

Enhancement of Immunoprotective Effect of CpG-ODN by Formulation with Polyphosphazenes Against *E. coli* Septicemia in Neonatal Chickens

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Abstract: Synthetic oligodeoxynucleotides (ODN) containing CpG motifs (CpG-ODN) have been shown to be effective immunoprotective agents and vaccine adjuvants in a variety of bacterial and protozoan diseases in different animal species. The objective of this study was to investigate the immunoprotective effect of formulated CpG-ODN with polyphosphazene, liposome or oil-in-water emulsion against *E. coli* infections in neonatal chickens. Eighteen-day-old embryonating eggs were inoculated with 50 µg CpG-ODN or formulated CpG-ODN with polyphosphazene, liposome or oil-in-water emulsion. Four days after exposure to formulated CpG-ODN or day-1 post-hatch, 1×10^4 or 1×10^5 cfu of a virulent isolate of *E. coli* was inoculated by the subcutaneous route in 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 following *E. coli* infection was 0% in groups receiving either non-CpG-ODN or saline. In contrast, birds receiving either CpG-ODN or CpG-ODN formulated with polyphosphazene had significantly higher survival of 55% ($P < 0.0001$). The relative risk of mortality was significantly reduced for birds treated with CpG-ODN formulated in PCPP (0.25), in PCEP (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 oil-in-water emulsion did not increase the immunoprotective effect against *E. coli* infection, no adverse reactions or poor hatchability 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 extra cellular bacterial infection in neonatal broiler chickens following *in ovo* delivery.

Keywords: CpG-ODN formulation, immunoprotection, *in ovo* delivery, polyphosphazenes, liposomes, oil-in-water emulsion.

INTRODUCTION

The innate immune system of vertebrates recognizes structurally conserved pathogen-associated molecular patterns (PAMPs) and allows immediate host immune responses to limit infection [1]. Bacterial DNA is among a series of PAMPs that have recently been found to stimulate the innate immune system and afford immune protection against microbial infections in vertebrate species [2-4]. In contrast to vertebrate DNA, bacterial DNA contains relatively abundant unmethylated CpG dinucleotides [5]. These unmethylated CpG dinucleotides within specific flanking bases (referred to as CpG motifs) are now known to be the molecular basis that contributes to the immunostimulatory activity of bacterial DNA [6]. Synthetic oligonucleotides containing the CpG motif (CpG-ODNs) mimicking bacterial DNA have been demonstrated to retain immunostimulatory activities [7]. CpG-ODN has direct stimulatory effects on monocytes and macrophages, which secrete IL-12 and other cytokines [8]. It activates NK cells to

have increased lytic activity and to secrete IFN- γ [8]. CpG-ODN has no apparent direct stimulatory effect on T-cells, but it enhances the ability of antigen presenting cells (APCs) to activate T-cells [9]. These effects have been seen in a variety of viral, bacterial and protozoan infections, among a range of vertebrate species including poultry [10-12].

Little is known about the effects of CpG-ODNs on immune responses in chickens. He, *et al.*, [13] used an avian macrophage cell line (HD11) and peripheral blood mononuclear cells (PBMC) to evaluate the ability of CpG-ODN to stimulate nitric oxide (NO), IL-1 β , and IFN- γ production. These workers showed that stimulation of HD11 cells with CpG-ODN for 24 hours stimulated the production of NO [13]. In 2004, Dalloul, *et al.*, [14] showed that CpG-ODNs could be used as immunoprotective agent 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. Furthermore, CpG-ODN demonstrated its ability as an adjuvant in poultry against infectious bursal disease and *E. coli* infections [15,16]. Recently a new chicken TLR was suggested by Roach, *et al.* [17]. This TLR shares the same biology with the fish TLR 21-TLR23 family [17]. In order to develop the

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information regarding TLRs in chicken, Higgs, *et al.*, [18] conducted an *in vitro-in vivo* experiment using 5-week-old *Salmonella typhimurium* challenge model. These workers identified TLR15 in chicken. TLR15 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. TLR15 was detected in the chicken embryo fibroblast cells spleen, bursa, bone marrow, and cecum of healthy chickens and over expressed during *Salmonella typhimurium* infection in the cecum [18].

Over the past decades, considerable efforts were devoted to the development of new experimental adjuvant formulations to increase the immunogenicity of subunit and protein vaccines [19, 20]. Adjuvants are formulated compounds or additives that, when combined with antigens, help to direct or boost the body's immune system [21]. They are classified in two broad categories: (a) delivery systems including particulate adjuvants such as liposomes and emulsions [22], and (b) immunostimulatory adjuvants including CpG-ODN [23].

Polyphosphazenes are potent immunostimulatory adjuvants with high molecular weight, containing a long-chain backbone of alternating phosphorus and nitrogen atoms [24, 25]. Among polyphosphazenes, PCPP or Poly [di (carboxylatophenoxy phosphazene)] is a potent immunostimulant via not only parenteral, but also mucosal vaccine delivery, against diseases such as influenza, tetanus toxoid, hepatitis B surface antigen, and herpes simplex virus [26]. The adjuvant activity of a new polyphosphazene electrolyte (PCEP or Poly [di (sodium carboxylatoethylphenoxy) phosphazene]) as a potent enhancer of antigen-specific immune responses was studied in mice inoculated with influenza virus [27]. This study revealed that the adjuvant activity of PCEP is higher than PCPP, as PCEP induces higher levels of Th1, Th2 type cytokines, and IgG1, IgG2 associated with production of both IFN- γ and IL-4.

Efficacy of CpG-ODN in combination with liposomes was also previously investigated. Liposomes are the smallest artificial vesicles that can be produced from natural non-toxic phospholipids and cholesterol [28]. It has been demonstrated that encapsulating CpG-ODN into liposomes improves the incorporation of CpG-ODN into dendritic cells, enhances IL-12, activates natural killer cells, and produces IFN- γ [29]. Cationic lipid (CL) is another example of a liposomal adjuvant [30]. 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 [31]. These findings provide support the use of stabilized cationic lipids significantly enhance the therapeutic efficacy of CpG-ODN [31, 32].

Emulsigen® is commonly used in veterinary vaccines [33]. This adjuvant acts by forming a mobile depot of antigen, which can target immune effectors cells such as, IFN- γ , Th 1, and IgG [32-34]. The depot effect, which results in slow release of Emulsigen®, improves the presentation of antigens, thereby enhancing the immune response [33, 35]. Emulsigen® is a unique oil-in-water adjuvant, without components of animal origin [33, 35].

We have previously demonstrated that *in ovo* delivery of unformulated CpG-ODNs were potent immunostimulants against bacterial infections in neonatal chickens [36]. The

objective of this study was to investigate the immunoprotective effect of formulated CpG-ODN with polyphosphazenes, liposomes or oil-in-water emulsion against *E. coli* infections in neonatal chickens.

MATERIALS AND METHODS

E. coli Culture and Animal Model

A field isolate of *E. coli* from a turkey with septicemia, was used as the challenge strain. This *E. coli* was serogroup O2, which is nonhemolytic, serum resistant, and produced aerobactin, a K1 capsule, and type 1 pili. Aliquots of bacteria were stored at -70° C in 50% brain heart infusion (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 h at 37° C. Two to three colonies were added to 200 ml of BHI broth in a 1-liter Erlenmeyer flask. The culture was grown at 37° C for 18-20 h with shaking at 200 rpm. This stationary phase culture was diluted to an absorbance of 0.4 at 600 nm. At this absorbance, the culture contained approximately 2.5×10^8 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, incubating for 18 h 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 in accordance with regulations of the Canadian Council for Animal Care. Inoculation of CpG-ODN in 18-day-old incubating eggs and this *E. coli* animal model in neonatal broilers was previously described [36]. Briefly, 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. CpG-ODN formulated with polyphosphazenes, liposomes, oil-in-water emulsions or cationic lipids were inoculated into 18-day-old incubating eggs in 100 μ l by the *in ovo* route into the amniotic cavity through the air cell of the egg using a 20 gauge, one inch needle. At the time of hatch birds were individually identified by neck tags (Swiftack Poultry Tags, Heartland Animal Health Inc., MO). Treatments were randomly allocated to groups of 20 birds that were housed in animal isolation rooms at Animal Care, Western College of Veterinary Medicine, University of Saskatchewan. Water and commercial broiler ration were provided *ad libitum*. Air from each room was exhausted through a HEPA filter and non-recirculated intake air was provided at a rate of 18 changes/h. Air pressure differential and strict sanitation was maintained in this isolation facility. Photoperiod of 24 h per day for the first 3 days and 8 h per day for the remaining 5 days was established. Room temperature was maintained at 30 - 32 °C. Birds were challenged with 1×10^4 or 1×10^5 cfu of *E. coli* by the subcutaneous route in the neck at day 1 post hatch (equivalent to day 4 post administration of treatment material).

Birds were observed for 8 days for clinical signs after *E. coli* challenge. Daily clinical scores for each individual bird were assigned as follows: 0 = normal; 1 = hesitate to move

and tire quickly; 2 = unable to stand or forage for food and was euthanized; 3 = dead. A cumulative clinical score (CCS) for each bird (CCS/Bird) and for each group of 20 (CCS/Group) were calculated as follows: Clinical scores were summed for each bird across the 10 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. 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 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 [37].

Synthetic CpG-ODN

The sequence of CpG ODN used was TCGTTCGTTGT CGTTTTGTCGTT⁽²⁰⁰⁷⁾ and the sequence of non-CpG ODN was TGCTGCTTGTGCTTTTGTGCTT⁽²⁰⁰⁷⁾. Both ODN's were free of endotoxin and produced with a phosphorothioate backbone (Qiagen GmbH, Hilden Germany). Synthetic CpG-ODN was diluted in sterile, pyrogen free saline.

Polyphosphazenes

Polyphosphazene adjuvants PCPP and PCEP were designed and synthesized by Parallel Solutions Inc. (Cambridge, MA). Aqueous solutions of both polymers were stored at room temperature in the dark, and were found to retain activity over several months under these storage conditions. Batches of polyphosphazenes were tested and found to have endotoxin levels that were 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 in a total volume of 100 µl of phosphate buffered saline (PBS; 0.2 M Na₂HPO₄, 1.8%, NaCl-pH 7.8).

Experimental Design

Formulation of CpG-ODN with Polyphosphazenes for In Ovo Delivery against E. coli Infection in Neonatal Chickens

In ovo administration has been proven to be a safe, efficacious and convenient method of vaccination in modern poultry production facilities [38]. Groups of embryonated eggs were randomly allocated to treatment, each containing 25 eggs. Groups were injected with CpG-ODN, non-CpG-ODN (50 µg), PCPP, PCEP, liposome, cationic lipids, oil-in-water emulsion (EMULSIGEN[®], MVP Laboratories Inc., Omaha, NE) alone or in combination, on day 18 of incubation. (Incubation period of the chicken embryo is 21 days). A dose of 50 µg of CpG-ODN was chosen for all the formulations in this study since this dose has demonstrated a significant protection against *E. coli* infection in either adult or neonatal chickens by intramuscular, subcutaneous or *in ovo*

routes [11, 36]. All the groups with above treatments were duplicated and challenged by either 1x10⁴ (n=20) or 1x10⁵ (n=20) cfu of *E. coli* at day 1 post-hatch (4 days after *in ovo* treatments). Because many variables affect the response of chickens to *E. coli* infection, duplicate groups were challenged with either 1x10⁴ or 1x10⁵ cfu in order to ensure that an appropriate interaction of host and pathogen was observed in at least one of the duplicates. Clinical scoring and bacterial isolations were conducted as described above.

In order to study whether the formulation of CpG-ODN with polyphosphazenes enhances the duration of protection against *E. coli* challenge, additional groups of birds were inoculated with the above formulations by the *in ovo* route and birds were challenged with 1x10⁴ cfu of *E. coli* at day-4 post-hatch (7 days after *in ovo* treatment). Clinical scoring and bacterial isolations were conducted as described above.

Statistical Analysis

The survival pattern and median survival of neonatal chickens were first evaluated by Cox's proportional hazards regression, and significant variables were compared using the log-rank test. The effects of treatment on relative risk of mortality were analysed using proportional hazards regression and Pearson's Chi-square, and the effects on cumulative clinical scores and bacteria isolated from air sacs were analysed by ranking the data prior to analysis of variance with Tukey comparison of mean ranks. The homogeneity of the relative frequency distribution of the level of bacteria in air sacs after various treatments was tested using the Chi-square test. Data were analysed using Statistix (Analytic Software, Tallahassee, FL USA, www.statistix.com) and Prism (Prism 4.0, GraphPad Software Inc., San Diego, CA, USA www.graphpad.com) with a significance level of p = 0.05.

RESULTS

a) Formulation of CpG-ODN with Polyphosphazenes for In Ovo Delivery Against E. coli Infection in Neonatal Chickens

The relative risk of mortality was significantly reduced for birds treated with CpG-ODN formulated in PCPP (relative risk 0.25) or in PCEP (relative risk 0.33) or unformulated (relative risk 0.39) in comparison to the group treated with saline (p<0.01). Indeed, the PCEP treatment, without CpG-ODN, also provided a modest degree of protection with relative risk of mortality of 0.57 (p<0.05) in comparison to the saline treated group. These results are reflected by the occurrence of mortality that is shown in the survival curves (Fig. 1). This immunoprotection occurred in groups challenged with either 1x10⁴ or 1x10⁵ cfu of *E. coli*, and the data are combined for clarity of presentation and analysis, because Cox's proportional hazards regression showed there was no significant difference (p=0.68) in group mortality between these challenge doses. The estimated risk of birds dying following treatment with CpG-ODN formulated in PCPP was 0.68 times lower than those treated with unformulated CpG-ODN (p=0.04), which shows a significant enhancement of benefit due to this formulation. The mortality of birds treated with CpG-ODN formulated in PCEP was lower than those treated with unformulated CpG-ODN; however, the difference in this trial was not statistically significant (p=0.35).

Groups of birds challenged with 1×10^4 and 1×10^5 cfu of *E. coli* that received CpG-ODN together with PCPP had a total cumulative clinical score (CCS) of 354 compared to birds that received CpG-ODN together with PCEP (449), CpG-ODN (501), non-CpG (804), PCPP (758), PCEP (601), or saline (777) (Fig. 2). Overall, the effect of treatment group on CCS was highly significant ($p < 0.001$), and groups with significantly different mean ranks are shown in Fig. (2). Birds that died peracutely within 24 h of challenge did not have gross lesions, but many bacteria were cultured from the air sacs (Fig. 1 and Table 1). In groups treated with CpG-ODN formulated with polyphosphazine or CpG-ODN alone, fewer bacteria were isolated from the air sacs compared to the other groups (Table 1). ($\chi^2=51.02$; $p < 0.001$). 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 and CpG-ODN formulated with PCPP ($p > 0.05$).

In order to study whether the formulation of CpG-ODN with polyphosphazenes enhanced the duration of protection against *E. coli* challenge, additional groups of birds were challenged with 1×10^4 cfu's of *E. coli* at day 4 post hatch (7 days after *in ovo* treatment). None of the groups that were treated with CpG-ODN formulated with polyphosphazenes or CpG-ODN alone showed protection for this duration against *E. coli* mortality compared to the control group treated with saline ($p > 0.05$) (Data not shown).

When CpG-ODN was formulated with liposomes, Emulsigen® or cationic lipids there was no increase in survival rates of neonatal chickens seen over that seen with CpG-ODN alone (Data not shown). The survival rate for the liposome, cationic lipids or Emulsigen® alone also were not different from the group treated with saline (Data not shown).

DISCUSSION

Synthetic oligodeoxynucleotides containing CpG motifs act as immunostimulants and are capable of accelerating and boosting innate and adaptive immunity. CpG-ODN induces maturation and activation of vertebrate immune cells, which then produce Th1-type cytokines including TNF- α , IL-12, and IFN- γ . Therefore, CpG-ODN activation contributes to the development of Th1 – type immune responses [39-40]. The immunoprotective effect of CpG-ODN against *E. coli* septicemia in chickens has been demonstrated previously [11, 41]. However, the effect of formulating CpG-ODN with other delivery systems to enhance the activity of CpG-ODN against bacterial diseases in poultry has not been described. This study demonstrates that formulating CpG-ODN with polyphosphazenes enhances the effectiveness of CpG-ODN against *E. coli* septicemia in neonatal chickens. In contrast, formulation in liposome, cationic lipids or oil-in-water combinations did not enhance the protective effect of CpG-ODN in this animal model. Although, our formulations of CpG-

Survival of birds after 1×10^4 and 1×10^5 cfu of *E. coli* challenge

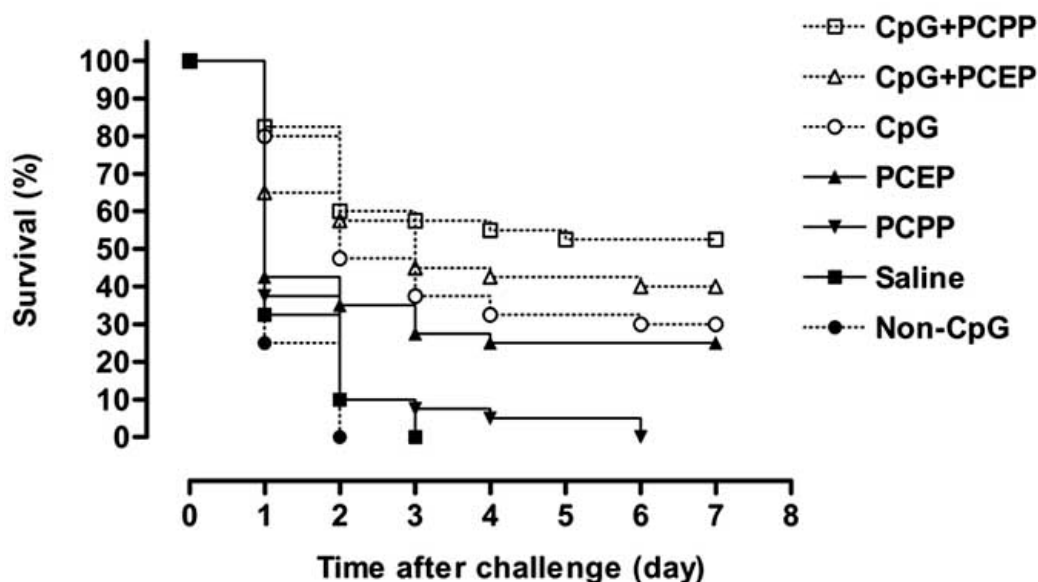


Fig. (1). Survival of neonatal chickens following *in ovo* treatment with formulated CPG-ODN⁽²⁰⁰⁷⁾. Survival of chickens following *E. coli* challenge in groups treated with CpG-ODN (○), non-CpG-ODN (●), PCPP (▼), PCEP (▲), CpG-ODN together with PCPP (◻) CpG-ODN together with PCEP (◻) or saline (■). Duplicate groups were challenged with either 1×10^4 (n=20) or 1×10^5 (n=20) cfu of *E. coli*, and Cox's proportional hazards regression showed there was no significant difference ($p=0.68$) in group mortality between these challenge doses. Hence, the data for the duplicate groups are combined for clarity of presentation (n = 40 for each treatment). Birds that received CpG-ODN or CpG-ODN formulated with PCPP or PCEP showed significantly higher survival rate compared to groups received non-CpG-ODN or saline ($p < 0.01$).

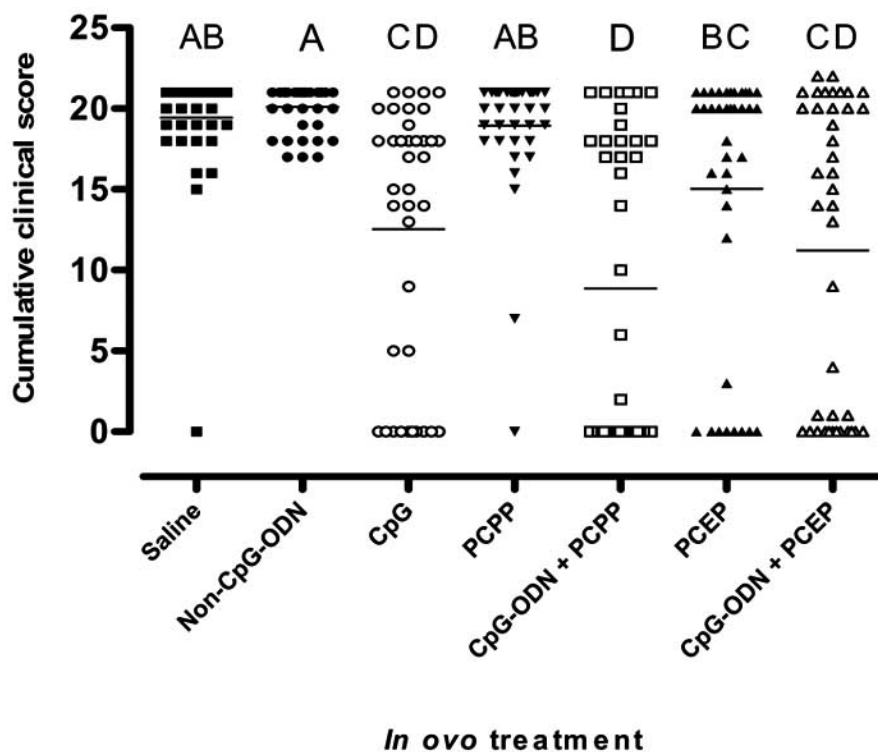


Fig. (2). Cumulative clinical scores (CCSs) in birds following *in ovo* treatment with CpG-ODN formulated in PCPP and PCEP. Cumulative clinical score of individual birds following challenge with 1×10^4 (A) and 1×10^5 (B) cfu of *E. coli* via subcutaneous route in groups treated with CpG-ODN (○), non-CpG-ODN (●), PCPP (▼), PCEP (▲), CpG-ODN together with PCPP (□) CpG-ODN together with PCEP (Δ) or saline (■). Birds that received CpG-ODN formulated with PCPP had lowest CCS among groups. Groups with different letters above the cluster of symbols are significantly different ($p < 0.05$); likewise, groups with the same letter (such as saline, non-CpG-ODN and PCPP, which all have an A) are not significantly different. (Bar = mean) The immunoprotective effects were similar in groups challenged with 1×10^4 and 1×10^5 cfu of *E. coli*, so data are pooled for groups given the two challenge (n=40 for each treatment).

Table 1. Number of Birds at Each Level of *E. coli* Isolation from Air Sacs of Neonatal Chickens Following *In Ovo* Treatment with CpG-ODN⁽²⁰⁰⁷⁾, and Subcutaneous Bacterial Challenge

	<i>E. coli</i> Growth (air sac) Score ^a	Saline	Non CpG -ODN	CpG -ODN	PCPP	PCEP	CpG-ODN + PCPP	CpG-ODN + PCEP
Significance^b		AB	ABC	BCD	A	ABC	D	CD
	0	0	0	7	0	5	19	7
	1+	4	2	8	1	4	4	11
	2+	12	17	9	13	14	8	7
	3+	12	12	10	18	10	3	10
	4+	12	9	6	8	7	6	5

^a Level of *E. coli* in lesions was estimated on MacConkey agar as described in *E. coli* animal model.

^b Groups with different letter designations are significantly different ($p < 0.05$); likewise, groups with the same letter (such as saline, non-CpG-ODN and PCPP, which all have an A) are not significantly different.

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 ($p < 0.001$) (n = 40 for each treatment).

ODN with liposomes, cationic lipids or oil-in-water emulsions did not increase the immunoprotective effect, we did not see any adverse effects or poor hatchability of chicken embryos by these formulations (data not shown).

The present investigation is the first to demonstrate that the polyphosphazenes PCPP and PCEP can enhance CpG-induced innate immunity and protection against bacterial infections in neonatal chickens. It may be possible to further improve this synergy by optimizing the ratio of CpG-ODN

to PCPP or PCEP. We did not study the optimization of CpG-ODN with polyphosphazenes in these experiments. Innate immunity against various infectious agents requires distinct types of immune responses. Polarized Th1 type immunity can be achieved by addition of complete Freund's adjuvant and CpG-ODN to an antigen [23, 42]. Defense against intracellular pathogens tends to involve Th1-like immune responses dominated by the production of cytokines (IFN- γ and TNF), IgG2a antibodies and CTL, while resistance to extracellular pathogens is often associated with humoral responses dominated by high levels of IgG1 and IgE [43], but *in ovo* delivery of CpG-ODN without formulation was able to significantly protect neonatal chickens against bacteria with either an extra-cellular phase of survival (*E. coli*) or an intracellular phase of survival (*Salmonella typhimurium*) (Taghavi *et al.* – Avian Dis. *In press*) in the avian host. Although, CpG-ODN formulated with polyphosphazenes improves the immunoprotective activity against *E. coli* septicemia in neonatal chickens, this combination was not able to lengthen the duration of protection against *E. coli* infection. 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 [11], and this duration of protection was not lengthened by polyphosphazene, liposome or oil-in-water emulsion formulations of CpG-ODN (data not shown). We did not study the biochemistry and chemical composition of CpG-ODN with polyphosphazene to understand the duration of protection. It is possible that optimization of CpG-ODN with polyphosphazene formulation might lengthen the duration of protection.

In conclusion, CpG-ODN formulated with polyphosphazenes and delivered by the *in ovo* route to 18 day incubating eggs was able to increase the immunoprotective effect against *E. coli* infections of neonatal chickens. Protection of neonatal chickens against *E. coli* infection by administration of CpG-ODN is related to Th1 polarized IFN- γ and IL-18 mediated pathways [44]. In addition, we have demonstrated that *in ovo* administration of CpG-ODN to incubating eggs is a safe and industrially feasible method since no increased embryo mortality was observed with CpG-ODN formulations (data not shown). There are clear advantages to using noninvasive delivery methods that can be automated. Many immunizations in the poultry industry in the past were subcutaneous injections in day-old chickens but those techniques were replaced by *in ovo* technology in the modern poultry industry. Further work is needed to understand the mechanisms of action by which these formulations enhance the immunoprotective activity of CpG-ODNs in the mucosal immune system of the amniotic cavity of chicken embryos.

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ada; Agriculture, Food and Rural Revitalization, Saskatchewan; and Saskatchewan Chicken Industry Development Fund. LAB is a holder of the Canadian Research Chair in Vaccinology. The ODNs were supplied by Merial Limited, USA.

ABBREVIATIONS

CpG-ODN	=	Cytosine-phosphodiester-guanine oligodeoxynucleotides
PAMP	=	Pathogen-associated molecular patterns
cfu	=	Colony-forming units
IFN- γ	=	Interferon gamma
IL	=	Interlukin
TNF- α	=	Tumor necrosis factor alpha
NK	=	Natural killer cells
CL	=	Cationic lipid
PCPP	=	Poly [di(carboxylatophenoxy phosphazene)]
PCEP	=	Poly [di (sodium carboxylatoethylphenoxy) phosphazene]
BHI	=	Brain-heart infusion broth
HEPA	=	High efficiency particulate arresting
CCS	=	Cumulative clinical score
APC	=	Antigen-presenting cells

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