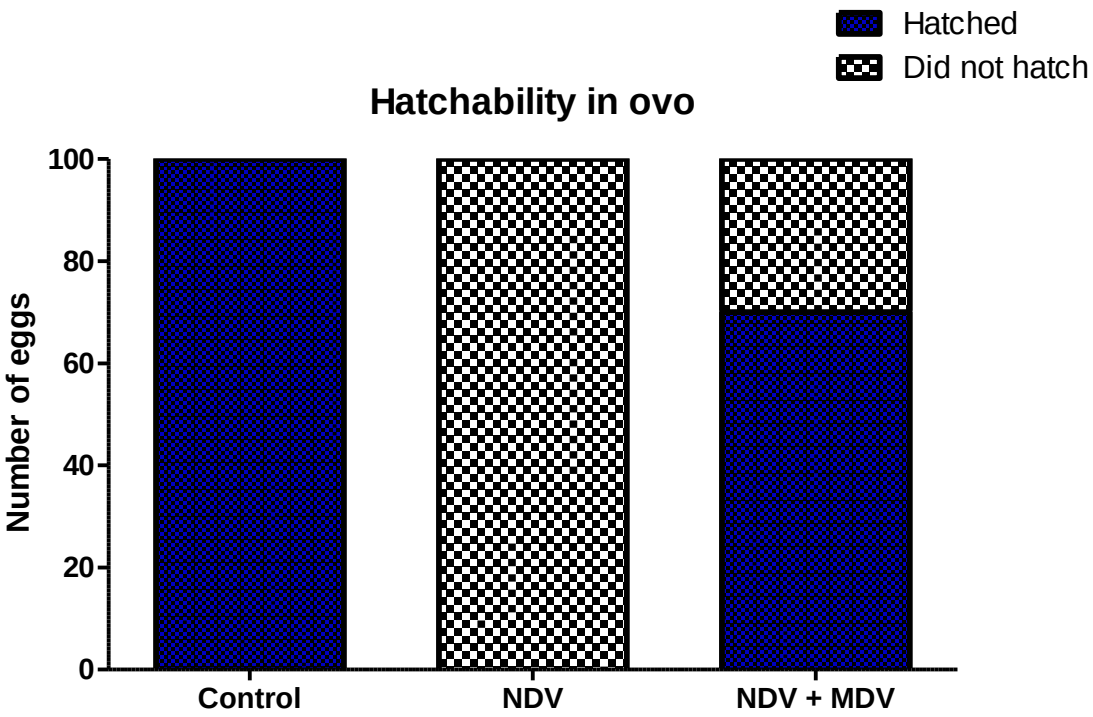


Figure 8: Hatchability of eggs vaccinated with NDV and MDV.

Discussion of Results

ND was first described in 1926 and, notwithstanding advances made in vaccination for the disease, it continues to have undesirable consequences for poultry producers. As such, ND remains a persistent threat to poultry producers worldwide, despite the availability of vaccines. Vaccination is a vital aspect of control (Seal et al., 2000), nevertheless, sterilizing immunity has not yet been achieved with NDV vaccines. Therefore, new vaccination strategies that may provide more effective protection are urgently required. Similarly, as pressure continues to mount on vaccine producers with regards to increasing demand, pricing burden and greater cost, innovative methods of vaccination are essential.

Large scale use of live vaccines is often a cost effective approach due to the faster application compared to having to administer individual vaccines to each bird of a flock. Regrettably, circumstances on farm are not always optimal with mass vaccination potentially protecting as little as half of the flock when given by spray or drinking water. *In ovo* vaccination of 18-day-old embryos has become a somewhat common way of vaccinating for some vaccines, such as Marek's disease virus (MDV). Yet, there have been some difficulties in using this method for the delivery of some live vaccines, in particular NDV vaccine. The Hitchner NDV vaccine, a commonly used vaccine for hatched birds, when used *in ovo* leads to complications in embryos and therefore its use is not recommended. With this in mind, industry has struggled with providing an efficacious NDV vaccine that can be delivered *in ovo*.

This 12 month project had 3 main aims which involved the determination of a potential optimal dose of novel V4 NDV virus for *in ovo* vaccination, which involved finding a safe dose that infected all SPF embryos at 18 days of embryonation without killing them. Importantly, we would need to observe that the vaccine was safe for embryos that may come from different SPF flocks. Subsequent to this, the testing of a vaccination in a challenge protection model and a combined NDV, MDV vaccination strategy was to be carried out. Critical to effective protection and utility, the delivery of an appropriate amount of virus that can induce an immune response yet not induce mortalities is the most important first step. However, a number of factors can influence the response of chickens to vaccination against ND, for example maternal or residual immunity, age, the virus strain and vaccine concentration and some of these factors, and others, have important consequences for *in ovo* vaccination.

Antibody mediated immunity induced by vaccination is critical to NDV control. The efficacy of any vaccination is correlated with the production of anti-viral antibodies. With this in mind, we measured the protective antibodies produced in response to NDV vaccination in the 10^6 EID50 group as compared to PBS controls. Figure 7 shows that birds vaccinated *in ovo* had an average HI titre of >24 at 2 weeks post vaccination and >25 at 8 weeks post vaccination. A protective HI titre for a flock of chickens is considered to

be 23, suggesting that the vaccinated birds had a protective titre at the 2 week time point and the co-housed birds also achieved a protective titre by the 8 week time point. It has been previously described that a beneficial antibody response to NDV may be associated with less valuable production traits (Lwelamira et al., 2009). The antibody response is vital to protection against NDV, nevertheless, another important aspect is the differences in resistance to NDV due to genetic variation (Kapczynski et al., 2013).

Various aspects of this research have supported the idea that acceptable hatchability and seroconversion may be attained with *in ovo* inoculation of NDV vaccines, however, the potential for this to be obtained consistently clearly requires a greater amount of research. In this study we aimed to further examine the impact of vaccinating *in ovo* with the V4 strain for NDV. We previously had shown in a small pilot study that when we vaccinated with either 10^1 or 10^3 EID50 *in ovo* that we did not see a significant reduction in hatchability or liveability. Nonetheless, we repeated the pilot study and analysed the hatchability (figure 3), liveability and 7 day mortality (figure 4). Hatchability studies showed that combining the bird losses from both the hatch and 7 day mortality (figure 4) the PBS control group had a total survival of 82.5% compared to 72.5% and 77.5% for the 10^1 and 10^3 EID50 groups, respectively. The total survival of the 10^6 EID50 group was 60%, which was significantly lower than that of the PBS group. Furthermore, if NDV were to become a commercially successful *in ovo* vaccine it may be co-administered with the *in ovo* MDV vaccine. We investigated the impact of vaccinating with both MDV and 10^3 EID50 (as this dose caused no significant loss of eggs). We observed drastically different results with a 100% hatch rate in our PBS injected group, a 0% hatch of the NDV vaccinated eggs and a 30% hatch rate of NDV + MDV vaccinated eggs (figure 8). This was highly unexpected. Clearly, a great deal more work needs to be done to determine the reasons for these observations and this outlines the vitally important aspect of the need for consistency in vaccine studies. Furthermore, there are many factors to take into consideration when analysing this data. One critical aspect to this experiment was the SPF stock from which we had obtained these eggs. The birds used in this experiment were from an import of a new breeding stock, an event which may have had a significant impact on our outcomes. The genetics of a bird has an intense impact on the ability to respond to vaccination and disease. The genetics influence the type of response made, beneficial or deleterious, and the performance outcomes. It is most important that careful attention is paid to genetics of the line of birds used and this has implications for disease management in a commercial setting. Specific immune gene families, for example MHC, play a role in disease resistance. Therefore, elucidating mechanisms regulating the immune response during NDV *in ovo* vaccination may be vitally important. The MHC is a gene family is involved in some of the important aspects of the immune response, such as antigen presentation and self-discrimination (Guillemot et al, 1988) and this leads particular MHC haplotypes to be associated with resistance or susceptibility to disease. Well established examples of this include Rous sarcoma virus (Heinzelmann et al, 1981), Marek's disease (Pevzner et al, 1981), and NDV (Dunnington et al, 1992). Although it is currently unknown if the change in SPF flock involved changes in the MHC of the test birds

between the various experiments, further investigation is required to determine if the dramatically different results seen were influence by genetic factors such as this.

In conclusion, NDV is an economically significant OIE identified worldwide virus with importance to commercial poultry producers. Control of NDV through use of vaccines is vital, however, cost effective approaches to vaccination are required. As we build on our understanding of how *in ovo* vaccines enhances the immune response, we will gain further insight on how to use *in ovo* vaccines to optimise the immune responses during vaccination. Newly emerging hypervirulent strains make existing vaccines less effective. With this in mind, a greater emphasis has been put on the development of *in ovo* vaccines that can provide effective immunity. At present, there are few NDV *in ovo* vaccines that can safely provide an appropriate level of immunogenicity to elicit wide-ranging and persistent immune responses. Consequently, there is a current lack of suitable, cost effective NDV *in ovo* vaccines for use in poultry, particularly broilers. Recent advances in our understanding of the immune response to pathogens have identified that the control of the direction of the immune reaction is critically dependant on the nature of the response. This information then supports the strategy of employing *in ovo* vaccines in a particular format, potentially with appropriate adjuvants, in an effort to influence the immune response in the proper direction to generate a protective response. Efforts are underway to enhance vaccine efficacy by use of adjuvants, particularly cytokines. The use of cytokines in vaccine formulation has been given serious consideration due to the ability of driving an appropriate immune response and ensuring a protective outcome.

Productivity increases in the poultry industry are becoming more difficult and the safe and effective delivery of vaccines is a key challenge for industry. The poultry industry relies on cost-effective methods of vaccine delivery and the continuing development of automated systems for vaccine administration on a commercial scale introduces a new approaches to the delivery of NDV vaccine for future research. NDV vaccines delivered *in ovo* could reduce the cost of vaccines by increasing the effectiveness of the vaccine and reducing labour costs associated with post-hatch vaccination.

Currently, there is an imperative and explicit requirement for renewed research in the immune response to NDV. A major challenge is to ascertain the molecular mechanisms of immunity associated with protection to infection. On the other hand, it is also very necessary that we identify the deleterious unwanted response and the implications for this on *in ovo* vaccination. Our improved knowledge of avian genetics and immune response to NDV means that we now have the tools to further progress our understanding and knowledge. Nevertheless, this must be implemented to develop advanced infection control approaches. New cost effective vaccination strategies assist in solving the major challenge of achieving sustainable, ethical poultry production and maintaining a supply of healthy and welfare conscious poultry products.

Implications

The results from this project have implications for the Australian poultry industry with regards to new approaches to dealing with viral infections. By taking a proactive approach to the investigation of the potential of novel vaccines the Australian poultry industry is boosting their preparedness and seeking alternative industry approaches. From this, future investigations may show that *in ovo* vaccination may be of use for NDV vaccination. Pressures on the poultry industry to produce high quality product at effective prices mean that there has been a need to augment vaccines to provide an effective and efficient approach. This means that approaches like *in ovo* administration of vaccine require characterisation and analysis of their impact on the embryo and their immune system to determine their potential to make a rational choice with regard to their use.

Productivity increases in the poultry industry are becoming more difficult and the safe and effective delivery of vaccines is a key challenge faced by the poultry industry. Improvement in the range and effectiveness of vaccines, diagnostics and therapeutics will result in reduced reliance on the use of antibiotics and chemicals in poultry production. Moreover, the production of new-generation poultry vaccines in collaboration with commercial partners is of critical importance to poultry producers. When using vaccines in livestock, special consideration must be taken with regards to delivery as the poultry industry relies on cost-effective methods of vaccine delivery. The use of *in ovo* vaccination has increased in recent years, and many studies have shown that a number of vaccines have been safely administered *in ovo*. The development of automated systems for vaccine and adjuvant administration on a commercial scale introduces a new method of delivery of vaccines for future research. Vaccines delivered *in ovo* could reduce the cost by increasing the effectiveness of the vaccine and reducing labour costs associated with post-hatch vaccination. Furthermore, the use of the vaccines will increase Australia's preparedness against disease outbreak risks. This then assists in solving the major challenge of achieving sustainable, ethical poultry production and maintaining a supply of healthy and welfare conscious poultry products.
With recent advances in our knowledge of poultry genetics, such as the genome sequence and a greater understanding of the function of avian immune responses, a better emphasis can be placed on the protective immune response to NDV. The elucidation of the immune response to NDV will be critically important for the development of better control strategies, including *in ovo* vaccination, to prevent outbreaks.

Recommendations

Within this project area we have identified some important steps in the evaluation of NDV vaccine delivered by *in ovo* injection as an alternative to post-hatch. The next steps for the best use of the outcomes of this project are:

1. Investigate the link to chicken MHC haplotype and outcome of vaccination. Our conflicting results suggest that there is a strong relationship between the host and the virus which is having an impact on vaccination outcome.

2. Immune response analysis of 18 day old embryos for enhanced *in ovo* delivery of vaccines:
We report here some very interesting findings with regards to the impact on the host immune system. These need further investigation to fully understand the interplay between the host and virus. Understanding what role the virus plays in reducing or enhancing the cytokine response may lead to new vaccination strategies.

3. Development of optimal methods for the safe and efficient delivery of vaccines under commercial conditions:
Our experiments have shown that the *in ovo* delivery of NDV vaccine can provide a protective immune response, however, this may be impacted by the strain, haplotype and disease state of the birds. We would recommend trialling this vaccination regime in commercial flocks as well as in specific haplotype birds to determine the extent of this relationship.

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POULTRY CRC

Plain English Compendium Summary

Sub-Project Title:	Analysis of the V4 strain of Newcastle disease virus (NDV) for use *in ovo* vaccination
Poultry CRC Sub-Project No.:	1.1.12
Researcher:	Andrew Bean
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Sub-Project Overview	
Background	The increasing demand for "clean and green" poultry products has increased the pressure to develop natural, non-chemical alternative strategies to manage infectious diseases in poultry. Compounding this is the observation that for many diseases, such as NDV, the current vaccine strategies offer less than complete protection. NDV is currently administered as a post hatch vaccine and attempts to deliver *in ovo* have previously been unsuccessful. We propose an *in ovo* vaccination regime to reduce cost and increase protection.
Research	We propose that these *in ovo* vaccination technologies be explored and developed by undertaking studies assessing the biological function of the NDV vaccine and assess their anti-viral potential and, similarly, to assess their ability to enhance protection. Furthermore, identification of the genetics associated in generating effective immune responses will provide mechanisms to manipulate the immune response to direct it towards an appropriate and controlled protective response.
Sub-Project Progress	• NDV *in ovo* vaccine was shown to reproducibly not reduce hatch; • The vaccination regime induced a protective immune response; • During the combined NDV/MDV vaccination studies it became apparent that host factors may play a role in susceptibility.
Implications	This project has developed a strong vaccine capability which has contributed to a number of other projects in the CRC and has ongoing applications in a wide range of projects of relevance to the poultry industry. This capability is not, in its own right, directed at producing specific commercial outcomes for the industry but is rather an enabling technology used by other more specifically focused projects. We see major opportunities in continuing *in ovo* vaccination studies, more focused work on monitoring the changes induced by vaccine treatments, and in monitoring and understanding the effects of more advanced genomics analysis.
Publications	This was a 12 month project and so far there has not been any publications from the research data. There is potential that a publication may eventuate in the future which may provide an important resource for the poultry industry.