Enhancement of Avian Influenza Virus vaccines in chickens through identification and application of novel mucosal adjuvants

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ABSTRACT

ENHANCEMENT OF AVIAN INFLUENZA VIRUS VACCINES IN CHICKENS THROUGH IDENTIFICATION AND APPLICATION OF NOVEL MUCOSAL ADJUVANTS

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The avian influenza virus (AIV) is a mucosal pathogen that is of relevance to the poultry industry and humans from economic and public health perspectives. Most commercially available AIV vaccines are of the inactivated type requiring parenteral co-administration with a water-in-oil adjuvant to generate an antigen-specific immune response. Limitations exist in the quantity and quality of antibody-mediated immune responses generated with this approach. This research was aimed at identifying novel, more efficacious adjuvants with the potential to be administered by the intramuscular and aerosol routes.

To evaluate the adjuvant potential of class B oligodeoxynucleotides (ODNs) in chickens, 2 doses of CpG ODN (oligodeoxynucleotides containing unmethylated CpG motifs) 2007 (CpG 2007) and 1826 were administered intramuscularly (IM) with a formalin-inactivated, whole, H9N2 avian influenza virus. We concluded that different members of class B ODNs displayed various levels of adjuvancy when combined with inactivated AIV in chickens based on neutralizing and virus-specific antibody responses generated by the 2 doses. Therefore, CpG 2007 was selected at a specific dose for poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticle encapsulation which was administered with inactivated AIV through the IM and aerosol routes; systemic and local

mucosal antibody-mediated responses were assessed. Significantly higher systemic and local mucosal antibody responses were observed after the administration of 3 doses of the nanoparticle-encapsulated CpG 2007 vaccine by the aerosol route compared to the formulation containing nonencapsulated CpG 2007. In contrast, significantly higher systemic and local mucosal antibody responses were induced with the nonencapsulated CpG 2007 formulation by IM administration.

To gain a better understanding of the antibody-mediated immune responses, the inactivated AIV vaccine component was encapsulated for IM administration. The protective efficacy of the vaccine and the ability to generate IgY antibodies of high avidity were assessed. Nonencapsulated AIV with encapsulated CpG 2007 elicited significantly higher magnitude antibody mediated responses and a reduction in shedding of cloacal virus compared to the encapsulated AIV and encapsulated CpG formulation. High avidity IgY antibodies were induced by both formulations.

In conclusion, the adjuvant potential of CpG 2007 and PLGA nanoparticles was demonstrated in the context of inactivated AIV using IM and aerosol routes of administration.

DEDICATION AND ACKNOWLEDGMENTS

I wish to dedicate this thesis to the memory of my late mother, Shirley V. Singh, whose words of wisdom and encouragement have inspired me to become the best version of myself. I also wish to dedicate this work to my family members who offered me unconditional love and support during this undertaking, my father, Kelvin, and sisters, Cressida and Nerissa.

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LIST OF ABBREVIATIONS

µg	microgram
µL	microlitre
AI	avian influenza
AID	activation induced cytidine deaminase
AIV	Avian influenza virus
$Al(OH)_3$	aluminium hydroxide
APCs	antigen presenting cells
ATF 2-c-Jun	activating transcription factor 2
BALT	bronchus associated lymphoid tissues
CALT	conjunctiva-associated lymphoid tissues
CD4+	cluster of differentiation 4 positive cells
CD8+	cluster of differentiation 8 positive cells
CFA	complete Freund's adjuvant
ChIFN-γ	chicken interferon gamma
chMDA5	chicken melanoma differentiation-associated protein 5
CpG ODN	oligodeoxynucleotides containing unmethylated CpG motifs
CpG 2007	CpG ODN 2007
CT-B	cholera toxin B
CTL	cytotoxic T lymphocyte
DCs	dendritic cells

DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
E. coli	*Escherichia coli*
ELISPOT	enzyme linked immunosorbent spot
FAE	follicle associated epithelium
Gal	galactose
GALT	gut associated lymphoid tissues
GLM	general linear model
H	heavy
HA	haemagglutinin
HALT	head associated lymphoid tissues
HI	haemagglutination inhibition
HLB	hydrophilic/lipophilic balance
HP	highly pathogenic
HPAIV	highly pathogenic avian influenza virus
IBDV	infectious bursal disease virus
IBV	infectious bronchitis virus
ID_{50}	50% infectious dose
IDO	indoleamine, 2, 3-dioxygenase
IFA	incomplete Freund's adjuvant
IFN-α	interferon alpha

IFN-β	interferon beta
IFN-γ	interferon gamma
Ig	immunoglobulin
IGSs	interferon stimulated genes
IL	interleukin
ILTV	infectious laryngotracheitis virus
IRF	IFN-regulatory factor
IRAK	IL-1 receptor associated kinase
ISCOMS	immune stimulating complexes
L	light
LGP2	laboratory of genetics and physiology
LP	low pathogenic
LPAIV	low pathogenic avian influenza virus
LPS	lipopolysaccharide
LRRs	leucine-rich repeats
M	matrix
MAMPS	microbe associated molecular patterns
MAP	mitogen-activated protein
MDV	Marek's disease virus
MHC	major histocompatibility complex

MyD88	myeloid differentiation primary response gene 88
Mx	myxovirus resistance
NA	neuraminidase
NALP3	NACHT, LRR and PYD domains-containing protein 3
NALT	nasal associated lymphoid tissues
NDV	Newcastle disease virus
NF-κβ	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRs	NOD-like receptors
NLRC5	NOD-like receptor family CARD domain containing 5
NP	nucleoprotein
OAS	oligoadenylate synthetase
OD	optical density
o/w	oil-in-water
pp38	phosphoprotein 38
PA	polymerase acidic protein
PB2	polymerase basic protein
PCR	polymerase chain reaction
pDCs	plasmacytoid dendritic cells
pIgA	polymeric immunoglobulin A (alpha)
pIgR	polymeric immunoglobulin receptor
PG	paranasal gland

PKR	protein kinase R
PLGA	poly(D,L-lactic-co-glycolic acid)
PRRs	pattern recognition receptors
RIG-I	retinoic acid inducible gene I
RLR	RIG-I-like receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
SA	sialic acid
S.D.	standard deviation
S.E.M.	standard error of mean
SPF	specific pathogen free
SQ or SC	subcutaneous
ssRNA	single stranded RNA
TAB	TGF-beta activated kinase
TAK	transforming growth factor-β-activated protein kinase
TGF	transforming growth factor
T_H1	T helper 1
T_H2	T helper 2
TIR	Toll-interleukin-1 receptor
TLRs	Toll-like receptors
TRAF	TNF receptor associated factor

TNF	tumor necrosis factor
V	variable
VN	virus neutralization
w/o	water-in-oil
w/o/w	water-in-oil-in-water

DECLARATION OF WORK PERFORMED

I declare that all experimental studies conducted in this thesis are original and were performed by myself or under my supervision with the exception of the item listed below:

Preparation of the nanoparticles used in the study was carried out by Dr. Tamiru Negash-Alkie, post-doctoral researcher at the Sharif Laboratory, Ontario Veterinary College, Department of Pathobiology, University of Guelph.

All research was conducted under the supervision of Dr. Shayan Sharif at the Department of Pathobiology, University of Guelph.

Shirene Melissa Singh

CHAPTER 1

INTRODUCTION

1.1. BACKGROUND AND RATIONALE

In recent times, there has been an increasing level of emphasis on emerging and re-emerging infectious diseases, such as avian influenza (AI). This trend primarily relates to the public health significance of the disease, intensification with respect to farming practices (necessary to sustain a growing global population) and an increase in the likelihood of virus spread, due to both enhanced transportation of goods and a higher frequency of travel by humans from one country to another (Kawai and Akira, 2006; Subbarao and Joseph, 2007).

The economic impact of AI on the poultry industry is difficult to estimate due to variability in factors such as the species of bird affected and the serotype of the virus involved in outbreaks; estimated losses can amount to values as high as $380 million Canadian dollars based on the gross economic costs associated with controlling the H7N3 subtype during the outbreak in British Columbia in the year 2004 (Pasick, Berhane, and Hooper-McGrevy, 2009). Therefore, the need for disease control in poultry cannot be over-emphasized.

Avian influenza vaccines constitute an integral component of control programs aimed at prevention, management or eradication of the avian influenza virus (AIV) in poultry. These vaccines elicit protective immune responses, ultimately preventing the development of clinical signs and reducing viral replication and shedding (van den Berg

et al., 2008). This, in turn, provides an opportunity for the virus transmission cycle to be broken. In order to maximize the impact of vaccination against AIV during an outbreak, it must be used alongside other elements of a control program. These elements include biosecurity measures, surveillance ("passive" and "active") strategies, reduction of host susceptibility to the virus, public awareness of the spread of the virus and proper disposal of infected birds by an acceptable means, both from a humane standpoint and from the perspective of prevention of environmental contamination with the virus (Swayne and Kapczynski, 2009).

Licensed, commercially available AIV vaccines used in poultry are those consisting of inactivated, whole virus, those generated by reverse genetics (H5N1 and H5N3 subtypes) or fowl poxvirus and Newcastle disease virus-vectored vaccines (van den Berg *et al*., 2008). Many of these commercially available vaccines are of the inactivated virus type and are co-administered with an adjuvant using the parenteral route of administration (Swayne and Kapczynski, 2009). Co-administration of adjuvants with inactivated vaccine antigens is necessary for the generation of antigen-specific immune responses (O'Hagan and Valiante, 2003). Parenterally administered inactivated influenza virus vaccines, although useful in generating systemic, circulating antibodies, are less capable of inducing local, neutralizing antibodies (Chen *et al*., 2001). This approach poses limitations such as the inability to elicit desirable mucosal immune responses (local, neutralizing, antibody-mediated immune responses) at the site of virus entry, and poor adaptability to mass vaccination schemes. Neutralizing antibody-mediated immune responses (systemic and local), induced by vaccination have a critical role in the

establishment of protective immunity against AIV. Immunogenicity can be enhanced through the co-administration of adjuvants with inactivated virus during vaccine administration (Swayne and Kapczynski, 2009). Therefore, vaccine adjuvants provide a potential means by which these limitations may be addressed. Firstly, they provide the opportunity to create a "tailored response" that is specific to selected infectious agents owing to their ability to skew the immune response toward a T helper 1-type (T_H1) or T helper 2-type (T_H2) direction (Marciani, 2003) or even a mixed response. Secondly, they can enhance the magnitude and duration of resulting antibody-mediated immune responses (Bode *et al*., 2011) which are necessary for a reduction in virus shedding in the context of AIV. Thirdly, the mucosal delivery of novel immune potentiators such as oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs (CpG ODN) incorporated into poly(D,L lactic-co-glycolic acid) (PLGA) microparticles (O'Hagan and Valiante, 2003) can allow for an enhanced adjuvant effect while minimizing the degree of adjuvant degradation upon *in vivo* administration in chickens.

Previous *in vivo* studies have demonstrated the immunogenicity of class B ODNs in chickens (Hung *et al*., 2011; Mallick *et al*., 2011). Class B ODNs were shown to have adjuvant effects in chickens when administered with inactivated AIV (Fu *et al*., 2013; Wang *et al*., 2009), and have been demonstrated as immunostimulatory for chicken B and T cells (Chrzastek, Piasecki, and Wieliczko, 2013). Dose-titration studies in chickens using CpG ODN 2007 delivered by the intramuscular route have been conducted with doses as low as 3.16 µg, followed by *Escherichia coli* (*E. coli*) challenge (Gomis *et al*., 2003). An earlier study conducted at our laboratory demonstrated the ability of high (50

μg) and low (10 μg) doses of CpG ODN 2007 to enhance the host immunity to a H4N6 AIV when administered intramuscularly; however, this was specifically in the context of prophylactic treatment (St Paul *et al*., 2012).

Therefore, we hypothesized that: a) class B ODN members have different degrees of adjuvanticity when co-administered with a formalin-inactivated H9N2 AIV during intramuscular administration which may affect their dose responses, b) differences in systemic and local mucosal immune responses may be enhanced by PLGA-encapsulation of CpG ODN 2007 in inactivated AIV vaccine formulations and by delivery routes and, c) higher protective immune responses may be induced in inactivated AIV vaccine formulations consisting of PLGA-encapsulated virus and PLGA-encapsulated CpG ODN 2007 compared to formulations containing PLGA-encapsulated CpG ODN 2007 with nonencapsulated virus.

The objectives described in this thesis were to (1) investigate the adjuvant potential of two different doses of class B CpG ODNs when combined with a formalin-inactivated H9N2 (A/Turkey/Ontario/1/66) virus using intramuscular administration, (2) compare the adjuvant potential of nonencapsulated CpG ODN 2007 with PLGA-encapsulated CpG ODN 2007 when combined with a formalin-inactivated H9N2 virus using intramuscular and aerosol routes of administration, (3) compare the protective systemic and local mucosal antibody responses generated by parenteral administration of a PLGA-encapsulated CpG ODN 2007 and PLGA-encapsulated H9N2 AIV vaccine formulation, to a PLGA-encapsulated CpG ODN 2007 and nonencapsulated H9N2 AIV formulation.

1.2. LITERATURE REVIEW

1.2.1. Avian influenza virus (AIV)

Avian influenza virus (AIV), an orthomyxovirus of the genus *Influenzavirus A*, is a single-stranded, negative-sense, RNA virus (Alexander, 2007) that contains several segmented genomes (PB2, PB1, PA, HA, NP, M and NS) (Webster *et al*., 1992). Influenza A viruses are assigned to distinct subtypes based on the presence of surface glycoproteins such as haemagglutinin (HA) and neuraminidase (NA). To date, most influenza A virus subtypes (H1-H16 and N1-N9) have been identified in wild birds, except for the recently discovered H17N10 and H18N11 subtypes associated with bats (Wu *et al*., 2014). The subtypes most commonly associated with domestic poultry are H3, H5, H6, H7 and H9 (Alexander, 2000; Peng *et al*., 2013; van den Berg *et al*., 2008). Based on levels of virulence, AIVs may be of the highly pathogenic (HP) pathotype or the low pathogenic/pathogenicity (LP) pathotype. Low pathogenic avian influenza viruses (LPAIVs) are characterized by the presence of basic amino acid residues at their HA cleavage sites; HA cleavage for these viruses occurs by trypsin-like enzymes, restricting LPAIV replication to anatomical areas like the respiratory and gastrointestinal tracts. In contrast, highly pathogenic avian influenza viruses (HPAIV) have multiple basic amino acids at the HA cleavage site; HA cleavage for HPAIVs occurs by ubiquitous enzymes, allowing these viruses to spread systemically (Soda *et al*., 2011; van den Berg *et al*., 2008). However, it is noteworthy that the systemic detection of LPAIV RNA has recently been demonstrated using polymerase chain reaction (PCR) for a few subtypes including H5N2, H7N1, H7N7 and H9N2 (Post *et al*., 2013).

1.2.2. Significance of the H9N2 virus subtype

The H9N2 virus subtype is significant to the poultry industry from an economic perspective; financial losses are attibuted to mortality rates, reduced egg production and, in some countries, vaccination programs (Fusaro *et al*., 2011). Furthermore, the H9N2 virus subtype is of public health significance as it is reported to be transmitted from poultry to humans (Butt *et al*., 2005).The first report of the H9N2 virus subtype in the United States of America was in the year 1966 when it was identified in turkeys (Homme and Easterday, 1970). Subsequent to undergoing widespead global circulation (Europe, Africa, Asia and the Middle East) (Fusaro *et al*., 2011) the H9N2 subtype became endemic in chickens in Asian and Middle Eastern countries (Lu *et al*., 2001; Nili and Asasi, 2002). Presently, three virus lineages are reported to be in global circulation, Chicken/Beijing/1/94,Quail/Hong Kong/G1/97 and Duck, Hong Kong/Y439/97 (Nang *et al*., 2013).

1.2.3. AIV tissue tropism

Avian influenza virus (AIV), a known mucosal pathogen, gains entry into its host via the respiratory and gastro-intestinal tracts (Villegas, 1998) (Webster *et al*., 1992). The HA glycoprotein mediates the attachment of AIV to surface receptors [sialic acid (SA) linked to galactose (GAL)] of target cells in the respiratory tract of chickens allowing the virus to gain entry into susceptible hosts (Kuchipudi *et al*., 2009; Yu *et al*., 2011). Avian influenza viruses display an affinity for SAα-2,3Gal-linkages while human influenza viruses show a preference for SAα-2,6 Gal-linkages (Baigent and McCauley, 2003).

After viral fusion with surface receptors of epithelial cells (target cells), access to internal cell compartments occurs through mechanisms such as receptor-mediated endocytosis. This results in the virus being contained in the endosomal compartment where the characteristic low pH within endosomes causes fusion of the viral envelope with the endosomal membrane, and the release of the virus core into the cytosol allowing for presentation of viral peptides along the major histocompatibility complex (MHC) class 1 pathway (Wilschut, 2009).

1.2.4. Host innate responses to AIVs

After initial entry of AIV into the host via the respiratory route or the oral route (Villegas, 1998), antigen processing and presentation become critical for the induction of an immune response against the virus. In humans and mice, regardless of the route of entry of AIV, virus particles are taken up by professional APCs such as dendritic cells (DCs). Uptake is mediated through the recognition of evolutionarily conserved microbe-associated molecular patterns (MAMPs) by germ-line encoded receptors for antigen presentation to antigen sensitive cells (Brownlie and Allan, 2011; Tizard, 1979). Studies with respect to the existence of DCs in chickens are limited, although the generation and characterization of bone-marrow derived dendritic cells have been reported in chickens (Wu *et al*., 2010); characterization of chicken DCs based on the expression of surface markers has been reported after the administration of fluorescently labeled antigen-coated beads (de Geus, Jansen, and Vervelde, 2012). Moreover, the actual antigen presenting cell type that can be attributed to the activation of naïve T cells in chickens is currently unknown. What is known is that innate responses constitute an integral element in the

development of local immunity in the (respiratory) mucosal system of birds (de Geus, Rebel, and Vervelde, 2012). Macrophages have an important role in this regard and are necessary for phagocytosis and the release of cytokines and prostaglandins (Qureshi *et al*., 1994). Chicken macrophages, although fewer in number in comparison to mammalian macrophages, are located in the large airway mucosa, connective tissues, parabronchial linings and interstitial tissues (de Geus and Vervelde, 2013). They are also found in the atria and infundibulae linings (Maina, 2002). Polymorphonuclear cells (PMNs) such as heterophils also contribute to the development of innate responses in birds. PMNs are important for phagocytosis, chemotaxis, adherence and bactericidal actions, with these functions being related to the age of the birds (Kogut *et al*., 1998).

1.2.4.1. Initial virus recognition by the host innate immune system

Pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) and cytoplasmic RNA helicases are essential for recognition of viral infections by the host innate immune system in mammals and avian species. These receptors are considered as virus replication independent and dependent, respectively (Kawai and Akira, 2006). The retinoic acid-inducible gene I (RIG-I) has not been characterized in chickens but has been identified in ducks with possible consequences such as increased levels of susceptibility to AIV in chickens (Barber *et al*., 2010). Evidence suggests that chicken melanoma differentiation-associated protein 5 (chMDA5) is responsible for sensing AIV through the recognition of double-stranded RNA (dsRNA) (Karpala *et al*., 2011). Laboratory of genetics and physiology (LGP2) has been identified in RIG-I-like receptor (RLR) signaling through the recognition of dsRNA, although knowledge in the context of

chickens remains limited. In chickens, LGP2 has been shown to have a greater affinity than RIG-I for dsRNA recognition (Chen, Cheng, and Wang, 2013). NOD-like receptors (NLRs), in general terms, are associated with the recognition of MAMPS of bacteria and hence are important in antibacterial host defenses (Inohara *et al*., 2005). However, their role in sensing RNA or DNA viruses in mammals must not be under-rated (Muruve *et al*., 2008). NOD-like receptor family CARD domain containing 5 (NLRC5) has been identified in humans (Benko *et al.*, 2010) and chickens (Lian *et al*., 2012); knowledge of its mechanism of action regarding antiviral immunity is limited. As previously mentioned, TLRs are responsible for the recognition of MAMPs which serve as "danger signals" alerting the host to the presence of an unwanted infection, thereby activating the relevant innate immune responses and ultimately resulting in the activation of B and T cells. The toll receptor, first identified in *Drosophila melanogaster*, was initially implicated in the dorso-ventral pattern of development of larvae (Steine *et al*, 1991; Werling and Jungi, 2003). Subsequently, Toll-mutant flies were shown to have a high level of susceptibility to fungal infections (Lemaitre *et al*., 1996). Toll-like receptors consist of three domains, an extracellular domain with leucine-rich repeats (LRRs), a transmembrane domain and a cytoplasmic TIR domain (Toll/IL-1 receptor domain), with the cytoplasmic domain is the area of origin for activation of signal transduction (Takeda and Akira, 2004).

Chickens, like mammals, exhibit differences in TLR expression for various cells (notably high in/on APCs and epithelial cells) as well as tissue types (highly expressed in spleen and lymphocytes in blood) (Kannaki *et al*., 2010). The significance of this relates

to the type of immune response that is generated. Thirteen TLR members have been identified in mammals while 10 have been described in chickens (Jungi *et al*., 2011). Of the chicken TLRs, TLR1LA and TLR1LB are the functional homologues of TLR1/6/10 in humans. TLR2 has been duplicated in the chicken resulting in TLR2A and TLR2B. Orthologues of human TLRs exist in chickens: TLR3 (recognizes double-stranded (ds) RNA), TLR4, TLR5 and TLR7 (recognizes single-stranded (ss) RNA). TLR8 exists as a pseudogene while TLR11 is notably absent in chickens. TLRs specific to chickens are TLR21 (the functional homologue to TLR9 in mammals), which recognizes oligodeoxynucleotides (ODN) containing unmethylated CpG motifs (CpG ODN) (Brownlie *et al*., 2009) and TLR15, which has recently been shown to be involved in the recognition of CpG ODN in macrophages (Ciraci and Lamont, 2011).

Recognition of viruses is accomplished through the identification of viral components such as genomic DNA and RNA, or dsRNA by cells of the innate immune system (Iwasaki and Medzhitov, 2004). After the initial recognition of viral components, a cascade of events ensues, culminating in the production of type 1 interferons (IFN-α and IFN-β) and pro-inflammatory cytokines, namely interleukin-1β (IL-1β), IL-6, tumor necrosis factor (TNF) and IL-12, resulting in the initiation of adaptive immune responses (Kawai and Akira, 2006). The induction of type 1 IFNs can be attributed to the activation of transcriptional factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ), activating transcription factor 2 (ATF 2-c-Jun), IFN-regulatory factor 3 (IRF 3) and IRF 7. These events are mediated via the recruitment of the Toll-interleukin-1 receptor (TIR) adaptor molecule, MyD88, through interactions with IL-1 receptor

associated kinase (IRAK) 4 and TNF receptor associated factor (TRAF) 6. TRAF6 results in the activation of TAK1, which, together with transforming growth factor (TGF)-beta activated kinase (TAB) 1, TAB2 and TAB3, lead to the activation of NF-κβ and ATF2-c-Jun (Takaoka and Taniguchi, 2003). Mitogen-activated protein (MAP) kinases 3 and 6 are also activated by TAK 1, leading to c-Jun N-terminal kinase and pp38 phosphorylation and ultimately activation of AFT2-c-Jun (Akira and Takeda, 2004). MyD88 associates with IRAK1 and IRF7, hence facilitating the phosphorylation of IRF7 by IRAK1 and resulting in the expression of type 1 IFNs. It should be noted that TRAF6-mediated ubiquitination is also needed to activate IRF7 (Kawai and Akira, 2006).

Interferons are important for the upregulation of antiviral genes during the early stages of viral infection, thus allowing the adaptive immune response enough time for development (Samuel, 2001). The existence of type 1 interferons (IFN-α and IFN-β) has been noted in both mammals and chickens (Lowenthal *et al*., 2001). The type II interferon, interferon-gamma (IFN-γ), has also been identified in mammals and birds (Kaiser, Wain, and Rothwell, 1998; Weining *et al*., 1996). Functions of IFN-γ include: 1) Defense against intracellular pathogens; it is associated with a T helper type 1 (T_H1) immune reponse (Schroder *et al*., 2004). 2) Induction of major histocompatibility complex (MHC) class I and MHC class II expression, antigen processing and presentation (Goossens *et al*., 2013; Zhou, 2009). 3) Regulation of nitric oxide production (Goossens *et al*., 2013). Type III IFNs have been identified in mammals and chickens. Paralogous type III IFNs are IFN-lambda 1 [IFN-λ (IL-29)], IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B). In chickens, one IFN-λ gene has been identified as opposed to 3 genes that

exist in humans. The antiviral activity of type III IFNs is similar to that displayed by type I IFNs (Kaiser *et al*., 2005; Karpala *et al*., 2008).

Interferon stimulated genes (ISGs) such as Myxovirus resistance proteins (Mx proteins) are associated with the inhibition of early stages of RNA virus replication, apoptosis and endocytosis (Jatiani and Mittal, 2004; Mibayashi, Nakade, and Nagata, 2002). The role of Mx variants in antiviral responses in chickens remains controversial (Schusser *et al*., 2011). Protein kinase R (PKR), an intracellular sensor which is constitutively expressed in an inactive form, has been identified in mammals and chickens (Goossens *et al*., 2013) and plays a role in the recognition of dsRNA, an intermediate in the replication of several viruses (Clemens and Elia, 1997). Recognition of dsRNA by PKR initiates a series of phosphorylation events and the activation of eukaryotic translation initiation factor 2 (eIF2α), which targets cellular and viral mRNA translation (Williams, 1999). An additional ISG involved in the recognition of dsRNA is 2′-5′ oligoadenylate synthetase (OAS), which, on activation, leads to the formation of 2′-5 linked oligoadenylates (2-5A). Binding of 2-5A to RNase L results in viral RNA and host RNA cleavage (Silverman, 2007).

1.2.5. TLR21 signaling pathways and CpG ODNs

Given the existence of various innate immune system signaling pathways, each of which is associated with different PRRs, the potential to drive the immune response in an advantageous direction must be considered. Extensive *in vitro* and *in vivo* studies have demonstrated the ability of CpG ODN to activate the immune system in species ranging from primates (human and nonhuman), ruminants, mice, pigs, horses, companion animals and chickens to fish (Mutwiri *et al*., 2004). Subsequent to engagement of CpG ODN by TLR9/21, signal transduction occurs, culminating in the production of pro-inflammatory cytokines. In humans, CpG ODN activation occurs through TLR9 which is expressed in B cells as well as plasmacytoid DCs (Bauer *et al*., 2001; Krug *et al*., 2001). The existence of plasmacytoid DCs has not been confirmed in chickens. Binding of CpG ODN to cell surface receptors allows entry into the cell, with the ultimate destination of CpG ODN being the endosomal compartment of the cell. The signaling mechanism after endocytosis of CpG ODN is mediated through the MyD88 adaptor protein and results in the initiation of the IRAK/TRAF6 cascade of events with activation of transcription factors such as NF_KB, fos-jun and pp38 (Mutwiri *et al*., 2003).

Immuno-stimulatory CpG DNA, by definition, are “short oligodeoxynucleotides containing cytosine-guanine dinucleotides” within particular base contexts (CpG motif) having the formula 5’-$X_1X_2\underline{CG}Y_1Y_2$-3’, where X_1X_2 are purines or thymine (T) and Y_1Y_2 are pyrimidines (Mutwiri *et al*., 2003). The type of immune response elicited by CpG ODN is believed to be a function of the base contexts of the CpG dinucleotides, in

conjunction with the spacing between the CpG motifs themselves. Spacing between the CpG motifs that includes two or more intervening bases, particularly “T”, is desirable. Bacterial CpG dinucleotides occur at an approximate frequency of 1 in 16 bases compared to a reduction of around a quarter of this frequency, which is present in the DNA of vertebrates. Furthermore, occurrence of the bases A, G or T before the CpG dinucleotides and A, C or T after the CpG dinucleotides is expected to be immune-stimulatory (Krieg and Davis, 2006; Mutwiri *et al*., 2003). Moreover, while mammalian DNA is usually methylated, the CpG dinucleotides found in bacteria tend to be unmethylated. These unmethylated CpG dinucleotides flanked by specific regions containing purines and pyrimidines constitute CpG motifs capable of eliciting both innate and adaptive immune responses. These immunostimulatory effects extend to synthetic oligodeoxynucleotides containing one or more CpG motifs (Krieg and Davis, 2006; Krieg *et al*., 1995; Mutwiri *et al*., 2003). Additional factors for consideration in selecting the most suitable type of CpG ODN are the inclusion of 2-3 CpG motifs, the presence of flanking regions and the nature of the backbone as CpG may be subject to degradation minutes after *in vivo* administration. A phosphorothioate backbone is therefore recommended (Krieg and Davis, 2006; Semple *et al*., 2000) as it is more resistant to degradation. Furthermore, the importance of using an appropriate route of administration for CpG ODN *in vivo* must be emphasized. The administration site of CpG ODN has been shown to influence whether resulting effects are immunostimulatory or immunosuppressive in mice. The induction of a strong, local, T cell response in lymph nodes was demonstrated after subcutaneous injection of antigen and CpG ODN; systemic administration of CpG ODN elicited a suppressive effect on T cell responses and effector

cell production in the spleen (Wingender *et al*., 2006). This immunosuppressive effect occurred in a dose-dependent manner and was shown to be a function of indoleamine, 2, 3-dioxygenase (IDO), which is responsible for the degradation of tryptophan. Tryptophan is a requirement for CD4+ and CD8+ T lymphocyte proliferation (Hayashi *et al*., 2004; Mellor and Munn, 2004). The immunosuppressive effect of IM administration of CpG ODN has not been well documented in chickens.

In general, CpG ODNs can be broadly classified into four types, class A (also known as D), class B (also known as K), class C and class P on the basis of their immuno-stimulatory properties according to studies conducted in mice and humans (Krieg and Davis, 2006). Members of class A, while known to be potent activators of natural killer (NK) cells and inducers of IFN-α secreted from pDCs, are poor stimulators of B cells. Other features of class A members include the presence of a mixed phosphodiester/ phosphorothioate backbone, a palindromic sequence formed by the CpG flanking region, a poly G tail located at the 3′ end of the sequence and the presence of a single CpG motif (Bode *et al*., 2011). This is in contrast to members of class B that are strong B cell activators but poor inducers of NK cells and IFN-α. Class B members also possess a phosphorothioate backbone and many CpG motifs that induce the production of TNF-α and IL-6. Members of class C possess a combination of properties characteristic of both classes A and B (Krieg and Davis, 2006); they promote the proliferation and differentiation of B cells and pDCs and are inducers of INF-α and IL-6. Class C and P members have multiple CpG motifs and a phosphorothioate backbone. Additionally,

members of class P contain two palindromic sequences, are known for their ability to stimulate B cells and pDCs, and promote the secretion of IFN-α (Bode *et al*., 2011).

In vivo studies investigating the ability of CpG ODN to elicit protection against viral infections in chickens are largely restricted to those conducted with infectious bursal disease virus (IBDV), Newcastle disease virus (NDV), AIV, infectious bronchitis virus (IBV), Marek's disease virus (MDV), avian reovirus and infectious laryngotracheitis virus (ILTV) (Gupta *et al*., 2014; St. Paul *et al*., 2013). Other studies have demonstrated the modulation of antigen-specific immune responses by CpG ODN in chickens and include studies conducted with *E. coli* and *Eimeria* sp. (Dalloul *et al*., 2004; Gomis *et al*., 2003). The protective effects of CpG ODN against NDV in chickens when administered with a commercially attenuated live NDV vaccine (Linghua, Xingshan, and Fengzhen, 2007) have been demonstrated. In another study, a T_H1-biased response was shown to occur with the parenteral coadministration of CpG ODN class B and an H5N1 AIV (Wang *et al*., 2009). Earlier *in vivo* studies conducted in different species using CpG ODN with various antigens are also suggestive of a T_H1-biased response (Ballas *et al*., 2001; Chu *et al*., 1997; Lipford *et al*., 1997). Furthermore, in chickens, immune responses to intracellular infectious agents have been shown to be dominated by a T_H1-like cytokine response characterized by the production of IFN-γ, IL-12 and IL-18; T_H2-like responses targeting extracellular pathogens are associated with the induction of IL-4, IL-13 and IL-19 (Kaiser, 2010).

More recently, in chickens, intranasal delivery of CpG ODN 2006 (a class B ODN) with a H5N1 (A/Duck/China/09) Re5 virus strain, either by itself or in combination with

the TLR3 agonist, polyinosinic: polycytidylic acid (poly I:C), was shown to elicit a virus-specific IgG response in serum and an IgA response in the respiratory tract. In this study, production of IL-12, IFN-γ and IL-6 was also promoted (Liang *et al*., 2013). Intramuscular coadministration of H5N2 (A/Duck/Taiwan/04) and a plasmid consisting of multiple copies of a CpG motif resulted in enhanced antibody responses and upregulation of cytokine gene expression in the spleen as indicated by haemagglutination inhibition (HI) titres and higher levels of mRNA expression for IFN-γ, IFN-α, TLR3, TLR7 and TLR 21. Moreover, this study demonstrated a survival rate of 100% post-challenge with live H5N1(A/Duck/China/E319-2/03) virus (Hung *et al*., 2011). The adjuvant efficacy of CpG ODN has also been observed when used in combination with other influenza vaccine subtypes such as H4N6 (A/Duck/Czech/56) (St. Paul *et al*., 2012) and H5N2 (A/Ostrich/Denmark/72420/96) (Xiaowen *et al*., 2009). Subsequently, antibody responses were shown to be enhanced in chickens that received CpG ODN 2007 in conjunction with H4N6 (A/Duck/Czech/56) virus via the subcutaneous (SQ or SC) route of administration compared to vaccine combinations that incorporated TLR3 or TLR4 agonists (lipopolysaccharide) with the virus. Findings from this study also indicated a potent induction of antibody- and cell-mediated responses when a combination of CpG ODN 2007 and poly I:C was used with inactivated H4N6 virus (St. Paul *et al*., 2014a). Induction of enhanced serological responses (HI titres, IgA and IgG antibody levels) was shown to occur after the SQ/SC administration of a virosome-based H4N6 vaccine was used in combination with CpG ODN (Mallick *et al*., 2011).

1.2.6. Chicken lymphoid organs and the generation of immune responses

Differences between the mammalian and the avian immune systems have been recognized and range from a lack of lymph nodes observed in chickens (Rose, 1979) and the presence of the chicken B locus (a minimal essential MHC) (Kaufman *et al*., 1999) to the presence of the bursa of Fabricius which plays a vital role in the development of chicken B cells (Ratcliffe, 2006). The primary lymphoid organs in chickens are the bursa of Fabricius and the thymus. The bursa of Fabricius, the site of B cell proliferation and differentiation, is located dorsal to the distal end of the cloaca (Swayne and Kapczynski, 2009). During the early stages of embryonic development, B cell precursors that were previously subjected to gene rearrangements colonize the bursa of Fabricius. Since this process of gene rearrangement occurs without the aid of a terminal deoxynucleotidyl transferase, the resulting antibody diversity is limited. At this time, diversification of antibody variable (V) region genes occurs in rapidly proliferating B cells. The process of diversification is necessary for B cell specificity; in chickens, the generation of immunoglobulin diversity occurs by means of somatic gene conversion (Ratcliffe, 2006). Somatic gene conversion involves the replacement of homologous gene sequences by those originating from upstream immunoglobulin heavy (H) and light (L) variable regions in genes already subjected to gene rearrangement in a process brought about by activation induced cytidine deaminase (AID) (Arakawa, Saribasak, and Buerstedde, 2004). The end result of gene conversion is the production of “daughter cells” with the ability to identify single antigens. The microenvironment of the bursa of Fabricius, although established as being necessary for the process of gene conversion, is not a

requirement for immunoglobulin gene rearrangement (Ratcliffe, 2006). Gene conversion can also occur in the splenic germinal centres (Arakawa *et al*., 1996).

The peripheral or secondary lymphoid organs of the chicken can be categorized as follows (Smialek *et al*., 2011): 1) Head-associated lymphoid tissues (HALT), which include the Harderian gland (HG), the conjunctiva-associated lymphoid tissues (CALT), the paranasal gland (PG), the nasal-associated lymphoid tissues (NALT) and the lymphoid cell infiltrates of the lamina propria (in the nasal cavity), 2) The bronchus-associated lymphoid tissues (BALT), and 3) The gut-associated lymphoid tissues (GALT).

Although the HG, CALT and PG are not directly connected anatomically to the respiratory system, their role in the development of local immunity, particularly as it applies to the upper airways must be considered. Secretions produced in the HG reach the conjunctival sac through the secretory duct. Transport to the beak cavity occurs through the lacrimal duct (Smialek *et al*., 2011). The HG can be divided into a head in which lymphoid aggregates and germinal centers can be found and a body filled with plasma cells. Most of the B cell receptors are of the IgA isotype but IgY-expressing (or positive) cells are also present (Reese, Dalamani, and Kaspers, 2006). It is noteworthy that IgA+ lymphocyte migration from the HG to the lacrimal glands and the intestinal lamina propria has been demonstrated in chickens (Akaki *et al*., 1997). This draws attention to the possibility of a “common mucosal immune system” in chickens with potential consequences for the design of vaccines aimed at inducing mucosal neutralizing antibodies. Therefore, one may consider that delivery of a mucosal vaccine at a particular

surface potentially renders some degree of protection at another mucosal surface in the host.

B cells constitute the lymphoid follicles of the NALT and are mainly of the IgY producing type, whereas those that produce IgA and IgM types occur at a lesser frequency. The B cells are surrounded by a “cap” of CD4+ cells, while CD8+cells can be found in the epithelium and mucosa of the nasal cavity, specifically in the lamina propria (Ohshima and Hiramatsu, 2000). The BALT are found at the level of the junction between the primary bronchus and the caudal bronchi, being constitutively expressed in the lung of the chicken (Bienenstock, Johnston, and Perey, 1973). B cells are not found in significant numbers in the lung of chickens prior to two weeks post-hatch (Jeurissen *et al*., 1989). At the age of six weeks and greater, developed BALT, composed of centrally located B cells surrounded by CD4+ cells are observed in birds. Antibody-secreting cells of various isotypes can also be seen during this time in the epithelium of both the BALT and non-BALT regions (de Geus, Rebel, and Vervelde, 2012). A characteristic feature of mature BALT in chickens is the presence of follicle-associated epithelium (FAE) encompassing aggregates of lymphocytes; this epithelial layer is devoid of specialized M cells which are observed in mammalian-associated lymphoid tissues (Fagerland and Arp, 1993). The function of the FAE relates to the active transport of bursal luminal contents to the inside of the bursal follicle. Through this mechanism, developing B cells located in the bursa of Fabricius are able to access gut-derived contents after the occurrence of hatching (Ratcliffe, 2006).

1.2.7. Chicken immunoglobulin isotypes and adaptive immune responses

Unlike mammals in which five classes of immunoglobulins exist (IgG, IgD, IgA, IgM and IgE), three isotypes of immunoglobulins are found in avian species, IgY, IgM and IgA (Lillehoj and Trout, 1996). These immunoglobulins are found in chicken serum at the following average concentrations, IgY, 300-700 mg/100 ml, IgM, 120-250 mg/100 ml and IgA, 30-60 mg/100 ml, respectively (Tizard, 1979). The IgA antibody isotype constitutes less than 4% of the serum immunoglobulins and exist as both monomeric (minor) and dimeric/polymeric (major) forms. While this isotype predominates in bile and intestinal secretions, this is not the case for saliva and lacrimal secretions in which IgY was the major occurring isotype (Lebacq-Verheyden, Vaerman, and Heremans, 1974). In mammals, during the primary immune response, antibodies of the IgM isotype predominate while IgG antibodies are mainly seen during the secondary immune response. This phenomenon is somewhat less pronounced in chickens (Benedict, Brown, and Hersh, 1963). The pentameric nature of the IgM molecule is linked to the feature of high valency and this allows for the simultaneous binding of ten antigen molecules. High valency, in turn, renders the IgM molecule very efficient at complement fixation and agglutination (Tizard, 1979).

The adaptive immune system can effectively generate specific immune responses against invading pathogens and simultaneously minimize the impact of collateral damage incurred by surrounding tissues (Palm and Medzhitov, 2009). The adaptive immune system accomplishes its function using randomly generated, diverse, clonally expressed and selected receptors. If the most effective outcome of infection is to occur, it is

imperative that the elements of the adaptive immune system work in concert with the innate immune system. It is noteworthy that the adaptive immune response is under the influence of PRR-induced signals. The origin of infection, as determined by the innate immune system, influences the type of adaptive immune response that follows; the degree and persistence of infection determines the magnitude and duration of the adaptive immune response, respectively. The PRR-mediated signals conveying the message that defense against infection is still an ongoing process or that the infection has been resolved, results in the generation of effector cells or memory cells, respectively (Palm and Medzhitov, 2009). Antibody-mediated responses are required for the production of neutralizing antibodies at the portals of virus entry. It should be noted that CD4+ T lymphocytes assist B cells in the generation of neutralizing antibodies (van den Berg *et al*., 2008).

Antibodies can be produced against various protein components of influenza viruses, namely, haemagglutinin (HA), neuraminidase (NA), matrix (M) and nucleoprotein (NP); however, only anti-HA antibodies are capable of neutralizing homologous viral antigens. The significance of anti-NA antibodies relates to the prevention of the efficient release of the virus after entry into target cells (van den Berg *et al*., 2008). Cell-mediated immune responses have been established as important for minimizing the severity of influenza virus infection in addition to having a role in clearance of the virus, although the exact mechanism by which CD8+ lymphocytes (CTL) perform this function in chickens requires further investigation (de Geus, Rebel, and Vervelde, 2012). Recognition of viral NP, PB2 and PA in conjunction with MHC

class 1 molecules by CD8+ T cells results in the production of IFN-γ, TNF and the release of perforin (Subbarao and Joseph, 2007). Molecules such as granzymes A and B (cytotoxic) and perforin (cytolytic) induce the death of virus-infected cells through apoptosis; the mode of action of granzyme B differs from that of granzyme A in its ability to 'cleave substrates at aspartic acid residues" and to utilize caspases in addition to the mitochondrial pathway (Pinkoski and Green, 2003). The serine protease, granzyme A, exerts its effects at a few levels. In addition to having anti-inflammatory activities, granzyme A causes a disruption in the cell mitochondrial metabolism and induces the production of reactive oxygen species (ROS), leading to the damage of ssDNA. Moreover, granzyme A results in the degradation of nuclear proteins and those associated with the repair of damaged DNA (Lieberman, 2010).

Generally, IgA antibodies are considered as important in the mucosal immune response against influenza viruses in humans and mice (van den Berg *et al*., 2008); however, data regarding the importance of an IgA mucosal response against AIV in chickens are limited. The polymeric immunoglobulin receptor (pIgR), which is responsible for transporting polymeric IgA (pIgA) from the basolateral aspect of mucosal epithelial cells to the apical region, has been identified in both mammals and chickens (Renegar *et al*., 2004; Wieland *et al*., 2004). Subsequent to receptor cleavage, secretory IgA is released into the mucociliary blanket where it serves to protect against mucosal pathogens. Although the role of secretory IgA in conferring protection against influenza viruses has been emphasized in humans and mice, the importance of serum IgG is not to be under-rated (Renegar *et al*., 2004). In humans (Wagner *et al*., 1987) and mice

(Renegar *et al*., 2004), the induction of sufficiently high levels of serum IgG followed by passive transudation is believed to be significant for protecting against influenza virus infections of the lower respiratory tract. In avian species, however, IgY antibodies generated in serum undergo transudation to lacrimal secretions and therefore play an important role in providing protection to the upper respiratory tract (Ohshima and Hiramatsu, 2000; Toro *et al*., 1993).

1.2.8. Challenges and the requirement for mucosal vaccines against AIV

Commercial vaccines against AIV in chickens consist mainly of inactivated virus, requiring parenteral (systemic) administration (Swayne and Kapczynski, 2009) and the use of an adjuvant for the generation of antigen-specific immune responses. This approach poses limitations such as the inability to elicit desirable mucosal immune responses at the site of virus entry, and, poor adaptability to mass vaccination schemes.

Despite this ever-growing need for effective mucosal vaccines, advances made in this area are somewhat limited and may be attributed to the following reasons (Neutra and Kozlowski, 2006): 1) Problems with the administration and delivery of a standardized or fixed dose of the vaccine using mucosal routes such as aerosol, feed or drinking water, 2) the complexity associated with measuring mucosal immune responses post administration (the inability to accurately quantify the level of antibodies in mucosal secretions and difficulties testing mucosal T cell responses), and, 3) vaccine components are subject to degradation by proteases/nucleases and mucociliary clearance mechanisms.

Available routes of administration for the mucosal delivery of vaccines in chickens are intranasal, intraocular, intratracheal, oral and aerosol. Of these options, oral (for

example, feed and water) and aerosol methods of delivery are conducive to mass administration. In this literature review, emphasis will be placed on the aerosol route of delivery. Aerosols can be regarded as solid or liquid particles suspended in air or gas, having small dimensions to allow them to remain airborne for extended periods of time due to the feature of low settling velocity (Tellier, 2006). Experimental studies in the context of delivery of AIV vaccines in chickens using the aerosol route of administration are limited yet ongoing and involve the H5N1 and H9N2 subtypes. A recent study conducted in 3-week-old chickens demonstrated that aerosol delivery of LP H9N2 virus was more efficient at generating infection compared to the intranasal and oral delivery methods based on virus recovery from oropharyngeal and cloacal swabs (50% infectious dose, ID_{50}) (Guan *et al*., 2013). These findings were supported by the upregulation of cytokine gene expression in the trachea and lung of chickens for aerosol delivery of the H9N2 virus compared to intranasal delivery (Guan, Fu, and Sharif, 2015). In another study in chickens conducted with HP H5N1 virus, the aerosol route of delivery was reported to result in a significantly lower ID_{50} (30 times) compared to the intranasal route (Sergeev *et al*., 2013). However, single aerosol administration of a whole, inactivated H9N2 virus adjuvanted with aluminium hydroxide [$Al(OH)_3$], chitosan, cholera toxin B subunit (CT-B) or Stimune did not elicit the production of virus-specific antibodies (de Geus et al., 2011).

Nevertheless, the need for increased levels of mucosal protection still exists and emphasis continues to be placed on the development of novel mucosal AIV vaccines and suitable delivery systems. Therefore, a thorough understanding of the respiratory immune

system in chickens is imperative not only for the design of novel mucosal vaccines and adjuvants but also for the efficient delivery to target sites and is discussed in the following section.

1.2.9. Respiratory tract of chickens

1.2.9.1. Structure of the avian lung

In the chicken, air enters through the nares or the mouth into the oro-nasal cavity. The larynx allows access to the trachea which branches into two primary bronchi at the level of the syrinx. The primary bronchi enter the ventral aspect of the lung, then courses dorsolaterally before opening into the abdominal air sacs. Secondary (medioventral, lateroventral, mediodorsal and laterodorsal) and tertiary bronchi (parabronchi) arise from the primary bronchi; gas exchange occurs at the level of the tertiary bronchi (de Geus, Rebel, and Vervelde, 2012). Air sacs are noted for their role in ventilation as opposed to gas exchange and are named as follows: clavicular air sac, cranial thoracic air sac, caudal thoracic air sac and the abdominal air sac. Expansion of the air sacs is a requirement for drawing air into the lungs during inspiration. The percentage of air entering the intrapulmonary primary bronchi and neopulmonic parabronchi (neopulmo) before reaching the abdominal and caudal thoracic air sacs is 50%. The other 50% enters the paleopulmonic parabronchi (paleopulm) (Corbanie *et al*., 2006). From the lumen of the parabronchi, air moves centrifugally through the atria, infundibula and the air capillaries. The characteristic ventilation flow pattern (a caudocranial direction) observed in the avian respiratory system relates to the fact that during inspiration, air does not enter the medioventral secondary bronchi but instead flows through the mediodorsal and

lateroventral secondary bronchi (Reese, Dalamani, and Kaspers, 2006). Consideration must be given to the unidirectional airflow pattern of the avian lung and the feature of continuous ventilation of the parabronchial area in the design of aerosolized vaccine systems. These are important determinants in the "strategic lodging" of antigens in target regions of the respiratory tract containing antigen presenting cells (APCs), thus increasing the likelihood of uptake and antigen presentation to CD4+ and CD8+ lymphocytes. It is also desirable to elicit an adequate mucosal antibody response in areas of the respiratory tract where SAα-2, 3-Gal and SAα-2, 6-Gal receptors are located. These sites include the tracheal epithelium, bronchi, bronchioles and chicken alveolar cells (Kuchipudi *et al*., 2009).

1.2.9.2. Particle deposition in the respiratory system

The ventilation flow pattern and size of particles play an important role in determining the site of deposition (due to sedimentation and impaction) of encapsulated materials in the respiratory tract as it pertains to the application of novel, vaccine delivery systems. Particles ranging in diameter from 3.7-7 µm have been shown to be deposited in the nasal cavity and proximal trachea as opposed to particles 1.1 µm in diameter which are lodged in the lung and posterior air sacs; particles that were 0.312 µm in diameter were found in the anterior air sacs (Hayter and Besch, 1974). In another study, microspheres 5 µm or greater in size were shown to be lodged in the lower airways of 4-week-old chickens while those that were ≥10 µm were scarcely deposited in the lungs and air sacs. In 2-week-old chickens, deposition of microspheres that were 5 µm or greater in size was significantly reduced in the lower airways, although particles of the

same size range were significantly higher in areas such as the nose and eyes, irrespective of the age of the chickens (Corbanie *et al*., 2006).

1.3. Commercial AIV vaccines for poultry

Currently, there are three types of AIV vaccines that have been licensed or approved for use in poultry (van den Berg *et al*., 2008). Inactivated vaccines, either whole or recombinant, require the use of an adjuvant and contain AIV viruses that have undergone propagation in eggs. Whole, inactivated AIV vaccines are either of the homologous (HA and NA are in common with the field subtype) or heterologous (only the HA glycoprotein is shared with the field subtype) category. Whole virus vaccines are reported to be of higher immunogenicity in comparison to other types (Lin *et al*., 2006) while inactivated vaccines are recognized for their ability to induce cross-protection in poultry (Swayne *et al*., 2000); inactivated vaccines are administered via the parenteral route with emulsions for the induction of potent antibody responses. The second commercially available option is the vaccines generated through reverse genetics (H5N1 and H5N3 subtypes) and, the third option includes fowlpoxvirus or Newcastle disease virus-vectored vaccines (van den Berg *et al*., 2008).

1.4. Vaccine adjuvants

A vaccine adjuvant is a substance used to elicit or enhance an antigen-specific immune response when administered in conjunction with a vaccine antigen *in vivo* (O'Hagan and Valiante, 2003). Desirable properties of an adjuvant include non-toxicity or minimal levels of toxicity, the capacity to elicit potent antibody-mediated and cell

mediated immune responses, the ability to induce immunological memory, minimal side effects (for example, non-mutagenic and non-carcinogenic) and stable under different temperatures and pH conditions for a considerable time period (Marciani, 2003).

Potentially protective antigens such as proteins, carbohydrates and lipids require co-administration with adjuvants in order to generate an adequate immune response. Administration of a killed, inactivated vaccine without the presence of an adjuvant can induce tolerance in naïve antigen-specific T cells (Schijns, 2000). Despite the fact that these immunomodulatory agents have been considered as essential components of "non-replicating" vaccines, knowledge of their mechanism of action is somewhat limited (Schijns, 2000). This is no surprise since an adjuvant can have more than one mechanism of action (Swayne and Kapczynski, 2009) creating a complex scenario of immunological consequences.

Broadly speaking, vaccine adjuvants have been traditionally classified on the basis of their origin, chemical criteria, physical properties or mechanisms of immunogenicity (Marciani, 2003). For purposes of this literature review, the initial focus will be on classification based on the concept of immunogenicity, followed by more modern approaches to classification. The following modes of action exist under the category of immunogenicity (Schijns, 2000):

1) The "geographical concept of immune reactivity," stems from the notion that initiation of an immune response is dependent on the ability of an antigen to reach or access naïve T cells present in peripheral lymphoid organs in order to provide signal 1 (antigen presentation) (Bachmann, Zinkernagel, and Oxenius, 1998). It is

thought that signal 2 (co-stimulation) is present at sufficient levels within the lymphoid organs and hence up-regulation is not a requirement, provided that signal 1 is present at the necessary levels (Schijns, 2000). Adjuvants that potentially mediate their effects via the "geographical concept" include immune stimulating complexes (ISCOMS), Quil A, $Al(OH)_3$, liposomes, cochleates and poly-lactic-co-glycolic acid (PLGA) microspheres (Schijns, 2000).

2) The concept of the depot effect is based on the idea that antigen persistence at the injection site or in the lymph node is ultimately responsible for the process of prolonged presentation of antigens (signal 1) to APCs. Examples of adjuvants in this category are oil emulsions, gels, microspheres and non-ionic co-polymers (Schijns, 2000).

3) The "signal 0 concept" refers to the recognition of adjuvants by PRRs on cells of the innate immune system with the subsequent activation of APCs. Such adjuvants include CpG rich motifs, LPS (monophosphoryl lipid A), *Mycobacterium* (muramyl dipeptide), cholera toxin, yeast extracts and complement (Schijns, 2000).

4) "Danger molecules"- the "danger model" (Matzinger, 1994) suggests that damaged cells or cells subjected to stress provide a "danger signal" that initiates an immune response through the activation of APCs such as DCs which are located in peripheral tissues. Moreover, it is suggested that these "danger signals" promote the expression of co-stimulatory molecules on APCs. This model promotes the view that the immune system has the ability to differentiate between

"dangerous" and "harmless" signals, as opposed to the more conventional immunological concept of distinguishing "self" from "non-self" (Matzinger, 1994). As such, it has been suggested recently that stress proteins induced as a result of cell damage can serve as "chaperones" for antigens, enhancing antigen capture and presentation by APCs (Colaco, 1999). Adjuvants in this category include oil emulsions, $Al(OH)_3$, IFNs and heat-shock proteins (Schijns, 2000).

5) Natural adjuvants (signal 2 molecules) - refer to the concept that the induction of inflammatory cytokines at the site of injection of an antigen with an adjuvant is potentially critical to the ability of the adjuvant to exert its effects. IL-1 is induced by $Al(OH)_3$, muramyl dipeptides and saponins (Schijns, 2000). IL-1, IL-6, IL-12 and TNF-α are induced by ISCOMS (Villacres-Eriksson et al., 1997; Watson *et al*., 1989). Some cytokines have undergone evaluation for potential use as adjuvants (Nohria and Rubin, 1994).

Recently, another approach is being explored for viewing the role of vaccine adjuvants, specifically, in terms of immune potentiators and (particulate) delivery systems. Immune potentiators (for example, TLR agonists) are compounds that, through the use of innate immune receptors, drive the activation of cells of the immune system in order to produce a pro-inflammatory response that is required for triggering an antigen-specific immune response. The presence of TLRs on B cells enables them to respond to immune potentiators in order to maximize the magnitude and type of immune response that follows (Liu *et al*., 2003). Details pertaining to the TLR9/21 agonist, CpG ODN have been discussed previously in the literature review.

Delivery systems (particulates) aim to contain vaccine components (both antigens and immune potentiators) in a localized, targeted administration site whereby they can be easily accessed by APCs while being released in a sustained manner (Panyam and Labhasetwar, 2003) (O'Hagan and Valiante, 2003). Delivery systems also provide an opportunity by which the frequency of vaccine administration can be minimized (dose-sparing effect) (Panyam and Labhasetwar, 2003). In addition to APCs being a major target for vaccine adjuvants, focus must also be given to B cells. Particulate adjuvants can enhance the activation of B cells by providing them with B cell epitopes that are repetitive in their nature (Bachmann *et al*., 1993; Jegerlehner *et al*., 2002). Furthermore, B cells that are activated by vaccine components are able to engage in more efficient antigen presentation with enhanced levels of cytokines being produced (O'Hagan and Valiante, 2003). Another mechanism by which delivery systems can induce immune responses is through the cross-presentation of exogenously derived antigens along the MHC class I pathway due to antigen leakage into the cytosol of cells subsequent to phagocytosis (Smith, Simon, and Baker Jr, 2013).

Vaccine carriers such as PLGA-based microparticles (MPs)/nanoparticles (NPs) have found widespread applications in the pharmaceutical industry. A distinction must be made between MPs and NPs on the basis of size; MPs range in size from 1 to 250 µm while NPs are between 10 and 1000 nm (Mundargi *et al*., 2008). Immune responses arising from particulate adjuvants are believed to be a function of particle size. The optimal size range for efficient phagocytosis is 200 nm to 1 µm (Kuroda, Coban, and Ishii, 2013). It is noteworthy that additional factors such as surface charge,

hydrophobicity, route of administration and the release kinetics also influence the ability of particulate adjuvants to induce adequate immune responses (Zhang *et al*., 2014). Antibody-mediated immune responses are reported to occur with the use of PLGA particles (MPs and NPs). In addition to the mechanisms of action outlined in the preceding paragraph, PLGA particles are also reported to exert effects at the level of the NLR pyrin domain containing 3 (NLRP3) inflammasome which induces the production of the active forms of IL-1β and IL-18 (Kuroda, Coban, and Ishii, 2013). To date, applications of a PLGA-based vaccine carrier has been restricted to a Newcastle disease virus DNA vaccine in chickens (Zhao *et al*., 2013); there have been no reports regarding applications to AIV.

Nanoemulsions are composed of two immiscible components in which the first phase, known as the dispersed phase, is incorporated into the second phase called the continuous phase and stabilized with the aid of surfactants or emulsifiers (Guy, 2007). Vaccine emulsions can be oil-in water (o/w) or water-in oil (w/o) as determined by the surfactants used during preparation and the fractional proportions of the incorporated phases. Surfactants are categorized based on their hydrophilic/lipophilic balance (HLB). Low HLB surfactants are associated with w/o emulsions, such as complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), Montanide ISA51 (50;50 o:w ratio) or Montanide ISA720 (70:30 o:w ratio) while the use of high HLB surfactants result in the formation of o/w emulsions (for example, MF59) (Guy, 2007). The advantages of using w/o emulsions relate to the induction of long term immune responses and a reduction in the quantity of vaccine required to generate an immune response (antibody-

mediated or cell mediated); o/w emulsions are associated with the induction of potent short term immune responses. Water-in-oil-in-water (w/o/w) emulsions display properties reminiscent of both w/o and o/w emulsions. The kinetics of release of vaccine emulsion components is determined by the properties of the emulsion and the rate of release from the administration site. The mechanisms of action of vaccine emulsions are believed to be varied. In addition to the depot effect, emulsions prevent the rapid degradation of vaccine components, induce inflammatory responses, recruit cells to sites of vaccine administration, assist in the uptake of antigenic components by APCs and facilitate the mechanism of lymphocyte trapping and cytokines secretion (Aucouturier, Dupuis, and Ganne, 2001).

CHAPTER 2

SYSTEMIC IMMUNE RESPONSES TO AN INACTIVATED, WHOLE H9N2 AVIAN INFLUENZA VIRUS VACCINE USING CLASS B CPG OLIGONUCLEOTIDES IN CHICKENS

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2.1. Abstract

Commercial vaccines against avian influenza viruses (AIV) in chickens consist mainly of inactivated AIV, requiring parenteral administration and co-delivery of an adjuvant. Limitations in T helper 1 or T helper 2 biased responses generated by these vaccines emphasize the need for alternative, more efficacious adjuvants. The Toll-like receptor (TLR) 21 ligand, CpG oligodeoxynucleotides (ODN), has been established as immunomodulatory, causing a T_H1-biased response in chickens. Therefore, the objective of this study was to investigate the adjuvant potential of high (20 µg) and low (2 µg) doses of CpG ODN 2007 (CpG 2007) and CpG ODN 1826 (CpG 1826) when administered to chickens with a formalin-inactivated H9N2 AIV (selected doses were based on previous dose titration studies (Gomis *et al*., 2007) and prophylactic studies conducted in chickens (St. Paul *et al*., 2012)). Antibody responses in sera were evaluated in 90 specific pathogen free (SPF) chickens after intramuscular administration of vaccine formulations at 7 and 21 days post-hatch. Antibody responses were assessed based on haemagglutination inhibition (HI) and virus neutralization (VN) assays; virus-specific IgM and IgY antibody responses were evaluated by ELISA. The results suggest that the vaccine formulation containing low dose CpG 2007 was significantly more effective at generating neutralizing (both HI and VN) responses than formulations with high or low doses of CpG 1826 or high dose CpG 2007. Neutralizing responses elicited by low dose CpG 2007 significantly exceeded those generated by a squalene-based adjuvanted vaccine formulation during peak observed responses as defined over a 42 day period. A significantly higher IgM response was elicited by the formulation containing low dose CpG 2007 compared to high and low doses of 1826. The magnitude of the IgY response

elicited by the low dose of CpG 2007 was not statistically significant.compared to that of CpG 1826 In conclusion, 2 µg of CpG 2007 is potentially promising as a vaccine adjuvant when delivered intramuscularly with inactivated H9N2 virus to chickens. Future studies may be directed at determining the mucosal antibody responses to the same vaccine formulations.

Keywords: Toll-like receptor 21, low pathogenic avian influenzavirus, H9N2 subtype, CpG oligodeoxynucleotides, chickens, antibody response, hemagglutination inhibition, virus neutralization, ELISA

2.2. Introduction

Avian influenza virus (AIV), a single-stranded, negative-sense RNA virus, is a member of the family *Orthomyxoviridae*. Influenza viruses have been classified as types A, B or C with type A being important in avian species (de Geus, Rebel, and Vervelde, 2012). Influenza A viruses are further categorized into subtypes based on the haemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. Currently, all existing influenza A subtypes have been isolated from wild birds (Costa *et al*., 2011) with the exception of the recently identified influenza-like viruses (H17N10 and H18N11) found exclusively in bats (Wu *et al*., 2014). Influenza A subtypes frequently (but not solely) associated with domestic poultry include H5, H7 and H9 (Alexander, 2000).

Avian influenza virus infections are of significance to the poultry industry from economic and public health perspectives. In recent years, there have been increasing concerns regarding virus transmission from birds to humans with the low pathogenic H9N2 AIV being implicated in sporadic outbreaks in Europe and Asia (Perdue and Swayne, 2005). The H9N2 subtype that displays differences in the levels of pathogenicity (Gharaibeh, 2008) remains endemic in Asia and the Middle East (Tse *et al*., 2013).

Vaccination is an important and effective means of controlling AIV in poultry when used in conjunction with other elements of an AIV control program. The majority of commercially available AIV vaccines are of the whole virus, inactivated type (Swayne and Kapczynski, 2008) administered by parenteral routes and requiring adjuvants for the induction of antigen-specific immune responses. Inactivated influenza virus vaccines, when administered intramuscularly (IM) in humans, elicit serum antibodies believed to

confer protection from lower respiratory tract challenges through a mechanism of lung transudation (Chen *et al*., 2001).

The adjuvant effects of synthetic or natural Toll-like receptor (TLR) ligands make these ligands promising candidates to induce serum and mucosal responses. Toll-like receptors are the most widely studied members of pattern recognition receptors (PRRs) that recognize pathogen associated molecular patterns (PAMPs) (Akira, Uematsu, and Takeuchi, 2006). Of the ten TLRs identified in chickens to date (Jungi *et al*., 2011), TLR21, the functional homologue of TLR9 in humans and mice, is responsible for recognition of CpG-oligodeoxynucleotides (ODN) (Bode *et al*., 2011). The induction of cytokine responses as a result of binding of CpG-ODN to TLR-9/21 (Herbáth *et al*., 2014) enhances host innate responses and subsequent adaptive immune responses. Generally, class B CpG ODNs are known to be strong activators of B cells (Krieg *et al.*, 1995) and are thus capable of enhancing production of neutralizing antibodies. B cells treated with class B ODN show upregulation of transcripts associated with antigen presentation, such as CD80 and major histocompatibility complex (MHC) class II molecules (St Paul, Paolucci, and Sharif, 2012).

The adjuvant effects of synthetic CpG-ODNs in the context of AIV vaccines have been studied in chickens to a limited extent (Wang *et al*., 2009). Although the efficacy of CpG-ODNs has been demonstrated in a few chicken vaccine trials, further studies are needed to explore the potential of these compounds as adjuvants (Fu *et al*., 2013). The adjuvant potential of two members of class B CpG ODN, namely, CpG ODN 2007 (CpG 2007) and CpG ODN 1826 (CpG 1826) was therefore investigated in combination with a formalin-inactivated H9N2 virus after intramuscular administration to SPF chickens.

2.3. Materials and methods

2.3.1. Chickens

Ninety, one-day-old specific-pathogen free (SPF) White Leghorn chickens were obtained from the Canadian Food Inspection Agency (Ottawa, Canada) and were kept in the isolation facility of the Ontario Veterinary College, University of Guelph. Feed and water were provided ad libitum during the entire experiment. Experimental procedures were approved by the University of Guelph Animal Care Committee and conducted in compliance with the guidelines of the Canadian Council on Animal Care.

2.3.2. Avian influenza virus

The H9N2 virus (A/Turkey/Ontario/1/66) was propagated in 10-day-old embryonated SPF chicken eggs. The egg-derived virus was inactivated with formalin (final concentration 0.02%) for 72 hours at 37°C as indicated previously (St. Paul *et al*., 2014b). The complete inactivation of the virus was confirmed through repeated passage in 10-day-old embryonated SPF chicken eggs and Madin-Darby canine kidney (MDCK) cells as previously described (St. Paul *et al*., 2014b). The MDCK cells were sourced from the Animal Health Laboratory, University of Guelph. The protein content of the inactivated virus preparation was determined using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL) as per manufacturer's recommendation. Finally, a hemagglutination (HA) assay was used to assess the surface integrity of the hemagglutinin protein of the AIV after formalin inactivation (St. Paul *et al*., 2014b).

2.3.3. Immunization studies

The immunogenicity of an inactivated H9N2 vaccine was tested in two parallel experiments to confirm repeatability of results (replicates 1 and 2) after intramuscular administration of the vaccine formulations to 7-day-old SPF chickens (n=5 to 7 per group). Primary and secondary immunizations were done at days 7 and 21 post-hatch. Each bird received 15 µg of the inactivated virus diluted with phosphate buffered saline (PBS) in various combinations as indicated below. A dose of 15 µg of virus was selected based on the dose used for pandemic and epidemic influenza vaccines (Kreitz, Osterhaus, and Rimmelzwaan, 2009)

Phosphorothioate backbone modified CpG 2007 (5′-TCGTCGTTGTCGTTTTGTCGTT-3′) and CpG 1826 (5′-TCCATGACGTTCCTGACGTT-3′) (CpG motifs underlined) and their non-CpG ODN controls were purchased from Sigma-Aldrich Canada and used at a high dose (20 µg/chicken) and a low dose (2 µg/chicken). Non-CpG ODN sequences were 5′-TGCTGCTTGTGCTTTTGTGCTT-3′ and 5′-TCCATGAGCTTCCTGAGCTT-3′ for 2007 and 1826, respectively. To establish the effects of range of doses, a high dose (20 µg/chicken) was selected based on preliminary experiments conducted at our laboratory (St. Paul *et al*., 2012) while a 10-fold decrease was used as the low dose (2 µg/chicken). All CpG ODNs were reconstituted in sterile endotoxin-free water. A squalene-based, oil-in-water emulsion, AddaVax^{TM}, (InvivoGen, San Diego, California, USA) was used as a positive control adjuvant according to the manufacturer's recommendations.

Chickens in group 1 were vaccinated with AIV and AddaVax^{TM}. Chickens in groups 2 and 3 were vaccinated using AIV with 2 µg and 20 µg of CpG 2007, respectively. Chickens in groups 4 and 5 received AIV with CpG 1826 (2 µg) and AIV with CpG 1826 (20 µg), respectively. Chickens in groups 6 and 7 were vaccinated with non-CpG ODN 2007 and non-CpG ODN 1826.

2.3.4. Haemagglutination inhibition (HI)

Sera were collected on days 7, 14, 21, 28, 35 and 42 days post-hatch to evaluate the kinetics of the antibody-mediated immune responses. Twenty-five microlitres (25 µl) of two-fold serially diluted serum samples from each time-point per chicken were incubated with an equal volume of H9N2 virus (4 haemagglutinating units (HAUs)) for 30 minutes at room temperature in 96-well V-bottom plates (Costar® Corning Inc.) Washed chicken red blood cells (RBCs) (Lampire Biologicals) at a concentration of 0.5% were added and the plates were further incubated for 30 minutes. The HI titres were expressed as the $\log_2$ of the reciprocal of the highest serum dilution resulting in complete inhibition of haemagglutination of RBCs.

2.3.5. IgM and IgY ELISAs

Virus-specific antibody titres in sera were determined using an indirect ELISA as described previously (Mallick *et al*., 2011) with modifications. Briefly, 96 well polystyrene plates (Nunc, Maxisorp) were coated overnight at 4°C with whole, inactivated H9N2 virus using an optimally determined concentration of 0.8 µg/100 µl in carbonate-bicarbonate buffer (pH 9.6). The range of coating concentrations evaluated

included: .05 µg/100 µl, 0.2 µg/100 µl, 0.8 µg/100 µl, 3.2 µg/100 µl and 5 µg/100 µl The plates were washed three times with 250 µl (per well) of PBS containing 0.05% Tween 20 (Sigma-Aldrich, St. Louis, Missouri, USA), then blocked with 100 µl (per well) of 0.25% fish skin gelatin (Sigma-Aldrich) and 0.05% Tween 20 in PBS (blocking buffer) and incubated for 1 hour at room temperature. After removal of the blocking buffer, 100 µl of serum diluted 1/50 in a solution consisting of 1.5% Tween-20 in PBS (with 0.29 M NaCl) (Hodgins and Shewen, 2000), was added to each well and further incubated at room temperature for 1 hour. Each serum sample was assayed in duplicate. The plates were then washed 3 times prior to addition of 100 µl of goat anti-chicken IgY (Fc specific) or goat anti-chicken IgM (µ chain specific) antibodies conjugated to horse-radish peroxidase (HRP) (Bethyl Laboratories, Montgomery, Texas), as previously described (Mallick *et al.*, 2011). The plates were incubated for 1 hour at room temperature. Positive (hyper-immune serum from a separate experiment) and negative controls (phosphate buffered saline and sera from non-immunized chickens) were included for each plate The plates were washed again and 100 µl of an HRP substrate solution (ABTS peroxidase substrate system; Kirkegaard and Perry Laboratories Gaithersburg, Maryland, USA) were added to each well, followed by an incubation period of 15-20 minutes in the dark at room temperature before addition of 100 µl of a 1% sodium dodecyl sulfate (SDS) stop solution. The optical density (OD) was determined at 405 nm using an ELISA plate reader (Bio-Tek Instruments, Winooski, Vermont USA). The data were reported as a sample to positive (S/P) ratio using the following calculation: S/P= (Mean OD405 of the sample minus the mean OD405 of the

blank control)/ Mean OD405 of the positive control minus the mean OD405 of the blank control).

2.3.6. Hyper-immune serum for optimization of ELISAs

Five, one-day-old SPF chickens were used for the production of hyper-immune serum against the formalin inactivated A/Turkey/Ontario/1/66/H9N2 virus. Each chicken received three IM injections of the formalin-inactivated virus (15 µg/bird) with AddaVax^{TM} adjuvant (as per manufacturer's recommendation) in 200 µl of PBS buffer. The IM injections were administered at 7, 21 and 35 days post-hatch. Sera were collected at days 14, 21, 27, 35 and 42 post-hatch, separated by centrifugation at 3,000 x *g* at 20°C for 15 minutes and stored at -80°C until further use.

2.3.7. Virus neutralization

Virus neutralization titres for serum samples were determined as outlined in Current Protocols in Immunology, Support Protocol 11 (Cottey, Rowe, and Bender, 2001) with modifications. Serial 2-fold dilutions of sera (starting dilution, 1:10) were made in 96-well round-bottom plates using Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS) containing 50 µg/ml gentamicin, 100 U/ml penicillin and 100 µg/ml streptomycin. An equal volume (50 µl) of diluted virus containing 50 tissue culture infectious dose 50 (50 $TCID_{50}$) was added to the diluted sera and incubated for 1 hour at 37°C. One hundred microlitres of this mixture were transferred to 96-well plates containing a 90% confluent monolayer of MDCK cells and incubated for 72 hours at 37°C and 5% CO_2. Each plate included control wells with serum only (no virus), virus

only (no serum) and uninfected cells (cell control). The VN titres were expressed as the $\log_2$ of the reciprocal of the highest serum dilution resulting in an absence of cytopathic effects.

2.3.8. Statistical analyses

Data from the two replicate experiments were pooled for statistical analyses using general linear models (GLMs) in SAS (version 9.2). Antibody titres for HI and VN assays were expressed as the $\log_2$ of the reciprocal of the endpoint dilution, for purposes of analysis. When significant differences were evident among groups, Duncan's multiple range test was used to determine which group means differed significantly. A GLM procedure was also used for S/P ratios for ELISA data analysis. Results were considered significant if $p<0.05$. The measure of goodness-of-fit of linear regression, R^2 was determined between virus neutralization titres and hemagglutination titres at 42 days of age using Pearson's correlation procedure. Seroconversion and seroprotection rates based on HI assays were compared for each vaccine treatment group using Fisher's exact test.

2.4. Results

2.4.1 Haemagglutination inhibition and neutralization antibodies in sera

Data from two replicate experiments were pooled for statistical evaluation to increase statistical power and improve the accuracy of the estimates of variance. All vaccinated groups attained a pre-vaccination (day 7) to post-vaccination (day 42) geometric mean increase of ≥ 2.5, based on HI titres (Fig. 1). The seroconversion (a 4-fold or greater increase in HI titre between pre-vaccination and post-vaccination sera) rate of chickens

receiving the vaccine containing 2 µg of CpG 2007 was significantly higher ($p<0.05$) (100%) than other treatment groups by day 28, including the squalene adjuvanted vaccine (50%) treatment group (Table 1). At 42 days post-hatch, the seroconversion rates for the low dose (2 µg) CpG 2007 and squalene adjuvanted vaccine groups were equal and significantly higher (100%) than the rate for high dose (20 µg) CpG 2007 (58%). The highest seroprotection rate (the percentage of chickens attaining a post-vaccination titre of 1/40 in each treatment group) at day 28 post-hatch was attained with low dose (2 µg) CpG 2007 (83%). The seroprotection rates of high dose (20 µg) CpG 2007 (58%) and low dose (2 µg) CpG 1826 (50%) treatment groups were significantly lower than that of low dose (2 µg) CpG 2007 (100%) at day 42 post-hatch (Table 1). The vaccine formulation containing the inactivated virus with low dose CpG 2007 induced the highest mean HI antibody titres of all formulations from days 21 to 42 post-hatch. Mean HI antibody responses to AIV vaccine containing low dose CpG 2007 were significantly higher than responses to vaccine containing high dose CpG 2007 and significantly higher than responses to vaccines adjuvanted with CpG 1826 (low or high dose) at days 28, 35 and 42 post-hatch. Mean HI antibody titres generated by the vaccine formulation containing low dose CpG 2007 were higher than those for the squalene adjuvanted vaccine at days 28, 35 and 42 post-hatch (significantly higher on days 28 and 42). The high dose of the CpG 1826 vaccine formulation (20 µg) elicited higher serum antibody responses compared to the low dose of CpG 1826 from day 28 to 42 but the difference was only significant at day 42.

To gain insight into the functionality of the antibodies elicited by vaccination, a virus neutralization assay using MDCK cells was used as an adjunct to the HI assay. Based on

the virus neutralization assay, the vaccine formulation containing CpG 2007 (2 µg) resulted in significantly higher mean titres compared to those for CpG 1826 at days 28, 35 and 42 (Fig. 2). The same vaccine formulation elicited significantly higher mean titres compared to the squalene formulation at days 28 and 42. There was a high correlation between HI and virus neutralization titres for day 42 (R^2 =0.945).

2.4.2. Serum IgM and IgY ELISA antibody responses

To gain further understanding of the dynamics of the antibody responses, IgM and IgY antibodies in serum were quantified by ELISA using S/P ratios. The positive control for the S/P ratio calculations was from an independent experiment as described in section 2.3.6. Mean S/P ratios for IgM antibodies peaked at day 28 (1 week post-secondary immunization, Fig. 3). At day 28, IgM S/P ratios were significantly higher for chickens receiving the vaccine formulation containing 2 µg of CpG 2007 than for chickens receiving CpG 1826 or squalene adjuvants.

Serum IgY antibody concentrations were also determined. At day 28 mean S/P ratios were significantly higher for chickens receiving the vaccine formulation containing 2 µg of CpG 2007 compared to the high dose CpG 2007 group (Fig. 4). Peak mean S/P ratios occurred at day 42; at days 35 and 42, low dose CpG 2007 and AddaVax™ vaccine formulations had the highest mean S/P ratios but differences among the groups were not statistically significant.

2.5. Discussion

A few studies have demonstrated the immunomodulatory properties of the Toll-like receptor 21 adjuvant, CpG, in chickens (St. Paul *et al*., 2013; Wang *et al*., 2009); however, limited data are available regarding the evaluation of various doses of this adjuvant for enhancing the immunogenicity of AIV vaccines. Here we report that a dose of 2 µg of CpG 2007, when administered intramuscularly with a formalin-inactivated H9N2 virus induces significantly higher serum antibody responses (HI and VN) compared to 20 µg of CpG 2007, although the exact dose-dependent mechanism of action requires further investigation. Furthermore, CpG 2007 at a dose of 2 µg was more efficacious than vaccine formulations containing equivalent high and low doses of another class B CpG member, CpG 1826. The "human" CpG motif, GTCGTT (characteristic of CpG 2007), has been demonstrated to be more effective at stimulating lymphocyte proliferation than the "mouse" motif, GACGTT (found in CpG 1826) in various species such as chickens (Mutwiri *et al*., 2003). In our study, differences observed in CpG ODN dose-response mechanisms may be related to an expansion of CD4+T regulatory cells due to higher doses of CpG, which have been described in mice and humans (Kulkarni, Behboudi, and Sharif, 2011; Negash, Liman, and Rautenschlein, 2013).

Evaluation of influenza vaccine immunogenicity is largely based on haemagglutination inhibition antibody titres, seroconversion and seroprotection rates (the proportion of vaccinated human subjects attaining a post-vaccination titre of 1/40) (Ainai *et al*., 2013; Beyer *et al*., 2011); however, seroprotection rates are less well-defined in chickens. In this study, the impact of time on the emergence of the immune responses

must also be considered. By 42 days of age, peak levels of neutralizing antibodies were detected using the HI and virus neutralization assays, which were shown to be highly statistically correlated. IgM antibody titres were shown to peak at day 28 (1 week post-secondary vaccination). The vaccine formulation containing the low dose of CpG 2007 elicited the highest IgM antibody titre at day 28 and significantly exceeded the immune responses generated by the AddaVax^{TM} (squalene) and CpG 1826 vaccine formulations. IgM antibodies are particularly important in providing early protection against influenza virus infections (Skountzou *et al*., 2014). While information on the importance of influenza induced IgM antibody responses in chickens is lacking, Qui and colleagues (Qiu *et al*., 2011) have suggested that in humans the extent of this response may be important in predicting virus clearance rates and can influence later IgG antibody responses. Although, the highest IgY antibody titres generated at day 42 were produced with the vaccine formulations containing the low dose of CpG 2007 and squalene, these were not statistically different from other treatment groups at the same time-point. However, these formulations were able to elicit higher titres than the high dose of CpG 2007 at day 28.

In conclusion, we showed that a lower dose (2 µg) of CpG 2007 was able to elicit higher serum antibody responses compared to a higher dose of the same adjuvant in chickens. Additionally, we showed that a dose of 2 µg of CpG 2007 generated higher antibody responses than another class B CpG ODN member, 1826. Future studies may be aimed at determining the mucosal antibody responses of these vaccine formulations when administered with inactivated virus.

Table 1: **Seroconversion and seroprotection rates expressed as percentages for vaccine groups**

	Seroconversion rates (% post-hatch)			Seroprotection rates (% post-hatch)		
Group	Day 28	Day 35	Day 42	Day 28	Day 35	Day 42
AddaVax	50	100	100	42.9	71.4	71.4
Low CpG 2007	100	100	100	83.3	100	100
High CpG 2007	50	58.3	58.3	50	58.3	58.3
Low CpG 1826	37.5	71.4	71.4	14.3	28.6	50
High CpG 1826	37.5	64.3	85.7	35.7	50	78.6

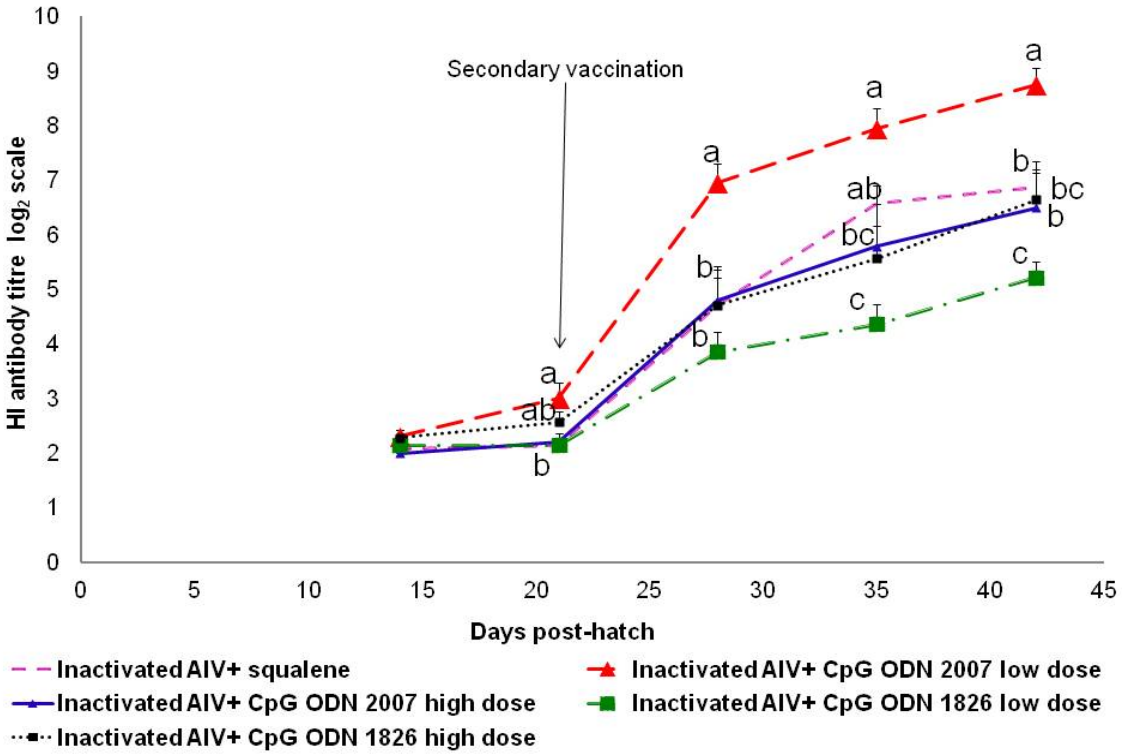

Figure 1: Mean serum hemagglutination inhibition (HI) antibody titres.

Chickens were vaccinated at 7 and 21 days post-hatch using formalin-inactivated H9N2 virus with low or high doses of CpG 2007 or CpG 1826 as adjuvants. A commercial adjuvant containing squalene in an oil-in-water emulsion was used as a positive control adjuvant. Within a single timepoint, means with the same letter do not differ significantly. Error bars indicate standard errors of the mean. Non-CpG 2007 and CpG 1826 without inactivated avian influenza virus (AIV) did not generate detectable antibody titres (titre of 1/4 [i.e. 2 in the figure, in $\log_2$ scale]) of the HI assay (data not shown). Data were analyzed using Proc GLM with Duncan's Multiple Range test, post ANOVA ($p< 0.05$).

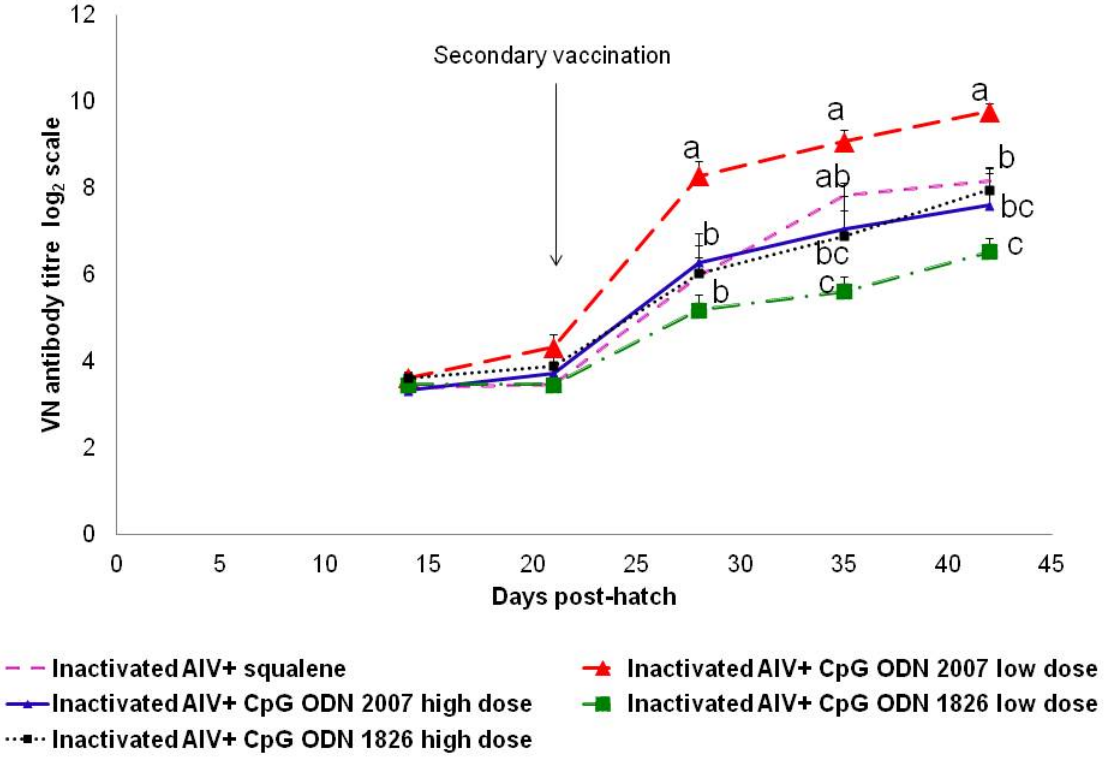

Figure 2: Serum virus neutralization antibody titres.

Chickens were vaccinated at 7 and 21 days post-hatch with formalin-inactivated H9N2 virus with low or high doses of CpG 2007 or CpG 1826 as adjuvants. Means with the same letter do not differ significantly. Error bars indicate standard errors of the mean. Data were analyzed using Proc GLM with Duncan's Multiple Range test, post ANOVA ($p< 0.05$).

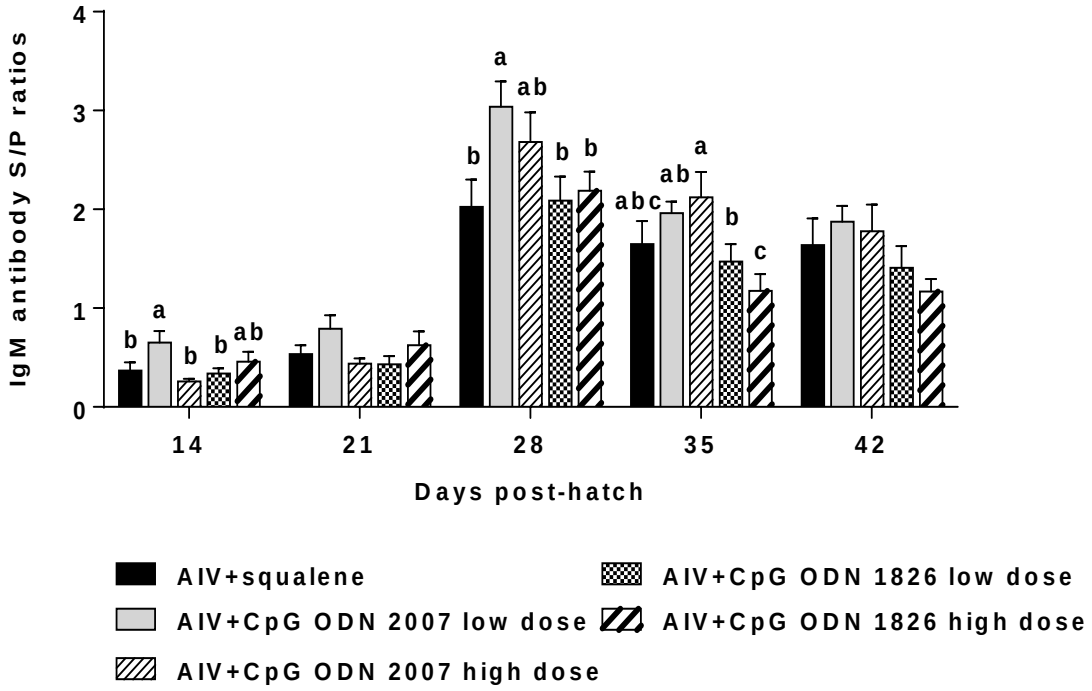

Figure 3: Serum IgM antibody sample to positive (S/P) ratios for avian influenza virus (H9N2) adjuvanted with low or high doses of CpG 2007 and CpG 1826.

Within a single time-point, means with the same letter do not differ significantly ($p < 0.05$).

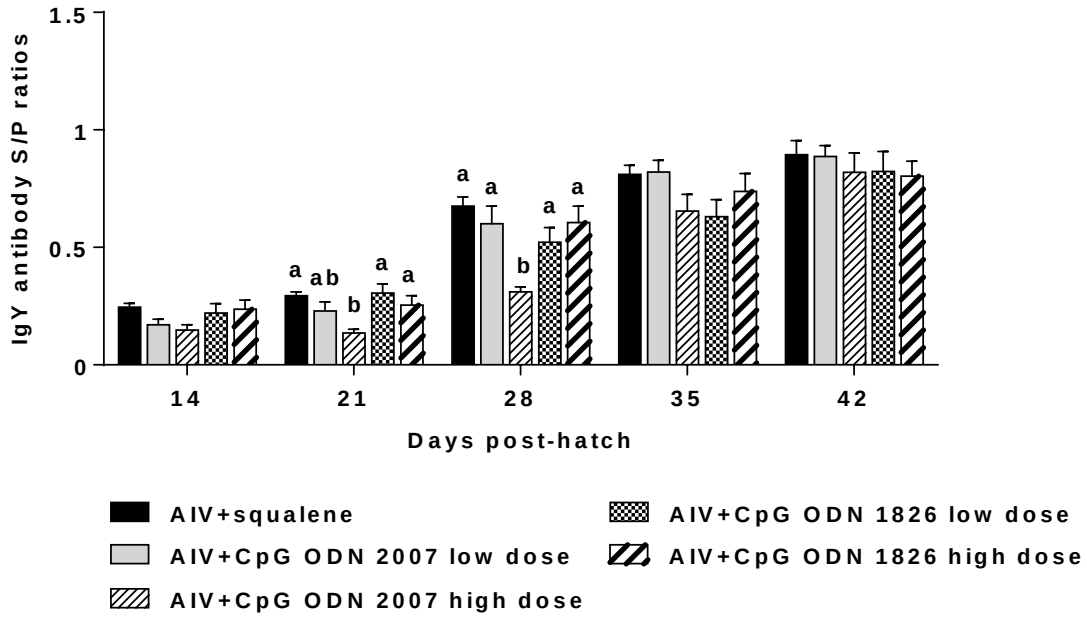

Figure 4: Serum IgY antibody sample to positive (S/P) ratios for avian influenza virus (H9N2) adjuvanted with low or high doses of CpG 2007 and CpG 1826.

Within a single time-point, means with the same letter do not differ significantly ($p < 0.05$).

2.6. Acknowledgements

This research was funded by the Poultry Industry Council and the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA). We wish to thank staff members at the Ontario Veterinary College Isolation Facility for rendering assistance with the housing and daily care of the chickens.

CHAPTER 3

CHARACTERIZATION OF IMMUNE RESPONSES TO AN INACTIVATED AVIAN INFLUENZA VIRUS VACCINE ADJUVANTED WITH NANOPARTICLES CONTAINING CPG ODN

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3.1. Abstract:

Avian influenza virus (AIV), a mucosal pathogen, gains entry into host chickens through respiratory and gastrointestinal routes. Most commercial AIV vaccines for poultry consist of inactivated, whole virus with adjuvant, delivered by parenteral administration. Recent advances in vaccine development have led to the application of nanoparticle emulsion delivery systems, such as poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles to enhance antigen-specific immune responses. In chickens, the Toll-like receptor (TLR) 21 ligand, CpG oligodeoxynucleotides (ODNs), have been demonstrated to be immunostimulatory. The objective of this study was to compare the adjuvant potential of CpG ODN 2007 encapsulated in PLGA NPs with nonencapsulated CpG ODN 2007 when combined with a formalin-inactivated H9N2 virus, via intramuscular and aerosol delivery routes. Chickens were vaccinated at days 7 and 21 post-hatch for the intramuscular route and, at days 7, 21 and 35 for the aerosol route. Antibody- mediated responses were evaluated weekly in sera and lacrimal secretions in specific pathogen free (SPF) chickens. The results indicate that nonencapsulated CpG ODN 2007 in inactivated AIV vaccines administered by the intramuscular route generated higher antibody responses compared to the encapsulated CpG ODN 2007 formulation by the same route. Additionally, encapsulated CpG ODN 2007 in AIV vaccines administered by the aerosol route elicited higher mucosal responses compared to nonencapsulated CpG ODN 2007. Future studies may be aimed at evaluating protective immune responses induced with PLGA encapsulation of AIV and adjuvants.

Keywords: CpG-ODN, vaccine, PLGA, chicken, antibody response, avian influenza virus, H9N2 subtype.

3.2. Introduction

Avian influenza virus (AIV), a single-stranded RNA virus of low pathogenicity (LP) or high pathogenicity (HP), enters potentially susceptible hosts through mucosal routes such as the respiratory and gastrointestinal tracts. Despite the fact that mucosal routes serve as portals of entry for AIV into susceptible hosts, many of the AIV vaccines approved for use in poultry are inactivated, whole virus vaccines, delivered with water-in-oil emulsion adjuvants (Swayne and Kapczynski, 2009) through parenteral routes. The ability to identify an efficacious mucosal adjuvant that elicits an antibody response when administered with an inactivated virus by the aerosol route could be advantageous for improved safety and for mass administration. This approach, however, has not been successfully employed in chickens although a previous attempt was made to investigate the immunogenicity of whole, inactivated H9N2 AIV delivered by single aerosol administration with aluminum hydroxide, chitosan, cholera toxin B subunit and Stimune (de Geus *et al*., 2011). Recently, the ability of aerosolized H9N2 AIV to cause infection in the respiratory tract of chickens has been demonstrated (Guan, Fu, and Sharif, 2015), further emphasizing the need to explore the potential of aerosol delivery of vaccine.

Adjuvants play a critical role in enhancing vaccine efficacy by improving antigen-specific immune responses through modulation of the magnitude and type of adaptive immune responses generated (Coffman, Sher, and Seder, 2010). Pathogen-associated molecular patterns (PAMPs), ligands for pattern recognition receptors (PRRs), are considered to be adjuvants since activation of cells of the innate immune system is a pre-requisite for induction of an adaptive immune response (Vilaysane and Muruve, 2009).

Toll-like receptor (TLR) ligands have been established as immunostimulatory in several species including chickens (St Paul *et al*., 2013) and therefore can be exploited to enhance immune responses to AIV vaccines in the capacity of prophylactic administration (St. Paul *et al*., 2012) or as vaccine adjuvants (Gupta *et al*., 2014) (St. Paul *et al*., 2014a; St. Paul *et al*., 2014b). Toll-like receptor 21 is regarded as the pattern recognition receptor for CpG ODN in chickens (Keestra *et al*., 2010). Recent evidence suggests that TLR15 is significant in the recognition of CpG ODN by chicken macrophages (Ciraci and Lamont, 2011), however, the implication of this finding is currently unknown. The evolution of vaccine development has resulted in increased emphasis on application of novel adjuvants like CpG ODNs and particulates such as polymeric nanoparticles [for example, poly(D,L-lactic-co-glycolic acid) (PLGA)], that function mechanistically as immune potentiators and delivery systems, respectively (Huang et al., 2010; Jain, O'Hagan, and Singh, 2011). Gomis *et al*. (2003) have demonstrated local and systemic protective effects of CpG ODN 2007 (a class B CpG ODN) against *Escherichia coli* infection in chickens. Furthermore, CpG ODN 2006, another class B ODN, has been shown to enhance host immunity in chickens when delivered intranasally with inactivated H5N1virus, resulting in the generation of antigen-specific antibodies in the respiratory tract and serum (Liang *et al*., 2013). The immunogenicity of a formalin-inactivated H9N2 virus administered intramuscularly with CpG ODN 2007 has also been shown in chickens (Singh *et al*., 2015). A previous study by Mallick and colleagues (Mallick *et al*., 2011) has also demonstrated the induction of enhanced immunogenicity (antibody-mediated and cell-mediated) of an H4N6 virosome-based vaccine containing CpG ODN as an adjuvant, delivered subcutaneously in

chickens. Nanoparticles, particulate materials ranging from 1-1000 nm in size, possess features of biodegradability and biocompatibility and can function as delivery systems and immunostimulants (Zhao *et al*., 2014). Nanoparticle emulsion-containing vaccines confer advantages such as enhanced immunogenicity, prevention of degradation of encapsulated contents, persistence and prolonged release (depot effect) at the site of administration, facilitating targeted delivery to antigen-presenting cells (APCs) with uptake by endocytosis, and an overall dose-sparing effect (Jain, O'Hagan, and Singh, 2011; Zhao *et al*., 2014). Non-condensing synthetic polymers such as PLGA are particularly useful for targeting APCs due to their neutral or slightly negative charge (Bhavsar and Amiji, 2007). The size of nebulized nanoparticles can be optimized for deposition at specific sites along the respiratory tract, maximizing the possibility of interaction with APCs. Small particles in the range of 1-3 µm have been shown to have a more homogenous distribution in the respiratory tract of chickens (Corbanie *et al*., 2006).

The immunogenicity of these novel adjuvants and delivery systems in combination with inactivated AIV in chickens has been investigated to a limited degree, particularly in the context of different routes of administration. We have previously demonstrated the adjuvant potential of CpG ODN 2007 (CpG 2007) at a dose of 2 µg, in combination with a formalin-inactivated H9N2 virus delivered intramuscularly (Singh *et al*., 2015). Therefore, the present study was aimed at comparing the adjuvant potential of nonencapsulated CpG 2007 (2 µg) with PLGA-encapsulated CpG 2007 (2 µg) in combination with a formalin-inactivated H9N2 virus using intramuscular and aerosol routes of administration.

3.3. Materials and Methods

3.3.1. Chickens

Specific pathogen-free (SPF) chickens were acquired from the Canadian Food Inspection Agency (Ottawa, Canada). All animal procedures were approved by the University of Guelph Animal Care Committee and conducted under strict compliance with the guidelines of the Canadian Council on Animal Care.

3.3.2. CpG 2007 and non-CpG 2007 sequences

CpG 2007 containing a modified phosphorothioate backbone (5′-TCGTCGTTGTCGTTTTGTCGTT-3′) and non-CpG 2007 (5′-TGCTGCTTGTGCTTTTGTGCTT-3′) were purchased from Sigma-Aldrich Canada and reconstituted in sterile endotoxin-free water.

3.3.3. Avian influenza virus strain and immunization studies

Formalin (final concentration 0.02%) inactivated H9N2 virus (A/Turkey/Ontario/1/66) was prepared as described previously (Singh *et al*., 2015). Ninety-six, one-day-old SPF White Leghorn chickens were randomly allocated to 16 treatment groups with each treatment group containing 6 chickens. Chickens in groups 1-8 were vaccinated intramuscularly while those in groups 9-16 were vaccinated through the aerosol route using the same vaccine formulations as shown in Table 2. Primary vaccination was performed on day 7 post-hatch while secondary vaccination occurred on day 21 post-hatch. In the case of aerosol administration, a third vaccination was carried out on day 35 post-hatch. Each chicken received 15 µg of formalin-inactivated virus with or without 2 µg (selected dose based on a previous study) (Singh *et al*., 2015) of various

encapsulated or nonencapsulated ODN formulations (except for negative control chickens). The aerosol chamber set up for administering vaccine formulations in this experiment was previously established and tested for delivery of cell-free aerosols of Marek's disease virus in our laboratory (Abdul-Careem *et al*., 2009). The dimensions of the chambers were 40 cm x 20 cm x 25 cm and aerosols (1.91 µm in size) were nebulized into the chamber as previously described (Abdul-Careem *et al*., 2009). Sera and lacrimal secretions were collected weekly until day 42 post-hatch for evaluation of the dynamics of serum and mucosal immune responses after administration of vaccine formulations using two routes of delivery.

3.3.4. Encapsulation of CpG ODN 2007 and non-CpG ODN 2007 in nanoparticles (NPs)

The polyethylenimine (PEI)-ODN (CpG 2007 or non-CpG) complex was formed by mixing 434 µg of ODN/125 µl of DNAse/RNAse free water with a solution of PEI (low molecular weight, Sigma-Aldrich) prepared in 150 mM NaCl as described by Boussif and colleagues (Boussif *et al*., 1995). This complex was sonicated in 56 mg of PLGA Resomer® RG 503H (acid terminated, 24-38 kD; Sigma-Aldrich), then dissolved in 1.25 ml of dichloromethane (Sigma-Aldrich) using a tip sonicator (Ultrasonic processor, 3 mm probe diameter, Fisher scientific) for 1 min (10 second sonication and 5 second pause at 40% amplitude). Subsequently, the resulting emulsion was sonicated with 3.25 ml of 2% polyvinyl alcohol (PVA) and 1% poloxamer 407 (30-70 kD and 87-90% hydrolyzed PVA and Poloxamer; Sigma-Aldrich) as described above. This emulsion was poured into 50 ml of 2% PVA/1% poloxamer solution and stirred for evaporation of dichloromethane. The particles were collected by centrifugation at 20,000

x g for 30 minutes at 4°C, washed three times in HyClone, Molecular-Biology Grade water (GE, Healthcare Life Sciences) lyophilized and stored at 4°C until use. Blank PLGA NPs (mock-NPs) were produced using a similar method.

The loading and encapsulation efficiency of ODNs was determined by dissolving a known quantity of lyophilized NPs (1 mg/ml in Tris-EDTA buffer) in 1 ml dichloromethane (Peine et al., 2013). The amount of ODNs released into the aqueous solution was measured by Quant-iT™ OliGreen® ssDNA reagent and kit system (Invitrogen) and with a GloMax®-Multi Detection System-Fluorometer (Promega, Madison, WI). The size of the NPs was determined by the dynamic light scattering method (Zetasizer Nano, Malvern Instruments, Worcestershire, UK).

3.3.5. IgM, IgY and IgA enzyme-linked immunosorbent assays (ELISAs)

Serum IgM and IgY antibody titres were determined as reported previously (Singh *et al*., 2015). Assay conditions remained unchanged for determining virus-specific IgY antibody titres in lacrimal secretions; however, modifications were made for IgA. Briefly, a whole, inactivated H9N2 virus concentration of 0.5 µg/100 µl of carbonate-bicarbonate buffer (pH 9.6) was used for coating 96 well polystyrene plates (Nunc, Maxisorp) at 4°C overnight. The plates were washed and blocked as previously described (Singh *et al*., 2015) prior to addition of 100 µl of a 1/10 dilution of lacrimal secretions to duplicate wells, followed by a 1-hour incubation period. After washing, 100 µl of mouse anti-chicken IgA (AbD Serotec, Kidlington, Oxford, UK), was added to each well at a 1/500 dilution. The plates were washed and 100 µl of goat-anti-mouse IgG (H/L) [HRP

(STAR 117P) AbD Serotec; Kidlington, Oxford, UK)] was added at a dilution of 1/10,000. After incubation for 1 hour, the plates were washed and 100 µl of an HRP substrate solution (ABTS peroxidase substrate system, Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA) was added and the plates were incubated for 2 hours at room temperature in the dark. One hundred microlitres of a stop solution [1% sodium dodecyl sulfate (SDS)] was added prior to determining the optical density at a wavelength of 405 nm and calculating the sample to positive ratios as described previously (Singh *et al*., 2015).

3.3.6. Haemagglutination inhibition (HI)

The HI assay was performed as described previously (Mallick *et al*., 2011) and antibody titres were determined based on the reciprocal of the highest serum dilution resulting in complete inhibition of haemagglutination of red blood cells (RBCs) (Lampire Biologicals).

3.3.7. Statistical analyses

General linear models (GLMs) were used in SAS (version 9.2 Cary, N.C., USA) to compare immune responses among experimental groups. The interaction of ODN type with encapsulation was examined. Haemagglutination inhibition antibody titres were expressed as the $\log_2$ of the reciprocal of the endpoint dilution; S/P ratios were used for analysis of ELISA data. Duncan's multiple range test was used to compare multiple groups post-GLM. A p value of <0.05 was considered significant.

3.4. Results

3.4.1. Generation of HI antibody responses by different vaccines delivered intramuscularly

To assess antibody responses generated with intramuscular administration of vaccine formulations, HI antibody titres were determined in serum samples. Mean HI antibody titres (Fig.5) of all vaccine formulations peaked at 1 week post-secondary vaccination, based on the duration of the study. Vaccine formulations containing nonencapsulated CpG 2007 elicited significantly higher HI antibody titres compared to vaccine adjuvanted with PLGA nanoparticles or the inactivated AIV vaccine formulation (without adjuvant) at 1 week post-secondary vaccination. Antibody titres measured by the HI assay were compared between the vaccine adjuvanted with nonencapsulated CpG 2007 and that containing encapsulated CpG 2007. The interaction term "ODN type*encapsulation" was not significant ($p>0.05$), indicating that encapsulation of CpG 2007 and encapsulation of non CpG 2007 had similar effects on HI responses. Significantly higher HI antibody titres were observed with vaccines adjuvanted with non encapsulated CpG 2007 compared to encapsulated CpG 2007 from 1 to 3 weeks post-secondary vaccination.

3.4.2. Virus-specific IgY and IgM antibody S/P ratios in serum generated by intramuscular administration

Virus-specific IgY and IgM antibody responses in serum generated by intramuscular administration of vaccine formulations were determined. At 3 weeks post-

secondary vaccination, no statistically significant differences were observed in mean serum IgY antibody S/P ratios between vaccine formulations containing nonencapsulated CpG 2007 (group 2) and nonencapsulated non-CpG 2007 (group 4) (Fig. 6a). However, mean serum IgY S/P ratios induced by the vaccine adjuvanted with nonencapsulated non-CpG 2007 were significantly higher than those for vaccines containing squalene (group 1), encapsulated CpG 2007 (group 3) or inactivated AIV only (group 7). No significant differences were observed in mean serum IgY S/P ratios between vaccines containing nonencapsulated or encapsulated CpG 2007 at 3 weeks post-secondary vaccination. At 1 week post-primary vaccination (day 14), mean IgM S/P ratios in chickens induced by the vaccine containing squalene were significantly higher than those elicited by the vaccine adjuvanted with nonencapsulated CpG 2007 (Fig. 6b). A decrease in mean IgM S/P ratios was observed in chickens receiving the vaccine containing non-CpG 2007 at 2 weeks post- primary vaccination (day 21), followed by an increase until 3 weeks post-secondary vaccination (day 42). At 1 week post-secondary vaccination (day 28), mean IgM S/P ratios in chickens receiving the vaccine adjuvanted with nonencapsulated CpG 2007 were statistically similar to those attained in chickens treated with vaccines adjuvanted with squalene or encapsulated CpG 2007.

3.4.3. Virus-specific IgY antibody S/P ratios in lacrimal secretions generated by intramuscular administration

At 2 weeks post-secondary vaccination (day 35), mean IgY S/P ratios in lacrimal secretions (Fig. 7) induced by the vaccine containing nonencapsulated CpG 2007 were significantly higher than ratios generated by the vaccine adjuvanted with encapsulated

CpG 2007. No significant differences were observed between the vaccines adjuvanted with squalene or nonencapsulated CpG 2007 and the AIV only formulation at 2 weeks post-secondary vaccination. At 3 weeks post-secondary vaccination (day 42), mean IgY S/P ratios in lacrimal secretions elicited by the vaccine containing nonencapsulated CpG 2007 were higher than ratios generated by the vaccine adjuvanted with encapsulated CpG 2007; however, this difference was not significant. Mean IgY S/P ratios generated by the vaccine adjuvanted with squalene were not significantly higher than those of AIV only; however, these IgY responses were significantly higher than those induced by the vaccine containing encapsulated CpG 2007.

3.4.4. Serum HI and IgY antibody responses generated by vaccines administered via aerosol route

To determine if the administration route had an effect on immunogenicity of the encapsulated formulation versus the nonencapsulated vaccine formulation, vaccines were delivered by aerosol route. Significantly higher HI antibody titres (Fig. 8) were generated at 1 week post-tertiary vaccination (day 42) in chickens receiving the vaccine adjuvanted with encapsulated CpG 2007 compared to the formulation with nonencapsulated CpG 2007. No significant differences were observed between inactivated AIV vaccines adjuvanted with nonencapsulated CpG 2007 and nonencapsulated non-CpG 2007 at 1 week post-tertiary vaccination. Although the encapsulated CpG 2007 vaccine formulation elicited higher mean serum IgY S/P ratios (Fig. 9a) compared to the nonencapsulated CpG 2007 formulation at 1 week post-tertiary vaccination, results were not significant. Mean IgY S/P ratios induced by the vaccine containing encapsulated non-CpG 2007 were

significantly higher compared to the nonencapsulated formulation at 1 week post-tertiary vaccination.

3.4.5. Virus-specific IgY and IgA S/P ratios in lacrimal secretions generated by aerosol administration

The vaccine formulation adjuvanted with encapsulated CpG 2007 generated higher IgY S/P ratios in lacrimal secretions compared to the formulation with nonencapsulated CpG 2007 at 1 week post-tertiary vaccination (day 42) although these results were not statistically significant (Fig. 9b); however, when compared to the inactivated AIV only formulation, significantly higher IgY S/P ratios were observed. The correlation between serum IgY antibody levels (Fig. 9a) and IgY antibodies in lacrimal secretions (Fig 9b) was evaluated using Pearson's correlation at 1 week post-tertiary vaccination (day 42) for nonencapsulated and encapsulated vaccine formulations delivered by the aerosol route [$R^2=0.447 (p<0.02)$]. Mean IgA S/P ratios (Fig. 9c) were significantly higher in lacrimal secretions of chickens receiving the AIV vaccine adjuvanted with encapsulated CpG 2007 compared to the vaccine formulation containing nonencapsulated CpG 2007 at 1 week post-tertiary vaccination (day 42).

3.5. Discussion

We previously reported the vaccine efficacy of nonencapsulated CpG 2007 at a dose of 2 µg, when administered intramuscularly with 15 µg of formalin inactivated H9N2 virus (Singh *et al*., 2015). Here, we examined the effect of PLGA encapsulation of

the CpG ODN adjuvant component on the kinetics of the immune response to formalin-inactivated AIV vaccine formulations using intramuscular and aerosol routes of administration.

The encapsulated CpG 2007 vaccine formulation, when administered by aerosol route, generated significantly higher antibody responses based on HI titres compared to the formulation adjuvanted with nonencapsulated CpG 2007 at 1 week post-tertiary vaccination (day 42 post-hatch). Furthermore, the encapsulated CpG 2007 vaccine formulation induced significantly higher IgA S/P ratios in lacrimal secretions at 1 week post-tertiary vaccination compared to the nonencapsulated CpG 2007 formulation. Although mean serum IgY S/P ratios generated by the encapsulated CpG 2007 vaccine formulation were higher than IgY S/P ratios generated with the nonencapsulated CpG 2007 formulation, these findings were not significant. Serum IgY S/P ratios were moderately correlated ($R^2=0.447$) with IgY S/P ratios in lacrimal secretions for vaccines delivered by aerosol route at 1 week post-tertiary vaccination. Therefore, the majority of IgY antibody levels in lacrimal secretions was likely due to local production as opposed to serum transudation. A previous report by de Geus and colleagues (de Geus *et al*., 2011) suggests that the lack of detectable antibodies against inactivated influenza virus in chickens when administered via aerosol, may be explained by insufficient virus concentrations or a requirement for booster vaccination. Our findings suggest that PLGA encapsulated, adjuvanted, inactivated AIV vaccine formulations were more efficacious than nonencapsulated, adjuvanted formulations when administered by the aerosol route. An immune response was induced with the inactivated AIV vaccine formulations after 2

boosters when administered by the aerosol route. It is also noteworthy that, in mice, particulate vaccine adjuvants have been shown to promote NALP3 inflammasome activation and the production of pro-inflammatory cytokines (Sharp *et al*., 2009). Albeit, the existence of the NALP3 inflammasome in chickens remains undetermined, we must consider the potential implication of the combination of encapsulated CpG 2007 and PLGA nanoparticles in terms of overall enhancement of antibody mediated immune responses. The size of the encapsulated ODNs used in these experiments was estimated to be 674.5 nm based on dynamic light scattering. We speculate that enhanced vaccine efficacy observed with PLGA encapsulated formulations delivered by aerosol route may be attributed to increased residential time at respiratory mucosal surfaces providing the opportunity for maximum interaction with antigen presenting cells. Considering the size of the nanoparticles, it is likely that deposition occurred at the level of the lower respiratory tract, allowing access to APCs located in the subepithelium, connective tissue and parabronchial linings (Corbanie *et al*., 2006; de Geus, Jansen, and Vervelde, 2012).

In contrast to aerosol administration, inactivated vaccines containing nonencapsulated CpG 2007 induced significantly higher immune responses compared to encapsulated formulations based on serum HI titres from 1 to 3 weeks post-secondary vaccination, and at 2 weeks post-secondary vaccination for lacrimal IgY S/P ratios when delivered by the intramuscular route. Local environmental conditions at both sites of administration such as pH, presence of enzymes and temperature (Chadwick, Kriegel, and Amiji, 2010) might account for some observed differences. Furthermore, although *in vivo* nanoparticle decomposition is required for release of encapsulated contents,

predicting *in vivo* behavioral characteristics of nanoparticles is still in its infancy. Additionally, it must be noted that administration of CpG ODN through different routes (systemic versus local) has been demonstrated to result in immunosuppressive or immunostimulatory effects at some dosage levels in other species (Wingender *et al*., 2006).

Unexpected findings included vaccine immunogenicity in the context of encapsulated non-CpG 2007 and nonencapsulated non-CpG 2007 for both routes of administration. With respect to the aerosol route of delivery, the encapsulated non-CpG 2007 vaccine formulation generated significantly higher mean IgY S/P ratios in both serum and lacrimal secretions compared to the respective nonencapsulated adjuvanted formulation at 1 week post-tertiary vaccination. Furthermore, although the vaccine containing nonencapsulated non-CpG 2007 elicited an early IgM response compared to nonencapsulated CpG 2007 during intramuscular administration, the exact mechanism of action of non-CpG ODNs remains to be investigated in chickens. Interestingly, St. Paul and colleagues (St Paul *et al*., 2011) have observed that *in vivo* administration of non-CpG 2007 at a dose of 50 µg induced levels of cytokine gene expression that were similar to those in response toCpG 2007 in chicken spleens. These cytokine transcripts were IFN-α, IFN-γ, IL-1β, IL-8, MyD88 and major histocompatibility complex II (St Paul *et al*., 2011). Recent studies demonstrated the ability of non-CpG ODN to influence early T cell and late B cell activation in mice (Herbath *et al*., 2015). Moreover, non-CpG ODNs have also been shown to costimulate mouse and human CD4+T cells through a MyD88-independent mechanism (Landrigan, Wong, and Utz, 2011). Finally, it has been reported

that non-CpG ODNs can act synergistically when combined with specific antigen resulting in the activation of the NF-κB pathway and B cell proliferation in mice (Wang and Krieg, 2003).

In this study, PLGA nanoparticles were used to encapsulate CpG 2007 by a water-in-oil-in-water (double emulsion) technique for aerosol and intramuscular delivery with inactivated H9N2 avian influenza virus. We showed that nonencapsulated CpG 2007 in inactivated AIV vaccines induced higher immune responses when delivered intramuscularly in chickens. Furthermore, we showed that encapsulated CpG 2007 in inactivated AIV vaccines generated higher responses than nonencapsulated AIV vaccines with aerosol administration. An implication of these results is that encapsulated vaccine formulations may be more applicable to mucosal administration than to systemic administration in chickens. Future studies may be aimed at investigating protective effects of these vaccine formulations against homologous and heterologous virus challenge.

Table 2: **Vaccine formulations for specific pathogen free chickens administered through the intramuscular and aerosol routes.**

Vaccine formulations	Group (Intramuscular route)	Group (Aerosol route)
AIV and AddaVax™ (squalene)	1	9
AIV and CpG 2007 (nonencapsulated)	2	10
AIV and PLGA encapsulated CpG 2007	3	11
AIV and non-CpG 2007 (nonencapsulated)	4	12
AIV and PLGA encapsulated non-CpG 2007	5	13
AIV and PLGA nanoparticles (PLGA NPs)	6	14
AIV (without adjuvant)	7	15
PBS only	8	16

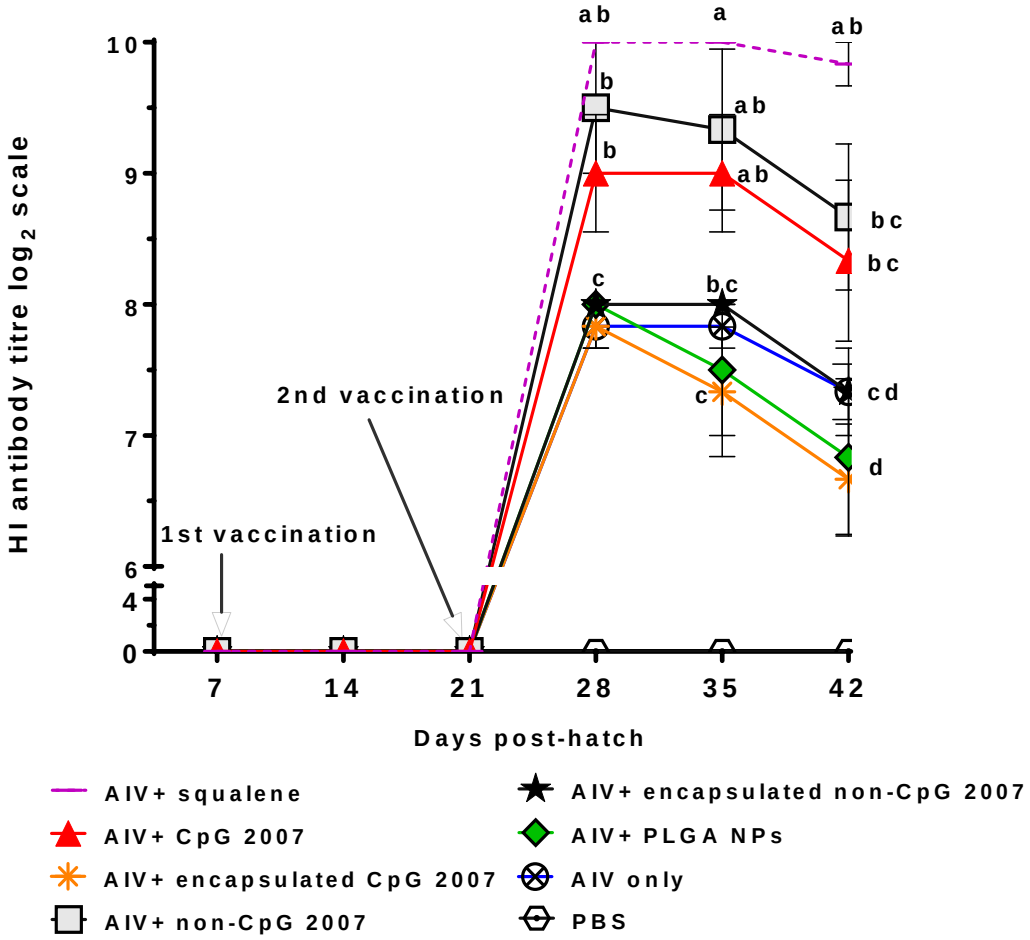

Figure 5: Mean serum haemagglutination inhibition (HI) titres in chickens vaccinated intramuscularly at 7 and 21 days post-hatch using encapsulated or nonencapsulated CpG ODN 2007 with formalin-inactivated H9N2 virus. A squalene-containing commercial oil-in water emulsion (AddaVax^{TM}) was used as a positive control adjuvant. Standard errors of the mean are represented by error bars. Means with the same letter are not statistically different. A Proc GLM with Duncan's Multiple Range test was used for data analysis with Duncan's Multiple Range test, ANOVA ($p<0.05$).

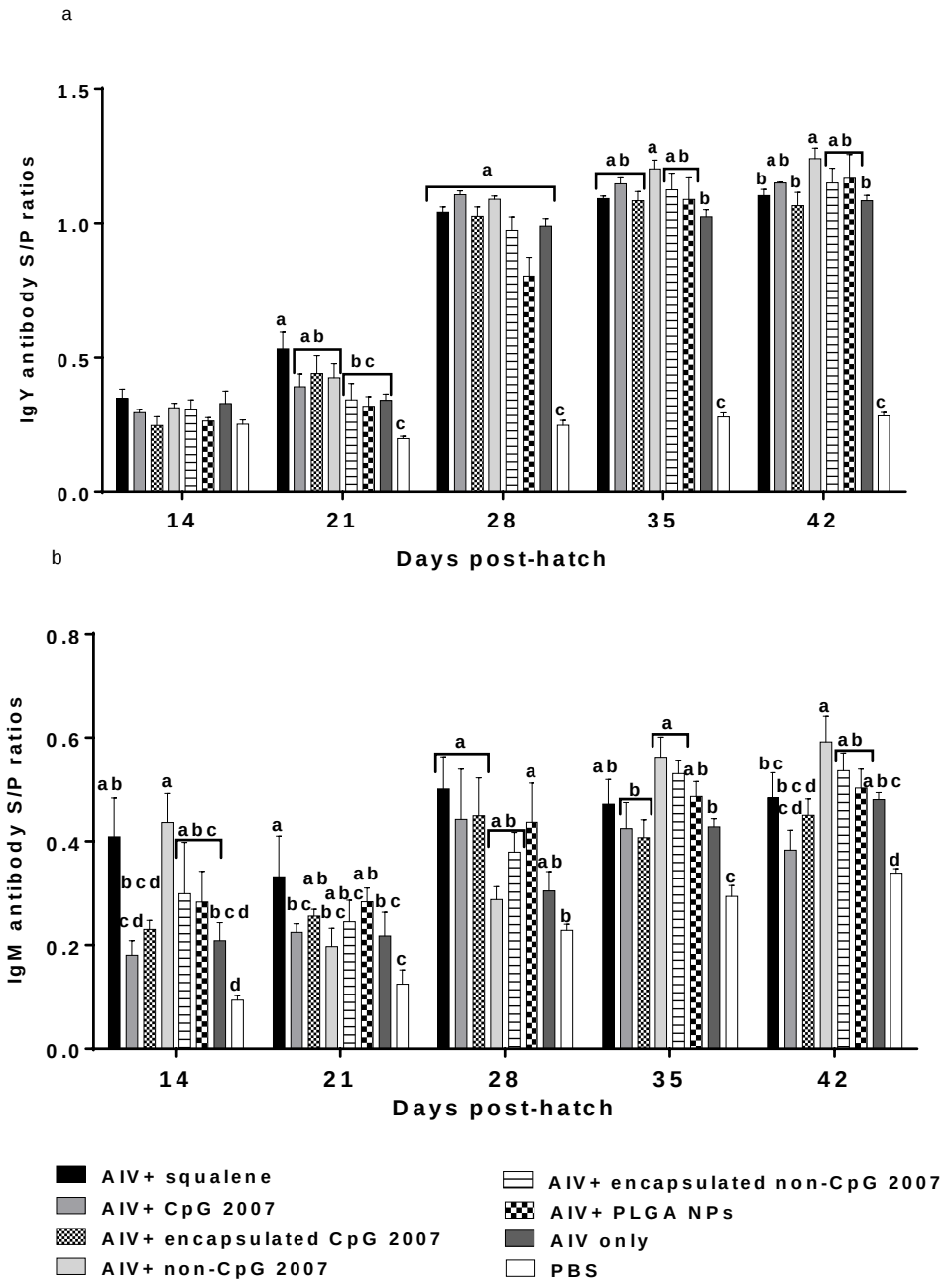

Figure 6: Serum IgY (a) and IgM (b) on days 14 to 42 post-hatch. Primary and secondary vaccinations occurred on days 7 and 21, respectively by intramusular route. Chickens received encapsulated or nonencapsulated CpG ODN 2007 with formalin-inactivated H9N2 virus. Control group was treated with phosphate buffered saline (PBS). Data represent mean S/P ratios per treatment group. Means with the same letter do not differ significantly, at each time-point ($p<0.05$).

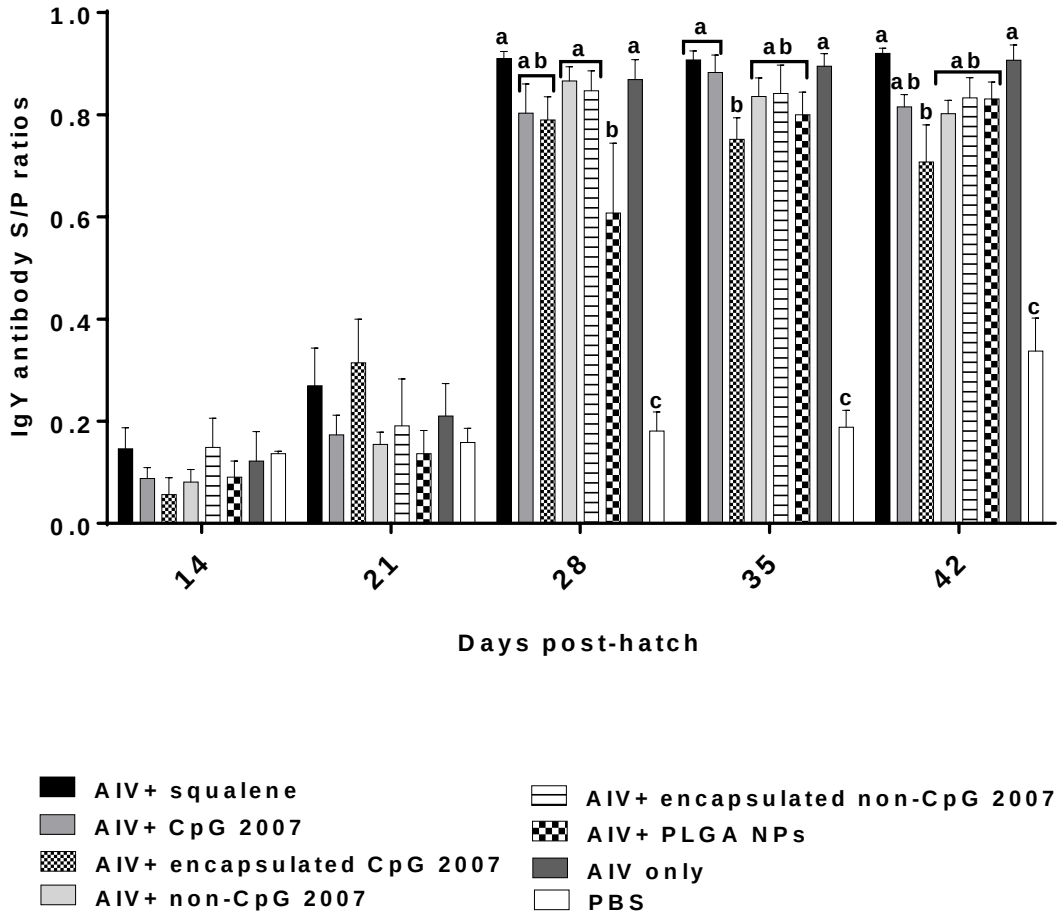

Figure 7: IgY S/P ratios in lacrimal secretions for chickens vaccinated intramuscularly with encapsulated or nonencapsulated CpG ODN 2007 with formalin-inactivated H9N2 virus. Means with the same letter do not differ significantly, at each time-point ($p<0.05$).

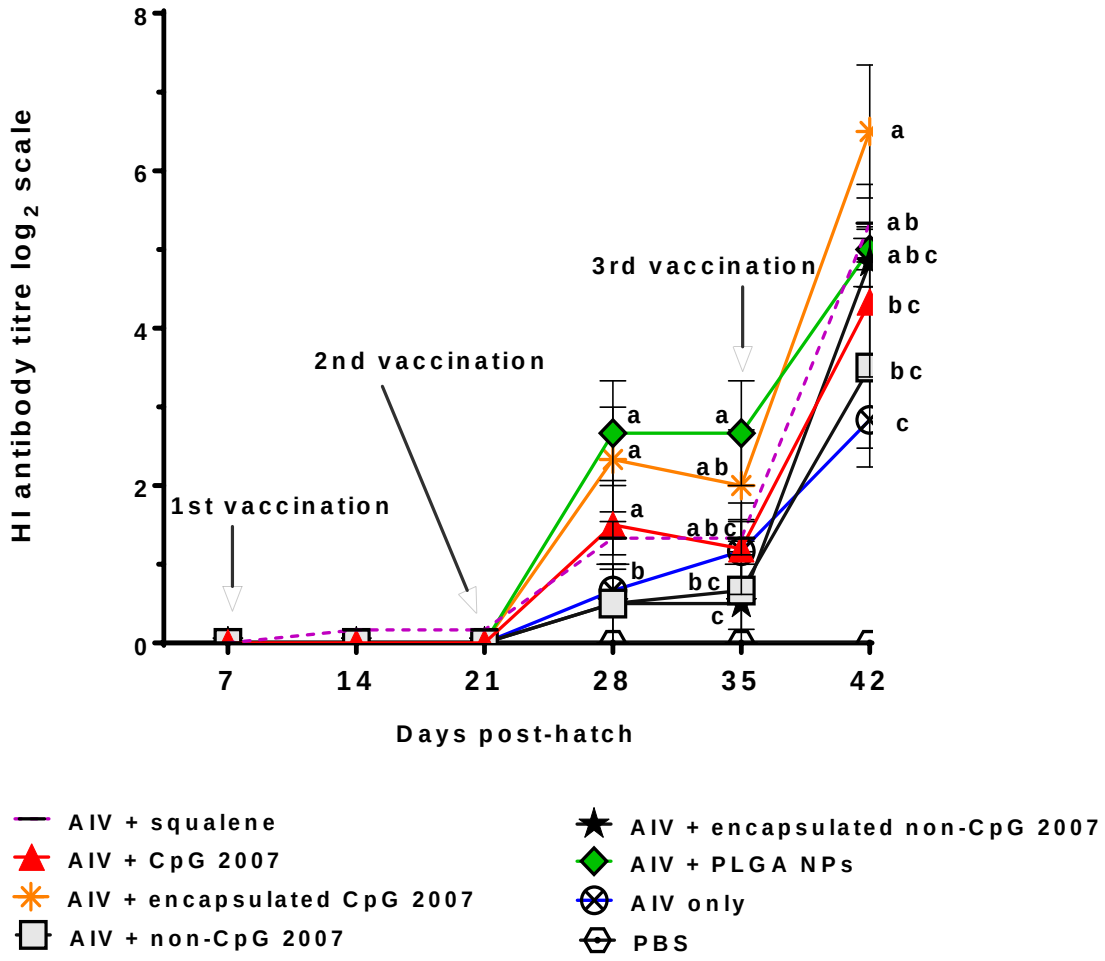

Figure 8: Mean serum hemagglutination inhibition (HI) titres in chickens vaccinated at 7, 21 and 35 days post-hatch using the aerosol route with formalin-inactivated H9N2 with encapsulated or nonencapsulated CpG ODN 2007. Means with the same letter are not statistically different ($p<0.05$).

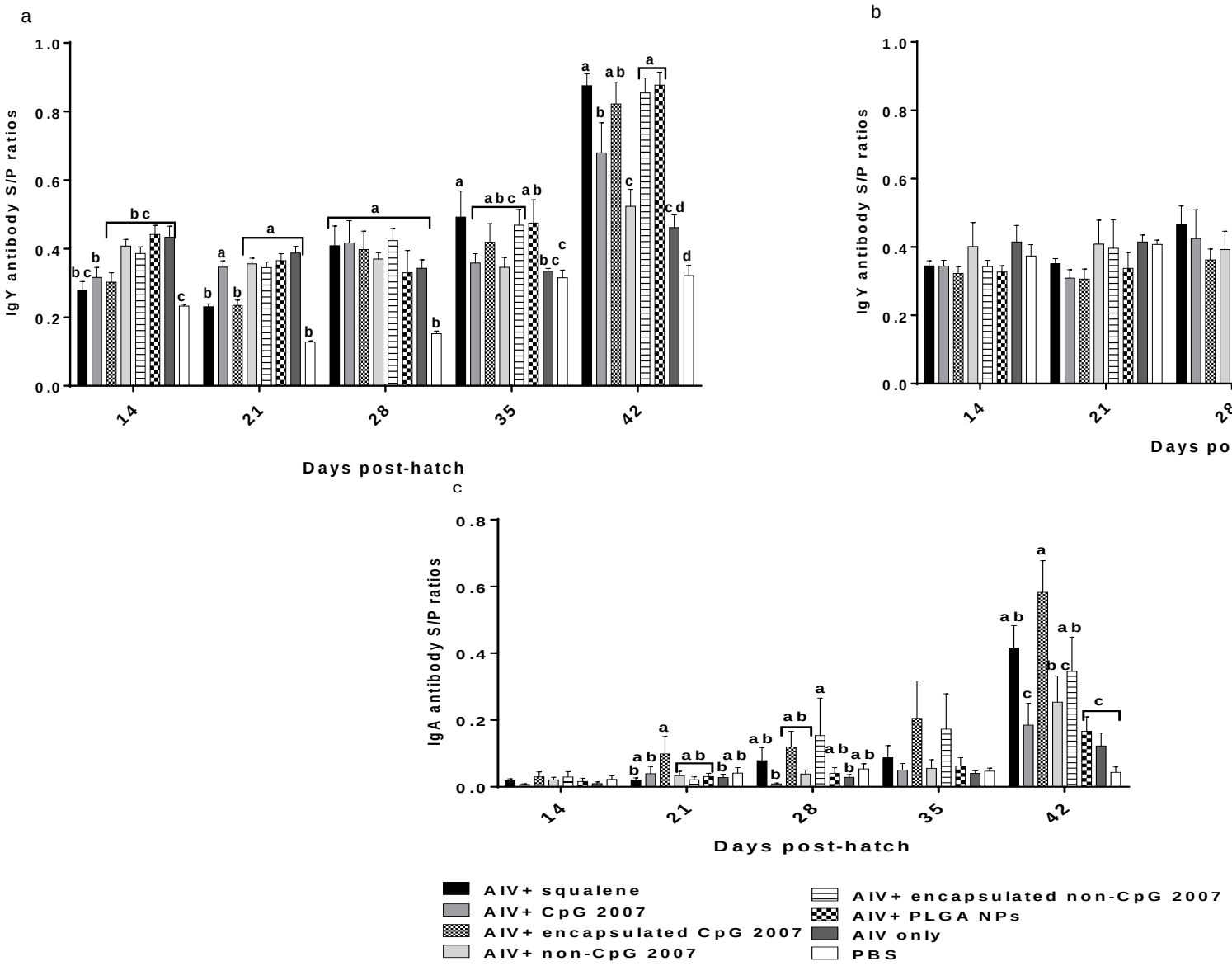

Figure 9: Serum IgY S/P ratios (a) and, IgY S/P ratios (b) and IgA S/P ratios (c) in lacrimal secretions from days 14 to 42 post-hatch. Chickens received 3 vaccinations through the aerosol route at days 7, 21 and 35 post-hatch. Means with the same letter are not statistically different ($p<0.05$).

3.6. Acknowledgements

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CHAPTER 4

PROTECTIVE ANTIBODY RESPONSES TO INTRAMUSCULAR DELIVERY OF POLY(D,L-LACTIC-CO-GLYCOLIC ACID) ENCAPSULATED OR NONENCAPSULATED INACTIVATED AIV VACCINES WITH CPG ODN 2007 IN CHICKENS

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4.1. Abstract

In poultry, parenteral administration of vaccines consisting of inactivated avian influenza virus (AIV) requires the simultaneous delivery of an adjuvant (water-in-oil emulsion). This type of vaccine is often limited in its ability to induce quantitatively better local (mucosal) antibody responses capable of curtailing virus shedding. Therefore, more efficacious adjuvants with the ability to provide enhanced immunogenicity and protective anti-AIV immunity in chickens are needed. While the Toll-like receptor (TLR) 21 agonist, CpG oligodeoxynucleotides (ODNs), has been recognized as a potential vaccine adjuvant in chickens, poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles, successfully tested as vaccine delivery systems in other species, have not been extensively explored. The present study, therefore, assessed both systemic and mucosal antibody-mediated responses following intramuscular vaccination (7 and 21 days post-hatch) of chickens with PLGA encapsulated H9N2 AIV plus encapsulated CpG ODN 2007 (CpG 2007), and nonencapsulated AIV plus PLGA encapsulated CpG 2007 vaccine formulations along with appropriate controls. Virus challenge was performed at 2 weeks post-secondary vaccination using the oculo-nasal route. Our results showed that chickens vaccinated with nonencapsulated AIV plus PLGA encapsulated CpG 2007 developed significantly higher systemic IgY and local (mucosal) IgY antibodies as well as haemagglutination inhibition antibody titres compared to PLGA encapsulated AIV plus encapsulated CpG 2007 vaccinated chickens. Additionally, chickens receiving nonencapsulated AIV plus PLGA encapsulated CpG 2007 showed significantly reduced virus shedding compared to controls. Furthermore, chickens that received CpG 2007 as an adjuvant in the vaccine formulation had antibodies exhibiting higher affinity,

indicating that the TLR21-mediated pathway may enhance antibody affinity maturation qualitatively. Collectively, our data indicate that vaccination of chickens with nonencapsulated AIV plus PLGA encapsulated CpG 2007 results in qualitatively and quantitatively augmented antibody responses leading to a reduction in virus shedding.

Keywords: avian influenza virus, chickens, CpG ODN 2007, poly(D,L-lactic-co-glycolic acid), immune response, intramuscular vaccination.

4.2. Introduction

Avian influenza virus (AIV) is a mucosal pathogen associated with economic losses (Pasick, Berhane, and Hooper-McGrevy, 2009) and public health (Katz *et al*., 2009) implications in poultry and humans, respectively. Vaccination constitutes an important component of AIV control programs in poultry. Vaccination, when complemented by other elements of a control program effectively prevents the development of clinical signs, minimizes the frequency of disease-associated deaths, and reduces virus shedding, thus exerting an effect on the virus transmission cycle (van den Berg *et al*., 2008). A few types of AIV vaccines have been evaluated experimentally for potential use in poultry, however, existing commercial vaccines are predominantly of the inactivated, whole virus type, delivered with a water-in-oil-emulsion which requires parenteral administration (Swayne and Kapczynski, 2008). Such vaccines, while effective at generating systemic antibodies, are limited by the type (T helper 1 or T helper 2-type responses) and magnitude of resulting immune responses and are less capable of inducing local, mucosal antibodies (Swayne and Kapczynski, 2008). Furthermore, the generation of high avidity systemic antibodies can result in enhanced virus neutralizing abilities. This highlights the need to identify novel, more efficacious AIV vaccine adjuvants that can potentially improve immunogenicity. In this regard, Toll-like receptor (TLR)-based adjuvants are promising candidates for application in chickens and mammals (Gupta *et al*., 2014). These adjuvants exert their effects at the level of the innate response, which, in turn, influences the course of the adaptive immune response in terms of the magnitude and type of response that ensues (Coffman, Sher, and Seder, 2010). In chickens, the TLR21 agonist, synthetic, unmethylated oligodeoxynucleotides with CpG motifs (CpG ODN)

has been demonstrated as a potential adjuvant candidate for AIV vaccines (Gupta *et al*., 2014; St. Paul *et al*., 2014a; St. Paul *et al*., 2014b). We have previously demonstrated the immunogenicity of 2 µg of CpG 2007, when administered parenterally (intramuscularly) with inactivated H9N2 virus (15 µg) in specific pathogen free (SPF) chickens (Singh *et al*., 2015).

A recent advancement in vaccine development involves the application of particulate delivery systems such as poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles aimed at improving vaccine immunogenicity. Particulate delivery systems influence vaccine immunogenicity through several mechanisms such as the slow release of encapsulated vaccine components at the administration site, their immunostimulatory capability and enhanced antigen stability (Zhao *et al*., 2014). Additional mechanisms include co-delivery of both encapsulated antigen and adjuvant to the same antigen presenting cell for enhanced antigen processing and repetitive presentation of B-cell epitopes (Gerdts *et al*., 2006; Sharma and Hinds, 2012; Spickler and Roth, 2003).

Generally, inactivated vaccines render protection against AIV through antibody-mediated immune responses (Swayne and Kapczynski, 2008). Haemagglutinin (HA)-specific neutralizing antibodies act mechanistically by blocking initial virus attachment and preventing the establishment of infection or inhibiting virus fusion (Subbarao and Joseph, 2007), impacting the virus transmission cycle. Limited information is available on whether anti-AIV antibodies of a specific isotype confer enhanced levels of protection against AIV in chickens. However, in mice, the antibody isotype known to contribute the most to influenza virus neutralization is IgG (Benne *et al*., 1997). In humans, serum-derived antibodies of the IgG isotype are mainly transudated into the respiratory tract and

are associated with long-term protection against influenza infection (Chen *et al*., 2001; Kreijtz, Fouchier, and Rimmelzwaan, 2011). Harderian gland secretions, characterized by the presence of IgM, IgA and IgY antibody isotypes (Jeurissen, Vervelde, and Janse, 1994; Olah, Kupper, and Kittner, 1996; Scott, Savage, and Olah, 1993), afford protection to the upper respiratory tract region in chickens with the IgY component arising as a result of the combination of local contribution from gland secretions and "non-localized" serum transudation (de Geus, Rebel, and Vervelde, 2012; Toro *et al*., 1993).

Therefore, in this study, immune responses (systemic and mucosal) generated by parenteral administration of a PLGA encapsulated H9N2 AIV plus PLGA-encapsulated CpG 2007 vaccine formulation were compared to those of a nonencapsulated H9N2 AIV plus PLGA encapsulated CpG 2007 formulation.

4.3. **Materials and methods**

4.3.1. Chickens

One-day-old SPF White Leghorn chickens were obtained from the Canadian Food Inspection Agency (Ottawa, Canada). Experimental procedures were approved by the University of Guelph Animal Care Committee and conformed to specifications of the Canadian Council on Animal Care.

4.3.2. Avian influenza virus

Low pathogenic H9N2 virus (A/Turkey/Ontario/1/66) used in this experiment was propagated in 10-day-old embryonated SPF chicken eggs and subsequently inactivated using formalin as described previously (St. Paul *et al*., 2014b).

4.3.3. Synthetic CpG ODN and non-CpG

CpG 2007 (5′-TCGTCGTTGTCGTTTTGTCGTT-3′) (CpG motifs underlined), consisting of a phosphorothioate backbone and non-CpG 2007 (5′-TGCTGCTTGTGCTTTTGTGCTT-3′) were purchased from Sigma-Aldrich Canada and reconstituted as previously described (Singh *et al*., 2015).

4.3.4. Experimental design

Fifty-four chickens were randomly distributed into nine treatment groups (n=6/group) as shown in Table 3. Chickens in group 1 received encapsulated AIV without an adjuvant while those in group 2 received encapsulated AIV plus encapsulated CpG 2007. Chickens in groups 3 and 4 received nonencapsulated AIV without adjuvant and nonencapsulated AIV plus encapsulated CpG 2007, respectively. Chickens in group 5 received PLGA nanoparticles (blank nanoparticles) while those in group 6 received PBS (received challenge on day 35 with live virus) Chickens in group 7 received encapsulated AIV plus encapsulated non-CpG; chickens in group 8 were vaccinated with PLGA nanoparticles plus encapsulated AIV. Chickens in group 9 received PBS (without challenge). Vaccine formulations were administered at days 7 (primary vaccination) and

21 (secondary vaccination) post-hatch using the intramuscular route. Serum and lacrimal secretions were collected on a weekly basis for serological analyses and evaluation of mucosal immune responses. Chickens in all groups, with the exception of group 9 were challenged on day 35 post-hatch with 200 µl of 1×10^6 50% tissue culture infectious dose ($TCID_{50}$/ml) of H9N2 virus using the oculo-nasal route. Cloacal swabs were collected on days 3, 5 and 7 post-challenge for virus titration in Madin-Darby canine kidney (MDCK) cells to assess the viral shedding as previously described (Szretter, Balish, and Katz, 2005). Virus titres were expressed as the $\log_{10}TCID_{50}$ per ml of cloacal sample.

4.3.5. Encapsulation of AIV and ODNs in PLGA nanoparticles (PLGA NPs)

A polyethylenimine (PEI; 2.5 kD, Sigma-Aldrich)-ODN complex was prepared according to Boussif and colleagues (Boussif *et al*., 1995). Four hundred and thirty four micrograms of CpG 2007 (or non-CpG 2007) in 125 µl nuclease free water was mixed with 125 µl PEI (0.29 mg) dissolved in 150 mM NaCl. The PEI/ODN complex was sonicated with 56 mg of PLGA Resomer® RG 503H (acid terminated, 24-38 kD; Sigma-Aldrich) dissolved in 1.25 ml of dichloromethane (Sigma-Aldrich) for 1 minute [10 second sonication, 5 second pause at 40% amplitude (Ultrasonic processor, 3 mm probe diameter, Fisher scientific)]. The PLGA solution was ultrasonicated with AIV (250 µl containing 1250 µg of inactivated AIV). The primary emulsions of PLGA-AIV and PLGA-ODN were sonicated for 2 minutes (10 second sonication and 5 second pause at 60% amplitude) in 3.25 ml of 2% PVA/1% poloxamer to form secondary emulsions, which were then poured into 50 ml 2% PVA/1% poloxamer (30-70 kD and 87-90% hydrolyzed PVA and Poloxamer; Sigma-Aldrich) solution under constant stirring.

Poly(D,L-lactic-co-glycolic acid) NPs were pelleted at 25,000 x *g* for 30 minutes at 4°C, washed 3 times with HyClone pure water (GE, Healthcare Life Sciences), snap frozen for lyophilisation then stored at 4°C until use.

The AIV content in NPs was determined by dissolving 5 mg of PLGA AIV-NPs in 1 ml of 0.1 N sodium hydroxide solution at 37°C with overnight agitation. Protein concentration was determined using a BCA protein assay kit (Pierce). Encapsulation efficiency and loading efficiency of CpG 2007, and the sizes of NPs were determined as described by Peine and colleagues (Peine *et al*., 2013).

4.3.6. Serology

Haemagglutination inhibition (HI) assays and virus neutralization (VN) assays were performed as previously described (Mallick *et al*., 2011) to evaluate serum antibody titres in chickens on a weekly basis until 3 weeks post-secondary vaccination; HI titres were expressed as the $\log_2$ of the reciprocal of the endpoint dilution while VN titres were based on the $\log_{10}$ of the reciprocal of the highest dilution resulting in an absence of cytopathic effects. Serum IgM and IgY S/P ratios were determined as indicated previously (Singh *et al*., 2015). Serum IgY antibody avidity indices were determined through resistance to 8 M urea as described by Kulkarni and colleagues (Kulkarni *et al*., 2014). Urea disrupts protein-protein bonds resulting in weakly bound antibodies being dislodged while high affinity antibodies remain bound to the antigen coated plates. A higher avidity index represents antibodies of greater affinity. The ELISA specifications stated previously (Singh *et al*., 2015) were used, however, with modifications: 1) Serial 2-fold dilutions were used instead of a 1:50 serum dilution, and, 2) a urea wash step was performed prior

to addition of 100 µl of goat anti-chicken IgY (Fc specific) antibodies conjugated to horse-radish peroxidase (Bethyl Laboratories, Montgomery, Texas). One hundred microlitres/well of 8 M urea in PBS was added and incubated at room temperature for 5 minutes, followed by washing as described previously (Singh *et al*., 2015). The IgY avidity index was calculated as the average urea-treated OD_{405} divided by the average PBS-Tween treated OD_{405}×100 (Scott, Savage, and Olah, 1993).

4.3.7. Mucosal immune responses in lacrimal secretions

Virus-specific IgY and IgA antibody titres were evaluated in lacrimal secretions until 3 weeks post-secondary vaccination. An indirect ELISA was performed as described previously for IgY (Singh *et al*., 2015), with modifications for IgA. A concentration of 0.5 µg/100 µl of carbonate-bicarbonate buffer (pH 9.6) was used for plate (Nunc, Maxisorp) coating at 4°C overnight. Subsequent to washing and blocking, 100 µl of a 1/10 dilution of lacrimal secretions were added to the wells in duplicate and incubated for 1 hour. Lacrimal secretions were diluted in 1.5% Tween-20 in PBS with 0.29 M NaCl (Hodgins and Shewen, 2000) before addition to wells. After washing, 100 µl of mouse anti-chicken IgA (AbD Serotec, Kidlington, Oxford, UK) was added at a 1/500 dilution. Plates were washed and 100 µl of goat-anti-mouse IgG (H/L): HRP [STAR 117P) AbD Serotec, Kidlington, Oxford, UK)] were added at a dilution of 1/10,000 to each well and incubated for 1 hour prior to washing. All subsequent steps remained as described previously (Singh *et al*., 2015).

4.3.8. Statistical analyses

Statistical differences in immune responses among vaccine groups were determined using general linear models (GLMs) in SAS (version 9.2 Cary, N.C., USA). The Reed-Muench formula was used to determine the $\log_{10}TCID_{50}$/ml of virus in cloacal swab samples per chicken (Szretter, Balish, and Katz, 2005). A p value of <0.05 was considered as significant. Pearson's correlation coefficient was used to quantify the association between serum and lacrimal immune responses, and virus shedding, $p<0.05$ using SAS.

4.4. Results

4.4.1. HI and VN antibody responses

Mean HI antibody titres (Figure 10) elicited by the vaccine containing nonencapsulated AIV and encapsulated CpG 2007 were significantly higher at 1 week post-primary and post-secondary vaccinations, and 2 weeks post-secondary vaccination compared to the nonencapsulated AIV formulation (AIV-only group). Mean HI titres elicited by nonencapsulated AIV with encapsulated CpG 2007 were significantly higher at 1 and 2 weeks post-primary and post-secondary vaccinations compared to the encapsulated AIV and encapsulated CpG 2007 formulation. At 1 week post-secondary vaccination, significantly higher mean HI titres were induced by the vaccine containing encapsulated AIV adjuvanted with encapsulated CpG 2007 compared to the formulation with encapsulated AIV without adjuvant. Significantly higher mean HI titres were elicited by the nonencapsulated AIV vaccine at 2 weeks post-primary vaccination, and at

1 and 2 weeks post-secondary vaccination compared to the encapsulated AIV formulation.

Mean VN antibody titres generated by the vaccine containing nonencapsulated AIV adjuvanted with encapsulated CpG 2007 were higher than those of the nonencapsulated AIV group but results were not significant (Figure 11); however, VN titres were significantly higher for the group receiving nonencapsulated AIV with encapsulated CpG 2007 at 1 and 2 weeks post-secondary vaccination compared to the encapsulated AIV and encapsulated CpG 2007 group. No significant differences were observed in VN titres between the encapsulated AIV with encapsulated CpG 2007 formulation and encapsulated AIV only. Significantly higher VN antibody responses were generated in the nonencapsulated AIV group versus the encapsulated AIV group. At 2 weeks post-secondary vaccination, prior to virus challenge, a strong correlation [r=*0.908 ($p<0.0001$)*] was demonstrated between serum HI titres and VN titres.

4.4.2. Virus-specific IgM and IgY antibody responses in serum

Mean serum IgM S/P ratios induced by the vaccine containing nonencapsulated AIV adjuvanted with encapsulated CpG 2007 were significantly higher at 1 week post-primary vaccination compared to those of the nonencapsulated AIV, and encapsulated AIV with encapsulated CpG 2007 vaccine formulations, respectively (Figure 12a). At 1 week post-secondary vaccination, mean IgM S/P ratios elicited by the formulation containing encapsulated AIV adjuvanted with encapsulated CpG 2007 were significantly higher than that of encapsulated AIV only. Significantly higher IgM S/P ratios were induced at 1

week post-primary vaccination in the nonencapsulated AIV vaccine group compared to the encapsulated AIV group.

Significantly higher mean IgY S/P ratios (Figure 12b) were induced at 2 weeks post-primary vaccination in the nonencapsulated AIV with encapsulated CpG 2007 group compared to that of encapsulated AIV adjuvanted with encapsulated CpG 2007. Mean serum IgY S/P ratios were significantly higher at 1 week post-secondary vaccination for chickens vaccinated with encapsulated AIV in combination with encapsulated CpG 2007 compared to those of chickens vaccinated with encapsulated AIV without adjuvant. At 2 weeks post-secondary vaccination, a moderate correlation [r=*0.528 ($p<0.0001$)*] was observed between serum HI titres and serum IgY titres.

4.4.3. IgY antibody avidity indices

At 7 days post-secondary vaccination, the avidity indices (Figure 13) for the nonencapsulated AIV plus encapsulated CpG 2007 and nonencapsulated AIV groups, were 70% and 55%, respectively. High avidity IgY antibodies (>50%) were generated by vaccine formulations containing nonencapsulated AIV plus encapsulated CpG 2007 (70%), and encapsulated AIV plus encapsulated CpG 2007 (65%) at 7 days post-secondary vaccination. The IgY avidity index for encapsulated AIV was determined as 45% at 7 days post-secondary vaccination.

4.4.4. IgY and IgA S/P ratios in lacrimal secretions

At 2 weeks post-primary vaccination, mean IgY S/P ratios (Figure 14a) generated by the vaccine containing nonencapsulated AIV with encapsulated CpG 2007 were

significantly higher than those of the encapsulated AIV and encapsulated CpG 2007 formulation. At 1 week post-secondary vaccination, significantly higher IgY S/P ratios were induced by the vaccine containing encapsulated AIV adjuvanted with encapsulated CpG 2007 compared to the vaccine with the encapsulated AIV formulation. No significant differences in IgA S/P ratios (Figure 14b) were detected among the vaccine groups prior to 2 weeks post-secondary vaccination. At 2 weeks post-secondary vaccination, a moderate correlation [r=*0.537 ($p<0.0001$)*] was observed between serum IgY and lacrimal IgY S/P ratios.

4.4.5. AIV challenge and subsequent virus shedding from vaccinated chickens

The nonencapsulated AIV with encapsulated CpG 2007 vaccine resulted in the lowest quantity of virus being shed on days 5 and 7 p.i. (Figure 15) with a $\log_{10}$ $TCID_{50}$/ml of 3.34 ($p<0.05$) and 3.75 ($p<0.05$), respectively. The nonencapsulated AIV and encapsulated CpG 2007 vaccine group generated significantly lower viral titres at 5 days p.i. compared to titres with encapsulated AIV adjuvanted with encapsulated non-CpG 2007 (4.08 $\log_{10}TCID_{50}$/ml, $p<0.05$), PLGA nanoparticles (4.78 $\log_{10}TCID_{50}$/ml, $p<0.05$) and PBS with virus challenge (4.63 $\log_{10}TCID_{50}$/ml, $p<0.05$), but was not significantly different from the nonencapsulated AIV group (3.5 $\log_{10}TCID_{50}$/ml, $p>0.05$). At 7 days p.i., a similar trend was observed among the same groups with a $\log_{10}$ $TCID_{50}$/ml of 4.89 ($p<0.05$) for encapsulated AIV adjuvanted with encapsulated non-CpG 2007, a viral titre of 5.91 $\log_{10}TCID_{50}$/ml ($p<0.05$) for PLGA nanoparticles and 5.96 $\log_{10}TCID_{50}$/ml ($p<0.05$) for PBS with virus challenge. Virus shedding was significantly reduced in the nonencapsulated AIV group adjuvanted with encapsulated CpG 2007

compared to that of encapsulated AIV with encapsulated CpG 2007 (4.37 $\log_{10}TCID_{50}$/ml, $p<0.05$). No significant differences were observed between the nonencapsulated AIV and encapsulated CpG 2007, and the nonencapsulated AIV groups (3.91 $\log_{10}TCID_{50}$/ml, $p>0.05$). At 2 weeks post-secondary vaccination, a moderate correlation [r=*-0.542 ($p<0.0001$)*] was observed between serum IgY S/P ratios and cloacal virus shedding at 7 days p.i.

4.5. Discussion

Poly(D,L-lactic-co-glycolic acid) nanoparticle delivery systems have been successfully applied to inactivated vaccines (Garg *et al*., 2010; Panyam and Labhasetwar, 2003) due to their ability to induce superior antibody responses through the activation of innate responses (Kuroda, Coban, and Ishii, 2013). In mice, the separate encapsulation of antigen and immune potentiators (TLR agonists) in PLGA nanoparticles has been shown to elicit enhanced antibody responses compared to co-encapsulation in the same nanoparticle (Kasturi *et al*., 2011). Here, we investigated the ability of PLGA nanoparticle-encapsulated AIV and CpG 2007 to enhance protective systemic and local antibody responses compared to nonencapsulated vaccine combinations in chickens. Our findings indicate that nonencapsulated AIV plus encapsulated CpG 2007 generated significantly higher systemic antibody responses (HI and VN titres and IgY S/P ratios) and local (lacrimal) IgY antibody responses, thereby causing a significant reduction in virus shedding compared to the vaccine containing both encapsulated AIV plus CpG 2007.

Although HI titres are useful indicators of AIV vaccine efficacy, the ability to predict the required protective quantities of HI antibodies remains a challenge, particularly for subtypes of low pathogenicity such as the H9N2 virus in chickens. For highly pathogenic AIVs, an HI titre of 1/128 or greater is considered sufficient for protection in chickens (Swayne *et al*., 2015). In the present study, a mean HI titre of 1/1024 was attained by 1 week post-secondary vaccination in chickens that received the nonencapsulated AIV plus encapsulated CpG 2007 vaccine formulation.

At 2 weeks post-secondary vaccination, a moderate correlation was seen between serum HI titres and serum IgY as well as between serum IgY and lacrimal IgY levels while a moderate, inverse correlation was observed between serum IgY and virus shedding. In chickens, the passive transudation of serum IgY to lacrimal secretions plays an important role in conferring protection to the upper respiratory tract against AIV (Ohshima and Hiramatsu, 2000; Toro *et al*., 1993). The findings of the present study also suggest that through the generation of sufficient quantities of serum IgY, transudation to lacrimal secretions can occur, which, we speculate, may impact virus replication at an earlier stage and result in an overall reduction in virus shedding. The LP H9N2 virus displays a tendency to establish infections in the upper respiratory tract of chickens (Nili and Asasi, 2002) and although parenteral vaccination may be limited to some extent by the level of mucosal immunity that is generated, the induction of high quantities of neutralizing antibodies can facilitate passive transudation of these antibodies into the lumen of mucosal tissues (Belyakov and Ahlers, 2009). Notably, in the present study, intramuscular administration of the vaccines did not elicit a lacrimal IgA antibody response, possibly because serum IgA quantities were too low to be transported to

lacrimal secretions via the chicken polymeric immunoglobulin receptor (Wieland *et al*., 2004). However, the ability to induce serum IgA in high enough quantities to be transported to lacrimal secretions is possible through other routes of administration (subcutaneous) using alternative delivery systems such as virosomes plus CpG 2007 as indicated by Mallick and colleagues (Mallick *et al*., 2012). In the present study, nonencapsulated AIV plus encapsulated CpG 2007 was shown to induce high avidity IgY antibodies at 1 week post-secondary vaccination. This finding is supported by the observation that chickens in this group showed significantly reduced levels of virus shedding. This is perhaps due to the fact that augmented antibody affinity maturation arising from the germinal centre reaction results in antibodies that exert efficient HA and virus neutralization capabilities in contrast to low avidity antibodies (Feng *et al*., 2009; Kasturi *et al*., 2011). Since direct B cell activation and subsequent plasma cell proliferation result from CpG DNA stimulation (Bode *et al*., 2011), PLGA encapsulation of CpG 2007 is expected to enhance this effect owing to its gradual release, hence providing sustained levels of signal 2 to APCs (Schijns, 2000). The production of long-lived plasma cells is a requirement for the generation of high-affinity antibodies and is brought about by the germinal centre reaction (Kasturi *et al*., 2011).

Encapsulation of AIV in our vaccine formulation did not confer a significant enhancement to antibody responses or a significant reduction in virus shedding when compared with nonencapsulated AIV plus encapsulated CpG 2007. These observations may relate to the instability of antigens (for instance, hydrolysis of peptide bonds) during the process of encapsulation which can potentially influence the dynamics of the immune response (Jiang *et al.*, 2005). Reduced levels of antigenicity may be further compounded

through encapsulation as a result of protein unfolding due to organic solvent exposure (Jain, O'Hagan, and Singh, 2011). A number of parameters, some of which are difficult to predict, influence the ability of the encapsulated antigen and adjuvant to be co-delivered to the same APC (Chadwick, Kriegel, and Amiji, 2010; Jain, O'Hagan, and Singh, 2011). One such important parameter is NP size which was estimated in our study as 682.03 nm +/- 4.56 for PLGA encapsulated AIV and 675 nm +/- 9 for PLGA encapsulated CpG 2007 using dynamic light scattering. Particles of 10 μm or smaller are regarded as effective in eliciting potent immune responses *in vivo* (Chadwick, Kriegel, and Amiji, 2010).

In summary, intramuscular administration of nonencapsulated AIV adjuvanted with encapsulated CpG 2007 induced enhanced systemic and local immune responses and caused a reduction in virus shedding compared to the vaccine containing PLGA encapsulated AIV plus PLGA encapsulated CpG 2007. These findings indicate that the application of PLGA nanoparticles to inactivated AIV vaccine formulations can enhance the generation of systemic and local antibody responses in chickens. Future studies may address systemic and local immune responses to PLGA nanoparticle surface adsorbed inactivated AIV in combination with CpG 2007.

Table 3: Vaccine formulations for specific pathogen free chickens administered through the intramuscular route.

Vaccine formulations	Group
Encapsulated AIV (without CpG 2007)	1
Encapsulated AIV and encapsulated CpG 2007	2
Nonencapsulated AIV (without CpG 2007)	3
Nonencapsulated AIV and encapsulated CpG 2007	4
PLGA NPs	5
PBS (with virus challenge)	6
Encapsulated AIV and encapsulated non-CpG 2007	7
PLGA NPs and encapsulated AIV	8
PBS (without virus challenge)	9

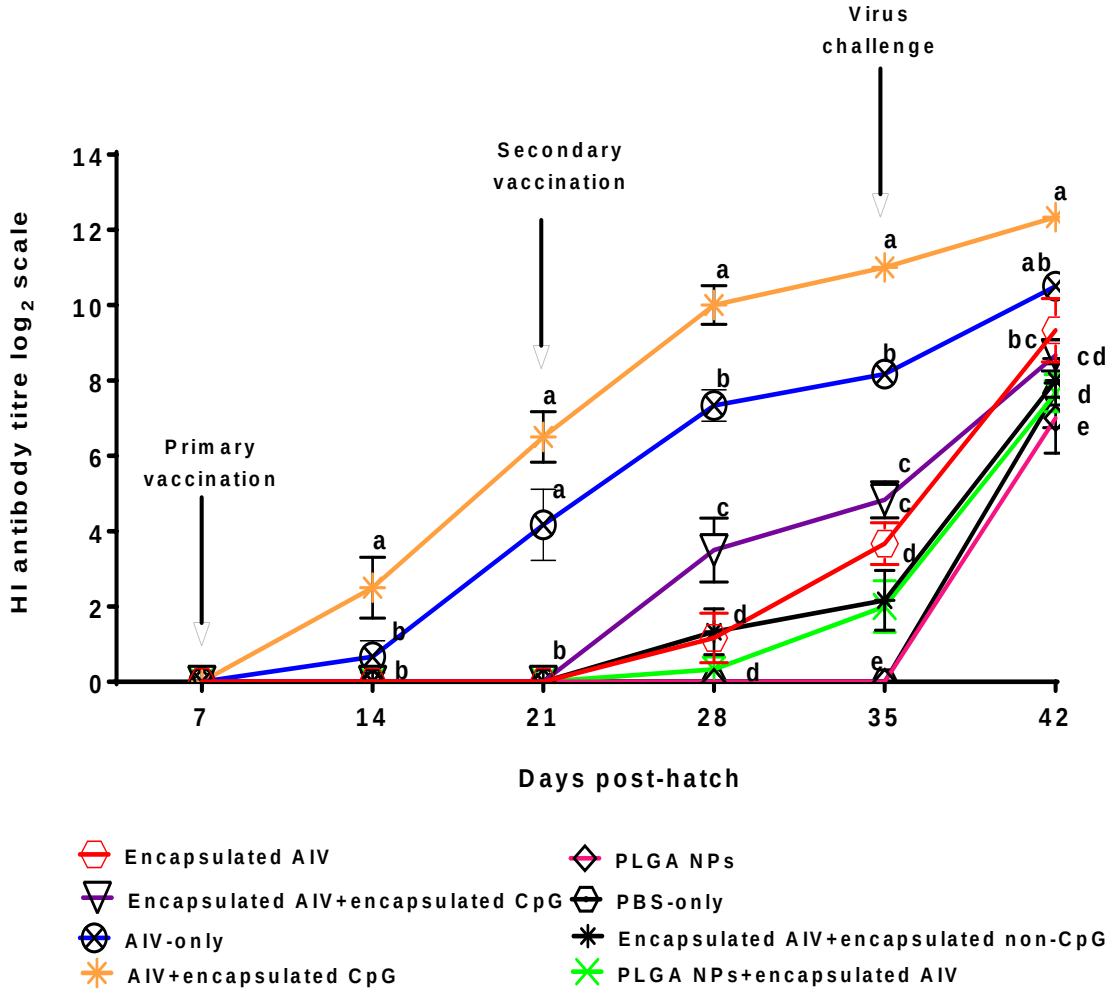

Figure 10: Mean serum haemagglutination inhibition (HI) titres in chickens vaccinated intramuscularly at days 7 and 21 post-hatch. Negative controls included PBS and PLGA NPs (blank) without adjuvant. Statistical significance among vaccinated groups was determined using a Proc GLM with Duncan's Multiple Range test, post ANOVA (p<0.05). Different alphabetical letters at each time-point represent significant differences among groups. Error bars represent standard error of the mean.

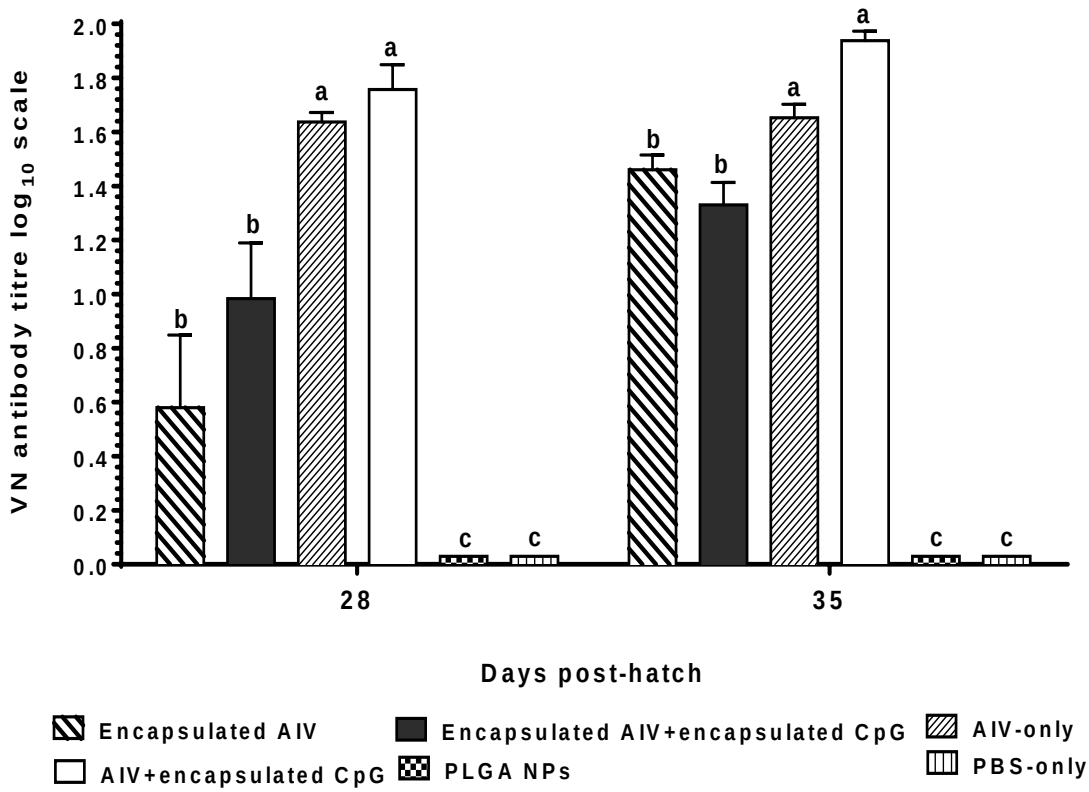

Figure 11: Mean serum virus neutralizing antibody titres generated at days 28 and 35 post-hatch. Primary vaccination occurred at day 7 post-hatch and secondary vaccination at day 21 post-hatch. Control groups received PBS and PLGA NPs (blank). Means with the same alphabetical letter at a single time-point do not differ significantly ($p<0.05$); error bars represent standard error of the means.

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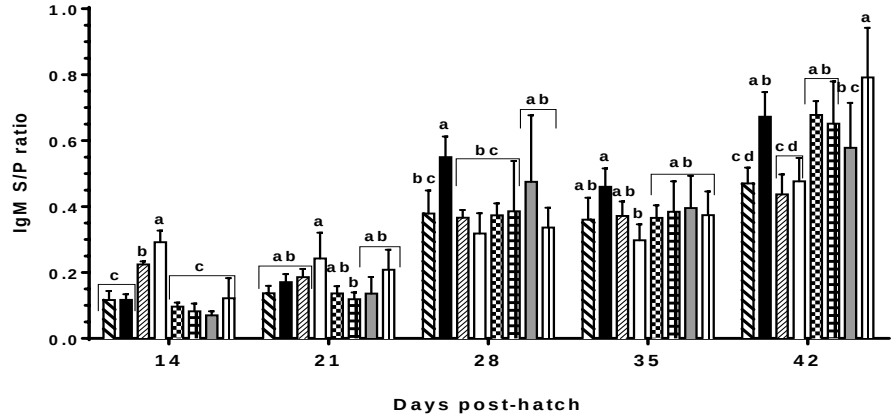
a



b

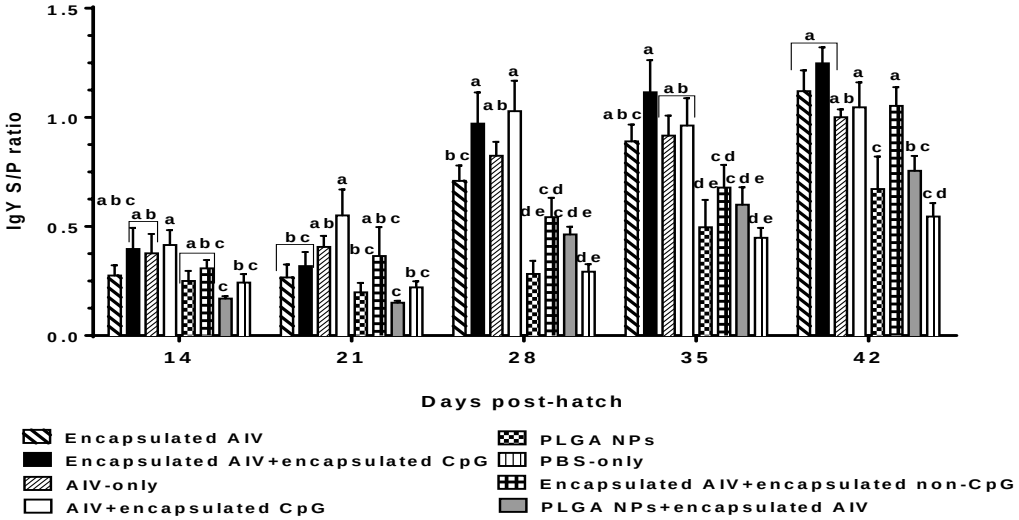


Figure 12: Serum IgM (a) and IgY (b) S/P ratios. Chickens were vaccinated intramuscularly at days 7 and 21 post-hatch with PLGA nanoparticle encapsulated and unencapsulated vaccine formulations. Control groups received PBS and PLGA NPs (blank). Significance is represented by different alphabetical letters per time-point (p<0.05). Error bars represent standard error of the mean.

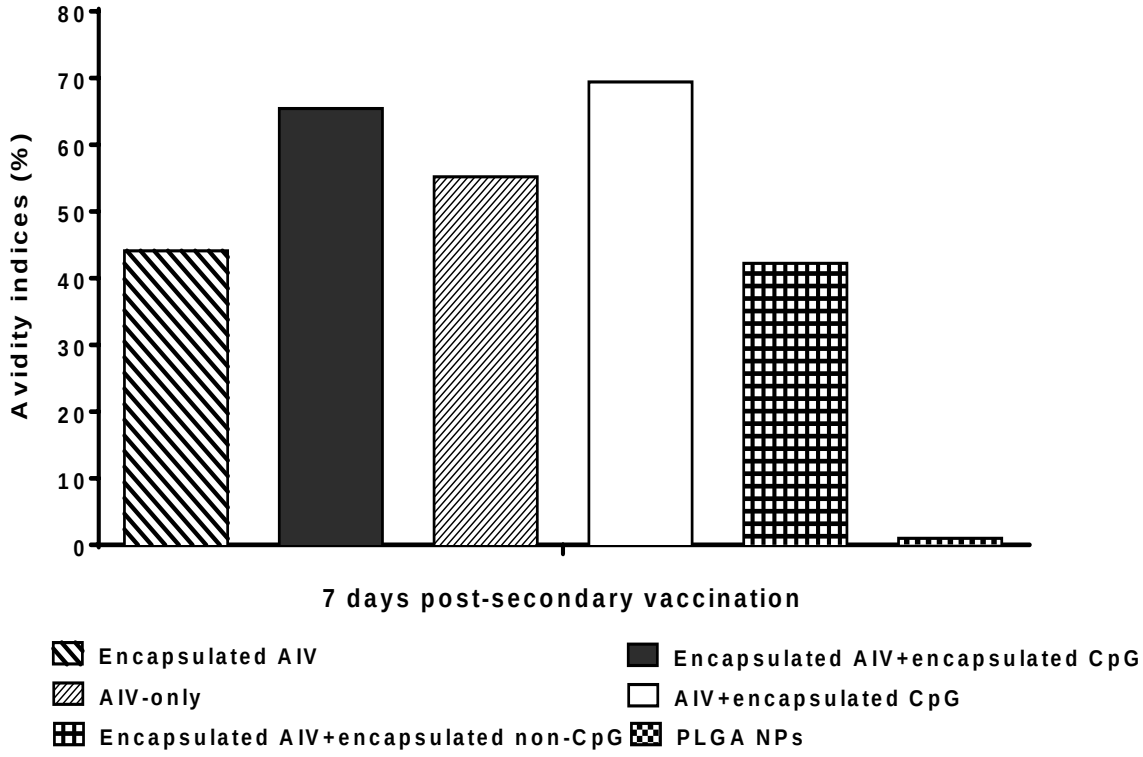


Figure 13: IgY antibody avidity indices (%) of serum from chickens at 7 days post-secondary vaccination (day 28 post-hatch). Primary and secondary vaccinations were administered at days 7 and 21 post-hatch, respectively. An avidity index of >50% represents antibodies of high avidity; values between 30% and 50% represent those of intermediate avidity, and values of <30% represent low avidity antibodies.

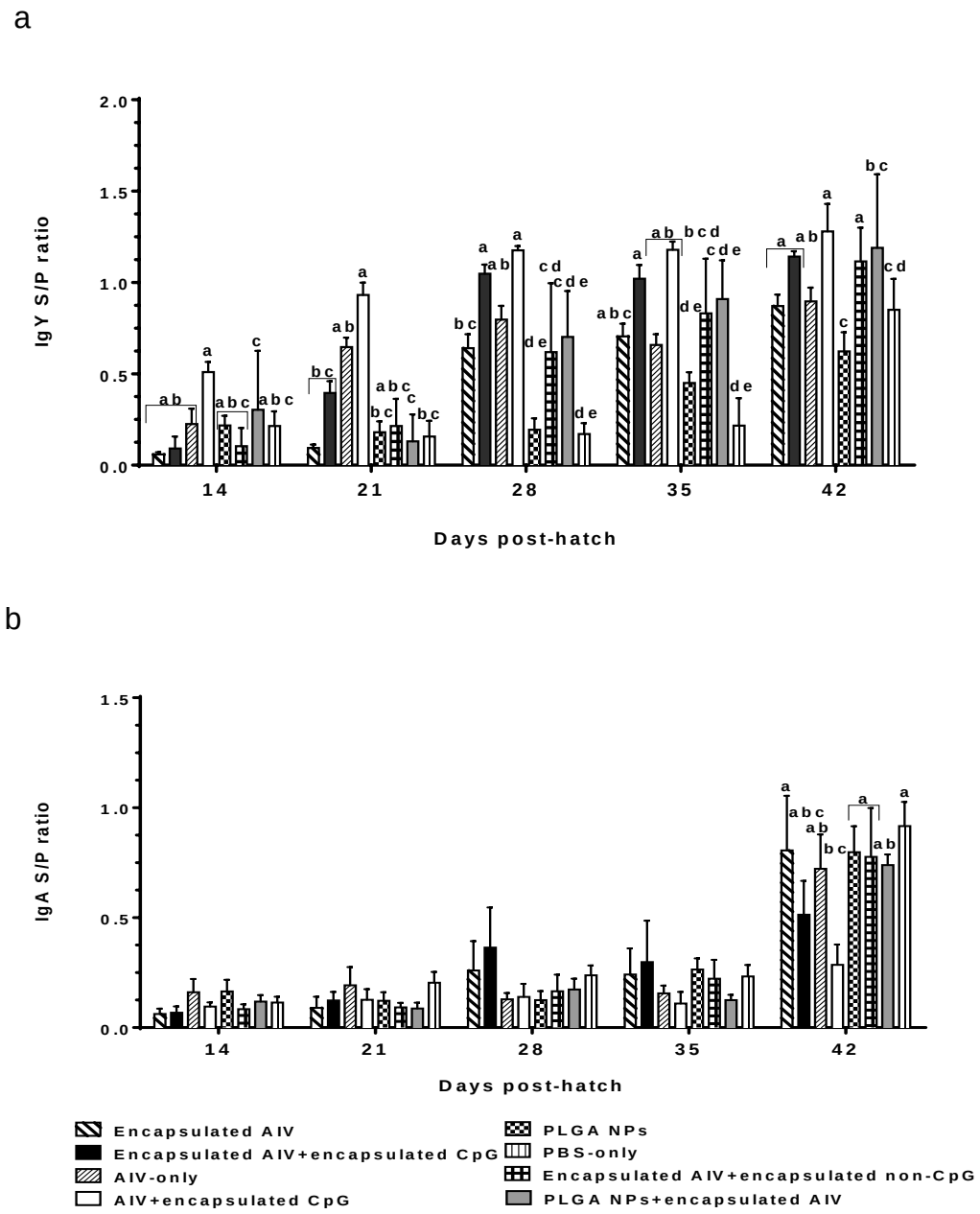


Figure 14: Mean IgY S/P ratios (a) and IgA S/P ratios (b) in lacrimal secretions of chickens vaccinated at days 7 and 21 post-hatch with PLGA nanoparticle encapsulated and unencapsulated vaccine formulations. Control groups received PBS and PLGA NPs (blank). Significance is represented by different alphabetical letters per time-point (p<0.05). Error bars represent standard error of the mean.

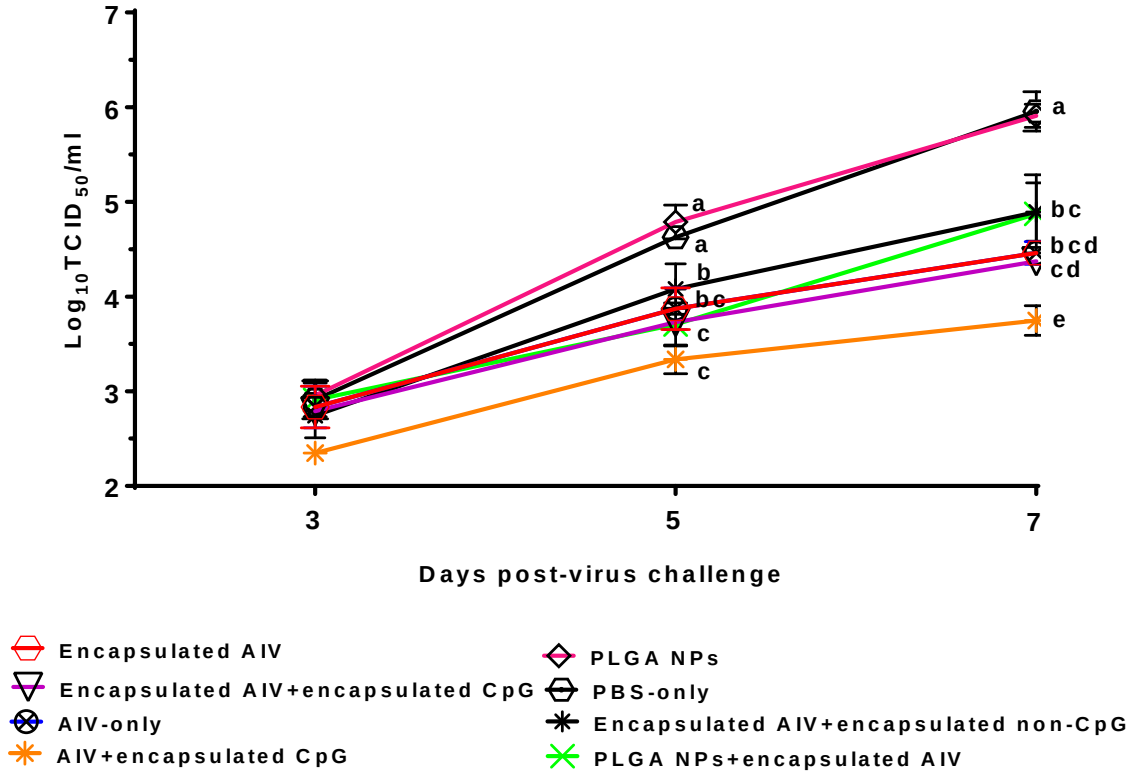


Figure 15: Virus titres of H9N2 AIV detected in cloacal swab samples on days 3, 5 and 7 post-challenge (virus quantitation by TCID50 in MDCK cells). Primary and secondary vaccinations occurred at days 7 and 21 post-hatch, respectively, with PLGA nanoparticle encapsulated and nonencapsulated vaccine formulations. Control groups received PBS and PLGA NPs (blank). Virus challenge occurred at 2 weeks post-secondary vaccination. Graphed values depict the mean $log_{10}TCID_{50}$/ml per vaccine group. Within a time point, means that do not have an alphabetical letter in common differ significantly ($p<0.05$). Error bars represent standard errors of the means.

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CHAPTER 5

5.1. GENERAL DISCUSSION

This thesis reports the investigation of novel, efficacious, vaccine adjuvants for their suitable application to inactivated, whole AIV vaccines by parenteral and mucosal routes of administration designed to curtail virus shedding in chickens. Recently, there has been a shift from the traditional or empirical approach to adjuvant selection for vaccine design to one based on immunological criteria (Guy, 2007). This is attributed to knowledge that the adaptive immune responses, which involve the generation of antibody-mediated responses, immunological memory and T cell-mediated immune responses are under the influence of the innate immune system (O'Hagan and Valiante, 2003). While T cell-mediated immune responses undoubtedly have a vital role in clearance of AIV after establishment (van den Berg *et al*., 2008), the induction of systemic and local mucosal, high-binding, neutralizing antibodies is imperative for the prevention of initial virus establishment in the host and, is therefore, a main focus in this thesis.

Class B ODNs are immunostimulatory to cells of the immune system in chickens, including B cells (Chrzastek, Piasecki, and Wieliczko, 2013; Mutwiri *et al*., 2003). Therefore, the first objective of the study was to compare the adjuvant potential of co-administered selected high and low doses of two representative class B ODN members, CpG 2007 and CpG 1826, given intramuscularly with a formalin-inactivated H9N2 AIV. Our study evaluated serum neutralizing antibody responses (HI and VN titres) and virus-specific antibody responses (serum IgM and IgY) until the termination of the experiment (6 weeks post-hatch) due to logistical issues associated with housing the chickens in the

Isolation Facility. Therefore, assessment of peak antibody-mediated responses was restricted to this time period. In our study, primary vaccination was administered at day 7 post-hatch and secondary vaccination occurred at day 21 post-hatch. Ideally, to induce optimum antibody responses using an inactivated AIV vaccine formulation, the recommended age of vaccine administration is after 14 days post-hatch (Swayne and Kapczynski, 2009). However, this was not a practical option in the design of our study, given the fact that the maximum duration of the study was set at 6 weeks. Regarding dose selection for our study, there was a 10-fold difference between the selected high (20 µg) and low doses (2 µg) based on previous studies in chickens: dose-titration with *E. coli* (Gomis *et al*., 2007) and prophylactic administration of CpG 2007 with inactivated AIV (St. Paul *et al*., 2012). Other criteria for dose selection involved economic feasibility and implications for long term, practical applications. In these previous studies, 10 µg was one of the doses used and was shown to exhibit immunostimulatory properties, therefore, 20 µg was considered to be a safe compromise for the high dose at the time of study design. Previous dose-titration studies used a dose as low as 3.16 µg (Gomis *et al*., 2007) for intramuscular delivery in chickens. Building on this theme, a dose of 2 µg was selected to provide a 10-fold difference compared to the high dose on the basis of its being not substantially different from 3.16 µg. The backbones of the ODNs used in our study consisted of phosphorothioate linkages as opposed to phosphodiester bonds so as to minimize the effects of *in vivo* degradation after intramuscular administration. The results of our study showed that, of the two members of class B ODNs used, CpG 2007 was the more efficacious adjuvant *in vivo*, and resulted in higher mean serum HI titres, VN titres and IgM S/P ratios from as early as 1 week post-secondary vaccination. This trend was

also observed for seroconversion and seroprotection rates at 1 week post-secondary vaccination which are important predictive parameters for evaluating protection. We observed that the administration of higher doses of adjuvants in the inactivated vaccine formulations did not necessarily confer higher levels of efficacy, indicating that class B ODN members have different degrees of adjuvanticity when combined with inactivated AIV during intramuscular administration as indicated by their dose responses. Although the exact mechanisms contributing to dose-related differences in adjuvanticity are unknown and require further investigation, suppressive effects associated with activation of subsets of T cells like CD4+ T regulatory cells (Kulkarni, Behboudi, and Sharif, 2011; Negash, Liman, and Rautenschlein, 2013), may be of importance.

The synthetic biodegradable polymer, PLGA, has been used in the development of nanoparticles and microparticles which can aid in the design of improved, novel, efficacious vaccines by eliciting optimum immune responses (Danhier *et al*., 2012). Generally, stronger antibody-mediated immune responses are generated by nanoparticles compared to microparticles, a feature that is likely to be attributed to their smaller size (Lin *et al*., 2015) with consequences for more efficient uptake by APCs. Therefore, PLGA nanoparticles were selected for our study. Based on our first experiment, the more efficacious dose of CpG 2007 was selected, encapsulated in PLGA nanoparticles then co-administered with inactivated AIV using the intramuscular and aerosol routes. The respiratory route of administration, although useful in terms of being a major point of entry for AIV, is associated with inherent challenges for the induction of mucosal antibodies including the low residence time, inadequate absorption and degradation of

vaccine components. Encapsulation of the adjuvant component of the vaccine formulation was done to enhance the potency of CpG by minimizing exposure to the harsh mucosal environment and to potentially improve immune responses. In the present study, we showed that the induction of significantly higher systemic (HI titres) and local (lacrimal IgA) antibody responses was possible with the PLGA nanoparticle encapsulated CpG vaccine formulation compared to the nonencapsulated formulation using the aerosol route. While this administration route is conducive to mass application, multiple vaccinations may be required to elicit antibody responses, an argument also suggested by de Geus and colleagues (de Geus *et al*., 2011). A plausible explanation for the observations made in our study is that by establishing contact with the mucus present in the respiratory tract, the PLGA nanoparticles became wet and swollen followed by an increase in mucoadhesive properties (Chaturvedi, Kumar, and Pathak, 2011). One major limitation of the study was the inability to determine the exact dose of the vaccine administered to each chicken as entry of aerosols was expected to occur through inhalation, open-mouth breathing, intraocular means and possibly by feather pecking. However, it is likely that aerosol entry through ocular mucosal surfaces contributed to the local IgA antibody response (Albini *et al*., 1974; Olah *et al*., 1992). Currently, the most significant neutralizing antibody isotype found in lacrimal secretions of chickens in the context of AIV is debatable. Another limitation of the study was the inability to predict with certainty, the way in which the nanoparticles exerted an influence on the immune response since this is determined by multiple factors: nanoparticle size, surface charge, shape, chemical structure, surface hydrophobicity, type of adjuvant encapsulated and the kinetics of release in mucosal and systemic environments. Limited data are currently

available on these parameters and mainly pertain to studies in humans (Danhier *et al*., 2012; Lin *et al*., 2015). In our study, a double emulsion solvent evaporation, w/o/w technique was used for PLGA encapsulation of CpG, which is hydrophilic (and lipophilic) (Karbach *et al*., 2012) by its nature. Negatively charged DNA is expected to result in the formation of a neutral nanoparticle when used with a polycationic polymer, allowing for improved intracellular delivery (Bhavsar and Amiji, 2007). In the present study, the size of the ODN containing nanoparticles was estimated to be 674.5 nm +/- 4.56 based on dynamic light scattering; the encapsulation efficiency was determined to be 74% based on the indirect method of quantification. The surface charge, as determined by the zeta potential was negative and the shape of the nanoparticles was spherical. In the present study, in contrast to the aerosol route, we showed that intramuscular delivery of the nonencapsulated CpG vaccine formulation induced significantly higher systemic (serum HI titres) and local (lacrimal IgY) antibody responses compared to the PLGA encapsulated vaccine after 2 vaccinations (days 7 and 21 post-hatch). Based on the mechanisms of release as described by Kumari and colleagues (Kumari, Yadav, and Yadav, 2010), CpG was likely to be released from the nanoparticles through diffusion, erosion or a combination of both. Consideration must be given to possible mechanisms of biological clearance that are applicable to the intramuscular route as this affects the period in which nanoparticles are expected to remain in the bloodstream (biodistribution). A possible explanation for the results observed in our study with the PLGA encapsulated vaccine formulation was that nanoparticle uptake by APCs was mediated by opsonization through the binding of antibodies, complement or fibrinogen thus increasing their

biological clearance (Aggarwal *et al*., 2009). Mechanisms of biological clearance were not pursued in our study.

In chapter 4, the immunogenicity and protective efficacy of PLGA nanoparticle encapsulated AIV and PLGA nanoparticle encapsulated CpG 2007 were assessed after intramuscular administration. The ultimate goal was to determine if encapsulation of the inactivated AIV component of the vaccine would induce more robust systemic and local mucosal antibody mediated immune responses compared to the formulation in which only the CpG component was encapsulated. Our experimental approach involved PLGA encapsulation of each vaccine component (inactivated AIV and CpG, separately) followed by mixing prior to intramuscular co-administration. Antigens have been successfully used with nanoparticles in other species to elicit immune responses through several approaches, including simple mixing (Zhang *et al*., 2014). Our results indicated that encapsulation of AIV did not offer an advantage by significantly enhancing the quantity of HI, VN, IgM or IgY antibody responses in serum and lacrimal secretions, and therefore, did not have an effect on cloacal virus shedding compared to the vaccine formulation containing nonencapsulated AIV adjuvanted with encapsulated CpG. We demonstrated that both encapsulated and nonencapsulated AIV, when adjuvanted with encapsulated CpG, were able to induce IgY antibodies of high avidity at 1 week post-secondary vaccination. This indicated that although our vaccine possessed the ability to induce a strong germinal centre reaction which is important for the production of memory B cells, the observed reduction in cloacal virus shedding was likely to be a function of the generation of neutralizing antibodies. The results generated in the present study were

consistent with findings in Chapter 3, which suggest that PLGA encapsulation of the CpG component of the vaccine formulation generated neutralizing antibodies in quantities high enough to be transuded to lacrimal secretions when administered intramuscularly. One limitation of our study was the negative charge of the nanoparticles which is associated with a tendency to localize within lysosomes with potential implications for degradation (Danhier *et al*., 2012). Surface modifications were not undertaken in our study to create a positively charged nanoparticle. Another limitation was the inability to predict the release pattern of the encapsulated vaccine contents as degradation rates are determined by a number of factors *in vivo* including the composition and molecular weight of the polymers used; in our study, PLGA 50:50 was used which was shown to be associated with slow release in a study conducted by Lin and colleagues (Lin *et al*., 2015). Additionally, in our study, protein (AIV) instability during the encapsulation process was difficult to assess and, studies of cross-reactions using homologous and heterologous viruses were not conducted. Finally, a "head to head" comparison was not attempted with the aerosol route using our vaccine formulations due to logistical reasons.

Major novel aspects of the research pertaining to chickens in this thesis include: a) Evaluation of vaccine adjuvancy through antibody-mediated immune responses subsequent to systemic (intramuscular) administration of high and low doses of class B ODNs with inactivated H9N2 AIV, b) assessment of the adjuvant potential of CpG 2007 (an immune potentiator) encapsulated in PLGA NPs (a particulate delivery system), administered with inactivated H9N2 virus through aerosol (mucosal) and intramuscular (systemic) routes, and, c) comparison of immunogenicity and protective efficacy of

vaccine formulations containing PLGA-encapsulated CpG 2007, with or without PLGA-encapsulated H9N2 virus after systemic administration (intramuscular route) as determined by antibody-mediated immune responses and their effects on virus shedding.

Despite the novel aspects of the work, some general caveats exist. Systemic and local, mucosal, neutralizing antibody responses are undoubtedly critical for the prevention of the initial establishment of AIV, however, to appreciate the full extent of vaccine immunogenicity, addressing some aspects of cell-mediated immunity might have been useful. However, in chickens, quantifying T cell responses remains a challenge due to the existence of very few assays. Secondly, the duration of the antibody mediated immune responses was not assessed due to the limited time period in which the experiments had to be conducted. Thirdly, although the formation of anti-CpG antibodies has been detected in humans to another class B ODN, CpG 2006 (CpG 7909) (Karbach *et al*., 2012), very little is known about the limiting effect on the establishment of immune responses and currently, no data exist for chickens. An important consideration in the design of any novel vaccine is the cost of production but a cost-benefit analysis was not undertaken in our study.

Future research directions should be aimed at gaining a holistic view of the immune responses of chickens to PLGA nanoparticle encapsulated vaccine formulations by addressing certain aspects of T cell-mediated immune response to supplement the knowledge gained on the kinetics of the antibody-mediated immune responses. More specifically, the expression of ChIFN-γ can be investigated through the use of an intracellular cytokine staining assay to gain an appreciation of the T_H1-like responses or

ChIFN-γ producing cells can be enumerated by an enzyme linked immunosorbent spot (ELISPOT). Future areas of exploration may also include PLGA encapsulation of different combinations of TLR agonists aimed at selectively enhancing different cytokines that augment antibody responses in chickens. This approach has been successfully demonstrated in mice (Kasturi *et al*., 2011). Biodistribution and immunogenicity studies may be attempted using fluorescent polymer nanoparticles (fluorophore-NP conjugate) (Robin and O'Reilly, 2015) containing encapsulated CpG with surface adsorbed inactivated AIV using aerosol and intramuscular modes of delivery. Another area requiring further investigation involves the mechanisms of action of non CpG ODNs in chickens, particularly when used in conjunction with inactivated AIV vaccines as preliminary and unexpected findings from our study showed that generation of antibody mediated immune responses was possible.

Overall, the findings presented in this thesis provide evidence regarding the use of CpG 2007 and PLGA nanoparticles as potential adjuvants in chickens when co-delivered with inactivated H9N2 AIV through parenteral and mucosal routes. The main theme of this work pertained to the establishment of systemic and local mucosal, neutralizing antibody responses; future directions should entail the assessment of aspects of immune responses, including T cell-responses. Furthermore, these studies should emphasize a more immunological basis and logical dose-sparing approach to the design of future vaccines and less of an empirical based one.

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