Enhancing the Immunogenicity of Whole Inactivated H9N2 Influenza Virus Vaccines in Chickens

By

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AVIAN INFLUENZA VIRUS (AIV) H9N2 SUBTYPE IS A LOW PATHOGENIC INFLUENZA VIRUS THAT COMMONLY INFECTS POULTRY IN CERTAIN PARTS OF THE WORLD. INACTIVATED AIV VACCINES CAN BE USED TO DECREASE INFECTION. OUR OBJECTIVE WAS TO STUDY THE IMMUNOGENICITY OF INACTIVATED H9N2 AIV VACCINES IN CHICKENS. TO THIS END, WE PERFORMED STUDIES THAT EXAMINED: 1) THE COMBINATION OF TOLL-LIKE RECEPTOR (TLR) LIGANDS AS ADJUVANTS, 2) THE INACTIVATION METHOD USED TO PRODUCE H9N2 AIV VACCINES, AND 3) THE ADMINISTRATION OF INACTIVATED H9N2 AIV VACCINES IN OVO TO CHICKEN EMBRYOS. WE OBSERVED THAT THE TLR21 LIGAND, CpG ODN 2007, IS AN EFFECTIVE VACCINE ADJUVANT FOR INACTIVATED H9N2 AIV INTRAMUSCULAR CHICKEN VACCINES, Beta-propiolactone inactivated H9N2 AIV LEADS TO INCREASED IMMUNE RESPONSES IN CHICKENS, AND IN OVO VACCINATION WITH INACTIVATED H9N2 AIV VACCINES IS PLAUSIBLE. IN CONCLUSION, THIS THESIS PROVIDES INFORMATION ON VARIOUS FACTORS WHICH IMPACT THE IMMUNOGENICITY OF INACTIVATED H9N2 AIV VACCINES IN CHICKENS.
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List of Abbreviations

Ab    Antibody
AIV   Avian influenza virus
APC   Antigen presenting cells
BPL   Beta-propiolactone
CD    Cluster of differentiation
CD4+  Cluster of differentiation 4 positive cells
CD8+  Cluster of differentiation 8 positive cells
CLR   C-type lectin receptors
cRNA  Complementary RNA
DAMP  Damage associated molecular pattern
DNA   Deoxyribonucleic acid
dsRNA Double stranded RNA
HA    Hemagglutinin
HI    Hemagglutination inhibition
IFN   Interferon
Ig    Immunoglobulin
IKK   IκB kinase
Il    Interleukin
ILTV  Infectious laryngotracheitis virus
IM    Intramuscular
IRAK  Interleukin-1 receptor-associated kinase 1
IRF   Interferon regulatory factor
kb    Kilobase
kGy   Kilogram
LPAIV Low pathogenic AIV
LPS   Lipopolysaccharide
LRR   Leucine rich repeats
M     Matrix
MAPK  Mitogen-activated protein kinase
MDA5  Melanoma differentiation-associated protein
MDCK  Madin-Darby canine kidney cell
MDV   Marek’s disease virus
MHC   Major histocompatibility complex
mRNA  Messenger RNA
MX1   Myxovirus resistance 1
MyD88 Myeloid differentiation primary response gene 88
NA    Neuraminidase
NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR   Nucleotide oligomerization domain like receptor
NLS   Nuclear localization sequence
nm    Nanometer
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>OAS</td>
<td>Oligoadenylate synthetase</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
</tr>
<tr>
<td>PA</td>
<td>Polymerase acidic protein</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PB1</td>
<td>Polymerase basic protein 1</td>
</tr>
<tr>
<td>PB2</td>
<td>Polymerase basic protein 2</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly (lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RIG-1</td>
<td>Retinoic acid-inducible gene I</td>
</tr>
<tr>
<td>RLR</td>
<td>Retinoic acid inducible gene I like receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>SFV</td>
<td>Semliki forest virus</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single stranded RNA</td>
</tr>
<tr>
<td>STING</td>
<td>Stimulator of IFN genes</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper cell</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/interleukin receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor associated factor 6</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>WIV</td>
<td>Whole inactivated virus</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>γ</td>
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Introduction

Avian influenza viruses (AIV) are enveloped, single-stranded segmented negative-sense RNA viruses that infect a wide variety of hosts. Low pathogenic AIVs (LPAIV), are a class of AIV which infect their hosts and often do not cause any signs of clinical disease. H9N2 is a LPAIV that is thought to be the most widespread influenza virus in poultry (Nagy et al., 2017). Although infection typically does not show any signs in chickens, it can lead to economical losses, attributed to factors such as decreases in egg production in laying hens, and increased mortality and morbidity during co-infection with other respiratory pathogens (Shen et al., 2014; Sun and Liu, 2014). There is also serological evidence that shows that human contact with H9N2 AIV is prevalent in countries such as India and China, especially among poultry workers (Wang and Fu, 2009; Pawar et al., 2012). This human contact highlights the increased chances for future human infection with the virus.

Controlling H9N2 AIV infection in chickens can be achieved through a variety of strategies including but not limited to increased biosecurity, culling of infected animals, and prophylactic strategies, namely vaccination. Many types of vaccines exist for viruses, and different types of vaccines confer different benefits and disadvantages. When considering immunogenicity, inactivated virus vaccines are not optimal and often require assistance of some kind to induce effective immune responses. However, whole inactivated virus (WIV) influenza vaccines are relatively easy and cheap to produce, and they are considered stable and are very unlikely to revert to a virulent state. The act of inactivating influenza viruses for vaccine purposes is approximately 70-80 years old (Krammer and Palese, 2015), yet there still remains questions about how the inactivation of viruses affects the immune response after vaccination.
Various strategies can be used for influenza virus inactivation, including the use of chemicals, such as formaldehyde and beta-propiolactone (BPL), or the application of electromagnetic radiation, such as gamma-radiation. Different methods induce inactivation in different ways with each having their own advantages and disadvantages. In chickens, there is little understanding whether the inactivation method of H9N2 influenza viruses has any effects on immune response when administered as a vaccine. For this reason, one objective of this thesis was to inactivate an H9N2 virus using three mechanistically distinct methods (formaldehyde, BPL, and gamma radiation) and compare antibody- and cell-mediated immune responses after administering these WIVs as intramuscular (IM) vaccines to chickens. It was hypothesized that different inactivation methods used for the same H9N2 AIV would result in differences in elicited immune responses.

Enhancing the immune response to WIV vaccines is possible by the addition of adjuvants. One type of vaccine adjuvant that has been studied in chickens is the Toll-like receptor (TLR) ligands. TLRs are group of receptors that are classically characterized as part of the innate immune system. They are present both on the surface and in the endosome of cells (De Nardo, 2015). Upon recognition of their cognate ligand, an intracellular signalling cascade is initiated culminating in an increase in the expression and production of factors that lead to rapid innate responses, and importantly for the purpose of vaccine adjuvants, this also leads to the development of adaptive immune responses (St. Paul et al., 2013). TLR5 and 21 ligands, flagellin and CpG ODN 2007 respectively, are both effective adjuvants when administered with inactivated AIV vaccines in chickens (St Paul et al., 2014b; Singh et al., 2015). Another objective in this thesis was to compare the adjuvant capabilities of flagellin and CpG ODN 2007, in addition to comparing five-fold different doses of each ligand, and to compare the ligands
when administered together in the same vaccine for chickens. In this study, the ligands were administered alongside a formaldehyde inactivated H9N2 AIV and antibody-mediated immune responses were quantified. We hypothesized that ligand combination would induce additive or synergistic immune responses in chickens.

The first two studies described in this thesis focused on the immune response to IM vaccines, administered twice to chickens as a primary and secondary vaccine. Chicken embryos can be vaccinated in ovo, as technology already exists to do this and is used for other viruses such as Marek’s disease virus (MDV) (Avakian et al., 2007). In our third and final objective, we aimed to quantify the immune response to inactivated AIV vaccines that were administered to chickens as a primary in ovo vaccination, followed by a secondary IM vaccination. We hypothesized that this strategy of vaccination would induce adaptive immune responses in chickens, and that the addition of CpG ODN 2007 to vaccines would enhance those adaptive immune responses.
Influenza A virus

Physical characteristics

Influenza A, B, C, and D viruses are species of viruses and are members of the family Orthomyxoviridae. Influenza A and B are two species of enveloped viruses which contain a single stranded, segmented, negative-sense RNA genome consisting of eight different RNA segments (Zheng and Tao, 2013). Influenza A and B virions range from 80 to 120 nm in diameter and display three different proteins on their lipid membrane; a trimeric hemagglutinin (HA) glycoprotein, a tetrameric neuraminidase (NA) glycoprotein, and a tetrameric M2 ion channel (Wise et al., 2012; Zheng and Tao, 2013). Influenza C viruses contain only seven RNA segments and lack NA on their surface (Garman, 2015). The interior of the influenza virus envelope is where the most abundant protein in the virion resides, the matrix protein (M). Here it interacts with RNA segments in the interior of the virion. The RNA segments exist as ribonucleoprotein (RNP) complexes and are coated with many monomeric nucleoproteins (NP). Also in the RNP complex are three unique polymerases; PB1, PB2, and PA (Boivin et al., 2010).

Influenza A viruses can by subtyped by the HA and NA proteins which are embedded in the viral envelope. To date, 18 different HA proteins and 11 different NA proteins have been identified from circulating influenza A viruses. H17N10 and H18N11 were recently isolated in two different species of bat, both viruses express newly discovered HA and NA molecules (Mehle, 2014). The other 16 HA and 9 NA proteins can be found on influenza A viruses which
are capable of infecting numerous organisms, but avian species are a primary reservoir for all subtypes. In human populations H1N1, H2N2, and H3N2 viruses have all caused pandemics (Mehle, 2014), however other subtypes of influenza A are capable of human infection (Wang and Fu, 2009; Sun and Whittaker, 2013). Influenza A viruses can also be classified as highly pathogenic or low pathogenic based on molecular characteristics of the virus and the ability to cause disease in chickens in a laboratory setting (Suguitan et al., 2012). Highly pathogenic viruses are thought to exhibit increased virulence due to the presence of basic amino acids at a cleavage site in the HA protein which must be cleaved prior to infection of a cell. A basic cleavage site can be cleaved by a greater range of proteases, including intracellular proteases like furin. This expands the tissue tropism of these viruses in birds and mammals (Suguitan et al., 2012). H5 and H7 subtype viruses are commonly classified as highly pathogenic viruses, although not all influenza viruses expressing these proteins are highly pathogenic.

**Replication cycle**

Cellular replication of influenza A viruses can be temporally organised. First, the virus must attach to host cells, followed by internalization, transcription, translation, replication, and finally release of newly produced virions from the cell. The HA proteins on the influenza virion bind to sialic acid residues found on host cells. AIV subtypes (HA1-16) are capable of binding to α2,3 glycosidic bonds formed between galactose and sialic acid which are present on avian epithelial cells. The HA glycoproteins present on influenza viruses which are capable of human infection also recognize α2,6 glycosidic bonds which are mainly found in the human trachea. Internalization of the cell bound influenza virion was traditionally thought to be due to clathrin-dependant mechanisms, but clatherin-independent internalization was later demonstrated to also occur (Sun and Whittaker, 2013). Recent research using polarized MDCK II cells demonstrated
that the presence of cellular polarity increased the proportion of clatherin-dependent influenza internalization, suggesting this mechanism is more commonly used during \textit{in vivo} influenza infection (Zhang and Whittaker, 2014). Following internalization into endosomes, HA mediated fusion occurs between the endosomal and viral membranes (Sun and Whittaker, 2013). The acidic environment of the endosome is essential for this process; it is also required for the release of RNPs from the virion interior. The M2 ion channel facilitates an influx of hydrogen ions into the virion, decreasing the internal pH which decreases RNP affinity to the matrix protein, freeing the RNPs from the virion (Boivin et al., 2010). Transcription and replication of influenza genes occur in the nucleus and transport of RNPs from the cytoplasm into the nucleus is dependent on active transport. All proteins in the RNP complex (NP, PB1, PB2 and PA) contain a nuclear localization sequence (NLS) although the importance of each NLS for collective RNP nuclear transport varies for each protein and NP mediated active transport is the most used during influenza A replication (Zheng and Tao, 2013). Synthesis of viral mRNA for protein production and viral cRNA for gene replication is mediated by the replication complex of PB1, PB2 and PA; each polymerase has designated functions to facilitate these processes (Boivin et al., 2010).

Virion assembly occurs following complete replication of all eight RNA gene segments and production of all structural proteins. Influenza A virions must contain all eight individual RNPs in order to be infectious, although the exact mechanism which accomplishes this feat is not completely understood (Isel et al., 2016). Electron microscopy has revealed the RNP complex to be arranged in a “7 + 1” configuration in which one RNP is surrounded by seven unique others. Currently, it is suggested that the eight RNPs coordinate this through interactions in signalling regions in non-protein coding portions of the RNA sequence (Isel et al., 2016). Virions bud out from host cell membranes and at this point NA functions to enzymatically cleave sialic acid.
residues on the host cell, inhibiting the HA protein from re-binding to the same cell again, and allowing for successful release of new infectious virions (Szewczyk et al., 2014).

*H9N2 low pathogenic avian influenza virus (LPAIV)*

Most of the AIV subtypes are of low pathogenic type. LPAIVs are usually localized to the respiratory tract and alimentary canal during infection of poultry, often resulting in no signs of infection. LPAIV infection can however elevate levels of poultry mortality if other bacterial infections occur simultaneously. H9N2 virus infection alone is able to result in clinical signs which are consistent with respiratory problems and a temporary decrease in egg production (Pantin-Jackwood and Swayne, 2009). Numerous factors affect egg production in laying hens, including H9N2 virus infection. For example, following experimental H9N2 virus oculo-nasal infection in laying hens, viral replication is detectable in the oviduct for five to seven days (Wang et al., 2015). Following H9N2 virus infection, histological changes can be found in sections of the oviductal parts, including degeneration of the uterine and vaginal epithelial layers. These histological changes are accompanied by increased apoptosis and elevated expression of inflammatory cytokines (Wang et al., 2015). Also, following infection with H9N2 virus, viral load in the uterus has been demonstrated to correlate with a decrease in expression of the calbindin-D28k gene, which codes for a calcium transport protein. The decrease in calbindin-D28k mRNA expression has also been shown to correlate with a decrease in eggshell calcium content and eggshell thickness in eggs laid by H9N2 virus infected hens (Qi et al., 2016). H9N2 virus infection in laying hens not only decreases egg production, but also decreases the eggshell quality in laid eggs, potentially decreasing the physical strength of the shell and increasing chance of microbial invasion (Qi et al., 2016). Vaccination of laying hens with a killed H9N2
virus vaccine is able to limit the decrease in egg production and decrease the production of abnormal eggs following H9N2 virus infection (Shin et al., 2016).

Since initial isolation in 1966 in the USA, and the first reported outbreak in 1994 in China, H9N2 virus has become one of the predominant AIVs circulating in poultry around the world. In China H9N2 virus infection of avian species occurs primarily in live poultry markets and poultry farms (Shen et al., 2014). Although over 100 isolates of H9N2 virus have been uncovered in China since 1994, a predominant genotypic group of H9N2 AIV (G57) has been linked to increased prevalence in chickens (Su et al., 2015). Since first identification in 2007, H9N2 G57 virus prevalence in chickens has drastically increased and it is now the most common circulating genotype (Pu et al., 2015). The frequency of H9N2 G57 virus in chickens also explains the discovery of a reduction of genetic diversity among H9N2 AIVs isolated from chickens from 2010 to 2013 in China, suggesting a population bottleneck which selected the more fit G57 group. There is also evidence of H9N2 G57 virus infection in swine, domestic aquatic birds, wild birds, and one case of human infection (Pu et al., 2015). Inactivated H9N2 virus vaccines have been in use in China since 1998 and although initially successful, H9N2 virus infection is still common in vaccinated chickens (Zhang et al., 2008; Su et al., 2015). The success H9N2 virus has had replicating in poultry from 2010 to 2013 is thought to have allowed it to contribute various gene segments to a novel H7N9 reassortant virus which has caused two outbreaks and 610 human deaths since 2013 (Pu et al., 2015).

The omnipresent nature of H9N2 virus in China is both a threat to economic loss and to human health. Human contact with H9N2 virus is evident among Chinese poultry workers; serological analysis demonstrates varying frequencies of workers who have serum anti-H9 antibodies, peaking at 15.5 % in poultry retailers. Poultry retailers handle live birds, suggesting
the transmission of H9N2 virus is mainly poultry-to-human and not human-to-human, highlighting the need to control H9N2 virus infection in chickens in both live poultry markets and farms (Wang and Fu, 2009). H9N2 virus transmission to dogs also more than doubled from 2010 to 2012, demonstrating its capacity to adapt to different hosts (Sun and Liu, 2014). Phylogenetic analysis of H9N2 viruses in China has revealed an increased frequency of mutations in the HA and NA proteins which can enhance HA cleavage and viral release from red blood cells. This mutation also has been shown to increase virulence associated with infection in chickens and mice. Additionally, increased proportions of isolated H9N2 viruses have been found to express an HA protein with a substitution mutation which experimentally shows increased tropism and replication efficacy for cultured human epithelial airway cells (Wan and Perez, 2007). To summarize, in China there is a high level of H9N2 virus infection in poultry. Decreasing the threat of human infection and disease from H9N2 virus infection is important and one way to possibly facilitate this is decreasing the transmission of H9N2 virus in poultry. One possible method which can be used to decrease transmission is vaccinating chickens with efficacious immunologically designed vaccines.

*Inactivated AIV vaccines*

A popular kind of influenza vaccine used in mammals and chickens involves inactivated influenza viruses. Human vaccines against influenza were first used in the 1940s and are similar to the vaccines used today. These original vaccines were created by growing influenza viruses in embryonated chicken eggs and inactivating the virus with formaldehyde, creating a whole-virus inactivated vaccine antigen (Krammer and Palese, 2015). Formaldehyde inactivated influenza viruses have also been studied as vaccines for chickens (St. Paul et al., 2011; St Paul et al., 2014b; Singh et al., 2015). Along with inactivated virus vaccines, many other types of vaccine
strategies exist; including attenuated viral vaccines, subunit vaccines and DNA vaccines. Furthermore, inactivation of influenza viruses can be achieved in different ways, including chemical inactivation and inactivation by application of different forms of radiation (Nunnally et al., 2015)

Formaldehyde is an aldehyde which when exposed to proteins will crosslink various amino acids affecting protein structure, leading to an inhibition of protein function and as a result, can lead to virus inactivation. Formaldehyde alkylates sulfhydryl and amino groups of proteins in addition to affecting the nucleotide bases adenine and guanine (Pawar et al., 2015). Prior to being used to inactivate viruses, formaldehyde was first used to decrease the toxicity of toxins, such as the tetanus toxin. These structurally altered toxins could then be safely administered as toxoid vaccines. In addition to influenza, inactivation with formaldehyde has also historically been used for other medically important viruses, such as poliovirus in the 1950s by Jonas Salk (Nunnally et al., 2015). Chemicals like formaldehyde which affect protein structure can potentially alter immunogenic epitopes on proteins such as the HA protein on the influenza virus envelope (Pawar et al., 2015).

Another chemical used for virus inactivation is beta-propiolactone (BPL). BPL is an alkylating agent which is capable of inducing changes to the nucleic acids of viruses. BPL can induce strand breaks in nucleic acids; it can also form linkages between nucleic acids and protein and between two nucleic acid molecules (Pawar et al., 2015). BPL mainly reacts with nitrogen atoms on guanosine and adenosine. These BPL-modified nucleotides can induce mutations during polymerization reactions effectively which also contributes to pathogen inactivation (Nunnally et al., 2015). More recently it has been demonstrated that BPL does interact with certain amino acids which contain nucleophilic groups, following interaction with BPL amino
acid polarity and side-chain structure can change drastically (She et al., 2013). Using ellipsometry, it has been shown that BPL inactivation can actually alter the HA protein of influenza leading to decreased adsorption and fusion with an experimentally derived membrane containing ganglioside receptors (Desbat et al., 2011). Although only BPL inactivated influenza viruses were used in the experiment, it is likely that adsorption and fusion are further inhibited for formaldehyde inactivated influenza viruses. Budimir et al. (2012) suggested that formaldehyde inactivation inhibits membrane fusion of whole inactivated influenza viruses significantly more than BPL inactivation does after vaccinating mice with both BPL and formaldehyde inactivated viruses, and observing significantly greater levels of cell-mediated immune responses in mice which received BPL inactivated influenza. This difference was attributed to the inability of the formaldehyde inactivated influenza virus to fuse with cell membranes.

In addition to inactivation using chemical treatments, influenza and other pathogens can be inactivated by applying different forms of radiation over a period of time. A popular type of radiation used for influenza virus inactivation is gamma-radiation. Gamma-radiation is capable damaging the nucleic acids of influenza, leading to strand breaks and nucleotide damage (Alsharifi and Müllbacher, 2010). Gamma-radiation is quantified by kilograys (kGy) and is defined as a unit of absorbed radiation. The Manual on Radiation Sterilization of Medical and Biological Material from the International Atomic Energy Agency initially stated that influenza virus requires 0.65 kGys of radiation for total loss of infectivity, and structural changes to the hemagglutinin protein only occurs after the 200 kGy mark. Since protein structures on the influenza virus are not altered by gamma-radiation in a major fashion, this method is thought to be an effective way of virus inactivation, resulting in an immunogenic
product when included in a vaccine. Gamma-radiation inactivated viruses have been administered experimentally in mice, revealing some interesting findings; first, gamma-radiation inactivated influenza viruses elicit type-1 interferon (IFN) responses (Furuya et al., 2011) and also that gamma-radiation inactivated influenza viruses have adjuvant like capabilities when co-administered with a gamma-radiation inactivated Semliki forest virus (SFV), resulting in elevated antibody titers against SFV (Babb et al., 2014). These findings suggest gamma-radiation is a good option for the inactivation of influenza viruses for inclusion in vaccines. Nonetheless, different inactivation methods, including chemical and radiation treatments have different effects on the immunogenicity of the resultant inactivated influenza virus, and all could potentially be used in an immunogenic and protective inactivated H9N2 chicken vaccine. Testing these methods of inactivation via in vivo chicken experiments is essential.

**Pattern recognition receptors**

**Overview**

Pattern recognition receptors (PRR) are a class of receptors which are present at the cellular level in various organisms (Takeuchi and Akira, 2010). PRRs are traditionally considered receptors of the innate immune system and are expressed by various cells in the cytoplasm, endosome, and cell surface (Akira et al., 2006). PRRs recognize conserved molecular epitopes present on microorganisms, collectively termed pathogen associated molecular patterns (PAMP). PRRs are also involved in the recognition of molecules which arise from damaged cells such as histones or heat shock proteins which are classified as damage associated molecular patterns (DAMP) (Takeuchi and Akira, 2010; He et al., 2015). Binding of PAMPs and DAMPs to PRRs leads to activation of unique intracellular signalling cascades which result in various pathogen protection responses (Akira et al., 2006). PRR- ligand binding
can lead to a change in the level of transcription of certain genes, inducing the production of various proteins such as type-1 IFNs, chemokines, antimicrobial peptides, and pro-inflammatory cytokines (Takeuchi and Akira, 2010). Multiple classes of PRRs have been discovered, including TLRs, nucleotide oligomerization domain like receptors (NLR), retinoic acid inducible gene I (RIG-I) like receptors (RLR) and C-type lectin receptors (CLR) (Kumagai and Akira, 2010).

*Toll-like receptors: structure and signalling characteristics*

TLRs are expressed by numerous organisms, including some from the plant phyla (Brownlie and Allan, 2011). The first class of innate receptors to be defined and most characterized class of PRRs is the TLRs (De Nardo, 2015). The Toll gene was initially discovered over 30 years ago to function as a crucial regulator of embryo development in drosophila (Anderson et al., 1985). Years later, it was shown that mutations in Toll signalling pathways had an impact on the expression of antibacterial genes in drosophila and survival was hindered when drosophila which carried these mutations were challenged with bacteria or fungus (Lemaitre et al., 1996). Soon after a human homologue of the Toll protein was discovered and shown to function similarly to Toll in drosophila (Medzhitov R et al., 1997). TLR expression is not limited to humans and drosophila and most vertebrate species express 10 to 13 different TLR receptors (St. Paul et al., 2013).

In the immune system cells in which they are expressed, TLRs can be found in two different areas; the plasma membrane or the endosomal/lysosomal membrane (De Nardo, 2015). Regardless of location, all TLRs are type one transmembrane proteins which can be segmented into three distinct regions; an extracellular ligand binding domain, a transmembrane hydrophobic domain consisting of one single alpha-helix, and an intracellular domain (Gay and Gangloff,
The multitude of ligands bound by TLRs demands a diverse array of receptors. The extracellular domain of TLRs consists of approximately 19 - 27 leucine rich repeats (LRR) (Keestra et al., 2013) which are constructed from 24-amino acid sequences characterized by hydrophobic residues (Gay and Gangloff, 2007). The LRRs assume beta-sheets as secondary structures and this creates a tertiary curved ‘horseshoe’ appearance (De Nardo, 2015). The intracellular domain of TLRs resembles the intracellular domain of the cytokine IL-1 receptor and activation of both receptor classes is hypothesized to stimulate a similar subsequent signalling cascade (Gay and Gangloff, 2007). Location of the N-terminal or extracellular/intra-endosomal domain of the TLR coincides with the class of ligand it binds. Plasma membrane TLRs bind to lipids and/or proteins of microbial origin as expected as contact between these receptors and ligands is favoured. TLRs which reside in the endosome or lysosome recognize microbial nucleic acids as this is a common site of exposure to these ligands (De Nardo, 2015).

Once bound to their specific ligand, TLRs form either a homodimeric or heterodimeric complex depending on the receptors involved. This dimerization brings the intracellular activation domains together facilitating a docking site for adaptor proteins to bind to (Keestra et al., 2013). Two major adaptor proteins exist, Myeloid differentiation primary response 88 (MyD88), which functions as an adaptor in all TLR signalling pathways except for TLR3, which uses the adaptor protein TIR-domain-containing adapter-inducing interferon-β (TRIF) (Keestra et al., 2013). Upon MyD88 docking onto activated TLR intracellular domains, Interleukin-1 receptor-associated kinase 1 (IRAK) proteins associate before being phosphorylated and subsequently dissociating and binding to TNF receptor-associated factor 6 (TRAF6). A series of subsequent interactions between signalling proteins eventually activates the IκB kinase (IKK) complex and mitogen-activated protein kinase (MAPK) proteins which phosphorylate IκB.
proteins, blocking the ability of IκB to inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) translocation into the nucleus. NF-κB is a transcription factor which enters the nucleus and upregulates the transcription of many genes including pro-inflammatory cytokine genes (Kawai and Akira, 2007). TRIF signalling occurs exclusively for TLR3 and partially for TLR4 (not in chickens) and results in the activation of NF-κB and IRFs, the latter which promotes the production of type-1 IFNs (Keestra et al., 2013).

TLR signalling leads to a local pro-inflammatory response which acts as an initial innate barrier to invading microbes (St. Paul et al., 2013). TLR signalling also has immunomodulatory effects which can alter the outcome of an adaptive immune response. Antigen presenting dendritic cells possess PRRs, including TLRs, and these dendritic cells can respond to TLR signalling in various ways (Mogensen, 2009). Dendritic cells become activated when TLRs encounter their cognate ligand, leading to the upregulation of important proteins such as antigen presenting molecules including major histocompatibility complex proteins one and two, (MHC-I/II), co-stimulatory molecules for T cell activation (CD80, CD86), and chemokines. TLR signalling at the time of infection will also impact the cytokines which are produced by activated dendritic cells. The cytokines produced by dendritic cells will influence the development of certain T helper (Th) cell subsets during naïve T cell activation and thus can direct the adaptive immune response based on the microbial derived ligands present during infection (Mogensen, 2009). For example, during malaria infection in mammals, parasite derived DNA-protein complexes are bound via TLR9 and this interaction leads to Th1 cell differentiation and antibody isotype switching. TLR4 signalling is also essential in the formation of adaptive immune responses against malaria and this has been demonstrated in mice (Zhang et al., 2016).
Chicken immune system

Innate responses

Initial recognition and defence from invading pathogens are roles of the innate immune system. Avian species such as the chicken employ an innate immune system which has numerous features similar to the mammalian innate immune system; however, there are certain characterized differences between the two. Less is understood about chicken innate response mechanisms compared to those of human and mouse, although due to the agricultural importance of the chicken most avian immunology discoveries are made using chickens (Brownlie and Allan, 2011). The initial recognition of pathogens is a function of various PRRs (Mogensen, 2009). In mammals, 13 TLRs have been discovered and humans express 10 of them (TLR1-10). Chickens share some of these TLRs as orthologues yet numerous differences exist in their TLR repertoire. Chickens express a total of 10 TLRs (TLR1La, TLR1Lb, TLR2a, TLR2b, TLR3, TLR4, TLR5, TLR7, TLR15 and TLR21), 5 of which are orthologues of receptors found in humans and mice (TLR2a/b, TLR4, TLR5 and TLR7) (Brownlie and Allan, 2011). TLR1L is described as “TLR1-like” when compared to mammalian TLR1, but it independently emerged from an ancestral gene which also gave rise to TLR6 and TLR10 in mammals (Temperley et al., 2008). TLR1L and TLR2 genes underwent separate duplication events an estimated 147 million years ago and 65 million years ago, respectively (Brownlie and Allan, 2011). In mammalian species, TLR2 dimerizes with TLR1 or TLR6 to facilitate binding to their cognate ligand and initiate signalling; these heterodimers recognize triacylated (TLR2/TLR1) and diacylated (TLR2/TLR6) lipopeptides (Keestra et al., 2013). Chicken recognition of triacylated and diacylated lipopeptides has been demonstrated by various combinations of TLR1La, TLR1Lb, TLR2a and TLR2b, although homodimers do not facilitate binding and signalling. The synthetic
molecule Pam3CSK4, which is a TLR1/2 ligand, has been used as an adjuvant in experimental vaccines for chicken (St Paul et al., 2014b). Unlike TLR1 and TLR2, TLR3 is present in endosomal compartment of chicken cells. TLR3 in chickens is missing 9 amino acids in the 12th LRR yet is still considered a mammalian orthologue; like TLR3 in mammals it forms a homodimer and recognizes dsRNA motifs (Karpala et al., 2012). The synthetic ligand poly I:C can also bind TLR3 and has been used experimentally as a vaccine adjuvant in chicken experiments (St Paul et al., 2014a). TLR3 signalling activates an IFN regulatory factor (IRF), which affects the rate of transcription of pro-inflammatory cytokines and type-1 IFNs (Brownlie and Allan, 2011; Karpala et al., 2012; Keestra et al., 2013). Chicken TLR7 binds RNA like TLR3; however it binds ssRNA, in addition to recognizing synthetic ligands such as Imiquimod, Gardiquimod and R848 (Brownlie and Allan, 2011). TLR8 in mammals is a paralog of TLR7 as a result of a duplication event and it also recognizes ssRNA. Chickens, along with all birds, do not have TLR8 and only small sequences of TLR8 DNA can be identified in the chicken genome (Magor et al., 2013). Chickens express TLR4 and the associated MD2 protein; this protein complex binds LPS, signals through MyD88 and leads to the upregulation of pro-inflammatory cytokines IL-1β and IL-8. Unlike mammalian TLR4, chicken TLR4 does not use TRIF in addition to MyD88 as an intracellular signalling molecule, abolishing the subsequent IRF mediated type 1 IFN response (Keestra et al., 2013). This difference in signalling cascades is suggested to be responsible for the decreased sensitivity chickens have to LPS and other endotoxins, compared to mammals which show toxic responses to LPS (Brownlie and Allan, 2011). Chickens also express a novel TLR unique from mammalian species which is TLR15, unique to birds and reptiles (Brownlie and Allan, 2011). Screening many ligands eventually revealed that TLR15 can recognize proteolytic activity at the cell surface. TLR15 has been
shown to recognize some fungal and bacterial proteases, and it does this without the need for any other specific chicken molecules. Initiation of TLR15 signalling happens after receptor cleavage and homodimerization (Keestra et al., 2013).

TLR21 is found in chickens and it does not share an orthologue with any mammalian species, however it recognizes unmethylated CpG ODNs which are also bound by TLR9 in mammals; TLR9 is non-existent in the chicken (Keestra et al., 2013). TLR21 is suggested to exist in the endo-lysosome of cells where it utilizes the adaptor protein MyD88 (Keestra et al., 2013). Expression of TLR21 has been demonstrated in chicken B cells and macrophages (St. Paul et al., 2013). Class B CpG ODNs, such as CpG ODN 2007 and 1826, are able to induce B cell proliferation. In vivo experiments in chickens have demonstrated that splenocytes from chickens which were previously intramuscularly administered a class B CpG ODN increased production of IFN-γ and MHC-II mRNA transcripts while decreasing production of IL-13 transcripts, suggesting TLR21 stimulation could result in Th1 favoured adaptive immune responses (St. Paul et al., 2011). In addition to promoting B cell proliferation and regulating the adaptive immune response, administration of CpG ODNs also stimulates innate immune system cells to produce pro-inflammatory cytokines and enhances their function (St. Paul et al., 2013). CpG ODN 2007 and 1826 when applied prophylactically either via intranasal or intramuscular routes have been demonstrated to decrease shedding of H4N6 AIV 4 and 7 days after experimental infection (Barjesteh et al., 2015b).

TLR5 in chicken and mammalian cells is located on the cell surface where it is able to recognize and bind the bacterial protein flagellin (Keestra et al., 2013). TLR5 transcripts have been identified via RT-PCR in numerous tissues and immune system cells in chickens. These tissues include the lungs, spleen, small intestine and kidneys (Iqbal et al., 2005). Cell subsets
expressing TLR5 include heterophils, B cells and macrophages (Kogut et al., 2005). TLR5 signalling is mediated by the adaptor protein MyD88. In vitro experiments with chicken heterophils have shown that stimulation with flagellin increased the production of reactive oxygen species and promoted degranulation in a concentration dependant manner. Heterophil stimulation with flagellin also increased IL-1β, IL-6 and IL-8 expression which will induce the attraction of other immune system cells to the area of infection (Kogut et al., 2005).

Chickens possess other PRRs which also function as part of the innate immune system. Two other receptors in the RLR class which are present in the cytoplasm of mammalian cells are able to recognize dsRNA, namely MDA5 and RIG-I. These receptors vary in that they recognize dsRNA of different lengths; RIG-I recognizes dsRNA less than 1 kb in length, while MDA5 recognizes larger segments (Karpala et al., 2012). Upon ligand recognition, both receptors signal through a mitochondria-mediated mechanism which eventually results in IRF3/7 induced transcription of type 1 IFNs (Magor et al., 2013). MDA5 is present in chicken cells but the same cannot be said for RIG-I, although RIG-I downstream signalling molecules have been identified (Karpala et al., 2012). Not all avian species lack RIG-I, duck cells produce RIG-I and the gene can be transfected into chicken cells resulting in a functioning protein in vitro (Barber et al., 2013; Magor et al., 2013). Following in vitro transfection of duck RIG-I into chicken DF-1 cells, and subsequent infection with influenza virus, a significantly greater amount of type-1 IFN induced transcripts can be detected. These include antiviral transcripts which produce proteins such as MX1, OAS, and PKR (Barber et al., 2013). Chickens also do not appear to have IRF3 and transcriptional modulations are mediated by IRF7 (Magor et al., 2013). This indicates that the duck RIG-I transfected DF-1 cells utilised existing downstream molecular mechanisms to induce signalling. There is debate as to whether the lack of RIG-I in chicken cells is responsible
for a deficiency in innate immunity or whether MDA5 signalling makes up for this deficit. Cheng et al. (2015) cloned the chicken adaptor protein gene, STING, and transfected it into chicken DF-1 cells. Overexpression of STING in DF-1 cells lead to an increase in virus induced IRF7 and type-1 IFN production, while knockdown of STING entirely inhibited virus induced increases in IRF7 and type-1 IFNs. They also demonstrated that MDA5 interacts with STING. Lastly, they suggested that there may be a separate cytosolic sensor of nucleic acid which signals through STING and this combined with MDA5-STING mediated detection decreases the need for RIG-I in chickens (Cheng et al., 2015).

An important cell type which is traditionally classified as an avian innate immune system cell is the heterophil, a cell which has a corresponding role to the neutrophil found in mammals (Brownlie and Allan, 2011). Heterophils are able to directly kill infectious pathogens by mechanisms such as phagocytosis, release of cytotoxic granular contents, and through the production of reactive oxygen species (ROS). However heterophils are less proficient in the production of ROS and rely more heavily on the production of antimicrobial proteins and peptides (Genovese et al., 2013). The mechanisms employed by heterophils can be initiated by PRR mediated recognition of PAMPS. Heterophils have been shown to express mRNA transcripts for TLRs 1La, 1Lb, 2a, 2b, 3, 4, 5, 7, 15 and 21 (Kogut et al., 2005; Genovese et al., 2013).

Adaptive immune system

Chickens employ an adaptive immune system which is capable of forming a diverse array of memory responses that are antigen specific yet also capable of recognizing self from non-self. Antigen processing and presentation are crucial stages in the formation of an adaptive immune response, and these processes have been studied extensively in chickens, second in knowledge...
only to select mammalian species (Kaufman, 2013). The MHC region in the chicken genome is arranged differently and is much smaller than it is in mammalian species. The chicken MHC region undergoes little recombination which results in stable haplotypes consistent for certain polymorphisms of MHC-I and MHC-II alleles. Chickens only express one MHC-I and MHC-II allele at a high level, and the allele which is expressed leads to either resistance or susceptibility to infectious disease. In humans, MHC alleles undergo a higher rate of recombination and average resistance against most infectious diseases is conferred (Kaufman, 2013). The relationship between tapasin and MHC in the chicken genome is thought to explain the limited expression of MHC alleles in chickens. Tapasin is a heterodimeric protein which is involved in the transport of peptides into the endoplasmic reticulum; humans rely on a monomorphic tapasin protein for loading peptide onto all MHC-I proteins. Chickens have numerous polymorphic tapasin alleles and certain alleles produce proteins which have evolved to favourably interact with a specific MHC-I allele. This pairing of MHC-I alleles to tapasin alleles has occurred because of the short distance between these two groups of genes on the chromosome, decreasing the rate of recombination (Walker et al., 2011). The single MHC-I allele that chickens express does vary however in function and expression; the MHC-I protein tends to be either expressed at high levels on the cell surface with a very specific peptide preference, or expressed at lower levels but with a greater range of peptides in which it will bind (Kaufman, 2015). The lack of MHC-I variability causes some chickens to respond well to vaccination or challenge with infectious pathogens such as MDV while causing others to respond poorly (Kaufman, 2000).

Chickens can also generate antibody-mediated immune responses to combat infection. Humans express five isotypes of immunoglobulins; IgA, IgD, IgE, IgG and IgM, while all birds only express three, lacking IgD and IgE (Magor et al., 2013). In chickens, IgG is also replaced
by IgY, a molecule which has evolutionary ties to both IgG and IgY. IgY has 4 constant heavy domains in the heavy chain similar to mammalian IgE, unlike the three found in IgG; IgY is however the most abundant immunoglobulin in chickens and it is transferred as maternal protection to the developing embryo in the egg (Schreiner et al., 2012). Maternal IgY from the hen is stored in the yolk before being taken up by the developing embryo, IgY transfer has been shown to be highest at day 19-21 of embryonic development, corresponding to peak serum maternal antibodies on days 1-3 post-hatch (Avakian et al., 2007). IgA and IgM of maternal origin can also be detected in the egg white as well as in the gut of the embryo (Rose, 1979).

Chickens also possess a different organisation of primary and secondary lymphoid organs relative to humans. In humans, the primary lymphoid organ responsible for B cell development is the bone marrow yet this is not true for chickens. Initial experiments in the 1950s revealed that the bursa of Fabricius is the primary site of B cell production and development in the chicken. Secondary lymphoid tissue is the site of the initiation and formation of adaptive immune responses. Major secondary lymphoid tissues in mammalian species include the lymph nodes and spleen. Chickens do have spleens, however they do not possess encapsulated lymph nodes, and have secondary lymphoid tissue present in other areas such as the gastrointestinal tract (Rose, 1979).

**Employing TLR ligands as immunostimulatory molecules**

TLR signalling is a critical and ubiquitous part of recognition and successful defense against invading microbial organisms. This naturally occurring phenomenon can be re-created and used advantageously in an extensive number of ways, mainly having to do with prophylactic treatments against pathogenic infection and disease. TLR ligands can be used prophylactically as stand-alone anti-microbial agents and as adjuvants in vaccines.
**Employing TLR ligands as prophylactic anti-microbial agents**

The anti-microbial prophylactic effects of TLR ligands have been demonstrated *in vitro*, *in vivo*, and *in ovo*. When administered to the chicken macrophage cell line MQ-NCSU, TLR ligands have been demonstrated to decrease the *in vitro* replication capacity of H4N6 LPAIV. PAM3CSK4, LPS, and class B CpG ODNs were all shown to decrease H4N6 titers 14 hours after infection, but the time of TLR ligand application to MQ-NCSU cells had ligand-specific effects. PAM3CSK4 application prior to, simultaneously, and post-infection significantly decreased H4N6 titer recovered from MQ-NCSU supernatants. LPS only had this effect when applied prior and post-infection, while class B CpG ODNs decreased H4N6 virus replication when applied prior to infection (Barjesteh et al., 2014). Macrophages are an important part of the chicken innate immune system; following TLR ligand stimulation, macrophages can produce pathogen-damaging substances such as reactive nitrogen species which can inhibit viral replication (He et al., 2011; Barjesteh et al., 2014). Stimulation of chicken embryo fibroblasts with poly I:C has been demonstrated to significantly decrease *in vitro* MDV replication following infection, and this interaction was also shown to be dependent on TLR3 (Hu et al., 2015).

The prophylactic capabilities of TLR ligands have also been demonstrated *in vivo*. In mice, this effect has been demonstrated with various TLR ligands and numerous types of microorganisms. Intranasal administration of an oligodeoxynucleotide TLR9 ligand in anthrax susceptible mice one day prior to infection with *Bacillus anthracis* increased survival from zero to 50 percent (Wu et al., 2008). Wu et al. (2014) demonstrated a similar increase in survival rate (40%) in *Bacillus anthracis* susceptible mice after bi-weekly intranasal administration of an adenine based TLR7 adjuvant. However, they challenged mice four weeks after the final TLR7
ligand treatment and still observed this increase in survival. Using the same TLR7 ligand administered intranasally one and three days prior to infection, Wu et al. (2014) also demonstrated an 80% and 100% increase in survival in BALB/c mice following lethal challenge with Venezuelan equine encephalitis virus and H1N1 influenza virus, respectively. TLR ligands have been demonstrated as functional prophylactic agents against MDV and AIV in chickens. TLR4 and TLR21 ligands (LPS and CpG ODN 2007) were shown to decrease the onset of disease induced by MDV infection when applied via the intra-air sac route one day prior to infection and subsequently applied intramuscularly13 days after infection (Parvizi et al., 2014). Specifically, LPS decreased tumor incidence in MDV infected chickens from 100% to 37.5% on day 21 post-infection, and both LPS and CpG ODN 2007 were able to significantly decrease clinical signs of disease caused by MDV by day 21 post-infection. Another chicken study displayed the anti-viral effects LPS, CpG ODN 2007, and poly I:C have on H4N6 virus replication following infection (St. Paul et al., 2012). In the latter study, TLR ligands were injected intramuscularly in chickens 24 hours prior to oculo-nasal challenge with H4N6 LPAIV. Analysis of cloacal and oropharyngeal swabs revealed that all three ligands were able to significantly decrease H4N6 titer in oropharyngeal secretions on day four post-infection and in cloacal swabs on day seven post-infection. Barjesteh et al. (2015) furthered this research by utilizing a greater range of TLR ligands to also include a TLR2 and TLR7 ligand as well as other types of CpG ODN, and by administering the ligands both intramuscularly and intranasally. Both methods of administration and all TLR ligands were able to significantly decrease oropharyngeal and cloacal shedding of H4N6 virus compared to control, yet intranasal treatment of CpG 1826 (class B CpG ODN) lead to the greatest decrease in oropharyngeal virus titer at
four days post-infection, and intranasal treatment of LPS lead to the greatest decrease in cloacal virus titer at seven days post-infection.

TLR ligand administration has been also tested during chicken embryonic development by \textit{in ovo} injection. \textit{In ovo} administration of TLR ligands was shown to significantly decrease AIV titers in experimentally infected eggs, inhibiting AIV replication \textit{in ovo} (Barjesteh et al., 2015a). The ligands in this experiment were injected into the allantoic fluid; however injection into the amniotic fluid has been demonstrated beneficial for the embryo/chick pre- and post-hatch. Injection of CpG ODN into embryonic day 18 (ED18) eggs decreased \textit{in ovo} replication of infectious laryngotracheitis virus (ILTV) in the lungs of chicken embryos. Injection of CpG ODN into ED18 eggs also decreased ILTV titer in tracheal swabs and significantly reduced mortality and morbidity in chickens following post-hatch experimental infection (Thapa et al., 2015). Post-hatch challenge with ILTV has also been studied following injection of ED18 eggs with lipoteichoic acid, a TLR2 ligand. This treatment also decreased ILTV titer in the lungs of chickens at one, three and five days after challenge (Thapa et al., 2015).

\textit{Employing TLR ligands as vaccine adjuvants}

The innate response following TLR ligand administration extends beyond short-term antimicrobial functions. TLR ligands also induce longer lasting immunomodulatory effects which can augment the way in which an immune response is formed and hence serve as candidate vaccine adjuvants. A vaccine must induce an immune response against the vaccine antigen and this response is dependent on processing and presentation of vaccine antigens to T cells. Signalling through TLRs on antigen presenting cells (APC) matures these cells and promotes the expression of co-stimulatory molecules like CD80 and CD86 which are crucial for activation of T cells in the formation of an immune response (St. Paul et al., 2013). TLR
signalling can also affect cytokine expression by these APCs, impacting the type of immune response generated. Intramuscular injection of TLR ligands in chickens has been shown to have an effect on the subsequent expression of cytokine genes in the spleen. St. Paul et al. (2011) injected LPS and CpG ODN 2007 intramuscularly and observed various changes in splenic cytokine gene expression. Specifically, LPS induced high upregulation of IFN-γ, IL-10, IL-1β and IL-8 two hours after injection, while CpG ODN 2007 to a lesser extent upregulated IFN-γ and IL-1β. IFN-γ is a cytokine which induces a Th1 cell style immune response, while IL-8 and IL-1β are pro-inflammatory cytokines (St. Paul et al., 2011). Numerous experiments have examined TLR ligands as vaccine adjuvants in mammals and chickens, the following examples will highlight studies performed in chickens, mainly focusing on inactivated influenza vaccines. A summary which includes the TLR ligand doses and results is provided in Table 1.

A considerable number of different TLR ligands have been tested as adjuvants co-administered as part of inactivated influenza vaccines. Poly I:C, CpG ODN, and spores of *Bacillus subtilis* (TLR2/4 ligand) tested as adjuvant for an inactivated intranasal H5N1 AIV vaccine were all individually capable of significantly increasing anti-H5N1 IgA titers in chicken nasal and tracheal cavities at three, five and, seven weeks post vaccination when compared to a non-adjuvanted H5N1 vaccine (Liang et al., 2013). In addition to amplifying mucosal immune responses, all three adjuvants increased serum hemagglutination inhibition (HI) antibody titers and CpG ODN significantly increased serum IgG three and five weeks post vaccination. CpG ODNs have been further shown to be efficacious inducers of systemic antibody-mediated immune responses when combined with an inactivated H5N1 vaccine, and that they can be physically modulated to enhance these responses (Fu et al., 2013). When considering an inactivated H4N6 AIV vaccine, the addition of flagellin resulted in significantly higher IgY and
HI antibody titers four weeks after primary vaccination, while the addition of PAM3CSK4 resulted in significantly higher lachrymal IgA titers four weeks after primary vaccination. In this experiment both ligands did however significantly reduce the level of H4N6 virus shed in cloacal fluids seven days after challenge (St Paul et al., 2014b). A similar study using the same inactivated H4N6 virus compared poly I:C, LPS and CpG ODN 2007 as vaccine adjuvants (St Paul et al., 2014a). In this study TLR ligands were dually combined in one vaccine formulation in addition to being used alone. Overall, CpG ODN 2007 elevated serum HI titers four weeks after primary vaccination, but the combination of CpG ODN 2007 plus poly I:C resulted in a significant difference. Recent research has examined the efficacy of CpG ODNs as vaccine adjuvants for inactivated H9N2 AIV vaccines (Singh et al., 2015). Past work with CpG ODNs (St Paul et al., 2014a; Barjesteh et al., 2015b) has shown that class B CpG ODNs (CpG 1826 and 2007) are immunostimulatory in chickens, and therefore these ligands were compared by Singh et al. (2015). They demonstrated that CpG ODN 2007 at a dose of 2 μg per bird per vaccination was a more immunogenic adjuvant than CpG 1826. Specifically, chickens that received CpG ODN 2007 produced significantly greater systemic HI antibody titers and H9N2 AIV neutralizing antibody titers compared to chickens which received CpG 1826. Lastly, TLR ligands have also been studied in ovo as adjuvants for vaccines delivered pre-hatch (Radomska et al., 2016). Flagellin of *Campylobacter jejuni* was modified to harbor the epitope which chicken TLR5 is capable of binding. This modified flagellin protein was able to induce systemic antibodies in hatched chickens after being delivered in ovo, thus the flagellin protein served as antigen and adjuvant. To conclude, there is vast evidence to support the use of TLR ligands as vaccine adjuvants for inactivated AIV chicken vaccines. Initial work has made multiple
findings, but more research should be done to form conclusions about which TLR ligands are optimal for inclusion in said vaccines.
Table 1. Summary of TLR ligands as vaccine adjuvants for inactivated AIV chicken vaccines.

<table>
<thead>
<tr>
<th>TLR Ligand</th>
<th>TLR</th>
<th>Dose</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly I:C</td>
<td>TLR3</td>
<td>50 μg</td>
<td>-Increased mucosal IgA titers against H5N1 virus</td>
<td>(Liang et al., 2013)</td>
</tr>
<tr>
<td>CpG ODN 2006</td>
<td>TLR21</td>
<td>50 μg</td>
<td>-Increased HI (antibody) Ab titers against H5N1 virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Increased serum IgY titers against H5N1 virus</td>
<td></td>
</tr>
<tr>
<td>Flagellin</td>
<td>TLR5</td>
<td>2 μg</td>
<td>-Increased serum HI and IgY titers against H4N6 virus</td>
<td>(St Paul et al., 2014b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Decreased H4N6 virus shedding after challenge</td>
<td></td>
</tr>
<tr>
<td>Pam3CSK4</td>
<td>TLR2</td>
<td>100 μg</td>
<td>-Increased lachrymal IgA titers against H4N6 virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Decreased H4N6 virus shedding after challenge</td>
<td></td>
</tr>
<tr>
<td>CpG ODN 2007</td>
<td>TLR21</td>
<td>10 μg</td>
<td>-Increased serum HI Ab titers against H4N6 virus</td>
<td>(St. Paul et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Combination with 100 μg Poly I:C enhanced HI Ab titers against H4N6 virus</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>2 μg</td>
<td>-Increased HI and neutralization Ab titers against H9N2 virus</td>
<td>(Singh et al., 2015)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Significantly greater Ab titers induced compared to CpG 1826</td>
<td></td>
</tr>
</tbody>
</table>
**Statement of rationale**

Previous research has demonstrated the immunomodulatory effects of TLR ligands, and that TLR ligands are candidate vaccine adjuvants for inactivated AIV vaccines administered to chickens. The addition of these ligands to vaccines has resulted in greater levels of antibody-mediated immune responses against AIV vaccines in chickens. Yet to be elucidated is which of these ligands are most efficacious at inducing systemic antibody-mediated immune responses, and whether combinations of these ligands in vaccine formulations will have synergistic effects on immune response.

Also, most of the experiments performed using TLR ligands as inactivated AIV vaccine adjuvants in chickens have used a formaldehyde inactivated influenza virus. Other methods of inactivation can be used to inactivate influenza viruses. The antibody and cell-mediated immune responses to these inactivated viruses have not been quantified and compared in chickens.

Lastly, the intramuscular administration method in which these vaccines have been experimentally delivered is not practical for large numbers of chickens. *In ovo* delivery is a far more efficient method of vaccine delivery for chickens. The adjuvant capability of TLR ligands should also be tested *in ovo*.

**Statement of hypotheses**

For the work to be performed, the first hypothesis is that TLR5 and TLR21 ligands can be combined as adjuvants in a formaldehyde inactivated H9N2 vaccine resulting in elevated systemic antibody-mediated immune responses in chickens. The second hypothesis is that different methods of H9N2 AIV inactivation will yield differences in cell-mediated and antibody-mediated immune responses when administered to chickens. The final hypothesis is
that in ovo delivery of an inactivated H9N2 AIV vaccine can induce systemic antibody-mediated and cell-mediated immune responses in hatched chickens and that this immune response can be enhanced by the addition of a TLR21 ligand, CpG ODN 2007.

**Experimental approach**

**Objective 1**

Compare flagellin (TLR5 ligand) and CpG ODN 2007 (TLR21 ligand) alone and in combination as adjuvants for a formaldehyde inactivated H9N2 AIV vaccine for chickens.

*Outline of research plan*

- Vaccinate chickens IM on day 7 and day 21 post-hatch with a formaldehyde inactivated H9N2 virus along with either a low/high dose of CpG ODN 2007 or flagellin or a combination of both. Control groups will receive PBS or inactivated H9N2 AIV plus Addavax™ as adjuvant.
- Collect serum weekly following primary vaccination to compare systemic antibody-mediated immune responses by HI and ELISA assays.

**Objective 2**

Compare 3 different inactivation methods of H9N2 AIV and administer the inactivated viruses alone or with CpG ODN 2007 to chickens.

*Outline of research plan*

- Vaccinate chickens IM on day 7 and day 21 post-hatch with either a formaldehyde inactivated, BPL inactivated, or gamma-radiation inactivated H9N2 virus either alone, with CpG ODN 2007, or with Addavax™. Control groups will receive PBS.
- Collect serum weekly following primary vaccination to compare systemic antibody-mediated immune responses by HI and ELISA assays.

- Analyze cell-mediated immune responses following booster vaccination.
  - 10 days post secondary vaccination whole spleen will be collected.
  - Splenocytes will be stimulated with inactivated virus at a concentration 1 μg/ml.
  - Splenocyte gene expression will be analyzed 12 and 24 hours post stimulation.
  - Supernatant IFN-γ will be quantified 48 and 72 hours post stimulation.

Objective 3

Study an inactivated H9N2 AIV administered with or without CpG ODN 2007 as an in ovo vaccine for chickens.

Outline of research plan

- Vaccinate chicken embryos on ED18 in ovo with inactivated H9N2 AIV alone, or combined with CpG ODN 2007. Control groups will receive PBS only or inactivated H9N2 AIV plus Addavax™ as adjuvant. Chickens will receive an identical booster vaccination delivered via IM injection on day 14 post-hatch.

- Collect serum weekly following hatch to compare systemic antibody-mediated immune responses by HI and ELISA assays.

- Analyze cell-mediated immune responses following booster vaccination.
  - 10 days post secondary vaccination whole spleen will be collected.
  - Splenocytes will be stimulated with inactivated virus at a concentration 1 μg/ml.
  - Splenocyte gene expression will be analyzed 12 and 24 hours post stimulation.
  - Supernatant IFN-γ will be quantified 48 and 72 hours post stimulation.
Chapter 2

TLR5 and TLR21 Ligands as Adjuvants for a Formaldehyde Inactivated H9N2 Influenza Virus Vaccine for Chickens

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Abstract

Low pathogenic AIV infection in chickens can result in economic loss and can lead to human infection. Poultry vaccination is a tool that can be used to decrease infection and transmission of AIVs. Prior research has demonstrated that Toll-like receptor ligands (TLR) can act as vaccine adjuvants and their addition to inactivated AIV vaccines can enhance the immune response elicited in chickens. The objective of this study was to compare the adjuvant abilities of TLR5 ligand (flagellin) and TLR21 ligand (CpG ODN 2007) either alone or in combination when administered with a formaldehyde inactivated H9N2 whole virus vaccine in chickens. Chickens were administered an H9N2 vaccine containing either a single dose of CpG ODN 2007 (2 μg or 10 μg), flagellin (0.4 μg or 2 μg) or a combination of both ligands. An additional group received AddaVax™, an oil emulsion adjuvant commonly used for poultry influenza virus vaccination. Chickens were vaccinated twice and blood samples were collected weekly following the primary vaccination. Hemagglutination inhibition (HI) and IgM and IgY ELISA assays were used to assess systemic antibody-mediated immune responses. Results suggest that vaccines containing CpG ODN 2007 induce significantly greater systemic antibody responses than vaccines containing flagellin or AddaVax™. Combinations of flagellin and CpG ODN 2007 did not demonstrate an inhibitory, additive, or synergistic effect on systemic antibody-mediated immune response. This suggests there is little benefit for combining these ligands in inactivated influenza virus chicken vaccines, and CpG ODN 2007 is equally effective as a stand-alone adjuvant. Additionally, for both flagellin and CpG ODN 2007, a five-fold higher dose induced antibody-mediated immune responses that were not significantly different compared to the lesser dose. Therefore, even lower doses of flagellin and CpG ODN 2007 could be effective, but further research is needed. Future studies should examine the induction of cell-
mediated immune responses when flagellin and CpG ODN 2007 are administered alone and together as adjuvants for inactivated H9N2 AIV vaccines. Additionally, other combinations of TLR ligands should be studied as adjuvants for inactivated H9N2 influenza virus vaccines for chickens.

**Introduction**

The H9N2 subtype of avian influenza (AIV) is a low pathogenic influenza virus which is endemic in poultry in China and other Eurasian countries (Sun and Liu, 2014). Poultry in these areas are commonly infected with H9N2 AIV, and this leads to significant economic losses attributed to decreases in egg production and elevated mortality during co-infection with other respiratory pathogens (Shen et al., 2014; Sun and Liu, 2014). Although only 30 cases of H9N2 virus infection have been diagnosed in humans, there is serological evidence showing that human contact with H9N2 viruses is common in China, especially among those who work in the poultry industry (Wang and Fu, 2009; Li et al., 2017). H9N2 viruses isolated from chickens in China have also demonstrated intraspecies transmission in pigs and minks, highlighting mammal to mammal transmission capabilities (Shi et al., 2016; Yong-feng et al., 2017). In addition to risk of direct human infection with H9N2, the virus has also contributed internal genes to a triple reassortant H7N9 virus which has caused 610 human deaths since February 2013 (Pu et al., 2015; WHO, 2017). Different strategies exist to control H9N2 infection in poultry and vaccination is one potential mechanism which can facilitate this.

To be successful, vaccines must elicit potent immune response against the target pathogen; there are different ways to help induce these responses and the use of adjuvants is a common and efficient strategy. TLR ligands are a well-studied, safe and effective group of molecules which demonstrate a broad range of adjuvant capabilities (Steinhagen et al., 2011).
TLR ligands are bound by TLRs, which are a class of pattern recognition receptors expressed in endosomes and on the surface of various immune system cells (De Nardo, 2015). Chickens express 10 TLRs; this differs from human expression patterns as TLR repertoires vary between species (St. Paul et al., 2013). TLRs bind unique conserved structures present in or on microbes or pathogens, collectively called pathogen-associated molecular patterns (PAMP). Binding of PAMPs and TLRs induces intracellular signalling cascades which leads to increased expression and subsequent production of certain proteins essential for both immediate innate responses, and future adaptive immune responses (Kawai and Akira, 2007; St. Paul et al., 2013).

TLR5 in chickens recognizes bacterial flagellin and has been demonstrated to enhance systemic antibody titers in chickens when administered alongside a formaldehyde inactivated H4N6 vaccine (St Paul et al., 2014b). TLR21 in chickens recognizes oligonucleotides including CpG ODN 2007 which is a synthetic molecule that has been shown to increase antibody-mediated immune responses in chickens when combined with a formaldehyde-inactivated H9N2 vaccine (Singh et al., 2015). Both flagellin and CpG ODN 2007 have separately shown beneficial effects at a 2 μg dose as part of an intramuscular vaccine for chickens (St Paul et al., 2014b; Singh et al., 2015). What remains to be elucidated is whether these ligands are more or less effective at different doses, and whether combining these ligands in one inactivated AIV vaccine would have any effect on the immune response elicited. Therefore, we chose to investigate both flagellin and CpG ODN 2007 as stand-alone and combination adjuvants for a formaldehyde-inactivated H9N2 vaccine. The results demonstrated CpG ODN 2007 to be a superior adjuvant compared to flagellin and do not suggest that combining adjuvants has an additive or inhibitory effect on systemic antibody responses following vaccination.
Materials and Methods

Chickens

Eighty, one-day-old specific-pathogen free (SPF) chickens were purchased from the Canadian Food Inspection Agency (CFIA) (Ottawa, Canada) and were housed in the isolation facility at the Ontario Veterinary College at the University of Guelph. Sampling methods and experimental procedures were approved by the University of Guelph Animal Care Committee and were conducted with compliance to the guidelines provided by the Canadian Council on Animal Care.

Formaldehyde inactivation of H9N2 AIV

An H9N2 AIV (A/Turkey/Ontario/1/66) was propagated in 10-day-old embryonated SPF chicken eggs. Inoculated eggs were incubated at 35 °C for 72 hours before allantoic fluid was collected. Allantoic fluid was mixed well with formaldehyde (final concentration 0.02%) and was incubated at 37 °C for 72 hours while being constantly shaken. Inactivation of the virus and stability of the hemagglutinin protein was confirmed by inoculation of 10-day-old embryonated chicken eggs with the inactivated virus and hemagglutination assay respectively. Inactivated H9N2 virus was then purified by filtering the formaldehyde treated allantoic fluid with a 40 µm cell strainer to remove debris before ultra-centrifugation at 65,000 x g (SW32 Ti rotor, Optima™ L-80 XP - Beckman Coulter, Inc.) through a 20% sucrose cushion at 4°C for 2 hours. The pellet was re-suspended in TNE buffer (50 mM Tris, 140 mM NaCl, 5 mM EDTA) and the ultra-centrifugation was repeated without sucrose to wash the inactivated virus. The concentrated virus was stored at -80°C. Total virus protein concentration was determined with a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL) following manufacturer’s recommendations.
**TLR ligands**

Ultrapure flagellin (>95% purity) isolated from *S. typhimurium* and synthetic class B CpG ODN 2007 were purchased from Invivogen (San Diego, California, USA). The ligands were suspended in endotoxin free water prior to use.

**Vaccine formulation and experiment timeline**

Eighty SPF chickens were randomly divided into 10 groups, each consisting of 8 chickens. Chickens were vaccinated via intramuscular injection in the thigh muscle on days 7 and 21 post-hatch receiving 100 and 200 μl total volume respectively. All groups excluding the PBS control group received 15 μg of formaldehyde inactivated H9N2 virus per vaccination. Chickens received either a high or low dose of flagellin or CpG ODN 2007, or they received a combination of both. A total of 8 groups were required to accommodate all possible combinations. The two remaining groups were used as a negative and positive control, and consisted of a group receiving only PBS and a group receiving formaldehyde inactivated H9N2 virus and AddaVax™, a squalene-based oil in water emulsion adjuvant. Vaccine formulations are summarized for all groups in Table 2. Blood was collected from all chickens from the wing vein every 7 days beginning 7 days post primary vaccination (ppv) and ending 28 days after primary vaccination.
**Table 2. Groups for in vivo vaccination study.** Chickens were vaccinated on days 7 and 21 post-hatch.

<table>
<thead>
<tr>
<th>Group</th>
<th>Inactivated H9N2</th>
<th>Flagellin</th>
<th>CpG ODN 2007</th>
<th>AddaVax™</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. PBS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2. AddaVax™</td>
<td>15 μg</td>
<td>-</td>
<td>-</td>
<td>1/2 volume</td>
</tr>
<tr>
<td>3. Flagellin Low</td>
<td>15 μg</td>
<td>0.4 μg</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4. Flagellin High</td>
<td>15 μg</td>
<td>2 μg</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5. CpG Low</td>
<td>15 μg</td>
<td>-</td>
<td>2 μg</td>
<td>-</td>
</tr>
<tr>
<td>6. CpG High</td>
<td>15 μg</td>
<td>-</td>
<td>10 μg</td>
<td>-</td>
</tr>
<tr>
<td>7. Flagellin Low CpG Low</td>
<td>15 μg</td>
<td>0.4 μg</td>
<td>2 μg</td>
<td>-</td>
</tr>
<tr>
<td>8. Flagellin Low CpG High</td>
<td>15 μg</td>
<td>0.4 μg</td>
<td>10 μg</td>
<td>-</td>
</tr>
<tr>
<td>9. Flagellin High CpG Low</td>
<td>15 μg</td>
<td>2 μg</td>
<td>2 μg</td>
<td>-</td>
</tr>
<tr>
<td>10. Flagellin High CpG High</td>
<td>15 μg</td>
<td>2 μg</td>
<td>10 μg</td>
<td>-</td>
</tr>
</tbody>
</table>

**Hemagglutination inhibition (HI) assay**

Freshly collected blood was kept at 37°C for one hour before being centrifuged at 5000 x g for 30 minutes; the top layer of serum was then collected. For the HI assay, serum samples were serially diluted in two-fold manner in 96 well V-bottom plates. Twenty five μl (four HA units) of H9N2 AIV was then added to each well. The plate was shaken for 30 seconds, covered and then incubated for 45 minutes at room temperature. Then 50 μl of 0.5 % chicken red blood cells (1% FBS) was added to each well and the plates were again shaken, covered and incubated for 45 minutes at room temperature. The HI titer was expressed as the reciprocal of the greatest dilution to show inhibition of red blood cell agglutination (log2 scale). Rates of seroconversion and seroprotection, defined as post-vaccination HI titers that increase by 4-fold or greater, and HI titers > 40, respectively (Singh et al., 2015), were calculated using HI titers.
Enzyme linked immunosorbent assay (ELISA)

Serum samples were analyzed for the presence of anti-H9N2 virus IgY and IgM by ELISA. Maxisorp flat bottom 96 well plates were coated with 800 ng of heat killed H9N2 AIV in 100 μl per well and incubated overnight at 4°C. Plates were washed three times with 200 μl wash buffer (0.05 % Tween 20 in PBS) and then blocked with 100 μl fish skin gelatin (0.25% final concentration in wash buffer) for one hour followed by the addition of 100 μl of diluted chicken serum. After one hour of incubation plates were washed three times and 100 μl of goat anti-chicken IgY or IgM antibody conjugated to horseradish peroxidase (1:5000) was added (Bethyl Laboratories, Montgomery, Texas). One hour later plates were washed three times and horseradish peroxidase substrate (ABTS peroxidase substrate system, Kirkegaard and Perry Laboratories Gaithersburg, Maryland, USA) was added. Colour development was stopped after 20 minutes with 1% SDS and absorbance at 405 nm was determined using an ELISA plate reader (Bio-Tek Instruments, Winooski, Vermont USA). Relative antibody titer was calculated in relation to a serially diluted high titer serum sample that was used on every plate using methods described previously (Sacks et al., 1988).

Statistical analysis

Group means for antibody titers, IFN-γ concentration, and relative gene expression were compared using Duncan’s Multiple Range Test using R© software and differences in means were considered significant if p < 0.05

Results

Seroconversion, seroprotection and HI antibody titer

Serum was collected weekly following primary vaccination on day 7 post-hatch. In total,
this resulted in 4 time points of serum collection, 2 of which occurred after the secondary vaccination, which was administered 21 days post-hatch and 14 days ppv. All vaccine formulations induced significantly elevated HI antibody titers against H9N2 relative to the PBS control group at all time points post-vaccination. Seroconversion rate was calculated for all groups on day 7 and 14 ppv. On day 7 post-vaccination, the group that received a 2 μg dose of CpG ODN 2007 in addition to one group that received a combination of adjuvants displayed 100% seroconversion, double what was observed in the group receiving AddaVax™ (Table 3). By day 14 ppv only the groups that received CpG ODN 2007 either alone or with flagellin demonstrated 100% seroconversion (Table 3). Seroprotection rate was also calculated for all chickens on day 21 and 28 ppv (day 7 and 14 post-secondary vaccination). On both day 21 and 28 ppv all chickens that received CpG ODN 2007 alone or in combination demonstrated 100% seroprotection; the group that received 0.4 μg of flagellin also demonstrated 100% seroprotection on both days. The group that received the AddaVax™ adjuvant displayed seroprotection rates of 25% and 75% on days 21 and 28 ppv respectively (Table 3).

Mean serum HI titers against H9N2 virus were also compared between groups for all 4 time points ppv. The groups of chickens that received CpG ODN 2007 alone (2 or 10 μg) demonstrated significantly greater HI titers than chickens receiving either dose of flagellin (0.4 or 2 μg) alone or AddaVax™ on day 14 and 28 ppv ($p < 0.05$) (Fig 1b and 1d). On day 14 ppv, all groups that received CpG ODN 2007 alone or in combination demonstrated significantly greater serum HI titers than groups that received flagellin ($p < 0.01$) or AddaVax™ ($p < 0.05$) (Fig 1b). This trend was also observed on day 28 ppv except for 2 groups that received different combinations of CpG ODN 2007 and flagellin. Combining adjuvants had no significant additive or synergistic effects on HI titers compared to CpG ODN 2007 alone; on day 14 and 21 ppv,
separate individual groups which received a combination of adjuvants had significantly lower HI titers compared to the group that received a 10 μg dose of CpG ODN 2007 alone as adjuvant \((p < 0.05)\) (Fig 1b and 1c). For both flagellin and CpG ODN 2007, a 5-fold difference in dose when administered alone did not result in any significant differences in mean serum HI antibody titers (Fig 1a, 1b, 1c and 1d).

**Serum Ig\(Y\) and Ig\(M\) antibody titers**

After analyzing HI titers, ELISA was performed to gain a better understanding of the isotype makeup of serum antibody responses. Relative Ig\(M\) and Ig\(Y\) titers were calculated referencing a serially diluted control sample for serum samples collected on days 7, 14, 21 and 28 ppv. On day 7 ppv the group that received a 2 μg dose of CpG ODN 2007 had significantly greater titers of Ig\(M\) compared to groups receiving flagellin alone or AddaVax\(^{TM}\) \((p < 0.05)\) (Fig 2a). On day 21 ppv all groups that received CpG ODN 2007 either alone or combined with flagellin demonstrated significantly greater serum Ig\(M\) titers compared to groups that received flagellin alone or AddaVax\(^{TM}\) \((p < 0.05)\) (Fig 2c). Also, on day 14 ppv, all groups which received CpG ODN 2007 alone or in combination had significantly higher titers of serum Ig\(M\) antibodies than groups which received flagellin alone \((p < 0.05)\) (Fig 2b). Serum Ig\(M\) titers peaked on day 21 ppv for all groups and by day 28 ppv Ig\(M\) titers began to decrease.

Relative serum Ig\(Y\) titers were also determined for all groups on days 7, 14, 21 and 28 ppv. On days 21 and 28 ppv all groups that received CpG ODN 2007 either alone or in combination with flagellin demonstrated mean serum Ig\(Y\) titers which were significantly higher than groups that received flagellin alone or AddaVax\(^{TM}\) \((p < 0.05)\) (Fig 3c and 3d); a similar trend was observed on day 14 ppv as well, but Ig\(Y\) titers from groups that received CpG ODN 2007 were only significantly greater than the groups than received flagellin alone and not
AddaVax™ \( (p < 0.05) \) (Fig 3b). The combination of CpG ODN 2007 and flagellin had no consistent effects on serum IgY titers when compared to CpG ODN 2007 alone; the only significant difference noted occurred on day 28 ppv between a group which received a combination of adjuvants and the group which received a 10 μg dose of CpG ODN 2007 as adjuvant \( (p < 0.05) \) (Fig 3d). Again, for both CpG ODN 2007 and flagellin there were no significant differences observed in average serum IgY titers between low and high doses at any time points.

**Discussion**

In the present study, we demonstrated the adjuvant capabilities of TLR5 and TLR21 ligands, flagellin and CpG ODN 2007, respectively. Specifically, we examined systemic antibody-mediated immune responses against an H9N2 virus when these ligands were administered as adjuvants alongside a formaldehyde inactivated H9N2 virus given to chickens. Previous work has demonstrated both of these ligands to be effective at enhancing systemic antibody-mediated immune responses in chickens (St Paul et al., 2014a, 2014b; Singh et al., 2015). In addition to comparing the ligands at previously studied doses (2 μg), we also compared 5-fold different doses of each ligand to see if antibody-mediated immune responses following vaccination would be affected.

Our findings from this study suggest CpG ODN 2007 is a superior adjuvant to flagellin and AddaVax™ when considering the induction of antibody-mediated immune responses. Antibody titers against H9N2 AIV were consistently elevated in chickens which received CpG ODN 2007, either alone or combined with flagellin, when compared with AddaVax™. CpG ODN 2007 has been shown to induce superior HI antibody responses against an inactivated H9N2 vaccine when compared to Addavax™ in the past (Singh et al., 2015). Here in this study
we confirmed this finding and demonstrated that serum IgY and IgM antibody titers were also increased more by CpG ODN 2007 than AddaVax™. We also compared antibody responses generated by the addition of different doses of flagellin and CpG ODN 2007 to inactivated H9N2 vaccines. Overall, we observed that a 5-fold lower dose of both ligands was equally effective at inducing antibody-mediated immune responses as was the higher dose. When comparing the ligands together, CpG ODN 2007 was clearly superior to flagellin at inducing antibodies against the H9N2 vaccine. This finding agrees with the results of previous research showing that in chickens, CpG ODN 2006, a class B CpG ODN similar to CpG ODN 2007, induces greater HI antibody titers against an inactivated H5N1 vaccine than a TLR2/4 (Bacillus subtilis spores) and a TLR3 ligand (poly I:C) do (Liang et al., 2013). Injection of CpG ODN 2007 in chickens enhances subsequent expression of IFN-γ and major histocompatibility complex II transcripts in the spleen (St. Paul et al., 2011). Formation of adaptive immune responses in the spleen could be enhanced by the increased expression and production of these proteins which are important for the development of adaptive immune responses. Research has shown that CpG ODN 2006 induces in vitro proliferation of human naïve B cells but this was not observed for flagellin (Jiang et al., 2007). The ability to stimulate B cell proliferation could be a mechanism to explain why CpG ODN 2007 is able to induce significantly greater antibody responses compared to flagellin in the present study.

Another main question to be addressed in this study was whether combining CpG ODN 2007 and flagellin in an inactivated H9N2 AIV vaccine would have any effects on the induction of antibody-mediated immune responses. There were 4 combinations of these ligands to cover potential differences attributed to dose. Flagellin is recognized by TLR5 which is present on the surface of cells and CpG ODN 2007 is recognized by TLR21, which is present in endosomes
(Keestra et al., 2013). After ligand recognition, both receptors utilize the intracellular adaptor protein MyD88 to eventually activate the transcription factor NF-κB, which leads to rapid innate responses and future adaptive immune responses (Kawai and Akira, 2007; St. Paul et al., 2013). We hypothesized that combining these ligands may have some level of additive or possibly synergistic effect on antibody-mediated immune responses. However, the results of the study did not provide convincing evidence for additive, synergistic or inhibitory effect of combining these ligands on antibody-mediated immune responses. This could possibly be due to the similarities in signalling pathways these ligands share once bound to their cognate receptor. It has however been shown that in vitro co-stimulation of human peripheral blood mononuclear cells (PBMC) with flagellin and CpG leads to a synergistic increase in production of pro-inflammatory cytokines, IL-6 and IL-1β (Timmermans et al., 2013), and a significant increase in production of IFN-γ (Merlo et al., 2007). In vitro experiments with chicken PBMCs have demonstrated a synergistic relationship between TLR3 and TLR21 stimulation, specifically co-stimulation of chicken monocytes with CpG ODN and poly I:C synergistically increased production of nitric oxide and expression of inducible nitric oxide synthase compared to stimulation with either ligand alone (He et al., 2011). Additionally, co-stimulation of chicken monocytes in vitro with these ligands synergistically upregulated the expression of IFN-γ (He et al., 2012). CpG ODN and poly I:C seem to synergistically stimulate cells of the chicken immune system. This could be explained by the usage of separate signalling adaptor proteins following receptor stimulation, MyD88 for TLR21 and TRIF for TLR3. This relationship suggests these ligands could be an effective adjuvant combination for chicken vaccines. Nevertheless, vaccination experiments in chickens using an inactivated H4N6 AIV vaccine have shown that combining CpG ODN 2007 and poly I:C as adjuvants does not lead to synergistic or additive increases in antibody-mediated
immune responses compared to administering either ligand alone (St Paul et al., 2014a). The combination of poly I:C and CpG ODN 2007 should still be studied in chickens as an adjuvant combination for inactivated H9N2 AIV vaccines.

In conclusion, we have demonstrated that CpG ODN 2007 is more effective than flagellin at induction of antibody-mediated immune responses against an inactivated H9N2 AIV vaccine in chickens. It was also shown that a 2 μg dose could exert these effects equally compared to a 10 μg dose, and that combining CpG ODN 2007 with flagellin did not have any additive, synergistic or inhibitory effects on antibody responses. Future experiments should examine the effects CpG ODN 2007 has on immune response when combined with H9N2 virus vaccines using different inactivation methods.
Table 3. Summary of seroconversion and seroprotection rates following primary vaccination. Chickens were vaccinated on day 7 and 21 post-hatch.

<table>
<thead>
<tr>
<th>Group</th>
<th>Seroconversion Rate (%)</th>
<th>Seroprotection Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7 PPV</td>
<td>Day 14 PPV</td>
</tr>
<tr>
<td>1. PBS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. AddaVax™</td>
<td>50</td>
<td>75</td>
</tr>
<tr>
<td>3. Flagellin Low</td>
<td>75</td>
<td>75</td>
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<tr>
<td>4. Flagellin High</td>
<td>50</td>
<td>62.5</td>
</tr>
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<td>5. CpG Low</td>
<td>100</td>
<td>100</td>
</tr>
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<td>6. CpG High</td>
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<td>100</td>
</tr>
<tr>
<td>7. Flagellin Low CpG Low</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>8. Flagellin Low CpG High</td>
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<td>100</td>
</tr>
<tr>
<td>9. Flagellin High CpG Low</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>10. Flagellin High CpG High</td>
<td>87.5</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 1. Serum HI antibody titers against H9N2 AIV

Average serum HI antibody titers of all groups from (A) day 7 (B) day 14 (C) day 21 and (D) day 28 post primary vaccination. Secondary vaccination occurred on day 14 post primary vaccination immediately following blood collection. Chickens were vaccinated with 15 μg of formaldehyde inactivated H9N2 AIV along with either AddaVax™, a low or high dose of flagellin or CpG ODN 2007 alone, or a combination of flagellin and CpG ODN 2007. One group received just PBS as a negative control. Group means which share the same letter do not differ significantly. Standard error of the mean is indicated with error bars. Data was analyzed in R using Duncan’s Multiple Range Test and differences in means were considered significant if $p < 0.05$. 


Average serum IgM antibody titers of all groups from (A) day 7 (B) day 14 (C) day 21 and (D) day 28 post primary vaccination. Secondary vaccination occurred on day 14 post vaccination immediately following blood collection. Chickens were vaccinated with 15 μg of formaldehyde inactivated H9N2 AIV along with either AddaVax™, a low or high dose of flagellin or CpG ODN 2007 alone, or a combination of flagellin and CpG ODN 2007. One group received just PBS as a negative control. IgM titer of the PBS group was removed from all figures as it was a measure of background. Group means which share the same letter do not differ significantly. Standard error of the mean is indicated with error bars. Data was analyzed in R using Duncan’s Multiple Range Test and differences in means were considered significant if p < 0.05.
Figure 3. Serum IgY antibody titers against H9N2 AIV

Average serum IgY antibody titers of all groups from (A) day 7 (B) day 14 (C) day 21 and (D) day 28 post primary vaccination. Secondary vaccination occurred on day 14 post vaccination immediately following blood collection. Chickens were vaccinated with 15 μg of formaldehyde inactivated H9N2 AIV along with either AddaVax™, a low or high dose of flagellin or CpG ODN 2007 alone, or a combination of flagellin and CpG ODN 2007. One group received just PBS as a negative control. IgY titer of the PBS group was removed from all figures as it was a measure of background. Group means which share the same letter do not differ significantly. Standard error of the mean is indicated with error bars. Data was analyzed in R using Duncan’s Multiple Range Test and differences in means were considered significant if p < 0.05.
Chapter 3

Examination of the Effects of Virus Inactivation Methods on the Induction of Antibody- and Cell-Mediated Immune Responses Against Whole Inactivated H9N2 AIV Vaccines in Chickens

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Abstract

Several types of AIV vaccines exist, including live-attenuated, vectored, and whole inactivated virus (WIV) vaccines. Inactivated vaccines offer some advantages compared to other types of vaccine, including ease of production and lack of ability to revert to a virulent state. However, WIV are poorly immunogenic, especially when these vaccines are delivered to mucosal surfaces. There are several factors that contribute to immunogenicity of vaccines, one of which is the method used to inactivate viruses. There are several methods for producing influenza WIVs, including formaldehyde, which is a chemical that affects protein structures leading to virus inactivation. Other methods include treatment with the chemical beta-propiolactone (BPL) and the application of gamma radiation, both of which have less effects on protein structures compared to formaldehyde, and instead alter nucleic acids in the virion. In this study, we sought to determine the effect of the above inactivation methods on immunogenicity of AIV vaccines. To this end, chickens were vaccinated with three different H9N2 WIVs using formaldehyde, BPL, and gamma radiation for inactivation. In addition to administering these three WIVs alone as vaccines, we also included CpG ODN 2007, a synthetic ligand recognized by TLR21 in chickens, as an adjuvant for each WIV. Subsequently, antibody- and cell-mediated immune responses were measured following vaccination. Antibody-mediated immune responses were increased in chickens which received the BPL WIV compared to the formaldehyde WIV. CpG ODN 2007 was found to significantly increase antibody responses for each WIV compared to WIV alone. Furthermore, we observed the presence of cell-mediated immune responses in chickens which received the BPL WIV combined with CpG ODN 2007. Based on our results, the BPL WIV + CpG ODN 2007 combination was the most effective vaccine in this study at
inducing adaptive immune responses against H9N2 AIV. Future studies could attempt to characterize mucosal adaptive immune responses to these vaccines.

**Introduction**

Low pathogenic avian influenza viruses (LPAIV) are a class of AIV which commonly infect wild birds without causing any signs of clinical disease (van Dijk et al., 2018). H9N2 AIV is a LPAIV which has become a concern for economical and health related reasons. H9N2 virus infection in chickens can decrease egg production, and co-infection with other infectious pathogens can result in immunosuppression and pathological changes (Shin et al., 2016; Gu et al., 2017). There is strong evidence of human exposure to the H9N2 virus among poultry workers in certain regions of the world including China and India (Wang and Fu, 2009; Pawar et al., 2012), although not many cases of infection are reported. H9N2 AIV is also responsible for contributing its internal genes to a reassortant H7N9 virus which has demonstrated a 40% mortality rate in human cases in China (Zhu et al., 2016).

Vaccination is a potential strategy that can be used to control H9N2 infection in poultry. Many types of influenza vaccines exist, including live-attenuated vaccines, subunit vaccines, and inactivated vaccines. Whole-inactivated virus (WIV) vaccines for influenza were first explored in the 1940’s, when formaldehyde and other compounds were used to inactivate influenza viruses (Krammer and Palese, 2015). Since then, many different ways to produce influenza WIV vaccines have emerged, including other chemical treatments or the application of different forms of electromagnetic radiation (Nunnally et al., 2015).

Two common chemicals used to produce influenza WIVs include formaldehyde and beta-propiolactone (BPL). As an aldehyde, formaldehyde reacts with protein structures, crosslinking
various amino acids. This in turn affects the function of proteins and can lead to a loss of virus infectivity (Pawar et al., 2015). Altering protein structures is an effective way to induce virus inactivation, however, this can change the outcome of the elicited immune response following vaccination due to changes on epitopes of immunogenic proteins, such as the influenza hemagglutinin (HA) protein (Pawar et al., 2015). BPL is an electrophilic compound which reacts with nucleic acids, mainly with the nucleotides guanosine and adenosine (Nunnally et al., 2015). These reactions alter the structure of nucleic acids inducing changes such as strand breaks and improper linkages between nucleic acids and protein, and between nucleic acids and other nucleic acids (Pawar et al., 2015). Although nucleic acids are the main target of BPL, there is evidence demonstrating that BPL affects protein structures of influenza virus (She et al., 2013). Also, it has been shown that BPL inactivated influenza viruses have a diminished ability to fuse with lipid membranes due to changes in the HA protein structure (Desbat et al., 2011).

Nevertheless, formaldehyde inactivated influenza viruses have also been shown to lack the ability to fuse with lipid membranes (Geeraedts et al., 2012).

Another method used to inactivate viruses is by the application of various forms of electromagnetic radiation. Inactivation is achieved through direct effects on nucleic acids, resulting in strand breaks, linkages, and nucleotide damage (Alsharifi and Müllbacher, 2010). Initial reports on using gamma radiation to inactivate pathogens stated that a complete loss of influenza infectivity can be achieved after application of 0.65 kiloGrays (kGy) of gamma radiation, and this must be increased to 200 kGy before the HA protein structure is compromised (International Atomic Energy Agency, 1973). This makes gamma radiation an effective way to inactivate influenza viruses without altering potentially immunogenic proteins in the process. In the present study, we chose to compare three different methods for inactivation of H9N2 AIV,
namely formaldehyde, BPL, and gamma radiation. Immunogenicity of these preparations was assessed in a series of in vivo studies. In addition to the type of vaccine administered, vaccine adjuvants can also affect the immune response after vaccination. CpG ODN 2007 is a synthetic oligodeoxynucleotide recognized by TLR2 in chickens that has been shown to be an effective adjuvant when added to inactivated influenza virus chicken vaccines (St Paul et al., 2014a; Singh et al., 2015). For this reason, CpG ODN 2007 was also combined with each of the H9N2 WIVs (formaldehyde, BPL and gamma radiation) to examine its effects on antibody- and cell-mediated immune responses following vaccination in chickens with different H9N2 WIVs.

Material and Methods

Chickens

One-hundred-thirty-one-day-old SPF chickens were purchased from the CFIA (Ottawa, Canada). The chickens were kept in the isolation facility at the Ontario Veterinary College at the University of Guelph in Ontario Canada. The chickens were kept for 42 days and during this time they were sampled and treated using protocols which were approved by the University of Guelph Animal Care Committee and were conducted with compliance to the guidelines provided by the Canadian Council on Animal Care.

Inactivation of H9N2 AIV

H9N2 AIV (A/Turkey/Ontario/1/66) was propagated in SPF embryonated-chicken eggs which had been incubating for 10 days at 37°C. Virus rich allantoic fluid was harvested after 72 hours of incubation at 35°C. For formaldehyde inactivation, allantoic fluid was mixed with formaldehyde to attain a final concentration of 0.02% formaldehyde before being incubated on a shaker at 180 rpm for 72 hours at 37°C. BPL inactivation was carried out using a previously
described protocol (Pawar et al., 2015). Briefly, allantoic fluid was mixed with 10% BPL diluted in PBS to obtain a final concentration of 0.1% BPL. This mixture was incubated for 16 hours at 4°C and then 37°C for 2 hours to hydrolyze remaining BPL. Formaldehyde and BPL treated allantoic fluid were then ultra-centrifuged at 23,000 rpm for 2 hours at 4°C. Formaldehyde inactivated H9N2 was centrifuged again to wash residual formaldehyde. Both inactivated virus pellets were re-suspended in TNE buffer (50 mM Tris, 140 mM NaCl, 5 mM EDTA). For gamma-radiation of H9N2, virus rich allantoic fluid was first concentrated by ultracentrifugation at 65,000 x g for 2 hours at 4°C. The virus pellet was resuspended in TNE buffer and then frozen at -80°C before being lyophilized. Lyophilized concentrated H9N2 was then subjected to 12.5 kGy of gamma-radiation at the Southern Ontario Centre for Atmospheric Aerosol Research at the University of Toronto. All 3 inactivated viruses were stored at -80°C. Just before vaccination, the gamma-radiated lyophilized virus was resuspended in TNE buffer. Total protein concentration was determined for each inactivated H9N2 virus vaccine using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL) following manufacturer recommendations.

CpG ODN 2007

CpG ODN 2007 was purchased from Invivogen (San Diego, California, USA). Prior to vaccination, CpG ODN 2007 was resuspended in sterile PBS.

Vaccine production and experimental outline

Chickens were divided into 10 groups summarized in Table 4. The abbreviated group names in Table 4 will be used to describe the vaccine which that group of chickens received. On day 7 post-hatch all chickens were vaccinated via intramuscular injection in the thigh muscle with 15 μg of one of the three WIVs. WIVs were administered alone, or in combination with an oil-emulsion adjuvant (AddaVax™) or 2 μg of CpG ODN 2007. On day 21 post-hatch the
chickens received a second vaccine dose. One group received PBS and served as a negative control group. Serum was collected weekly and spleens were harvested 10 days after the second vaccination.

Table 4. Vaccination groups. All birds were vaccinated on day 7 and 21 post-hatch.

<table>
<thead>
<tr>
<th>Group</th>
<th>Inactivated Virus</th>
<th>Adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. PBS</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2. Form</td>
<td>15 ug formaldehyde inactivated H9N2</td>
<td>None</td>
</tr>
<tr>
<td>3. Form + Add</td>
<td>15 ug formaldehyde inactivated H9N2</td>
<td>AddaVax™</td>
</tr>
<tr>
<td>4. Form + CpG</td>
<td>15 ug formaldehyde inactivated H9N2</td>
<td>2 ug CpG ODN 2007</td>
</tr>
<tr>
<td>5. BPL</td>
<td>15 ug BPL inactivated H9N2</td>
<td>None</td>
</tr>
<tr>
<td>6. BPL + Add</td>
<td>15 ug BPL inactivated H9N2</td>
<td>AddaVax™</td>
</tr>
<tr>
<td>7. BPL + CpG</td>
<td>15 ug BPL inactivated H9N2</td>
<td>2 ug CpG ODN 2007</td>
</tr>
<tr>
<td>8. Gamma</td>
<td>15 ug gamma radiation inactivated H9N2</td>
<td>None</td>
</tr>
<tr>
<td>9. Gamma + Add</td>
<td>15 ug gamma radiation inactivated H9N2</td>
<td>AddaVax™</td>
</tr>
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Hemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA)

Blood was collected from the wing vein and kept at 37°C for one hour before being centrifuged at 5000 g for 30 minutes at room temperature. Serum was then collected from the tubes and used for HI and ELISA assays. For the HI assay, 96-well v-bottom plates were used to prepare 2-fold serial dilutions of serum in PBS. 4 HA units of H9N2 in 25 μl of PBS was then added to each well and the plate was shaken, covered, and incubated for 45 minutes at room temperature. Then 50 μl of a 0.5% chicken red blood cell suspension (1% FBS) was added to each well, shaken, and incubated until red blood cells had settled in control wells that did not contain H9N2. The HI titer was represented as Log₂ of the reciprocal of the last dilution which
demonstrated inhibition of red blood cell agglutination. For the ELISA, 96-well Maxisorp flat bottom plates were coated overnight at 4°C with 800 ng of heat-killed H9N2 in 100 μl per well. Plates were washed 3 times with wash buffer (0.05% Tween 20 in PBS) and blocked for 1 hour at room temperature with blocking buffer (0.25% fish skin gelatin in wash buffer (Sigma-Aldrich)). Next, diluted serum samples were added to each well and incubated at room temperature for 1 hour. Plates were washed 3 times and 100 μl of goat-anti chicken IgY or IgM conjugated to horseradish peroxidase (1/5000 in blocking buffer) was added to each well (Bethyl Laboratories, Montgomery, Texas). Plates were incubated at room temperature for 1 hour, followed by 3 washes and then 100 μl of horseradish peroxidase substrate was added (ABTS peroxidase substrate system Kirkegaard and Perry Laboratories Gaithersburg, Maryland, USA). Colour development was stopped after 20 minutes with 100 μl of 1% SDS and absorbance at 405 nm was measured using a plate reader (Bio-Tek Instruments, Winooski, Vermont USA). Titers were estimated using a previously described method [17].

Analysis of cell-mediated immune response

Ten days after secondary vaccination, spleens were collected from 5 chickens from each group. Spleens were suspended in HBSS immediately and kept on ice. Splenocyte single cell suspensions were prepared in supplemented RMPI medium and centrifuged at 400 g at 4°C on top of histopaque (Sigma). The monolayer interface between the histopaque and media was collected and resuspended in media to yield single-cell splenocyte suspensions. Splenocytes were counted using a Moxi Z mini automated cell counter (Orflo Technologies) and seeded in 48-well flat bottom plates at a density of 5x10^6 cells/500 μl per well and incubated at 41°C (5% CO₂). Cells were then stimulated with inactivated H9N2 virus (1 μg/ml); cells were stimulated with the same inactivated virus in which the corresponding chicken was vaccinated with. Cells
were collected and lysed with TRIzol™ (Invitrogen™) 12 and 24 hours post stimulation to subsequently measure gene expression. Supernatant was collected 48 and 72 hours post stimulation and IFN-γ was quantified using a chicken IFN-γ sandwich ELISA kit following manufacturer recommendations (Invitrogen™).

**Gene expression analysis**

RNA extraction and cDNA synthesis were performed as previously described (St Paul et al., 2014b). RNA was extracted from splenocytes. Primers were synthesized by Sigma Aldrich (Table 5). Relative expression of each gene was calculated relative to the housekeeping gene β-actin using software on the Light-Cycler® 480 II (Roche Diagnostics GmbH), as previously described (Taha-abdelaziz et al., 2016). Each reaction started with a pre-incubation at 95°C for 10 min, followed by 45 cycles of denaturation (95°C for 10 s), annealing (60°C for 5 s) and extension (72°C for 10 s). Subsequent melt curve analysis was performed by heating to 95°C for 10 s, cooling to 65°C for 1 min, and heating to 97°C.

**Table 5. Primer sequences used for real time PCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-Actin</td>
<td>F 5’ – CAACACAGTGCTGTCTGGTGTA - 3’</td>
<td>(Brisbin et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>R 5’ – ATCGTACTCCTGCTTGCTGATCC - 3’</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>F 5’ – ACACTGAAAGTCAAAGCCGCACA - 3’</td>
<td>(Brisbin et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>R 5’ – AGTCGTTCCATCGGGAGGTTGGC - 3’</td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>F 5’ – TGCAGTTACCTGGGAGAAGTGGT - 3’</td>
<td>(Yitbarek et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>R 5’ – ACTTCCGGTGTGATTAGACCGGT - 3’</td>
<td></td>
</tr>
</tbody>
</table>
Statistical analysis

Group means for antibody titers, IFN-γ concentration, and relative gene expression were compared using Duncan’s Multiple Range Test using R© software and differences in means were considered significant if p < 0.05.

Results

Systemic antibody-mediated immune responses

Serum was collected weekly following the primary vaccination until 28 days post primary vaccination (ppv), totalling four time points to analyze systemic antibody-mediated immune responses. HI antibody titers were first analyzed from sera of vaccinated chickens. For non-adjuvanted WIVs, the Gamma vaccine induced significantly greater HI antibody titers on day 7 ppv (4-fold greater) than the BPL and Form vaccines (p < 0.05) (Fig 4a). The BPL vaccine induced significantly greater HI antibody titers compared to the Form vaccine on day 14 (4-fold greater) and 28 (2-fold greater) ppv (p < 0.05) (Fig 4b,4d). Following day 7 ppv, the BPL and Gamma vaccines did not differ significantly in their respective induction of HI antibodies. When 2 µg of CpG ODN 2007 was included with any of the WIVs, it led to significant increases in HI antibody titers at various times ppv when compared to the WIVs administered alone or with Addavax™ (p < 0.05). Excluding day 14 ppv, all vaccines that contained CpG ODN 2007 (Form + CpG, BPL + CpG, and Gamma + CpG) induced the highest HI antibody titers relative to their counterpart WIV vaccines (no adjuvant or AddaVax™). Generally, (excluding day 14 ppv) there were no significant differences in HI antibody titer so long as 2 µg of CpG ODN 2007 was included in the vaccine formulation (p < 0.05) (Fig 4a-d).
Interestingly, serum IgY antibody titers resembled HI antibody titers throughout the experiment. The Gamma vaccine induced significantly greater IgY antibody titers than the Form and Form + Add vaccines (23-fold and 6-fold greater, respectively) on day 7 ppv (p < 0.01), while the BPL vaccine induced higher IgY antibody titers than the Gamma vaccine on day 14, 21 and 28 ppv, and significantly higher IgY antibody titers than the Form vaccine on all time points ppv (p < 0.05) (Fig 5a-d). On day 21 and 28 ppv (day 7 and 14 post-secondary vaccination), serum IgY antibody titers were increased for all WIV vaccines when CpG ODN 2007 was included, relative to unadjuvanted or Addavax™ adjuvanted vaccines (Fig 5c, 5d). This difference was significant (p < 0.05) for all groups except for the BPL WIV vaccine on day 21 ppv, as the BPL + CpG vaccine only induced significantly greater serum IgY antibody titers relative to the BPL vaccine (p < 0.05) (Fig 5c).

Finally, we checked serum IgM antibody titers following vaccination. The Gamma and BPL vaccines induced significantly greater IgM antibody titers (11-fold and 6-fold greater, respectively) relative to the Form vaccine on day 7 ppv (p < 0.01) (Fig 6a), while the BPL vaccine induced significantly greater IgM antibody titers (5-fold and 3-fold greater, respectively) relative to the Form and Gamma vaccines on day 14 ppv (p < 0.05) (Fig 6b). On day 21 ppv, all vaccines which contained CpG ODN 2007 induced significantly greater IgM antibody titers compared to nonadjuvanted or AddaVax™ adjuvanted vaccines (p < 0.05), with one minor exception (Form + CpG and Form) (Fig 6c). By day 28 ppv, IgM antibody titers regressed and only two groups (Form + CpG and Form) displayed titers significantly greater than non-vaccinated chickens (p < 0.05) (Fig 6d).
Cell-mediated immune responses

To analyze cell-mediated immune responses, chickens were euthanized 10 days post-secondary vaccination, and spleens were harvested. Splenocytes were re-stimulated in vitro with either formaldehyde, BPL, or gamma radiation inactivated H9N2 WIVs, using the same WIV that was used to vaccinate chickens. IFN-γ production and IFN-γ and IL-2 gene expression were quantified. IFN-γ production in splenocytes from vaccinated chickens was elevated relative to non-vaccinated stimulated splenocytes (Fig 7a-f). However, the only significant difference in IFN-γ production occurred 72 hours post stimulation (p < 0.05), as splenocytes from chickens vaccinated with the BPL + CpG vaccine produced significantly more IFN-γ than splenocytes from chickens that were vaccinated with the PBS and BPL + Add vaccines (Fig 7d). Relative expression of IFN-γ reflected the initial observations on protein production (Fig 8a-f). At both 12 and 24 hours post-stimulation, a significant upregulation (minimum 7-fold increase and 4-fold increase, respectively) of IFN-γ expression was noted in splenocytes from chickens which received the BPL + CpG vaccine relative to splenocytes from non-vaccinated chickens or chickens which received the BPL or BPL + Add vaccines (p < 0.05) (Fig 8c, 8d). Similarly, IL-2 expression was upregulated in splenocytes from chickens which received the BPL + CpG vaccine. At 12 hours post stimulation, IL-2 expression in splenocytes from chickens which received the BPL + CpG vaccine was significantly greater than expression in splenocytes from chickens which received a control vaccine or received the BPL and BPL + Add vaccines (p < 0.05) (Fig 8c). We also observed a significant 3-fold upregulation of IL-2 expression in splenocytes from chickens which received the Form + CpG vaccine compared to splenocytes from chickens in the control group, at 24 hours post stimulation (p < 0.05) (Fig 9b). We did not observe any other significant changes in IL-2 expression (Fig 9a, 9d-f).
Discussion

In the present study, we have formulated three different types of H9N2 WIVs and administered them to chickens intramuscularly in order to compare their immunogenicity. We chose to use formaldehyde, BPL, and gamma radiation to inactivate H9N2 AIV due to their unique mechanisms of virus inactivation.

Antibody-mediated immune responses are crucial for defense against influenza virus infection. Antibodies curb influenza virus replication by binding to virus proteins which in turn can inhibit their function (Han and Marasco, 2011). We initially noted that the Gamma vaccine induced superior anti-H9N2 antibody titers compared to the Form and BPL vaccines. Following the first time point it became evident that the BPL vaccine was equal if not greater than the Gamma vaccine, and both were superior to the Form vaccine at inducing antibody-mediated immune responses. Formaldehyde can alter immunogenic proteins of influenza virus, in particular the HA protein, during inactivation (Nunnally et al., 2015); this likely influenced antibody-mediated immune responses after vaccination. In a study comparing influenza inactivation strategies, formaldehyde inactivation decreased the HA unit titer of an H9N2 virus by a factor of 4, while BPL treatment did not alter the HA unit titer at the same chemical concentration (Pawar et al., 2015). Both BPL and gamma radiation are thought to have minimal effect on protein structure, and in the current study, there were no consistent differences in antibody-mediated immune responses which favoured either inactivation method. However, BPL inactivation has practical and favourable characteristics compared to gamma radiation, as structural problems with gamma radiation inactivated influenza viruses have been reported after radiation of non-frozen virus preparations (David et al., 2017). The present study also highlighted the capabilities of the TLR21 ligand, CpG ODN 2007, to act as an effective adjuvant
when incorporated with all WIVs. It has been demonstrated that CpG ODN 2006 and 2007, which are both class B CpG ODNs, can enhance antibody-mediated immune responses when administered with inactivated influenza vaccines for chickens (Liang et al., 2013; St Paul et al., 2014a; Singh et al., 2015).

Cellular immune responses, mediated by CD4+ and CD8+ T-cells, are important components of the immunity against influenza (Seo and Webster, 2001). CD8+ T-cells are major effector cells and kill influenza virus infected cells which display viral proteins on major histocompatibility complex one (MHC-I) proteins, while CD4+ T-cells produce the necessary cytokines for the development of adaptive immune responses (La Gruta and Turner, 2014). To assess the presence of cell-mediated immune responses, we stimulated splenocytes from vaccinated chickens with H9N2 WIVs and examined the recall response by analyzing the expression of IFN-γ and IL-2 as well as the production of IFN-γ. We have shown here that the BPL + CpG vaccine induced a cell-mediated immune response in vaccinated chickens. However, we did not observe significant differences in IFN-γ production for any other vaccine formulation. These results are in line with a previous study comparing BPL and formaldehyde WIV vaccines in mice which showed that a BPL WIV led to significantly greater levels of influenza nucleoprotein-specific CD8+ T-cells (Budimir et al., 2012). This difference was attributed to the ability of the BPL WIV to undergo endosomal membrane fusion in professional antigen presenting cells (APC). This allows influenza proteins to enter the cytoplasm and undergo cross-presentation, a process that lead to exogenous peptides being processed and presented via the MHC-I pathway, which is essential for the activation of CD8+ T-cells. CpG ODN 2007 could have also had a role in inducing cell-mediated immune responses. In chickens, TLR21 binding of CpG ODN 2007 in APCs leads to increased gene expression and production
of proteins which are essential for T-cell activation, including co-stimulatory proteins such as CD80/86 (St. Paul et al., 2013).

In chickens the main cellular producers of IFN-γ include CD8+ T-cells as well as CD4+ T helper 1 (Th1) cells (Kapczynski, 2014). Th1 cells produce certain cytokines including IFN-γ and IL-2, which support cell-mediated immune responses (Teng et al., 2006). We detected significantly increased IL-2 expression in those groups of chickens which received CpG ODN 2007 as vaccine adjuvant (Form + CpG and BPL + CpG). This suggests that CpG ODN 2007 could have led to the activation of Th1 cells. If CD8+ T-cells were also activated after vaccination, they could then become further activated by cytokine secreting Th1 cells. Given that BPL inactivated influenza WIVs promote CD8+ T-cell activation, it is understandable that cell-mediated immune responses were induced by the BPL + CpG vaccine. However, IL-2 expression in the Form + CpG group was also elevated, but we did not observe any significant increases in IFN-γ production or expression. CpG ODN 2007 in the vaccine could have led to the activation of Th1 cells, but the formaldehyde WIV likely did not lead to the activation of CD8+ T-cells, perhaps because of the loss of WIV membrane fusion.

In conclusion, we have demonstrated the effects that inactivation method of an H9N2 virus can have on the immune response following vaccination in chickens. The BPL WIV + CpG ODN 2007 combination was the best vaccine formulation in this study at inducing adaptive immune responses against H9N2 AIV. Future studies should examine the effects that inactivation method has on the mucosal immune response following vaccination.
Figure 4. Serum HI antibody titer against H9N2 AIV

Average serum HI ab titers from (A) day 7, (B) day 14, (C) day 21, and (D) day 28 ppv. Secondary vaccination occurred on day 14 ppv immediately following blood collection. Chickens were vaccinated with 15 μg of one of the 3 H9N2 WIVs alone or with either AddaVax™ or 2 μg of CpG ODN 2007. One group received just PBS as a negative control. Group means which share the same letter do not differ significantly. Standard error of the mean is indicated with error bars. Data was analyzed using Duncan’s Multiple Range Test and differences in means were considered significant if p < 0.05.
Figure 5. Serum IgY antibody titer against H9N2 AIV

Average serum IgY ab titers from (A) day 7, (B) day 14, (C) day 21, and (D) day 28 ppv. Secondary vaccination occurred on day 14 ppv immediately following blood collection. Chickens were vaccinated with 15 μg of one of the 3 H9N2 WIVs alone or with either AddaVax™ or 2 μg of CpG ODN 2007. One group received just PBS as a negative control. Group means which share the same letter do not differ significantly. Standard error of the mean is indicated with error bars. Data was analyzed using Duncan’s Multiple Range Test and differences in means were considered significant if p < 0.05.
Figure 6. Serum IgM antibody titer against H9N2 AIV

Average serum IgM antibody titers from (A) day 7, (B) day 14, (C) day 21, and (D) day ppv. Secondary vaccination occurred on day 14 ppv immediately following blood collection. Chickens were vaccinated with 15 μg of one of the 3 H9N2 WIVs alone or with either AddaVaxTM or 2 μg of CpG ODN 2007. One group received just PBS as a negative control. Group means which share the same letter do not differ significantly. Standard error of the mean is indicated with error bars. Data was analyzed using Duncan’s Multiple Range Test and differences in means were considered significant if p < 0.05.
Splenocytes were stimulated with 1 µg/ml of BPL, formaldehyde, or gamma-radiation inactivated H9N2 virus. Individual graphs represent groups of chickens vaccinated with either A) formaldehyde inactivated H9N2, C) BPL inactivated H9N2 or E) gamma-radiated H9N2, 48 hours after stimulation. IFN-γ in supernatant was also quantified 72 hours post stimulation for the same vaccine groups in B), D), and F). Splenocytes from chickens which received PBS acted as controls and were stimulated and average IFN-γ concentrations were compared to the vaccinated groups. Group means which share the same letter did not differ significantly. Standard error of the mean is indicated with error bars. Data was analyzed using Duncan’s Multiple Range Test and differences in means were considered significant if p < 0.05.
Splenocytes were stimulated with 1 μg/ml of BPL, formaldehyde, or gamma-radiation inactivated H9N2 virus. Individual graphs represent relative IFN-γ expression 12 hours post stimulation from groups of chickens vaccinated with either A) formaldehyde inactivated H9N2, C) BPL inactivated H9N2, or E) gamma-radiated H9N2. IFN-γ expression was also quantified after 24 hours for the same vaccine groups in B), D), and F). Splenocytes from the PBS group were stimulated and average IFN-γ expression was compared to the vaccinated groups. Group means which share the same letter did not differ significantly. Standard error of the mean is indicated with error bars. Data was analyzed using Duncan’s Multiple Range Test and differences in means were considered significant if p < 0.05.
Figure 9. IL-2 expression in splenocytes following stimulation

Splenocytes were stimulated with 1 μg/ml of BPL, formaldehyde, or gamma-radiation inactivated H9N2 virus. Individual graphs represent relative IL-2 expression 12 hours post stimulation from groups of chickens vaccinated with either A) formaldehyde inactivated H9N2, C) BPL inactivated H9N2, or E) gamma-radiated H9N2. IL-2 expression was also quantified after 24 hours for the same vaccine groups in B), D), and F). Splenocytes from the PBS group were stimulated and average IL-2 expression was compared to the vaccinated groups. Group means which share the same letter did not differ significantly. Standard error of the mean is indicated with error bars. Data was analyzed using Duncan’s Multiple Range Test and differences in means were considered significant if p < 0.05.
Chapter 4

Determining the Immunogenicity of BPL Inactivated H9N2 Virus Vaccines Administered In Ovo to Chicken Embryos

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Abstract

Infection of chickens with low pathogenicity AIV, such as H9N2 virus, culminates in decreased production and increased mortality and morbidity if co-infection with other respiratory pathogens occurs. Research has shown that chicken vaccination results in the formation of adaptive immune responses, and \textit{in ovo} vaccination is a practical option for vaccination against H9N2 AIV in chickens. Chicken embryos are already vaccinated against some viral infections such as Marek’s disease virus \textit{in ovo}. We have previously observed the induction of cell- and antibody-mediated immune responses after intramuscular (IM) administration of a beta-propiolactone (BPL) whole inactivated virus (WIV) H9N2 vaccine to chickens. In the current study, we set out to characterize the immune response in chickens against a BPL H9N2 WIV vaccine after primary vaccination \textit{in ovo} on embryonic day 18, and secondary IM vaccination on day 14 post-hatch. We also included the TLR21 ligand, CpG ODN 2007, and an oil emulsion adjuvant, AddaVax™, as adjuvants for the vaccines. Antibody mediated immune responses were observed after administering the secondary IM vaccine. Cell-mediated immune responses were observed in chickens that received the BPL H9N2 WIV combined with AddaVax™. Our results demonstrate that adaptive immune responses can be induced in chickens after a primary \textit{in ovo} vaccination and secondary IM vaccination. Future studies should examine increased doses of BPL H9N2 WIV for \textit{in ovo} administration, and periods greater than 14 days should be sampled to study the induction of systemic antibody response after \textit{in ovo} vaccination alone.

Introduction

AIV H9N2 subtype is an enveloped, negative-sense single-stranded segmented RNA virus in the family \textit{Orthomyxoviridae} that is now the most widespread AIV subtype found in poultry (Nagy et al., 2017). During infection, chickens are often free of clinical signs, but
infection can increase morbidity and mortality during co-infection with other respiratory pathogens (Hassan et al., 2017). Poultry vaccination against H9N2 virus is a possible way to decrease infection and transmission among birds. *In ovo* administration is a potential vaccination route for chickens, as technology already exists that can facilitate mass vaccinations, and commercial *in ovo* vaccines against various pathogens including MDV already exist (Avakian et al., 2007).

*In ovo* vaccines are typically delivered on embryonic day 18 (ED18) to the amniotic cavity, during this time the embryo is typically imbibing amniotic fluid (Avakian et al., 2007). Early research demonstrated that administration of an inactivated H5N9 AIV vaccine *in ovo* resulted in post-hatch (ph) seroconversion and induction of immune responses in hatched chicks against H5N9 virus (Stone et al., 1997). Various other types of AIV vaccines have been studied for *in ovo* administration, including; non-replicating adenovirus vectors expressing hemagglutinin (HA) proteins (Toro et al., 2007; Toro and Tang, 2009; Mesonero et al., 2011), attenuated vaccines (Cai et al., 2011), and recombinant attenuated vaccines (Steel et al., 2008). Attenuated viral vaccines generally induce strong immune responses, however there is always the risk of mutation back to the virulent form (Yadav et al., 2013). Vector virus vaccines for influenza virus can lead to the induction of immune responses against the HA protein, however, internal influenza proteins in the virion are absent in the vaccine, and peptides from these proteins are often the target of cell-mediated immune responses that can be cross-protective to other subtypes of AIV (La Gruta and Turner, 2014).

In chickens, TLR ligands have adjuvant capabilities when combined with inactivated AIV vaccines administered *in vivo* (St Paul et al., 2014a, 2014b; Singh et al., 2015). CpG ODNs have also been shown to be immunostimulatory when administered to the chicken embryo. *In
administration of CpG ODN has been shown to decrease in ovo replication of H4N6 AIV and infectious bronchitis virus (Dar et al., 2014; Barjesteh et al., 2015a), in addition to having protective effects following experimental challenge with Salmonella and infectious laryngotracheitis virus ph (MacKinnon et al., 2009; Simrika Thapa et al., 2015). In the present study, embryos were vaccinated in ovo with H9N2 beta-propiolactone (BPL) whole inactivated virus (WIV) vaccines, followed by a secondary intramuscular (IM) vaccination ph. Subsequently, cell- and antibody-mediated immune responses were quantified in chickens. CpG ODN 2007 was also included as an adjuvant in this study to determine its adjuvant effects when administered in ovo.

Materials and Methods

Eggs and hatching conditions

Sixty eight SPF eggs were purchased from the CFIA (Ottawa, Canada) and were incubated at the recommended temperature and relative humidity until hatch at Arkell Poultry Research Station (University of Guelph). After 22 days of incubation, hatched chicks (n=64) were transported to the isolation facility at the Ontario Veterinary College at the University of Guelph for the remainder of the experiment. All sampling and treatment protocols were approved by the University of Guelph Animal Care Committee and were conducted with compliance to the guidelines provided by the Canadian Council on Animal Care.

BPL inactivation of H9N2 influenza virus

H9N2 AIV (A/Turkey/Wisconsin/1/66) was propagated in SPF chicken eggs at 35 °C for 72 hours. Allantoic fluid containing H9N2 virus was harvested and viable virus was inactivated with BPL. Thirty eight parts allantoic fluid were first combined with one part 0.5 M disodium
phosphate (DSP), then 1 part of a 4% BPL solution was added in dropwise manner to 38 parts of an allantoic fluid-DSP mixture, resulting in a final concentration of 0.1% BPL. After mixing well, the allantoic fluid solution was kept on ice for 30 minutes, followed by 2 hours of incubation at 37°C to hydrolyze remaining BPL. The pH of the solution was then adjusted to ~7.4 with a 7% sodium bicarbonate. The BPL treated allantoic fluid was centrifuged on low speed (500 x g) to remove debris before being pooled and ultra-centrifuged at 65000 x g (SW32 Ti rotor, Optima™ L-80 XP - Beckman Coulter, Inc.) for 2 hours at 4 °C on top of a 30% sucrose cushion. The concentrated WIV pellet was then resuspended in TNE buffer (50 mM Tris, 140 mM NaCl, 5 mM EDTA) and total virus protein was determined using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL) following the manufacturer’s recommendations.

CpG ODN 2007

CpG ODN 2007 was purchased from Invivogen (San Diego, California, USA) and was resuspended in sterile PBS.

Experiment design and vaccine groups

On ED18 all embryos were vaccinated in ovo via the amniotic cavity. Amniotic injection totalled 100 µl and was performed using a 25-gauge needle. The experiment consisted of four groups, three of which received 15 µg of BPL inactivated H9N2 virus and one that received PBS and was a negative control group. Of the three groups that received the BPL inactivated H9N2 virus, one received it alone, one received it combined with an oil emulsion adjuvant (AddaVax™), and one received it combined with 2 µg of CpG ODN 2007. Vaccine groups are summarized in table 6 along with names that will be used to describe each vaccine group are listed. Seventeen eggs were vaccinated for each group and hatchability varied ranging from 15 –
17 hatched chicks. On day 14 post-hatch chickens received a secondary IM vaccine in the thigh muscle identical to the first vaccine in 200 µl total volume.

Table 6. Vaccination groups. All birds were vaccinated in ovo on ED18, and received an identical secondary vaccine administered in the thigh muscle on day 14 post-hatch

<table>
<thead>
<tr>
<th>Group</th>
<th>Inactivated Virus</th>
<th>Adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. PBS</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2. BPL</td>
<td>15 µg beta-propiolactone (BPL) inactivated H9N2</td>
<td>None</td>
</tr>
<tr>
<td>3. BPL + Add</td>
<td>15 µg BPL inactivated H9N2</td>
<td>Addavax™</td>
</tr>
<tr>
<td>4. BPL + CpG</td>
<td>15 µg BPL inactivated H9N2</td>
<td>2 µg CpG ODN 2007</td>
</tr>
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Serum collection and hemagglutination inhibition (HI) assay

Serum samples were collected from the wing vein of 10 chickens per group in 1.5 ml microcentrifuge tubes. Blood was kept at 37 °C for 1 hour and was then centrifuged at 5000 x g for 30 minutes. Serum collected from the tubes was used for HI and ELISA assays. For the HI assay, 2-fold serial dilutions of serum were prepared in PBS in 96-well v-bottom plates. Four HA units of H9N2 virus in 25 µl of PBS were added to each well and plates were covered, shaken, and left for 45 minutes at room temperature. Then, 50 µl of 0.5% chicken red blood cells (1 % FBS) were added to each well (total volume 100 µl per well) and plates were covered, shaken, and left for 45 minutes at room temperature. HI antibody titers were recorded as the reciprocal of the greatest dilution that inhibited red blood cell agglutination (Log₂ scale).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed for both IgY and IgM isotypes. All washes were 200 µl in volume, and any other addition to the plate was 100 µl in volume. Ninety-six well Maxisorp
plates (BioLegend®) were coated overnight at 4°C with 800 ng of heat inactivated H9N2 AIV per well. Plates were then washed with wash buffer (0.05% tween 20 in PBS) three times and blocking buffer (0.25% fish skin gelatin in wash buffer) was added for one hour. Blocking buffer was dumped and diluted serum was added to the plate for one hour of incubation at room temperature. Then the plate was then washed three times and goat-anti chicken IgY or IgM conjugated to horseradish peroxidase (1/5000 in blocking buffer) was added to each well (Bethyl Laboratories, Montgomery, Texas). After one hour of incubation, plates were washed 3 times and horseradish peroxidase substrate was added (ABTS peroxidase substrate system Kirkegaard and Perry Laboratories Gaithersburg, Maryland, USA). After 20 minutes, 1% SDS was added to each well to stop colour development. Plates were read with a plate reader (Bio-Tek Instruments, Winooski, Vermont USA) at 405 nm. Relative antibody titer was calculated in relation to a serially diluted high titer serum sample that was used on every plate using methods described by (Sacks et al., 1988).

Analysis of cell-mediated immune response

Ten days after the secondary vaccination, 5 chickens from each group were euthanized and spleens were collected and kept ice cold in HBSS. Then, spleens were crushed and filtered through 40 µm cell strainers and resuspended in supplemented RMPI medium, creating single cell suspensions. Mononuclear cells were isolated by centrifuging single cell suspensions at 400 x g on a histopaque gradient at room temperature. Mononuclear splenocytes were collected from the histopaque interface and washed 3 times in supplemented RPMI medium. Cells were counted using a Moxi Z mini automated cell counter (Orflo Technologies) and seeded at a density of 1x10^7 cells/ml in 48 well plates (400 µl/well). Cells were stimulated with 1 µg/ml of BPL inactivated H9N2 virus. At 12 and 24 hours post-stimulation, cells were collected for
analysis of cytokine gene expression and at 48 and 72 hours supernatant was collected to assess IFN-γ production in supernatant using an IFN-γ ELISA kit (Invitrogen™).

**Gene expression analysis**

RNA extraction and cDNA synthesis were performed as previously described (St Paul et al., 2014b). Primers were synthesized by Sigma Aldrich (Table 5). Relative expression of each gene was calculated relative to the housekeeping gene, chicken β-actin, using software on the Light-Cycler® 480 II (Roche Diagnostics GmbH), as previously described (Taha-abdelaziz et al., 2016). Reactions were as follows: pre-incubation at 95°C for 10 min, followed by 45 cycles of denaturation (95°C for 10 s), annealing (60°C for 5 s) and extension (72°C for 10 s). Melt curve analysis was performed by heating to cooling to 65°C for 1 min, and heating to 97°C.

**Statistical analysis**

Group means for antibody titers, IFN-γ concentration, and relative gene expression were compared using Duncan’s Multiple Range Test using R© software and differences in means were considered significant if p < 0.05.

**Results**

**Antibody-mediated immune responses**

The first evidence of antibody responses occurred on day 21 ph (day 7 post-secondary vaccination), and all chickens that received a vaccine demonstrated the presence of antibody-mediated immune responses compared to none in the PBS group. There were significant differences in HI titers between vaccinated groups on day 21 ph, as both the BPL + Add and BPL + CpG vaccines resulted in 2-fold higher HI titers compared to the BPL vaccine (p < 0.05) (Fig 10). By day 28 ph, HI titers increased for all vaccinated groups, but there were no
significant differences evident between groups (Fig 10). Serum IgY titers were also first seen on day 21 ph, although there were no significant differences in IgY titers at this time point between vaccinated groups (Fig 11a). However, on day 28 ph, serum IgY titers were significantly increased in chickens that received the BPL + Add vaccine relative to the BPL vaccine ($p < 0.01$) (Fig 11b). IgM titers were also first evident on day 21 ph. The BPL + CpG vaccine induced significantly higher IgM titers that were 3-fold and 10-fold higher than those induced by the BPL + Add and BPL vaccines, respectively ($p < 0.01$) (Fig 11c). Also, on day 21 ph the BPL + Add vaccine induced 3-fold significantly higher serum IgM titers than the BPL vaccine ($p < 0.01$) (Fig 11c). By day 28 ph, serum IgM titers had decreased dramatically, and only the chickens that received the BPL + Add vaccine demonstrated serum IgM titers that were significantly higher than the PBS control group ($p < 0.05$) (Fig 11d).

**Cell-mediated immune responses**

IFN-$\gamma$ production in supernatants of stimulated splenocytes was assessed at 48 and 72 hours post-stimulation. At both time points, significantly more IFN-$\gamma$ production (4 fold higher) was observed from splenocytes from chickens that received the BPL + Add vaccine compared to all other groups ($p < 0.05$) (Fig 12a). Gene expression analysis indicated that at 12 hours post-stimulation, there were no significant differences in IFN-$\gamma$ expression (Fig 12b). By 24 hours post-stimulation, the highest level of IFN-$\gamma$ expression occurred in splenocytes from chickens that received the BPL + Add vaccine, although this difference was not significant (Fig 12c). Lastly, expression of IL-2 was also quantified in splenocytes 12 and 24 hours post-stimulation. We observed a significant increase in expression of IL-2 in splenocytes from chickens that received the BPL + Add vaccine, relative to splenocytes from chickens that received the BPL
vaccine or PBS (2-fold and 6-fold greater, respectively) \((p < 0.05)\) (Fig 12d). At 24 hours post-stimulation there were no significant differences in IL-2 expression (Fig 12e).

**Discussion**

There is little data on *in ovo* whole inactivated virus (WIV) influenza vaccines, however our findings concerning the induction of antibody-mediated immune responses contrast with some of the previously published results. Previously, a group vaccinated ED18 embryos with BPL inactivated H5N9 AIV and observed serum HI titers in chickens by 4 weeks of age (Stone et al., 1997), however it is difficult to compare the dose of inactivated influenza virus administered between studies, due to differences in dose quantification. *In ovo* vaccine antigen dose appears to be important for the induction of antibody responses, and this relationship has been demonstrated for *in ovo* administered adenovirus vector vaccines expressing HA proteins (Toro et al., 2007; Toro and Tang, 2009; Mesonero et al., 2011). Additionally, the time for antibody responses to appear in hatched chickens following *in ovo* vaccination seems to take longer compared to vaccines administered ph in chickens. For example, in a study where chicken embryos were administered \(10^9\) infectious units of an adenovirus vector influenza vaccine, less than one-percent of chickens displayed serum HI titers by ten days ph (Toro and Tang, 2009). By 20 and 40 days ph, the percentage rose to 65 and 85 percent, respectively; notably, this dose was the highest in the study and a ten-fold lower dose displayed a similar trend but at lower magnitudes. This suggests that our 14 day time frame prior to the second vaccine could have been too short to detect antibodies induced by *in ovo* vaccination alone.

Past research has shown that CpG ODN 2007 can increase systemic antibody titers in chickens when combined with a formalin inactivated H9N2 virus vaccine administered intramuscularly (Singh et al., 2015). We have also demonstrated that CpG ODN 2007 has the
same effects on antibody response when combined with a BPL inactivated H9N2 virus vaccine (J. Astill et al., manuscript submitted). In the present study, consistent differences in serum IgY and HI titers were not evident when comparing non-adjuvanted to CpG ODN 2007-adjuvanted vaccines. Nevertheless, following the secondary IM vaccination, serum IgM titers were the highest in chickens which received vaccines with CpG ODN 2007 as an adjuvant. This further demonstrates the adjuvant capabilities of CpG ODN 2007 as an adjuvant for inactivated IM influenza vaccines.

Cell mediated-immune responses have been demonstrated to exhibit protective responses against AIV in chickens following in ovo vaccination. For example, in a study where chicken embryos were vaccinated in ovo with a recombinant attenuated H5N1 vaccine, only 30% of chickens developed antibodies to the H5 protein, yet 80% of vaccinated chickens survived a lethal challenge, compared to no survival in the control group (Steel et al., 2008). Although not experimentally demonstrated, the enhanced survival was partially attributed to cell-mediated immune responses. Our observations suggest that BPL inactivated H9N2 virus vaccines can induce cell-mediated immune responses after a primary in ovo and secondary IM vaccination. Research in mice has suggested that influenza BPL WIV vaccines induce cell-mediated immune responses, specifically CD8+ T cell responses, because of structural characteristics that BPL inactivated influenza viruses possess (Budimir et al., 2012). Using IM vaccination, our group made a similar finding in chickens (J. Astill et al., manuscript submitted), however we only observed cell-mediated immune responses when CpG ODN 2007 was combined with the BPL WIV vaccine. Strikingly, CpG ODN 2007 did not enhance cell-mediated immune responses in the present study. There is ample evidence that demonstrates that CpG ODNs are immunostimulatory when administered to chicken embryos (MacKinnon et al., 2009; Dar et al.,
2014; Barjesteh et al., 2015a; Simrika Thapa et al., 2015). Despite this, our results suggest that CpG ODN 2007 may not be suited to induce cell-mediated immune responses as an in ovo vaccine adjuvant, however different doses should be studied. The oil emulsion adjuvant used in the study, Addavax™, proved to be an effective adjuvant for in ovo vaccination, but future studies should examine this further.

In conclusion, the present study has demonstrated the immune responses induced in chickens after a primary in ovo and secondary IM vaccination with a BPL WIV H9N2 vaccine. Future studies should determine if greater doses of H9N2 WIV vaccines administered in ovo can induce serum antibody responses.
Figure 10. Serum HI antibody titers against H9N2 AIV

Average serum HI titers 21 and 28 days ph, from 10 chickens per group. Chickens were vaccinated *in ovo* on ED18 and received a second vaccination 14 days ph. Vaccines consisted of 15 µg of BPL inactivated H9N2 virus administered alone (BPL), with AddaVax™ (BPL + Add), or with 2 µg CpG ODN 2007 (BPL + CpG). One group received just PBS as a negative control (PBS). Serum was collected weekly following hatch. HI titers were first observed in serum 7 days post secondary vaccination. Group means that share the same letter did not differ significantly. Standard error of the mean is indicated with error bars. Data were analyzed using Duncan’s Multiple Range Test and differences in means were considered significant if $p < 0.05$. 
Figure 11. Serum IgY and IgM titers in serum against H9N2 AIV

Average serum IgY titers A) 21 days and B) 28 days ph and average serum IgM titers C) 21 days and D) 28 days ph, from 10 chickens per group. Chickens were vaccinated \textit{in ovo} on ED18 and received a second vaccination 14 days ph. Vaccines consisted of 15 µg of BPL inactivated H9N2 virus administered alone (BPL), with AddaVax\textsuperscript{TM} (BPL + Add), or with 2 µg CpG ODN 2007 (BPL + CpG). One group received just PBS as a negative control (PBS). Serum was collected weekly following hatch. Antibody titers were first observed in serum 7 days post secondary vaccination. Group means that share the same letter did not differ significantly. Standard error of the mean is indicated with error bars. Data were analyzed using Duncan’s Multiple Range Test and differences in means were considered significant if \( p < 0.05 \).
Figure 12. IFN-γ production in stimulated splenocyte supernatants and IFN-γ and IL-2 expression in stimulated splenocytes

Splenocytes were isolated and seeded in 48 well plates and stimulated with 1 μg/ml of BPL inactivated H9N2 virus. Supernatants were collected A) 48 and 72 hours after stimulation and supernatant IFN-γ concentrations were quantified. IFN-γ expression in splenocytes was quantified at B) 12 hours and C) 24 hours post-stimulation and IL-2 expression was quantified at D) 12 hours and E) 24 hours post-stimulation. Gene expression was quantified relative to β-actin expression. Group means that share the same letter did not differ significantly. Standard error of the mean is indicated with error bars. Data was analyzed using Duncan’s Multiple Range Test and differences in means were considered significant if $p < 0.05$. 

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Chapter 5

General Discussion

Vaccination is a potential way to control AIV infection in chickens. Decreasing chicken infection with influenza virus, especially H9N2 virus, could benefit poultry producers economically, decrease the threat of human infection with H9N2 virus and could indirectly decrease human infection with pathogenic H9N2 reassortant viruses (Pu et al., 2015). For vaccines to decrease infection and transmission in chickens, effective adaptive immune responses must be elicited. Both the antibody- and cell-mediated arms of the adaptive immune system play a role in inhibiting influenza virus infection. Antibodies that recognize and bind to specific epitopes of viral proteins act in a variety of ways to stop or slow infection. Examples include antibodies that are specific for epitopes of the HA protein that bind to sialic acid or epitopes that are involved in conformational changes that allow for membrane fusion in the endosome during early stages of infection. Additionally, antibodies that recognize the NA protein can stop NA mediated cleavage of sialic acid which is necessary for the release of new virions from infected cells (Han and Marasco, 2011). An important distinction between cell- and antibody- mediated immune responses is that only the latter can block initial infection from occurring. Nevertheless, cell-mediated immune responses are important for inhibiting ongoing influenza infection, specifically, CD8+ T cells can clear virally infected cells that display viral peptides on MHC-I proteins (van den Berg et al., 2008). Therefore, our studies were aimed at quantifying immune responses in chickens following vaccination with various vaccine formulations. In this thesis, HI and ELISA assays were used to determine anti-HA and anti-total H9N2 virus antibody titers. To study cell-mediated immune responses, spleen cells from
vaccinated chickens were stimulated with inactivated H9N2 virus antigens, and IFN-γ production was quantified along with its expression and the expression of IL-2.

Adjuvants can be described as a compound that is combined with a vaccine antigen in order to enhance the immune response directed against that antigen. TLR ligands are a well studied class of adjuvants, and their immunostimulatory properties have been illustrated in chickens (St. Paul et al., 2013). TLRs are stimulated by their cognate ligands that naturally occur as macromolecules which are associated with pathogens, referred to as PAMPS. Stimulation leads to rapid innate responses which are meant to clear the invading pathogen which initiated stimulation, however, there are further downstream effects of TLR signalling and they are highlighted by the enhancement of adaptive immune responses. The connection between TLR stimulation and adaptive immune responses is suggested to be bridged by professional APCs, as TLR stimulation in these cells leads to an increase in the production of various factors essential for the activation of T cells, such as cell surface receptors like MHC-II and CD80/86, and various immunomodulatory cytokines (St. Paul et al., 2013). For this reason, TLR ligands have been studied as adjuvants for inactivated AIV chicken vaccines. Recent work done in our laboratory has highlighted various TLR ligands as effective adjuvants, however, the TLR5 ligand, flagellin, and the TLR21 ligand, CpG ODN 2007, stand out due to the their ability to elicit elevated levels of antibody-mediated immune responses against inactivated AIV vaccines (St Paul et al., 2014b; Singh et al., 2015). For this reason, one of our objectives in this thesis was to compare flagellin and CpG ODN 2007 as adjuvants when administered with a formaldehyde inactivated H9N2 virus. In previous work in our laboratory, a 2 µg dose for both ligands was studied. Therefore, in this study a 2 µg dose in addition to 5-fold different doses were studied for both ligands, resulting in a 2 µg and 10 µg dose for CpG ODN 2007, and a 0.4 µg and 2 µg
dose for flagellin. We hypothesized that combining the ligands would have additive or synergistic effects on antibody-mediated immune responses against H9N2 virus, however, this relationship was not observed. Instead we observed that CpG ODN 2007 was superior to flagellin, and CpG ODN 2007 alone was equally effective as any combination of the two ligands. Singh et al. (2015) previously discovered that a 20 µg dose of CpG ODN 2007 was equally effective or less effective than a 2 µg dose at inducing antibodies against an inactivated H9N2 AIV vaccine. In chapter 2, evidence is provided to support this finding, as a 2 µg dose of CpG ODN 2007 was consistently able to produce serum antibody titers that were not significantly different compared to titers produced by chickens that received a 10 µg dose. This suggests that future studies could determine if even lower doses of CpG ODN 2007 are still able to effectively exert adjuvant effects for inactivated H9N2 AIV vaccines, as this could decrease the cost per dose of vaccine. The primary focus of the study described in chapter 2 was to quantify antibody-mediated immune responses. As a result, it is not clear whether these ligands are able to promote cell-mediated immune responses. St Paul et al., (2014b) demonstrated that splenocytes from chickens that received an inactivated H4N6 virus vaccine adjuvanted with flagellin proliferated significantly more than splenocytes from chickens that did not receive flagellin following in vitro re-stimulation. This suggests that flagellin might have induced cell-mediated immune responses in chickens in our study, nevertheless, the presence of cell-mediated immune responses likely does not make up for the lower antibody responses that were observed. Future studies should look at whether combinations of these ligands, or other TLR ligands, lead to the induction of cell-mediated immune responses in addition to enhancing serum antibody titers.

Typically, inactivated virus vaccines need adjuvants due to their poor immunogenicity. However, other aspects of vaccine production can be altered to enhance immune responses.
Specifically, for inactivated viral vaccines, the method used for inactivation can drastically alter the immune response elicited due to structural and functional differences of the inactivated virion. Concerning antibody-mediated immune responses to inactivated influenza vaccines, antibodies must be specific for epitopes that are present on the native virus. Influenza proteins can be altered during inactivation and this leads to the disruption of important immunogenic epitopes and subsequently could affect antibody responses, rendering them ineffective at inhibiting influenza virus infection. Formaldehyde and BPL are two chemicals commonly used to inactivate influenza viruses, with each chemical having different effects on protein structures. For example, treatment of an H9N2 virus with same chemical concentration (0.1 %) of BPL and formaldehyde led to a four times greater decrease in the HA titer of the virus treated with formaldehyde (Pawar et al., 2015). This indicates that the HA protein undergoes structural changes during formaldehyde inactivation, and antibody responses could then be directed against improper epitopes should these inactivated viruses be administered as a vaccine. That led us to our second objective, which was to compare the effect that inactivation method of H9N2 AIV has on its immunogenicity following intramuscular vaccination in chickens. The inactivation methods using BPL, formaldehyde, and gamma-radiation were chosen due to their unique mechanisms of inactivation; formaldehyde mainly alters protein structures, BPL affects nucleic acids but also has some effects on protein, while gamma-radiation specifically affects nucleic acids. We hypothesized that different inactivation methods used for H9N2 AIV would culminate in differences in immune responses after vaccinating chickens. The results of the study described in chapter 3 demonstrate that formaldehyde inactivated H9N2 AIV led to decreased antibody-mediated immune responses compared to BPL and gamma-radiation inactivated H9N2 AIV. Interestingly, beside one initial time point in the study, BPL and gamma-radiation
inactivated H9N2 AIV vaccines did not differ consistently when considering antibody-mediated immune responses, even though theoretically gamma-radiation induces less effects on protein structures during inactivation. This suggests BPL inactivation might have less of an effect on protein structures which are immunogenic, or some other aspect of the BPL WIV in our study enhanced immunogenicity in chickens. Also notable was that when 2 µg of CpG ODN 2007 was added to each H9N2 WIV, antibody titers were significantly elevated in chickens compared to the WIVs administered without adjuvant, and antibody titers were not significantly different among groups that received CpG ODN 2007. The inactivation method used for influenza virus can also affect the functional abilities of the WIV which in turn can alter the outcome of vaccination. For instance, inactivation of influenza virus impairs its ability to undergo membrane fusion as a replicating virus is normally capable of (Desbat et al., 2011; Geeraedts et al., 2012). After administration, the H9N2 WIVs are taken up by professional APCs where they will be processed and presented on MHC-II proteins leading to adaptive immune responses. However, after being internalized, H9N2 WIVs are potentially capable of fusion with the endosomal membrane if this function has not been abolished as result of inactivation. Endosomal membrane fusion is important as this leads to the release of viral components into the cytoplasm, where the protein constituents can be processed and enter the MHC-I presentation pathway, which is essential for the activation of CD8+ T cells. In mice, vaccination with a BPL and formaldehyde inactivated H5N1 WIV vaccine showed that the BPL WIV led to significantly greater numbers of nucleoprotein-specific CD8+ T cells, and this was attributed to decreased membrane fusion abilities of the formaldehyde WIV. Similarly, in our study we only observed cell-mediated immune responses in chickens that were vaccinated with a BPL inactivated H9N2 virus. Strikingly, cell-mediated immune responses were only observed in our study when 2 µg
of CpG ODN 2007 was included with the BPL WIV. This puts forward the idea that the BPL inactivated H9N2 virus vaccine induced cell-mediated immune responses as it led to the priming of naïve CD8\(^+\) T cells, but CpG ODN 2007 was required to fully induce responses, likely through the activation of professional APCs, which eventually activated CD8\(^+\) T cells. Nevertheless, our study highlights the effects that inactivation method has on antibody- and cell-mediated immune responses following vaccination with H9N2 WIV vaccines in chickens.

The first two studies described in chapters 2 and 3 followed an identical vaccination schedule complying with previous research done in our laboratory, whereby chickens received a primary vaccination on day 7 post-hatch and a secondary vaccination on day 21 post-hatch. In both studies, both vaccinations were administered via IM injection in the thigh. These studies were designed to uncover relationships between immunogenicity, inactivated influenza virus vaccine antigens, and TLR ligand adjuvants. As mentioned previously, two doses of an inactivated virus vaccine are typically required to produce effective adaptive immune responses, however, administering multiple vaccines to chickens on a large poultry operation is difficult and likely not plausible. Vaccinating chicken embryos in ovo using inactivated influenza vaccines is a possible method to vaccinate large numbers of birds against H9N2 AIV. First, there is technology which makes injecting thousands of eggs possible, and chicken embryos are already vaccinated in ovo against pathogens such as MDV (Haq et al., 2013). This led to the final objective of this thesis described in chapter 4, which was to quantify the immune response to BPL inactivated H9N2 vaccines administered to chicken embryos first in ovo on ED18, and later on day 14 post-hatch with a secondary vaccine administered to hatched chickens via IM administration in the thigh. This vaccination schedule decreases the number of doses administered post-hatch, yet still allows chickens to receive two doses. In this study, CpG ODN
2007 and the oil-emulsion adjuvant, AddaVax™, were also included as adjuvants to determine their adjuvant capabilities when administered in ovo. We hypothesized that immune responses would be induced in chickens following the above described vaccinations, and that CpG ODN 2007 would enhance these immune responses. Antibody- and cell-mediated immune responses were observed in chickens after the secondary vaccination, however they were not observed after the primary in ovo vaccination. Other research studying in ovo administered influenza vaccines have consistently demonstrated serum antibody responses in chickens around 3-4 weeks post-hatch (Toro et al., 2007; Toro and Tang, 2009; Mesonero et al., 2011), highlighting that our 2 week time period prior to administering the secondary vaccine could have been too short to observe antibodies induced from the in ovo vaccine alone. Additionally, research has demonstrated that there is a relationship between the occurrence of antibody-mediated immune responses and the in ovo influenza vaccine dose (Toro and Tang, 2009; Mesonero et al., 2011). This suggests our dose might have been below the threshold needed to induce antibody responses. CpG ODN 2007 did not enhance antibody- or cell-mediated immune responses, however when AddaVax™ was included in the vaccine we observed the presence of cell-mediated immune responses. IgM antibody titers were significantly increased in sera of chickens which received CpG ODN 2007 7 days after the secondary IM vaccine. This specific result provides even more evidence to suggest that CpG ODN 2007 is a very effective adjuvant when administered intramuscularly, however, more research needs to be done to determine its in ovo adjuvant potential. Additionally, for future WIV H9N2 in ovo vaccine studies, greater doses of inactivated virus should be included, and serum sampling should be extended to include more times post-hatch for the quantification of antibody-mediated immune responses.
In this thesis, several novel findings are reported. One major discovery was that the inactivation method used to produce H9N2 WIVs affects immune responses after vaccination in chickens. Specifically, BPL inactivation was shown to enhance cell- and antibody-mediated immune responses compared to formaldehyde and gamma-radiation inactivation methods. In addition to providing further evidence that CpG ODN 2007 is an effective adjuvant for inactivated H9N2 AIV IM chicken vaccines, we have also demonstrated it to be superior to flagellin. This thesis also reports that combinations of CpG ODN 2007 and flagellin do not induce superior levels of immunogenicity compared to CpG ODN 2007 alone when administered with inactivated H9N2 AIV IM chicken vaccines. Also, this thesis contains new findings about the induction of cell- and antibody mediated immune responses to BPL inactivated H9N2 virus vaccines administered as a primary in ovo vaccination and secondary IM vaccination.

The results described in this thesis come with some caveats. First, in chapter 2, CpG ODN 2007 and flagellin were compared for their induction of antibody-mediated immune responses, yet we did not quantify cell-mediated immune responses. Also, these studies focused on systemic antibody responses. Mucosal responses are also very important for protection against influenza virus infection and these were not quantified. Additionally, for all experiments performed we quantified immune responses for a maximum of 4 weeks post primary vaccination. We could not determine memory responses to vaccination due to experimental housing time restrictions.

Future research can continue to elucidate immunogenicity to inactivated H9N2 AIV vaccines in chickens. Specifically, the induction of cell-mediated immune responses against inactivated H9N2 AIV vaccines when administered with combinations of TLR ligands should be examined further. Assessing the release of IFN-γ from re-stimulated splenocytes from
vaccinated chickens is a possible method to study cell-mediated immune responses. Future studies should continue to study *in ovo* vaccines against H9N2 AIV for chickens, however greater doses of inactivated H9N2 virus and CpG ODN 2007 should be tested in vaccine formulations, and longer periods of time should be allocated to assess antibody responses induced by *in ovo* vaccination alone. Future studies could also focus on alternatives to intramuscularly delivered secondary vaccines following *in ovo* vaccination. One alternative route for secondary vaccination is the aerosol route due to its ease of application to large numbers of chickens, similar to *in ovo* administration.

In conclusion, this thesis has shed some light on how various factors impact the immunogenicity of H9N2 WIV vaccines in chickens. Specifically highlighted were the use of different inactivation strategies, the use of TLR ligand adjuvants, and the route of vaccine administration. Future research should examine the immunogenicity of other TLR ligand combinations with BPL inactivated H9N2 vaccines in chickens. Additionally, *in ovo* inactivated H9N2 vaccine studies in the future should include greater doses of H9N2 WIVs and should extend the period of sampling for the detection of antibody-mediated immune responses.
References


