Immunostimulatory effects and delivery of oligodeoxynucleotides containing CpG motifs (CpG-ODN) in neonatal broiler chickens

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ABSTRACT

Oligodeoxynucleotides containing CpG motifs (CpG-ODN) have been shown to stimulate the innate immune system against a variety of bacterial, viral, and protozoan infections in a variety of vertebrate species. The objectives of this study were to investigate the immunostimulatory effect of CpG-ODN against *Salmonella* Typhimurium infection and the formulation and delivery of CpG-ODN by the *in ovo* route. Day-old broiler chicks or embryonated eggs (day 18^{th} of incubation) received either 50 μg of CpG-ODN, 50 μg of non-CpG-ODN, or saline. At day four-post hatch, all birds were subcutaneously inoculated by *Salmonella* Typhimurium. Clinical signs, pathology, bacterial isolations from the air sacs, and mortality were observed for ten days following challenge. The survival rate of the birds that received CpG-ODN via *in ovo* or *in vivo* treatments was significantly higher than the control group. *Salmonella* Typhimurium level in the peripheral blood and pathology were significantly lower ($p < 0.001$) in CpG-ODN group compared to the control group. In order to investigate the effect of formulation of CpG-ODN, embryonated eggs (day 18^{th} of incubation) were inoculated with either 50 μg of CpG-ODN alone or CpG-ODN formulated with polyphosphazene, liposome, or Emulsigen®. Four days after administration of CpG-ODN formulations, the birds were challenged with *E. coli* by subcutaneous injection. Clinical signs, pathology, bacterial isolations from the air sacs, and mortality were observed for seven days following challenge. The birds that received either CpG-ODN or CpG-ODN formulated with polyphosphazene had significantly higher survival rates (30 and 60%) compared to the birds in groups receiving either non-CpG-ODN or saline.

Bacterial loads in the air sacs were lower in groups treated with formulated CpG-ODN compared to the CpG-ODN alone or control groups. However, formulation of CpG-ODN with liposomes or Emulsigen® did not increase the immunoprotective effect against *E. coli* infection. We showed that treatment with CpG-ODN protects neonatal chickens against an intracellular bacterial infection and that co-treatment of CpG-ODN with polyphosphazene enhances the immunoprotective effect of CpG-ODN.

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LIST OF ABBREVIATIONS

Ag	antigen
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell
APEC	avian pathogenic *E. coli*
BC	B cell
BCR	B cell receptor
bDNA	bacterial deoxyribonucleic acid
BHI	brain heart infusion
BSA	bovine serum albumin
CCS	cumulative clinical score
CD	cluster of differentiation
cfu	colony-forming units
CL	cationic lipid
CMI	cell-mediated immunity
CpG	cytosine-phosphodiester guanine
CTL	cytotoxic T lymphocyte
DC	dendritic cell
ERK	extracellular receptor kinase
GM-CSF	granulocyte macrophage-colony stimulating factor
HBV	hepatitis B virus
HIV	human immunodeficiency virus

IBD	infectious bursal disease
IBDV	infectious bursal disease virus
IDO	indoleamine 2, 3-dioxygenase
IFA	incomplete Freund's adjuvant
Ig	immunoglobulin
IL	interleukin
INF	interferon
iNOS	inducible nitric oxide synthase
JKN	c-Jun NH_2-terminal kinase
L	liter
LB	luria broth
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MRL	minimum residual level
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenosine dinucleotide phosphate
ND	newcastle disease
NFκB	nuclear factor kappa B
NK	natural killer
NO	nitric oxide
ODN	oligodeoxynucleotides
OVA	ovalbumin

PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PCEP	poly [di (sodium carboxylatoethylphenoxy) phosphazene]
PCPP	poly [di (sodium carboxylatophenoxy) phosphazene]
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PMN	polymorphonuclear
PO	phosphodiester
PRR	pattern recognition receptor
PS	phosphorothioate
ROS	reactive oxygen species
SCID	severe combined immunodeficiency disease
SPI	*Salmonella* pathogenicity island
TC	T cell
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
TSIA	triple sugar iron agar
T3SS	type three secretion system
vDNA	vertebrate DNA
VTA	vaccine targeting adjuvant

INTRODUCTION

Many *Salmonella* serotypes can infect poultry. Some serotypes, such as *Salmonella enterica* subspecies *enterica* serotype Pullorum and *Salmonella enterica* subspecies *enterica* serotype Gallinarum, are host specific for chickens while other serotypes, such as *Salmonella enterica* subspecies *enterica* serotype Typhimurium, *Salmonella enterica* subspecies *enterica* serotype Enteritidis, and *Salmonella enterica* subspecies *enterica* serotype Heidelberg are able to infect a range of hosts (Gast 2003). *Salmonella* infections are responsible for many acute and chronic diseases in poultry, including fowl typhoid and pullorum disease (Gast 2003). In chickens, the cecum is the primary colonization site of *Salmonella* (Xu *et al.* 1988), although the cloaca (Xu *et al.* 1988) and the crop (Impey and Mead 1989) are also commonly colonized. Cross contamination with *Salmonella* between birds can occur during transit to market. There is evidence that poultry products are one of the primary sources of *Salmonella* infection in humans (Hald *et al.* 2004), where it can cause severe disease. Contamination of poultry products with *Salmonella* is therefore a major public health problem, and results in substantial economic loss every year.

Salmonella Typhimurium is a common cause of salmonellosis among humans and animals in many countries, including Canada (Leon-Velarde *et al.* 2004). *Salmonella* Typhimurium has been reported to be responsible for 40 to 70% of human salmonellosis cases (Khakhria *et al.* 1997; Leon-Velarde *et al.* 2004), and has been one of the most common bacteria isolated from hatching eggs in Canada in recent decades (Poppe *et al.* 1998). Contamination of broiler chicks with *Salmonella* Typhimurium may

occur by vertical or horizontal transmissions (Gast 2003). In poultry rearing barns, a number of environmental factors can contribute to the contamination of naïve chicks, including air, litter, feed, unclean facilities, and vectors such as insects, humans, rodents and wild birds (Gast 2003). Multiple virulence factors in the genome of *Salmonella* Typhimurium facilitate efficient colonization, invasion of the gastrointestinal tract, and intracellular survival in the host (Libby 2004). The genomes of pathogenic *Salmonella* species (spp.) have large groups of preserved virulence genes that are not present in the similar region of nonpathogenic species. These regions of chromosomal DNA are referred to as *Salmonella* pathogenicity islands (SPIs) (Blum *et al.* 1994). These SPIs include SPI-1, which encodes a type three secretion system (T3SS) associated with invasion; SPI-2, which encodes a T3SS that contributes to intracellular survival; SPI-3, which encodes genes associated with intracellular proliferation; SPI-4, which encodes genes for toxin secretion and apoptosis; and SPI-5, which encodes a number of different T3SS-associated proteins (Libby 2004). Most *Salmonella* Typhimurium isolates also have virulence plasmids that encode genes required for causing systemic disease (Poppe *et al.* 1998). A *Salmonella* plasmid, which contains genes for fimbriae is important for replication in extraintestinal sites such as the liver and spleen (Ahmer *et al.* 1999). *Salmonella* Typhimurium induces high mortality in neonatal chickens, especially those under three days of age (Gast and Beard 1989). High mortality rates in neonatal chickens are due to an immature immune system that permits multiplication of bacteria in blood, and replication in the reticuloendothelial tissue of the liver and spleen (Gast and Beard 1989; Gast 2003). Birds inoculated with *Salmonella* at seven to ten days of age show lower mortality and fewer clinical signs, due to maturation of gut associated

lymphoid tissues (GALT) (Gast and Beard 1989).

Salmonella Typhimurium infection is difficult to control, as bacteria can be introduced to poultry farms from many sources (mentioned above). In order to control the disease, biosecurity practices must prevent the infection from all sources and must be verified by periodic testing (Gast 2003), which is costly for producers. Probiotic therapy can limit bacterial colonization of the intestinal tract by increasing levels of interferon (IFN)-γ and interleukin (IL)-2, thereby activating innate immunity (Dalloul *et al.* 2004). Probiotic therapy of chicks increases the number of Ig-producing cells (IgM, IgG) detected in Peyer's patches and cecal tonsils, and also increases the length of the cecal tonsils (Yurong *et al.* 2005). However, probiotics are only useful prior to infections, and therefore not useful for concurrent neonatal infections (Toro *et al.* 2005). Vaccinating breeders with live and killed vaccines may reduce the susceptibility of their progeny to *Salmonella* Typhimurium infection. Nevertheless, vaccines have not been effective in consistently preventing infection (Gast 2003).

It has been shown that *Salmonella* Typhimurium is capable of developing resistance to antibiotic therapy (Verma *et al.* 2004). The emergence of resistance in *Salmonella* has been associated with the use of antibiotic agents in livestock including chickens (Gray *et al.* 1996). Therefore, replacement of antibiotics with alternative measures to prevent salmonellosis should be a priority for poultry research.

Over recent decades, many studies have been conducted to identify and understand biochemical events and signals that stimulate immune responses to pathogens. In 1992, Yamamoto *et al.* first reported that bacterial DNA (bDNA) has immune stimulatory effects and can activate natural killer (NK) cells, while vertebrate

DNA (vDNA) has no such effect (Yamamoto *et al.* 1992). Later, Krieg *et al*. (1995) showed that bDNA has a specific molecular structure, cytosine phosphodiester guanine (CpG), in which the cytosine is unmethylated (Krieg *et al.* 1995) and is recognized as foreign by the host. Synthetic CpG-Oligodeoxynucleotides (ODN) mimic pathogen associated molecular patterns (PAMPs) (Krieg 2002). They are recognized by pathogen recognition receptors (PRRs) of the immune system (Krieg 2002). In mammals, CpG-ODN can directly or indirectly activate immune cells such as B and T lymphocytes and plasmacytoid dendritic cells (pDCs). It can also induce a variety of proinflammatory and anti-inflammatory cytokines, chemokines, and antibodies such as IL-1, IL-6, IL-10, IL-12, IL-18, tumor necrosis factor (TNF)-α, INF-γ, and IgG mediated by toll-like receptor (TLR)-9 (Krieg *et al.* 1995).

The immunostimulatory effect of CpG-ODN against many different infections has been studied in a variety of species such as fish (Jorgensen *et al.* 2001) , mammals, including food animals such as pigs (Kamstrup *et al.* 2001), cattle (Zhang *et al.* 2001), and sheep (Mena *et al*. 2003). Previously a chicken model has been used to investigate the immunoprotective effect of CpG-ODN against *E. coli*, an extracellular bacterial infection that causes high mortality in neonatal chickens (Gomis *et al.* 2004). Further studies have shown that CpG-ODN stimulate the innate, and later the adaptive immune system of chickens against a pathogen by stimulating the degranulation and oxidative activities of heterophils, and upregulation of nitric oxide (NO) production (He *et al.* 2005). Therefore, consideration of CpG-ODN as an alternative treatment for other important infections in poultry such as salmonellosis caused by *Salmonella* Typhimurium.

An optimal dose of CpG-ODN is required for induction of immune responses (Mutwiri *et al.* 2004). CpG-ODN are rapidly cleared from circulation through absorption by serum proteins such a albumin, and degradation by serum nucleases (Mutwiri *et al.* 2004). Therefore, in some cases, repeated administration of CpG-ODN is required (Molenkamp *et al.* 2007). Previously, it has been reported that adjuvants such as liposomes such as Poly[di (carboxylatophenoxphosphazene)] (PCPP) (Alcon *et al.* 2006; Jaafari *et al.* 2007 Payne *et al.* 1998; Mutwiri *et al.* 2007), , and oil-in-water emulsion (Emulsigen®) (Aucouturier *et al.* 2001) can stimulate immune cells of the host against pathogens. It has also been shown that the immunostimulatory effect of CpG-ODN can be enhanced by adding adjuvants such as Emulsigen® (Ioannou *et al.* 2002) and liposomes (Alcon *et al.* 2005a; Alcon *et al.* 2005b). However, it is not known whether the CpG-ODN with PCPP will enhance the immunostimulatory effect of CpG-ODN in chickens.

Objectives were:

1. To develop an animal model of *Salmonella* Typhimurium in neonatal broiler chickens.
2. To investigate the effect of CpG-ODN in the neonatal broiler model.

    a. To determine the duration of protection against *Salmonella* Typhimurium in neonatal broilers.

    b. To determine the effect of CpG-ODN on *Salmonella* Typhimurium septicemia.

    c. To determine the effect of *in ovo* delivery of CpG-ODN against *Salmonella* Typhimurium infection.

3. To determine if the immunoprotective effect of CpG-ODN was enhanced by addition of PCPP or Emulsigen® using an *E. coli* septicemia model in neonatal chickens**.**

1.0. LITERATURE REVIEW

1.1. *Salmonella* nomenclature

Before 2005, the nomenclature of genus *Salmonella* had been in a state of confusion. Two systems were commonly used. The system that confirmed the Rule of the Bacteriologic Code was used by a minority of bacteriologists, while a second system, which was proposed in 1987 by Le Minor and Popoff, was accepted by a majority of bacteriologists, despite the fact it was not consistent with the rules (Euzéby 1999).

In the system outlined by the Rule of the Bacteriologic Code, *Salmonella cholereasuis* had been considered as the only species of genus *Salmonella* and categorized into seven different subspecies (subsps). *Salmonella choleraesuis* subsp. *arizonae*; *Salmonella choleraesuis* subsp. *bongori*; *Salmonella choleraesuis* subsp. *choleraesuis*; *Salmonella choleraesuis* subsp. *diarizonae*; *Salmonella choleraesuis* subsp. *houtenae*; *Salmonella choleraesuis* subsp. *indica*; and *Salmonella choleraesuis* subsp. *salamae*, *Salmonella enteritidis*, *Salmonella paratyphi*, *Salmonella typhi*, and *Salmonella typhimurium* (Euzéby 1999).

Le Minor and Popoff recommended that *Salmonella enterica* (type strain LT2) be considered the only species of the genus *Salmonella* (Le Minor 1987). LeMinor

and Popoff proposed that the name *Salmonella choleraesuis* was a heterotypic synonym of "*Salmonella enterica*", and that *Salmonella enteritidis*, *Salmonella typhi* and *Salmonella typhimurium* were heterotypic synonyms of "*Salmonella enterica* subsp. *Enterica. Salmonella enterica* subspecies, which were suggested by these authors, were: *Salmonella enterica* subsp. *arizonae*", *"Salmonella enterica* subsp. *bongori*", "*Salmonella enterica* subsp. *enterica*", "*Salmonella enterica* subsp. *diarizonae*", "*Salmonella enterica* subsp. *houtenae*", "*Salmonella enterica* subsp. *indica*", and "*Salmonella enterica* subsp. *salamae*"(Le Minor 1987).

In 1999, Euzéby requested that the Judicial Commission of the International Committee for Systematic of Prokaryotes alter the nomenclature of *Salmonella* to be based on the Le Minor and Popoff's system (Euzéby 1999). In 2005, the Judicial Commission issued an Opinion (the Judicial Opinion 80) that stated *Salmonella enterica* was the species of the genus *Salmonella* and accepted the combinations proposed by LeMinor and Popoff (Truper 2005). The Judicial Commission also agreed that the names *Salmonella choleraesuis*, *Salmonella enteritidis*, *Salmonella paratyphi*, *Salmonella typhi*, and *Salmonella typhimurium* are not conserved specific epithetic in the names of bacteria. *Salmonella* nomenclature proposed by the Judicial Opinion 80 that is relevant is as follows:

Salmonella enterica subsp *enterica* serotype Enteritidis,

Salmonella enterica subsp. *enterica* serotype Typhi,

Salmonella enterica subsp. *enterica* serotype Typhimurium,

Salmonella enterica subsp. *enterica* serotype Pullorum,

and *Salmonella enterica* subsp. *enterica* serotype Gallinarum.

1.1.1. Salmonellosis

Salmonella are gram-negative, facultative, intracellular bacteria that are widespread in nature and have the ability to infect a variety of mammalian and avian hosts (Gast 2003). Most potentially pathogenic bacteria in the genus *Salmonella* of veterinary importance belong to species *Salmonella enterica* subspecies *enterica* (Carter 1990). Two major forms of the disease caused by this subspecies are septicemia and acute, sub-acute or chronic enteritis. Animals with the septicemic form of the disease experience high mortality, while animals with chronic enteritis experience low mortality with mild diarrhea (Gast 2003).

1.1.2. Typhoid and paratyphoid salmonellosis in poultry

More than 2,400 serotypes of *Salmonella enterica* subspecies *enterica* have been identified (Le Minor *et al.* 1982). Serotypes that infect poultry are grouped into two categories (Gast 2003). The first category contains the non-motile serotypes. They include *Salmonella* Pullorum, which infects young chickens and turkeys up to two to three weeks of age and causes pullorum disease, and *Salmonella* Gallinarum, which affects mature birds and causes fowl typhoid disease (Gast 2003). These serotypes are host-specific for avian species and have caused significant economic losses in the commercial poultry industry over recent decades (Gast 2003). The second category contains the motile serotypes, or paratyphoid *Salmonella* (Carter 1990). They can cause food borne diseases in humans. The most commonly reported serotypes belonging to paratyphoid *Salmonella* are *Salmonella* Enteritidis and *Salmonella* Typhimurium (Barrow 2000). Paratyphoid *Salmonella* serotypes are of most concern for

the poultry industry, as they can cause mortality in poultry and readily enter the food chain where they may cause human disease (Barrow 2000).

1.1.3. *Salmonella* Typhimurium in neonatal chickens

Chickens of one to two days of age are highly susceptible to *Salmonella* infection (Libby 2004). In the septicemic form of the disease, *Salmonella* replicates in the reticuloendothelial tissues of the liver and spleen and disseminates to a variety of internal organs, resulting in high mortality and morbidity (Barrow 1987). When orally administered, *Salmonella* Typhimurium adheres to the ceca of two-day-old chickens, causing low mortality but high morbidity (Gast 2003). Birds that recover from infection become carriers of *Salmonella* and remain as the main source of infection for other birds. Carrier birds shed *Salmonella* in their feces (Gast 2003).

1.1.3.1. Sources, vectors, transmission and pathogenesis of S*almonella* Typhimurium

Salmonella can be transmitted horizontally via environmental contamination, fomites, feed, rodents, insects, and wild birds (Gast 2003; Strother 2005). *Salmonella* can also be introduced to neonatal poultry by vertical transmission from their infected parents (Gordon and Tucker 1965). In addition, *Salmonella* Typhimurium inside or on the surface of eggs can spread through hatcheries via air, land on hatching cabinets, and subsequently inoculate other neonatal chickens (Bailey *et al*. 1994).

Pathogenesis of salmonellosis follows several steps, including adherence to intestinal epithelial cells, invasion, and intracellular survival. *Salmonella* serotypes have

numbers of short filamentous structures or fimbria for adherence to Peyer's patches and intestinal villi (Libby 2004). Virulence factors encoded in SPI-1 (Blum *et al.* 1994), SPI-4 (Ahmer *et al.* 1999), and *Salmonella* virulence plasmids (Gulig 1990) are thought to play a role in invasion. Virulence factors encoded in SPI-1 provide accumulation of fluid in the intestine (Blum *et al.* 1994). *Salmonella* Typhimurium that survive in the intestine move to macrophages in the lymph nodes and then move into the circulatory system via the thoracic duct (Libby 2004). SPI-2 (Hensel *et al.* 1995; Vazquez-Torres *et al.* 2000) and virulence factors encoded by SPI-3 (Blanc-Potard and Groisman 1997) are essential for intra-macrophage survival. Virulence factors encoded in SPI-2 interferes with movement of nicotinamide adenosine dinucleotide phosphate (NADH) oxidase to *Salmonella* thereby preventing phagocyte dependent oxidative killing. Therefore, SPI-2 is required for systemic phase of infection (Fang and Vazquez-Torres 2002).

1.1.3.2. Clinical signs associated with *Salmonella* Typhimurium in neonatal chickens

Infection of eggs with *Salmonella* Typhimurium can lead to high mortality in embryos and newly hatched birds without clinical signs (Nakamura *et al.* 1993). Typical signs of *Salmonella* Typhimurium infection in older chickens and poults are anorexia, closed eyes, drooping wings, ruffled feathers and emaciation (Barrow *et al.* 1987). Watery diarrhea leading to dehydration and pasting of the vent is often seen in neonatal chickens (Gast 2003). Blindness (Padron 1990) and lameness (Gast 2003) have been observed sporadically.

1.1.3.3. Pathology associated with *Salmonella* Typhimurium in chickens

On gross examination, spleens and livers are swollen, congested, and hemorrhagic with necrotic foci (Gast 2003). Enlarged and congested kidneys may also be seen (Hoop and Pospischil 1993). Fibrinopurulent perihepatitis, pericarditis, airsacculitis and polyserositis have commonly been associated with salmonellosis in neonatal poultry (Padron 1990; Hoop and Pospischil 1993). Unabsorbed or coagulated yolk and yolk sacculitis may also be seen (Padron 1990). On histological examination, bacterial colonies, infiltration of inflammatory cells (heterophils, lymphocytes, and macrophages) and cell debris is observed (Holt and Porter 1992; Porter and Holt 1993).

1.1.3.4. Immunity against *Salmonella* in chicken

Host defense against pathogens such as *Salmonella* is initiated by the innate immune system, which in turn directs the development of the acquired immune response. Polymorphonuclear (PMN) leukocytes are cellular components of innate response, with the primary cell type in poultry being the heterophil. Heterophils have been previously shown to be the primary responders to *Salmonella* infections in chickens (Kogut *et al.* 1994). The importance of the avian heterophil in innate immune responses to *Salmonella* infections has been highlighted by the description of the expression of Toll-like receptors (TLRs) by avian heterophils (Kogut *et al.* 2005a). Kogut *et al.* showed that avian heterophils are able to express TLR 1, TLR 2, TLR 3, TLR 4, TLR 5, TLR 6, TLR 7, and TLR 10 (Kogut *et al.* 2005a), but not TLR 9 (He *et al.* 2006; Kogut *et al.* 2006) during *Salmonella* septicemia. Recently, bacterial flagellin has been described as a potent stimulator of avian heterophils as well

(Genovese *et al.* 2007). Flagellin leads to cell signaling events through TLR 5 that induces a high level of peripheral blood heterophils and the accumulation of leukocytes at the site of infection (Genovese *et al.* 2007).

Macrophages are central effectors of the innate immune defense against *Salmonella.* These cells are able to kill the pathogen by oxygen and nitrogen reactive activities (Alter-Koltunoff *et al*. 2007). In addition, macrophages induce macrophage inflammatory proteins (MIP) in the spleen and liver, followed by production of IFN-γ, accompanied by increased numbers of both CD4 (+) and CD8 (+) T cells (TCs) against *Salmonella* (Okamura *et al.* 2005). Proinflammatory cytokines, which are induced following *Salmonella* infection include, IL-1-β, IL-6, IL-8, and IL-18 (Kogut *et al.* 2005b).

Salmonella infection can produce strong antibody responses by increasing the levels of IgG, IgA, and IgM in serum, intestinal contents, and bile of infected birds (Gast 2003). Unlike IgA, IgG is only present in systemic sites; therefore, greater induction of *Salmonella*-specific IgG by *Salmonella* Typhimurium can be a response to a higher level of systemic infection (Beal 2006).

Cell-mediated immunity against *Salmonella* Typhimurium infection and a strong delayed hypersensivity reaction in chickens two and five weeks after inoculation have also been reported (Hassan *et al.* 1991).

1.2. *Escherichia coli* infection in poultry

E. coli belongs to the family *Enterobacteriaceae* and is a gram-negative,

rod-shape bacterium that grows rapidly in bacteriological media. Many strains of *E. coli* have peritrichous flagella and are motile (Gyles 2004). Unlik*e Salmonella*, *E. coli* is an extracellular pathogen.

E. coli infection (colibacillosis) in poultry is usually a secondary infection that causes localized or systemic disease when a host's defenses have already been damaged by either a primary disease (Barnes 2003) or by poor management and biosecurity practices (Dho-Moulin and Fairbrother 1999). However, *E.* coli can cause a primary infection in very young poultry at or soon after hatching. Pathogenic *E. coli* are isolated from the intestinal tracts of neonatal chicks more often than from eggs, which suggests that *E. coli* is transmitted horizontally and can spread rapidly after hatching (Harry and Hemsley 1965). Most *E. coli* serotypes isolated from poultry are pathogenic only to avian species (Harry and Hemsley 1965).

1.2.1. Virulence factors of *E. coli* in chicken

Avian pathogenic *E. coli* (APEC) are present in the normal intestinal microflora and the bird's environment. Biochemical characteristics and drug sensitivity of pathogenic and nonpathogenic isolates of *E. coli* are similar (Cloud *et al*. 1985). There is no single virulence factor that can differentiate all pathogenic and nonpathogenic strains of *E. coli* (Barnes 2003). Factors that generally do not correlate with virulence are hemolysis, heat-stable toxin, metabolic activity, motility, R-plasmids, and phage resistance (Vidotto *et al.* 1990; Fantinatti *et al.* 1994). Aerobic production, the presence of fimbriae (type1-curli-p) and flagella, or capsule (K1), and serum resistance are among virulence factors that have been described so far for APEC (La Ragione and Woodward 2002).

Aerobactin- The effects of low levels of free iron on the growth of APEC in chicken are well established (Payne 1988; Martinez *et al*. 1990). Payen and Martinez showed that the majority of *E. coli* from septicemic chickens had the aerobic iron hunting system. This aerobactin system allowed APEC to grow under iron-limiting conditions, and it strongly correlated with mortality in neonatal chickens (Dho and Lafont 1984; Payne 1988; Martinez *et al.* 1990).

Type 1 fimbria- Type 1 fimbria are long and thread-like filaments (Krogfelt and Klemm 1988). The expression of type 1 fimbriae by *E. coli* is mainly observed in the trachea, lungs, and air sacs of the birds (Pourbakhsh *et al.* 1997a; Pourbakhsh *et al.* 1997b). They are significantly associated with adhesion of APEC to epithelial cells, colonization, invasion, and persistence in chicken (La Ragione and Woodward 2002). These findings extended those of Malaviya *et al.* who suggested that type 1 fimbria could be mast cell activators, and that this activation would result in phagocytosis and total recall of neutrophils to the site of infection in mice (Malaviya *et al.* 1996).

Curli fimbriae- Curli fimbriae are thin, hair-like filaments, which are found in most *E. coli* isolates (Olsen *et al.* 1989). Herwald *et al.* (1998) suggested that hypotension and coagulopathies, which are seen in severe *E. coli* infections, are associated with the ability of curli fimbriae to interact with number of host proteins such as fibronectin and fibrinolytic proteins, and coagulation cascades. Fibronectin is known to interact with many extracellular substances such as collagen, fibrin and heparin and also with specific membrane receptors on responsive cells (Herwald *et al.* 1998). La Ragione *et al.* (2000) recently showed that curli fimbriae of *E. coli* are important in colonization, invasion, and persistence of APEC in chickens (La Ragione *et al.* 2000).

P fimbriae- P fimbriae or F11 have been described in a minority of APEC (Pourbakhsh and Fairbrother 1994). However, receptors for p fimbriae are absent in the trachea and they do not mediate adhesion to avian respiratory tissues. Therefore, P fimbriae might be involved in presence and persistence of pathogenic microorganism, its toxins in the blood or septicemia (Pourbakhsh *et al.* 1997b).

Flagella- Flagella are fine hair-like filaments, which are found on the most motile APEC (La Ragione and Woodward 2002). Flagella support bacterial penetration of intestinal mucus by passing through the underlying epithelial cells. They are important in colonization, invasion and persistence of APEC in chickens (La Ragione *et al.* 2000).

K1 antigen- The most common K antigen is K1. The K1 capsule is composed of polysaccharide. It is anti-phagocytic, and is involved in extra-intestinal infections and serum resistance (La Ragione and Woodward 2002).

Serum resistance- Cell surface structures such as capsule, LPS or outer membrane protein mediate resistance to killing by complement (the factor that causes immune cytolysis, the lysis of antibody coated cells) in serum (Ellis *et al.* 1988). It has been shown that serum resistance is associated with avian septicemic strains of *E. coli*, and complement resistance is highly correlated with lethality in the chicken and moderately associated with chicken embryo lethality (Nolan *et al.* 1992; Wooley *et al.* 1992).

1.2.2. Pathogenesis of respiratory form of *E. coli* in chickens

E. coli infections occur when skin or mucosal barriers of chickens are damaged(e.g. unhealed navel, wounds, disruption of normal flora, tracheitis) (Gyles 2004). APEC mucosal infection results in the septicemic form of *E. coli* in chickens

with early changes characterized by airsacculitis (Dho-Moulin and Fairbrother 1999). APEC adhere to respiratory epithelial cells by F1 pili (Dho-Moulin and Fairbrother 1999; Arp 1980) and P pili (Dho-Moulin and Fairbrother 1999; Dozois *et al.* 2000), after which they enter the bloodstream via the lungs (Ackermann and Cheville 1991) and air sacs (Pourbakhsh *et al.* 1997a) to reach internal organs.

1.2.3. Sources, vectors, and transmission of *E. coli* in chickens

Eggshell contamination with pathogenic *E. coli* is a common source of infection and can result in high chick mortality (Giovanardi *et al.* 2005). Eggshell contamination may also result from ovarian infection or salpingitis (Barnes 2003). Poultry house dust may contain $1x10^5$-$1x10^6$ *E. coli*/g that can survive and are considered as a source of infection for long periods, especially under dry conditions (Harry and Hemsley 1965).

1.2.4. *E. coli* infections in neonatal chickens

Hens inoculated experimentally may shed *E. coli* in up to 26% of their eggs and cause the death of embryos late in the incubation period (Siccardi 1975). The clinical signs associated with colibacillosis are nonspecific and vary with age and concurrent diseases. *E. coli* causes death in newly hatched birds at or shortly after hatching (within 24 - 48 hours) due to septicemia (Pourbakhsh *et al.* 1997a). The common form of *E. coli* in neonatal chickens is yolk sacculitis and omphalitis (Pourbakhsh *et al.* 1997a). Panophthalmitis is an uncommon consequence of *E. coli* septicemia and is usually unilateral (Gyles 2004).

1.2.5. Pathology associated with *E. coli* infections

Gross lesions are omphalitis, yolk sacculitis, fibrinopurulant airsacculitis, pericarditis perihepatitis (Gyles 2004), and peritonitis (Cheville and Arp 1978). Acute septicemia with splenomegaly, congested muscles, and multiple necrotic foci of the liver occurs occasionally (Gyles 2004). Microscopically, caseous exudates consisting of heterophils, bacterial colonies, giant cells and macrophages may be visible (Cheville and Arp 1978). In severe cases of omphalitis, necrosis of the body wall and underlying skin may be seen (Gyles 2004). Yolk sacculitis and enlargement of yolk sacs are associated with delayed absorption of the yolk sacs (Gyles 2004).

1.3. Overview of the avian immune system

The overall organization and mechanisms of the avian immune system are similar to those in mammals. In poultry, immune cells are located in primary lymphoid organs such as the thymus and the bursa of Fabricius, or secondary lymphoid organs such as the spleen, bone marrow, gland of Harder (located ventral and posteriomedial to the eyeball), and conjunctival-associated, bronchial-associated, and gut-associated lymphoid tissues. Primary lymphoid organs are the site for differentiation and maturation of T and B lymphocytes, while secondary lymphoid organs are principal sites of antigen-induced immune response (Sharma 1991).

The first line of defense against pathogens that enter the body is the innate immune system. The innate immune system includes phagocytic cells such as heterophils and macrophages (Qureshi *et al*. 2000); a complement system that produces proteins, which bind to bacteria in order to kill the bacteria, or enhance bacterial

phagocytosis (Koppenheffer *et al*. 1998); and NK cells. Although NK cells are neither - T nor B lymphoid cells, they are cytotoxic for virus-infected and tumor cells (Sharma 1981).

Pathogens that can pass through host's physical barriers or innate immune defense mechanisms induce a specific immune response (adaptive immunity) (Sharma 1991). Adaptive immunity is mediated by a variety of cells, the most important of which are T cell receptors (TCR), B cell receptors (BCR), and macrophages (Schauenstein *et al*. 1988). T lymphocytes (TC) recognize foreign Ags after the antigens have been processed by antigen presenting cells (APCs) (Sharma 1991). B lymnphocytes (BC) are able to recognize foreign Ags via immunoglobulin (IgM, IgG, and IgA) that project from the cell surface (Parvari *et al.* 1988).

TCs, BCs, macrophages, and dendritic cells (DC) all secrete cytokines (Sharma 1991). Cytokines produced by T helper cells (Th) can be separated into two functional types, Th 1 and Th 2 based cytokines (Sharma 1991). Cytokines secreted by Th 1 cells help the cell-mediated branch of immune responses, such as: IFN-γ, which activates macrophages (Golemboski *et al.* 1992); IL-2, which promotes proliferation of TCs, CTL, NK cells, and BCs (Lawson *et al.* 2000); and TNF-α , IL-12, and Il-18. Cytokines produced by Th 2 cells, such as IL-4, IL-5, promote BC activation and antibody production (Sharma 1991).

1.4. Cytosine-phosphodiester-Guanine Oligodeoxynucleotides (CpG-ODN)

1.4.1. Early applications of CpG-ODN

More than a century has passed since William Coley introduced the deliberate

use of bacterial extracts for successful treatment of cancer (Wiemann and Starnes 1994). Since then, extracts of the attenuated *Mycobacterium bacillus* Calmette Guerin (BCG) have become standard therapy for human bladder cancer (Morales 1978). DNA of *Mycobacterium bovis* strain BCG, which is composed of 70.0% DNA, 28.0% RNA, 1.3% protein, 0.20% glucose, and 0.1% lipid. The DNA of *Mycobacterium bovis* was found to be the component of BCG that activates NK cells and induces tumor regression (Tokunaga 1984). It was later confirmed that purified BCG DNA induces NK cell activity and the production of type 1 and type 2 interferon *in vitro* (Yamamoto *et al.* 1988). Investigators cloned mycobacterial genes or synthesized oligodeoxynucleotides (ODN), and found that certain nucleic acid sequences (palindromes) in these ODN were responsible for immune stimulatory effects (Yamamoto *et al.* 1992). Methylation of the cytosine phosphodiester guanine (CpG) was reported to have no influence on the immune stimulatory activities of the DNA (Kuramoto *et al.* 1992). Messina *et al.* (1991) reported that purified bDNA induced murine BC proliferation and immunoglobulin secretion, but that vDNA did not. In contrast, they suggested that methylation of CpGs disrupted the unique higher ordered structure of bDNA molecules.

1.4.2. The location of CpG motifs

Today, most of the information regarding CpG-ODN is based on studies using human cells and mice. A long history of human therapy with CpG-ODN encouraged further explorations of CpG-ODN using the human cell model. All information presented in this chapter is based on the studies using these two species as model.

After synthesizing and testing several hundred ODNs, it became clear that an

adequate sequence element for inducing BC activation was a CpG dinucleotide in a particular base context (Krieg 2002). It is interesting that an increase in the number of stimulatory CpG motifs in ODN increases the activity of the ODN, although the addition of a CpG into the terminal end of an ODN may actually reduce the degree of the BC activation (Krieg *et al.* 1995). Stimulatory activities of CpG were lost when CpG dinucleotides from ODN were eliminated (Krieg *et al.* 1995).

1.4.3. Immune recognition of CpG motifs as a defense mechanism

The CpG content of vDNA and bacterial or viral DNA varies markedly (Krieg 2002). The frequency of CpG dinucleotides in vertebrate genomes is only 25% of the predicted frequency for it to occur (Bird 1993). The most common base that precedes a CpG in vDNA is a cytosine (C) and the most common base that follows a CpG is a guanine (G) (Han *et al.* 1994)**.** In addition to differences in CpG content between vDNA and bDNA or viral DNA, 70% of CpG dinucleotides in vertebrates are methylated at five positions, while fewer CpG dinucleotides are methylated in bacterial or viral DNA (Bird 1993)**.** Krieg et *al*. (2002) showed that the immune system recognizes CpG motifs using protective pathways that are also activated by (PRRs).

TLRs are among PRRs that mediate host recognition of PAMPs in pathogens (Janeway and Medzhitov 2002). TLRs trigger an innate immune response that promotes the removal of foreign pathogens and supports the development of adaptive immunity (Akira *et al.* 2001). The effects of CpG-ODN on human and mouse cells have been well studied. CpG motifs must interact with independent receptor molecules (TLR9) presented in endosomal vesicles (Ishii *et al.* 2002) before triggering an innate immune

response (Katso *et al.* 2001). This interaction leads to the swelling and acidification of endocytic vesicles and generation of reactive oxygen species (Takeshita *et al.* 2001). BCs and plasmacytoid DCs (pDC) are the main cell types that express TLR9 and respond directly to stimulation by CpG-ODN (Takeshita *et al.* 2001; Gursel *et al.* 2002). Activation of these cells by CpG-ODN initiates an immunostimulatory cascade that induces maturation, differentiation and proliferation of NK cells, TCs and monocytes/macrophages (Klinman *et al.* 1996; Sun *et al.* 1998). Together these cells secrete cytokines and chemokines that create a proinflammatory (IL-1, IL-6, IL-18, and TNF) and Th 1-baised (interferon-γ, IFN-γ, and IL-12) immune environment (Ballas *et al.* 1996; Klinman *et al.* 1996). DNA with methylated CpG motifs (vDNA) does not result in cytokine/chemokine secretion (Klinman *et al.* 1996; Gursel *et al.* 2002).

1.4.5. Effect of the ODN backbone On CpG-mediated immune activity

Zhao *et al.* (1993) used fluorescein-conjugated oligonucleotides to study the effects of phosphorothioate (PS) or phosphodiester (PO) backbone on surface binding, uptake, and degradation of mouse spleen cells (Zhao *et al.* 1993). They showed that binding and uptake of BCs was higher in PS than in PO. Phosphorothioate completely blocked PO binding because of its higher affinity for oligonucleotide binding sites on the cell membrane (Zhao *et al.* 1993). The level of CpG-induced BC activation was 200 times greater with the PS backbone than with the PO backbone (Krieg 2002). On the other hand, PS was less susceptible to DNase than PO (Stein *et al.* 1988). Stability against serum degradation can be noticeably enhanced by adding a PS backbone, which maintains an increased immunostimulatory effect of CpG-ODN (Ballas *et al.* 1996).

Oligodeoxynucleotides with normal PO backbone degrades within four hours especially in lymphocytes. This significantly reduces its ability to cause BC proliferation (Krieg 2001). Overall, ODN with a double-stranded palindrome are likely more stable against serum degradation than ODN without a duplex (Ballas *et al.* 1996).

Ballas *et al.* found that in mice, all of the ODN lytic activity is held in the NK cells, which are a valuable cell immune population against pathogens (Ballas *et al.* 1996). Including a PS backbone in ODN may reduce stimulation of macrophage and NK cell activity against pathogens. This reduction and decreased immune response may be due to less binding of PS backbone ODN to the macrophage receptors compared with an ODN in which at least part of the backbone is PO (Ballas *et al.* 1996). However, the effect of PS on NK cells can be increased by adding poly G sequences at the 5' and 3' ends of the ODN (Ballas *et al.* 1996). The effects of different ODN backbones on CpG-mediated immune activities are summarized in Table 1.1.

Table 1.1. Comparison of PS and PO backbone on CpG-mediated immune activities

Immunomodulatory activity	**PS**	**PO**
Binding and uptake of BCs	+++	+
Susceptibility to DNase	+	+++
NK cell activity	+++	+

1.4.6. Identifying distinct classes of immune stimulatory CpG-ODN

1.4.6.1. Characteristics of class A CpG-ODN (CpG-A ODN)

Oligodeoxynucleotides that contain PO backbones are particularly effective at activating NK cells and inducing IFN-α production from pDCs, and are accordingly known as CpG-A ODN (Vallin *et al.* 1999) (Krieg 2001). CpG-A ODN contain poly-G motifs with PS connections at the 5' and 3' ends and a PO at the ODN center and single CpG motif. These properties induce the highest degree of NK cell activation and IFN-α production in virus-free four- to six-week-old mice (Ballas *et al.* 1996). Krug *et al.* (2001) showed that CpG-A ODN directly induce the secretion of IFN-α from pDCs, which indirectly support the subsequent maturation of antigen presenting cells (APCs) using human cell model.

However, immunostimulatory effects of CpG-A ODN with no poly-G motifs can be enhanced by administering cationic lipids (CLs) intravenously to mice (Mui *et al.* 2001), or by administering antibodies collected from patients with systemic lupus erythematosus (Vallin *et al.* 1999). Plasma concentrations of IL-12, IFN-γ, IL-6, monocyte chemoattractant protein-1 and TNF-α was higher when CpG-ODN plus CLs was given intravenously to mice than when the same dose of free CpG-ODN was given. CpG-A ODN and bDNA had similar effects on activation of NK cells, but CpG-A ODN had a stronger effect on induction of IFN-α production and a lower effect on activation of BCs than bDNA (Krieg 2002). The immunostimulatory effects of CpG-A ODN are shown in Table 1.2.

1.4.6.2. Characteristics of class B CpG-ODN (CpG-B ODN)

CpG-B ODN encode multiple CpG motifs on a PS backbone, trigger the differentiation of APCs and induce the proliferation and activation of BCs (Krug *et al.* 2001a) (Hartmann and Krieg 2000). CpG-B ODN enhance BC stimulatory properties, such as secretion of IL-6 and IL-10, but reduce NK cell stimulation (Krieg 2001). They also produce TNF-α and IgM (Klinman 2004). CpG-B ODN have no poly-G motifs (Krieg 2001). In addition to secreting cytokines and immunoglobulin, BCs activated by CpG-B ODN express increased levels of molecules such as class two major histocompatibility complex (II MHC), cluster of differentiation (CD) 80 and CD 86 (Krieg *et al.* 1995; Davis *et al.* 1998). The immune stimulatory effects of CpG-B ODN are shown in Table 1.2.

1.4.6.3. Characteristics of class C CpG-ODN (CpG-C ODN)

CpG-C ODN are composed entirely of PS backbones (Klinman 2004). They can contain a TCGTCG sequence at the 5' end, and internally a GTCGTT sequence (Hartmann *et al.* 2003). This class of ODN contains multiple CpG motifs and is able to stimulate BCs to secrete IL-6 and IgM, and stimulate pDCs to produce IFN-α, thereby combining stimulatory properties of both CpG-A ODN and CpG-B ODN classes (Hartmann *et al.* 2003). The immune stimulatory effects of CpG-C ODN are shown in Table 1.2.

1.4.6.4. Characteristics of CpG-S ODN

This class of ODN blocks TLR 9, TLR 7 and / or TLR 8 and RNA-mediated

Table 1.2. Characteristics of immune stimulatory effects of different classes of CpG-ODN

ODN Type	Structure characteristics	Immune activity
CpG-A ODN	-Contain poly-G motifs and single CpG motif (CG) -Mixed PS and PO backbone	1-Activation of NK cells 2-Induction of IFN-α from pDC 3-APC maturation, mediated by IFN-α
CpG-B ODN	-Contain no poly-G motifs and multiple CpG motifs -PS backbone	1-Proliferation and activation of BCs 2-Secretion of IL-6, IL-10, TNF, IgM 3-Increase level of II MHC, CD 80, and CD 86
CpG-C ODN	-Contain multiple CpG motifs -PS backbone	1-Stimulation of BCs to secret IL-6 2-Stimulation of DCs to produce IFN-α
CpG-S ODN	-Contain CCT motifs near the 5' end, and at least one G-rich area near the 3' end	1-Blocks TLR 9- TLR 7 and/or TLR 8, and RNA-mediated immune activation

immune activation (Barrat *et al.* 2005). However, CpG-S ODN do not block activation of immune cells through TLR 4, CD 40 or the BCR (Lenert *et al.* 2001). To be most effective at blocking CpG-induced TLR 9 activation, this class should contain a CCT motif near the 5' end, and at least one G-rich area near the 3' end with three to five bases (Lenert *et al.* 2003). The immune stimulatory effects of CpG-S ODN are shown in Table 1.2.

1.4.7. Mechanism of actions of CpG-ODN

CpG-ODN bind to cell membranes, likely due to strong bonds between G motifs

in the ODN and lipid bi-layers of the cell membrane (Hughes *et al*. 1994; Kimura *et al.* 1994; Krieg 2002). It is not clear whether cells take up CpG-ODN through a cell surface receptor or an intracellular signaling pathway (Manzel and Macfarlane 1999). BCs and monocytic cells have the highest rates of CpG-ODN uptake, while TCs and neutrophils have a lower rate of uptake. Following uptake into lymphocytes, ODN move to the endosomal compartment where CpG-ODN send primary signals to stimulate immune system (Zhao *et al.* 1994). Endocytosis and endosomal acidification/maturation are both required for CpG-ODN to stimulate production of TNF-α.

CpG-ODN are among the PAMPs that are recognized by TLR 9 (Krieg 2002). Among different CpG-ODN classes, CpG-B ODN are the only class that have been shown to directly activate human BCs and pDCs via TLR 9 (Bauer *et al.* 2001). The preferred CpG motif in humans is CTCGTT, and in mice is CACGTT (Bauer *et al.* 2001). It is not clear if TLR 9 is present in endosomes, but evidence suggests that TLR 9 expression improves the endosomal uptake of CpG-ODN (Takeshita *et al.* 2001). CpG-ODN activates BCs following activation of mitogen-activated protein kinase (MAPKs) within seven minutes, which in turn contributes to IL-12 production (Yi and Krieg 1998a). On the other hand, CpG-ODN triggers the activation of nuclear factor kappa B (NFκB) in human BCs and murine macrophages, which induces the production of reactive oxygen species (ROS) within five minutes as well as the protection of BCs against apoptosis (Yi *et al.* 1996; Sparwasser *et al.* 1997; Yi and Krieg 1998b; Hartmann and Krieg 2000). Activated NFκB also trigger multiple transcription factors, which are important regulators for proinflammatory cytokines (Sweet *et al.* 1998).

1.4.8. Cellular responses to CpG-ODN

In general, CpG-ODN stimulates different immune cells, regardless of whether the DNA is in the form of genomic bDNA or in the form of synthetic ODN with a nuclease-resistant PS backbone. Different cellular responses to CpG-ODN are described in the next sections. The effects of CpG-ODN on different immune cells are shown in Fig. 1.1.

1.4.8.1. B cells (BCs)

In 1998, Jakob *et al*. showed that both bDNA and CpG-ODN stimulated BCs enter the G1 phase of the cell cycle and secrete proinflammatory cytokine (IL-6) and anti-inflammatory cytokine (IL-10) within a few hours (Jakob *et al.* 1998). The BC proliferation activity of CpG-ODN seemed to be a signal–dependent process, as it could greatly be enhanced if the BCR was engaged at the same time (Krieg *et al.* 1995; Tasker and Marshall-Clarke 2003).

Low concentrations of CpG-ODN in mature peripheral BCs can be strongly synergistic with signals through BCRs, which results in a ten fold increase in BC proliferation, antigen-specific immunoglobulin secretion, and IL-6 secretion (Krieg *et al.* 1995). Further studies showed that the CpG-induced IL-6 expression was required for BCs to subsequently secrete IgM (Yi *et al.* 1998). Davis *et al.* (1998) found that IgM levels in mice immunized with hepatitis B virus (HBV) surface antigen and CpG-ODN were five times higher than IgM levels in mice immunized with Ag and a standard adjuvant such as alum.

Mice that did not produce IFN-γ induced less than half of the normal IL-6 and IgM response to CpG–ODN (Yi *et al.* 1996). However, *in vivo* administration of CpG-

ODN stimulated NK cells to secrete IFN-γ, which enhanced BC responses in these mice (Cowdery *et al.* 1996; Yi *et al.* 1996). Yi *et al.* (1999) showed that CpG-ODN had anti-apoptosis activity and could prevent the normal spontaneous apoptosis of isolated mature murine primary BCs.

1.4.8.2. Dendritic Cells (DCs)

TLRs allow DCs to induce different patterns of immune responses in humans and mice depending on the pathogen (Krieg 2002). Among human DCs, only pDCs have been directly activated by CpG-ODN, through TLR 9 (Krieg 2002). CpG-ODN induces activation of plasmacytoid DCs which, increases the surface expression of II MHC and the co-stimulation of molecules CD 86 and CD 40, secretes cytokines (IL-12, IL-18, TNF-α), INF-α (through type 2 DC precursors) and chemokine (IL-8), and activates TCs (Hartmann and Krieg 1999; Kadowaki *et al.* 2001a; Kadowaki *et al.* 2001b; Krug *et al.* 2001a). Plasmacytoid DCs are also known for their role as the primary source of the IFN-α (Krieg 2002). Human monocyte-derived DCs do not express TLR 9, but do express IL-12 and IL-18 mRNA, which means that responses to CpG-ODN in humans are due to indirect effects (Krieg 2002). Like human pDCs, murine bone marrow-derived DCs and Langerhans cells can be activated by CpG-ODN. This activation occurs through maturation of immature DCs and activation of mature DCs to trigger APCs rich in II MHC and to produce high levels of cytokines such as IL-12, IL-6, and TNF-α (Jakob *et al.* 1998; Sparwasser *et al*. 1998; Ban *et al.* 2000). CpG-ODN induce murine monocyte- derived DCs to produce IL-12 and IL-18, but do not have any effect in upregulating co- stimulatory molecules (Krieg 2000).

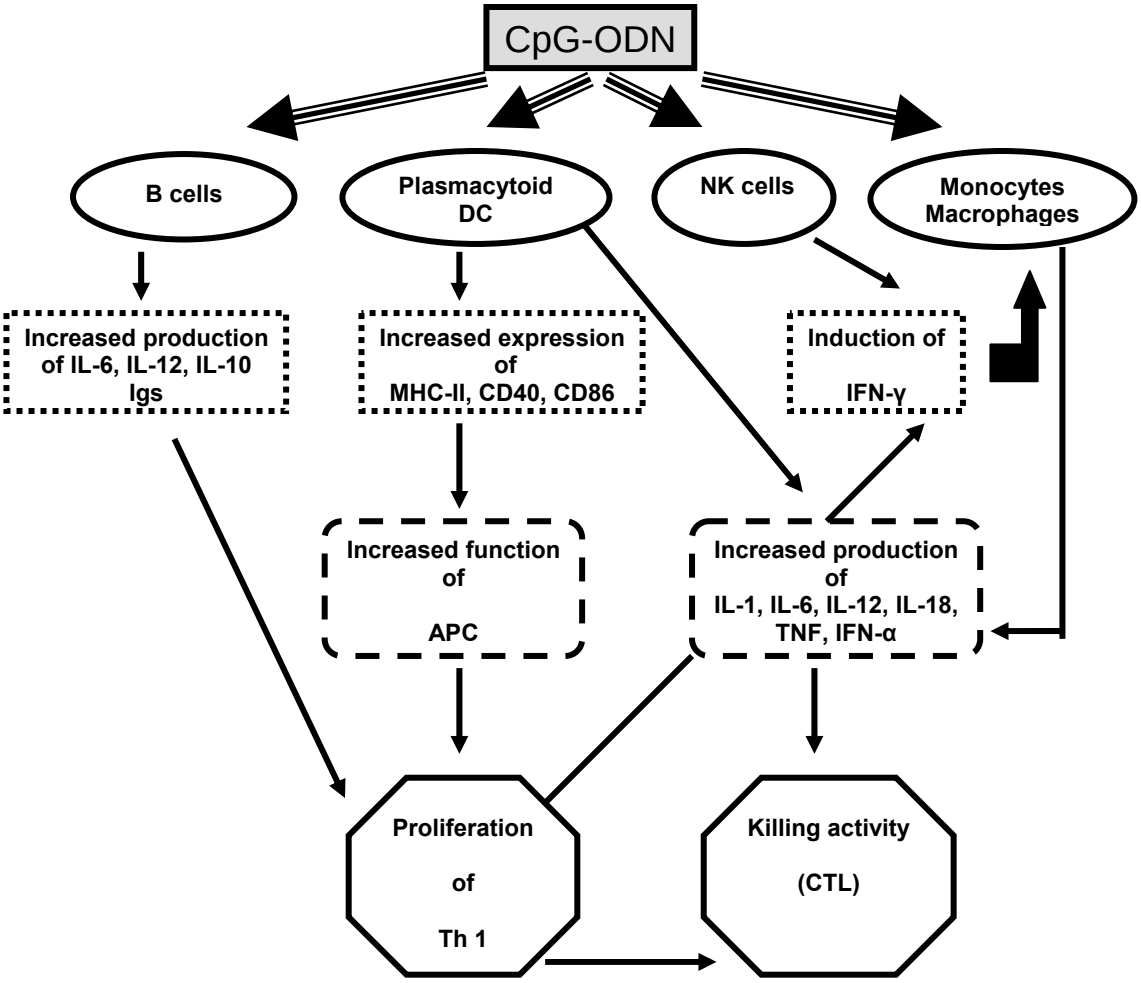


Fig. 1.1. The effects of CpG-ODN on different immune cells

1.4.8.3. Monocytes and macrophages

Kadowaki *et al.* (2001b) showed that while plasmacytoid pre-DC strongly expressed TLR 7 and 9, monocytes preferentially expressed TLR 1, 2, 4, 5, and 8. However, treatment of human PBMC with CpG-ODN stimulated the monocytes to express increased levels of CD 40 are found on matures B-lymphocytes and activates BCs. In addition, the treatment caused monocytes to produce IL-6 and TNF-α with delayed kinetics when compared to LPS (Hartmann and Krieg 1999). Among monocyte-

related cells, murine macroglial cells and astrocytes were also activated by CpG-ODN (Deng *et al.* 2001; Schluesener *et al.* 2001).

Murine macrophages/monocytes do not respond directly to CpG-ODN, as their activation requires a primary cytokine expression that includes IFN-γ (Sparwasser *et al.* 1998). This means that the production of nitric oxide (NO), expression of the inducible nitric oxide synthase (iNOS) gene, and TNF-α release from macrophages and bone marrow-derived macrophages in response to bDNA are absolutely dependent on IFN-γ activities (Sweet *et al.* 1998).

Chu *et al.* (1999) demonstrated that treatment of murine macrophages for 18 hours with CpG-ODN decreased surface expression of II MHC (no APC expression). However, these inhibitory effects of CpG-ODN on Ag processing did not occur after short treatments (e.g., two hours) with CpG-ODN. Moreover, CpG-ODN treatment did not change the endocytotic activity of macrophages (Chu *et al.* 1999).

1.4.8.4. Natural killer cells (NK cells)

Many studies have confirmed that NK cells are activated by bDNA in murine, human, and other species (Kuramoto *et al.* 1992; Tokunaga *et al.* 1992; Yamamoto *et al.* 1992). These studies have shown that the effects of CpG-ODN on murine NK cells are not direct and require either IFNs type 1 or the CpG-ODN-induced IFN-α induction in APCs, which influence the expression of IFN-γ (Ballas *et al.* 1996; Cowdery *et al.* 1996). Therefore, CpG-ODN act as a co-stimulatory signal for murine NK cells (Ballas *et al.* 1996). In contrast, CpG-OPDN with CG palindromes and ACCGGT sequences can stimulate human NK cells directly (Ballas *et al.* 1996).

1.4.8.5. T cells (TCs)

In general, CpG-ODN are not known to stimulate TCs directly (Sun e*t al.* 1998). This is not surprising, as TCs do not express TLR 9 (Sun *et al.* 1998). In the human immune system, pDCs and BCs express TLR 9 and thus are sensitive to CpG-ODN. In contrast, monocytes, myeloid DCs, and TCs, are activated indirectly by stimulated pDCs and BCs (Krug *et al.* 2001b; Hornung *et al.* 2002). However, CpG-ODN can stimulate murine APCs, and they produce IFN-α and activate IFN type 1 receptors and TCs (Sun *et al.* 1998).

1.4.8.6. Neutrophils

In humans, CpG-ODN did not activate neutrophils against *Mycobacterium tuberculosis* (Neufert *et al.* 2001). However, CpG-ODN indirectly activated murine neutrophils (Krieg 2002). A large dose of CpG-ODN administered to mice increased the immune system's ability to resist acute polymicrobial sepsis (Weighardt *et al.* 2000). While CpG-ODN did not change systemic levels of IL-12, IL-18 and IL-10 in mice, administration of CpG-ODN strongly enhanced accumulation of neutrophils at the primary site of infection. This was likely due to upregulation of phagocytic receptors and increased phagocytic activity (Weighardt *et al.* 2000). Thus, even though CpG-ODN do not appear to activate neutrophils directly, they do increase the effectiveness of neutrophils (Krieg 2002).

1.4.9. Therapeutic application of CpG-ODN

1.4.9.1. Activation of innate immune defense against infection

In the case of an infection, the host's immune system can be activated when a specific molecular structure in a pathogen, known as PAMP, is recognized by PRRs (TLR 9) (Krieg 2002). Krieg *et al*. (1998) showed that CpG-ODN might be such a molecular structure, and could be recognized by PRRs as well. They administered CpG-ODN two days prior to *Listeria monocytogenes* challenge, and found that it could fully protect mice against infection. Protection was induced by increasing serum IL-12 and IFN-γ, and by activating DCs. The protective effect of CpG-ODN in this experiment lasted for at least two weeks. A number of studies have provided further evidence that CpG-ODN, given orally or by injection, can protect many species against a wide range of protozoan, bacterial, and viral pathogens (Krieg *et al.* 1998).

Recently, *in vivo* studies showed that CpG-ODN can protect chickens against the intracellular bacterial infection *Salmonella* Enteritidis (He *et al.* 2005b) Protection against *Salmonella* Enteritidis was due to enhanced secretion of IL-6, IL-1β, IFN-γ; and NO production (He *et al.* 2005b). Oxidative burst activity in chicken monocytes and heterophils also played a role in the chickens' immune system against bacteria (He *et al.* 2007). Later, Gomis *et al*. (2004) showed that *in ovo* delivery of CpG-ODN could protect neonatal chickens against *E. coli* septicemia (Gomis *et al.* 2004).

The effect of CpG-ODN against viral infections such as HBV has been studied in the mice genetically deficient in type 1 IFN receptors. Therefore, the hepatocytes of these mice could not express TLR 9 (Olbrich *et al.* 2002). The antiviral effect of CpG-ODN in this model was likely due to secretion of IFN-α in response to

stimulation by CpG-ODN and activation of TLR 9.

In addition to providing protection against infections, CpG-ODN has an effect on hematopoietic function of mice as well (Krieg 2002). Treatment of mice with high doses of PS CpG-ODN induced massive splenomegaly and increased spleen granulocyte-macrophage colony forming units and early erythroid progenitors (burst-forming units-erythroid) (Sparwasser *et al.* 1999).

In conclusion, CpG-ODN can be recognized by TLR 9 and stimulate innate immune system of the host by activation of BCs and DCs. Activation of these cells increase the production of IgM and cytokines, and increase APC function, which subsequently activate adaptive immune system by activation of T and NK cells, and production of IFN-γ. The immunostimulatory effect of CpG-ODN has been shown against different viral, bacterial, and protozoan diseases in different species.

1.4.9.2. Role of CpG-ODN as a vaccine adjuvant and in DNA vaccines

Adjuvant activity of CpG-ODN in DNA vaccines have been studied by many researches. Mechanisms that induce adjuvant activities of CpG-ODN are:

I- Synergy between TLR 9 and BCRs followed by stimulation of antigen-specific BCs (Krieg *et al.* 1995);

II- Inhibition of BC apoptosis (Yi *et al.* 1998);

III- Enhanced DCs maturation and differentiation, resulting in enhanced activation of Th 1 cells and strong cytotoxic T lymphocyte (CTL) generation (Lipford *et al.* 2000; Sparwasser *et al.* 2000). CpG-A ODN compared to CpG-B ODN may give weaker antibody, but stronger CTL response (Krieg 2002).

Recently, Ioannou *et al*. (2003) studied the safety of CpG-ODN as a vaccine adjuvant. They showed that subcutaneous injection of CpG-ODN induced less perivascular and necro-dermatitis reactions at the site of injection than did other adjuvants. Other adjuvants used in the study included Freund's complete adjuvant (FCA), alum, Emulsigen®, and VTA. Systemic administration of CpG-ODN also did not cause significant inflammatory responses (Ioannou *et al.* 2003).

Vaccines containing CpG-ODN can be administered via different parental and mucosal routes. CpG-ODN are an effective mucosal vaccine adjuvant as they induce both local and systemic humoral and cellular immune responses (Gallichan *et al.* 2001). For example, intranasal immunization of mice with influenza vaccines containing CpG-ODN enhanced the production of influenza specific antibodies in mice (Moldoveanu *et al.* 1998). Intravaginal immunization of mice with herpes simplex virus type 2 plus CpG-ODN could induce specific immunity and protection against herpes (Kwant and Rosenthal 2004). Conjunctival, oral, and even transcutaneous vaccination with DNA vaccines could enhance protection against herpes simplex virus type 1 (Nesburn *et al.* 2005), rotavirus (Dong *et al.* 2005) and *Chlamydia muridarum* in mice (Berry *et al.* 2004).

The adjuvant effects of CpG-ODN were improved markedly by ensuring that the ODN remained close to the Ag (Davis *et al.* 1998; Gursel *et al*. 2001). Researches showed that specific IgG responses could be increased by 10-100 folds by cross-linking the ODN and Ag with alum, lipid emulsions or vesicles (Davis e*t al.* 1998; Klinman *et al.* 1999a; Gursel *et al.* 2001). Recently, Jaafari *et al*. (2007) found that encapsulation of CpG-ODN in liposomes improved the immunogenicity of *Leishmania* Ag. This

improved-immunogenicity effect is responsible for lower spleen parasite burden and higher IgG and IFN-γ production.

Overall, using CpG-ODN as vaccine adjuvant leads to a decrease in the amount of Ag needed (Tighe *et al.* 2000).

1.4.9.3. Other immunotherapeutic activities of CpG-ODN

Anti-tumor effects- Anti-tumor effects of CpG-ODN have been recognized for decades**.** The immune cascade that is obtained by CpG-ODN results in the activation of NK cells and CLTs that assists the treatment of cancer (Klinman 2004).

Anti-allergic effect- Allergic diseases such as asthma which is an inflammatory disease of the airways, result from Th 2 type immune responses against environmental Ags (Barnes 2001). When CpG-ODN is combined with an allergen, the ODN stimulate an antigen-specific Th 1-cell response that prevent the development of Th 2-cell mediated allergic asthma (Klinman 2004).

1.5. Mechanism by which CpG-ODN act on the avian immune system

Little is known about the effects of CpG-ODN on immune responses in chickens. Investigations of the effectiveness of CpG-ODN in chickens can be divided into two types:

I- Mechanism of CpG-ODN as adjuvant and

II- Activities of CpG-ODN as stand alone immune stimulant.

1.5.1. Mechanism of CpG-ODN as an adjuvant on the avian immune system

1.5.1.1. Studies without a pathogen challenge

These *in vivo* studies evaluated antibody response to a protein Ag such as bovine serum albumin (BSA) with CpG-ODN as an adjuvant. In order to investigate whether the immunostimulatory actions of CpG-ODN occur in avian species as in mammals, 15-day-old chickens were orally immunized with BSA alone or BSA with CpG-ODN and the serum antibody response was followed (Vleugels *et al.* 2002). Vleugels *et al.* (2002) observed significantly higher and more persistent BSA-specific responses in the CpG-treated group. These results suggested that CpG-ODN were very promising adjuvant in chickens and opened further investigations.

Later, Ameiss *et al.* (2006) showed that a single dose of CpG-ODN administered early in neonatal life could significantly enhance antigen-specific humoral responses to BSA delivered orally to the chicken. This oral administration of CpG-ODN specifically enhanced both the IgM and IgA responses in the serum, distal ileum and cecum. These results suggested that oral administration of CpG-ODN might be very attractive for generating protection against enteric pathogens in commercial poultry (Ameiss *et al.* 2006).

Recently, the ability of IFA in combination with CpG-ODN to enhance the immunostimulatory effect of CpG-ODN was studied as well. This combination could maximize the production of IgY (specific antibody in the egg yolks) of laying hens against the fimbrial adhesion F 4 of porcine enterotoxigenic *E. coli*. In addition, CpG-ODN were suggested to be safe in commercial conditions for the immunization of laying hens on a larger scale without any adverse effect on the health of the birds or on the egg

yield for at least one year. (Levesque *et al.* 2007).

1.5.1.2. Studies with a pathogen challenge

Previously, Vleugels *et al.* (2002) proved that CpG-ODN could be valuable adjuvant for poultry vaccines. Many studies have been conducted using CpG-ODN as vaccine adjuvant against some of the poultry viral and bacterial disease. Wang *et al.* (2003b) found that chicken immunized with an infectious bursal disease virus (IBDV) - DNA vaccine had higher antibody titers than chickens immunized with the regular IBDV vaccine. Later, chickens were successfully immunized against IBD in Pakistan using the IBDV-DNA vaccine (Mahmood *et al.* 2006). The immunoadjuvant effects of CpG-ODN to other viral vaccines such as, Newcastle disease (ND) vaccine and its protective effects against ND virus in chicken were recently investigated as well (Linghua *et al.* 2007). The results demonstrated that the titer of serum IgG to ND vaccine and the proliferation of lymphocytes, and therefore the level of protection against ND were significantly stronger in chickens that received CpG-ODN than in the birds that received only ND vaccine.

Recent results stimulated research into the effectiveness of CpG-ODN as an adjuvant to vaccines against some important poultry bacterial diseases, such as *E. coli* (Gomis *et al.* 2007) and *Chlamydophila psittaci* (Loots *et al.* 2006). Birds that received *E. coli* bacterin formulated with CpG-ODN had higher survival rates, lower numbers of *E. coli* isolation from internal organs, and higher IgG levels in comparison with the birds that received vaccines alone (Gomis *et al.* 2007). However, *Chlamydophila psittaci*-CpG-ODN combined vaccine *in vivo* created no beneficial effect on the protective

immune response of the birds against chlamydiosis (Loots *et al.* 2006)

1.5.2. Activities of CpG-ODN as a stand alone immune stimulant of the avian immune system

1.5.2.1. *In vitro* studies

Studies have been conducted *in vitro* to investigate the mechanism by which CpG-ODN act at the cellular level in chicken. He *et al.* (2003) used an avian macrophage cell line (HD11) and peripheral blood mononuclear cells (PBMCs) to evaluate the ability of CpG-ODN to stimulate NO, IL-1β, and IFN-γ production. These researches showed that stimulation of HD11 cells with 10 μm phosphodiester CpG-ODN for 24 hours stimulated the production of NO (He *et al.* 2003). Maximum production of NO was seen when the cell line was stimulated with CpG-ODN containing GTCGTT, and enhancement of stimulatory effect was observed following increase the number of GTCGTT. Optimal NO stimulatory CpG-ODN also strongly stimulated the IL-1β gene expression in the macrophage HD11 cells and the IFN-γ gene expression in PBMCs (He *et al.* 2003).

Later, it was shown that the CpG-ODN that induced NO production characteristically required a clathrin-dependent endocytosis following endosomal maturations (clathrin is a protein that coats vesicles formed during endocytosis of materials on the surface of cells) (He and Kogut 2003).

The first study on serum-dependent activation of leukocytes by CpG-ODN in chickens was done by He *et al.* (He *et al.* 2005a). CpG-ODN were found to be an immune stimulant of chicken heterophils. Recognition of CpG-ODN by chicken

heterophils led to the release of granules. This serum-dependent heterophil degranulation was a phenomenon specific to CpG-ODN, as other microbial agonists such as LPS, lipoteichoic acid (found in the walls of most gram-positive bacteria), phorbol esters (activators of protein kinase C), and formalin-killed *Salmonella* Enteritidis did not induce heterophil degranulation (He *et al.* 2005a).

Further studies were performed to investigate the *in vitro* stimulation of leukocytes by CpG-ODN in neonatal chickens (He *et al.* 2005b). These studies found that when ODNs stimulated heterophils or monocytes, the oxidative burst that generates ROS (an innate immune response of immune cells) increased (He *et al.* 2005b). The inflammatory immune response of chicken monocytes in response to CpG-ODN was also found to be enhanced by combining of bDNA and double-stranded RNA (He *et al*. 2007).

1.5.2.2. *In vivo* / *In ovo* studies

The immunoprotective effect of CpG-ODN has been investigated using *in vivo* studies against pathogens. The immunoprotective effect of CpG-ODN was first observed in broiler chickens using an *E. coli* septicemia model (Gomis *et al.* 2003). Treating birds with a minimum of 10 μg of CpG-ODN at day 22 of age was protective against an *E. coli* septicemia infection (at day 25 of age). Chicken treated with CpG-ODN had reduced number of bacterial colonies isolated from peripheral blood, increased survival rates, and protection against *E. coli* infection for nearly six days (Gomis *et al.* 2003). Later, it was shown that CpG-ODN had protection against *E. coli* infection of not only mature, but also neonatal broiler chickens (Gomis *et al.* 2004). Neonatal chickens treated with 50 μg of CpG at two days of age, and challenged three days later with *E.*

coli, were significantly protected from infection and had a higher survival rate than the control group. These results stimulated research into the immunostimulatory effect of CpG-ODN against other bacterial infections in chickens.

He *et al.* showed that 25 or 50 μg of CpG-ODN injected (intraperitoneal) to two-day-old birds had significantly lower level of organ invasion (the liver and spleen) by *Salmonella* Enteritidis (inoculated the birds 24 hours post CpG-ODN treatment) compared with the control group (He *et al.* 2005b). The effect of CpG-ODN on the level of peritoneal *Salmonella* Enteritidis infection in neonatal chickens has also been investigated. Significant reduction of mortality was observed in chickens treated with 25 or 50 μg of CpG-ODN injected intraperitoneally at day two of age. These birds were challenged with *Salmonella* Enteritidis four hours post treatment with CpG-ODN by intraperitoneal injection. The protective effects of CpG-ODN were demonstrated throughout the seven-day-period of experiment (He *et al.* 2005b).

Recently, the modern broiler industry adapted to *in ovo* vaccination for control of diseases such as Marek's disease. This method reduces the costs of poultry production by injecting compounds in precisely calibrated volumes to large numbers of eggs per day, without causing trauma to developing embryos (Krieg 1999). The benefits of this method encouraged research into the potential of *in ovo* delivery CpG-ODN for enhancing immune protection of neonatal chickens against infections during the first week of life (Gomis *et al.* 2004). Gomis *et al.* (2004) showed that 50 μg of CpG-ODN injected into the chicken eggs at day 18 of incubation was protective against *E. coli* (inoculated the birds at three days of age).

The immunostimulatory effect of CpG-ODN against protozoan diseases has also

been studied. In 2004, Dalloul *et al.* (2004) showed that CpG-ODN could be used as immunoprotective agents against the protozoan infection *Eimeria* in chickens, resulting in increased protection. Both intravenous and subcutaneous injections of 10 and 50 µg of CpG-ODN reduced the number of oocysts shed in birds, while oral delivery did not. Recent work has also showed that adding a PS backbone to CpG-ODN increases resistance to *Eimeria* infection in birds injected *in ovo* (Dalloul *et al.* 2004).

1.5.3. Chicken TLRs

Many studies have demonstrated that chicken cells respond to pathogens with the same activities known for mammalian TLRs. As in mammals, TLRs are PRRs of chickens and are able to recognize a specific molecular structure in foreign infectious microorganisms. These molecular structures are known as PAMPs and serve as a link between innate and adaptive immunity (Krieg 2002). CpG-ODN are among PAMPs that are recognized by TLRs (Krieg 2002). In mammals, intracellular localization of TLR 9 suggests CpG-ODN must be recognized by this receptor in order to activate cellular function, as mice genetically deficient in this molecule show no CpG-ODN–induced activation of BCs, DC, or NK cells (Hemmi *et al.* 2000). It was previously described that CpG-ODN act at the cellular level and have strong immunoprotective effect against many bacterial infection in chicken. However, there is no evidence suggesting the existence of TLR 9 in chicken in recent studies. The question is, which avian TLR acts as a link between CpG-ODN and cellular signaling pathway to boost immune system against infection? Eight TLRs have been well described. TLRs 1, 2, 3, 4, 5, 6, 7 and 10 (Boyd *et al.* 2001; Fukui *et al.* 2001; Leveque *et al.* 2003; Iqbal *et al.* 2005; Philbin *et*

al. 2005; He *et al.* 2006; Kogut *et al.* 2006). The TLR 4 of chickens is involved in resistance to *Salmonella* infection (Leveque *et al.* 2003), while the TLR 5 controls the entry of flagellated *Salmonella* into systemic sites (Iqbal *et al.* 2005). Recently, a new chicken TLR (TLR 21) was identified by Roach *et al*. (2005). This TLR shares the same biology with the fish TLR 21-TLR 23 family (Roach *et al.* 2005). TLR 8 has also been described in just galliform bird species such as turkeys, grouse, chickens, quails, and pheasants (Philbin *et al.* 2005).

Information from the human and mouse TLR pathways allowed Lynn *et al.* (2003) to begin to reconstruct the TLR pathway in chicken (Lynn *et al.* 2003). These researches could detect a non-mammalian TLR (TLR 15). TLR 15 seemed to be specific to avian species as it differs from TLRs known in other vertebrates, and might respond to pathogens whose the host is the chickens. Later, Higgs *et al.* (2006) conducted an *in vitro-in vivo* experiment using five-week-old *Salmonella* Typhimurium challenge model to locate this receptor (Higgs *et al.* 2006). TLR 15 was detected in the chicken embryo fibroblast cells of the spleen, bursa, bone marrow, and cecum of healthy chickens and over expressed during *Salmonella* Typhimurium infection in the cecum (Higgs *et al.* 2006). Further studies are required to point out the possibility of recognition of CpG-ODN by chicken TLR 15 during infection.

1.6. Adjuvants

Adjuvants are formulated compounds or additives that, when combined with Ags, help to direct or boost the body's immune system (Gupta 1998). Adjuvants are classified in two broad categories: I- delivery systems that include particulate adjuvants

such as liposomes and emulsions (Singh and O'Hagan 2002), and II-immunostimulatory adjuvants such as CpG-ODN (Davis *et al.* 1998). The ability of an adjuvant to qualitatively affect the outcome of the immune response is an important consideration, because of the need for vaccines against infections (Pashine *et al.* 2005). Adjuvants increase immunogenicity of weak Ags, modulate antibody isotypes, and enhance the speed and duration of immune responses (O'Hagan *et al.* 2001).

Adjuvants activate the release of ILs by macrophages and some of them are administered by creating a depot at the site of injection to prolong the release and interaction of Ags with APCs. (Gregoriadis 1990).

1.6.1. Liposomes

Liposomes are the smallest artificial vesicles that can be produced from natural non-toxic phospholipids and cholesterol (Jaafari *et al.* 2007). Liposomes are attractive candidates for the delivery of Ags due to their non-toxic nature, biodegradability, biocompatibility, adjuvanticity, and induction of cellular immunity. Early studies have shown that liposomes are good adjuvants due to their ability to induce cell-mediated immunity (CMI) (Manesis *et al.* 1979), to increase internalization of Ags by macrophages, to increase Ag presented to TCs, and to induce IgG subclasses (Davis *et al.* 1987).

Recent studies suggest that the adjuvant effect of liposomes is due to depot formation at the site of injection, selective uptake of liposomal Ags into the regional lymph nodes and more efficient Ag presentation. Efficiency of Ag presentation

increases due to Ag clustering and massive delivery to APCs (Gregoriadis 1990; Jaafari *et al.* 2007).

CLs potentiate a noticeable activation of innate immunity by the TLR 9 stimulants, bDNA, and CpG–ODN (Ishii *et al.* 1997; Gursel *et al.* 2001; Sellins *et al*. 2005). The ability of CL to potentiate activation of innate immunity by DNA is due to protection of DNA from extracellular degradation and enhanced entry of DNA into the endosomal compartment, where TLR 9 is selectively expressed (Briane *et al.* 2002; Rocha *et al.* 2002). The ability of CLs to promote entry into the cell via the endosomal compartment may also be important in activation by TLR 3. This is because TLR 3 is also expressed primarily in the endosomal compartment (Iwasaki and Medzhitov 2004).

Liposomes have also been used to generate CD 8+ TC responses by introducing protein Ags into the cytosol and stimulating the I MHC pathway (O'Hagan and Valiante 2003). Verma *et al*. (2002) showed that when CpG-ODN are encapsulated in liposomes, their incorporation into DCs are increased and IL-12 enhanced. Moreover, NK cells are activated and IFN-γ is produced. Liposomal adjuvants used in this study are vaccine targeting adjuvant (VTA) and cationic lipid (CL).

1.6.1.1. Vaccine Targeting Adjuvant (VTA)

Biphasic lipid vesicles are liposomal adjuvant and formulations suitable for the delivery of proteins, peptides, and oligo/polynucleotides (Alcon *et al.* 2005a). These vesicles are made up of:

I- An outer membrane composed of two layers of vesicle-forming lipids

II- A core compartment containing an oil-in-water emulsion and

III- A dose of an immunogen effective to elicit an immune response (Foldvari 1998).

These formulations, known as VTAs, are designed for vaccine application and have been shown to enhance the adjuvant activity of CpG-ODN (Babiuk *et al.* 2004). Babiuk *et al*. (2004) showed that mice injected subcutaneously with CpG-ODN formulated in VTA had significantly higher serum IgG levels than mice injected with CpG-ODN alone. Additionally, mice given CpG-ODN formulated in VTA by intrperitonial injection had increased serum levels of IL-12 compared to the mice that received CpG-ODN alone (Babiuk *et al.* 2004). Moreover, mice injected intravenously with CpG-ODN formulated in VTA had higher serum levels of IL-6, and IFN-γ than the mice that received CpG-ODN alone (Mui *et al.* 2001). This formulation protected mice from a lethal herpes simplex virus type 1 challenge with minimal clinical signs using a scratch model (Irie *et al.* 1993; Babiuk *et al.* 2004). Systemic administration of CpG-ODN encapsulated in VTA protected pigs against porcine pleuropneumonia infection (Alcon *et al.* 2003). Protection of pigs was attributed to an increased immune response induced by the outer membrane lipoprotein A of *Actinobacillus pleuropneuminiae* (Alcon *et al.* 2003).

1.6.1.2. Cationic Lipid (CL)

CL is another liposomal adjuvant that was studied in this thesis. A number of studies have investigated the effects of CL and CL formulated with CpG-ODN on the immune system. CLs were capable of binding strongly to vaccine Ags against influenza and induced robust anti-influenza immune responses both after subcutaneous and

intranasal administration in mice (Guy *et al.* 2001). Both routes activated innate immune responses such as induction of proinflammatory cytokines and immune cell activation (Guy *et al.* 2001). D'Souza *et al.* (2002) also showed that CL increased the level of IgG by three to ten-fold in immunized mice with tuberculosis vaccines that included CL. CL also increased production of splenic TC-derived Th 1-type cytokines such as IL-2 and IFN-γ (D'Souza *et al.* 2002).

Encapsulation of CpG-ODN in a CL increased Ag-specific IFN-γ production, and IFN-γ-dependent IgG2a production by 10-40 fold in mice (Gursel *et al.* 2001). These increases are consistent with the preferential induction of a Th 1-based immune response compared to CpG-ODN alone (Gursel *et al.* 2001). Recently, it has been shown that this formulation enhanced DNA binding and up taking in murine DCs, and also induced proinflammatory cytokine secretion (Yoshinaga *et al.* 2007). Cytokines included TNF-α and IL-12 secreted from granulocyte–macrophage colony–stimulating factor (GM-CSF)-cultured bone marrow-derived DCs (Yoshinaga *et al.* 2007). Mice injected intrarectally or intranasally with CpG-ODN formulated with CL generated higher levels of IgG and IgA antibodies than did mice injected with CpG-ODN intramuscularly (Joseph *et al.* 2006). Increased antibody levels occurred both in mucosal (Peyer's patches, lamina propria, colon) and peripheral (spleen, serum) sites of the intrarectally and intranasally immunized mice (Joseph *et al.* 2006).

Intravenous administration of CL is likely to cause more severe side effects than intrapulmonary administration, and can be lethal at higher doses of the complex (Yew and Scheule 2005).

1.6.2. Polyphosphazenes

Polyphosphazenes are potent immunostimulant adjuvants with a high molecular weight. They contain a long-chain backbone of phosphorus and nitrogen atoms with organic side groups attached to each phosphorus (Payne and Andrianov 1998). Combining this phosphorus-nitrogen backbone with appropriate side groups leads to almost unlimited possibilities for preparing materials (Allcock 1990). The ability of hydrophobic polymer matrices to degrade slowly in aqueous solutions is one of the most critical properties in determining the potential of these materials as controlled release systems (Langer 1990).

One of the most important potential applications of polyphosphazenes relates to their activity as immunoadjuvant compounds that enhance the immune response (Payne *et al.* 1998; Andrianov *et al.* 2004). Lu *et al.* (1996) showed that polyphosphazenes are effective adjuvants in a vaccine against human immunodeficiency virus type 1 (HIV-1) in intramuscularly and intranasally immunized rhesus monkeys, rabbits, and mice. Therefore, polyphosphazenes have the potential to become important adjuvants to vaccine candidates in human clinical trials.

The effects of CpG-ODN formulated with polyphosphazene against influenza infection in mice have been studied recently (Mutwiri *et al.* 2007). Results indicated that higher levels of Th 1, Th 2 type cytokines, and IgG1, IgG2 were associated with production of both IFN-γ and IL-4 in mice immunized with CpG-ODN plus polyphosphazenes compared to mice immunized with CpG-ODN alone (Mutwiri *et al.* 2007).

The biological performance of polyphosphazenes depends on their molecular

weight and composition. It is therefore of critical importance to consider the properties of these polymers when using them as adjuvants (Payne *et al.* 1998). The polyphosphazene adjuvants used in this study are Poly [di (carboxylatophenoxy phosphazene)] (PCPP) and Poly [di (sodium carboxylatoethylphenoxy) phosphazene] (PCEP).

1.6.2.1. Poly [di (carboxylatophenoxy phosphazene)] (PCPP)

PCPP is a water-soluble phosphazene polyelectrolyte. PCPP is of considerable interest because of their unique physicochemical and biological properties and their important applications as materials for microencapsulation and immunoadjuvats (Payne *et al.* 1995). PCPP has three desirable properties. First, its size and physical characteristics facilitate uptake into mucosal lymphoid tissue where immune responses are stimulated. Second, PCPP is synthesized under very mild conditions so that antigenic integrity is maintained. Third, the hydrogel properties of PCPP allow a sustained Ag release over a long period (Allcock 1990; Andrianov 1998). Water-solubility of PCPP salts, combined with a main chain that is easily degraded by hydrolytic reactions, enable PCPP to move from the injection site, thereby making PCPP attractive candidate for vaccine applications. PCPP combined with Ags is a potent immunoadjuvant for many vaccines such as influenza (Payne *et al.* 1998) and cholera vaccines (Wu *et al.* 2001). Mice immunized with vaccines containing PCPP, had IgM, IgG1, IgG2a, IgG2b, and IgG 3 ELISA antibody titers to influenza and cholera that were two to ten times higher than in mice immunized with the regular vaccine (Payne *et al.* 1998; Wu *et al.* 2001). In mice infected with influenza, immune responses induced by PCPP were predominated by high levels of IL-4 and IgG 1 (Mutwiri *et al.* 2007).

Mutviri *et al.* (2007) also showed that adjuvant activities of PCPP were greater than adjuvant activities of alum and were not influenced by the dose of Ag used.

1.6.2.2. Poly [di (sodium carboxylatoethylphenoxy) phosphazene] (PCEP)

PCEP is a new polyphosphazene polyelectrolyte. PCEP significantly increases both IgG1 and IgG2a levels, which involve Th 1 and Th 2 humoral immune responses associated with production of both IFN-γ and IL-4 (Mutwiri *et al.* 2007). Andrianov *et al.* (2006) showed that PCEP could be used as a vaccine adjuvant as, like PCPP, it is capable of producing micro spheres as well. Microspheres are water-soluble and can extend PCEP utility in areas of mucosal immunization and controlled vaccine release (Andrianov *et al.* 2006). In addition, studies investigating the adjuvant activity of PCEP provide evidence that this polymer generates high immune responses. For example, in a study using influenza and hepatitis B surface Ags, mice injected with these Ags plus PCEP had IgG levels that were ten times higher than mice injected with PCEP (Andrianov *et al.* 2006). It has been shown that the adjuvant activities of PCEP are greater than those of PCPP (Mutwiri *et al.* 2007).

PCEP's degradability has been studied recently (Andrianov *et al.* 2006). This polymer degradation involves separation of side groups and formation of an unstable phosphazene resulting in the breakdown of the backbone (Andrianov *et al.* 2006).

1.6.3. Emulsigens®

Emulsigen® is oil-in-water emulsion that contains no components of animal origin. Emulsigen® creates a depot at the injection site from which the

Ag is released slowly, leading to prolonged stimulation of the immune system. At the same time, Emulsigen® also causes undesirable reactions at injection sites. Damage to tissues caused by Emulsigen® probably contributes to its adjuvant effects, as reducing the amount of Emulsigen® reduces both tissue damage and adjuvant activity (Vogel 1995).

Emulsigen® is widely applied in pharmaceuticals such as ointments and injections (Wedlock *et al.* 2005). The high solubilizing capacity of organic compounds in Emulsigen® combined with stability has made it an attractive vehicle for parental administrations (Park and Kim 1999). Emulsigen® is a licensed adjuvant that is extensively used in commercial veterinary vaccines. For example, piglets injected with a *streptococci sp.* vaccine that included Emulsigen® had higher levels of antigen-specific IFN-γ and lower levels of IL-4 in PBMCs than piglets injected with the regular vaccine (Linghua *et al.* 2006). These results suggest that TCs are activated and Th 1-based immune responses are induced (Linghua *et al.* 2006). Moreover, chickens showed increased antibody titers against ND when injected with an ND vaccine containing Emulsigen® compared with the regular ND vaccine (Hilgers *et al.* 1998).

The efficiency of combining CpG-ODN and Emulsigen® to combat a variety of diseases in different species has been studied recently. A formulation combining CpG-ODN with Emulsigen® enhanced antibody responses to an equine influenza virus vaccine (Lopez *et al.* 2006). This formulation also induced higher levels of IgG 1, IgG 2, and IFN-γ in response to *streptococci sp.* vaccine in swine than the regular vaccine (Linghua *et al.* 2006). Strong IFN-γ responses were measured in cattle vaccinated with Emulsigen® and CpG-ODN (Ioannou *et al.* 2002; Wedlock *et al.* 2005) *Mycobacterium*

bovis and herpes virus glycoprotein vaccines that had been formulated.

1.7. Summary and objectives

Infections with *Salmonella* and *E. coli* in humans and domestic animals are a worldwide problem. *Salmonella* Typhimurium and *E. coli* cause high mortality in neonatal chickens. The wide variety of sources of infection can contribute to the fact that these diseases are difficult to control with biosecurity practices, antibiotics, probiotics, and vaccination programs. Alternatives for antibiotics are needed, making novel prophylactic and therapeutic agents a priority for research in poultry. To fulfill this demand, the poultry industry is seeking new disease prevention and therapeutic agents to diminish the effects of these bacterial diseases.

Unmethylated CpG-ODN have been known to express TLR 9 and stimulate innate immunity. It activates DCs, APCs, BCs proliferation, and macrophages and increases the production of IgM, IL-6, IL-12, and TNF. CpG-ODN have no apparent direct stimulatory effect on TCs, but they enhance the ability of APCs to activate TCs. Owing to the strength and nature of these effects, CpG-ODN have been tested for immune therapeutic and protective use. Immunostimulatory effects of CpG-ODN have been seen in a variety of viral, bacterial, and protozoan infections, among a range of vertebral species, including poultry. In 2003, for the first time Gomis *et al.* (3003) demonstrated that CpG-ODN had both local and systemic protective effects in broiler chicken against *E. coli*, a bacterium with an extracellular phase of survival, despite the fact that poultry does not express TLR 9. However, the effect of CpG-ODN against an interacellular bacterium remained to investigate. The first objective of this study was to

develop an animal model of *Salmonella* Typhimurium, an intracellular bacteria, in neonatal broiler chickens with a mammalian or avian isolates of *Salmonella* Typhimurium. This would facilitate the investigation of the immunostimulatory effect of CpG against this pathogen. The second objective of this study was to investigate the effect of CpG-ODN treatment followed by dose titration of CpG-ODN and to study the duration of protection of CpG-ODN against *Salmonella* Typhimurium septicemia in neonatal broiler chickens. Thirdly, *in ovo* delivery of CpG-ODN in embryonated eggs against *Salmonella* Typhimurium in neonatal chicken was studied.

Previously, it has been demonstrated that unformulated CpG-ODN delivered *in ovo* were potent immunostimulants against bacterial infections in neonatal chickens. The last objective of this study was to investigate if the immunoprotective effect of CpG-ODN in neonatal chickens could be enhanced by formulating CpG-ODN with polyphosphazenes, liposomes or Emulsigen®. CpG-ODN are rapidly eliminated from the circulation due to absorption onto serum proteins and degradation by serum nucleases. Formulating CpG-ODN with these adjuvants might protect CpG-ODN from serum elimination and increase their duration of protection against pathogens. This was done using an *E. coli* challenge model.

2.0. DEVELOPMENT OF A CHALLENGE MODEL OF *SALMONELLA* TYPHIMURIUM IN NEONATAL BROILER CHICKENS

2.1. Abstract

Salmonella Typhimurium infection is a significant problem in the modern broiler industry due to high mortality in neonatal broiler chickens. The objective of this study was to develop a model of systemic *Salmonella* Typhimurium infection in neonatal broiler chickens with mammalian or avian isolates of *Salmonella* Typhimurium. Isolates of *Salmonella* Typhimurium from a mature chicken, a house sparrow (*Passer domesticus*), and a human were inoculated into groups of three-day-old broiler chickens. Groups of chickens received approximately $1x10^6$, $1x10^7$, or $1x10^9$ cfu in the stationary or the logarithmic growth phase of *Salmonella* Typhimurium either by subcutaneous or oral routs (n = 20). Chickens were observed for ten days post-infection, during which clinical signs, mortality, and pathological lesions were observed and bacteria isolated. The mortality rate was 70% in neonatal broilers inoculated with either $1x10^6$ or $1x10^7$ cfu of stationary phase of the human or chicken *Salmonella* Typhimurium isolate. In contrast, the mortality rate was 25% in broilers inoculated with either $1x10^6$ or $1x10^7$ cfu of stationary phase of the house sparrow *Salmonella* Typhimurium isolate. Oral administration of all three strains of *Salmonella* Typhimurium caused 5-30% mortality. Mortality, clinical disease, and pathological

lesions were caused by live *Salmonella* Typhimurium, not LPS. In broiler chickens, the human or mature chicken isolates of *Salmonella* Typhimurium reproduced the clinical disease of salmonellosis with high repeatability, while the house sparrow isolate of *Salmonella* Typhimurium rarely reproduced clinical salmonellosis.

2.2. Introduction

Salmonella Typhimurium is a gram negative, facultative intracellular pathogen. It can cause large economic losses in the poultry industry, primarily due to high mortality in neonatal chickens (Gast and Beard 1989), and epizootics in wild passerines (Refsum *et al.* 2003). *Salmonella* Typhimurium can be vertically transmitted to broiler chickens from infected parents (Gordon and Tucker 1965). Vertical transmission of *Salmonella* Typhimurium may result in increased mortality of embryos and neonatal chicks (Libby 2004). Horizontal transmission of *Salmonella* Typhimurium in poultry is associated with contaminated feed, rodents, insects and other vectors (Zecha 1977) (McAllister *et al.* 1994). Eggshells contaminated with *Salmonella* Typhimurium can spread bacteria widely in the hatchery, thereby contaminating equipment and infecting newly hatched chickens (Gordon and Tucker 1965).

Clinical signs of *Salmonella* Typhimurium infection in neonatal chickens include closed eyes, drooping wings, ruffled feathers (Gast 2003), anorexia, and emaciation (Barrow *et al.* 1987). Watery diarrhea accompanied by dehydration and pasting of the vent is also observed frequently (Gast 2003). Blindness (Padron 1990) and lameness (Barrow *et al.* 1987) occur occasionally. Once a poultry flock has been infected with *Salmonella* Typhimurium, survivors serve as carriers of the bacteria, which colonize the

intestinal tract and are shed in feces for several weeks post infection (Gast and Beard 1989; Beal *et al*. 2004).

In contrast to neonatal chickens, older chickens infected with *Salmonella* Typhimurium can have enteric infections with mild or no clinical disease. At the time of processing, carcasses can therefore serve as a major source of *Salmonella* Typhimurium contamination of meat for human. Unfortunately, *Salmonella* Typhimurium control measures include strict biosecurity practices, use of antibiotics, and vaccination do not completely work and have limited success in older birds (Gast and Beard 1989; Beal *et al*. 2004).

Salmonella Typhimurium causes high mortality in the genus Passeridae, especially in house sparrows (*Passer domesticus*) (Pennycott *et al.* 2006). It appears that house sparrows acquire the infection primarily from their environment or other infected carriers (Refsum *et al.* 2003). There is a risk that house sparrows infected with *Salmonella* Typhimurium could in turn transmit the disease to humans, other domestic animals or poultry (Pennycott *et al.* 2006).

Salmonellosis is an important food borne disease in humans in both developed and developing countries. In industrialized countries, paratyphoid *Salmonella* are an important cause of bacterial gastroenteritis (Haque *et al.* 2004). For example, *Salmonella* is estimated to cause 1.4 million illnesses and 600 deaths annually in the United States (Mead *et al.* 1999; Rice *et al*. 2003). Most human infections originate from contaminated food sources such as meat, milk, and egg. However, non food-borne infections occur due to contact with infected animals, contaminated water, or environmental contamination (Mead *et al.* 1999).

We have demonstrated previously that CpG-ODN stimulate innate immunity in neonatal broiler chickens against *E. coli*, a bacterium with an extracellular phase of survival (Gomis *et al.* 2004). In order to investigate the possible immunostimulatory effect of CpG-ODN against *Salmonella* Typhimurium, a bacterium with an intracellular phase of survival, we developed an animal model in neonatal broiler chicks with mammalian and avian isolates of *Salmonella* Typhimurium.

2.3. Materials and methods

2.3.1. Bacteria

Field isolates of *Salmonella* Typhimurium from a 25-week-old broiler breeder (D05-03438) and a house sparrow (D04-02959) with ingluveitis, cloacitis, hepatitis, and splenitis were obtained from the Prairie Diagnostic Services, University of Saskatchewan, Saskatoon, SK, Canada. A copy of the human isolate of *Salmonella* Typhimurium SL1344 was obtained from the Vaccine and Infectious Disease Organization, University of Saskatchewan, Canada. Aliquots of the bacteria were stored at –70^{o}C in a 50% brain heart infusion broth (BHI) (Difco, Detroit, MI) supplemented with 25% (w/v) glycerol (VWR Scientific Inc., Montreal, QC). Bacteria to be used as the challenge were cultured on a Triple Sugar Iron Agar [(TSIA II with 5% sheep's blood) Becton, Dickinson and Company Sparks, MD] for 18-24 hours at 37^{o}C. Two to three colonies of bacteria were added to 200 ml of Luria Broth (LB) (Miller-BDH Inc.) in a one-L Erlenmeyer flask. To obtain *Salmonella* Typhimurium in the stationary phase, cultures were grown at 37^{o}C for 16-18 hours with shaking at 200 rpm. The stationary phase cultures contained approximately 1x10^{9} cfu/ml of *Salmonella*

Typhimurium after this incubation period. To prepare *Salmonella* Typhimurium in the logarithmic growth phase, a 1:1000 dilution of the stationary phase culture was added to 100 ml of Luria Broth in a 500-ml Erlenmeyer flask and incubated at 37^{o}C for three hours with shaking at 200 rpm. After shaking, the culture contained approximately 1x10^{9} cfu per ml of bacteria. These cultures were further diluted with saline to the concentration of bacteria required for challenge experiments. Serial dilutions were plated in duplicate on TSIA plates and incubated for 18-24 hours at 37^{o}C. Following incubation, bacterial colonies on the plates were counted to validate the challenge dose.

2.3.2. Animal model development

All procedures involving animals were done according to a protocol approved by the University of Saskatchewan, Committee on Animal Care and Supply in accordance with regulations of the Canadian Council for Animal Care. Day-old broiler chickens were obtained from a local hatchery in Saskatchewan, Canada, and identified with individual neck tags (Swiftack Poultry Tags, Heartland Animal Health Inc., MO.). Groups of 20 chickens were randomly allocated to treatments and housed at the Animal Care Unit, Western College of Veterinary Medicine, University of Saskatchewan. Water and commercial broiler rations were provided *ad libitum*. Air was not re-circulated through rooms. The photoperiod was set at 24 hours per day for the first three days and 16 hours per day for the remaining seven days. Room temperature was maintained at 30 -32 $^{\circ}$C for the first week and 28-30 $^{\circ}$C for the second week. Three-day-old chickens were challenged with *Salmonella* Typhimurium isolates subcutaneously in the neck or

orally using 18-gauge gavage needle.

Birds were observed for ten days following challenge. Daily clinical scores for each bird was given using the following categories: 0 = normal; 1 = hesitant to move and easily fatigued; 2 = unable to stand or forage for food and therefore euthanized; 3 = dead. Cumulative clinical scores (CCSs) for each bird (CCS/Bird) and for each group of 20 (CCS/Group) were calculated as follows:

CCS/Bird- Clinical scores were summed for each bird across the ten days of the trial. Birds that had been euthanized were given a score of 2 for the day of euthanasia and 3 for each day remaining in the trial. Dead birds were given a score of 3 for each day remaining in the trial, including the day of death.

CCS/Group- Clinical scores for each bird were summed across groups of 20 birds to give a CCS for each group.

Mortality was recorded daily. Dead or euthanized birds were necropsied immediately. Gross lesions of pericarditis, perihepatitis, airsacculitis and polyserositis were recorded. Bacterial swabs were taken from air sacs and cultured on TSIA plates. Growth on the plates was recorded as follows: 0 = no growth; +1 = growth of bacteria on area 1; +2 = growth of the bacteria on areas 1 and 2; +3 = growth of bacteria on areas 1, 2 and 3; and +4 = growth of bacteria on areas 1, 2, 3 and 4 (Barry 1972).

2.3.3. Experimental design

(a) Virulence of *Salmonella* Typhimurium isolated from avian and mammalian hosts

Three groups of 60 chickens were inoculated with *Salmonella* Typhimurium isolated from chicken, house sparrow or human subjects (Table 2.1). Each treatment group was divided into four sub-groups of 20 chickens; I- two groups were injected subcutaneously (neck) with either $1x10^6$ or $1x10^7$ cfu /ml of stationary phase *Salmonella* Typhimurium, II- the third group was injected subcutaneously (neck) with approximately $1x10^6$ cfu of logarithmically growing *Salmonella* Typhimurium, III- the fourth group was orally administered approximately $1x10^9$ cfu of logarithmically growing *Salmonella* Typhimurium. As described above, clinical signs were observed and bacteria cultured daily.

Table 2.1. Groups of neonatal broiler chickens challenged with different isolates of *Salmonella* Typhimurium

***Salmonella* Typhimurium isolate / group**	**Treatment**	**Phase of *Salmonella* Typhimurium**	**Dose of *Salmonella* Typhimurium cfu / ml**	**Route of administration**	**Number of birds / group**
Chicken	1	Stationary	$2.0x10^6$	Subcutaneous	20
	2	Stationary	$2.0x10^7$	Subcutaneous	20
	3	Logarithmic	$2.0x10^6$	Subcutaneous	20
	4	Logarithmic	$2.0x10^9$	Oral	20
House sparrow	1	Stationary	$1.1x10^6$	Subcutaneous	20
	2	Stationary	$1.1x10^7$	Subcutaneous	20
	3	Logarithmic	$1.1x10^6$	Subcutaneous	20
	4	Logarithmic	$1.1x10^9$	Oral	20
Human	1	Stationary	$1.5x10^6$	Subcutaneous	20
	2	Stationary	$1.5x10^7$	Subcutaneous	20
	3	Logarithmic	$1.5x10^6$	Subcutaneous	20
	4	Logarithmic	$1.5x10^9$	Oral	20

(b) Dose titration of *Salmonella* Typhimurium isolated from a chicken

Three-day-old broiler chicks were randomly allocated into four groups, each containing 20 birds. Birds in each group were inoculated subcutaneously (neck) with $1.2x10^5$, $1.2x10^6$, $1.2x10^7$, or $1.2x10^8$ cfu /ml of stationary phase culture of *Salmonella* Typhimurium isolated from a chicken. Clinical signs were recorded and bacteria cultured as described above

To determine if the clinical salmonellosis and pathological lesions observed in chickens inoculated with the chicken isolate were associated with live *Salmonella* Typhimurium septicemia and not LPS, the following trial was conducted. At three days age, ten birds were challenged subcutaneously (neck) with $1.5x10^8$ cfu of killed *Salmonella* Typhimurium. To kill the bacteria, LB containing $1.5x10^8$ cfu of *Salmonella* Typhimurium was left at room temperature for three days. It was then incubated for one hour at 65°C and kept in the fridge for another two days. The broth was cultured on TSIA plates to confirm the death of the bacteria. Birds were observed for ten days post inoculation.

2.3.4. Statistical analysis

The survival pattern and median survival were compared using log-rank tests and chi-square statistics. Survival data was analyzed using Prism (Prism 4.0, GraphPad Software Inc., San Diego, CA. USA www.graphpad.com). The clinical score for each bird was summed over the ten-day observation period. Median scores were compared among groups using a Kruskal-Wallis test.

2.4. Results

(a) Virulence of *Salmonella* Typhimurium isolated from avian and mammalian hosts

Chicken isolate-

Mortality was similar in birds inoculated subcutaneously with logarithmically growing *Salmonella* Typhimurium and those inoculated by the same route with stationary *Salmonella* Typhimurium (Fig. 2.1). The lowest mortality rate was observed in chickens orally challenged with logarithmically growing *Salmonella* Typhimurium (Fig. 2.1). Groups inoculated subcutaneously with either $2x10^6$ and $2x10^7$ cfu of stationary, or $1x10^6$ cfu of logarithmically growing *Salmonella* Typhimurium had cumulative clinical scores (CCS/Group) of 226, 389 and 317 respectively. The CCS of the group orally inoculated with $1x10^9$ cfu of *Salmonella* Typhimurium was 163 (Fig. 2.4). *Salmonella* Typhimurium isolates from the air sacs of neonatal broiler chickens in the groups inoculated using subcutaneous or oral routes with different doses and different phases of culture are shown in Table 2.2.

Human isolate- The group that was subcutaneously inoculated in the neck with logarithmically growing phase of *Salmonella* Typhimurium had a survival rate that was between the survival rates of the groups that were inoculated subcutaneously with either $1.5x10^6$ or $1.5x10^7$ cfu of stationary phase *Salmonella* Typhimurium. The mortality rate was the lowest for the group that was orally challenged with logarithmically growing *Salmonella* Typhimurium (Fig. 2.2). Birds that were inoculated subcutaneously with either $1.5x10^6$ and $1.5x10^7$ cfu of stationary phase or $1.5x10^6$ cfu of logarithmically growing *Salmonella* Typhimurium had CCS (per group) of 157, 406 and 331 respectively (Fig. 2.4). The CCS of the group that was orally inoculated with $1.5x10^9$

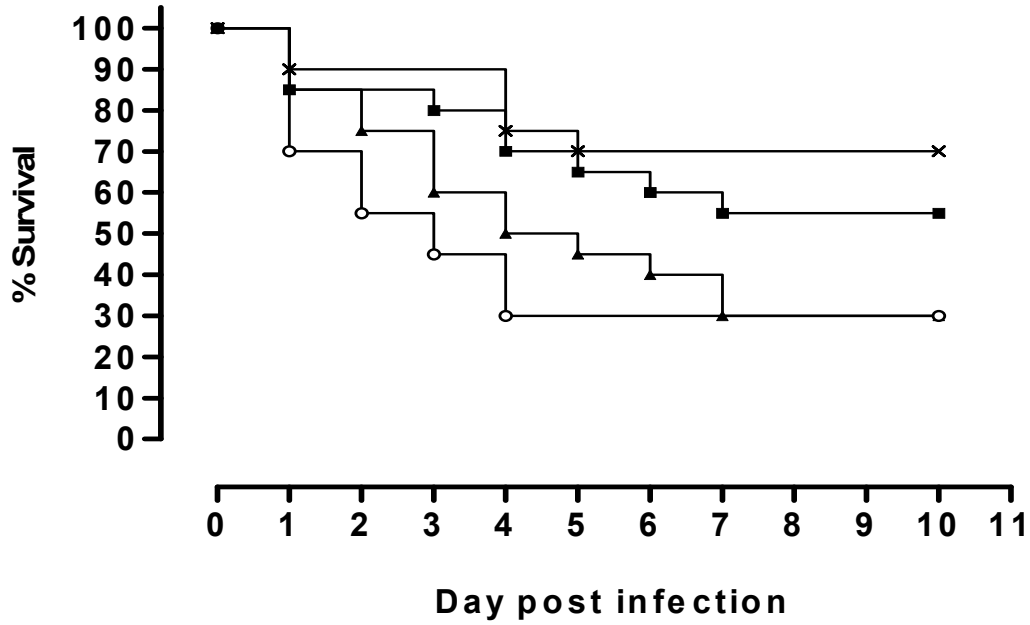


Fig. 2.1. Survival of chickens following challenge with a chicken isolate of *Salmonella* Typhimurium. Birds were inoculated subcutaneously with $2x10^6$ cfu (■) or $2x10^7$ cfu (○) of stationary phase, $1x10^6$ cfu (▲) of log phase, or orally with $1x10^9$ (x) log phase of cultures (n = 20).

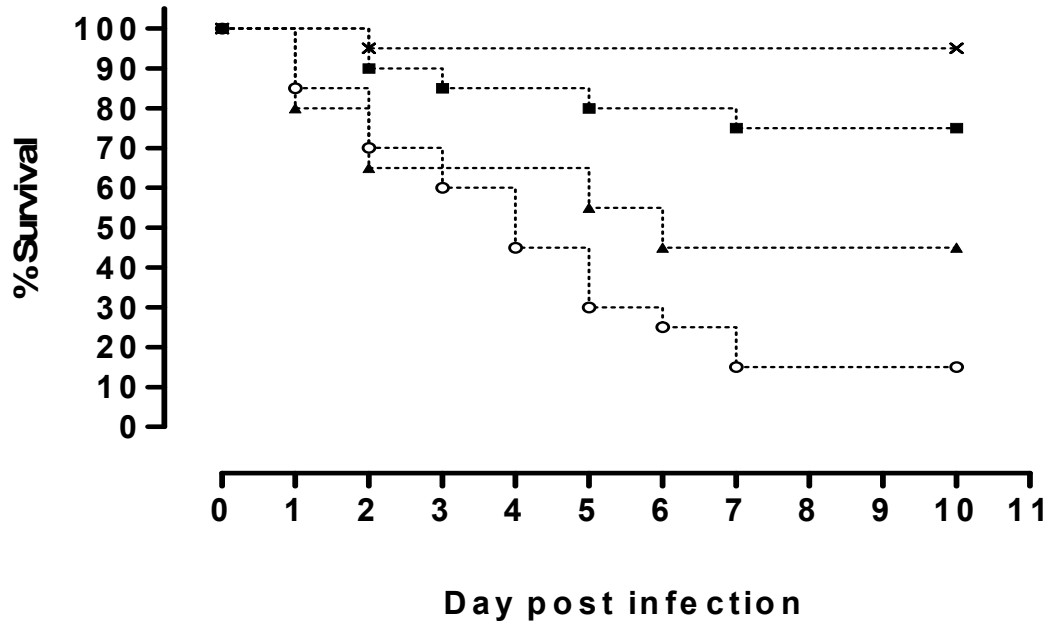


Fig. 2.2. Survival of chickens following challenge with a human isolate of *Salmonella* Typhimurium. Birds were inoculated subcutaneously with $1.5x10^6$ cfu (■) or $1.5x10^7$ cfu (○) of stationary phase, $1.5x10^6$ cfu (▲) of log phase, or orally with $1.5x10^9$ (x) log phase of cultures (n = 20).

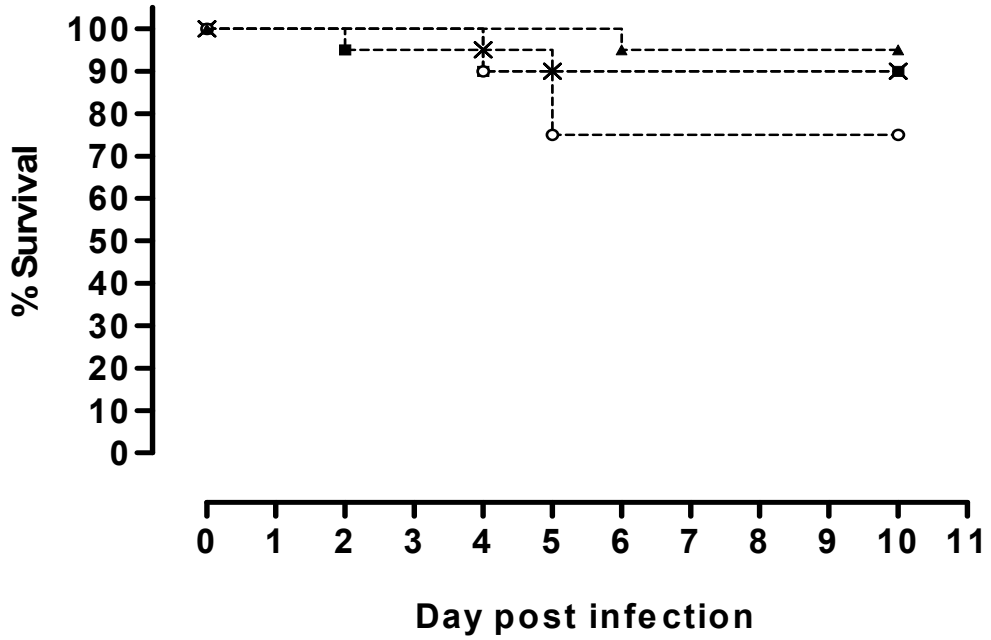


Fig. 2.3. Survival of chickens following challenge with a house sparrow isolate of *Salmonella* Typhimurium. Birds were inoculated subcutaneously with $1.1x10^6$ cfu (■) or $1.1x10^7$ cfu (○) of stationary phase, $1.5x10^6$ cfu (▲) of log phase, or orally with $1.5x10^9$ (x) log phase of cultures (n = 20).

Table 2.2. Level of *Salmonella* Typhimurium isolated from the air sacs of the birds following challenge with chicken, house sparrow, and human isolates.

Salmonella **Typhimurium isolate/ group/**	**Phase of *Salmonella* Typhimurium**	**Dose of *Salmonella* Typhimurium cfu / ml**	**Route of administration**	**Growth (Score [A])**				
				0	**+1**	**+2**	**+3**	**+4**
Chicken	Stationary	$2.0x10^{6}$	Subcutaneous	5 *	8	6	1	0
	Stationary	$2.0x10^{7}$	Subcutaneous	1	5	10	2	2
	Logarithmic	$2.0x10^{6}$	Subcutaneous	3	9	6	0	2
	Logarithmic	$2.0x10^{9}$	Oral	10	7	3	0	0
House sparrow	Stationary	$1.1x10^{6}$	Subcutaneous	10	8	1	1	0
	Stationary	$1.1x10^{7}$	Subcutaneous	2	16	2	0	0
	Logarithmic	$1.1x10^{6}$	Subcutaneous	8	11	1	0	0
	Logarithmic	$1.1x10^{9}$	Oral	14	6	0	0	0
Human	Stationary	$1.5x10^{6}$	Subcutaneous	3	12	5	0	0
	Stationary	$1.5x10^{7}$	Subcutaneous	0	8	11	1	0
	Logarithmic	$1.5x10^{6}$	Subcutaneous	3	8	6	1	2
	Logarithmic	$1.5x10^{9}$	Oral	15	5	0	0	0

Salmonella Typhimurium isolated from air sacs of neonatal broiler chickens. Groups were inoculated by subcutaneous or oral routes with different doses and different phases of culture, as shown in this table.

[A] *Salmonella* Typhimurium growth on the TSIA plates was scored as follows: 0 = no growth; +1 = growth of bacteria on area 1; +2 = growth of the bacteria on areas 1 and 2; +3 = growth of bacteria on areas 1, 2, and 3; +4 = growth of bacteria on areas 1, 2, 3, and 4 (n = 20).

* Number of birds.

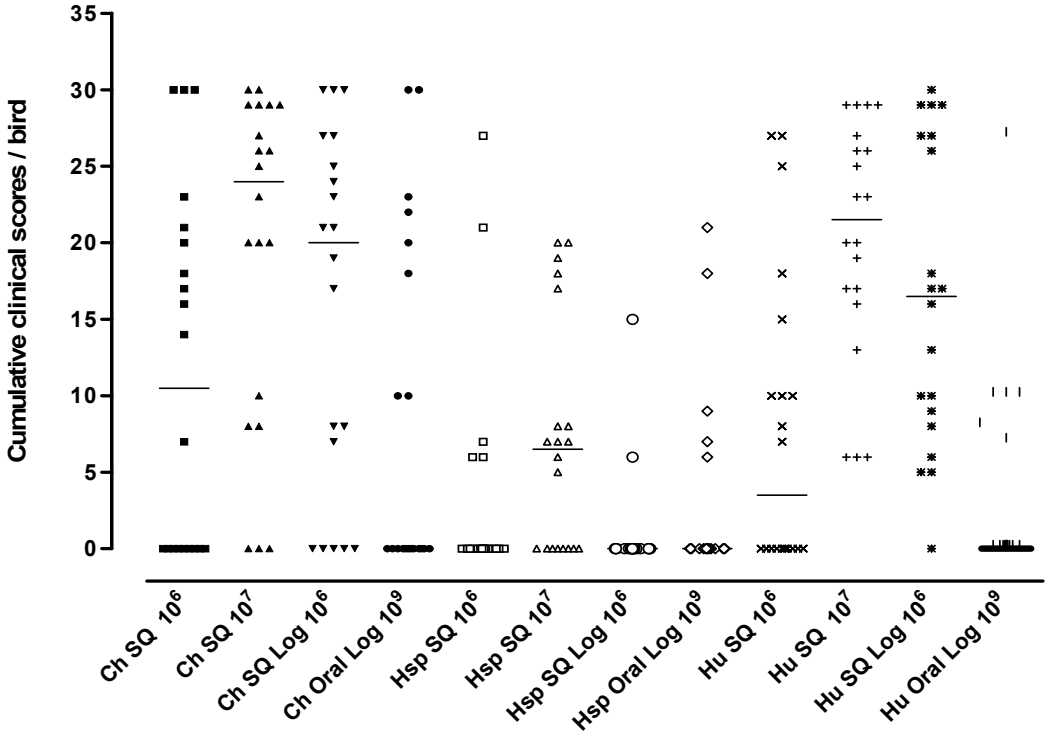


Fig. 2.4. Cumulative clinical scores (CCSs) for individual birds following subcutaneous challenge with different isolates of *Salmonella* Typhimurium. CCSs of birds inoculated with $1x10^6$ (■,□,x), $1x10^7$ (▲,Δ,+) cfu of stationary phase culture, $1x10^6$ (▼,○, *) and $1x10^9$ (●,◊, ׀) cfu of log phase culture of *Salmonella* Typhimurium [Ch = chicken, Hsp = house sparrow, Hu = human, SQ = subcutaneous, Log = logarithmic] (Bar = median) (n = 20).

cfu *Salmonella* Typhimurium was 72 (Fig. 2.2).

House sparrow isolate- Chickens inoculated subcutaneously (neck) with stationary phase *Salmonella* Typhimurium had the highest mortality rate of 25%. Birds subcutaneously inoculated with logarithmically growing *Salmonella* Typhimurium hadthe lowest mortality rate (Fig. 2.3). The CCS of groups inoculated subcutaneously with either $1.1x10^6$ and $1.1x10^7$ cfu of stationary phase or $1.5x10^6$ cfu of logarithmic phase *Salmonella* Typhimurium were 67, 142 and 21, respectively. The CCS of the group orally inoculated with $1.5x10^9$ cfu *Salmonella* Typhimurium was 61 (Fig. 2.4).

(b) Dose titration of *Salmonella* Typhimurium isolated from a chicken

Typically, birds inoculated with $1.2x10^8$ cfu of *Salmonella* Typhimurium had acute septicemia and died or were euthanized 24-72 hours post-challenge. Pathological lesions that were observed in this animal model were pericarditis, airsacculitis, perihepatitis and polyserositis. Birds inoculated with $1.2x10^7$ cfu of *Salmonella* Typhimurium had moderate to severe lesions in the air sacs and other serosal surfaces (mentioned above). In contrast, birds that received either $1.2x10^5$ or $1.2x10^6$ cfu of *Salmonella* Typhimurium had mild to moderate lesions in the air sacs and other serosal surfaces (mentioned above) (Fig. 2.7). *Salmonella* Typhimurium was isolated from 100% of lesions in the air sacs of birds that died of septicemia between days one and ten post-challenge. The CCS of the group inoculated with $1.2x10^8$ cfu of *Salmonella* Typhimurium was high (563), compared to groups inoculated with $1.2x10^5$ (70), $1.2x10^6$ (95), and $1.2x10^7$ (193) cfu of *Salmonella* Typhimurium (Fig. 2.6). Bacterial isolation results are shown in Table 2.3.

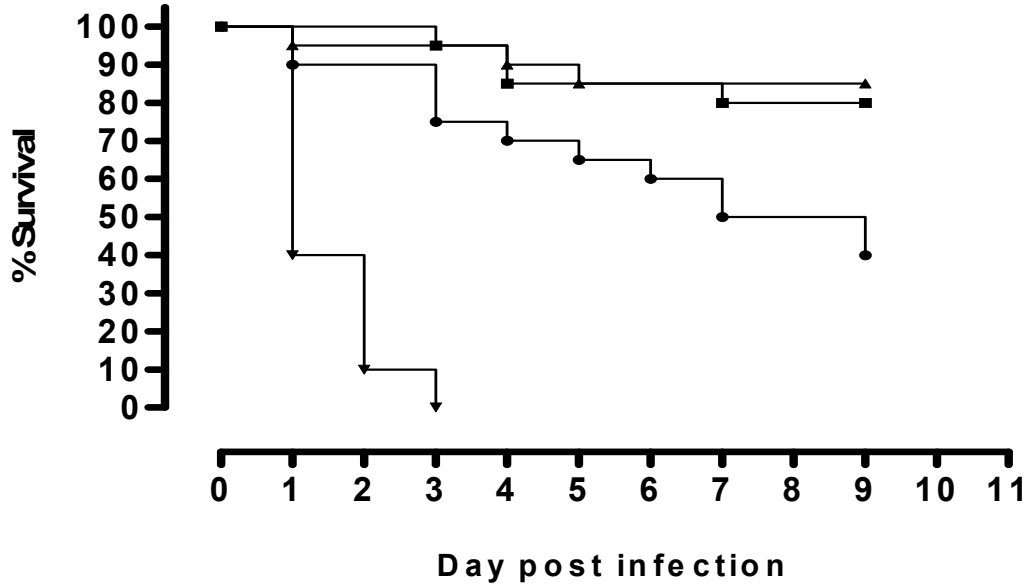


Fig. 2.5. Survival of neonatal chickens following challenge with stationary phase *Salmonella* Typhimurium isolated from a chicken**.** Survival of three-day-old chickens following challenge with $1.2x10^5$ (▲), $1.2x10^6$ (■), $1.2x10^7$ (●) or $1.2x10^8$ (▼) cfu of a chicken isolate of *Salmonella* Typhimurium by subcutaneous injection in the neck (n = 20).

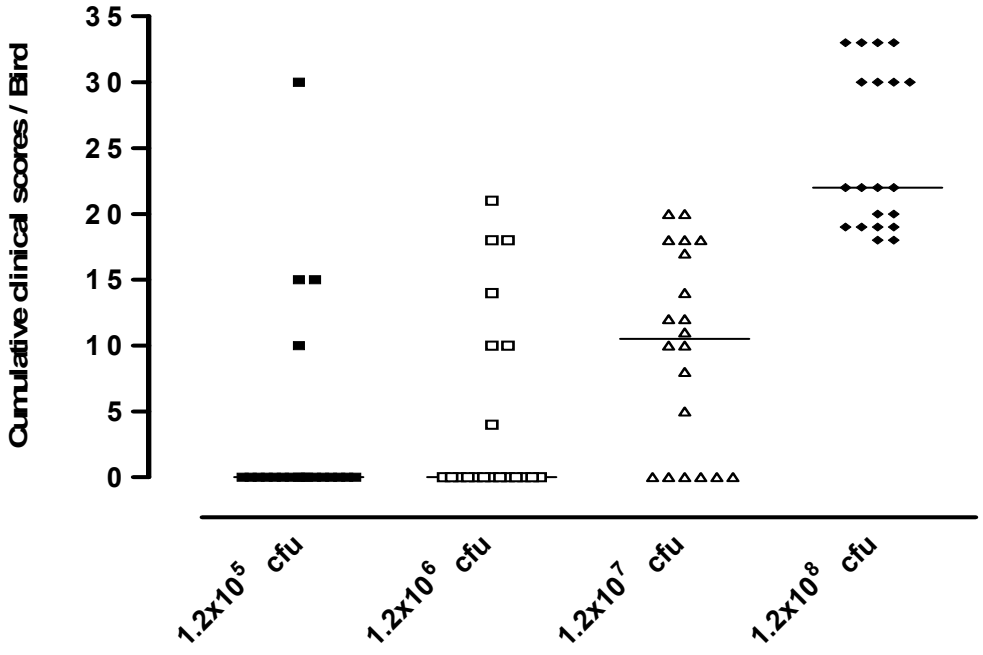


Fig. 2.6. Cumulative clinical scores (CCSs) of individual birds following subcutaneous challenge with a chicken isolate of *Salmonella* Typhimurium. The CCS of individual birds that had been subcutaneously inoculated with different doses of stationary phase *Salmonella* Typhimurium is shown in this graph (Bar = median) (n = 20).

Table 2.3. Level of *Salmonella* Typhimurium isolated from the air sacs of neonatal chickens following challenge with various doses of *Salmonella* Typhimurium isolated from a chicken

***Salmonella* Typhimurium challenge dose (cfu)**	**Number of birds with *Salmonella* Typhimurium growth (score [A])**				
	0	**+1**	**+2**	**+3**	**+4**
$1.2x10^5$	13	4	2	1	0
$1.2x10^6$	11	4	3	2	0
$1.2x10^7$	5	6	5	3	1
$1.2x10^8$	0	0	5	10	5

Birds were subcutaneously inoculated with stationary phase *Salmonella* Typhimurium at day three of age. Isolation of *Salmonella* Typhimurium from the air sacs of the neonatal broiler chickens in the groups inoculated with different doses of cultures are shown in this table.

[A] *Salmonella* Typhimurium growth on the TSIA plates were scored as follows: 0 = no growth; +1 = growth of bacteria on area 1; +2 = growth of the bacteria on area 1 and 2; +3 growth of bacteria on area 1, 2, and 3; +4 = growth of bacteria on area 1, 2, 3, and 4 (n = 20).

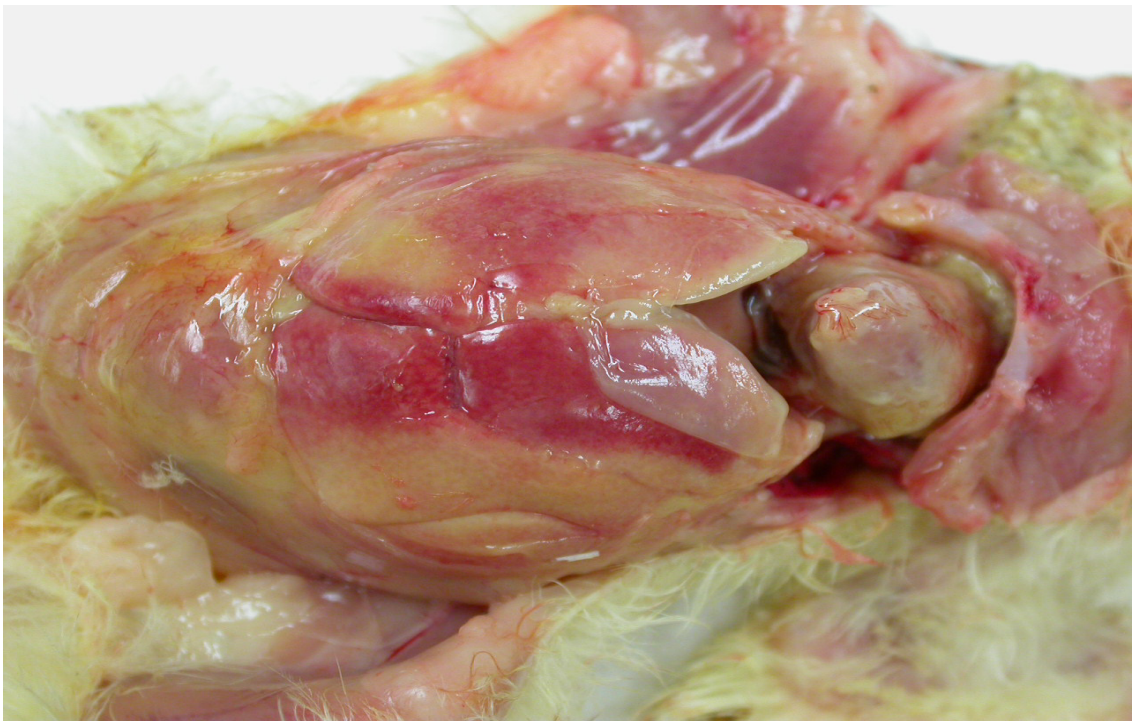
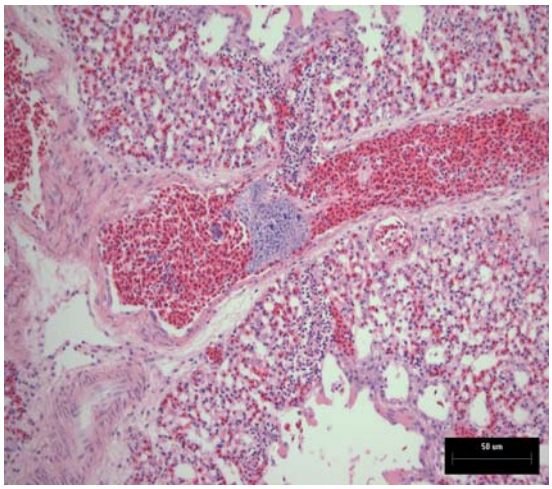
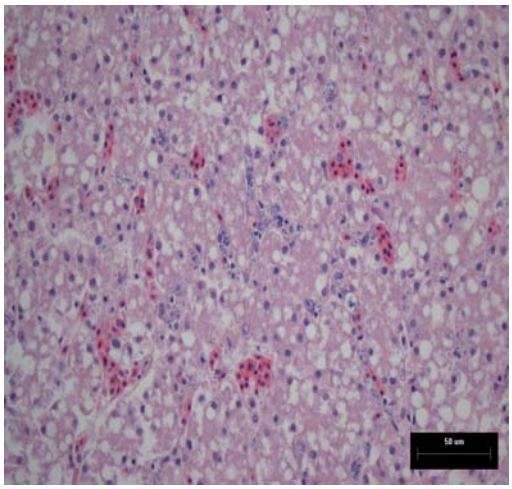


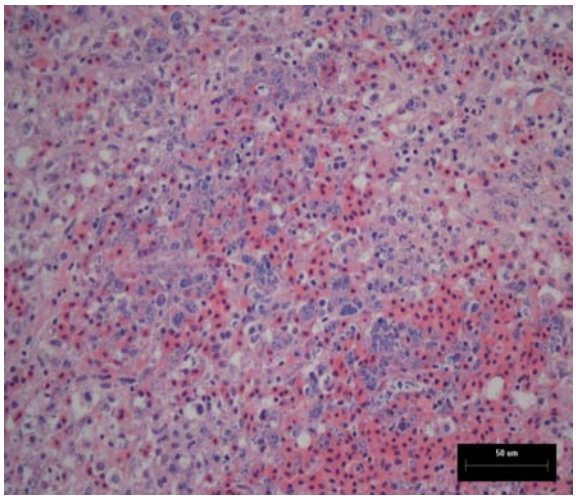
Fig. 2.7. Pericarditis, perihepatitis, and airsacculitis (polyserositis) of a neonatal broiler chicken following $1x10^6$ cfu of *Salmonella* Typhimurium challenge. *Salmonella* Typhimurium isolate originated from a broiler chicken with a field case of salmonellosis.



(a) Lung



(b) Liver



(c) Spleen

Fig. 2.8. Histopathological lesions of the lung, liver and spleen in a bird inoculated with *Salmonella* Typhimurium. Colonies of bacteria in the blood vessels of the lung (a), sinusoids of the liver (b), and red and white pulp of the spleen (c) in neonatal broiler chickens following subcutaneous inoculation with $1.2x10^6$ cfu of stationary phase *Salmonella* Typhimurium.

The survival of the birds that had been subcutaneously inoculated with killed *Salmonella* Typhimurium was 100%. No bacteria were isolated from TSIA plates.

2.5. Discussion

Salmonella Typhimurium is a common serotype associated with high mortality in neonatal broiler chickens (Gast and Beard 1989). In contrast, *Salmonella* infection in adult poultry leads to colonization of the gastrointestinal tract and shedding with no apparent clinical disease or mortality but increased risk of carcass contamination (Henzler *et al.* 1994; Libby 2004). Controlling *Salmonella* in the poultry industry is a priority due to its association with food borne infections in humans. Moreover, control of *Salmonella* with antibiotics has been linked to the emergence of antibiotic resistant strains of bacteria (Ngwai *et al.* 2006). Although *Salmonella* control programs such as biosecurity, probiotics and vaccination have been implemented in broiler breeder, broiler grow-out and processing, success of control programs has been limited (Gast 2003).

The objective of this study was to develop an animal model of *Salmonella* Typhimurium in neonatal broiler chickens with mammalian or avian isolates. *Salmonella* Typhimurium isolates from a chicken, a house sparrow and a human were used in this study. We were able to reproduce *Salmonella* Typhimurium septicemia in neonatal broiler chickens with both chicken and human isolates. The gross and microscopic lesions, mortality and clinical signs observed in this study are typical findings in field cases of *Salmonella* Typhimurium. In contrast, the house sparrow isolate of *Salmonella* Typhimurium did not reproduce clinical disease, regardless of

dose, route of administration or phase of growth. It is possible that the house sparrow isolate of *Salmonella* Typhimurium lacks virulent factors necessary to elicit clinical disease in neonatal broiler chickens (Skyberg *et al.* 2006). Polymerase chain reaction (PCR) analysis of 15 genes identified as putative virulence factors revealed that all genes tested were present in human and broiler isolates of *Salmonella* Typhimurium. In contrast, the house sparrow isolate lacked the prgH gene found in the SPI-1 and sifA genes, which is a secreted effecter protein (personal communication-Allan, B). If this isolate is typical of other isolates from wild birds, then there is little risk that wild birds would transmit salmonellosis to poultry. However, it is impossible to speculate on the characteristics of *Salmonella* caused by wild birds based on a single isolate. Clinical salmonellosis and pathological lesions in this animal model were associated with live *Salmonella* Typhimurium septicemia and not with LPS of *Salmonella* Typhimurium as neonatal chickens were inoculated with 100 times more killed *Salmonella* Typhimurium than the normal challenge dose. No clinical signs or pathological lesions were observed.

All birds inoculated with $1x10^8$ cfu of *Salmonella* Typhimurium isolated from a chicken died within three days due to septicemia. The mortality rate in this group was considered too high to be useful for further studies. In contrast, the survival rate of the $1x10^7$ cfu group was 40%, which could be useful in developing an animal model for future studies. As the survival rates of groups given $1x10^5$ and $1x10^6$ cfu were similar (80 and 85%), $1x10^6$ and $1x10^7$ cfu with one log difference were chosen for the animal model.

Both stationary and logarithmic phases of *Salmonella* Typhimurium produced disease in neonatal chicken. However, survival rates of chickens injected with

logarithmic phase *Salmonella* Typhimurium ($1x0^6$ cfu) of different isolates were either similar or intermediate to the two doses of stationary phase *Salmonella* Typhimurium ($1x10^6$ and $1x10^7$ cfu).

This animal model mimicked the clinical signs and pathological lesions seen in commercial poultry attributed to *Salmonella* Typhimurium infection. This model provides a valuable tool for studying pathogenesis and control of salmonellosis in young poultry. Although the mortality rate using this animal model is higher than that for field cases of salmonellosis, the gross and histopathological lesions are representative of the natural disease of salmonellosis in poultry. This animal model will facilitate studies on the interactions between *Salmonella* Typhimurium and the innate immune system of neonatal broiler chickens.

3.0. DELIVERY OF CPG-ODN TO EMBRYONATED EGGS TO PROTECT NEONATAL CHICKENS AGAINST *SALMONELLA* TYPHIMURIUM INFECTION

3.1. Abstract

Oligodeoxynucleotides (ODN) containing CpG motifs (CpG-ODN) have been shown to stimulate the innate immune system against viral, bacterial and protozoan infections in many vertebrate species. The objective of this study was to investigate the immunostimulatory effect of CpG-ODN against *Salmonella* Typhimurium septicemia in neonatal broilers. Day-old broiler chicks, or embryonated eggs that had been incubated for 18 days, received 50 μg of CpG-ODN, 50 μg of non-CpG-ODN, or saline. Four days after exposure to CpG-ODN or day two-post hatch, $1x10^6$ or $1x10^7$ cfu of a virulent *Salmonella* Typhimurium isolate was inoculated subcutaneously into the neck. Clinical signs, pathology, bacterial isolations from air sacs, and mortality were observed for ten days following challenge with *Salmonella* Typhimurium. The survival rate of birds in groups receiving either non-CpG-ODN or saline following *Salmonella* Typhimurium infection was 40-45%. In contrast, birds receiving CpG-ODN had a significantly higher survival rate of 80-85% ($P < 0.0001$). Bacterial loads and pathology were lower in groups treated with CpG-ODN than groups treated with saline or non-CpG-ODN.

Colony forming units of *Salmonella* Typhimurium in the peripheral blood were significantly lower in birds treated with CpG-ODN than in the group treated with saline. This is the first time that immunoprotective effect of CpG-ODN against an intracellular bacterial infection has been demonstrated in neonatal broiler chickens following *in ovo* delivery.

3.2. Introduction

The innate immune system of vertebrates recognizes structurally conserved PAMPs and allows immediate host immune responses to limit infection (Medzhitov and Janeway 2002). Bacterial and viral DNA are among PAMPs recently found to stimulate the innate immune system and afford immune protection against microbial infections in vertebrate species (Ashkar and Rosenthal 2002; Krieg 2002; Mutwiri *et al.* 2003). In contrast to vDNA, bDNA contains relatively abundant unmethylated CpG dinucleotides (Bird 1993). These unmethylated CpG dinucleotides within specific flanking bases (overall referred to as CpG motif) are now known to be the molecular basis that contributes to the immunostimulatory activity of bDNA (Ahmad-Nejad *et al.* 2002). Synthetic oligonucleotides containing the CpG motif (CpG-ODN) mimicking bDNA have been demonstrated to retain immunostimulatory activities (Yamamoto *et al.* 1992). TLR 9, a member of an innate immune receptor family, has been identified and is known to recognize bDNA or CpG-ODN and initiates the signaling cascade leading to various innate immune responses in murine and human immune cells (Hemmi *et al.* 2000; Bauer *et al*. 2001). Recently, studies have also shown the immune stimulatory activities of CpG-ODN in other vertebrate species including fish (Jorgensen *et al.* 2001; Meng *et al.* 2003; Carrington and Secombes 2006), chickens (Vleugels *et al.* 2002;

Gomis *et al.* 2003; He *et al.* 2003; He and Kogut 2003; Gomis *et al.* 2004; He *et al.* 2005a; He *et al.* 2005b), pigs (Kamstrup *et al.* 2001), cattle (Zhang *et al.* 2001), and sheep (Mena *et al.* 2003).

CpG-ODN stimulate the immune system to mount a rapid innate immune response and promotes the development of Th 1- type protective immunity. Due to the strength and nature of this stimulation, CpG-ODN have been tested for immune therapeutic and protective use (Klinman *et al.* 1999a; Krieg 2002). For example, use of CpG-ODN in a murine model has been shown to have immunoprotective and immunotherapeutic effects against bacterial infections caused by *Listeria monocytogenes* (Krieg *et al.* 1998; Klinman *et al.* 1999b), *Francisella tularensis* (Klinman *et al.* 1999b), *Klebsiella pneumoniae* (Deng *et al.* 2004), and *Burkholderia pseudomallei* (Wongratanacheewin *et al.* 2004). In addition, immunoprotective effect of CpG-ODN against parasitic protozoan infections caused by *Leishmania* major (Zimmermann *et al.* 1998), *Plasmodium yoelii* and *Plasmodium falciparum* (Gramzinski *et al.* 2001), and *Leishmania donovani* (Datta *et al.* 2003) has been investigated in mice as well. Several studies have also demonstrated that CpG-ODN have antiviral activities (Harandi *et al.* 2003; Schlaepfer *et al.* 2004).

Salmonella Typhimurium is a facultative intracellular pathogen capable of causing disease in a wide range of host species (Gast 2003). It is the primary cause of high mortality in neonatal chickens (Gast 2003). In older chickens, however, this serotype causes enteric infection, inflammatory diarrhea with a mild mortality and no clinical disease (Gast 2003). Primary infection of chickens one to three days of age with *Salmonella* Typhimurium has pronounced effects on the persistence of enteric infection

(Beal *et al.* 2004). Control of *Salmonella* Typhimurium in young infected birds is complicated because it leads to colonization of the intestinal tract and shedding of *Salmonella* Typhimurium in feces for several weeks without clinical disease. As a result, *Salmonella* is present in the intestine when chicken are processed, thereby increasing the risk that carcasses be contaminated, potentially transmitting *Salmonella* Typhimurium to humans. Control measures, including strict biosecurity, the use of antibiotics, probiotics, and vaccination programs increase production expenses and do not successfully eliminate *Salmonella* from contaminated houses (Zecha *et al.* 1977).

It has been shown that chemokines and cytokines play a key role in the protective response to *Salmonella*. Clearing primary infections of *Salmonella* Typhimurium in chickens appears to involve IFN-γ mediated TC (primarily Th 1) responses (Withanage *et al.* 2005). However, the mucosal immune system of chickens does not fully mature until some weeks after hatching (Libby 2004). Indeed, the enteric immune system is poorly developed in newly hatched chickens, which experience severe systemic disease if challenged with *Salmonella* Typhimurium under three days of age (Barrow *et al.* 1987). Therefore, stimulating the innate immune system of neonatal chickens against bacterial pathogens would be beneficial to the poultry industry.

Recently, it has been demonstrated that CpG-ODN have an immunoprotective effect against *E. coli,* which causes an extracellular bacterial infection in neonatal broiler chickens (Gomis *et al.* 2004). However, little is known about the effect of CpG-ODN against intracellular bacterial infections in chickens (He *et al.* 2005). Specifically, the use of CpG-ODN in embryos to fight intracellular bacterial infections has not been

studied. The objective of this study is to investigate the imunostimulatory effect of CpG-ODN against *Salmonella* Typhimurium infections in neonatal chickens.

3.3. Materials and methods

3.3.1. *Salmonella* Typhimurium culture and animal model development.

A field isolate of *Salmonella* Typhimurium taken from a 25-week old broiler chicken (Prairie Diagnostic Services number (05-03438) was used as the challenge strain. Aliquots of bacteria were stored at –70^{o}C in 50% BHI (Difco, Detroit, MI) supplemented with 25% (wt/vol) glycerol (VWR Scientific Inc., Montreal, Quebec, Canada). Bacteria for use as the challenge were cultured on a Triple Sugar Iron Agar plate (TSIA) with 5% sheep's blood (Becton Dickinson and Company Sparks, MD) for 18-24 hours at 37^{o}C. Two to three colonies of bacteria from the agar plate were added to 200 ml of Luria Broth (LB) (Miller, BDH Inc.) in a one-L Erlenmeyer flask. The culture was grown at 37^{o}C for 16-18 hours with shaking at 200 rpm. At this time, the culture contained approximately 1 x 10^{9} cfu/ml of stationary phase *Salmonella* Typhimurium. The cultures were further diluted in saline to the concentration of bacteria required for challenge experiments. Birds were challenged subcutaneously in the neck with either $1x10^{6}$ or $1x10^{7}$ cfu of *Salmonella* Typhimurium in 250 μl of saline. Serial dilutions of the culture were plated on TSIA plates in duplicate and incubated for 18-24 hours at 37^{o}C. The number of colonies was then counted to validate the challenge dose.

All procedures involving animals were done according to the protocol approved by the University of Saskatchewan Committee on Animal Care. Day-old broiler chickens or eggs were obtained from a local hatchery in Saskatchewan, Canada, and

identified individually with neck tags (Swiftack Poultry Tags, Heartland Animal Health Inc., MO). Groups containing 20 day-old chickens were randomly assigned to individual animal isolation chambers at the Animal Care Unit, Western College of Veterinary Medicine, University of Saskatchewan. Water and commercial broiler ration were provided *ad libitum*. Air from each room was removed by a HEPA filter and replaced with non-recirculated air at the rate of 18 changes/hour. Air pressure differentials were maintained and strict sanitary practices followed for the duration of the experiment. A photoperiod of 24 hours per day for the first three days and 16 hours per day for the remaining seven days was established. Room temperature was maintained at 30-32 $^{\circ}$C in the first week and 28-30 $^{\circ}$C in the second week.

Clinical signs in birds were observed twice daily for ten days following challenge with *Salmonella* Typhimurium. Daily clinical scores for each bird were assigned as follows: 0 = Normal; 1= hesitant to move and rapidly fatigued; 2 = unable to stand or forage for food and therefore euthanized; 3 = found dead. Cumulative clinical scores (CCSs) for each bird (CCS/Bird) and for each group of 20 (CCS/Group) were calculated as follows:

CCS/Bird- Clinical scores were summed for each bird across the ten days of the trial. Birds that had been euthanized were given a score of 2 for the day of euthanasia and 3 for each day remaining in the trial. Dead birds were given a score of 3 for each day remaining in the trial, including the day of death.

CCS/Group- Clinical scores for each bird were summed across groups of 20 birds to give a CCS for each group.

Mortality was recorded every day. Dead or euthanized birds were necropsied immediately. Gross lesions such as pericarditis, perihepatitis, airsacculitis and polyserositis were recorded. Bacterial swabs were taken from the air sacs and cultured on TSIA plates. Growth on the plates was recorded as follows: 0 = no growth; +1 = growth of bacteria on area 1; +2 = growth of the bacteria on areas 1 and 2; +3 = growth of bacteria on areas 1, 2 and 3; and +4 = growth of bacteria on areas 1, 2, 3 and 4 (Barry 1972).

3.3.2. Synthetic CpG-ODN

The sequence of CpG-ODN used was TCGTCGTTGTCGTTTTGTCGTT and the sequence of non-CpG-ODN was TGCTGCTTGTGCTTTTGTGCTT. Both ODNs were free of endotoxin and produced with a phosphorothioate backbone (provided by Qiagen-GmbH, Hilden, Germany). Synthetic CpG-ODN was diluted in sterile, pyrogen free saline and administered in a 100 µl volume either *in ovo* into the amniotic cavity through the air cell of the egg using a 20-gauge one-inch needle or intramuscularly into the leg of hatched chicks. The volume injected and the needle length were selected to simulate *in ovo* technology used in the poultry industry.

3.3.3. Experimental design

(a) Effect of treatment with CpG-ODN and dose titration of CpG-ODN on *Salmonella* Typhimurium infection in neonatal broilers

The objective of this experiment was to investigate the protective effect of CpG-ODN and the dose titration of CpG-ODN against *Salmonella* Typhimurium infection.

Based on previous data on the effectiveness of CpG-ODN against *E. coli* (Gomis *et al.* 2004), 50 μg of CpG-ODN was selected as the standard dose. Day-old broiler chickens were randomly allocated to one of seven groups, each containing 20 birds. Birds in each group were identified with neck band and injected in the leg with CpG-ODN (50, 10, or 1 μg per bird), non-CpG-ODN (50, 10, or 1 μg per bird), or saline. All groups were challenged at four days of age with $1x10^7$ cfu *of Salmonella* Typhimurium, as described previously. Clinical evaluations were made and bacteria isolated as described above.

(b) Duration of CpG-ODN protection against *Salmonella* Typhimurium infection in neonatal broiler chickens

Day-old broiler chicks were randomly allocated to one of eight groups, each containing 20 birds. Birds in each group were individually identified with neck bands, and injected with 50 μg of CpG-ODN intramuscularly in the leg on one of the following days of age: 1, 2, 3, 4, 6, 8, or 9. All groups were challenged on day 9 of age with $1.1x10^8$ cfu of *Salmonella* Typhimurium as described above. Clinical evaluations were made and bacteria isolated as described above.

(c) Effect of CpG-ODN on *Salmonella* Typhimurium septicemia

Birds were randomly allocated to one of two groups of 80 birds and housed in an animal isolation room. At one day of age, groups were injected intramuscularly in the leg with 50 μg of CpG-ODN or saline. All birds were challenged on day three with $1.2x10^6$ cfu of *Salmonella* Typhimurium as described above. One ml of blood was collected from the suboccipital cavernous sinus (atlantooccipital sinus) of three

anesthetized birds into three ml syringes containing 250 µl heparin (Organon Teknika, Toronto, Canada) at 1, 6, 12, 18, 24, 36, 48 hours, 3, 4, 5, 6, and 7 days post-challenge with *Salmonella* Typhimurium. The birds were subsequently euthanized with halothane (Halocarbon Products Corporation Rivin Edge, NJ) at each time point. The number of bacteria in individual blood samples was determined by plating ten-fold serial dilutions in duplicate on TSIA plates and incubating them at 37^{o}C for 24 hours. The threshold of detection was 100 cfu per ml. During the course of this experiment, mortality was observed in both the treatment and control groups. Birds that survived were randomly selected at each time point.

(d) Effect of CpG-ODN administered *in ovo* at day 18 of incubation followed by challenge with *Salmonella* Typhimurium at hatching

In ovo administration is known to be a safe, efficacious and convenient method for vaccinating poultry (Ricks *et al.* 1999). Embryonated eggs were randomly allocated to one of three groups, each containing 25 eggs. On day 18 of incubation, groups were inoculated with 50 µg of CpG-ODN, 50 µg of non-CpG-ODN or saline. All groups were challenged with 1.1x10^{6} cfu of *Salmonella* Typhimurium on day two post-hatch as described above. Clinical scores were assigned and bacteria isolated as in previous experiments.

3.3.4. Statistical analysis

Survival patterns and median survival times were compared using log-rank tests and chi-square statistics. Survival data was analyzed using Prism (Prism 4.0, GraphPad

Software Inc., San Diego, CA. USA www.graphpad.com) with $\alpha = 0.05$. Clinical scores for each bird were summed over the ten-day observation period and the differences among groups were tested using Krukal-Wallis non-parametric analysis of variance. Blood samples taken simultaneously from treatment and control groups were compared using the non-parametric Wilcoxon signed rank test. The effect of CpG treatment on development of septicemia was evaluated using Wilcoxon rank sum tests and proportional hazards regression analysis (Statistic 7, Analytical Software, Tallahassee FL).

3.4. Results

(a) Effect of treatment with CpG-ODN and dose titration of CpG-ODN on *Salmonella* Typhimurium infection in neonatal broilers

The survival rate of birds treated with 50 μg of CpG-ODN was higher than in groups treated with 10 or 1 μg of CpG-ODN, 50, 10, or 1 μg of non-CpG-ODN or saline ($p< 0.01$) (Fig. 3.1). Cumulative clinical score (CCS) differed significantly among groups ($p<0.001$). Comparison of mean CCS ranks by group revealed that the CCS of 50 μg CpG-ODN group differed significantly from the CCS of other groups ($p<0.05$) (Fig. 3.2). The group treated with 50 μg of CpG-ODN (CCS of 195) had a significantly lower CCS than other groups (10 μg of CpG-ODN, CCS of 315; 1 μg of CpG-ODN, CCS of 349; 50 μg non-CpG-ODN, CCS of 360; 10 μg of non-CpG-ODN, CCS of 431; 1 μg of non-CpG-ODN, CCS of 454; or saline, CCS of 503). More birds in all groups other than the group treated with 50 μg CpG-ODN developed airsacculitis, pericarditis and perihepatitis, either alone or in combination (polyserositis). Birds that

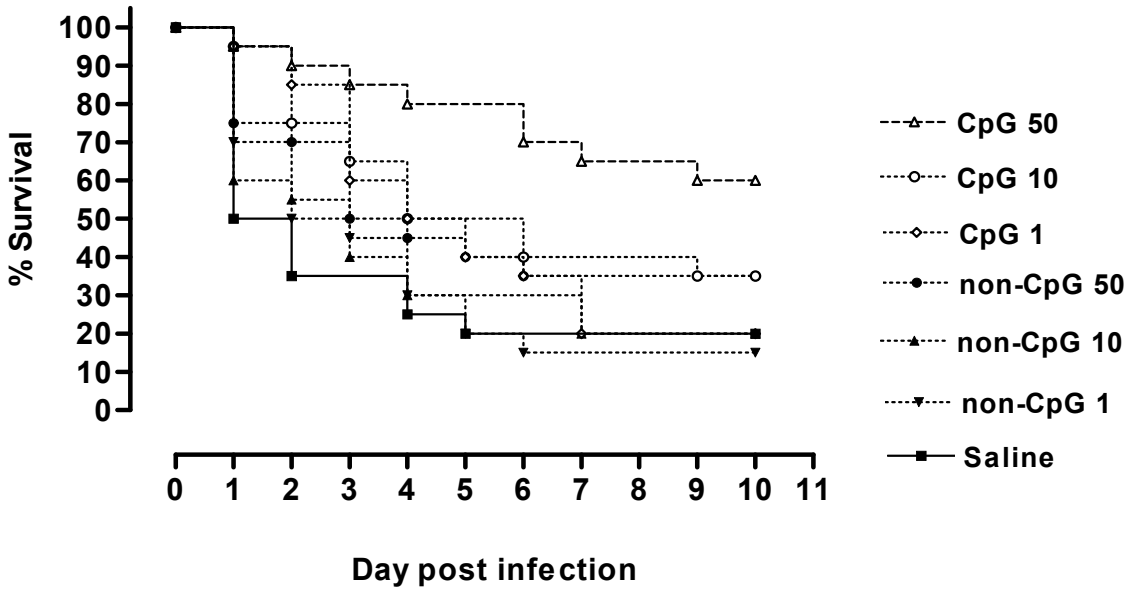


Fig. 3.1. Survival of neonatal chickens following treatment with different doses of CpG-ODN. Groups were treated intramuscularly with 50 (Δ), 10 (○), or 1(◊) µg of CpG-ODN, 50 (●), 10 (▲), or 1(▼) µg of non-CpG-ODN or saline (■). All birds were challenged subcutaneously with $1x10^7$ cfu of *Salmonella* Typhimurium three days post treatment. Birds that received 50 µg of CpG-ODN showed significantly higher survival rates than groups treated with saline, 10 or 1 µg of non-CpG-ODN ($p < 0.01$). Birds treated with 10 or 1 µg of CpG-ODN were not protected against *Salmonella* Typhimurium infection. ($p > 0.05$) ($n = 20$).

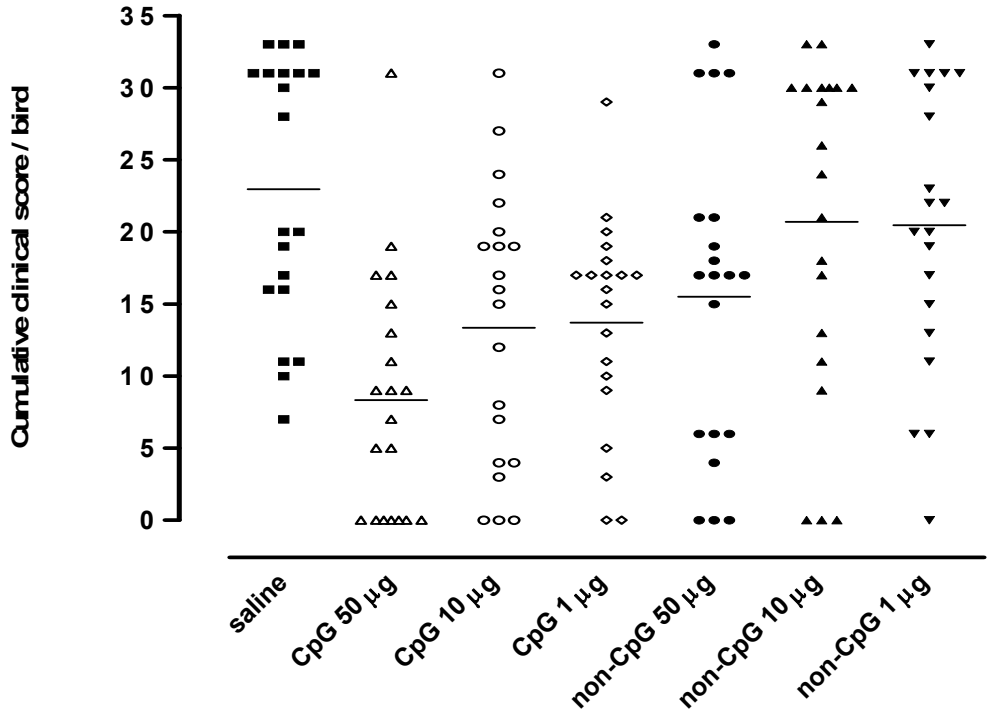


Fig. 3.2. Cumulative clinical scores (CCSs) for neonatal chickens following treatment with different doses of CpG-ODN. The cumulative clinical scores of individual birds following subcutaneous challenge with $1x10^7$ cfu of *Salmonella* Typhimurium three days post treatment with 50 (Δ), 10 (○), 1 (◊) µg of CpG-ODN, 50 (●), 10 (▲), 1 (▼) µg of non-CpG-ODN or saline (■) are shown in this graph. Birds intramuscularly injected with 50 µg of CpG-ODN had the lowest CCS among groups (Bar = median) (n = 20).

Table 3.1. Level of *Salmonella* Typhimurium isolated from the air sacs of neonatal chickens treated with different doses of CpG-ODN

Salmonella Typhimurium challenge dose	*Salmonella* Typhimurium growth (Score [A])	saline	non-CpG-ODN 50 µg	CpG-ODN 50 µg	non-CpG-ODN 10 µg	CpG-ODN 10 µg	non-CpG-ODN 1 µg	CpG-ODN 1 µg
	0	**1** [B]	**6**	**6**	**5**	**2**	**3**	**5**
$1x10^7$	+1	**12**	**6**	**12**	**7**	**12**	**6**	**6**
cfu / bird	+2	**3**	**3**	**1**	**5**	**4**	**7**	**7**
	+3	**3**	**3**	**1**	**2**	**2**	**4**	**2**
	+4	**1**	**2**	**0**	**1**	**0**	**0**	**0**

In the groups that were intramuscularly treated with different doses of CpG-ODN at day one of age and subcutaneously inoculated with $1x10^7$ cfu of *Salmonella* Typhimurium three days later, bacteria were isolated as frequently from the air sacs at necropsy regardless of treatment ($p = 0.28$).

A *Salmonella* Typhimurium growth on the TSIA plates were scored as follows: 0 = no growth; +1 = growth of bacteria on area 1; +2 = growth of the bacteria on area 1 and 2; +3 growth of bacteria on area 1, 2, and 3; +4 = growth of bacteria on area 1, 2, 3, and 4 ($n = 20$).

B Number of birds.

died per acutely within 24 hours of challenge did not have gross lesions but did have bacteria in the air sacs, as determined by culture (Table 3.1). In groups treated with 50 μg of CpG-ODN, bacteria were isolated less frequently from the air sacs than in groups treated with 10 and 1 μg of CpG-ODN, all non-CpG-ODN groups or saline; however the bacteria isolation scores were not significantly different ($p = 0.28$) (Table 3.1).

(b) Duration of CpG-ODN protection against *Salmonella* Typhimurium infection in neonatal broiler chickens

Birds were treated with CpG-ODN at different times prior to challenge to determine the duration of the protective effect. Groups of birds that received CpG-ODN on days 1, 2, 3, 5, 7 and 9 prior to challenge had significantly better survival than the control group ($p< 0.01$) (Fig. 3.3). However, treatment with CpG-ODN one to two days prior to challenge with *Salmonella* Typhimurium induced the highest protection in neonatal chickens. The group that received CpG-ODN at the time of challenge was not significantly protected ($p = 0.09$). Patterns in clinical signs and bacterial isolations were similar to those in previous experiments.

(c) Effect of CpG-ODN on *Salmonella* Typhimurium septicemia

To evaluate the protective effect of CpG-ODN against systemic infection, the level of bacteremia was monitored following challenge. *Salmonella* Typhimurium was isolated from birds in the control group, which received saline, at a higher levels (cfu/100 μl) than the group that received CpG-ODN ($P<0.001$) (Table 3.2). Moreover,

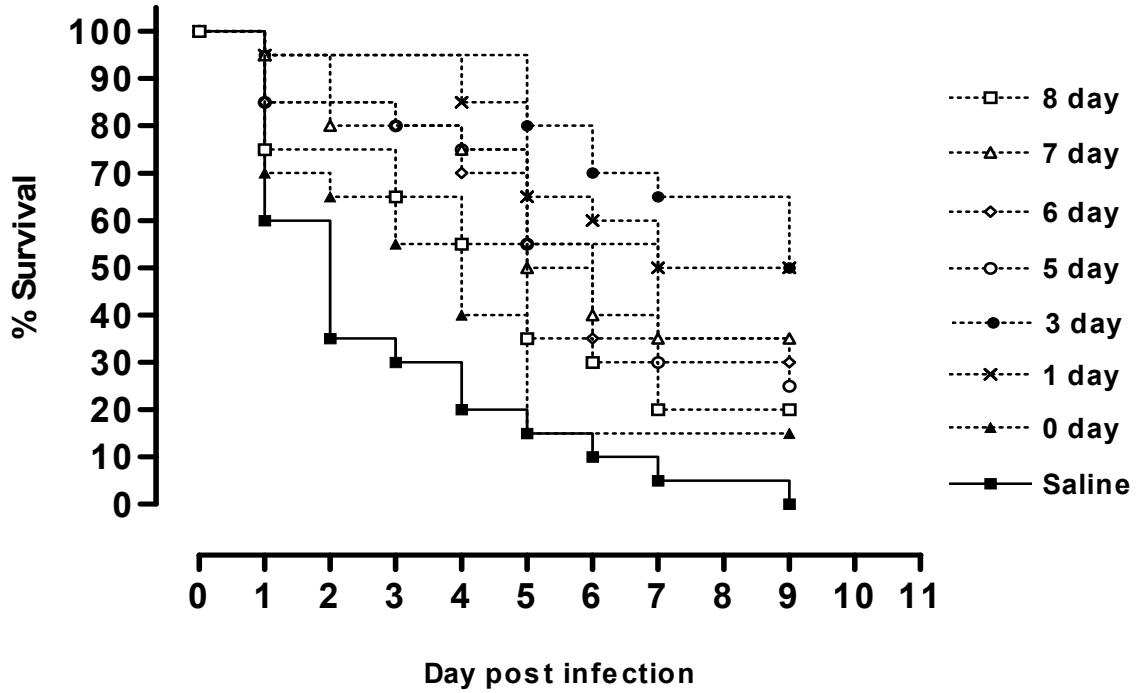


Fig. 3.3. Duration of protection induced by CpG-ODN against *Salmonella* Typhimurium infection. Percent survival of chickens following a single intramuscular administration of 50 μg of CpG-ODN at the age of 1, 2, 3 4, 6, 8, or 9 days followed by *Salmonella* Typhimurium challenge ($1.1x10^8$ cfu) at day nine of age. Groups of birds challenged subcutaneously with *Salmonella* Typhimurium at days 1, 3, 5, 6, 7 or 8 post-CpG-ODN-treatment were significantly protected ($p < 0.01$). However, the highest protection occurred in groups that received CpG-ODN one to three days prior to the challenge ($p > 0.05$) ($n = 20$).

Table 3.2. *Salmonella* Typhimurium isolated from blood of the birds treated with either saline or CpG-ODN

Time of blood collection / hour	Saline group (cfu)	CpG-ODN (50 μg) group (cfu / ml)
	$3.0x10^2$	**0**
1	**$2.0x10^3$**	**0**
	$2.5x10^5$	**0**
	$2.0x10^3$	**0**
6	**$3.5x10^5$**	**0**
	$1.5x10^5$	**0**
	$5.5x10^7$	**0**
12	**$2.0x10^4$**	**0**
	$2.0x10^4$	**0**
	$3.0x10^4$	**0**
18	**$2.5x10^6$**	**0**
	0	**0**
	$4.5x10^6$	**$2.0x10^3$**
24	**$1.5x10^5$**	**$2.0x10^3$**
	$4.5x10^7$	**0**
	$8.5x10^7$	**$1.0x10^3$**
48	**$5.5x10^7$**	**$1.0x10^2$**
	$2.0x10^5$	**0**
	$4.0x10^6$	**$1.0x10^4$**
72	**$4.5x10^8$**	**$1.0x10^4$**
	$7.0x10^6$	**0**
	$5.5x10^8$	**$2.0x10^4$**
96	**$4.5x10^8$**	**$3.0x10^4$**
	$2.0x10^6$	**0**
	$7.5x10^6$	**$1.0x10^2$**
120	**$2.0x10^3$**	**0**
	$2.5x10^5$	**0**
	$1.5x10^4$	**0**
144	**0**	**0**
	0	**0**
	0	**0**
168	**0**	**0**
	0	**0**

One ml of blood was collected from the suboccipital cavernous sinus of three anesthetized birds at each time. The number of bacteria in blood samples was determined by plating ten-fold serial dilutions in duplicate on TSIA agar followed by incubation at 37^oC for 24 hours (n = 3).

the relative risk of having bacteria in the blood was lower in birds treated with CpG-ODN than in birds treated with saline. Treatment also delayed the detection of bacteria in the control group. All birds with bacteria were positive at one hour post-challenge in the control group. In contrast, birds treated with CpG-ODN did not have detectable bacteremia until 24 hours post-challenge. No bacteria were isolated from birds treated with CpG-ODN on days six and seven post-challenge, while birds were found positive in the control group (Table 3.2).

(d) Effect of *in ovo* administration of CpG-ODN at day 18 of incubation and challenge with *Salmonella* Typhimurium at hatch

A dose of 50 μg of CpG-ODN was effective in protecting neonatal chickens against *Salmonella* Typhimurium infection when delivered intramuscularly. A similar trial was conducted to evaluate the effect of *in ovo* delivery of CpG-ODN on day 18 of incubation against *Salmonella* Typhimurium. Birds given CpG-ODN by the *in ovo* route had significantly greater survival ($p < 0.01$) than birds in control groups that received non-CpG-ODN or saline (Fig. 3.4). Non-CpG-ODN treatment did not protect birds against death following *Salmonella* Typhimurium challenge ($p > 0.05$) and was similar to that of saline. There was a significant difference among CCS of groups given treatment *in ovo* ($p = 0.02$). Comparison of mean ranks of CCS by group showed that two sets of groups differed significantly ($p < 0.05$) (Fig. 3.5). Only the group treated *in ovo* with 50 μg of CpG-ODN (CCS of 95) did not belong to the set of unprotected groups, which had higher CCS (50 μg of non-CpG-ODN *in ovo*, CCS of 157; or saline *in ovo*, CCS of 306). Birds treated with CpG-ODN had a lower number of

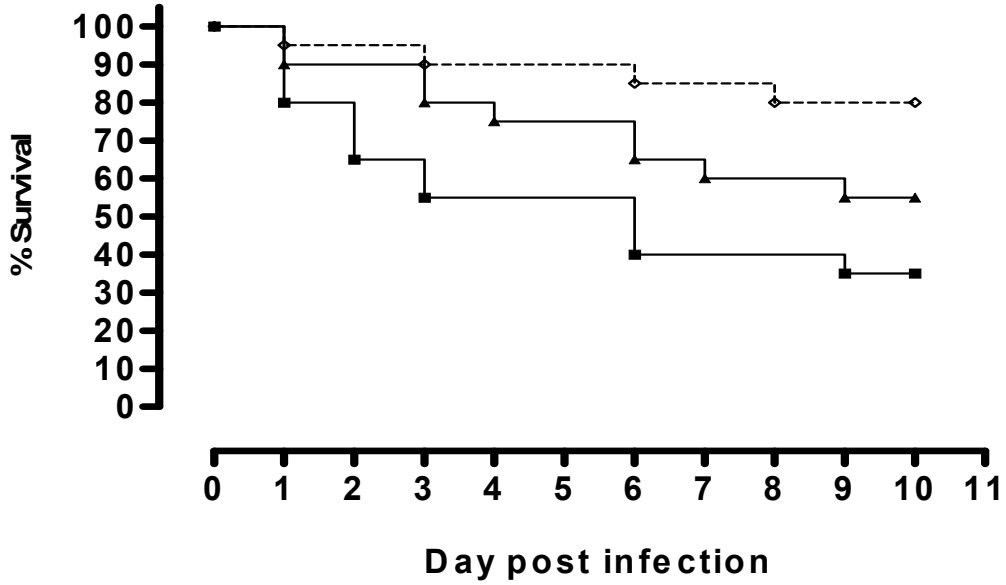


Fig. 3.4. Survival of neonatal chickens following *in ovo* treatment with CpG-ODN against *Salmonella* Typhimurium infection. Survival of chickens following *in ovo* treatment with 50 μg of CpG-ODN (◊), 50 μg non-CpG-ODN (▲), or saline (■) into the amniotic cavity of the eggs at day 18 of incubation. All birds were challenged subcutaneously with $1.1x10^6$ cfu of *Salmonella* Typhimurium at day two of age. Birds that received CpG-ODN showed significantly higher protection against *Salmonella* Typhimurium than birds that received non-CpG-ODN or saline ($p < 0.01$). Birds that received 50 μg of non-CpG-ODN were not protected against *Salmonella* Typhimurium infection ($p > 0.05$) ($n = 20$).

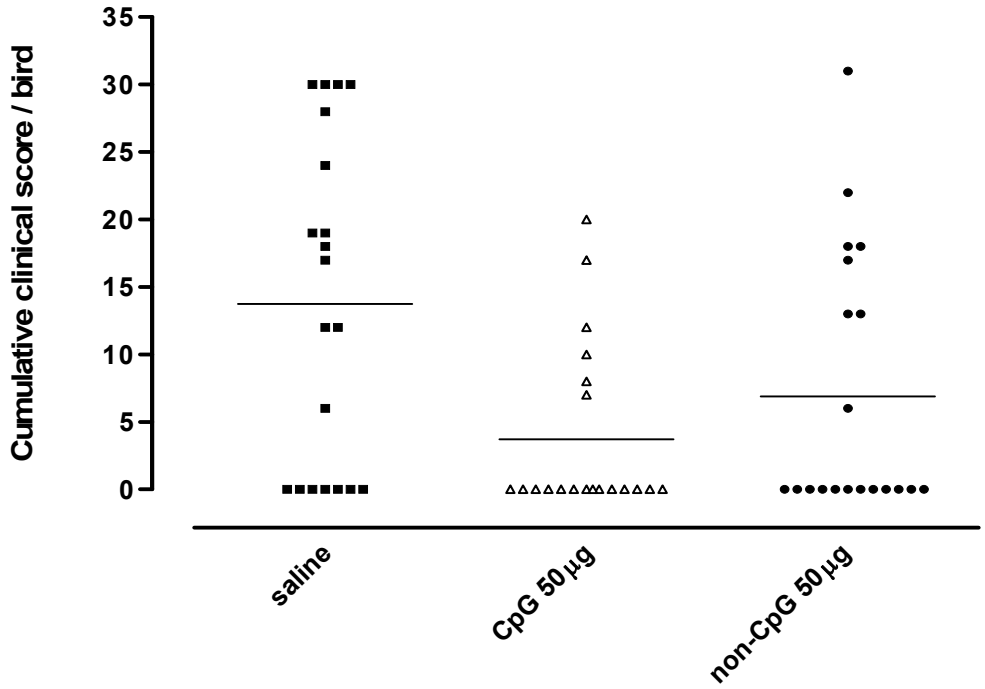


Fig. 3.5. Cumulative clinical scores (CCSs) of neonatal chickens following *in ovo* treatment with CpG-ODN prior to inoculation with *Salmonella* Typhimurium. Eggs received 50 µg of CpG-ODN (Δ), 50 µg of non-CpG-ODN (●), or saline (■) on day 18 of incubation. All groups were challenged with $1.1x10^6$ cfu of *Salmonella* Typhimurium at day two of age. The median CCS of birds treated with 50 µg was lower than that of other groups (Bar = median) (n = 20).

Table 3.3. Level of *Salmonella* Typhimurium isolated from air sacs of neonatal chickens following *in ovo* treatment with CpG-ODN

Salmonella **Typhimurium challenge dose**	***Salmonella*** **Typhimurium growth (score *)**	**saline** [A]	**non-CpG-ODN 50 µg** [AB]	**CpG-ODN 50 µg** [B]
$1.1x10^6$ cfu	**0**	4 ■	7	13
	+1	8	7	3
	+2	4	2	2
	+3	2	2	2
	+4	2	2	0

[A B] Groups with different superscripts are significantly different ($p<0.05$).

Birds treated with CpG-ODN had a lower number of bacteria isolated from air sacs compared to birds treated with saline.

* *Salmonella* Typhimurium growth on the TSIA plates was scored as follows: 0 = no growth; +1 = growth of bacteria on area 1; +2 = growth of the bacteria on areas 1 and 2; +3 = growth of bacteria on areas 1, 2, and 3; +4 = growth of bacteria on areas 1, 2, 3, and 4 (n = 20).

■ Number of birds.

bacteria isolated from air sacs compared to birds treated with saline (Table 3.3) ($p < 0.05$).

3.5. Discussion

Synthetic CpG-ODN are known to be effective stimulators of the immune system and potent adjuvants in a number of species, including mice, primates and humans (Davis *et al.* 2000; Weeratna *et al.* 2000). Previously, CpG-ODN have been shown to protect neonatal and mature chickens against *E. coli*, a bacterium with extracellular survival (Gomis *et al.* 2004). In the present study, this finding has been extended to include the effect of CpG-ODN against *Salmonella* Typhimurium, a bacterium with an intracellular phase of survival.

The innate immune system is highly developed and has been well conserved over the course of evolution for rapid recognition and response to pathogens through recognition of PAMPs. One example of such recognition of PAMPs is that the vertebrate innate immune system (found in fish, birds and mammals) can discriminate bDNA from self-DNA by recognition of unmethylated CpG motifs present in the pathogen's DNA (Krieg 2002). CpG-ODN are therefore recognized as danger signal by the host and evoke innate immune responses, which in turn promote the host's adaptive immune system to combat potential infections. It has been demonstrated here that CpG-ODN are able to protect neonatal chickens against lethal infection with *Salmonella* Typhimurium. Protection by CpG-ODN was associated with lower clinical signs and decreased number of *Salmonella* Typhimurium in the blood. In previous studies, it was demonstrated that CpG-ODN stimulated a murine hepatocyte cell line to inhibit intracellular growth of *Salmonella* Typhimurium (Sanchez-Campillo *et al.* 2004) and

Salmonella Enteritidis infection in chickens (He *et al.* 2005b). Furthermore, a recent study suggested that chemokines and cytokines play a key role in the protective response against salmonellosis. Clearance of primary infection of *Salmonella* Typhimurium in the chicken appeared to involve IFN-γ-mediated TC responses (Withanage *et al.* 2005). These responses indicate that clearance of the systemic infection is primarily Th 1 mediated (Carter 1990).

Treatment with CpG-ODN provided effective protection when given one to three days prior to infection in an *E. coli* model, and appeared to have some effect for almost six days (Gomis *et al.* 2003). The duration of this effect represents a significant proportion of the neonatal lifespan of a broiler chicken, which commonly becomes infected with *E. coli* or *Salmonella* species. These infections result in a significant mortality and production loss for the poultry industry (Barnes 2003). Thus, injection of CpG-ODN *in ovo* to the developing embryo would be a feasible method to control these infections in the poultry industry. Currently, antibiotics are used in neonatal chickens as a preventative measure against bacterial infections. There is a potential to use CpG-ODN given by the *in ovo* route to reduce the incidence of bacterial infection in neonatal poultry and replace the use of antibiotics. *In ovo* delivery of CpG-ODN at day 18 of incubation significantly protected neonatal birds against *Salmonella* Typhimurium infection. This protection is associated with minimizing bacteremia (Table 3.3). Birds treated with CpG-ODN showed delayed onset of septicemia and faster recovery from infection than birds in the control group (Table 3.2). The mechanism of bacterial clearance remains to be determined but likely is associated with IFN-γ and IL-18 (Patel *et al.* 2007).

It is generally accepted that disease prevention by prophylactic measures or by immune stimulation is preferable to the use of therapeutic agents in the food animal industry. This has become especially important due to a number of food safety and human health issues. Therefore, the animal production industry is seeking ways of reducing the use of antibiotics such as vaccination, strict bio-security and immune stimulation. Therefore, novel immunostimulants may serve as alternative, desirable ways to prevent disease.

In conclusion, CpG-ODN were effective in protecting chickens against *Salmonella* Typhimurium infection. A large-scale field study is necessary to determine the commercial utility of CpG-ODN in preventing bacterial infections in neonatal birds. Future studies may develop the potential of CpG-ODN as stimulant of the innate immune system against infections in neonatal birds and an alternative to routine use of antibiotics in the poultry industry.

4.0. ENHANCEMENT OF IMMUNOPROTECTIVE EFFECT OF CPG-ODN BY FORMULATION WITH POLYPHOSPHAZENES AGAINST *E. COLI* SEPTICEMIA IN NEONATAL CHICKENS

4.1. Abstract

Synthetic oligodeoxynucleotides (ODN) containing CpG motifs (CpG-ODN) have been shown to be effective immunoprotective agents and vaccine adjuvants against a variety of bacterial, viral and protozoan diseases in different animal species. The objective of this study was to investigate the immunoprotective effect of CpG-ODN formulated with polyphosphazenes, liposomes or an oil-in-water emulsion against *E. coli* infections in neonatal chickens in separated trials. Eighteen-day-old embryonated eggs were inoculated with 50μg non-CpG-ODN, 50μg CpG-ODN or CpG-ODN formulated with polyphophazenes, liposomes or an Emulsigen®. Four days after exposure to CpG-ODN, non-CpG-ODN or formulated CpG-ODN, at one day post-hatch, approximately $1x10^4$ or $1x10^5$ cfu of a virulent isolate of *E. coli* was inoculated subcutaneously into the neck. Clinical signs, pathology, bacterial isolations from the air sacs, and mortality were observed for eight days following challenge with *E. coli*. The survival rate of birds in groups receiving either non-CpG-ODN or saline following *E. coli* infection was 0%. In contrast, birds given either CpG-ODN or CpG-ODN

formulated with polyphosphazene had a significantly higher survival rate of 55% ($P<0.0001$). The relative risk of mortality was lower for birds treated with CpG-ODN formulated in PCPP (poly [di (sodium carboxylatophenoxy) phosphazene]) (0.25), CpG-ODN formulated in PCEP (poly [di (sodium carboxylatoethylphenoxy) phosphazene]) (0.33), or unformulated CpG-ODN (0.39) in comparison to the group treated with saline ($p<0.01$). Although formulation of CpG-ODN with liposomes or an oil-in-water emulsion did not increase the immunoprotective effect against *E. coli* infection, no adverse reactions such as decreased hatchability or increased mortality were observed in embryos. This is the first time that CpG-ODN formulated with polyphosphazene has been demonstrated to have an immunoprotective effect against an extracellular bacterial infection in neonatal broiler chickens following *in ovo* delivery.

4.2. Introduction

The innate immune system of vertebrates recognizes a specific molecular structure presented in pathogens that are known as PAMPs. Following recognition of PAMPs, the host immune system immediately responses to infection (Medzhitov and Janeway 2002). Recently, it has been found that bDNA is among PAMPs that can stimulate the innate immune system of different vertebrate species and afford immune protection against a variety of bacterial, viral, and protozoan infections (Ashkar and Rosenthal 2002; Krieg 2002; Mutwiri *et al.* 2003). Bacterial DNA contains relatively abundant unmethylated CpG dinucleotides, while vDNA is methylated (Bird 1993). CpG motifs are now known to contribute to the immunostimulatory activity of bDNA (Ahmad-Nejad *et al.* 2002) and retain immunostimulatory activities (Yamamoto *et al.*

1992). CpG-ODN has direct stimulatory effects on monocytes and macrophages, which secrete IL-12 and other cytokines (Stacey *et al.* 2000). It activates NK cells to increase lytic activity and to secrete IFN-γ (Ballas *et al.* 1996). CpG-ODN have no apparent direct stimulatory effect on TCs, but they enhance the ability of APCs to active TCs (Krieg 1999). These effects have been seen in a variety of viral, bacterial and protozoan infections, among a range of vertebrate species, including poultry (Vleugels *et al.* 2002; Gomis *et al.* 2003; He *et al.* 2005a).

Over the past decades, considerable efforts were devoted to the development of new experimental adjuvant formulations that would increase the immunogenicity of subunit and protein vaccines (Gupta *et al.* 1993; Aguado *et al.* 1999). Adjuvants are formulated compounds or additives that, when combined with Ags, help to direct or boost the body's immune system (Gupta 1998). They are classified into two broad categories: I- delivery systems including particulate adjuvants such as liposomes and emulsions (Singh and O'Hagan 2002), and II- immunostimulatory adjuvants such as CpG-ODN (Davis *et al.* 1998).

Polyphosphazenes are potent immunostimulatory adjuvants with a high molecular weight, containing a long-chain backbone of alternating phosphorus and nitrogen atoms (Lu 1996a; Andrianov 1998). Among polyphosphazenes, PCPP is a potent immunostimulant via not only parenteral, but also mucosal vaccine delivery, against diseases such as influenza, tetanus toxoid, hepatitis B surface Ag, and herpes simplex virus (Payne *et al.* 1995). The adjuvant activity of a new polyphosphazene electrolyte (PCEP) as a potent enhancer of Ag-specific immune responses was recently studied in mice inoculated with influenza virus (Mutwiri *et al.* 2007). This study

revealed that the adjuvant activity of PCEP is higher than that of PCPP, as PCEP induces higher levels of Th 1, Th 2 type cytokines, and IgG 1, IgG 2 associated with production of both IFN- γ and IL-4.

In this study, the efficacy of combining CpG-ODN with liposomes was investigated. Liposomes are produced from natural non-toxic phospholipids and cholesterol. The adjuvant effect of liposomes is mostly due to: I- depot formation at the injection site, II- selective uptake of liposomal Ags into regional lymph nodes and, III- Ag presentation resulting from Ag clustering and massive delivery to APCs (Gregoriadis 1990) (Cox and Coulter 1997). It has been demonstrated that encapsulating CpG-ODN into liposomes improves the incorporation of CpG-ODN into DCs, enhances IL-12, activates NK cells, and produces IFN-γ (Verma *et al.* 2004).

Biphasic lipid vesicles of VTAs are composed of I- an outer membrane with two layers of vesicle-forming lipids, II- a core compartment containing an oil-in-water emulsion, and III- a dose of immunogen effective to elicit an immune response (Foldvari 1998). To improve the bioavailability and to optimize the immunostimulatory activity of CpG-ODN, Foldvari *et al.* (1998) developed a novel lipid-based delivery system, Biphasix^{TM} VTA, suitable for administering vaccine Ags and CpG-ODN (Foldvari 1998). This delivery approach provided extended release and optimum exposure of the Ag and CpG-ODN to cells of the immune system. There is evidence that encapsulation of CpG-ODN in a delivery system can potentiate the adjuvant effects VTA (Mui *et al.* 2001; Alcon *et al.* 2003; Babiuk *et al.* 2004).

Furthermore, it has been demonstrated that stabilized CLs (cationic lipids) improve the uptake and immunostimulatory activity of CpG-ODN

(Li *et al*. 2001; Mui *et al.* 2001). Liposomes stimulate both humoral and cell-mediated immunity, including CTL responses (Joseph *et al.* 2006; Yoshinaga *et al.* 2007). In an immunization model, encapsulation of CpG-ODN with the liposomal adjuvant CL increased IFN-γ and IgG responses by 15- to 40-fold compared with CpG-ODN alone (Gursel *et al.* 2001). These findings provide support that stabilized CLs enhance the therapeutic efficacy of CpG-ODN (Gursel *et al.* 2001).

Emulsigen® is commonly used in veterinary vaccines. This adjuvant acts by forming a depot of Ag, which can target immune effectors cells such as, IFN-γ, Th 1, and IgG (Linghua *et al.* 2006; Lopez *et al.* 2006). The depot effect, which results in slow release of Emulsigen® improves the presentation of Ags, thereby enhancing the immune response (Zaloga *et al.* 2007). Emulsigen® is a unique oil-in-water adjuvant, without components of animal origin (Zaloga *et al.* 2007).

Previously, we have demonstrated that unformulated CpG-ODN delivered *in ovo* were potent immunostimulants against bacterial infections in neonatal chickens. The objective of this study was to investigate if the immunoprotective effect of CpG-ODN in neonatal chickens could be enhanced by formulating CpG-ODN with polyphosphazenes, liposomes or Emulsigen®.

4.3. Materials and methods

4.3.1. *E. coli* culture and animal model

A field isolate of *E. coli* obtained from a turkey with septicemia was used as the challenge strain. This *E. coli* isolate was from serogroup O2, which is non-hemolytic, serum resistant, and produced aerobactin and has a K1 capsule and type one pili

(Ngeleka *et al.* 1996). Aliquots of bacteria were stored at 70°C in 50% BHI (Difco, Detroit, MI) supplement with 25% (v/w) glycerol (VWR Scientific, Inc., Montreal, Quebec). Bacteria for use as the challenge were cultured on BHI agar for 18-24 hours at 37°C. Two to three colonies were added to 200 ml of Luria Broth (LB) (Miller-BDH Inc.) in a one-L Erlenmeyer flask. The culture was grown at 37°C for 18-20 hours with shaking at 200 rpm. The stationary phase culture was diluted to an optical density of 0.4 at 600 nm. At this concentration, the culture contained approximately 2.5 x 10^8 colony-forming units (cfu) of bacteria. The cultures were further diluted in BHI to the concentration of bacteria required to challenge birds. The *E. coli* challenge dose was confirmed by plating serial dilutions of the diluted culture in duplicate on BHI plates, followed by incubating for 18 hours at 37°C, and counting the number of colonies. All procedures involving animals were done according to a protocol approved by the University of Saskatchewan, Committee on Animal Care. CpG-ODN was administered to eggs after 18 days of incubation, as described previously for the *E. coli* model developed in neonatal broilers by Gomis *et al.* (Gomis *et al.* 2004). Hatching eggs were obtained from a local hatchery in Saskatchewan, Canada. Eggs were hatched at the Poultry Hatchery, Department of Animal and Poultry Science, University of Saskatchewan. At the time of hatch birds were identified with individual neck tags (Swiftack Poultry Tags, Heartland Animal Health Inc., MO). Groups of 20 chickens were randomly assigned and placed in separate rooms at the Animal Care Unit, Western College of Veterinary Medicine, University of Saskatchewan. Water and commercial broiler ration were provided *ad libitum*. Each room had non-recirculated air. Photoperiods of 24 hours per day for the first three days and 16 hours per day for the

remaining seven days were established. Room temperature was maintained at 30-32 $^{\circ}$C for the first week and 28-30°C for the second week. Birds were challenged subcutaneously into the neck with $1x10^4$ or $1x10^5$ cfu of *E. coli* at day one post hatch or day four-post administration of formulated CpG-ODN.

Birds were observed for clinical signs for eight days following challenge with *E. coli*. Daily clinical scores for individual birds were assigned as follows: 0 = normal; 1 = hesitant to move and easily fatigued; 2 = euthanized; 3 = dead. Cumulative clinical scores (CCSs) for each bird (CCS/Bird) and for each group of 20 (CCS/Group) were calculated as follows:

CCS/Bird- Clinical scores were summed for each bird across the eight days of the trial. Birds that had been euthanized were given a score of 2 for the day of euthanasia and 3 for each day remaining in the trial. Dead birds were given a score of 3 for each day remaining in the trial, including the day of death.

CCS/Group- Clinical scores for each bird were summed across groups of 20 birds to give a CCS for each group.

Dead or euthanized birds were necropsied immediately. Gross lesions such as pericarditis, perihepatitis, airsacculitis and polyserositis were recorded. Bacterial swabs were taken from the air sacs and cultured on MacConkey agar plates (Becton, Dickinson and Company Sparks, MD). Growth of *E. coli* on the plates was recorded as follows: 0 = no growth; +1 = growth of bacteria on area 1; +2 = growth of the bacteria on areas 1 and 2; +3 = growth of bacteria on areas 1, 2 and 3; and +4 = growth of bacteria on areas 1, 2, 3 & 4 (Barry 1972).

4.3.2. Synthetic CpG-ODN

The sequence of CpG-ODN used was TCGTCGTTGTCGTTTTGTCGTT [(2007)] and the sequence of non-CpG-ODN was TGCTGCTTGTGCTTTTGTGCTT [(2007)]. Both ODNs were free of endotoxin (provided by Qiagen-GmbH, Hilden Germany) and were produced with a phosphorothioate backbone. Synthetic CpG-ODN was diluted in sterile, pyrogen free saline.

4.3.3. Preparation of Polyphosphazenes

Polyphosphazene polyelectrolytes PCPP and PCEP were designed and synthesized by Parallel Solutions Inc. (Cambridge, MA) using previously described procedures (Mutwiri *et al.* 2007). Aqueous solutions of both polymers were stored at room temperature in the dark. Batches of polyphosphazenes found to have endotoxin levels below 0.034 ng/ml, as assessed by Limulus Amebocyte Lysate assay (Biowhittaker, Walkersville, MD). Fifty μg of CpG-ODN was formulated with 50 μg of polyphosphazenes to obtain a total volume of 100 μl of PBS.

4.3.4. Preparation of biphasic lipid vesicles, vaccine targeting adjuvant (VTA) and cationic lipid (CL)

Biphasix™ Vaccine Targeting Adjuvant (VTA) (Formulation codes: VTA-4 and VTA-6), a lipid-based delivery system and cationic lipids (CL-16 and CL-18) were developed at PharmaDerm Laboratories Ltd. Saskatoon, Saskatchewan, Canada. VTA was prepared as described previously (Foldvari 1998; Foldvari *et al.* 1999; Gursel *et al.* 2001). Briefly, the lipid phase components were mixed together at 70°C and hydrated

with the microemulsion aqueous phase. The resulting biphasic vesicles (a multi-compartmental system consisting of aqueous, micellar, amphipathic bilayer, and hydrophobic compartments) were mixed with 50 μg CpG-ODN solution at 9:1 (v/v).

4.3.5. Preparation of Emulsigen®

Emulsigen® is a common adjuvant in poultry vaccines (Johnston *et al.* 1997). In this study, Emulsigen® was used to formulate CpG-ODN for delivery by the *in ovo* route. This adjuvant was obtained from MVP Laboratories Inc., Ralston, NE. On day 18 of incubation, eggs were inoculated with either: I- 90 μl of 30% Emulsigen® together with 50 μg CpG-ODN in 10 μl of saline, II- 90 μl of 10% Emulsigen® together with CpG-ODN 50 μg in 10 μl of saline, or III- 90 μl of 30% Emulsigen® in 10 μl of saline.

4.4. Experimental design:

(a) Formulation of CpG-ODN with polyphosphazenes for *in ovo* protection against *E. coli* infection in neonatal chickens

In ovo administration is known to be a safe, efficacious and convenient method for vaccinating poultry (Ricks *et al.* 1999). Thirteen groups of 25 embryonated eggs were randomly allocated to treatments. The groups were injected on day 18 of incubation with CpG-ODN, non-CpG-ODN, PCPP, PCEP, PCPP together with CpG-ODN, PCEP together with CpG-ODN, or saline. All groups of 20 birds were challenged with either $1.5x10^4$ or $1.5x10^5$ cfu of *E. coli* at day one post-hatch and kept separately in different rings (four days after *in ovo* treatments). Clinical scores were recorded and bacteria isolated as described above.

To determine if formulation of CpG-ODN with polyphosphazenes enhanced the duration of protection against *E. coli*, additional groups of birds were inoculated with the above formulations by the *in ov*o route. These birds were challenged with $1x10^4$ cfu of *E. coli* at day-four post-hatch (seven days after *in ovo* treatment). Clinical scores were recorded and bacteria isolated as described above.

(b) Formulation of CpG-ODN with VTA for *in ovo* protection against *E. coli* infection in neonatal chickens

Embryonated eggs were randomly allocated to six groups of 25. On day 18 of incubation, groups were injected with CpG-ODN, VTA-4, VTA-6, VTA-4 together with CpG-ODN, VTA-6 together with CpG-ODN, or saline. On day one post-hatch, all groups of 20 birds were challenged with $2x10^4$ or $2x10^5$ cfu of *E. coli* (four days after *in ovo* treatment) and kept in different rings. Clinical scores were recorded and bacteria isolated as described above.

(c) Formulation of CpG-ODN with cationic lipids for *in ovo* protection against *E. coli* infection in neonatal chickens

Embryonated eggs were randomly allocated into six groups of 25. On day 18 of incubation, groups were injected with CpG-ODN, CL-14, CL-16, CL-14 together with CpG-ODN, CL-16 together with CpG-ODN, or saline. All groups containing 20 birds each were challenged with $2x10^4$ or $2x10^5$ cfu of *E. coli* on day one post-hatch (four days after *in ovo* treatment) and kept in different rings. Clinical scores were recorded and bacteria isolated as described above.

(d) Formulation of CpG–ODN with Emulsigen® for *in ovo* protection against *E. coli* infection in neonatal chickens

Embryonated eggs were randomly allocated into five groups of 25. On day 18 of incubation, groups were inoculated with CpG ODN, 10% Emulsigen®, 10% Emulsigen® together with CpG-ODN, 30% Emulsigen® together with CpG-ODN, or saline. All birds were challenged with $1.5x10^4$ or $1.5x10^5$ cfu of *E. coli* on day one post-hatch (four days after *in ovo* treatment) and kept in separate groups. Clinical scores were recorded and bacteria isolated as described above.

4.5. Statistical analysis

The survival pattern and median survival were compared using the log-rank test. Survival data was analyzed using Prism (Prism 4.0, GraphPad Software Inc., San Diego, CA. USA www.graphpad.com) with $p = 0.05$. The effect of treatments on relative risk of mortality and on cumulative clinical scores was analyzed using proportional hazards regression and Kruskal-Wallis non-parametric analysis of variance with comparison of mean ranks using Statistic (Analytic Software, Tallahassee, FL USA, www.statistix.com). Chi-square tests in Statistic were used to test the homogeneity of the relative frequency distribution of the level of bacteria in air sacs after treatments.

4.6. Results

(a) Formulation of CpG-ODN with polyphosphazenes for *in ovo* protection against *E. coli* infection in neonatal chickens

The relative risk of mortality was significantly lower in birds treated with CpG-ODN formulated in PCPP (0.25), CpG-ODN formulated in PCEP (0.33), or

unformulated CpG-ODN (0.39) in comparison to the group treated with saline ($p < 0.01$) (Fig. 4.1). The PCEP treatment without CpG-ODN also provided a modest degree of protection compared to the saline treatment (relative risk of mortality = 0.57, $p < 0.05$). The immunoprotective effect and mortality rates were similar in groups challenged with $1.5x10^4$ and $1.5x10^5$ cfu of *E. coli*, so data were pooled for groups given the two challenge doses. The estimated risk of dying was 0.68 times lower in birds treated with CpG-ODN formulated in PCPP than in birds treated with unformulated CpG-ODN ($p = 0.04$). The proportion of birds treated with CpG-ODN formulated in PCEP that died was lower than those treated with unformulated CpG-ODN; however the difference not significant ($p = 0.35$).

Groups that received CpG-ODN together with PCPP had the lowest cumulative clinical score (354), followed by birds that received CpG-ODN together with PCEP(449), CpG-ODN (501), PCEP (601), or saline (777), PCPP (758), or non-CpG (804) (Fig. 4.2).

Overall, the effect of treatment group on CCS was highly significant ($p < 0.001$). Groups with significantly different mean ranks are shown in Fig. 4.2. The majority of birds in groups not protected against *E. coli* infection developed airsacculitis, pericarditis and perihepatitis, either alone or in combination (polyserositis) compared to birds in groups treated with CpG-ODN formulated with polyphosphazene or CpG-ODN alone. Birds that died per acutely within 24 hours of challenge did not have gross lesions, but bacteria were cultured from the air sacs (Table 4.1). In groups treated with CpG-ODN formulated with polyphosphazene or CpG-ODN alone, fewer bacteria were isolated from the air sacs compared to other groups (Table 4.1). ($\chi^2 = 51.02$; $p < 0.001$).

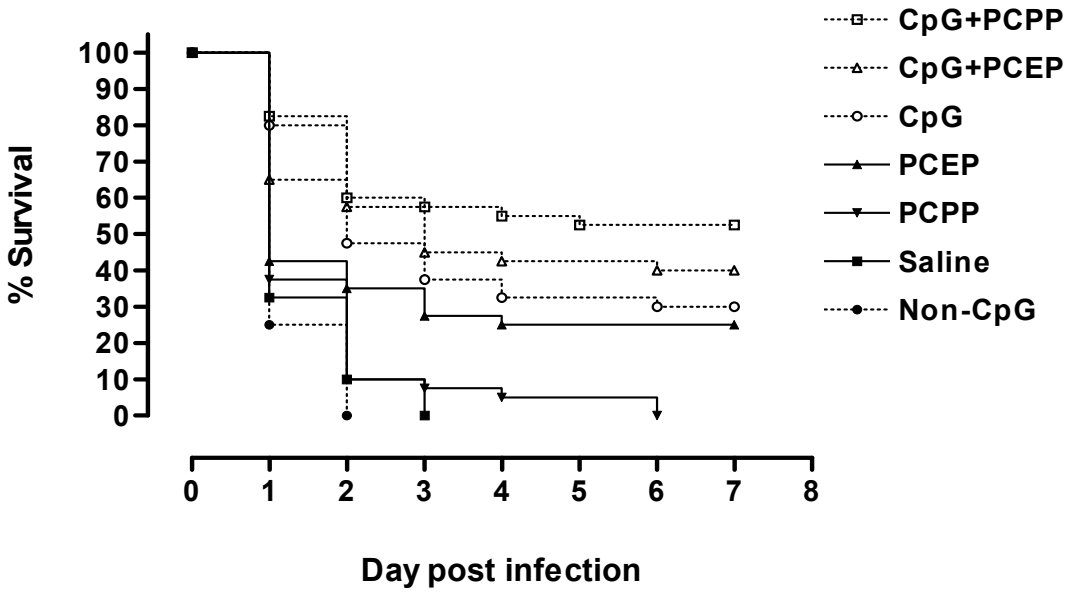


Fig. 4.1. Survival of neonatal chickens following *in ovo* administration of CpG-ODN formulated with polyphosophazenes. Chickens were subcutaneously challenged with $1.5x10^4$ and $1.5x10^5$ cfu of *E. coli* in groups treated *in ovo* with CpG-ODN (○), non-CpG-ODN (●), PCPP (▼), PCEP (▲), CpG-ODN together with PCPP (□) CpG-ODN together with PCEP (Δ) or saline (■). Birds that received CpG-ODN or CpG-ODN formulated with PCPP or PCEP showed a significantly higher survival rate ($p < 0.01$) compared to groups that received non-CpG-ODN or saline. The immunoprotective effect and mortality rates were similar in groups challenged with $1x10^4$ and $1x10^5$ cfu of *E. coli*, so data were pooled for groups given the two challenge (n = 40 for each treatment).

Although the highest immunoprotection was seen in the group treated with CpG-ODN formulated with PCPP, there was no significant difference in cumulative clinical score or bacterial isolation between groups treated with CpG-ODN alone and CpG-ODN formulated with PCPP ($p > 0.05$).

In order to determine if the formulation of CpG-ODN with polyphosphazenes enhances the duration of protection against *E. coli* challenge, additional groups of birds were challenged with $1x10^4$ cfu of *E. coli* at day four-post hatch (seven days after *in ovo* treatment). None of the groups treated with CpG-ODN formulated with polyphosphazenes or CpG-ODN alone showed protection of this duration against *E. coli* mortality, compared to the control group treated with saline ($p > 0.05$).

(b) Formulation of CpG–ODN with VTA for *in ovo* protection against *E. coli* infection in neonatal chickens

Survival of birds treated with CpG-ODN was significantly greater than survival of birds in groups treated with VTA-4, VTA-6 or saline ($p < 0.01$). Groups treated with CpG-ODN formulated either with VTA-4 or VTA-6 did not show higher immunoprotection against *E. coli* infection than the group treated with CpG-ODN alone($p > 0.05$) (Fig. 4.3). There were no significant differences in cumulative clinical score or bacterial isolation between groups treated with CpG-ODN alone and CpG-ODN formulated with VTA-4 or VTA-6 ($p > 0.05$).

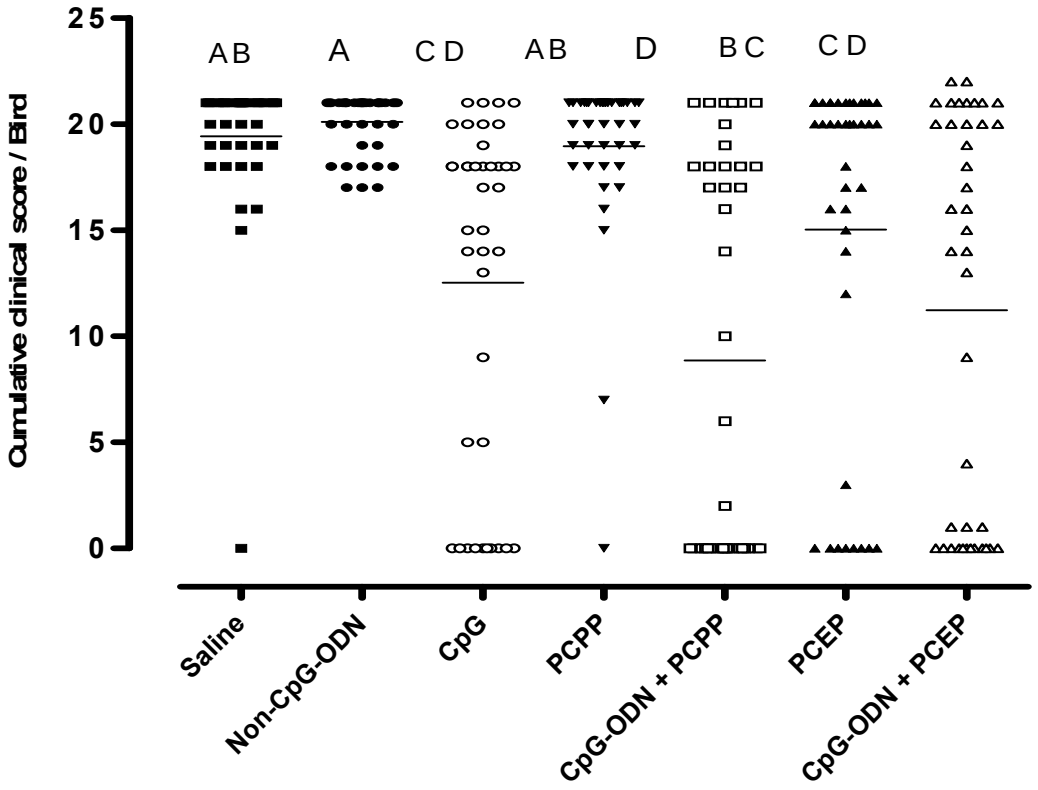


Fig. 4.2. Cumulative clinical scores (CCSs) in birds following *in ovo* treatment with CpG-ODN formulated in PCPP and PCEP. Cumulative clinical score of individual birds following challenge with $1.5x10^4$ and $1.5x10^5$ cfu of *E. coli* via subcutaneous route in groups treated with saline, CpG-ODN (○), non-CpG-ODN (●), PCPP (▼), PCEP (▲), CpG-ODN together with PCPP (□) CpG-ODN together with PCEP (Δ) or saline (■). Birds that received CpG-ODN formulated with PCPP had lowest CCS among groups. Groups with different letters above the cluster of symbols are significantly different ($p < 0.05$) (Bar = median). The immunoprotective effects were similar in groups challenged with $1x10^4$ and $1x10^5$ cfu of *E. coli*, so data were pooled for groups given the two challenge (n = 40 for each treatment).

Table 4.1. Level of *E. coli* isolated from air sacs of neonatal chickens following *in ovo* treatment with CpG-ODN formulated with PCPP and PCEP

***E. coli* Growth (scores *)**	**Saline [AB]**	**non-CpG-ODN [ABC]**	**CpG-ODN [BCD]**	**PCPP [A]**	**PCEP [ABC]**	**PCPP+ CpG-ODN [D]**	**PCEP+ CpG-ODN [CD]**
0	0 ▪	0	7	0	5	19	7
+1	4	11	8	1	4	4	11
+2	12	17	9	13	14	8	7
+3	12	12	10	18	10	3	10
+4	12	0	6	8	7	6	5

Groups with different superscript letters designations are significantly different ($p < 0.05$). Embryonated eggs incubated for 18 days received different formulations as shown above. Overall, these *in ovo* treatments had a significant effect on bacterial load in air sacs ($p < 0.001$). The immunoprotective effects were similar in groups challenged with $1x10^4$ and $1x10^5$ cfu of *E. coli*, so data were pooled for groups given the two challenge ($n = 40$ for each treatment).

* *E. coli* growth on the MacConkey plates were scored as follows: 0 = no growth; +1 = growth of bacteria on areas 1; +2 = growth of the bacteria on areas 1 and 2; +3 = growth of bacteria on areas 1, 2, and 3; +4 = growth of bacteria on areas 1, 2, 3, and 4.

▪ Number of birds.

(c) Formulation of CpG-ODN with cationic lipids for *in ovo* protection against *E. coli* infection in neonatal chickens

Survival of birds treated with CpG-ODN was significantly greater than survival in groups treated with CL-14, CL-16 or saline ($p < 0.01$). Groups treated with CpG-ODN formulated either with CL-14 or CL-16 did not have a greater immunoprotection against *E. coli* infection compared to the group treated with CpG-ODN alone ($p > 0.05$) (Fig. 4.4). There were no significant differences in cumulative clinical score or bacterial isolation between groups treated with CpG-ODN alone and CpG-ODN formulated with CL ($p > 0.05$).

(d) Formulation of CpG–ODN with Emulsigen® for *in ovo* protection against *E. coli* infection in neonatal chickens

Survival of birds treated with CpG-ODN was significantly greater than that of groups treated with 10% oil-in-water Emulsion® or saline ($p< 0.01$). CpG-ODN formulated either with 10% oil-in-water Emulsion® or 30% oil-in-water Emulsion® did not produce an enhanced immunoprotective effect against *E. coli* infection ($p > 0.05$), compared to the group treated with CpG-ODN alone (Fig. 4.5). There were no significant differences in cumulative clinical score or bacterial isolation between groups treated with CpG-ODN alone and CpG-ODN formulated with Emulsion® ($p > 0.05$).

4.7. Discussion

Synthetic oligodeoxynucleotides containing CpG motifs act as immunostimulants and are capable of accelerating and boosting innate and adaptive immunity. CpG-ODN

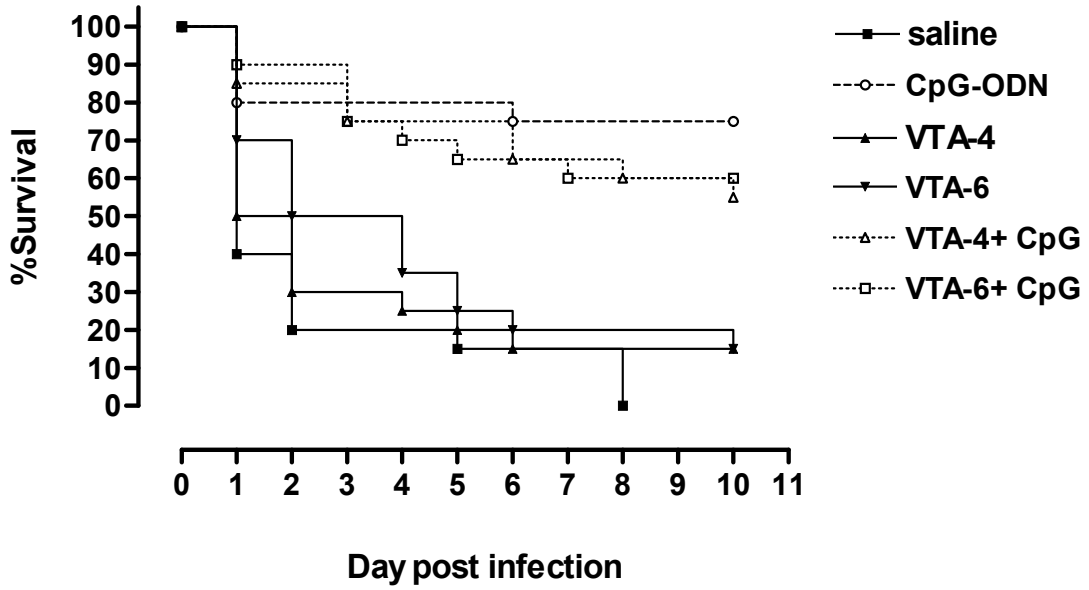


Fig. 4.3. Survival of neonatal chickens following *in ovo* treatment of CpG-ODN formulated with VTA. Chickens were subcutaneously challenged with $2x10^5$ cfu of *E. coli* and treated with CpG-ODN (○), VTA-6 (▼), VTA-4 (▲), CpG-ODN together with VTA-6 (□), CpG-ODN together with VTA-4 (Δ) or saline (■). Birds that received CpG-ODN or CpG-ODN formulated with VTA-6 or VTA-4 showed significantly higher survival rates ($p < 0.01$) compared to groups that received saline ($n = 20$).

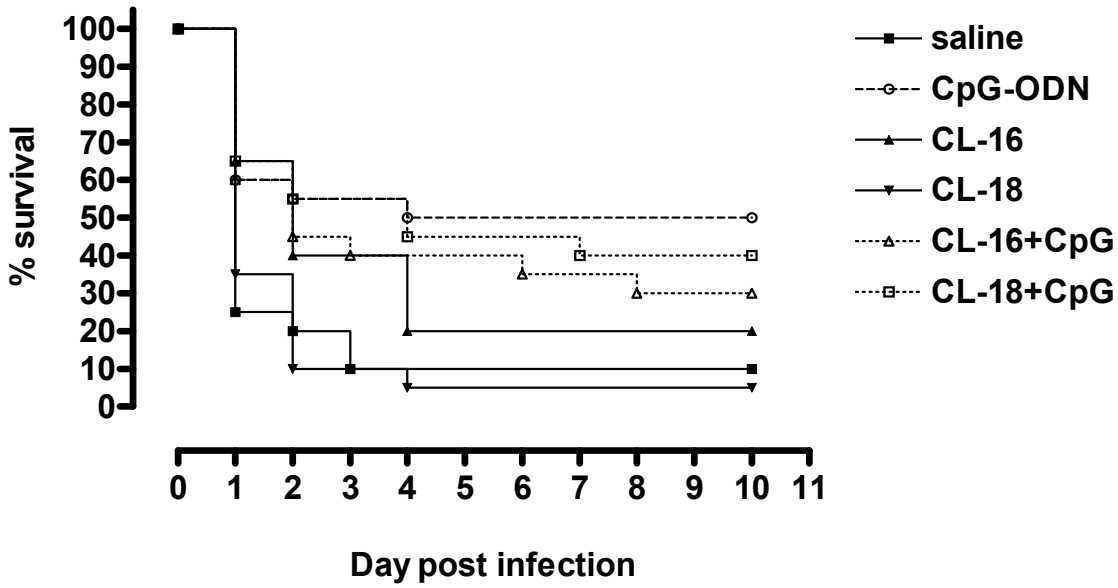


Fig. 4.4. Survival of neonatal chickens following *in ovo* treatment of CpG-ODN formulated with CL. Chickens were subcutaneously challenged with $2x10^4$ cfu of *E. coli* and treated with CpG-ODN (○), CL-18 (▼), CL-16 (▲), CpG-ODN together with CL-18 (□), GpG-ODN together with CL-16 (Δ) or saline (■). Birds that received CpG-ODN or CpG-ODN formulated with Cl-18 or CL-16 showed significantly higher survival rates ($p < 0.01$) compared to groups that received saline ($n = 20$).

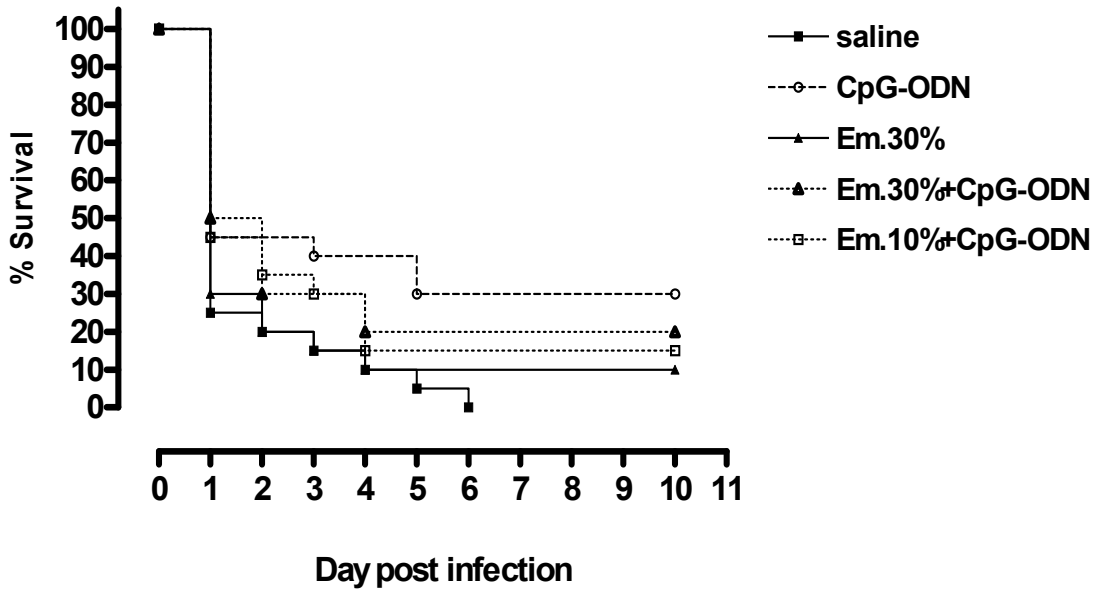


Fig. 4.5. Survival of neonatal chickens following *in ovo* treatment of CpG-ODN formulated with Emulsigen®. Groups of chickens were subcutaneously challenged with $1.5x10^5$ cfu of *E. coli* and treated with CpG-ODN (○), Emulsigen® 30% (▲), CpG-ODN together with Emulsigen® 30% (Δ), CpG-ODN together with Emulsigen® 10% (□) or saline (■). There were no significant differences among groups treated with CpG-ODN together with Emulsigen® 10% and Emulsigen® 30% and the saline group [Em = Emulsigen®] (n = 20).

induces maturation and activation of vertebrate immune cells, which then produce Th 1-type cytokines including TNF-α, IL-12, and IFN-γ. Therefore, CpG-ODN activation contributes to the development of Th 1-type immune responses (Yamamoto 2002; Klinman 2003). The immunoprotective effect of CpG-ODN against *E. coli* septicemia in chickens has been demonstrated previously (Gomis *et al.* 2003; He *et al*. 2005b). However, the effect of formulating CpG-ODN with other adjuvants to enhance activity against bacterial diseases in poultry has not been described. This study demonstrates that formulating CpG-ODN with polyphosphazenes synergizes the effectiveness of CpG-ODN in providing protection against *E. coli* septicemia in neonatal chickens.

Formulating CpG-ODN with appropriate adjuvant constitutes a way to potentate the effects of these immunostimulatory molecules. This effect is likely due to protection of CpG-ODN from degradation while increasing the uptake of CpG-ODN by cells of the immune system (Singh and O'Hagan 2002). The present investigation demonstrates that the polyphosphazenes PCPP and PCEP can enhance CpG-ODN-induced innate immunity and protection against bacterial infections in neonatal chickens. It may be possible to enhance immune stimulation of CpG-ODN by optimizing the ratio of CpG-ODN to PCPP or PCEP. Further studies are required to investigate this possibility. Innate immunity against different infectious agents requires distinct types of immune responses. Although CpG-ODN formulated with polyphosphazenes increase immunoprotective activity against *E. coli* septicemia in neonatal chickens, this combination did not lengthen the duration of protection. We have previously demonstrated that *in ovo* delivery of CpG-ODN without formulation had an immunoprotective effect against *E. coli* infection for a period of six days (Gomis *et al.*

2003). This period of protection was not lengthened by adding polyphospazene formulation of CpG-ODN (data not shown).

It has been described previously that CpG-ODN are effective mucosal adjuvants and that VTA formulations are suitable delivery systems for CpG-ODN by the intra-nasal route (Foss and Murtaugh 1999; McCluskie *et al.* 2000; Gallichan *et al.* 2001). On the other hand, CLs have been used to successfully deliver antisense oligonucleotides to inhibit protein expression in target tumor cells (Zhang *et al.* 2001) and anti-HIV therapy (Duzgunes *et al.* 2001) by facilitating cytoplasmic delivery and protecting against nuclease digestion. Combining CpG-ODN with CLs provides long *in vivo* stability that may enhance cytokine secretion (IL-12, IFN-γ and IL-6) and interaction with APCs (Gursel *et al.* 2001; Sellins *et al*. 2005). We did not see synergism of immunostimulatory activity of CpG-ODN formulated with VTA, CLs or Emulsigen® in our experiments or even an additive effect. It has been well documented that the CpG-ODN-lipid complex can potentially create a depot effect at the site of inoculation, from which CpG-ODN are slowly released and presented to the immune system over a prolonged period. (Foldvar*i et al.* 1999). It is possible that our formulations were not optimized for the amniotic environment and the lipid vesicles containing CpG-ODN, and VTA or CL did not facilitate uptake and transport to the APCs of the mucosal immune systems of the respiratory and intestinal tracts of the developing embryo. However, the mechanism by which VTA or CL formulations of CpG-ODN act in the amniotic cavity is not known and needs further studies. Although our formulations of CpG-ODN with liposomes and Emulsigen® did not increase the immunoprotective effect, we did not see any reduced hatchability of chicken embryos because of these

formulations.

In conclusion, CpG-ODN formulated with polyphosphazenes and delivered by the *in ovo* route to eggs at day 18 of incubation resulted in an increased immunoprotective effect against *E. coli* infections in neonatal chickens. Protection of neonatal chickens against *E. coli* infection by administration of CpG-ODN is likely mediated by IFN-γ and IL-18 mediated pathways (Patel *et al.* 2007). In addition, we have demonstrated that *in ovo* administration of CpG-ODN to incubating eggs is a safe and feasible method of delivery. There are clear advantages to using *in ovo* delivery methods. Historically, many immunizations in the poultry industry were subcutaneous injections administered to day-old chickens. Those techniques have now been replaced with *in ovo* technology, which is considered less invasive. Further work is needed to understand the mechanisms by which CpG-ODN and polyphosphazenes formulations enhance the immunoprotective activity of CpG-ODN in the amniotic cavity of chicken embryos.

5.0. GENERAL SUMMARY AND DISCUSSION

The chicken mucosal immune system does not fully mature until weeks after hatch. Indeed, the enteric immune system is very poorly developed in newly hatched chickens (Barrow *et al.* 1987). Therefore, chickens under three days of age, which experience severe systemic diseases, experience high morbidity and mortality. Among those diseases that target newly hatched chicks, salmonellosis due to *Salmonella* Typhimurium and colibacillosis due to *E. coli* are the most economically important (Barnes 2003; Withanage *et al.* 2005). Many efforts have been made to reduce mortality due to *Salmonella* Typhimurium and *E. coli* septicemias in neonatal chickens, but with limited success. Control measures which are recommended to reduce the economic loss includes strict biosecurity, probiotics, and antibiotic therapy. However, these measures also increase the production cost. Moreover, the use of antibiotics in reducing mortality of neonatal chickens increases the possibility of the emergence of antibiotic resistant strains of bacteria. Recently, poultry companies have started raising chickens without antibiotics. To achieve this goal, they must increasingly depend on vaccines (Souza 2007). This also increases production expenses. These increased costs are passed on to the consumer. Therefore, the poultry industry is looking for alternative approaches for controlling mortality in neonatal chickens that would simultaneously reduce the use of

antibiotics and production cost. Recently, immunostimulants have been considered as possible alternatives to minimize losses due to bacterial infection in newly hatched chicks.

Immune systems containing cells, chemokines, cytokines, and immunoglobulins play a key role in the protective responses against diseases such as, salmonellosis and colibacillosis in poultry (Kariyawasam *et al.* 2002; Withanage *et al*. 2005). Clearance of primary infection by *Salmonella* Typhimurium in the chicken appears to involve mostly IL-1-β, IL-6, and IFN-γ mediated TC (primarily Th 1) responses (He *et al.* 2003; Withanage *et al.* 2005). *E. coli* induce a Th 1 like response with abundant IFN-γ, IgG, IgA, IgM, and IL-12, but little or no IL-4, IL-13, and IL-5 and activate macrophages and heterophils, which play a key role in fighting *E. coli* infections in chickens (Kariyawasam *et al.* 2002; Mellata *et al.* 2003). Therefore, using innate immune stimulators that promote rapid responses to microbial pathogens in neonatal chickens is of great interest to the poultry industry.

For nearly a century it has been known that bDNA extracts have immunostimulatory and therapeutic effects (Morales 1978; Tokunaga *et al.* 1992). Bacterial DNA extracts activate NK cells and produce type 1 and type 2 interferons (Tokunaga *et al.* 1992; Yamamoto *et al.* 1992). Unmethylated CpG motifs in bDNA trigger protective pathways similar to those activated by PRRs and detect endotoxins and other microbial products (Krieg 2002). In addition, they are responsible for stimulation of lymphocyte proliferation and variable effects on immunoglobulin production in oligodeoxynucleotides with a PS backbone (Bird 1993). Immune recognition of CpG motifs are shown to increase production of BCs, NK cells, and DCs

(Krieg 2002). Due to the strength and nature of this stimulation, CpG-ODN has been investigated for immune therapeutic and protective use in a variety of animal species (Krieg 2002).

It has been demonstrated that CpG-ODN has an immunoprotective effect against *E. coli* that often infects neonatal broiler chickens (Gomis *et al.* 2004). However, studies on the effect of CpG-ODN against *Salmonella* in neonatal chickens were limited to *in vitro* studies of CpG-ODN against *Salmonella* Enteritidis (He *et al.* 2005b). In order to study the *in vivo* affect of CpG-ODN against *Salmonella* Typhimurium, an intracellular bacterium, we first developed an animal model in neonatal broiler chickens using avian and mammalian isolates of *Salmonella* Typhimurium. Both human and chicken isolates produced salmonellosis with high mortality and similar pathologic lesions of pericarditis, perihepatitis, airsacculitis, and polyserositis in neonatal chickens because the two isolates shared the same virulent factor genes (15 out of 15). In contrast, the house sparrow isolate failed to induce mortality in neonatal chickens, possibly due to the lack of two of 15 virulent factor genes present in the other isolates (personal communication-Allan, B.).

Stationary phase *Salmonella* Typhimurium was chosen as our challenge at a dose of $1x10^6$ and $1x10^7$ cfu. This challenge produced clinical disease and mortality at a level that was sufficient for our future experiments. We also demonstrated that clinical salmonellosis and pathological lesions in this animal model were not associated with the LPS of *Salmonella* Typhimurium, but rather with live *Salmonella* Typhimurium septicemia.

Administration of 50 μg of CpG-ODN intramuscularly (leg), one to three days

prior to challenge with *Salmonella* Typhimurium increased the protection against septicemia. This protection was shown to last for at least six days. Birds treated with CpG-ODN had lower bacterial loads of *Salmonella* Typhimurium and fewer associated pathologic lesions and clinical signs when compared to birds treated with saline. By day six of age, CpG-ODN completely cleared *Salmonella* Typhimurium from peripheral blood of treated chicks. Recently, it has been demonstrated that systemic clearance of *Salmonella* Typhimurium is partially associated with cellular responses such as secretion of IFN-γ mediated TCs in chickens (Withanage *et al.* 2005). Therefore, it can be implied that the immune stimulatory effect of CpG-ODN against *Salmonella* Typhimurium is primarily Th 1 type mediated.

Over the past two decades, the rapidly growing poultry industry has accepted *in ovo* injection to control Marek's disease and administer antibiotics as preventive measures (Johnston *et al.* 1997). A number of immune and antibody responses can be induced in embryos at 12-14 days of incubation, soon after the emergence of T and B lymphocyte cell progenitors (Houssaint *et al*. 1991; Cormier 1993). The *in ovo* method provides early accelerated immune response that may improve protection against poultry diseases (Gagic *et al.* 1999). Recently, it was demonstrated that *in ovo* delivery of CpG-ODN protected neonatal broiler chickens against *E. coli* septicemia (Gomis *et al.* 2004). The present investigation demonstrated that *in ovo* delivery of CpG-ODN at day 18 of incubation protected neonatal chickens against *Salmonella* Typhimurium infection. It is hoped that *in ovo* delivery of 50 μg of CpG-ODN could protect neonatal chickens against *E. coli* and *Salmonella* Typhimurium septicemia under field conditions. This would be very advantageous to the poultry industry.

During the last decade, the efficacy of combining CpG-ODN with adjuvants to enhance the immunoprotective effect of vaccines has been investigated. Our research is the first to demonstrate that CpG-ODN formulated with polyphosphazenes such as PCPP and PCEP enhances innate immunity against bacterial infections in neonatal chickens. However, further studies are required to investigate the mechanisms by which CpG-ODN formulated with PCPP or PCEP enhance immune protection against *E. coli* septicemia. While CpG-ODN formulated with PCPP or PCEP enhanced immune protection against *E. coli* septicemia, the duration of protection was not increased. On the other hand, there was no significant difference between the survival rates of birds that received CpG-ODN formulated with polyphosphazene groups and groups treated with CpG-ODN alone ($p = 0.35$). It is possible that the ratio of CpG-ODN to PCPP or PCEP used in this experiment was not optimal. Further research is required to determine the optimal ratio.

In our study, the respective effects of *in ovo* delivery of CpG-ODN formulated in liposomic adjuvants such as VTA and CLs, and CpG-ODN formulated with Emulsigen® were also investigated. VTA has been shown previously to improve the adjuvant activity of CpG-ODN following intranasal delivery (Babiuk *et al.* 2004) or systemic administration in pigs (Alcon *et al.* 2003). CLs are known to facilitate cytoplasmic delivery of CpG-ODN, as they protect CpG-ODN from nuclease digestion and potentiate activation of innate immunity (Briane *et al.* 2002; Rocha *et al.* 2002). Therefore, the incorporation of CpG-ODN with liposomes provides longer *in vivo* stability that may result in enhanced cytokine secretion (IL-12, IFN-γ and IL-6) (Lu 1996b). Emulsigen®, which has been known to have longer deposition at the site of

immunization, is used in inactivated bacterial or viral vaccines with the potential to elicit higher levels of antibodies (Foldvari 1998). In each of our experiments, *in ovo* delivery of formulated CpG-ODN with VTA, CL and Emulsigen® did not enhance protection against *E. coli* infection compared to CpG-ODN alone. It is likely that these adjuvants cannot improve the adjuvant activity of CpG-ODN following *in ovo* delivery and our formulations may not have been optimized for the environment in the amniotic cavity where chicken embryos were developing. However, we did not observe poor hatchability of chicken embryos using these formulations suggesting that they are safe *in ovo*.

In summary, CpG-ODN was a significant immunoprotective agent against *Salmonella* Typhimurium infection in neonatal broiler chickens. Intramuscular injection at day one of age or *in ovo* delivery of 50 µg of CpG-ODN at day 18 of incubation significantly increased the survival rate ($p < 0.01$) when compared to the control treatments groups. We have also demonstrated that *in ovo* delivery of 50 µg of CpG-ODN formulated with polyphosphazenes such as PCPP and PCEP is a safe method for increasing the immunoprotective effect against *E. coli* infections of neonatal chickens. These findings provide a foundation for the development of immune stimulants for the protection against bacterial infections that have the potential to replace or supplement antibiotic therapy.

6.0. REFERENCES

Ackermann, M.R. and Cheville, N.F. (1991) Ultrastructural studies of the lung of turkeys (Meleagris gallopavo) inoculated intratracheally with *Escherichia coli.* Vet Pathol **28**, 183-191.

Aguado, T., Engers, H., Pang, T., and Pink, R. (1999) Novel adjuvants currently in clinical testing November 2-4, 1998, Fondation Merieux, Annecy, France: a meeting sponsored by the World Health Organization. Vaccine **17**, 2321-2328.

Ahmer, B.M., Tran, M., and Heffron, F. (1999) The virulence plasmid of *Salmonella* Typhimurium is self-transmissible. J Bacteriol **181**, 1364-1368.

Akira, S., Takeda, K., and Kaisho, T. (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol **2**, 675-680.

Alcon, V., Baca-Estrada, M., Vega-Lopez, M., Willson, P., Babiuk, L.A., Kumar, P., Hecker, R., and Foldvari, M. (2005a) Mucosal delivery of bacterial antigens and CpG oligonucleotides formulated in biphasic lipid vesicles in pigs. AAPS J **7**, E566-571.

Alcon, V.L., Baca-Estrada, M., Vega-Lopez, M.A., Willson, P., Babiuk, L.A., Kumar, P., and Foldvari, M. (2005b) Intranasal immunization using biphasic lipid vesicles as delivery systems for OmlA bacterial protein antigen and CpG oligonucleotides adjuvant in a mouse model. J Pharm Pharmacol **57**, 955-962.

Alcon, V.L., Baca-Estrada, M.E., Potter, A., Babiuk, L.A., Kumar, P., and Foldvari, M. (2006) Biphasic lipid vesicles as a subcutaneous delivery system for protein antigens and CpG oligonucleotides. Curr Drug Deliv **3**, 129-135.

Alcon, V.L., Foldvari, M., Snider, M., Willson, P., Gomis, S., Hecker, R., Babiuk, L.A., and Baca-Estrada, M.E. (2003) Induction of protective immunity in pigs after immunisation with CpG oligodeoxynucleotides formulated in a lipid-based delivery system (Biphasix). Vaccine **21**, 1811-1814.

Allcock, H.R. (1990) Polyphosphazenes as new biomedical and bioactive materials In: Biodegradable polymers as drug delivery system eds. M. Chasin, R.Langer. pp.163-193. New York: Marcel Dekker.

Alter-Koltunoff, M., Goren, S., Nousbeck, J., Feng, C.G., Sher, A., Ozato, K., Azriel, A. and Levi, B.Z. (2007) Innate immunity to intraphagosomal pathogens is mediated by IRF-8 that stimulates the expression of macrophage specific NRAMP1 through antagonizing repression by C-MYC. J Biol Chem **5**, 2724-2733.

Ameiss, K.A., El Attrache, J., Barri, A., McElroy, A.P., and Caldwell, D.J. (2006) Influence of orally administered CpG-ODNs on the humoral response to bovine serum albumin (BSA) in chickens. Vet Immunol Immunopathol **110**, 257-267.

Andrianov, A.,K. and Le Golvan, P.M. (1998) Characterization of poly [di (carboxylatophenoxy) phosphazene] by an aqueous gel permeation chromatography. J Appl Polymer Sci **60**, 2289-2295.

Andrianov, A.K., Marin, A., and Chen, J. (2006) Synthesis, properties, and biological activity of poly [di (sodium carboxylatoethylphenoxy) phosphazene]. Biomacromolecules **7**, 394-399.

Andrianov, A.K., Svirkin, Y.Y., and LeGolvan, M.P. (2004) Synthesis and biologically relevant properties of polyphosphazene polyacids. Biomacromolecules **5**, 1999-2006.

Arp, L.H. (1980) Consequences of active or passive immunization of turkeys against *Escherichia coli* O78. Avian Dis **24**, 808-815.

Ashkar, A.A. and Rosenthal, K.L. (2002) Toll-like receptor 9, CpG DNA and innate immunity. Curr Molecular Med **2**, 545-556.

Aucouturier, J., Dupuis, L., and Ganne, V. (2001) Adjuvants designed for veterinary and human vaccines. Vaccine **19**, 2666-2672.

Babiuk, S., Baca-Estrada, M.E., Middleton, D.M., Hecker, R., Babiuk, L.A., and Foldvari, M. (2004) Biphasic lipid vesicles (Biphasix) enhance the adjuvanticity of CpG oligonucleotides following systemic and mucosal administration. Curr Drug Deliv **1**, 9-15.

Bailey, J.S., Cox, N.A., and Berrang, M.E. (1994) Hatchery-acquired *Salmonellae* in broiler chicks. Poul Sci **73**, 1153-1157.

Ballas, Z.K., Rasmussen, W.L., and Krieg, A.M. (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J Immunol **157**, 1840-1845.

Ban, E., Dupre, L., Hermann, E., Rohn, W., Vendeville, C., Quatannens, B., Ricciardi-Castagnoli, P., Capron, A., and Riveau, G. (2000) CpG motifs induce Langerhans cell migration *in vivo*. Int Immunol **12**, 737-745.

Barnes, H.J., Vallancourt, J., and Gross, W.B. (2003) Collibacillosis. In: Diseases of Poultry eds. Y. M. Saif, H.J.Barones, J. R. Gillson, A. M. Fadly, L. R. McDougald, and D. E. Swayne. pp.631-656. Ames, Iowa: BlackWell Publishing Co.

Barnes, P.J. (2001) Th 2 cytokines and asthma: an introduction. Respir Res **2**, 64-65.

Barrat, F.J., Meeker, T., Gregorio, J., Chan, J.H., Uematsu, S., Akira, S., Chang, B., Duramad, O. and Coffman, R.L. (2005) Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. J Exp Med **202**, 1131-1139.

Barrow, P.A. (2000) The paratyphoid *Salmonellae*. Rev Sci Tech **19**, 351-375.

Barrow, P.A., Huggins, M.B., Lovell, M.A., and Simpson, J.M. (1987) Observations on the pathogenesis of experimental *Salmonella* Typhimurium infection in chickens. Res Vet Sci **42**, 194-199.

Barry, A.L. (1972) Infectious diseases: a guide to understanding and management of infectious processes. In: Clinical Specimens for Microbiologic Examination ed. Haeprich, P.D. pp.103-107. New York, NY: Harper and Row Publishing Co.

Bauer, S., Kirschning, C.J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H. and Lipford, G.B. (2001) Human TLR 9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci USA **98**, 9237-9242.

Beal, R.K., Wigley, P., Powers, C., Barrow, P.A., and Smith, L.A. (2006) Cross-reactive cellular and humoral immune responses to *Salmonella enterica* serovars Typhimurium and Enteritidis are associated with protection to heterologous re-challenge. Vet Immunol Immunopathol **114**, 84-93.

Beal, R.K., Wigley, P., Powers, C., Hulme, S.D., Barrow, P.A., and Smith, A.L. (2004) Age at primary infection with *Salmonella enterica* serovar Typhimurium in the chicken influences persistence of infection and subsequent immunity to re-challenge. Vet Immunol Immunopathol **100**, 151-164.

Berry, L.J., Hickey, D.K., Skelding, K.A., Bao, S., Rendina, A.M., Hansbro, P.M., Gockel, C.M., and Beagley, K.W. (2004) Transcutaneous immunization with combined cholera toxin and CpG adjuvant protects against *Chlamydia muridarum* genital tract infection. Infect Immun **72**, 1019-1028.

Bird, A.P. (1993) Functions for DNA methylation in vertebrates. Cold Spring Harb Symp Quant Biol **58**, 281-285.

Blanc-Potard, A.B. and Groisman, E.A. (1997) The *Salmonella* selC locus contains a pathogenicity island mediating intramacrophage survival. EMBO J **16**, 5376-5385.

Blum, G., Ott, M., Lischewski, A., Ritter, A., Imrich, H., Tschape, H., and Hacker, J. (1994) Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. Infect Immun **62**, 606-614.

Boyd, Y., Goodchild, M., Morroll, S., and Bumstead, N. (2001) Mapping of the chicken and mouse genes for Toll-like receptor 2 (TLR 2) to an evolutionarily conserved chromosomal segment. Immunogenetics **52**, 294-298.

Briane, D., Lesage, D., Cao, A., Coudert, R., Lievre, N., Salzmann, J.L., and Taillandier, E. (2002) Cellular pathway of plasmids vectorized by cholesterol-based cationic liposomes. J Histochem Cytochem **50**, 983-991.

Carrington, A.C. and Secombes, C.J. (2006) A review of CpGs and their relevance to aquaculture. Vet Immunol Immunopathol **112**, 87-101.

Carter, M.E., and Chengappa, M. M. (1990) Entererobacteria. In: Diagnostic procedures in veterinary bacteriology and mycology. eds. CG. R. Carter, J.R.Cole, Jr. pp.107-128. San diego, CA: Harcourt Brace Jovanovich Publishers.

Cheville, N.F. and Arp, L.H. (1978) Comparative pathologic findings of *Escherichia coli* infection in birds. J Am Vet Med Assoc **173**, 584-587.

Chu, R.S., Askew, D., Noss, E.H., Tobian, A., Krieg, A.M., and Harding, C.V. (1999) CpG oligodeoxynucleotides down-regulate macrophage class II MHC antigen processing. J Immunol **163**, 1188-1194.

Cloud, S.S., Rosenberger, J.K., Fries, P.A., Wilson, R.A., and Odor, E.M. (1985) *In vitro* and *in vivo* characterization of avian *Escherichia coli.* I. Serotypes, metabolic activity, and antibiotic sensitivity. Avian Dis **29**, 1084-1093.

Cormier, F. (1993) Avian pluripotent haemopoietic progenitor cells: detection and enrichment from the para-aortic region of the early embryo. J Cell Sci **105**, 661-666.

Cowdery, J.S., Chace, J.H., Yi, A.K., and Krieg, A.M. (1996) Bacterial DNA induces NK cells to produce IFN-gamma *in vivo* and increases the toxicity of lipopolysaccharides. J Immunol **156**, 4570-4575.

Cox, J.C. and Coulter, A.R. (1997) Adjuvants--a classification and review of their modes of action. Vaccine **15**, 248-256.

D'Souza, S., Rosseels, V., Denis, O., Tanghe, A., De Smet, N., Jurion, F., Palfliet, K., Castiglioni, N., Vanonckelen, A., Wheeler, C., and Huygen, K. (2002) Improved tuberculosis DNA vaccines by formulation in cationic lipids. Infect Immun **70**, 3681-3688.

Dalloul, R.A., Lillehoj, H.S., Okamura, M., Xie, H., Min, W., Ding, X., and Heckert, R.A. (2004) *In vivo* effects of CpG oligodeoxynucleotide on *Eimeria* infection in chickens. Avian Dis **48**, 783-790.

Davis, D., Davies, A. and Gregoriadis, G. (1987) Liposomes as adjuvants with immunopurified tetanus toxoid: the immune response. Immunol Lett **14**, 341-348.

Davis, H.L., Suparto, II, Weeratna, R.R., Jumintarto, Iskandriati, D.D., Chamzah, S.S., Ma'ruf, A.A., Nente, C.C., Pawitri, D.D., Krieg, A.M., Heriyanto, Smits, W., and Sajuthi, D.D. (2000) CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. Vaccine **18**, 1920-1924.

Davis, H.L., Weeratna, R., Waldschmidt, T.J., Tygrett, L., Schorr, J., and Krieg, A.M. (1998) CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J Immunol **160**, 870-876.

Deng, G.M., Liu, Z.Q., and Tarkowski, A. (2001) Intracisternally localized bacterial DNA containing CpG motifs induces meningitis. J Immunol **167**, 4616-4626.

Deng, J.C., Moore, T.A., Newstead, M.W., Zeng, X., Krieg, A.M., and Standiford, T.J. (2004) CpG oligodeoxynucleotides stimulate protective innate immunity against pulmonary *Klebsiella* infection. J Immunol **173**, 5148-5155.

Dho-Moulin, M. and Fairbrother, J.M. (1999) Avian pathogenic *Escherichia coli* (APEC). Vet Res **30**, 299-316.

Dho, M. and Lafont, J.P. (1984) Adhesive properties and iron uptake ability in *Escherichia coli* lethal and nonlethal for chicks. Avian Dis **28**, 1016-1025.

Dong, J.L., Liang, B.G., Jin, Y.S., Zhang, W.J., and Wang, T. (2005) Oral immunization with pBsVP6-transgenic alfalfa protects mice against rotavirus infection. Virol **339**, 153-163.

Duzgunes, N., Simoes, S., Konopka, K., Rossi, J.J., and Pedroso de Lima, M.C. (2001) Delivery of novel macromolecular drugs against HIV-1. Exp Opin Biol Ther **1**, 949-970.

Ellis, M.G., Arp, L.H., and Lamont, S.J. (1988) Serum resistance and virulence of *Escherichia coli* isolated from turkeys. Am J Vet Res **49**, 2034-2037.

Euzéby, J.P. (1999) Revised *Salmonella* nomenclature: designation of *Salmonella enterica* (ex Kauffmann and Edwards 1952) Le Minor and Popoff 1987 sp. nov., nom. rev. as the neotype species of the genus *Salmonella* Lignieres 1900 (Approved Lists 1980), rejection of the name *Salmonella* Choleraesuis (Smith 1894) Weldin 1927 (Approved Lists 1980), and conservation of the name *Salmonella* Typhi (Schroeter 1886) Warren and Scott 1930 (Approved Lists 1980). Request for an Opinion. Int J Syst Bacteriol **49,** 927–930.

Fang, F. and Vazquez-Torres, A. (2002) *Salmonella* selectively stops traffic. Trends Microbiol **10**, 391-392.

Fantinatti, F., Silveira, W.D., and Castro, A.F. (1994) Characteristics associated with pathogenicity of avian septicaemic *Escherichia coli* strains. Vet Microbiol **41**, 75-86.

Foldvari, M., Baca-Estrada, M.E., He, Z., Hu, J., Attah-Poku, S., and King, M. (1999) Dermal and transdermal delivery of protein pharmaceuticals: lipid-based delivery systems for interferon alpha. Biotechnol Appl Biochem **30**, 129-137.

Foldvari, M. and Baca-Estrada, M. (1998) Biphasic lipid vesicle composition for transdermal administrtion of an immunogen. In: US Patent. pp.755-780. Canada: Pharma Derm Lab. LTD.

Foss, D.L. and Murtaugh, M.P. (1999) Mucosal immunogenicity and adjuvanticity of cholera toxin in swine. Vaccine **17**, 788-801.

Fukui, A., Inoue, N., Matsumoto, M., Nomura, M., Yamada, K., Matsuda, Y., Toyoshima, K., and Seya, T. (2001) Molecular cloning and functional characterization of chicken Toll-like receptors. A single chicken toll covers multiple molecular patterns. J Biol Chem **276**, 47143-47149.

Gagic, M., St Hill, C.A., and Sharma, J.M. (1999) *In ovo* vaccination of specific-pathogen-free chickens with vaccines containing multiple agents. Avian Dis **43**, 293-301.

Gallichan, W.S., Woolstencroft, R.N., Guarasci, T., McCluskie, M.J., Davis, H.L., and Rosenthal, K.L. (2001) Intranasal immunization with CpG oligodeoxynucleotides as an adjuvant dramatically increases IgA and protection against herpes simplex virus-2 in the genital tract. J Immunol **166**, 3451-3457.

Gast, R.K. (2003) Paratyphoid infections. In*:* Diseases of Poultry eds. Y. M. Saif, H.J.Barones, J. R. Glisson, A. M. Fadly, L. R. McDougald, and D. E. Swayne. pp.583-613. Ames, Iowa: BlackWell Publishing Co.

Gast, R.K. and Beard, C.W. (1989) Age-related changes in the persistence and pathogenicity of *Salmonella* Typhimurium in chicks. Poul Sci **68**, 1454-1460.

Genovese, K.J., He, H., Lowry, V.K., Nisbet, D.J., and Kogut, M.H. (2007) Dynamics of the avian inflammatory response to *Salmonella* following administration of the Toll-like receptor 5 agonist flagellin. FEMS Immunol Med Microbiol **51**, 112-117.

Giovanardi, D., Campagnari, E., Ruffoni, L.S., Pesente, P., Ortali, G., and Furlattini, V. (2005) Avian pathogenic *Escherichia coli* transmission from broiler breeders to their progeny in an integrated poultry production chain. Avian Pathol **34**, 313-318.

Golemboski, K.A., Bloom, S.E., and Dietert, R.R. (1992) Assessment of neonatal avian inflammatory macrophage function following embryonic cyclophosphamide exposure. Int J Immunopharmacol **14**, 19-26.

Gomis, S., Babiuk, L., Allan, B., Willson, P., Waters, E., Ambrose, N., Hecker, R., and Potter, A. (2004) Protection of neonatal chicks against a lethal challenge of *Escherichia coli* using DNA containing cytosine-phosphodiester-guanine motifs. Avian Dis **48**, 813-822.

Gomis, S., Babiuk, L., Allan, B., Willson, P., Waters, E., Hecker, R., and Potter, A. (2007) Protection of chickens against a lethal challenge of *Escherichia coli* by a vaccine containing CpG oligodeoxynucleotides as an adjuvant. Avian Dis **51**, 78-83.

Gomis, S., Babiuk, L., Godson, D.L., Allan, B., Thrush, T., Townsend, H., Willson, P., Waters, E., Hecker, R., and Potter, A. (2003) Protection of chickens against *Escherichia coli* infections by DNA containing CpG motifs. Infect Immun **71**, 857-863.

Gordon, R.F. and Tucker, J.F. (1965) The epizootiology of *Salmonella* Menston infection of fowls and the effect of feeding poultry food artificially infected with *Salmonella*. Br Poul Sci **6**, 251-264.

Gramzinski, R.A., Doolan, D.L., Sedegah, M., Davis, H.L., Krieg, A.M., and Hoffman, S.L. (2001) Interleukin-12- and gamma interferon-dependent protection against malaria conferred by CpG oligodeoxynucleotide in mice. Infect Immun **69**, 1643-1649.

Gray, J.T., Fedorka-Cray, P.J., Stabel, T.J., and Kramer, T.T. (1996) Natural transmission of *Salmonella* Choleraesuis in swine. Appl Environ Microbiol **62**, 141-146.

Gregoriadis, G. (1990) Immunological adjuvants: a role for liposomes. Immunol Today **11**, 89-97.

Gulig, P.A. (1990) Virulence plasmids of *Salmonella* Typhimurium and other *salmonellae*. Microb Pathog **8**, 3-11.

Gupta, R.K. (1998) Aluminum compounds as vaccine adjuvants. Adv Drug Deliv Rev **32**, 155-172.

Gupta, R.K., Relyveld, E.H., Lindblad, E.B., Bizzini, B., Ben-Efraim, S., and Gupta, C.K. (1993) Adjuvants--a balance between toxicity and adjuvanticity. Vaccine **11**, 293-306.

Gursel, I., Gursel, M., Ishii, K.J., and Klinman, D.M. (2001) Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligonucleotides. J Immunol **167**, 3324-3328.

Gursel, M., Verthelyi, D., Gursel, I., Ishii, K.J., and Klinman, D.M. (2002) Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide. J Leukoc Biol **71**, 813-820.

Guy, B., Pascal, N., Francon, A., Bonnin, A., Gimenez, S., Lafay-Vialon, E., Trannoy, E., and Haensler, J. (2001) Design, characterization and preclinical efficacy of a cationic lipid adjuvant for influenza split vaccine. Vaccine **19**, 1794-1805.

Gyles, C.L. and Fairbrother, J. M. (2004) *Escherichia coli*. In: Pathogenesis of bacterial infections in animal eds. C. L. Gyles, J.F.Prescott, J. G. Songer, and C. O. Thoen. pp.193-223. Ames, Iowa: BlackWell Publishing Co.

Hald, T., Vose, D., Wegener, H.C., and Koupeev, T. (2004) A Bayesian approach to quantify the contribution of animal-food sources to human salmonellosis. Risk Anal **24**, 255-269.

Han, J., Zhu, Z., Hsu, C., and Finley, W.H. (1994) Selection of antisense oligonucleotides on the basis of genomic frequency of the target sequence. Antisense Res Dev **4**, 53-65.

Haque, A., Bowe, F., Fitzhenry, R.J., Frankel, G., Thomson, M., Heuschkel, R., Murch, S., Stevens, M.P., Wallis, T.S., Phillips, A.D., and Dougan, G. (2004) Early interactions of *Salmonella enterica* serovar Typhimurium with human small intestinal epithelial explants. Gut **53**, 1424-1430.

Harandi, A.M., Eriksson, K., and Holmgren, J. (2003) A protective role of locally administered immunostimulatory CpG oligodeoxynucleotide in a mouse model of genital herpes infection. J Virol **77**, 953-962.

Harry, E.G. and Hemsley, L.A. (1965) The relationship between environmental contamination with septicemia strains of *Escherichia coli* and their incidence in chickens. Vet Rec **77**, 241-245.

Hartmann, G., Battiany, J., Poeck, H., Wagner, M., Kerkmann, M., Lubenow, N., Rothenfusser, S., and Endres, S. (2003) Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-alpha induction in plasmacytoid dendritic cells. Eur J Immunol **33**, 1633-1641.

Hartmann, G. and Krieg, A.M. (1999) CpG DNA and LPS induce distinct patterns of activation in human monocytes. Gene Ther **6**, 893-903.

Hartmann, G. and Krieg, A.M. (2000) Mechanism and function of a newly identified CpG DNA motif in human primary B cells. J Immunol **164**, 944-953.

He, H., Crippen, T.L., Farnell, M.B., and Kogut, M.H. (2003) Identification of CpG oligodeoxynucleotide motifs that stimulate nitric oxide and cytokine production in avian macrophage and peripheral blood mononuclear cells. Dev Comp Immunol **27**, 621-627.

He, H., Genovese, K.J., Nisbet, D.J., and Kogut, M.H. (2006) Profile of Toll-like receptor expressions and induction of nitric oxide synthesis by Toll-like receptor agonists in chicken monocytes. Mol Immunol **43**, 783-789.

He, H., Genovese, K.J., Nisbet, D.J., and Kogut, M.H. (2007) Synergy of CpG oligodeoxynucleotide and double-stranded RNA (poly I:C) on nitric oxide induction in chicken peripheral blood monocytes. Mol Immunol **44**, 3234-3242.

He, H. and Kogut, M.H. (2003) CpG-ODN-induced nitric oxide production is mediated through clathrin-dependent endocytosis, endosomal maturation, and activation of PKC, MEK1/2 and p38 MAPK, and NF-kappaB pathways in avian macrophage cells (HD11). Cell Signal **15**, 911-917.

He, H., Lowry, V.K., Ferro, P.J., and Kogut, M.H. (2005a) CpG-oligodeoxynucleotide-stimulated chicken heterophil degranulation is serum cofactor and cell surface receptor dependent. Dev Comp Immunol **29**, 255-264.

He, H., Lowry, V.K., Swaggerty, C.L., Ferro, P.J., and Kogut, M.H. (2005b) *In vitro* activation of chicken leukocytes and *in vivo* protection against *Salmonella* Enteritidis organ invasion and peritoneal *Salmonella* Enteritidis infection-induced mortality in neonatal chickens by immunostimulatory CpG oligodeoxynucleotide. FEMS Immunol Med Microbiol **43**, 81-89.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000) A Toll-like receptor recognizes bacterial DNA. Nature **408**, 740-745.

Hensel, M., Shea, J.E., Gleeson, C., Jones, M.D., Dalton, E., and Holden, D.W. (1995) Simultaneous identification of bacterial virulence genes by negative selection. Science **269**, 400-403.

Henzler, D.J., Ebel, E., Sanders, J., Kradel, D., and Mason, J. (1994) *Salmonella* Enteritidis in eggs from commercial chicken layer flocks implicated in human outbreaks. Avian Dis **38**, 37-43.

Herwald, H., Morgelin, M., Olsen, A., Rhen, M., Dahlback, B., Muller-Esterl, W., and Bjorck, L. (1998) Activation of the contact-phase system on bacterial surfaces--a clue to serious complications in infectious diseases. Nat Med **4**, 298-302.

Higgs, R., Cormican, P., Cahalane, S., Allan, B., Lloyd, A.T., Meade, K., James, T., Lynn, D.J., Babiuk, L.A., and O'Farrelly, C. (2006) Induction of a novel chicken Toll-like receptor following *Salmonella enterica* serovar Typhimurium infection. Infect Immun **74**, 1692-1698.

Hilgers, L.A., Nicolas, I., Lejeune, G., Dewil, E., and Boon, B. (1998) Effect of various adjuvants on secondary immune response in chickens. Vet Immunol Immunopathol **66**, 159-171.

Hoiseth, S.K. and Stocker, B.A. (1981) Aromatic-dependent *Salmonella* Typhimurium are non-virulent and effective as live vaccines. Nature **291**, 238-239.

Holt, P.S. and Porter, R.E., Jr. (1992) Microbiological and histopathological effects of an induced-molt fasting procedure on a *Salmonella* Enteritidis infection in chickens. Avian Dis **36**, 610-618.

Hoop, R.K. and Pospischil, A. (1993) Bacteriological, serological, histological and immunohistochemical findings in laying hens with naturally acquired *Salmonella* Enteritidis phage type 4 infection. Vet Rec **133**, 391-393.

Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorfer, B., Giese, T., Endres, S., and Hartmann, G. (2002) Quantitative expression of Toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol **168**, 4531-4537.

Houssaint, E., Mansikka, A., and Vainio, O. (1991) Early separation of B and T lymphocyte precursors in chick embryo. J Exp Med **174**, 397-406.

Hughes, J.A., Avrutskaya, A.V., and Juliano, R.L. (1994) Influence of base composition on membrane binding and cellular uptake of 10-mer phosphorothioate oligonucleotides in Chinese hamster ovary (CHRC5) cells. Antisense Res Dev **4**, 211-215.

Impey, C.S. and Mead, G.C. (1989) Fate of *salmonellas* in the alimentary tract of chicks pre-treated with a mature cecal microflora to increase colonization resistance.
J App Bacteriol **66**, 469-475.

Ioannou, X.P., Griebel, P., Hecker, R., Babiuk, L.A., and van Drunen Littel-van den Hurk, S. (2002) The immunogenicity and protective efficacy of bovine herpesvirus 1 glycoprotein D plus Emulsigen are increased by formulation with CpG oligodeoxynucleotides. J Virol **76**, 9002-9010.

Ioannou, X.P., Griebel, P., Mena, A., Gomis, S.M., Godson, D.L., Mutwiri, G., Hecker, R., Babiuk, L.A., and van Drunen Littel-van den Hurk, S. (2003) Safety of CpG oligodeoxynucleotides in veterinary species. Antisense Nucleic Acid Drug Dev **13**, 157-167.

Iqbal, M., Philbin, V.J., Withanage, G.S., Wigley, P., Beal, R.K., Goodchild, M.J., Barrow, P., McConnell, I., Maskell, D.J., Young, J., Bumstead, N., Boyd, Y., and Smith, A.L. (2005) Identification and functional characterization of chicken Toll-like receptor 5 reveals a fundamental role in the biology of infection with *Salmonella enterica* serovar Typhimurium. Infect Immun **73**, 2344-2350.

Irie, H., Shimeld, C., Williams, N., and Hill, T. (1993) Protection against ocular and cutaneous infection with herpes simplex virus type 1 by intragastric immunization with live virus. J Gen Virol **74,** 1357-1362.

Ishii, K.J., Takeshita, F., Gursel, I., Gursel, M., Conover, J., Nussenzweig, A., and Klinman, D.M. (2002) Potential role of phosphatidylinositol 3 kinase, rather than DNA-dependent protein kinase, in CpG DNA-induced immune activation J Exp Med **196**, 269-274.

Ishii, N., Fukushima, J., Kaneko, T., Okada, E., Tani, K., Tanaka, S.I., Hamajima, K., Xin, K.Q., Kawamoto, S., Koff, W., Nishioka, K., Yasuda, T., and Okuda, K. (1997) Cationic liposomes are a strong adjuvant for a DNA vaccine of human immunodeficiency virus type 1. AIDS Res Hum Retroviruses **13**, 1421-1428.

Iwasaki, A. and Medzhitov, R. (2004) Toll-like receptor control of the adaptive immune responses. Nat Immunol **5**, 987-995.

Jaafari, M.R., Badiee, A., Khamesipour, A., Samiei, A., Soroush, D., Kheiri, M.T., Barkhordari, F., McMaster, W.R., and Mahboudi, F. (2007) The role of CpG ODN in enhancement of immune response and protection in BALB/c mice immunized with recombinant major surface glycoprotein of *Leishmania* (rgp63) encapsulated in cationic liposome. Vaccine **25**, 6107-6117.

Jakob, T., Walker, P.S., Krieg, A.M., Udey, M.C., and Vogel, J.C. (1998) Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th 1 responses by immunostimulatory DNA. J Immunol **161**, 3042-3049.

Janeway, C.A., Jr. and Medzhitov, R. (2002) Innate immune recognition. Annu Rev Immunol **20**, 197-216.

Jorgensen, J.B., Johansen, A., Stenersen, B., and Sommer, A.I. (2001) CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (Salmo salar L.) leucocytes to produce supernatants with antiviral activity. Dev Comp Immunol **25**, 313-321.

Joseph, A., Itskovitz-Cooper, N., Samira, S., Flasterstein, O., Eliyahu, H., Simberg, D., Goldwaser, I., Barenholz, Y., and Kedar, E. (2006) A new intranasal influenza vaccine based on a novel polycationic lipid--ceramide carbamoyl-spermine (CCS) I. immunogenicity and efficacy studies in mice. Vaccine **24**, 3990-4006.

Kadowaki, N., Antonenko, S., and Liu, Y.J. (2001a) Distinct CpG DNA and polyinosinic-polycytidylic acid double-stranded RNA, respectively, stimulate CD11c-type 2 dendritic cell precursors and CD11c+ dendritic cells to produce type I IFN. J Immunol **166**, 2291-2295.

Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R.W., Kastelein, R.A., Bazan, F., and Liu, Y.J. (2001b) Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. J Exp Med **194**, 863-869.

Kamstrup, S., Verthelyi, D., and Klinman, D.M. (2001) Response of porcine peripheral blood mononuclear cells to CpG-containing oligodeoxynucleotides. Vet Microbiol **78**, 353-362.

Kariyawasam, S., Wilkie, B.N., Hunter, D.B., and Gyles, C.L. (2002) Systemic and mucosal antibody responses to selected cell surface antigens of avian pathogenic *Escherichia coli* in experimentally infected chickens. Avian Dis **46**, 668-678.

Katso, R., Okkenhaug, K., Ahmadi, K., White, S., Timms, J., and Waterfield, M.D. (2001) Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Annu Rev Cell Dev Biol **17**, 615-675.

Khakhria, R., Woodward, D., Johnson, W.M., and Poppe, C. (1997) *Salmonella* isolated from humans, animals and other sources in Canada, 1983-92. Epidemiol Infect **119**, 15-23.

Kimura, Y., Sonehara, K., Kuramoto, E., Makino, T., Yamamoto, S., Yamamoto, T., Kataoka, T., and Tokunaga, T. (1994) Binding of oligoguanylate to scavenger receptors is required for oligonucleotides to augment NK cell activity and induce IFN. J Biochem **116**, 991-994.

Klinman, D.M. (2003) CpG DNA as a vaccine adjuvant. Exp Rev Vaccines **2**, 305-315.

Klinman, D.M. (2004) Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat Rev **4**, 249-258.

Klinman, D.M., Barnhart, K.M., and Conover, J. (1999a) CpG motifs as immune adjuvants. Vaccine **17**, 19-25.

Klinman, D.M., Conover, J., and Coban, C. (1999b) Repeated administration of synthetic oligodeoxynucleotides expressing CpG motifs provides long-term protection against bacterial infection. Infect Immun **67**, 5658-5663.

Klinman, D.M., Yi, A.K., Beaucage, S.L., Conover, J., and Krieg, A.M. (1996) CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. Proc Natl Acad Scis USA **93**, 2879-2883.

Kogut, M.H., Iqbal, M., He, H., Philbin, V., Kaiser, P., and Smith, A. (2005a) Expression and function of Toll-like receptors in chicken heterophils. Dev comp Immunol **29**, 791-807.

Kogut, M.H., McGruder, E.D., Hargis, B.M., Corrier, D.E., and DeLoach, J.R. (1994) Dynamics of avian inflammatory response to *Salmonella*-immune lymphokines. changes in avian blood leukocyte populations. Inflammation **18**, 373-388.

Kogut, M.H., Rothwell, L., and Kaiser, P. (2005b) IFN-gamma priming of chicken heterophils upregulates the expression of proinflammatory and Th 1 cytokine mRNA following receptor-mediated phagocytosis of *Salmonella enterica* serovar Enteritidis. J Interferon Cytokine Res **25**, 73-81.

Kogut, M.H., Swaggerty, C., He, H., Pevzner, I., and Kaiser, P. (2006) Toll-like receptor agonists stimulate differential functional activation and cytokine and chemokine gene expression in heterophils isolated from chickens with differential innate responses. Microbes Infect **8**, 1866-1874.

Koppenheffer, T.L., Spong, K.D. and Falvo, H.M. (1998) The complement system of the marsupial Monodelphis domestica. Dev Comp Immunol **22**, 231-237.

Krieg, A.M. (1999) Mechanisms and applications of immune stimulatory CpG oligodeoxynucleotides. Biochem Biophysiol **1489**, 107-116.

Krieg, A.M. (2000) The role of CpG motifs in innate immunity. Curr Opin Immunol **12**, 35-43.

Krieg, A.M. (2001) Now I know my CpGs. Trends Microbiol **9**, 249-252.

Krieg, A.M. (2002) CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol **20**, 709-760.

Krieg, A.M., Love-Homan, L., Yi, A.K., and Harty, J.T. (1998) CpG DNA induces sustained IL-12 expression *in vivo* and resistance to *Listeria monocytogenes* challenge. J Immunol **161**, 2428-2434.

Krieg, A.M., Yi, A.K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A. and Klinman, D.M. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. Nature **374**, 546-549.

Krogfelt, K.A. and Klemm, P. (1988) Investigation of minor components of *Escherichia coli* type 1 fimbriae: protein chemical and immunological aspects. Microb Pathog **4**, 231-238.

Krug, A., Rothenfusser, S., Hornung, V., Jahrsdorfer, B., Blackwell, S., Ballas, Z.K., Endres, S., Krieg, A.M., and Hartmann, G. (2001a) Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. Eur J Immunol **31**, 2154-2163.

Krug, A., Towarowski, A., Britsch, S., Rothenfusser, S., Hornung, V., Bals, R., Giese, T., Engelmann, H., Endres, S., Krieg, A.M., and Hartmann, G. (2001b) Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD 40 ligand to induce high amounts of IL-12. Eur J Immunol **31**, 3026-3037.

Kuramoto, E., Yano, O., Kimura, Y., Baba, M., Makino, T., Yamamoto, S., Yamamoto, T., Kataoka, T., and Tokunaga, T. (1992) Oligonucleotide sequences required for natural killer cell activation. Jpn J Cancer Res **83**, 1128-1131.

Kwant, A. and Rosenthal, K.L. (2004) Intravaginal immunization with viral subunit protein plus CpG oligodeoxynucleotides induces protective immunity against HSV-2. Vaccine **22**, 3098-3104.

La Ragione, R.M., Sayers, A.R. and Woodward, M.J. (2000) The role of fimbriae and flagella in the colonization, invasion and persistence of *Escherichia coli* O78:K80 in the day-old-chick model. Epidemiol Infect **124**, 351-363.

La Ragione, R.M. and Woodward, M.J. (2002) Virulence factors of *Escherichia coli* serotypes associated with avian colisepticaemia. Res Vet Sci **73**, 27-35.

Langer, R. (1990) New methods of drug delivery. Science **249**, 1527-1533.

Lawson, S., Rothwell, L., and Kaiser, P. (2000) Turkey and chicken interleukin-2 cross-react in *in vitro* proliferation assays despite limited amino acid sequence identity. J Interferon Cytokine Res **20**, 161-170.

Le Minor, L., and Popoff, M. Y. (1987) Request for an Opinion. Designation of *Salmonella enterica* sp. nov., nom. rev., as the type and only species of the genus *Salmonella.* Int J Syst Bacteriol **37**, 465–468.

Le Minor, L., Veron, M., and Popoff, M. (1982) [A proposal for *Salmonella* nomenclature]. Ann Microbiol **133**, 245-254.

Lenert, P., Rasmussen, W., Ashman, R.F., and Ballas, Z.K. (2003) Structural characterization of the inhibitory DNA motif for the type A (D)-CpG-induced cytokine secretion and NK-cell lytic activity in mouse spleen cells. DNA Cell Biol **22**, 621-631.

Lenert, P., Stunz, L., Yi, A.K., Krieg, A.M., and Ashman, R.F. (2001) CpG stimulation of primary mouse B cells is blocked by inhibitory oligodeoxyribonucleotides at a site proximal to NF-kappaB activation. Antisense Nucleic Acid Drug Dev **11**, 247-256.

Leon-Velarde, C.G., Cai, H.Y., Larkin, C., Bell-Rogers, P., Stevens, R.W., and Odumeru, J.A. (2004) Evaluation of methods for the identification of *Salmonella enterica* serotype Typhimurium DT104 from poultry environmental samples. J Microbiol Methods **58**, 79-86.

Leveque, G., Forgetta, V., Morroll, S., Smith, A.L., Bumstead, N., Barrow, P., Loredo-Osti, J.C., Morgan, K., and Malo, D. (2003) Allelic variation in TLR4 is linked to susceptibility to *Salmonella enterica* serovar Typhimurium infection in chickens. Infect Immun **71**, 1116-1124.

Levesque, S., Martinez, G., and Fairbrother, J.M. (2007) Improvement of adjuvant systems to obtain a cost-effective production of high levels of specific IgY. Poult Sci **86**, 630-635.

Levin, A.A. (1999) A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. Biochim biophysiol **1489**, 69-84.

Li, W.M., Bally, M.B., and Schutze-Redelmeier, M.P. (2001) Enhanced immune response to T-independent antigen by using CpG oligodeoxynucleotides encapsulated in liposomes. Vaccine **20**, 148-157.

Libby, S.J., Halsey, T. A., Altier, C., Potter, J., and Gyles, C. L. (2004) *Salmonella.* In: Pathogenesis of Bacterial Infections in Animals eds. C. L. Gyles, J.F.Prescott., J. G. Songer, and C. O. Theon. pp.143-167. Ames, Iowa: BlackWell Publishing Co.

Linghua, Z., Xingshan, T., and Fengzhen, Z. (2006) The efficacy of CpG oligodinucleotides, in combination with conventional adjuvants, as immunological adjuvants to swine streptococcic septicemia vaccine in piglets *in vivo*. Int Immunopharmacol **6**, 1267-1276.

Linghua, Z., Xingshan, T., and Fengzhen, Z. (2007) Vaccination with Newcastle disease vaccine and CpG oligodeoxynucleotides induces specific immunity and protection against Newcastle disease virus in SPF chicken. Vet Immunol Immunopathol **115**, 216-222.

Lipford, G.B., Sparwasser, T., Zimmermann, S., Heeg, K., and Wagner, H. (2000) CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th 1 predisposition to antigen-driven responses. J Immunol **165**, 1228-1235.

Loots, K., Loock, M.V., Vanrompay, D., and Goddeeris, B.M. (2006) CpG motifs as adjuvant in DNA vaccination against *Chlamydophila psittaci* in turkeys. Vaccine **24**, 4598-4601.

Lopez, A.M., Hecker, R., Mutwiri, G., van Drunen Littel-van den Hurk, S., Babiuk, L.A., and Townsend, H.G. (2006) Formulation with CpG ODN enhances antibody responses to an equine influenza virus vaccine. Vet Immunol Immunopathol **114**, 103-110.

Lu, Y., Touzjian, N., Kushner , N., Chutkowski, C., Qian , H., Jenkins , S., Payne , L., and B. Roberts (1996a) HIV-1 secreted viral antigens (SVA), polyphosphazene adjuvant, and SHIV challenge mode. In: Int Conf AIDS. Cambridge, MA, USA: Virus Research Institute.

Lu, Y.C., and Touzjian, N. (1996b) HIV-1 secreted viral antigens (SVA) , polyphosphazene adjuvant , and SHIV challenge model. 11th Int AIDs Conf.

Mahmood, M.S., Siddique, M., Hussain, I., Khan, A., and Mansoor, M.K. (2006) Protection capability of recombinant plasmid DNA vaccine containing VP2 gene of very virulent infectious bursal disease virus in chickens adjuvanted with CpG oligodeoxynucleotide. Vaccine **24**, 4838-4846.

Malaviya, R., Ikeda, T., Ross, E., and Abraham, S.N. (1996) Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. Nature **381**, 77-80.

Manesis, E.K., Cameron, C.H., and Gregoriadis, G. (1979) Hepatitis-B-surface-antigen-containing liposomes enhance humoral and cell-mediated immunity to the antigen. FEBS Lett **1,** 107-111.

Manzel, L. and Macfarlane, D.E. (1999) Lack of immune stimulation by immobilized CpG-oligodeoxynucleotide. Antisense Nucleic Acid Drug Dev **9**, 459-464.

Martinez, J.L., Delgado-Iribarren, A., and Baquero, F. (1990) Mechanisms of iron acquisition and bacterial virulence. FEMS Microbiol Rev **6**, 45-56.

McAllister, J.C., Steelman, C.D., and Skeeles, J.K. (1994) Reservoir competence of the lesser mealworm (Coleoptera: Tenebrionidae) for *Salmonella* Typhimurium (Eubacteriales: Enterobacteriaceae). J Med Entomol **31**, 369-372.

McCluskie, M.J., Weeratna, R.D., and Davis, H.L. (2000) Intranasal immunization of mice with CpG DNA induces strong systemic and mucosal responses that are influenced by other mucosal adjuvants and antigen distribution. Mol Med **6**, 867-877.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., and Tauxe, R.V. (1999) Food-related illness and death in the United States. Emerg Infect Dis **5**, 607-625.

Medzhitov, R. and Janeway, C.A., Jr. (2002) Decoding the patterns of self and nonself by the innate immune system. Science **296**, 298-300.

Mellata, M., Dho-Moulin, M., Dozois, C.M., Curtiss, R., 3rd, Lehoux, B., and Fairbrother, J.M. (2003) Role of avian pathogenic *Escherichia coli* virulence factors in bacterial interaction with chicken heterophils and macrophages. Infect Immun **71**, 494-503.

Mena, A., Nichani, A.K., Popowych, Y., Godson, D.L., Dent, D., Townsend, H.G., Mutwiri, G.K., Hecker, R., Babiuk, L.A., and Griebel, P. (2003) Innate immune responses induced by CpG oligodeoxyribonucleotide stimulation of ovine blood mononuclear cells. Immunol **110**, 250-257.

Meng, Z., Shao, J., and Xiang, L. (2003) CpG oligodeoxynucleotides activate grass carp (Ctenopharyngodon idellus) macrophages. Dev Comp Immunol **27**, 313-321.

Messina, J.P., Gilkeson, G.S., and Pisetsky, D.S. (1991) Stimulation of *in vitro* murine lymphocyte proliferation by bacterial DNA. J Immunol **147**, 1759-1764.

Moldoveanu, Z., Love-Homan, L., Huang, W.Q., and Krieg, A.M. (1998) CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. Vaccine **16**, 1216-1224.

Molenkamp, B.G., van Leeuwen, P.A., Meijer, S., Sluijter, B.J., Wijnands, P.G., Baars, A., van den Eertwegh, A.J., Scheper, R.J., and de Gruijl, T.D. (2007) Intradermal CpG-B activates both plasmacytoid and myeloid dendritic cells in the sentinel lymph node of melanoma patients. Clin Cancer Res **13**, 2961-2969.

Morales, A. (1978) Adjuvant immunotherapy in superficial bladder cancer. Natl Cancer Inst Monogr, 315-319.

Mui, B., Raney, S.G., Semple, S.C., and Hope, M.J. (2001) Immune stimulation by a CpG-containing oligodeoxynucleotide is enhanced when encapsulated and delivered in lipid particles. J Pharmacol Exp Therapeutics **298**, 1185-1192.

Mutwiri, G., Benjamin, P., Soita, H., Townsend, H., Yost, R., Roberts, B., Andrianov, A.K., and Babiuk, L.A. (2007) Poly [di (sodium carboxylatoethylphenoxy) phosphazene] (PCEP) is a potent enhancer of mixed Th 1/Th 2 immune responses in mice immunized with influenza virus antigens. Vaccine **25**, 1204-1213.

Mutwiri, G., Pontarollo, R., Babiuk, S., Griebel, P., van Drunen Littel-van den Hurk, S., Mena, A., Tsang, C., Alcon, V., Nichani, A., Ioannou, X., Gomis, S., Townsend, H., Hecker, R., Potter, A., and Babiuk, L.A. (2003) Biological activity of immunostimulatory CpG DNA motifs in domestic animals. Vet Immunol Immunopathol **91**, 89-103.

Mutwiri, G.K., Nichani, A.K., Babiuk, S., and Babiuk, L.A. (2004) Strategies for enhancing the immunostimulatory effects of CpG oligodeoxynucleotides. J Control Release **97**, 1-17.

Nakamura, M., Nagamine, N., Suzuki, S., Norimatsu, M., Oishi, K., Kijima, M., Tamura, Y., and Sato, S. (1993) Long-term shedding of *Salmonella* Enteritidis in chickens which received a contact exposure within 24 hrs of hatching. J Vet Med Sci **55**, 649-653.

Nesburn, A.B., Ramos, T.V., Zhu, X., Asgarzadeh, H., Nguyen, V., and BenMohamed, L. (2005) Local and systemic B cell and Th1 responses induced following ocular mucosal delivery of multiple epitopes of herpes simplex virus type 1 glycoprotein D together with cytosine-phosphate-guanine adjuvant. Vaccine **23**, 873-883.

Neufert, C., Pai, R.K., Noss, E.H., Berger, M., Boom, W.H., and Harding, C.V. (2001) *Mycobacterium tuberculosis* 19-kDa lipoprotein promotes neutrophil activation. J Immunol **167**, 1542-1549.

Ngeleka, M., Kwaga, J.K., White, D.G., Whittam, T.S., Riddell, C., Goodhope, R., Potter, A.A. and Allan, B. (1996) *Escherichia coli* cellulitis in broiler chickens: clone relationships among strains and analysis of virulence-associated factors of isolats from diseased birds. Infec Immun **64**, 3118-3126.

Nolan, L.K., Wooley, R.E., and Cooper, R.K. (1992) Transposon mutagenesis used to study the role of complement resistance in the virulence of an avian *Escherichia coli* isolate. Avian Dis **36**, 398-402.

O'Hagan, D.T. and Valiante, N.M. (2003) Recent advances in the discovery and delivery of vaccine adjuvants. Nat Rev Drug Discov **2**, 727-735.

Okamura, M., Lillehoj, H.S., Raybourne, R.B., Babu, U.S., Heckert, R.A., Tani, H., Sasai, K., Baba, E., and Lillehoj, E.P. (2005) Differential responses of macrophages to *Salmonella enterica* serovars Enteritidis and Typhimurium. Vet Immunol Immunopathol **107**, 327-335.

Olsen, A., Jonsson, A. and Normark, S. (1989) Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. Nature **338**, 652-655.

Padron, M. (1990) *Salmonella* Typhimurium outbreak in broiler chicken flocks in Mexico. Avian Dis **34**, 221-223.

Patel, B.A., Gomis, S., Dar, A., Willson, Ph.J., Babiuk, L.A., Potter, A., Mutwiri, G. and Tikoo, S.K. (2007) Oligodeoxynucleotides containing CpG motifs (CpG-ODN) predominantly induce Th 1 type immune response in neonatal chicks. Dev Comp Immunol [In press].

Park, K.M. and Kim, C.K. (1999) Preparation and evaluation of flurbiprofen-loaded microemulsion for parenteral delivery. Int J Pharmaceutics **181**, 173-179.

Parvari, R., Avivi, A., Lentner, F., Ziv, E., Tel-Or, S., Burstein, Y., and Schechter, I. (1988) Chicken immunoglobulin gamma-heavy chains: limited VH gene repertoire, combinatorial diversification by D gene segments and evolution of the heavy chain locus. EMBO J **7**, 739-744.

Pashine, A., Valiante, N.M., and Ulmer, J.B. (2005) Targeting the innate immune response with improved vaccine adjuvants. Nat Med **11**, S63-68.

Payne, L.G. and Andrianov, A.K. (1998) Protein release from polyphosphazene matrices. Adv Drug Deliv Rev **31**, 185-196.

Payne, L.G., Jenkins, S.A., Andrianov, A., and Roberts, B.E. (1995) Water-soluble phosphazene polymers for parenteral and mucosal vaccine delivery. Pharmaceutical Biotechnol **6**, 473-493.

Payne, L.G., Jenkins, S.A., Woods, A.L., Grund, E.M., Geribo, W.E., Loebelenz, J.R., Andrianov, A.K., and Roberts, B.E. (1998) Poly [di (carboxylatophenoxy) phosphazene] (PCPP) is a potent immunoadjuvant for an influenza vaccine. Vaccine **16**, 92-98.

Payne, S.M. (1988) Iron and virulence in the family Enterobacteriaceae. Crit Rev Microbiol **16**, 81-111.

Pennycott, T.W., Park, A., and Mather, H.A. (2006) Isolation of different serovars of *Salmonella enterica* from wild birds in Great Britain between 1995 and 2003. Vet Rec **158**, 817-820.

Philbin, V.J., Iqbal, M., Boyd, Y., Goodchild, M.J., Beal, R.K., Bumstead, N., Young, J., and Smith, A.L. (2005) Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR 8 in chickens. Immunol **114**, 507-521.

Poppe, C., Duncan, C.L., and Mazzocco, A. (1998) *Salmonella* contamination of hatching and table eggs: a comparison. Can J Vet Res **62**, 191-198.

Porter, R.E., Jr. and Holt, P.S. (1993) Effect of induced molting on the severity of intestinal lesions caused by *Salmonella* Enteritidis infection in chickens. Avian Dis **37**, 1009-1016.

Pourbakhsh, S.A., Boulianne, M., Martineau-Doize, B., and Fairbrother, J.M. (1997a) Virulence mechanisms of avian fimbriated *Escherichia coli* in experimentally inoculated chickens. Vet Microbiol **58**, 195-213.

Pourbakhsh, S.A., Dho-Moulin, M., Bree, A., Desautels, C., Martineau-Doize, B., and Fairbrother, J.M. (1997b) Localization of the *in vivo* expression of P and F1 fimbriae in chickens experimentally inoculated with pathogenic *Escherichia coli.* Microb Pathog **22**, 331-341.

Pourbakhsh, S.A. and Fairbrother, J.M. (1994) Purification and characterization of P fimbriae from an *Escherichia coli* strain isolated from a septicemic turkey. FEMS Microbiol Lett **122**, 313-318.

Qureshi, M.A., Heggen, C.L., and Hussain, I. (2000) Avian macrophage: effector functions in health and disease. Dev Comp Immunol **24**, 103-119.

Refsum, T., Vikoren, T., Handeland, K., Kapperud, G., and Holstad, G. (2003) Epidemiologic and pathologic aspects of *Salmonella* Typhimurium infection in passerine birds in Norway. J Wildl Dis **39**, 64-72.

Rice, D.H., Hancock, D.D., Roozen, P.M., Szymanski, M.H., Scheenstra, B.C., Cady, K.M., Besser, T.E., and Chudek, P.A. (2003) Household contamination with *Salmonella enterica*. Emerg Infect Dis **9**, 120-122.

Roach, J.C., Glusman, G., Rowen, L., Kaur, A., Purcell, M.K., Smith, K.D., Hood, L.E., and Aderem, A. (2005) The evolution of vertebrate Toll-like receptors. Proc Natl Acad Sci USA **102**, 9577-9582.

Rocha, A., Ruiz, S., and Coll, J.M. (2002) Improvement of DNA transfection with cationic liposomes. J Physiol Biochem **58**, 45-56.

Schauenstein, K., Kromer, G., Hala, K., Bock, G., and Wick, G. (1988) Chicken-activated-T-lymphocyte-antigen (CATLA) recognized by monoclonal antibody INN-CH 16 represents the IL-2 receptor. Dev Comp Immunol **12**, 823-831.

Schlaepfer, E., Audige, A., von Beust, B., Manolova, V., Weber, M., Joller, H., Bachmann, M.F., Kundig, T.M., and Speck, R.F. (2004) CpG oligodeoxynucleotides block human immunodeficiency virus type 1 replication in human lymphoid tissue infected ex vivo. J Virol **78**, 12344-12354.

Schluesener, H.J., Seid, K., Deininger, M., and Schwab, J. (2001) Transient *in vivo* activation of rat brain macrophages/microglial cells and astrocytes by immunostimulatory multiple CpG oligonucleotides. J Neuroimmunol **113**, 89-94.

Sellins, K., Fradkin, L., Liggitt, D., and Dow, S. (2005) Type I interferons potently suppress gene expression following gene delivery using liposome (-) DNA complexes. Mol Ther **12**, 451-459.

Sharma, J.M. (1981) Natural killer cell activity in chickens exposed to Marek's disease virus: inhibition of activity in susceptible chickens and enhancement of activity in resistant and vaccinated chickens. Avian Dis **25**, 882-893.

Sharma, J.M. (1991) Overview of the avian immune system. Vet Immunol Immunopathol **30**, 13-17.

Siccardi, F.J. (1975) Considerations concerning the host-parasite relationship of *Escherichia coli* in poultry. Am J Vet Res **36**, 572.

Singh, M. and O'Hagan, D.T. (2002) Recent advances in vaccine adjuvants. Pharmaceutical Res **19**, 715-728.

Skyberg, J.A., Logue, C.M., and Nolan, L.K. (2006) Virulence genotyping of *Salmonella* spp. with multiplex PCR. Avian Dis **50**, 77-81.

Souza, K. (2007) Tyson foods touts antibiotic-free products. In: Business. Northwest

Arkansas. Sparwasser, T., Koch, E.S., Vabulas, R.M., Heeg, K., Lipford, G.B., Ellwart, J.W., and Wagner, H. (1998) Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur J Immunol **28**, 2045-2054.

Sparwasser, T., Miethke, T., Lipford, G., Erdmann, A., Hacker, H., Heeg, K., and Wagner, H. (1997) Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor-alpha-mediated shock. Eur J Immunol **27**, 1671-1679.

Sparwasser, T., Vabulas, R.M., Villmow, B., Lipford, G.B., and Wagner, H. (2000) Bacterial CpG-DNA activates dendritic cells *in vivo*: T helper cell-independent cytotoxic T cell responses to soluble proteins. Eur J Immunol **30**, 3591-3597.

Stacey, K.J., Sester, D.P., Sweet, M.J., and Hume, D.A. (2000) Macrophage activation by immunostimulatory DNA. Curr Top Microbiol Immunol **247**, 41-58.

Stein, C.A., Subasinghe, C., Shinozuka, K., and Cohen, J.S. (1988) Physicochemical properties of phosphorothioate oligodeoxynucleotides. Nucleic Acids Re*s* **16**, 3209-3221.

Stone, K.D., Zhang, H.Z., Carlson, L.K., and Donnenberg, M.S. (1996) A cluster of fourteen genes from enteropathogenic *Escherichia coli* is sufficient for the biogenesis of a type IV pilus. Mol Microbiol **20**, 325-337.

Strother, K.O. (2005) Reservoir competence of lesser mealworm (Coleoptera: Tenebrionidae) for *Campylobacter jejuni* (Campylobacterales: Campylobacteraceae). J Med Entomol **42**, 42-47.

Sun, S., Zhang, X., Tough, D.F., and Sprent, J. (1998) Type I interferon-mediated stimulation of T cells by CpG DNA. J Exp Med **188**, 2335-2342.

Suzuki, K., Mori, A., Ishii, K.J., Saito, J., Singer, D.S., Klinman, D.M., Krause, P.R., and Kohn, L.D. (1999) Activation of target-tissue immune-recognition molecules by double-stranded polynucleotides. Proc Natl Acad Sci USA **96**, 2285-2290.

Sweet, M.J., Stacey, K.J., Kakuda, D.K., Markovich, D., and Hume, D.A. (1998) IFN-gamma primes macrophage responses to bacterial DNA. J Interferon Cytokine Res **18**, 263-271.

Takeshita, F., Leifer, C.A., Gursel, I., Ishii, K.J., Takeshita, S., Gursel, M., and Klinman, D.M. (2001) Cutting edge: Role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. J Immunol **167**, 3555-3558.

Tasker, L. and Marshall-Clarke, S. (2003) Functional responses of human neonatal B lymphocytes to antigen receptor cross-linking and CpG DNA. Clin Exp Immunol **134**, 409-419.

Tokunaga, T., Yamamoto, H., Shimada, S., Abe, H., Fujisawa, Y., Furani, Y., Yano, O., Kataoka, T., and Sudo, T. (1984) Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. I. Isolation, physiocochemical characterization, and antitumor activity. J Nat Cancer Inst **72**, 955-962.

Tokunaga, T., Yano, O., Kuramoto, E., Kimura, Y., Yamamoto, T., Kataoka, T., and Yamamoto, S. (1992) Synthetic oligonucleotides with particular base sequences from the cDNA encoding proteins of *Mycobacterium bovis* BCG induce interferons and activate natural killer cells. Microbiol Immunol **36**, 55-66.

Toro, H., Price, S.B., McKee, A.S., Hoerr, F.J., Krehling, J., Perdue, M., and Bauermeister, L. (2005) Use of bacteriophages in combination with competitive exclusion to reduce *Salmonella* from infected chickens. Avian Dis **49**, 118-124.

Truper, H.G. (2005) The type species of the genus *Salmonella* Lignieres 1900 is *Salmonella enterica* (ex Kauffmann and Edwards 1952) Le Minoe and Popoff 1987, with the type strain LT2T, and conservation of the epithet *enterica* in *Salmonella enterica* over all earlier epithets that may be applied to this species. Opinion 80. Inter J Sys Evolutionary Microbiol **55**, 519-520.

Vallin, H., Perers, A., Alm, G.V., and Ronnblom, L. (1999) Anti-double-stranded DNA antibodies and immunostimulatory plasmid DNA in combination mimic the endogenous IFN-alpha inducer in systemic lupus erythematosus. J Immunol **163**, 6306-6313.

Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Holden, D.W., Lucia, S.M., Dinauer, M.C., Mastroeni, P., and Fang, F.C. (2000) *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science **287**, 1655-1658.

Verma, I., Pandey, R., and Khuller, G.K. (2004) Liposomes as adjuvant for anti-mycobacterial vaccine development. Ind J Exp Biol **42**, 949-954.

Vidotto, M.C., Muller, E.E., de Freitas, J.C., Alfieri, A.A., Guimaraes, I.G., and Santos, D.S. (1990) Virulence factors of avian *Escherichia coli.* Avian Dis **34**, 531-538.

Vleugels, B., Ververken, C., and Goddeeris, B.M. (2002) Stimulatory effect of CpG sequences on humoral response in chickens. Poul Sci **81**, 1317-1321.

Vogel, F.R. (1995) Immunologic adjuvants for modern vaccine formulations. Ann NY Acad Sci **754**, 153-160.

Wang, L., Prakash, R.K., Stein, C.A., Koehn, R.K. and Ruffner, D.E. (2003a) Progress in the delivery of therapeutic oligonucleotides: organ/cellular distribution and targeted delivery of oligonucleotides *in vivo*. Antisense Nucleic Acid Drug Dev **13**, 169-189.

Wang, X., Jiang, P., Deen, S., Wu, J., Liu, X., and Xu, J. (2003b) Efficacy of DNA vaccines against infectious bursal disease virus in chickens enhanced by coadministration with CpG oligodeoxynucleotide. Avian Dis **47**, 1305-1312.

Wedlock, D.N., Denis, M., Skinner, M.A., Koach, J., de Lisle, G.W., Vordermeier, H.M., Hewinson, R.G., van Drunen Littel-van den Hurk, S., Babiuk, L.A., Hecker, R., and Buddle, B.M. (2005) Vaccination of cattle with a CpG oligodeoxynucleotide-formulated mycobacterial protein vaccine and *Mycobacterium bovis* BCG induces levels of protection against bovine tuberculosis superior to those induced by vaccination with BCG alone. Infect Immun **73**, 3540-3546.

Weeratna, R.D., McCluskie, M.J., Xu, Y., and Davis, H.L. (2000) CpG DNA induces stronger immune responses with less toxicity than other adjuvants. Vaccine **18**, 1755-1762.

Weighardt, H., Feterowski, C., Veit, M., Rump, M., Wagner, H., and Holzmann, B. (2000) Increased resistance against acute polymicrobial sepsis in mice challenged with immunostimulatory CpG oligodeoxynucleotides is related to an enhanced innate effector cell response. J Immunol **165**, 4537-4543.

Wiemann, B. and Starnes, C.O. (1994) Coley's toxins, tumor necrosis factor and cancer research: a historical perspective. Pharmacol Therapeutics **64**, 529-564.

Withanage, G.S., Wigley, P., Kaiser, P., Mastroeni, P., Brooks, H., Powers, C., Beal, R., Barrow, P., Maskell, D., and McConnell, I. (2005) Cytokine and chemokine responses associated with clearance of a primary *Salmonella enterica* serovar Typhimurium infection in the chicken and in protective immunity to rechallenge. Infect Immun **73**, 5173-5182.

Wooley, R.E., Spears, K.R., Brown, J., Nolan, L.K., and Fletcher, O.J. (1992) Relationship of complement resistance and selected virulence factors in pathogenic avian *Escherichia coli*. Avian Dis **36**, 679-684.

Wu, J.Y., Wade, W.F., and Taylor, R.K. (2001) Evaluation of cholera vaccines formulated with toxin-coregulated pilin peptide plus polymer adjuvant in mice. Infect Immun **69**, 7695-7702.

Xu, Y.M., Pearson, G.R. and Hinton, M. (1988) The colonization of the alimentary tract and visceral organs of chicks with *Salmonellas* following challenge via the feed: bacteriological findings. Br Vet J **144**, 403-410.

Yamamoto, S. (2002) Discovery of immunostimulatory CpG-DNA and its application to tuberculosis vaccine development. Jpn J Infect Dis **55**, 37-44.

Yamamoto, S., Kuramoto, E., Shimada, S., and Tokunaga, T. (1988) *In vitro* augmentation of natural killer cell activity and production of interferon-alpha/beta and -gamma with deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. Jpn J Cancer Res **79**, 866-873.

Yamamoto, S., Yamamoto, T., Shimada, S., Kuramoto, E., Yano, O., Kataoka, T., and Tokunaga, T. (1992) DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. Microbiol Immunol **36**, 983-997.

Yew, N.S. and Scheule, R.K. (2005) Toxicity of cationic lipid-DNA complexes. Adv Genet **53**, 189-214.

Yi, A.K., Chace, J.H., Cowdery, J.S., and Krieg, A.M. (1996) IFN-gamma promotes IL-6 and IgM secretion in response to CpG motifs in bacterial DNA and oligodeoxynucleotides. J Immunol **156**, 558-564.

Yi, A.K., Chang, M., Peckham, D.W., Krieg, A.M., and Ashman, R.F. (1998a) CpG oligodeoxyribonucleotides rescue mature spleen B cells from spontaneous apoptosis and promote cell cycle entry. J Immunol **160**, 5898-5906.

Yi, A.K. and Krieg, A.M. (1998b) CpG DNA rescue from anti-IgM-induced WEHI-231 B lymphoma apoptosis via modulation of I kappa B alpha and I kappa B beta and sustained activation of nuclear factor-kappa B/c-Rel. J Immunol **160**, 1240-1245.

Yi, A.K. and Krieg, A.M. (1998) Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. J Immunol **161**, 4493-4497.

Yoshinaga, T., Yasuda, K., Ogawa, Y., Nishikawa, M., and Takakura, Y. (2007) DNA and its cationic lipid complexes induce CpG motif-dependent activation of murine dendritic cells. Immunol **120**, 295-302.

Yurong, Y., Ruiping, S., Shimin, Z. and Yibao, J. (2005) Effect of probiotics on intestinal mucosal immunity and ultrastructure of cecal tonsils of chickens. Arch Anim Nutr **59**, 237-246.

Zaloga, G.P., Dunham, A.J., Gonyon, T.M., Best, L.M., and Groves, M. (2007) Safety and stability of lipid emulsions. Nutr Clin Pract **22**, 367-369.

Zecha, B.C., McCapes, R.H., Dungan, W.M., Holte, R.J., Worcester, W.W., and Williams, J.E. (1977) The Dillon Beach Project--a five-year epidemiological study of naturally occurring *Salmonella* infection in turkeys and their environment. Avian Dis **21**, 141-159.

Zecha, B.C., McCapes, R. H., Dungan, W.M., Holte, R.J., Worcester, W.W., and Williams, J.E. (1977) The Dillon Beach Project--a five-year epidemiological study of naturally occurring *Salmonella* infection in turkeys and their environment. Avian Dis **21**, 141-159.

Zhao, Q., Matson, S., Herrera, C.J., Fisher, E., Yu, H., and Krieg, A.M. (1993) Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate, and mixed phosphorothioate and methylphosphonate oligonucleotides. Antisense Res Dev **3**, 53-66.

Zhao, Q., Waldschmidt, T., Fisher, E., Herrera, C.J., and Krieg, A.M. (1994) Stage-specific oligonucleotide uptake in murine bone marrow B-cell precursors. Blood **84**, 3660-3666.

Zimmermann, S., Egeter, O., Hausmann, S., Lipford, G.B., Rocken, M., Wagner, H., and Heeg, K. (1998) CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. J Immunol **160**, 3627-3630.