EFFECTS OF CYTOSINE-PHOSPHATE-GUANOSINE OLIGODEOXYNUCLEOTIDES (CPG-ODN) ON VACCINATION AND IMMUNIZATION OF NEONATAL CHICKENS

A Thesis

by

ADRIANA BARRI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2004

Major Subject: Poultry Science

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ABSTRACT

Effects of Cytosine-phosphate-Guanosine Oligodeoxynucleotides (CpG-ODN) on Vaccination and Immunization of Neonatal Chickens. (December 2004)

Adriana Barri, D.V.M., Universidad Nacional Autónoma de México

Chair of Advisory Committee: Dr. David J. Caldwell

The objective of this investigation was to evaluate the effects of administering CpG-ODN to commercial strain chickens as a potential adjuvant to vaccination against *Salmonella*, *Eimeria* spp., and Newcastle disease virus, or immunization to bovine serum albumin (BSA). During Experiment 1, which evaluated the dual application of CpG-ODN and a Newcastle disease virus vaccine, in the first of three replicate trials, on day 28 of the experiment, animals in the Vaccine + CpG 1& 14 experimental group were observed to have the highest levels of ($p<0.05$) anti-NDV IgG in serum. These levels were elevated above levels in animals from all other experimental groups. This suggestion for an adjuvant effect associated with CpG-ODN administration was not supported in the remaining two trials of experiment 1.

Experiment 2 evaluated the potential for CpG-ODN to adjuvant a commercial live oocyst coccidial vaccine when applied by an oral route to neonatal broiler chickens. Overall, when body weight gain during challenge, development of intestinal lesions, and anti-*Eimeria* IgG levels were evaluated, vaccine administration alone was demonstrated to provide the best measure of protection among animals in all

experimental groups, including those receiving either CpG-ODN or Non CpG-ODN.

Experiment 3 investigated the simultaneous administration of CpG-ODN or Non-CpG ODN and a commercially acquired *Salmonella typhimurium* vaccine to SCWL chickens. Similar to experiments 1 and 2, antigen specific IgG responses in serum and indices of protection against field strain *Salmonella* challenge were variable and inconsistent.

Anti-BSA IgG levels were compared in broiler and SCWL chickens immunized against BSA by a drinking water route of administration alone, or in combination with two different concentrations of CpG-ODN or Non CpG-ODN in experiment 4. The only observation where CpG-ODN and BSA co-administration resulted in anti-BSA IgG levels that were elevated above BSA alone immunized chickens was measured in broilers at day 19 post-final immunization.

Taken together, given the variable results reported in this investigation related to the co-administration of ODN and vaccine or protein antigen, these data are largely inconclusive for suggesting that CpG-ODN can effectively adjuvant humoral immune responses in commercial strain chickens.

DEDICATION

A Dios: Quien me ha llenado de una luz y fuerza interna maravillosa. Eres mi amigo y eterna compañia. Gracias a Ti, no estoy sola.

A mi adoradisima familia;
Queridisimos Papas.
Hermanos: Ana Beatriz, Leon, Syrlene y Eduardo
A los angelitos de la familia: Bruno, Leon David, Arianna y Juan Pablo
A los abuelos: sobre todo, a mi adorada Abuelita.

Con todo el cariño del mundo les dedico esta tesis; la cual significa mucho mas que el logro de un grado de maestria. Sin ustedes, no lo hubiera logrado. Ustedes fueron mi fuente de inspiracion y la fuerza para seguir adelante. Gracias por compartir, disfrutar y apoyarme en esta nueva etapa de mi vida. Los admiro, respeto y adoro con toda mi alma.

"Busquen con ardor los dones mas perfectos; pero tengan en cuenta que todos los dones, aún los más perfectos, nada son sin el AMOR de DIOS"

Sta. Teresita del niño Jesus

To God: Who has illuminated and blessed me with a wonderful internal strength. You are my friend and eternal companion. Thanks to you, I'm not alone.

To my beloved family:
My dearest Parents
Brothers and Sisters: Ana Beatriz, Leon, Syrlene, and Eduardo
To the small angels of the family: Bruno, Leon David, Arianna, and Juan Pablo
To my grandparents: over all to my beloved grandma.

With all my love I dedicate this thesis to you. This thesis means much more than the obtention of the MS degree. Without you, I would have never made it. You were my source of inspiration and the strength that kept me going on. Thanks for sharing, enjoying, and supporting me in this step of my life. I admire, respect and adore you with all my soul.

"Search with passion the most perfect gifts, but take into consideration that all the gifts, even the most perfect ones, are nothing without God's love"

Sta. Teresita del niño Jesus

ACKNOWLEDGEMENTS

First I would like to acknowledge my advisor. Dr. David Caldwell, thank you for all your support, guidance, patience, and good sense of humor. I am sincerely grateful for the opportunity you gave me by opening the doors of your lab to me and for helping me to fly. I would also like to thank the committee members, Drs. Audrey McElroy, Gale Wagner, Harry Danforth, and John El-Attrache. I greatly appreciate your suggestions, comments, and advice in this manuscript.

Dr. Alan Sams, I really appreciate all the support you have given me ever since I arrived in the U.S. Thank you for giving me such a good opportunity by allowing me to be part of your department at Texas A&M University. Thank you very much.

To the poultry health research lab family: Scott, Keith, Neil, Jason, Sid, Nancy and Phelue: I've been very lucky to work with you all. Lots of work! But hey, didn't we have fun? I am overwhelmed with gratitude for all of your patience, for not making fun of my accent, and for being so helpful and open to aid me in whatever doubt, complaint, or petition I had for you...or against you? Especially I'm glad you helped me in the early mornings while bleeding all those birds. If not, I would probably still be there. Thanks again. AZUL!

To the neighbor lab: Blair, Zack, Melannie, Blayne, and Jaime. Special thanks to Jaime: it was very nice of you to wake up at dawn and bleed chickens with us every time I needed help. Muchas gracias, and AZUL to you too!

Dale, thanks to you, it was a lot of feed, and too many technical complications all of which you solved for me. Thanks for your patience. Thanks a lot for your help!

To the people in the poultry science department, Jo Ann, Robert, Dana, and Jeanelle: You've been very helpful. Thanks.

To my friends in Mexico, Miguel, Claudia, and French, I certainly will always have you in my heart. For being there in the good and bad times, thanks.

Luis, thanks for your constant advice and criticism. Thanks for the laughs, and tears. I have never cried and laughed so much with someone in my life.

To my dear family: thanks for listening, and giving me so much advice, over all thanks for the long hours on the phone trying to shorten the distances between us.

Guayo lindo, thank you for everything, you were once again, my guardian angel.

Leon and Syrlene thank you for all your support and advice. You helped me a lot in starting my new life over here, it would have been very difficult without your help. Thank you. Guera, the laughs over the phone were the best. Thanks for sharing our drama, it made my life much more spicy! YES! I'm not the only crazy one!

Papito and Brujita linda, I respect and admire you so much, what else can I say! Thanks for giving me the living example of what love, honesty and hard work is. Thanks for letting me be, and for helping me be the person I am now. OH! And thanks for letting me know how everything is going over there. Please keep me posted until I get back! (in 5 more years?). **I LOVE YOU SO MUCH!**

And last to the <u>most important part of my life</u>, thanks to **GOD** for being my guide, light and strength throughout my life.

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CHAPTER I

INTRODUCTION

The poultry industry represents one of the most important sectors in all modern animal agriculture within in United States. It is considered to be a $22 billion industry that employs about 240,000 workers (U.S.D.A., 2004). It is one of the fastest growing segment of the animal industry, thus, regardless of the product produced, most of the systems employed by this industry involve intensive rearing were large numbers of birds, usually flocks with tens of thousands, are raised inside a closed house (Sharma, 1999). To control disease conditions under these dense and intensive rearing environments, vaccines represent an important and invaluable prophylactic and therapeutic strategy for commercial producers (Sharma, 2003). The commercial poultry industry relies heavily vaccines to protect chickens and turkeys against many pathogens that threaten poultry health and the subsequent overall economics of the industry. Vaccines in the poultry industry are needed in order to induce protection against infections by stimulating lymphocytes to produce antibodies or cytokines and differentiate into memory cells so that subsequent encounters with the same pathogens will be resolved quickly and efficiently (Abbas *et al*, 2000; Bermudez and Stewart-Brown, 2003; Sharma, 2003). The fundamental task of the immune system in such a scenario is to protect the host against invading microorganisms or pathogens through a

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This thesis follows the style and format of Poultry Science.

cooperative approach involving both innate and adaptive immunity. Innate immune responses are typically activated by innate system cells, such as macrophages, heterophils, or dendritic cells, which then act in concert to either kill the invading microbe or augment and trigger an appropriate adaptive immune response. Because innate immune cells lack the highly specific antigen receptors found on T and B cells, they rely on surface membrane proteins known as pattern recognition receptors (PRRs). PRRs have the ability to detect surface components of microbial pathogens known as pathogen associated molecular patterns (PAMPs). PAMPs recognition by PRRs in this setting is an indication of a threat to the host in the form of potential infection. Such recognition activates appropriate defense pathways in the innate system to either remove the pathogen or stimulate the adaptive system to act upon the pathogen for its ultimate removal. Cytosine-phosphate-guanosine (CpG) motifs naturally present in bacterial DNA represent one of the most recent examples of substances that influence adaptive immune responses by activating the innate immune system. CpGs are 6-8 base nucleotide motifs consisting of an unmethylated CpG dinucleotide that induces cells of the innate immune system to increase phagocytosis, pro-inflammatory cytokine production, activation of NK cells, and increase nitric oxide generation by macrophages.

The most critical line of defense against pathogens in an organism is the mucosal associated lymphoid tissues (MALT), which are comprised of the bronchial, salivary, genitourinary, nasopharyngeal, and gut associated lymphoid tissues (GALT) (Lillehoj and Trout, 1996; Yun *et al.*, 2000; Bar-Shira *et al.*, 2003). The last two, although separated, are important and integrated immune systems. They are considered

to be very important in the protection of the organism because these are the primary routes of entrance for any pathogen into the host (specifically digestive or respiratory) (Bar-Shira *et al.,* 2003). The ability of CpG-oligodeoxynucleotides (ODN) to act as vaccine adjuvants has been explored extensively in recent years in mammals but very little research has been conducted along these lines in commercial poultry. In recent years, DNA vaccines or DNA extracts from bacteria have been recognized as having tremendous potential for improving vaccination strategies in commercial poultry against different pathogens. The objective of this investigation was to evaluate the administration of CpG-ODN as a potential oral or mucosal adjuvant to commercially available vaccines against *Salmonella*, *Eimeria* spp., and Newcastle disease Virus, and a common protein antigen, bovine serum albumin (BSA), used frequently as an immunogen in poultry by our laboratory. Measured experimental outcomes included the level of antibodies produced and protection against subsequent clinical challenge in experimental animals following immunization and vaccination procedures evaluated in this study.

CHAPTER II

LITERATURE REVIEW

Basic Concepts of Immunology

The term immunity comes from the Latin word *immunitas* which originated in the Roman Empire where it was initially used to signify freedom from the taxes, services, and prosecution for Roman senators for which other citizens were forced to comply. The emperors frequently granted it to special classes of persons or to whole states (Abbas *et al,* 2000). Today, in medical terminology immunity refers to the condition in which an organism can resist disease. The immune system is the only defense of an organism against infectious diseases (Lynn *et al.,* 2003). Different factors may affect its response. The immune system consists of coordinated responses between a stimulus (antigen, antigen's nature, route and dose), external factors (diet or stress), and internal factors including genetics (MHC expression or not, T and B cell repertoire, etc), which make a living animal capable of establishing an effective immune response against the stimulus (Abbas *et al.,* 2000).

Any substance that is capable, under appropriate conditions, of inducing a specific immune response and of reacting with the products of that response, like antibodies or specifically sensitized T-lymphocytes, is an antigen (Ag). Antigens may be soluble substances, such as toxins, foreign proteins, or particles derived from bacteria or other infectious pathogens and tissue cells. Only the portion of the protein or

polysaccharide molecule known as the epitope reacts with the variable region of the antibody or a specific receptor on a lymphocyte. The ability for an epitope or antigen to elicit immune responses is known as immunogenicity (Abbas *et al.,* 2000).

Innate and adaptive are two integrated but distinct forms of immunity that exist in any organism or animal. Innate or nonspecific immunity is the organism's first generalized line of defense against all pathogens (Lynn *et al.,* 2003). Innate immunity is composed of 1) physical and chemical barriers including skin, tears, mucous, and saliva, 2) phagocytic cells such as heterophils, macrophages, and dendritic cells, and 3) inflammation of tissues once an organism is injured or after a pathogen has initiated an infection (Abbas *et al.,* 2000). These innate immune mechanisms usually help prevent the entrance and spread of different pathogens, but when the infection can not be controlled completely, specific or adaptive immunity is activated or engaged. The adaptive/acquired or specific immune system responds through lymphocytes and other accessory cells (Abbas *et al.,* 2000). Unlike innate immunity, it responds only after the pathogen is present, it is much more specific, and has memory facilitating it to react faster and stronger when a repeated exposure to the pathogen occurs. Importantly, a very important characteristic of adaptive immunity is that it does not attack normal body components, only those substances it recognizes as foreign and non-self. The most important cells of the adaptive immune system are lymphocytes or B and T cells (Abbas *et al.,* 2000).

It is well supported that the onset of the innate immune responses starts once an antigen has entered into the organism. The first cells to migrate to the site of infection

are the phagocytic cells, macrophages, dendritic cells, and heterophils, all of which are important components of the innate immune response and the inflammatory response (Qureshi, 1998; Ahmad-Nejad *et al.,* 2002)). Innate immune responses are initiated with the interaction between these cells and the pathogen. They can ingest the pathogen through pinocytosis, phagocytosis, or receptor mediated endocytosis (Qureshi, 1998; Thery and Amigorena, 2001). The receptors that activate innate cells and initiate these actions are known as pattern recognition receptors (PRRs). There are different kinds of PRRs: 1) Secreted (mannan binding lectin), 2) Endocytic (macrophage mannose receptor), and 3) Signaling, including the toll like receptors (TLRs), which associate with pathogen's surface structures called pathogen associated molecular patterns (PAMPs). PAMPS include mannan components of yeast cell walls, lipopolysaccharide (LPS) from Gram-negative bacteria, lipoproteins, peptidoglycans, and DNA containing unmethylated CpG-ODN (Medzhitov and Janeway, 1997; Aderem and Underhill, 1999; Thery and Amigorena, 2001; Lynn *et al.,* 2003). PRRs can also be expressed intracellularly like protein the kinase receptor produced under viral infection or some TLR like TLR9 (Janeway and Medzhitov, 2002). The PAMP-PRR interaction helps with the internalization of the antigen, its enzymatic degradation, and its further presentation through the MHC class I or II molecules to the surface, for activation of the adaptive immune response by stimulating naive T cells and B cells to proliferate and differentiate to eliminate the antigen (Aderem and Underhill, 1999; Abbas *et al.,* 2000). Internalization of the antigen/pathogen can be also triggered through the opsonization of the antigen with antibodies through the recognition of the antibody's constant region

receptor and the microbes' cell surface receptors, or through complement receptors that recognize complement fragments on the pathogen's cell surface (Abbas *et al.,* 2000).

The adaptive immune response consists of two separate responses: humoral and cell mediated responses. Humoral immunity consists of the activation of B cells which are responsible for the production of immunoglobulins. Immunoglobulins are antibodies and are present mainly in serum, cell surfaces, egg yolk, bile and mucosal secretions (Abbas *et al*., 2000). There are different means by which antibodies can react to a pathogenic organism: 1) Neutralization: prevent the pathogen from attaching to surface receptors of target cells thereby inhibit replication (virus), 2) Opsonization: antibodies attach to the microbes surface and facilitate macrophages and phagocytic cells to internalize and destroy such pathogens, and 3) Complement activation: (classic pathway), antibodies bind to the surface of the pathogen and activate the complement proteins, which are bound to phagocytes receptors facilitating the phagocytosis and destruction of it (Abbas *et al.,* 2000). There are three main isotypes of immunoglobulins in chickens: IgA, IgG(Y), and IgM. This differs with mammals in that most mammals have five standard isotypes. IgM is found on the surface of most B cells, and is the usually the antibody that predominates in a primary immune response. Class switching of an antibody occurs when IgM producing cells stop producing this isotype and "switch" to the production of another isotype like IgG or IgA. The cytokines that stimulate the B cells to do the class switch are IL-4 and IL5. IgG is the predominant immunoglobulin circulating in blood. IgA is involved in mucosal immunity (Castro and Powell, 1994; Staeheli *et al.,* 2001). T lymphocytes or T cells exist as two different

classes of cells 1) T helper Lymphocytes ($CD4^+$), whose main function is to secret cytokines for regulating immune responses; and 2) T cytotoxic Lymphocytes ($CD8^+$), which destroy viral infected cells (Abbas *et al.*, 2000).

Antigen presenting cells, such as dendritic cells, B cells and macrophages, are the cell populations that integrate the innate and adaptive immune systems. In their immature form, these cells phagocytose and degrade pathogens and express the degraded microbial peptides in major histocompatibility complex (MHC) class I or II molecules for presentation to lymphocytes (Abbas *et al.*, 2000; Janeway and Medzhitov, 2002). After the identification of the pathogen, the non-mature antigen presenting cells of the innate system, control the acute innate host response by phagocytosing and then killing the invading microbe by producing oxygen intermediates (superoxide anion, hydrogen peroxide, chloramines and hydroxyl radicals) and secreting proteolytic enzymes (Abbas *et al.*, 2000). The end product of this microbial recognition is the activation of intracellular signaling pathways that initiate maturation of the APC with cellular processes such as a) production of inflammatory cytokines such as TNF-α, and IL-1β, effector cytokines like IL-12 and type I interferon, b) up regulation of co-stimulatory molecules like CD80 and CD86 (regulated by transcription factor NFκB), and c) presentation of the antigenic peptides through their expression in MHC class I or II molecules for antigen presentation to the acquired immune system (Swaggerty *et al* 2004; Wagner, 2002).

The most important outcome from the activation of naive T cells through the presentation of the antigen-MHC complex is the proliferation of the antigen-specific T

cell and then the differentiation into effector and memory cells (Wagner, 2002). The origin of the pathogen will determine the class of MHC molecule used. Any extracellular antigen or protein, including bacteria (*Salmonella spp*) or parasites (*Eimeria* spp), will be presented through an MHC class II complex and recognized by a $CD4^+$ T cell. Any antigen or protein with an endogenous origin or presence in the cytosol, such as virus, will be presented through the MHC class I pathway and will be recognized by $CD8^+$ T cells. T cells become activated and initiate the production of different cytokines that will function in the effector stages of cell mediated immunity. T cells will also increase the expression of receptors for different cytokines as well once activated. One of the most important cytokines secreted for proliferation of T cells is IL-2, an autocrine cytokine, which acts as a growth factor (Abbas *et al.,* 2000; Thery and Amigorena, 2001). Depending on the T cell phenotype activated ($CD4^+$ or $CD8^+$); a specific differentiation pathway will follow (Castro and Powell, 1994). $CD4^+$ T cells differentiate into effector TH cell subsets that produce or secrete 2 different profiles of cytokines: Th1 and Th2 cells. Th1 cells, secretes IL-2 and IFNγ and therefore promote the cell mediated immune response. Th2 cells secrete IL-4, IL-5, IL-6, and IL-10 promoting the humoral immune response (Abbas *et al,* 2000; Staeheli *et al.,* 2001; Davison, 2003). When $CD8^+$ T cells recognize the antigen presented, they differentiate into cytotoxic cells (CTLs) which have the capacity to lyse and kill cells expressing foreign antigen in MHC I molecules. The most important function of the $CD4^+$ effector cells is to activate phagocytic cells and lymphocytes. The expression of co stimulators helps T lymphocytes to work in harmony; the co stimulators involved in T cell activation are CD28 (on T cell surface) which binds

to B7-1 or CD80 and B7-2 or CD86 expressed mainly on professional APC (Abbas *et al,* 2000).

B lymphocytes require stimulation similar to T cells in order to proliferate and differentiate into antibody producing cells, plasma cells, or memory cells (Castro and Powell, 1994). According to Bernasconi and Fagarasan, there are two subsets of B cells. Conventional B cells, the B2 subgroup which are activated with the participation of Th2 cells, and another subset referred to as B1 cells which are activated independently of T cell cooperation (Fagarasan and Honjo, 2000; Bernasconi, 2002). When B cells are activated, they proliferate into effector or plasma cells and into memory cells (Fagarasan and Honjo, 2000; Abbas *et al*, 2000; Bernasconi, 2002). Different pathways to stimulate B cells into proliferation and differentiation exist. First is the scenario where B naive cell activation occurs in the presence of the antigen. The antigen binds to the immunoglobulin present on the B cell's surface, and with the help of co stimulation by antigen-specific T cells through the interaction of CD40 with its ligand, and the secretion of cytokines (T-dependent pathway used by B2 cells), activation occurs. A second scenario occurs in absence of the antigen through microbial derived products such as LPS or CpG-ODN, which stimulate B cells via TLR4 and TLR9 (T-independent pathway used by B1 cells; (Bernasconi, 2002).

Cytokines are essential effector molecules of innate and acquired immunity that initiate and coordinate cellular and humoral responses directed to eradicating pathogens (Castro and Powell, 1994). Cytokines are active proteins that are secreted by different immune cells (T cells, B cells, macrophages, and dendritic cells) in order to regulate

other cells. They are classified in different groups, including, interferons (IFN), tumor necrosis factor (TNF), interleukins (IL), and chemokines. Cytokines can regulate immune responses by attaching to the surface of the cells and initiating signaling pathways (Paul *et al.,* 1994; Staeheli *et al.,* 2001). Some of the chicken cytokines that have been completely characterized to date are IFNα, IFN β, IFNγ, IL1β, IL2, IL6, IL8 IL15, IL16, IL17, and IL18 (Staeheli *et al.,* 2001; Lillehoj *et al.,* 2004). IL-6, is produced by T and B cells, endothelial cells, and macrophages, among others. Its secretion is induced by LPS, and can also be found in serum, ascites fluids, and in thymus tissue sections of chickens. As described above, T helper cells, depending on the kind of cytokines they secrete, are separated into two classifications: Th1 and Th2 cells. However, none of the Th2 cytokines homologs for avian cytokines (IL-4, IL-5, IL-10, or IL-13) have been characterized to date (Staeheli *et al.,* 2001; Davison, 2003). On the other hand, one of the cytokines secreted by Th1 cells, under stimulation of IL-12 and IL-18 (produced by macrophages, dendritic cells, and B cells) is IFNγ, which activates macrophages and enables the destruction of pathogens (Staeheli *et al.,* 2001). Studies have been performed evaluating cytokine expression during *Eimeria* spp infection in birds. Some reports show IL-6 is observed in serum of chickens that were infected with *Eimeria* (Staeheli *et al.,* 2001). Using standard PCR, there are also studies that report the increase of mRNA expression of IFNγ at the infection site in the gut of diseased birds. Other studies have shown a great inflammatory response during the infection with *Eimeria* mediated by chemokines, cytokines and TGF synthesized and secreted at the local site of infection (Staeheli *et al*., 2001). For viral infections, including infection by

Newcastle disease virus, reports describe that the oral administration of IFNα was able to diminish the state of the disease or at least improve the health in infected chickens. When IFNα was administered in a higher concentration through the drinking water, birds were reported to not lose weight due to the disease and the lesions in tracheal tissue were "strongly reduced" (Staeheli *et al.,* 2001).

Cytosine- phosphate- Guanosine Oligodeoxynucleotides (CpG-ODN)

Prior to 1984, bacterial DNA was largely considered to be immunologically non-reactive. Around this time, a series of investigations utilizing bacterial DNA isolated from *Mycobacterium bovis* first began to describe the potential stimulatory effects of bacterial DNA on the immune system (Hacker, 2002). By demonstrating direct and indirect stimulation of B cells and stimulation of NK cells to secrete INF-γ and the initiation of anti-tumor activity, CpG-ODN were first described as being the immunostimulatory component of bacterial DNA by Krieg and co-workers (Krieg *et al.,* 1995, 1996; 1999). In these investigations CpG-ODN were characterized as being specific sequence contexts of oligodeoxynucleotides (ODNs) with unmethylated cytosine-phosphate-guanosine (CpG) dinucleotides. CpG-ODN are 6-8 base nucleotide motifs consisting of CpG dinucleotides linked together by phosphodiester bonds and usually flanked by two 5' purines and two 3' pyrimidines. Since they are polyanions and cannot diffuse across cell membranes freely, ODN uptake into the cells requires the participation of a voltage-gated channel (Krieg, 2002). These unmethylated dinucleotides are expressed approximately 20 times more frequently in bacterial than in vertebrate DNA and the vertebrate cytosine residues are more frequently methylated at

position 5. These differences characterize CpG-ODN as PAMPs and thus enable vertebrates to protect themselves against invading pathogens (Klinman *et al.,* 1996). Hemmi and Bauer, respectively, reported that CpG DNA is recognized by a member of the Toll-like receptor (TLR) family which triggers the host's innate immune system. CpG-ODN present in bacterial DNA represents one of the most recent examples of PAMPs that influence adaptive immune responses by activating the innate immune system (Hemmi *et al.*, 2003).

The chemical structure of CpG-ODN greatly influences their ability to stimulate innate system cells. Changes in the bases flanking the CpG dinucleotides (purines and pyrimidines) have a great influence on the response because they influence recognition by the innate immune system. Although CpG-ODN uptake involves the binding to cell surface proteins with a non specific antigen-receptor, the immunostimulatory effects of CpG-ODN are activated by binding to an intracellular receptor, TLR9 (Krieg, 2002). Although this is not well understood, it is possible that TLR-9 may require a specific sequence in order to recognize CpG-ODN as a PAMP and bind to it as an antigen-dependent response according to the sequences that are surrounding the CpG dinucleotides (Zhao *et al.,* 1996; Pisetsky and Reich, 1999; Dalpke *et al*., 2002; Krieg, 2002). CpG-ODN in humans and mice, for example, must have specific nucleotide sequences in order to trigger an immune response. The sequence GACGTT has been shown to be the most immunostimulatory sequence in mice and rabbits, however it is inhibitory for other species and distinct from the most stimulatory sequence in humans GTCGTT (Rankin *et al., 2001;* Vleugels *et al.,* 2002). Research into the

immunostimulatory effects of CpG-ODN have not been limited to mice, rabbits, or humans, and have included sheep, horses, pigs, dogs, and cats, thus demonstrating that responsiveness to unmethylated bacterial DNA sequences is a phenomenon conserved across many species (Rankin *et al.,* 2001). Some research groups have proposed different consensus base sequences for immunostimulatory DNA sequences in different species. They found that the main characteristic of ODN stimulating an immune response was the presence of a GTCGTT motif. They reported that three GTCGTT motifs within the ODNs had a stronger and consistent stimulatory effect, while ODNs with only one or two did not activate the immune cells as consistently (Rankin *et al* 2001; Vleugels *et al.,* 2002; He *et al.,* 2003). They also found that some ODNs can have in the same structure, three distinct CpG motifs: GTCGTT + GACGTT + TGCGTT. This proposed sequence combination was shown to be stimulatory to immune cells from goats, horses, pigs, dogs, cats, chickens, rats, and rabbits, but not stimulatory for leukocytes from sheep or mice (Rankin *et al.,* 2001). Most of the immune enhancing potential in different species is strictly CpG dependent and inversions to GpC abolish this activation (Ahmad-Nehad *et al.,* 2002). Modifications made in the natural backbone chemistry of the CpG may also affect the immunostimulatory effects of the bacterial DNA by enhancing or deteriorating such effects. Natural ODNs are bound by phosphodiester bonds which make them very unstable and susceptible to endogenous nucleases found in biological fluids. These enzymes, present in responding immune system cells, quickly degrade CpG-ODNs by hydrolyzing the phosphodiester backbone, reducing the potential for immunostimulation. To improve stability necessary to allow

investigation into the immunostimulatory effects of CpG-ODN, synthetic CpG-ODN have been designed. However, such modification of CpG-ODN structure has proven to be complicated because many factors have been shown to influence both their activity and stability. Synthetic ODNs or analogs differ from the native ones by the substitution of a molecule of oxygen (O^-) at the phosphate group in the nucleotide. As such, these analogs are more resistant to nucleases, by increasing their stability against degradation. Successfully created analogs that have been reported to date include: a) Methylphosphonate ODN; b) Phosphoramidate ODN; c) Phosphorothioate ODN; and d) (N3'---- P5') Phosphoramidate (Gallo *et al.,* 2003). The most common ODN recently reviewed in the literature have contained the phosphorothioate backbone due to their ability to increased nonspecific binding to different proteins, increased efficiency of binding to cell membranes, and a higher degree of cellular uptake. ODN with this backbone are taken up much more efficiently than ODN with the phosphodiester backbone and are more efficiently endocytosed than ODN with a methylphosphonate, besides, their half life in vivo is 48 hours while the half life of a CpG-ODN with a phosphodiester backbone is 5 minutes (Pisetsky and Reich, 1999*;* Gallo *et al.,* 2003; Krieg, 2002). According to the susceptibility to degradation which is determined by the backbone, the ability to induce proliferation of B cells, activate macrophages, NK cells or other immune cells, will be either enhanced or decreased (Krieg, 2002; Zhao *et al.,* 1996; Pisetsky and Reich, 1999). Krieg describes that when phosphorothioate CpG-ODN are administered IV or SC they tend to concentrate in liver and kidney in high levels, and in less concentrations in spleen and bone marrow.

The following scenario can be used to describe how CpG-ODN stimulate the innate immune system during a bacterial infection. CpGs cross the cell membrane via sequence-non-specific receptor-mediated endocytosis. Once the bacterium is ingested by a macrophage and its DNA is degraded into ODN inside the cell, CpG-ODN is recognized by TLR-9. Recognition through TLR-9 will induce an immune response involving both innate and adaptive immune system cells (Krieg, 2002). The cellular activation by TLR-9 binding proceeds through a signaling cascade involving myeloid differentiation marker 88 (MyD88), IL-1R-associated kinase (IRAK), tumor necrosis factor receptor associated factor 6 (TRAF 6), nuclear factor kappa B (NF- κB) translocation, and up-regulation of genes involved in host defense. After this initial response, the triggering of TLR by specific CpG-ODN, results in TLR-9 specific immune responses (Takeshita *et al.,* 2001; Medzhitov, 2001; Kaisho and Akira, 2002). CpG-ODN can also act directly on B cells triggering cytokine production (IL-6, and IL-10), and immunoglobulin secretion. They've also been shown to activate dendritic cells to produce pro-inflammatory cytokines like IL-12 and stimulate antigen-specific T-cells, and proliferation of B cells (Medzhitov, 2001; Krieg, 2002). By such mechanisms, CpG-ODN are proposed to be important elements for eliciting mucosal and systemic immune responses in many animal species (Krieg *et al.,* 2001). CpG-ODN stimulates the production of IFNγ more than IL-12, and IL-10 functions as a counter-regulatory towards IL-12 secretion (Krieg, 2002). Activated B cells by CpG-ODN increase also cytokine expression of the Fcγ receptor, MHC II, CD80, and CD86 (Krieg, 2002).

Vaccines

The poultry industry is one of the most important sectors of all modern agricultural systems in United States. The value of broilers alone produced during 2001 was $16.7 billion (U.S.D.A., 2004). Regardless of the product produced, whether it be meat type chickens or turkeys, table eggs, or other specialty products, most of the systems employed by the industry involve intensive rearing were large numbers of birds, usually flocks with tens of thousands, are raised inside a closed house (Sharma, 1999). In such environments, the possibility of spreading disease among animals in a flock is much higher, therefore biosecurity measures must be followed and strict vaccination schedules must be maintained in order to diminish the possibility of disease outbreaks. Bacteria, virus, fungi, and parasites are potential pathogens of poultry. Some may cause severe clinical disease and death in less than 72 hours, while others may only cause sub clinical infections. In both instances, economic losses for growers and the industry in general are experienced (Bermudez and Stewart-Brown, 2003). To control disease conditions within these intensive rearing environments, vaccines represent an important and invaluable prophylactic and therapeutic strategy for commercial producers (Sharma, 2003). The commercial poultry industry relies heavily on vaccines to protect chickens and turkeys against many pathogens that threaten poultry health and the subsequent overall economics of the industry. Vaccines in the poultry industry are required to induce protection against infections by stimulating lymphocytes to produce antibodies or cytokines, and differentiate into memory cells so that subsequent encounters with the

same pathogens will be resolved quickly and efficiently (Abbas *et al,* 2000; Bermudez and Stewart-Brown, 2003; Sharma, 2003))

The term vaccine was first employed by Edward Jenner in 1796 after his successful prevention of infection by the smallpox virus. Approximately one hundred years later, in 1880, Louis Pasteur discovered that by allowing bacteria, in this case, *Pasteurella multocida* , the etiological agent of Fowl Cholera, to grow for a long period of time in the cell culture, it would become attenuated or weakened, and thus work effectively and without risk as a preventive vaccine for chicken and humans diseases (Gordon, 2003). Later, Pasteur extended this concept to other pathogens and found the cure for rabies. By inoculating a boy bitten by an infected dog with his vaccine, he was able to prevent the appearance of the clinical signs and symptoms of rabies. This procedure is still the only cure for this disease (Abbas *et al.,* 2000).

In some cases, a vaccine alone will cause a mild form of disease, but will usually not endanger the life of the animal. Vaccinations are used frequently and can start at day-of-hatch in poultry, and in some cases even before hatch, through *in ovo* vaccination (Bermudez and Stewart-Brown, 2003). Whenever a vaccine program is initiated, endemic diseases, flock history, distance from other birds or other houses or other productions, age, genetic background of the birds, and other environmental factors should be considered. It is important not to vaccinate when the pathogen is not endemic in order to avoid the introduction of a pathogen into a "clean" environment (Sharma, 1999; Brigitte, 2004). The protection of a flock can also be enhanced through vaccination of breeders in order to maximize maternal antibodies that will be passed

through the egg for protection in newly hatched chicks (Bermudez and Stewart-Brown, 2003). Maternal antibodies can circulate in the chick for up to 3 weeks, hopefully giving the neonatal animal time to allow for further maturation and development of the immune system (Bermudez and Stewart-Brown, 2003). While an initial administration of most vaccines usually generate immunologic memory, a secondary or boost administration of the same vaccine, is often required. Some vaccines however, like Marek's Disease virus vaccine, can elicit lifelong immunity following a single administration (Sharma, 1999).

Specific immune defenses against different pathogens are required. When a vaccine is being developed, different aspects should be considered, such as overcoming immune evasion strategies used by persistent pathogens, major histocompatibility complex molecule expression, integration of the pathogen genome in host DNA, antigenic variability of the pathogen, latency, age and genetic background of the host, among other things (Brigitte, 2004).

Vaccines also need to elicit strong $CD4^+$ and $CD8^+$ T cell responses. To do so, attenuated pathogens have traditionally been the choice for vaccines; unfortunately, such vaccines also expose potentially uncontrolled risks (Brigitte, 2004). The dose and route of administration of a vaccine are also very important, if applied through the incorrect route or in an insufficient concentration, the vaccine may not succeed in generating immunity. A vaccine may require several administrations as well. The different routes of administration of vaccines include intra muscular (IM), subcutaneous (SC), wing web puncture, and aerosol spray (Bermudez and Stewart-Brown, 2003). Oral (through drinking water or gavage), intranasal, and ocular (eye drop) immunizations are good

routes for generating mucosal immunity, and they can also elicit a systemic humoral responses (Abbas *et al.,* 2000). While in mammals, it is widely believed that oral routes of administration of antigen may lead to a state of systemic immune tolerance. More recent reports however, describe that in chickens of immunocompetent age, oral antigen administration with our without adjuvant results in robust systemic as well as local, mucosal immune responses (Klipper *et al.,* 2000, 2001; Ameiss *et al.,* 2004). Through the use of different antigen delivery systems that vary from soluble proteins with mucosal adjuvants to attenuated recombinant bacterial and viral vectors, immune responses with different effector characteristics can be induced.

There are several different types of vaccines currently used in commercial poultry operations. These include attenuated and inactivated vaccines that are usually used for bacterial and viral pathogens. These are vaccines where the intact pathogen is treated in such a way (prolonged growth in cell cultures, heat, or chemical substances) that it can no longer produce clinical disease. The goal of such vaccines is to persist in the host long enough to imitate the threatening signals normally expressed by pathogens and trigger both innate and adaptive immune response (Abbas *et al*, 2000). In addition to viral and bacterial vaccines of this type, several live and attenuated vaccines against coccidia species are commercially available that are composed of oocysts from distinct species of *Eimeria* (Lillehoj and Trout, 1996; Yun *et al.,* 2000). Another type of vaccine, consist of subunit vaccines, purified antigens. In these vaccines, antigenic portions of the pathogen are usually given together with an adjuvant or hapten- carrier conjugates to generate immunity in the host. These vaccines are used usually designed for bacterial

toxins or polysaccharide antigens. These purified protein vaccines are effective at stimulating TH cells and antibody responses by B cells, since they are acquired extracellularly as proteins and are expressed through the MHC II pathway of antigen processing and presentation. Recombinant proteins obtained from *Eimeria acervulina* have been shown to be good immunostimulatory antigens for cellular and humoral responses in natural and passive immunity (Lillehoj *et al*, 2004). Another type of vaccine includes synthetic antigen vaccines which are synthesized in a laboratory following the identification of the most immunodominant epitope(s) of a specific pathogen. This is possible through the use of recombinant DNA technology and by mutational analysis where the peptides are overlapped making it possible for them to be presented and recognized (Abbas *et al.,* 2000). According to Sharma (1999), live fowl pox virus and turkey herpes virus are common vectors with inserts of genes that encode for immunogenic proteins of different avian pathogens such as Newcastle disease virus, avian influenza, or infectious bronchitis disease virus (IBDV), to enhance protection in the host. In this scenario, live viral vectors represent yet another classification of vaccines. Such vaccines consist of the introduction of genes encoding pathogenic antigens of a certain pathogen into a vector (which is non pathogenic) and then infecting the organism to induce an immune response which typically includes the activation of $CD8^+$ cells. However, this can cause the lysis of host cells presenting this antigen even though the virus is non pathogenic. Another type of vaccine getting a great deal of attention in recent years is a DNA vaccine. These vaccines involve the internalization of a plasmid containing a cDNA that will encode the protein antigen of the pathogen within

the cytoplasm of transfected cells, and thus induce a good CTL response. Since bacterial DNA contains CpG-ODN, it will also be recognized as foreign and an innate immune response will be elicited stimulating at the same time a good adaptive immune response (Abbas *et al*, 2000).

Adjuvants

Adjuvants are substances that when are administered with an antigen can induce a stronger antigen-specific immune response (Sharma, 1999; McCluskie and Weeratna, 2001a). The selection of an appropriate adjuvant is often as important as the development of the vaccine. The most efficacious adjuvants successfully imitate the immunogenicity elicited by live pathogens (Kaufmann, 1996). Adjuvants can act in different ways, including: a) enhancing the immunological life of the vaccine; b) increasing antigen uptake; and c) by improving antigen presentation through MHC pathways in APCs. Some adjuvants can be used just as delivery systems with some immunostimulatory properties; however the best adjuvants are the ones that are immunostimulatory molecules *per se*, such bacterial toxins, hormones, synthetic CpG-ODN, and others. Different natural immune markers or ligands, including cytokines and chemokines, are now being used to regulate or enhance effector T cell function and memory T cell maintenance during vaccination (Kaufmann, 1996). Chemokines augment Th 1 and CTL responses increasing a vaccine's efficacy, however, they are still under development. IFNγ for example has shown to be a good adjuvant by increasing the antibody responses when administered in a plasmid during the immunization against *Eimeria acervulina*. When given orally IFNγ has also been shown to improve the health

in chickens infected with NDV (Staeheli *et al.,* 2001). The FDA will only approve those adjuvants that produce the minimal adverse effects in an immunized animal. For mucosal immunization the most potent adjuvants described to date appear to be *Vibrio Cholerae* toxin (Ct) and *Escherichia coli* enterotoxin (Et) (O'Hagan *et al.,* 2001). Adjuvants can also determine which type TH cell response will be stimulated, either a Th1 –like or a Th2 –like responses. For example, oral administration of a soluble protein with Ct as an adjuvant has shown a Th2-type immune response, while the use of CpG-ODN stimulates a Th1 –like type immune response (Staats *et al.,* 1996; McCluskie and Davis, 1998, 1999a; McCluskie *et al,* 2002; O'Hagan *et al.,* 2001).

Due to modifications done to pathogenic antigens while developing vaccines, some of them may induce a weaker immune response than the original pathogen when administered alone by either parenteral or mucosal routes. Under such circumstances, the generation of a successful immune response is often accomplished through the use of adjuvants. Studies evaluating adjuvant properties demonstrate that adjuvants have the capacity to affect the kinetics, duration, amount, avidity, and neutralization capacity of antibodies produced during immunization (Hinoshi *et al.,* 1996). It is also known that adjuvants affect the specificity of antibody responses by affecting the epitope to which the antibody is targeted (Hui *et al.,* 1991). Although, adjuvants have been studied and used for many years, the mechanism of action of some is still not well understood. Nonetheless, it is firmly believed that adjuvants are as important as the vaccines in some instances when it is necessary to induce protection of an animal against disease (Weeratna *et al*., 2000).

Some adjuvants exist as delivery systems and have the capacity to form deposits of antigens at the site of inoculation. This helps promote a slower release of the antigen, thus exposing immune cells to it for a longer period of time. Examples of these types of adjuvants are oil (lipid) emulsions and aluminum salts (Weeratna *et al.,* 2000). Another class of adjuvants: LPS, CPG-ODN, or monophosphoryl lipid A (MPL), are known as immunostimulatory adjuvants. These adjuvants represent PAMPS and can activate the innate immune system of the host when administered as described above (Hagan *et al.*, 2001). Other very effective adjuvants, usually only used for research purposes, are complete Freund's adjuvant (CFA) and incomplete Freund's Adjuvant (IFA). These represent a mixture of delivery, and immunostimulatory adjuvants. The mineral oil present in CFA forms antigen deposits at the injection site causing slow release or exposure to the immune system. The most active components of CFA are extracts of killed mycobacterium suspended in paraffin oil. This suspension is capable of very potently stimulating macrophages and ultimately induces a strong Th1 response in the immunized animal. The most immunostimulatory component of CFA is muramyl dipeptide (Roitt, 1994). The difference between CFA and IFA is that IFA does not contain the mycobacterial extracts. When using IFA, the antigen is usually suspended directly in paraffin oil prior to administering to the animal. Both adjuvants act as nonspecific stimulators of the immune cells thus enhancing humoral and cell mediated responses (Weeratna *et al.,* 2000). Complications arising from the use of CFA due to its toxicity from causing necrosis or damage from over stimulation of macrophages within

the tissue at the inoculation site prohibits the use of CFA in humans (Kiyono and McGhee, 1996).

Different studies have reported the successful use of CpG-ODN as vaccine adjuvants. Most of these have been performed in mice and thus the extrapolation of these data into other vertebrates needs to be performed carefully. Another concern related to some of these reports centers on much of this research being done *in vitro*, which obviously does not take into consideration the multiple different effects that could alter the response in an *in vivo* system. Weeratna and his group compared different adjuvants, when given individually or mixed, and evaluated their ability to enhance an immune response against hepatitis B surface antigen (HBsAg). They also evaluated the degree of damage these antigens could cause when injected into the muscle tissue of the mice. They found that the combination of Alum and CpG had the best results for inducing measured immune responses with the smallest degree of tissue injury (Weeratna *et al.,* 2000). Another group demonstrated that intranasal immunization of mice against influenza virus simultaneously with CpG-ODN in a concentration of 50µl/mouse was effective to stimulate systemic and mucosal immune responses (Moldoveanu *et al.,* 1998). Another laboratory evaluated the mucosal response of CpG-ODN as mucosal adjuvants in mice, when immunized against one or more of the following antigens: Tetanus toxoid, HBsAg, and the vaccine Fluviral, alone or in combination. When evaluating three distinct routes of administration, oral, intranasal, and intra-rectal, their results indicated that CpG-ODN are capable of inducing antibody production in serum as well as in mucosa (McCluskie and Davis, 2000a; McCluskie *et al,* 2001b). This group

also observed that Non CpG-ODN induced an immune response by producing antigen-specific IgG, as well as CpG or Ct (McCluskie and Davis,1999b; McCluskie *et al,* 2000b, 2001c).

As stated above, most experiments performed to date investigating the immunostimulatory effects of CpG-ODN have been done in mice, primates, or humans. Until recently, there were only three studies that evaluated the effects of CpG-ODN as potential adjuvants in poultry. These included a study into the efficacy of CpG-ODN against IFA by comparing the efficacy in producing IgG and IgM after being injected subcutaneously with BSA in chickens. Observed results showed that the antibody levels were higher in the chickens injected with both CpG-ODN and BSA, suggesting a possible adjuvant effect via the subcutaneous route of administration (Vleugels *et al.,* 2002). A second study evaluated the proliferation of PBMC in peripheral blood in 6 chickens with different sequences and backbones of CpG-ODN. Despite an obvious criticism of using such a limited number of experimental animals, the results of this experiment indicated that a sequence with 3 CPG motifs in the same ODN (sequence number 2135) was the most immunostimulatory (Rankin *et al.,* 2001). Another study by Gomis and co-workers represents essentially the only complete report on a study done *in vivo* in chickens. They evaluated the administration route and the specific protection against *E. coli* infection induced by CpG-ODN when inoculated to birds via SC or IM administration (Gomis *et al.*, 2003). Importantly, all studies investigating immunostimulatory properties of CpG-ODN that have been done in chickens to date

have not evaluated CpG-ODN as oral or mucosal adjuvants. Thus, exploration of CpG-ODN to adjuvant mucosal immune responses is warranted using chickens as a model.

Oral Immunization of Poultry

Bovine Serum Albumin (BSA), due to its broad-scale availability and ease of use, is a protein that has been frequently used in research settings to evaluate immune responses in mammals and chickens. Recently, it has been used as a food-protein antigen to assist researchers in understanding the phenomenon of oral tolerance in chickens, mice, and other mammals.

Oral tolerance is a condition of unresponsiveness of the immune system to an orally administered antigen (Miller and Cook, 1994). The avian immune system does become tolerant to orally ingested food antigens in a fashion similar to mammals, however, tolerance is usually only induced when the animal is a neonate, usually during the first week of life (3- 10 days post hatch). Following this time, fed antigens become immunostimulatory, and do not induce tolerance. Interestingly, this phenomenon occurring at 10 days of age in the chicken coincides with the appearance of the Peyer's patches (PP), more extensive B and T cell colonization, and subsequent cytokine expression at mucosal sites, all of which are required for the induction of humoral responses in the mucosa (Miller and Cook, 1994, Ameiss *et al.,* 2004, Klipper *et al.,* 2004). The ability of an orally administered antigen to either induce tolerance or immunity depends not only on the age of the animal, but on the frequency, dose and physical presentation of the antigen given. It has been shown that soluble antigen given orally in low concentrations on consecutive days of administration (Ameiss *et al.,* 2004)

induces a robust systemic and local antibody response. When administered in similar fashion in terms of concentration and route of administration, but using a dry or dry or pelletized form of antigen, tolerance is induced (Klipper *et al.*, 2000, 2001). Thus, after the birds become more immunologically mature, the ability of developing oral tolerance to soluble antigens is lost. According to Ameiss *et al.,* (2004) drinking water administration is effective in eliciting a humoral response than a direct oral gavage, and equally as good as intra-peritoneal antigen administration with adjuvant in Leghorn chickens. Klipper and co-workers demonstrated that the hyper immunization of breeding hens with BSA and the subsequent presence of maternal antigen-specific antibodies in progeny inhibits tolerance. These authors suggest this to be mechanisms to describe why some pathogens which are exposed to the neonatal chick do not induce tolerance (Klipper *et al.,* 2004).

Poultry Salmonellosis

According to the Food-borne Diseases Active Surveillance Network (FoodNet), in their final report for 2001; the bacterial pathogens with the highest relative incidence for causing bacterial food borne illness in humans during the period between 1996 and 2001 were *Campylobacter*, *Salmonella*, and *Shigella.* Even though there was a decline of *Salmonella* incidence for food-borne disease during the period of 1996 through 1999; the increase in the incidence of infections caused by *Salmonella* in 2000 and 2001 represents an emerging challenge to public health agencies. According to the CDC (CDC, 2004), human salmonellosis constituted 31 % of the food-related deaths in the United States between 1992 and 1997 (Mead *et al.*, 2004).

Salmonella are gram negative, aerobic, non-capsulated, and non-sporulating bacteria, which can infect mammals, birds, reptiles, and amphibian hosts (Gast, 2003). *Salmonella* are widely distributed in nature. They have been a great concern for the poultry and animal agriculture industries for quite some time. Two host specific, non motile strains of *Salmonella, S. pullorum* and *S. gallinarum* which cause Pullorum Disease and Fowl Typhoid, respectively, have been adequately controlled in most parts of the world and essentially eradicated from commercial flocks within the U.S., Canada, Australia, Japan, and Western Europe. At least within the U.S., no outbreaks have been reported since 1980 (Gast, 2003).

Of the 2400 different serotypes of *Salmonella* which exist, only a small fraction of these serotypes are known to be linked to both poultry reservoirs and human health. Infection in humans usually occurs when contaminated products including eggs, or raw /undercooked meat are consumed (Charlton *et al.,* 2000). To reduce human food-borne salmonellosis traced back to poultry as the source of infection, the poultry industry and many research laboratories are constantly exploring new methodologies that will help decrease *Salmonella* incidence in poultry. Poultry producers and processors must also comply with mandatory regulations imposed by federal regulatory agencies to keep the presence of *Salmonella* on processed poultry or poultry products to a minimum accepted standard.

Most serotypes of *Salmonella enterica* subspecies *enterica* responsible for gastrointestinal disease among humans are commonly grouped as paratyphoid (PT) *Salmonella*e. They consist of serotypes that include *S. enteritidis, S. oranienberg, S.*

montevideo, S. newport, S. typhimurium, S. anatum, S. derby, and S. bredeney. Of these, *S.* e*nteritidis, S. typhimurium, and S. montevideo* are frequently isolated in poultry (Charlton *et al.,* 2000).

In humans, gastroenteritis is the most common clinical sign of an infection by paratyphoid *Salmonella*e. Human salmonellosis can also manifest vomiting, diarrhea, fever, other systemic symptoms, or even death (Buck and Weker, 1998). Chickens infected with *S. enteritidis* and *S. typhimurium*, present minimal, if any clinical signs. However, when affected, chickens are usually 2 days old or less and often suffer high mortality related to systemic disease (Beal *et al.,* 2004). Other reports mention no mortality, but instead a large shedding of bacteria through feces due to massive bacterial replication in the gut (Zhang-Barber *et al.,* 1999). Regardless of the presence or absence of clinical signs in young or mature chickens, these paratyphoid serotypes can colonize the chicken oviduct, ceca, and other regions of the gastrointestinal tract, and are often isolated from internal organs such as liver and/or spleen which can be primary sites of replication along with the ceca (Gast, 2003). This extensive degree of colonization or infection in commercial poultry underscores the possible threat to human health associated with these bacteria. Transmission of PT *Salmonella*e to other poultry can occur through shedding of the bacteria in feces, within a hatchery from the presence of contaminated egg shells, through vectors such as personnel, birds, rats, insects, or through fomites like farm equipment (Gast, 2003).

Salmonella are intracellular facultative organisms. Depending on the infectious dose, age, and health condition of the animal, the development of the disease will result

either in gut colonization, invasion of different organs, or death. There are different possible routes of inoculation consisting of: oral, intra-tracheal, conjunctiva, intra-cloacal, aerosol, or through contaminated semen (Gast, 2003).

Salmonella spp are capable of inducing both systemic and mucosal antibody production in poultry. Serum antibodies, mainly the IgG isotype, have been reported to interfere with bacterial adherence to mucosal surfaces (Gallin and Fauci, 1985). Several distinct approaches in vaccine development which target the elicitation of a humoral response to *Salmonella* in poultry have been taken to date. Live, attenuated, and killed bacterins have all been shown to be effective in some respects, but often inconsistent. One of the most common approaches to the vaccination of commercial poultry against *Salmonella* through the years has involved the use of an oil-immersion bacterin. Two reports by Gast and co-workers evaluated the ability of two killed *S. enteritidis* bacterins, one acetone killed and one commercially available, administered orally or subcutaneously to laying hens. The ability to reduce intestinal colonization and shedding in the feces was evaluated. In these experiments bacterins only resulted in partial protection to hens vaccinated by either oral or subcutaneous routes (Gast *et al.,* 1992, 1993). Limited protection from the use of bacterins has resulted in other researchers developing modified live, or gene deletion vaccines. Studies with genetically modified *Salmonella* strains have also been reported and modification of both virulent and avirulent strains has been shown to induce humoral responses in chickens on a dose dependent basis (Curtiss III *et al.,* 1993). Curtiss and co-workers claim that using a live, avirulent *Salmonella typhimurium* vaccine strain can diminish colonization, shedding,

and result in progeny protection through transovarian antibody transmission against a number of different *Salmonella* serotypes (Curtiss III *et al.,* 1993; Curtiss III and Hassan, 1996; Hassan *et al.,* 1993, 1997; Hassan and Curtiss III, 1994). After immunization with this modified strain, good IgG levels are present in egg yolk, serum, and intestinal secretions of the neonatal chickens. Also, the immunization of different animals with this same *Salmonella* modified strain, is observed to produce a good mucosal, humoral and cellular immune response, against LPS, and outer membrane proteins (OMP; Curtiss III and Hassan, 1996). Many additional reports indicate an incremental increase antibody levels, mainly IgG and IgA in bile, intestinal mucosa, and serum occurs after oral immunization (Beal *et al.,* 2004). The knowledge of humoral kinetics in poultry is still not well understood, however, leading Zhang-Barber *et al.* (1999) to describe the development of new and effective vaccines as an empirical challenge.

While precisely unknown to what extent they may play at present, humoral responses may be an important component in the chicken immune response to overcome *Salmonella* colonization. The need to reduce the incidence of *Salmonella* in poultry is imperative; therefore there is still a need for developing improved and more successful strategies for vaccination. Successful vaccination that will bring better protective immunity to limit the invasiveness of the bacteria and reduce its multiplication in affected tissues is clearly needed.

Eimeria *Infections in Commercial Poultry*

Coccidiosis is an enteric parasitism infection of great economic importance in commercial poultry. Some estimates describe costs and losses to the U.S. poultry industry between 1 to 2 billion dollars annually (Yun *et al.,* 2000, Morris *et al*. 2004). Economic losses associated with this enteric disease condition are not only due to morbidity / mortality or decreases in performance, but also to costs associated with constant medication that must be given to essentially all floor-reared commercial poultry flocks for the duration of the productive cycle. The emergence of drug resistance in strains of coccidia, and the pressure applied to the industry to restrict the use of antibiotics or medications in products consumed by humans, is forcing researchers to evaluate and investigate alternative methods of control.

Coccidia parasites belong to the genus *Eimeria,* member of the family *Eimeriidae that* belongs to the subphylum Apicomplexa. There are seven species of *Eimeria* that affect chickens*: E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox,* and *E. tenella*. The most common species usually found within broiler production are *Eimeria acervulina, E. maxima, E. mitis,* and *E. tenella* (Crouch *et al.,* 2003). Each species can parasitize the intestine of commercial poultry individually or in combination. However, the *Eimeria* that infect poultry are host specific and show a definitive tissue tropism within the area of the gut they choose to invade. The morphology (size, shape, and color) of oocysts also varies among species, as well as the kind of macroscopic lesion they produce which facilitates species specific differential diagnosis (McDougald, 2003).

Oocysts shed through feces sporulate in the environment making them infective when ingested by other poultry. After such ingestion, the sporozoites are released from sporocyst and oocyst shells within the gizzard, and are then able to invade and establish in the intestinal mucosa or the lamina propria of the gut. Within these sites, sporozoites undergo multiplication until the liberation of schizonts, the most pathogenic phase of the protozoan. Within enterocytes, they multiply sexually, resulting in zygotes which mature into oocysts. Following two stages of sexual development, formed oocysts are liberated from host cells and release into the lumen for excretion to the environment. Oocysts are excreted for several days by this manner through the feces (Yun *et al.,* 2000). Although it depends on the species, the entire life cycle usually takes from 4 to 6 days to complete (McDougald, 2003).

Eimeria spp. can affect essentially all regions of the both the small and large intestine of commercial poultry with species specificity (Yun *et al.,* 2000; McDougald, 2003). Infection with coccidia can result in increased mucus secretion, cellular rupture, reduced nutrient absorption, hemorrhage, diarrhea, dehydration, weight loss, and occasionally death. Egg production in laying hens can also decrease with coccidiosis (Yun *et al*., 2000). The kind of lesions formed in the intestine as a result of infection can predispose for the establishment of other enteric pathogens, including *Clostridium perfringens* or *Salmonella typhimurium* (Gast, 2003; McDougald, 2003). In chickens the most common *Eimeria* to invade the upper small intestine or duodenum is *E. acervulina*. *E. maxima,* normally infects the lower duodenum, jejunum, and ileum. Whereas, *E. tenella* typically infects the cecum (McDougald, 2003). Long term survival of oocysts in

rearing environments requires certain environmental characteristics in order to survive and be viable; including high rearing density, low ammonia, higher levels of oxygen, and susceptible chickens (Chapman *et al.,* 2002).

Eimeria is highly immunogenic, but does not demonstrate cross-species protection, thus requiring vaccines to include all the endemic strains and species present in a particular poultry production complex. Since they are immunogenic, they have the capacity of enhancing a protective immunity for a long period of time after immunization; but some species require variable numbers of oocysts to induce such immunity. The exact mechanism of the humoral response in coccidia infection is still not well understood. Some studies report that the presence of antibodies inhibits sporozoite invasion by eliminating and destroying them within the lumen (Rose *et al.,* 1984; 1987). It is also known that the infection with *Eimeria* will induce parasite-specific antibody production locally and systemically (Lillehoj and Trout, 1996). It has been demonstrated with different approaches that the participation of T cells in responses against coccidia is of major importance (Lillehoj and Trout, 1996). There is an inverse proportion between presence of T cells and susceptibility of the chick to *Eimeria* infection. Whenever there is a decrease in T cells, resulting in a measurable decrease in IFNγ and IL2, there is a corresponding increase in susceptibility to *Eimeria* (Lillehoj *et al*., 2004). Lymphoproliferation assays have been very useful in helping to determine the importance of the activation of antigen specific T cells from chicken immunized with *Eimeria*. There are also differences in the T-cell population in the duodenum after primary and secondary immunization (Lillehoj and Trout, 1996). For example, after the

secondary infection, an important number of CD8+ IEL cells increases, higher than in the primary infection, and a reduced oocyst production is apparent. CD4+ IEL cells also increase 7 days post primary infection. Overall, there is a close relation between infected epithelial cells and the presence of this CTL cells in the gut. Hens immunized with gametocyte surface antigens of *E. maxima* are able to produce specific IgG antibodies and transfer them to their progeny (Lillehoj *et al.,* 2004). Lillehoj and Trout (1996) demonstrated in various studies that within the IEL, $CD8^+$ cells are actually involved in transporting sporozoites from villus enterocytes to crypt enterocytes where establishment and completion of the life cycle would continue. These experiments revealed that when depleting $CD8^+$ cells from a chicken, the oocyst production decreased to 55 % in the primary infection with *E. tenella* and *E. acervulina* (Lillehoj and Trout, 1996).

Newcastle Disease Virus

Newcastle Disease (NDV) is a permanent threat to the aviculturists and the commercial poultry industry in the United States and worldwide. According to the OIE (Office of International Epizootics) NDV is a disease on list A having the following characteristics: “a transmissible disease that has the potential for very serious and rapid spread, irrespective of national borders, that is of serious socio-economic or public health consequence, and that is of major importance in the international trade of animals and animal products.” During the last few years, a total of 2618 confirmed outbreaks with exotic NDV (END) have been reported in the U.S., including outbreaks in the states of California, Nevada, Arizona, and Texas. In previous decade, especially during 1995, 1997, and 1998, there were additional outbreaks with NDV, however in most cases, with

a less virulent serotype. The economic impact of NDV outbreaks is characterized by high mortality in END infections in commercial flocks, condemnation and eradication of other infected flocks, and trade restrictions associated with quarantine and surveillance of affected areas within individual states where outbreaks have been detected.

NDV is a *Paramyxovirus* that belongs to the family Paramyxoviridae and its genus is *Rubulavirus*. It exists as one serotype, giving it the name Avian Paramyxovirus type 1 (APMV-1) (Alexander., 2003). NDV consists of an enveloped, single molecule with single stranded RNA. Its genome encodes for six different proteins, and an RNA directed RNA polymerase. The most important of these proteins, in terms of diagnosis and virulence factors, are hemagglutinin and neuraminidase proteins (HN), and the fusion protein (F). HN is the responsible for hemagglutination of avian red blood cells (RBC), and is the antigenic structure of the virus. The fusion protein fuses with host cell receptors giving rise to virus replication. Both play a major role, as surface proteins, by inducing an immune response in the host (Alexander, 2003).

NDV infection begins with attachment to host cell receptors through the HN protein. The two membranes fuse by the action of the F protein which causes entrance of the virus into the cell. Within the cytoplasm the virus replicates. The RNA directed RNA polymerase transcribes a complementary transcript of positive sense of its own genome that will work as an mRNA helping the translation of its own proteins utilizing the hosts' cells. During its replication, NDV proteins are produced with the F protein, which is a precursor (F0) that has to be cleaved to F1 and F2 in order to be virulent. If no cleavage occurs, the result will be proteins with no virulence. The cleavage is determined by host

protease sensitivity to the amino acid sequence of the virulent strain. The amino acid sequence varies depending on the strain of virus. Virulent strains for example, present arginine and phenylalanine in specific locations of the amino acid sequence, while non-virulent strains present different sequences (Alexander, 2003).

Transmission among poultry can occur by ingestion or inhalation of infected secretions or contaminated feces. The transport or movement of people, equipment, feed, water, birds, or even vaccines from an endemic area into a "clean"zone is also known to result in infection. The incubation period of NDV varies form 2 to 15 days in poultry (Alexander, 2003).

Historically, the only effective means of controlling NDV has been through vaccination. The most common routes of inoculation of a vaccine to protect against NDV are oral, ocular, and intranasal. The humoral immune response to vaccination is first observed within 6-10 days post immunization where antibody production in the mucosa and serum is evident. IgM, IgG, and IgA have been observed in serum and tears after ocular immunization with the B1 strain of NDV, a mildly virulent strain live virus vaccine (Al Garib., *et al.,* 2003). It is well proven, that the ocular (eye drop) administration of a vaccine can induce antibody production at mucosal surfaces and in serum to neutralize the virus and prevent infection. The Hitchner B1 strain of NDV is also commonly used as a vaccine strain. Some reports suggest that the eye drop application of this vaccine similarly enhances IgM, IgA, and IgG production in serum and tears. Such elevated systemic and local antibody titers have been correlated with enhancing the protection against this pathogen (Russell and Ezeifeka, 1995). Overall,

vaccination protects flocks by diminishing the clinical signs that the virus produces within the host. Depending on the vaccine strain used however, replication and shedding of the virus may not be completely eliminated (Russell and Koch, 1993). While successful in many respects, the observation of vaccinated animals continuing to be susceptible to NDV suggests improvements in vaccines and methods of administration are still needed. Presently, researchers are in constant search for vaccines that will provide greater protection and improve animal health for aviculturists and the commercial poultry industry

Summary and Conclusions

A major research interest in our laboratory at present is investigating vaccine efficacy to improve enteric and respiratory disease resistance in commercial poultry by studying host immune responsiveness to vaccination and challenge. As such, the overall objective of the present investigation was to investigate the potential of CpG-ODN to act as an oral or mucosal adjuvant when administered to broilers and leghorns immunized with commercially available NDV, coccidial, or *Salmonella* vaccines, or BSA when administered by mucosal routes of immunization.

CHAPTER III

EFFECTS OF CPG OLIGODEOXYNUCLEOTIDES ON VACCINATION OR IMMUNIZATION STRATEGIES DIRECTED AGAINST NEWCASTLE DISEASE VIRUS, *EIMERIA*, *SALMONELLA*, OR BOVINE SERUM ALBUMIN (BSA) IN COMMERCIAL STRAIN CHICKENS

Introduction

Newcastle Disease virus (NDV) is a permanent threat to aviculturists and the commercial poultry industry in the United States and worldwide. According to the OIE (Office of International Epizootics) NDV is a disease on list A with the following characteristics: "a transmissible disease that has the potential for very serious and rapid spread, irrespective of national borders, that is of serious socio-economic or public health consequence, and that is of major importance in the international trade of animals and animal products." During the last few years, a total of 2618 confirmed outbreaks with exotic NDV (END) have been reported in the U.S., throughout the states of California, Nevada, Arizona, and Texas. In previous decade, especially during 1995, 1997, and 1998, there were additional outbreaks with NDV, however in most cases, with a less virulent serotype. The economic impact of NDV outbreaks is characterized by high mortality in END infections in commercial flocks, condemnation and eradication of other infected flocks, and trade restrictions associated with quarantine and surveillance of affected areas within individual states where outbreaks have been detected.

Coccidiosis is an enteric parasitic infection of great economic importance in commercial poultry. Some estimates describe costs and losses to the U.S. poultry industry that are between 1 to 2 billion dollars annually (Yun *et al.*, 2000, Morris *et al*. 2004). Economic losses associated with this enteric disease condition are not only due to morbidity / mortality or decreases in performance, but also to costs associated with constant medication that must be given to essentially all floor-reared commercial poultry flocks for the duration of the productive cycle. The emergence of drug resistant coccidia, and the pressure applied to the industry to restrict the use of antibiotics or medications to products consumed by humans, is forcing researchers to evaluate and investigate alternative control methods of control.

According to the Food-borne Diseases Active Surveillance Network (FoodNet) final report in 2001; the bacterial pathogens with the highest relative incidence for causing bacterial food borne illness in humans during the period between 1996 and 2001 were *Campylobacter*, *Salmonella*, and *Shigella.* Even though there was a decline of *Salmonella* incidence for food-borne disease during the period of 1996 through 1999; the increase in the incidence of infections caused by *Salmonella* in 2000 and 2001 represents an emerging challenge to public health agencies. According to the CDC (CDC, 2004), human salmonellosis constituted 31 % of the food-related deaths in the United States between 1992 and 1997 (Mead *et al.*, 2004). Poultry and poultry products are frequently implicated as sources of human foodborne salmonellosis.

A common strategy for controlling or minimizing the presence of each of these pathogens in commercial poultry flocks is vaccination. The commercial poultry industry

relies heavily vaccines to protect chickens and turkeys against pathogens that threaten poultry health and the subsequent overall economics of the industry. The fundamental task of the immune system in responding to vaccination is to generate protection in the host against invading microorganisms or pathogens through a cooperative approach involving both innate and adaptive immunity. Innate immune responses are typically activated by innate system cells, such as macrophages, heterophils, or dendritic cells, which then act in concert to either kill the invading microbe or augment and trigger an appropriate adaptive immune response. Because innate immune cells lack the highly specific antigen receptors found on T and B cells they often rely on surface membrane proteins known as pattern recognition receptors (PRRs). PRRs have the ability to detect surface components of microbial pathogens known as pathogen associated molecular patterns (PAMPs). PAMPs recognition by PRRs in this setting is an indication of a threat to the host in the form of potential infection. Such recognition activates appropriate defense pathways in the innate system to either remove the pathogen or stimulate the adaptive system to act upon the pathogen for its ultimate removal.

CpG motifs naturally present in bacterial DNA represent one of the most recent examples of substances that influence adaptive immune responses by activating the innate immune system. CpGs are 6-8 base nucleotide motifs consisting of an unmethylated CpG dinucleotide that induce cells of the innate immune system to increase phagocytosis, pro-inflammatory cytokine production, activation of NK cells, increase nitric oxide generation by macrophages, and stimulate B cell proliferation and differentiation. The ability of CpG-ODN to act as vaccine adjuvants has been explored

extensively in recent years in mammals, but very little research has been conducted along these lines in commercial poultry. In recent years, DNA vaccines or DNA extracts from bacteria have been recognized as having tremendous potential for improving vaccination strategies in commercial poultry against different pathogens. The objective of this investigation was to evaluate the administration of CpG-ODN as a potential oral adjuvant to commercially available vaccines against *Salmonella*, *Eimeria* spp., and Newcastle Disease Virus, and a common protein antigen, bovine serum albumin (BSA). Measured experimental outcomes included the level of antibodies produced and protection against subsequent clinical challenge in experimental animals following immunization and vaccination procedures evaluated in this study.

Materials and Methods

Experimental Animals

For experiment 1, which consisted of 3 replicate trials of 160 chickens each, fertile eggs from specific pathogen free breeder flocks were obtained from Charles River SPAFAS (SPAFAS Avian Products and Services, North Franklin, CT.), incubated, and hatched at the Texas A&M University Poultry Science Center. For experiment 2, 160 straight-run (Cobb X Cobb) broiler chicks were obtained from a local commercial hatchery on day-of-hatch. For experiments 3 (n=252) and 4 (n=132), single-comb-white-leghorn (SCWL) (Hy-Line W-36) chicks were obtained from a local commercial hatchery on day-of-hatch. Additionally in experiment 4, 132 straight-run (Cobb X Cobb) broiler chicks were obtained on day-of-hatch from the same hatchery used to obtain chicks for experiment 2. At the time of chick placement in all experiments, chicks were

provided *ad libitum* access to drinking water and a balanced, un-medicated, corn-soybean ration that met or exceeded National Research Council (NRC, 1994) guidelines for poultry. Chicks were wing banded, randomly assigned to specific experimental groups, and placed on either floor pens with clean pine shavings (experiments 1, 3, and 4), or wire starter batteries (experiment 2) with age-appropriate supplemental heat.

Vaccines and BSA

The vaccines for NDV, Coccidia, and *Salmonella* were obtained from the individual vaccine manufacturers for use in the present experiments. The vaccine used for NDV vaccination strategies was live virus B1 Strain, titer $10^{7.5}$ obtained from Merial Select (Gainesville, GA). The live oocyst coccidial vaccine used in experiment 2 was Coccivac®-B obtained from Schering-Plough Animal Health (Millsboro, DE). This vaccine contained four species of *Eimeria* (E*. tenella, E. maxima, E. acervulina, E. mivati*). In experiment 3, chickens were vaccinated with the modified live (deletion mutant) *Salmonella* vaccine Megan® Vac 1 manufactured by Lohmann Animal Health International (Gainesville, GA) for Megan Health, Inc (St. Louis, MO). Immunization of chickens in experiment 4 was performed with BSA-fraction V obtained from Sigma Chemical Company (St. Louis, MO).

CpG-ODN and Non CpG-ODN

In all experiments, either synthetic CpG-ODN or Non CpG-ODN were evaluated as oral or mucosal adjuvants respective to experimental group. The CpG-ODN that was used was the ODN 2007 (5’ TCG TCG TTG TCG TTT TGT CGT 3’; CpG motifs are underlined) as previously described (Rankin *et al.*, 2001). As a control to the use of

CpG-ODN, we also used Non CpG-ODN 2041 (5'CTG GTC TTT CTG GTT TTT TTC TGG 3') also as described previously (Rankin *et al.,* 2001). The CpG-ODN and the Non CpG-ODN were synthesized with a phosphorothioate backbone to increase resistance to hosts nucleases. Both ODN were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). Both ODN were resuspended in sterile pyrogen-free PBS immediately prior to administration. Each bird receiving CpG-ODN or Non CpG-ODN was administered a dose of 50μg through the specific route of administration (oral or intranasal) relative to experiment and experimental group.

Indirect Enzyme Linked Immunosorbent Assay (ELISA)

In experiment 1, a commercial ELISA kit (Idexx Laboratories, Inc., Westbrook, ME) was used to evaluate the specific NDV IgG levels in serum samples obtained from experimental animals. For experiments 2, 3, and 4, indirect ELISA was performed on all serum samples using flat-bottomed 96 well plates (Nalge Nunc International, Rochester, NY). Prior to addition of sample antiserum, plates were coated with antigen and allowed to incubate overnight. Respective to individual experiment, this consisted of adding the following to each well: 5.2 μl of recombinant protein of *E. acervulina* (EASZ240) per well for experiment 2, 10μl of LPS specific for *S. enteritidis* (Sigma L6011) or *S. typhimurium* (Sigma L6511) per well during experiment 3, or 5μl of BSA fraction V (Sigma 2960) per well during experiment 4. Following antigen binding, each well was rinsed with PBS-T and then blocked with a blocking buffer containing 0.5% of BSA for half an hour. Plates were rinsed again and then 150μl of diluted serum was added into each well. Serum dilutions for all experiments ranged between 1:320 to 1:2560 per

sample. Data presented represent serum dilutions of 1:640. All the dilutions were performed within each plate. Following sample addition to wells, plates were agitated for 2 hours. Following rinsing, 150µl of horseradish peroxidase (HRPO) conjugated goat anti-chicken IgG (dilution 1:10,000) was added to each well in experiments 2 and 4. In experiment 3, a conjugated rabbit anti-Chicken IgG (Sigma A9046) was used. For all the samples in all experiments, a rabbit conjugated anti-Chicken IgG (Sigma A 9046) was used at the same dilution (1:10,000). Plates were incubated for two hours, rinsed again, and then a substrate solution containing 100µl of DMSO with 1mg TMB in 10ml of sodium acetate buffer (pH 5.5) was added to all wells. Reactions were stopped after 15 min by adding 50 µl of 1M sulfuric acid. Plates were read in Magellan multi well plate reader at a wavelength of 450nm. Plates were read in the Magellan multi-well plate reader at a wavelength of 620 nm, according to specification of the own kit.

Experimental Design

Experiment 1. To evaluate the ability of CpG-ODN to adjuvant NDV vaccination on day-of-hatch ,all chicks were randomly separated into the following eight groups: 1) Vaccine alone (V), 2) Vaccine alone with boost on day 14 (V 1& 14), 3) Vaccine + CpG-ODN (V + CpG), 4) Vaccine + CpG-ODN with boost on day 14 (V + CpG 1 & 14), 5) Vaccine + Non CpG-ODN (V + Non CpG), 6) Vaccine + NON CpG-ODN with boost on day 14 (V + Non CpG 1 & 14), 7) challenge control and 8) negative control. CpG-ODN or Non CpG-ODN were co-administered with the vaccine (live virus Type B1 Strain titer $10^{7.5}$ from Merial Labs) intranasally and intraocularly to chicks respective to experimental groups. 14 days later, a booster administration of vaccine, vaccine +

CpG ODN, or vaccine + Non-CpG ODN was given respective to group. On day 28, chickens from all experimental groups, except negative control group, were challenged intranasally and intraocularly respective to experimental groups with a Newcastle Disease Virus La Sota Field strain at a dose of $10^{9.2}$. To evaluate antibody levels by ELISA, an approximate 1 ml sample of blood was obtained by jugular or bicipital vein venipuncture and allowed to clot for serum collection. Serum was transferred to clean micro centrifuge tubes, and frozen at -20 C until assay by ELISA. Samples were taken on days 14, 28, and 31 post-vaccination of each replicate experiment.

Experiment 2. To evaluate the potential adjuvant properties of CpG-ODN during coccidial vaccination, 160 day-of-hatch broiler chicks were obtained and randomly distributed into 4 different experimental groups of 40 birds each. Chicks were reared on wire in an electrically heated brooder battery unit with one experimental group per level. Specific experimental groups consisted of: 1) non-vaccinated control, 2) Vaccinated, 3) Vaccinated with CpG-ODN, and 4) Vaccinated with Non CpG-ODN. Vaccination was performed on one day post-hatch by oral co-administration of CpG-ODN (50μg/bird) or Non CpG-ODN (50μg/bird) and one manufacturer's recommended dose of Coccivac® B. On day 21 all chickens were weighed, transferred to two grower battery units, and further separated into eight experimental groups of 20 birds each for clinical challenge with *E. acervulina or E. tenella*. The heterologous mixed challenge on day 21 consisted of $5x10^5$ oocysts/bird of *E. acervulina* and $1 x10^5$ oocysts/bird of *E. tenella* was administered *per os* to chickens respective to experimental group. Day 6 post-challenge, all experimental animals were bled, weighed, and then killed for lesion score

determination according to the methods of Johnson and Reid (1970). Additionally, to determine antibody levels resulting from coccidial vaccination and challenge, blood was taken on days 10, 21, and 27 in similar fashion to the above description for obtaining and processing blood samples for experiment 1.

Experiment 3. To evaluate the ability of CpG-ODN to adjuvant vaccination strategies against *Salmonella*, day-of-hatch 252 SCWL chicks were randomly separated into 7 experimental groups. These groups consisted of: 1) Vaccine alone, 2) Vaccine alone with boost on day 14, 3) Vaccine + CpG-ODN, 4) Vaccine + CpG-ODN with boost on day 14, 5) Vaccine + Non CpG-ODN, 6) Vaccine + Non CpG-ODN with boost on day 14, and 7) negative control. CpG-ODN or Non-CpG ODN were orally co-administered with the *S. typhimurium* deletion mutant vaccine Megan® Vac 1 on day-of-hatch. A booster immunization with the same vaccine, vaccine + CpG ODN, or vaccine + NON-CpG ODN, was administered respective to experimental group on day 14. On day 28, chickens from all experimental groups were separated and mixed into three separate groups, consisting of a non-challenged group, and individual *S. enteritidis* or *S. typhimurium* challenged groups. Field strain challenge of experimental animals with *Salmonella enteritidis* and *Salmonella typhimurium* was performed by giving an oral dose of 5 X 10^8 cfu/ml to each chicken. All birds were bled for antibody level determination following vaccination and challenge, as described above, on days 14, 28, and 35 post-vaccination.

On day 35, seven days post-*Salmonella* challenge, all challenged animals were killed and liver, spleen, and ceca were enriched in tetrathionate broth for 24 h at 42 C for

specific recovery of either *S. typhimurium* or *S. enteritidis*. Additionally, the cecal contents from 5 birds per group were serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} dilutions in Butterfield's solution) and plated for direct enumeration of both challenge isolates. All samples were plated to brilliant green agar containing 20 µg/ml nalidixic acid and 25 µg/ml sodium novobiocin. All plates were incubated at 37 C for approximately 24 h and all suspect colonies were confirmed both biochemically and serologically.

Experiment 4. For this experiment, designed to simultaneously evaluate the efficacy of CpG-ODN in elevating antigen specific antibodies to BSA in both broiler and SCWL chickens, day-of-hatch chicks were randomly separated into the following six 6 different experimental groups: 1) BSA alone, 2) BSA + CpG (50 µg/chick), 3) BSA + CpG (75µg/chick), 4) BSA + Non CpG (50 µg/chick), 5) BSA + Non CpG (75 µg/chick), or 6) Negative control. Broiler and SCWL chicks were reared separately throughout the experiment. All birds designated for BSA immunization received *ad libitum* access to bell drinkers with water containing 1.4mg/ml of BSA on day 15 through day 21. On the first day of immunization, each animal respective to experimental group also received either 50 or 75 µg CpG-ODN or Non CpG-ODN along with 25 mg of BSA orally using a feeding needle. Chickens in negative control groups were provided bell drinkers with tap water only. All animals, except the experimental animals in the control groups, also received a BSA booster immunization without CpG-ODN or Non CpG-ODN on day 26 following the last day of drinking water immunization. The boost consisted of 24 hours access to bell drinkers containing a solution of 1.4mg/ml of BSA. Blood samples for antibody level determination were

taken and processed, as described above, on days 19 and 33 post final day of immunization.

Statistical Analysis

Serum samples form all experiments were read in duplicate and normalized for plate effects by dividing by a positive standard. The mean absorbance of serum samples were analyzed using the General Linear Model of analysis of variance (ANOVA) and statistically different means ($p<0.05$) were further separated using Duncan's Multiple Range Test. All analyses were performed using SPSS software (SPSS, v. 11.0, 2001). Differences in the $\log_{10}$ CFU value of *S. typhimurium* and *S. enteritidis* in the cecal contents were evaluated in the same way.

Results

Experiment 1.

Experiment 1, which evaluated the effectiveness of CpG-ODN to adjuvant vaccination of commercial SPF chickens with a commercially obtained NDV vaccine, consisted of 3 replicate trials conducted over time. In the first replicate trial, on day 28, animals in the Vaccine + CpG 1& 14 experimental group were observed to have the highest ($p<0.05$) anti-NDV IgG levels, which were elevated above levels in animals from all other experimental groups. The Vaccine + CpG-ODN, Vaccine + Non CpG-ODN, and Vaccine + Non CpG-ODN were significantly different ($p<0.05$) from vaccine alone, vaccine 1&14, and the challenge control (control 1) and negative control (control 2) groups. Further, vaccine alone and vaccine 1&14 were significantly different from the negative and challenge controls. On day 31, Vaccine + Non CpG 1&14 remained higher

and was markedly ($p<0.05$) different from Vaccine alone, Vaccine 1&14, Vaccine + CpG-ODN 1&14, negative and challenge control groups, but were not different from groups Vaccine + CpG-ODN and Vaccine + Non CpG-ODN (Table 1). Antibody levels in animals in the Vaccine + Non CpG-ODN experimental group were higher ($p<0.05$) than levels in animals from vaccine alone and the negative and challenge controls. Further, we observed that Vaccine 1&14, Vaccine + CpG, Vaccine + CpG 1&14, vaccine + Non CpG do not differ among themselves, but showed an elevation when compared with the negative and control experimental groups. These levels were not significantly different from the Vaccine alone group however.

For the second replicate trial, there were a few modifications to the experimental design as compared to replicate 1. Of these, two additional groups were added: a Vaccine + CpG on day-of-hatch group that received a boost of only CpG-ODN (administered in a PBS solution, through ocular and intranasal routes of administration) on day 14. A similar grouped that was added took the same approach but with only Non CpG-ODN only on day 14. In this replicate, an extra time point for blood collection, day 14, was also added. Results from day 14 bleedings during this trial revealed that the animals in the experimental group Vaccine + Non CpG-ODN 1& Non CpG-ODN 14 had the highest ($p<0.05$) antibody levels among all experimental groups, however these levels were not significantly different from Vaccine alone, vaccine + CpG-ODN, Vaccine + CpG-ODN 1&14, and Vaccine + Non CpG-ODN 1&14 (Table 1). Animals in the groups Vaccine alone, Vaccine + CpG-ODN, Vaccine + CpG-ODN 1&14 and Vaccine + Non CpG-ODN 1&14, were not significantly different among themselves, but

were different ($p<0.05$) when compared against Vaccine 1&14, Vaccine + CpG-ODN 1& CpG-ODN 14, Vaccine + Non CpG-ODN, and the control groups. Data from day 28 revealed that animals in the group immunized with only the vaccine showed the highest IgG antibody levels, a difference ($p<0.05$) that was distinct from all other experimental groups (Table 1)

Antibody levels in animals from Vaccine + CpG-ODN and Vaccine + Non CpG-ODN 1 & Non CpG-ODN 14, were significantly different ($p<0.05$) from Vaccine 1&14, Vaccine + CpG-ODN 1& CpG-ODN 14, Vaccine + Non CpG-ODN, and the negative and challenge control groups. Levels in chickens from Vaccine + CpG-ODN 1&14 and Vaccine + Non CpG-ODN 1& 14 were not significantly different between themselves, but were higher ($p<0.05$) than the negative and the challenge controls. Results for day 31 bleedings demonstrated that chicks which received the vaccine alone or the Vaccine + CpG-ODN on day-of-hatch and a boost with ODN and vaccine, show the highest levels of antibodies in peripheral blood, however they only differed ($p<0.05$) from Vaccine + CpG-ODN and the negative and challenge controls. Levels in animals in the rest of the experimental groups were not different from each other with the exception of the challenge and negative controls, which showed a lower IgG level.

The third and final replicate trial was essentially the same experimental design as in the first replicate in that it consisted of the same experimental groups. However, the extra time point for bleeding on day 14 was included similar to replicate trial 2.

TABLE 1. Effect of NDV vaccine with or without CpG-ODN or Non CpG-ODN administration on serum IgG in SPF chickens vaccinated on day-of-hatch with a commercial NDV vaccine

	Replica 1		Replica 2			Replica 3		
Group	Day 28	Day 31	Day 14	Day 28	Day 31	Day 14	Day 28	Day 31
Vaccine	0.870±0.110^{c}	1.132±0.145^{c}	0.358±0.072^{ab}	2.929±0.323^{a}	2.218±0.158^{a}	0.214±0.028^{b}	1.630±0.177^{a}	1.596±0.183^{a}
Vaccine 1&14	1.045±0.164^{c}	1.279±0.140^{bc}	0.034±0.006^{c}	1.226±0.199^{c}	1.742±0.246^{ab}	0.206±0.029^{b}	1.329±0.129^{ab}	1.392±0.090^{a}
V CpG	1.584±0.153^{b}	1.587±0.179^{abc}	0.311±0.076^{ab}	1.908±0.256^{b}	1.521±0.182^{b}	0.027±0.033^{b}	1.477±0.194^{ab}	1.277±0.141^{ab}
V CpG 1&14	3.316±0.167^{a}	1.479±0.159^{bc}	0.265±0.044^{ab}	1.436±0.181^{bc}	2.234±0.292^{a}	0.278±0.047^{b}	1.383±0.176^{ab}	1.298±0.134^{ab}
V CpG 1 & CpG 14			0.044±0.073^{c}	1.009±0.188^{c}	1.660±0.217^{ab}			
V N CpG	1.982±0.216^{b}	1.780±0.157^{ab}	0.028±0.057^{c}	1.007±0.143^{c}	1.864±0.153^{ab}	0.251±0.032^{b}	1.1777±0.102^{b}	0.982±0.078^{b}
V N CpG 1&14	1.796±0.200^{b}	2.047±0.302^{a}	0.271±0.063^{ab}	1.449±0.170^{bc}	2.035±0.276^{ab}	0.427±0.077^{a}	1.254±0.532^{ab}	1.308±0.115^{ab}
V N CpG 1 & Non CpG 14			0.474±0.111^{a}	1.836±0.204^{b}	1.917±0.193^{ab}			
Control 1	0.022±0.010^{d}	0.027±0.003^{d}	0.016±0.006^{c}	0.036±0.005^{d}	0.002±0.004^{c}	0.023±0.009^{c}	0.012±0.011^{c}	0.014±0.002^{c}
Control 2	0.004±0.013^{d}	0.0964±0.052^{d}	0.013±0.012^{c}	0.074±0.066^{d}	0.082±0.085^{c}	0.000±0.002^{c}	0.005±0.004^{c}	0.023±0.004^{c}

Data represent mean ±S.E. of antigen-specific IgG production 14, 28, or 31 post-vaccination in experimental animals for each experimental group.
$^{a,b.c}$Means ±S.E. in columns within each column with no common superscript differ significantly ($p<0.05$).
Treatments: Vaccine: Vaccine alone; Vaccine 1&14: Vaccine alone with boost on day 14; V CpG: Vaccine + CpG-ODN; V CpG 1&14: Vaccine + CpG-ODN with boost on day 14; V Non CpG: Vaccine + Non CpG-ODN; V Non CpG 1&14: Vaccine + CpG-ODN with boost on day 14; Control 1: Not vaccinated but challenged; Control 2: Not vaccinated not challenged.

Data from day 14 bleeding in this trial revealed that the chickens in the experimental group Vaccine + Non CpG-ODN 1& 14 had the highest ($p<0.05$) IgG level as compared to the remaining experimental groups (Table 1). Antibody levels in the challenge and negative experimental groups were significantly lower ($p<0.05$) than the remainder of the experimental groups. On day 28, collected data demonstrated that IgG levels from animals in the Vaccine alone group were the highest ($p<0.05$), but did not differ from the rest of the groups with the exception of Vaccine + Non CpG-ODN, and the controls. The rest of the groups do not differ from one another, but were higher ($p<0.05$) than observed levels in the control experimental groups. For the samples on day 31, levels in animals in Vaccine alone, and vaccine 1&14 experimental groups were different ($p<0.05$) from Vaccine + Non CpG-ODN and the negative controls. All remaining groups followed exactly the same trend as on day 28, with the exception that vaccine 1&14 differed ($p<0.05$) from the vaccine + Non CpG experimental group.

Experiment 2

Experiment 2 compared the response of chickens vaccinated orally with a commercial live oocyst coccidial vaccine, with or without CpG-ODN or Non CpG-ODN administration. Birds were challenged 21 days post immunization with *E. tenella* and *E. acervulina* to evaluate protection against field strain challenge. Data obtained from lesion scoring all challenged animals for *E. acervulina* revealed that the negative control with challenge was the group with the highest ($p<0.05$) lesion score as compared to all other experimental groups. All groups not receiving challenge were significantly lower ($p<0.05$) than the challenged experimental groups. Lesions in the Coccivac + Non CpG

with challenge group were higher($p<0.05$) than other experimental groups, with the exception of the Coccivac B + CpG with challenge group, which was not different with Coccivac B with challenge, but did vary ($p<0.05$) to the rest of the non challenged groups (Table 2). For *E. tenella*, the results show that all the lesions in animals from all challenged groups were markedly higher ($p<0.05$) when compared to the not challenged groups, but no difference was found when compared among challenged groups. All the non challenged groups did not differ from each other either (Table 2). Results in body weight gain from day 21 to day 27 showed that in the negative control without challenge, and the Coccivac B with challenge, there was a better ($p<0.05$) weight gain during challenge that what was observed in all other experimental groups, with the exception of Coccivac B group. Gain during challenge in the Coccivac B group was not different from Coccivac B + Non CpG-ODN with challenge. The rest of the experimental groups had a markedly lower ($p<0.05$) weight gain. *Eimeria spp*.-specific IgG antibodies on day 21 indicated that animals within the groups Coccivac B and Coccivac B with challenge had the highest ($p<0.05$) overall antibody levels in serum. Animals in the groups Coccivac B + CpG, Coccivac B + CpG with challenge, Coccivac B + Non CpG, and Coccivac B + Non CpG with challenge were all similar among themselves but differed ($p<0.05$) from the negative controls (challenged and not challenged). The negative controls had the lowest ($p<0.05$) observed values. On day 27 the negative control group with challenge had the highest ($p<0.05$) levels of IgG measured in peripheral blood. Levels of IgG did not differ in all other experimental

groups with the exception of animals from the Coccivac B alone group, which had the lowest overall measured values.

Experiment 3

In this experiment, day-of-hatch SCWL chickens were orally vaccinated and boosted with a commercial *Salmonella* vaccine consisting of a genetically modified *S. typhimurium,* with or without dual administration of CpG-ODN or Non CpG-ODN. Data obtained from day 14 bleedings revealed no measurable IgG level specific for either *S. enteritidis* or *S. typhimurium* in all experimental groups (data not shown). Antibody levels on day 28 and 35 increased in all groups. Observed values for *S. enteritidis* IgG levels for day 28 showed that the chickens within the Vaccine alone 1 & 14 experimental group had the highest ($p<0.05$) antibody levels as compared to other experimental groups(the table on page 59). Measured levels in Vaccine + CpG 1 &14 and Vaccine + Non CpG groups were notably higher ($p<0.05$) than values in vaccine alone and negative control groups, but not different from Vaccine + CpG and Vaccine + Non CpG 1 & 14 groups. The samples obtained for *S. typhimurium* on day 28 showed that Vaccine + Non CpG 1 &14 IgG levels were considerably higher ($p<0.05$) than levels in vaccine + CpG, Vaccine + CpG 1&14, and the negative control, but not different from the experimental groups vaccine alone, vaccine 1&14, and vaccine + Non CpG. Further, IgG levels in the experimental group Vaccine 1&14 were higher ($p<0.05$) than the vaccine + CpG group and the negative control. Vaccine alone, Vaccine + CpG, Vaccine + CpG 1&14, Vaccine + Non CpG were not different from negative controls. Samples obtained on day 35, when assayed for specific *S. enteritidis* IgG level, indicated

TABLE 2. Effect of a live oocyst coccidial vaccine with or without CpG-ODN or Non CpG-ODN administration on intestinal lesion score, body weight gain during challenge, or serum IgG response in broiler chickens vaccinated on day-of-hatch

	Lesion scores		Body Weight Gain*	Serum IgG levels	
Group	**EA**	**ET**	**EA&ET**	Day 21 **EAZS240**	Day 27
Negative Control	0±0^{e}	0.179±0.074^{b}	385.232±11.271^{a}	0.077±0.010^{c}	0.151±0.034^{b}
Negative Control With Challenge	2.154±0.1435^{a}	1.885±0.128^{a}	310.486±17.115^{c}	0.061±0.008^{c}	0.437±0.028^{a}
CoccivacB	0.222±0.082^{de}	0.482±0.098^{b}	367.404±7.889^{ab}	0.330±0.030^{a}	0.004±0.019^{c}
CoccivacB With Challenge	0.777±0.097^{c}	2±0.119^{a}	382.826±9.459^{a}	0.334±0.032^{a}	0.090±0.011^{b}
CoccivacB+ CpG	0.357±0.1056^{d}	0.429±0.095^{b}	329.196±8.707^{c}	0.146±0.024^{b}	0.093±0.010^{b}
CoccivacB + CpG With Challenge	0.929±0.135^{bc}	2.143±0.133^{a}	326.614±17.359^{c}	0.140±0.015^{b}	0.119±0.016^{b}
CoccivacB + Non CpG	0.214±0.079^{de}	0.321±0.089^{b}	323.064±14.559^{c}	0.165±0.021^{b}	0.119±0.015^{b}
CoccivacB + Non CpG-ODN With Challenge	1.214±0.157^{b}	2.036±0.081^{a}	337.364±9.533^{bc}	0.159±0.018^{b}	0.120±0.027^{b}

Data represent mean ±S.E. of intestinal lesions scores, body weight gain during challenge, or antigen-specific IgG production 21 or 27 days post-vaccination in experimental animals for each experimental group.

^{a,b,c}Means ±S.E. in columns within each column with no common superscript differ significantly ($p<0.05$).

animals in the Vaccine + CpG group had the highest antibody levels, but were not different from other experimental groups with exception ($p<0.05$) of Vaccine + CpG 1&14 and the negative control groups. Antibody levels in chickens in the Vaccine alone group differed ($p<0.05$) from the negative control group, but were not different from the remainder of the experimental groups. Additionally, there were no other differences among the rest of the groups. On day 35 when samples were analyzed for specific *S. typhimurium* IgG, levels in animals in the Vaccine 1&14, Vaccine + Non CpG, and Vaccine + Non CpG 1&14 groups were higher ($p<0.05$) than the negative controls, but not different from the remainder of the groups (Table 3). The rest of the groups were also not different from the negative control group. Bacterial recovery data revealed no differences among all experimental groups with regard to $\log_{10}$ CFU recovered from cecal contents for *Salmonella enteritidis* and *S. typhimurium.* Recovery following selective enrichment revealed differences in liver and spleen samples recovered from the *S. typhimurium* challenged birds from groups Vaccine 1 & 14, Vaccine + CpG-ODN 1 & 14, Vaccine + Non CpG-ODN, and Vaccine + Non CpG-ODN 1 & 14 when compared to the rest of the experimental groups. Differences among S. *typhimurium* challenged chickens were not observed following selective enrichment of cecal samples. Similarly, differences were also not observed in either enriched liver and spleen, or cecal samples in S. *enteritidis* challenged chickens. (Table 4).

TABLE 3. Effect of a gene modified-live *Salmonella* vaccine with or without CpG-ODN or Non CpG-ODN on serum IgG response in SCWL chickens vaccinated on day-of-hatch

	Day 28	Day 35	Day 28	Day 35
Group	**Serum IgG *S. enteritidis***		**Serum IgG *S. typhimurium***	
V	0.076±0.005^{c}	0.189±0.017^{ab}	0.072±0.023^{abc}	0.137±0.030^{ab}
V 1&14	0.120±0.007^{a}	0.159±0.011^{abc}	0.129±0.017^{ab}	0.230±0.038^{a}
V CpG	0.066±0.004^{bc}	0.211±0.026^{a}	0.055±0.007^{c}	0.144±0.037^{ab}
V CpG 1&14	0.085±0.006^{b}	0.141±0.016^{bc}	0.063±0.009^{bc}	0.149±0.033^{ab}
V Non CpG	0.086±0.014^{b}	0.168±0.019^{abc}	0.077±0.022^{abc}	0.174±0.033^{a}
V Non CpG 1&14	0.078±0.005^{bc}	0.175±0.018^{abc}	0.141±0.041^{a}	0.187±0.037^{a}
Control	0.054±0.014^{c}	0.120±0.015^{c}	0.049±0.015^{c}	0.051±0.012^{b}

Data represent mean ±S.E. of antigen-specific IgG production in serum 28 or 35 days post-vaccination in experimental animals for each experimental group.

^{a,b,c}Means ±S.E. in columns within each column with no common superscript differ significantly ($p<0.05$).

Treatments: V: Vaccine alone; V 1&14 Vaccine alone with boost on day 14; V CpG: Vaccine + CpG-ODN; V CpG 1&14: Vaccine + CpG-ODN with boost on day 14; V Non CpG: Vaccine + Non CpG-ODN; V Non CpG 1&14: Vaccine + CpG-ODN with boost on day 14; Control: Not vaccinated.

TABLE 4. Effect of a gene modified-live *Salmonella* vaccine with or without CpG-ODN or Non CpG-ODN on *S. enteritidis* or *S. typhimurium* cecal colonization or organ invasion response in SCWL chickens vaccinated on day-of-hatch

	S. enteritidis		***S. typhimurium***			
Group	**Liver & Spleen**	**Ceca**	**Liver & Spleen**	**Ceca**	***S. enteritidis* log$_{10}$**	***S. typhimurium* log$_{10}$**
V	3/11	11/11	4/12	12/12	2.323±0.55[a]	2.782±0.34[a]
V 1&14	1/12	11/12	2/12*	11/12	2.323±0.55[a]	2.782±0.34[a]
V CpG	1/12	12/12	6/12	12/12	2.924±0.58[a]	3.001±0.56[a]
V CpG 1&14	2/11	11/11	1/12*	12/12	2.951±0.20[a]	2.751±0.35[a]
V Non CpG	2/11	11/11	2/12*	11/12	3.034±0.42[a]	3.257±0.45[a]
V Non CpG 1&14	1/11	11/11	2/12*	12/12	2.958±0.22[a]	3.578±0.54[a]
Control	4/11	10/11	7/12	12/12	2.827±0.46[a]	2.532±0.47[a]

Data represent incidence of *S. enteritidis* or *S. typhimurium* cecal colonization or liver / spleen invasion (number of animals positive for each respective bacterium per the total number of animals challenged per experimental group).

*Indicates a statistically lower incidence level as compared to control ($p<0.05$)..

Treatments: V: Vaccine alone; V 1&14 Vaccine alone with boost on day 14; V CpG: Vaccine + CpG-ODN; V CpG 1&14: Vaccine + CpG-ODN with boost on day 14; V Non CpG: Vaccine + Non CpG-ODN; V Non CpG 1&14: Vaccine + CpG-ODN with boost on day 14; Control: Not vaccinated.

Experiment 4.

Experiment 4 was designed to evaluate two different doses for CpG-ODN: CpG-ODN 50 (50 μg/bird) or CpG-ODN 75 (75μg/bird), and appropriate Non CpG-ODN controls (50μg/bird, and 75μg/bird), for effectiveness in immunization to BSA when administered orally as adjuvants in the drinking water. This experiment concurrently measured BSA-specific IgG levels in two different lines of chickens, SCWL and broilers, when bled on day 19 and 33 following the last day of immunization. The results observed in day 19 samples from post immunization SCWL chickens revealed that the BSA + CpG 50 group had higher ($p<0.05$) IgG levels when compared to the BSA + Non CpG 50, BSA + Non CpG 75, and the negative controls, but not different from BSA alone and BSA CpG 75 experimental groups (please refer to the table on page 64). Samples from the broiler chicks from the same sampling day indicated that that chickens from the BSA + CpG 75 group had markedly higher ($p<0.05$) circulating IgG levels when compared to the rest of the experimental groups with the exception of BSA + CpG 50 and BSA+ Non-CpG 50. Levels in the BSA alone and BSA+ Non-CpG 75 were higher than levels measured in the negative controls, but not different than levels in the BSA +CpG 50 and BSA + Non CpG 50 groups. The negative control group was observed to have the lowest ($p<0.05$) IgG levels among all the experimental groups. Samples obtained from SCWL on day 33, revealed IgG levels in chickens in the BSA + CpG 75 to be higher than all other experimental group with the exception of BSA alone experiment group. However, the BSA alone group did not differ from BSA + CpG 50, but differed ($p<0.05$) from the rest of the experimental groups. BSA + CpG 50 levels

were not notably different from BSAN on CpG 50 and BSA Non CpG 75. Antibody levels in negative control animals were again the lowest ($p<0.05$) observed in all groups. Serum IgG levels measured in broiler chickens on day 33 post immunization reveled no significant differences among all experimental groups with the exception of the negative controls, which were the lowest ($p<0.05$) of the remainder of the experimental groups.

Discussion

Experiment 1 of the present study was designed to investigate the potential for CpG-ODN to adjuvant a commercial NDV vaccine when applied by an ocular and intranasal route to neonatal SPF chickens. With the exception of replicate trial 1, across the other replicate trials, data were variable in terms of describing any possible enhancement of a humoral response in CpG-ODN or Non CpG-ODN treated chickens. Replicate trial 1 clearly demonstrated an enhanced humoral response associated with CpG-ODN administration, especially on day 28 of this trial (Table 1). Both within this trial and throughout other experiments of this study, stimulatory effects of Non CpG-ODN administration were observed. These trends were not reinforced, however, in replicate trials 2 and 3 (Table 1). Surprisingly, in these trials, the highest overall NDV IgG levels in animals across all experimental groups were measured in chickens in the vaccine alone group that did not receive a booster vaccination on day 14 (Table 1). Even though we could speculate that there is interference of some of the vaccine components with the ODN, there is no explanation as to why the group vaccine alone with boost had different results even before receiving the boost. Therefore, after a great deal of consideration of these variable results from experiment 1, we can not provide a

reasonable explanation for these observations. Further studies need to be done. As an observation, a specific ELISA to measure different isotypes in serum, and possibly within respiratory mucosa should be performed instead of using a commercial kit.

Experiment 2 evaluated the potential for CpG-ODN to adjuvant a commercial live oocyst coccidial vaccine when applied by an oral route to neonatal broiler chickens. Overall, when body weight gain during challenge, development of clinical lesions, and anti-*Eimeria* IgG levels were evaluated, vaccine administration alone was demonstrated to provide the best measure of protection among animals in all experimental groups, including those receiving either CpG-ODN or Non CpG-ODN. Given the well documented emphasis on the involvement of cell mediated immunity (CMI) in the generation of complete immunity in chickens to *Eimeria* immunization and challenge (please see Chapter II), an obvious criticism of the experimental design of experiment 2 would be that a measure of some CMI index may have resulted in different, possibly more positive observations associated with ODN administration. It bears consideration however that this might be over speculation since measurement of performance parameters revealed that animals receiving ODN administration along with the vaccine did not perform as well as animals which received vaccine alone. Interestingly, the observation of anti-*Eimeria* IgG levels being depressed at six-days post- *E. acervulina* and *E. tenella* challenge, as compared to levels measured on day 21, while unexplainable, is interesting. The only experimental animals among all experimental groups that were observed to have increased the anti-*Eimeria* IgG levels during challenge were in the negative control and negative control with challenge groups.

TABLE 5. Effect of BSA immunization through the drinking water with or without CpG-ODN or Non CpG-ODN on serum IgG response in SCWL or broiler chickens

Group/Treatment	Day 19 Serum IgG vs. BSA SCWL	Day 33 Serum IgG vs. BSA SCWL	Day 19 Serum IgG vs. BSA BROILERS	Day 33 Serum IgG vs. BSA BROILERS
BSA Only	0.629±0.054^{ab}	0.954±0.040^{ab}	0.479±0.041^{b}	0.657±0.060^{a}
BSA. + CpG-ODN 50	0.730±0.040^{a}	0.871±0.050^{bc}	0.529±0.042^{ab}	0.756±0.060^{a}
BSA + CpG-ODN 75	0.610±0.098^{ab}	1.016±0.043^{a}	0.664±0.070^{a}	0.751±0.060^{a}
BSA + Non CpG-ODN 50	0.530±0.033^{b}	0.776±0.032^{c}	0.567±0.064^{ab}	0.771±0.067^{a}
BSA + Non CpG-ODN 75	0.550±0.048^{b}	0.768±0.036^{c}	0.465±0.050^{b}	0.778±0.050^{a}
Negative Control	0.024±0.029^{c}	0.003±0.001^{d}	0.049±0.008^{c}	0.049±0.017^{b}

Data represent mean ±S.E. of antigen-specific IgG production in serum 19 or 33 days post-last day of immunization in experimental animals for each experimental group.

^{a,b,c}Means ±S.E. in columns within each column with no common superscript differ significantly ($p<0.05$).

CpG-ODN 50 or Non CpG-ODN 50 refers to administration of 50 µg CpG-ODN or Non CpG-ODN, respectively.

CpG-ODN 75 or Non CpG-ODN 75 refers to administration of 75 µg CpG-ODN or Non CpG-ODN, respectively.

The fact that negative control animals, in this case responding within six days with such magnitude in terms of an IgG response, suggests some degree of cross-contamination among pens within the brooder battery by vaccine or challenge oocysts occurred. In support of this, five experimental animals in the non-challenged negative control experimental group were observed to have cecal lesions, albeit at a very low level, consistent with *E. tenella* infection. Nonetheless, additional investigation into these phenomena would likely add clarity to our poor understanding of the results of this specific experiment. Evaluation of different isotypes present in serum, gut and bile should be analyzed. A different way of housing in order to avoid cross contamination is also recommended.

Experiment 3 investigated the effectiveness of the co-administration of CpG-ODN or non-CpG ODN and a commercially acquired *Salmonella typhimurium* vaccine for effects on stimulating antigen-specific IgG levels and protection against field strain *Salmonella* challenge. Similar to experiments 1 and 2, experiment 3 also demonstrated variable results in IgG levels specific for *S. typhimurium* or *S. enteritidis* associated with either CpG-ODN or Non CpG-ODN and vaccine groups, when compared to vaccine alone groups. Examples include the single vaccine and CpG-ODN co-administration *S. enteritidis*-IgG levels being elevated on day 35 of this experiment, despite being not different ($p<0.05$) from vaccine alone controls (Table 3). Additionally, experimental animals in the vaccine + Non CpG-ODN experimental group had similarly high anti-*S. typhimurium* IgG levels on both days 28 and 35 of this experiment, but again, these levels were not distinguishable from vaccine alone controls (Table 3). Protection against

field strain challenge revealed a somewhat more positive observation associated with Non CpG-ODN and vaccine co-administration (Table 4). When protection against *S. typhimurium* organ invasion was assessed in animals in all experimental groups, significant protection was observed in the vaccine with boost, CpG-ODN with vaccine and boost, Non CpG-ODN with vaccine, and the Non CpG-ODN with vaccine and boost experimental groups. While most animals in these experimental groups were vaccinated twice, both initially on day-of-hatch and then with a booster administration on day 14, the non-CpG with vaccine experimental group was the only group associated with significant protection against *S. typhimurium* organ invasion that received vaccine only once (Table 4). Again, as with our conclusions related to observations from experiments 1 and 2, data from the present experiments demonstrated variable results related to the co-administration of ODN and vaccine, which were often indistinguishable from results observed in animals receiving vaccine alone.

Anti-BSA IgG levels were compared in broiler and SCWL chickens immunized against BSA by a drinking water route of administration alone, or in combination with two concentrations of CpG-ODN or Non CpG-ODN in experiment 4. Although there were a number of suggestive trends for increased antibody production due to co-administration of CpG-ODN + BSA, such as the difference in SCWL chickens associated with the administration of CpG-ODN at 50 μg/chicken for day 19 IgG levels and the CpG-ODN at 75 μg/chicken for day 33 IgG levels, these values were not distinguishable from animals administered BSA alone. The only observation where CpG-ODN and BSA co-administration resulted in anti-BSA IgG levels that were

elevated above BSA alone immunized chickens, was measured in broilers for the day 19 time point. While it is tempting to suggest that broilers and SCWL chickens responded differently to the administration of CpG-ODN in this experiment, since CpG-ODN and BSA co-administration to broilers revealed the only elevation in anti-BSA IgG level statistically distinguishable from BSA alone controls, further investigation into comparing these and similar effects is clearly needed before such characterizations can be made. Such differences in immunoresponsiveness are supported in the literatures as Leshchinsky and co-worker (Leshchinsky and Klasing, 2001) reported that two genetic types of chickens (broilers and SCWL) clearly exhibit different capacities for generating immune response. Taken together, while suggestive in certain specific areas within and across experiments in the present investigation, these data are largely inconclusive for suggesting that CpG-ODN can effectively adjuvant humoral immune responses to commercial vaccines or protein antigens in commercial strain chickens.

CHAPTER IV

CONCLUSIONS

To control disease conditions under the dense and intensive rearing conditions of commercial poultry production, vaccines represent an important and invaluable prophylactic and therapeutic strategy for commercial producers (Sharma, 2003). The commercial poultry industry relies heavily vaccines to protect chickens and turkeys against many pathogens that threaten poultry health and the subsequent overall economics of the industry. The fundamental task of the immune system in responding to vaccination is to generate protection in the host against invading microorganisms or pathogens through a cooperative approach involving both innate and adaptive immunity. Innate immune responses are typically activated by innate system cells, such as macrophages, heterophils, or dendritic cells, which then act in concert to either kill the invading microbe or augment and trigger an appropriate adaptive immune response. Because innate immune cells lack the highly specific antigen receptors found on T and B cells they rely on surface membrane proteins known as pattern recognition receptors (PRRs). PRRs have the ability to detect surface components of microbial pathogens known as pathogen associated molecular patterns (PAMPs). PAMPs recognition by PRRs in this setting is an indication of a threat to the host in the form of potential infection. Such recognition activates appropriate defense pathways in the innate system

to either remove the pathogen or stimulate the adaptive system to act upon the pathogen for its ultimate removal.

CpG motifs naturally present in bacterial DNA represent one of the most recent examples of substances that influence adaptive immune responses by activating the innate immune system. CpGs are 6-8 base nucleotide motifs consisting of an unmethylated CpG dinucleotide that induce cells of the innate immune system to increase phagocytosis, pro-inflammatory cytokine production, activation of NK cells, increase nitric oxide generation by macrophages, and stimulate B cell proliferation and differentiation. The ability of CpG-ODN to act as vaccine adjuvants has been explored extensively in recent years in mammals but very little research has been conducted along these lines in commercial poultry. In recent years, DNA vaccines or DNA extracts from bacteria have been recognized as having tremendous potential for improving vaccination strategies in commercial poultry against different pathogens. The objective of this investigation was to evaluate the administration of CpG-ODN as a potential oral adjuvant to commercially available vaccines against *Salmonella*, *Eimeria* spp., and NewcastleDisease Virus, and a common protein antigen, bovine serum albumin (BSA). Measured experimental outcomes included the level of antibodies produced and protection against subsequent clinical challenge in experimental animals following immunization and vaccination procedures evaluated in this study.

Experiment 1 of the present study was designed to investigate the potential for CpG-ODN to adjuvant a commercial NDV vaccine when applied by an ocular and intranasal route to neonatal SPF chickens. With the exception of replicate trial 1, across

the replicate trials, data were variable in terms of describing any possible enhancement of a humoral response in CpG-ODN or Non CpG-ODN treated chickens. Replicate trial 1 clearly demonstrated an enhanced humoral response associated with CpG-ODN administration, especially on day 28 of this trial (Table 1). Both within this trial and throughout other experiments of this study, stimulatory effects of Non CpG-ODN administration were observed. These trends were not reinforced, however, in replicate trials 2 and 3 (Table 1). Surprisingly, in these trials, the highest overall NDV IgG levels in animals across all experimental groups were measured in chickens in the vaccine alone group that did not receive a booster vaccination on day 14 (Table 1). Since the vaccine administered to each chicken during the three trials was given by the same person, under the same conditions with the same vaccine and the same dose. And since in some cases the vaccine alone experimental group had a good response, but the vaccine alone with a boost experimental groups, before receiving the boost had significantly different results, we can not provide a reasonable explanation for these observations.

Experiment 2 evaluated the potential for CpG-ODN to adjuvant a commercial live oocyst coccidial vaccine when applied by an oral route to neonatal broiler chickens. Overall, when body weight gain during challenge, development of clinical lesions, and anti-*Eimeria* IgG levels were evaluated, vaccine administration alone was demonstrated to provide the best measure of protection among animals in all experimental groups, including those receiving either CpG-ODN or Non CpG-ODN. Given the well documented emphasis on the involvement of cell mediated immunity (CMI) in the generation of complete immunity in chickens to *Eimeria* immunization and challenge

(please see Chapter II), an obvious criticism of the experimental design of experiment 2 would be that a measure of some CMI index may have resulted in different, possibly more positive observations associated with ODN administration. It bears consideration however that this might be over speculation since measurement of performance parameters revealed that animals receiving ODN administration along with the vaccine did not perform as well as animals which received the vaccine alone. Nonetheless, additional investigation into these phenomena would likely add clarity to our poor understanding of the results of this specific experiment.

Experiment 3 investigated the effectiveness of the co-administration of CpG-ODN or Non CpG-ODN and a commercially acquired *Salmonella typhimurium* vaccine for effects on stimulating antigen-specific IgG levels and protection against field strain *Salmonella* challenge. Similar to experiments 1 and 2, experiment 3 also revealed variable results in terms of elevated IgG levels specific for *S. typhimurium* or *S. enteritidis* associated with either CpG-ODN or Non CpG-ODN and vaccine co-administration, when compared to vaccine alone groups (Table 3). Protection against field strain challenge revealed a somewhat positive observation associated with Non CpG-ODN and vaccine co-administration (Table 4). While most animals in these experimental groups were vaccinated twice, both initially on day-of-hatch and then with a booster administration on day 14, the non-CpG with vaccine experimental group was the only group associated with significant protection against *S. typhimurium* organ invasion that received vaccine only once (Table 4). Again, as with our conclusions related to observations from experiments 1 and 2, data from the present experiments

demonstrated variable results related to the co-administration of ODN and vaccine which were often indistinguishable from results observed in animals receiving vaccine alone. Anti-BSA IgG levels were compared in broiler and SCWL chickens immunized against BSA by a drinking water route of administration alone, or along with the co-administration of two concentrations of CpG-ODN or Non CpG-ODN in experiment 4. Although there are a number of suggestive trends for increased antibody production due to co-administration of CpG-ODN and BSA the only observation where CpG-ODN and BSA co-administration resulted in anti-BSA IgG levels that were elevated above BSA alone immunized chickens was measured in broilers for the day 19 time point. Taken together, while suggestive in certain specific areas within and across experiments in the present investigation, these data are largely inconclusive for suggesting that CpG-ODN can effectively adjuvant humoral immune responses to commercial vaccines or protein antigens in commercial strain chickens.

Until recently, there were only a few experiments that evaluated the effects of CpG-ODN as potential adjuvants in poultry. These experiments included a study into the efficacy of CpG-ODN against IFA by comparing their efficacy in producing IgG and IgM after being injected subcutaneously with BSA in chickens. Observed results showed that antibody levels were higher in chickens injected with both CpG-ODN and BSA, suggesting a possible adjuvant effect via the subcutaneous route of administration (Vleugels *et al.,* 2002). A second study evaluated the proliferation of PBMC in peripheral blood in 6 chickens with different sequences and backbones of CpG-ODN. Despite an obvious criticism of using such a limited number of experimental animals,

the results of this experiment indicated that a sequence with 3 CPG motifs in the same ODN (sequence number 2135) was the most immunostimulatory (Rankin *et al.,* 2001). Another study by Gomis and co-workers represents essentially the only complete report on a study performed *in vivo* in chickens to date. This group evaluated the administration route and the specific protection against *E. coli* infection induced by CpG-ODN when inoculated to birds via SC or IM administration (Gomis *et al.*, 2003).

There were different deficiencies in the experimental approach taken in this investigation. Consideration of these for further studies need to be evaluated. For example, the evaluation of just a single sequence of CpG-ODN and Non CpG-ODN was inappropriate. Different sequences of CpG-ODN and Non CpG-ODN, including the ones utilized in this investigation, should be evaluated, since there was a similar response of Non CpG-ODN sequence with the CpG-ODN. Different timings of the administration of the ODN, prior and after immunization or vaccination need to be evaluated. The measurement of only a single isotype of secreted antibody in experimental animals resulted in an incomplete evaluation of the chicken's immune response towards the adjuvants and the vaccination or immunization, therefore different isotypes, local and systemic, also need to be investigated. Since our objective was to evaluate the CpG-ODN as a mucosal adjuvant, known adjuvants should be used as a control. Even though the performance of the birds was not improved, it would be interesting to measure some parameters of cell mediated immunity (evaluation of cytokine expression, specifically IFNγ, and IL6) to provide additional insight into observed responses elicited by ODN administration. Future studies should be designed with these observations in mind before

additional conclusions can be made related to the potential usefulness of ODN administration for immunostimulation of commercial poultry.

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