Aerosol delivery of synthetic DNA containing CpG motifs in broiler chicks at hatch under field conditions using a commercial-scale prototype nebulizer provided protection against lethal Escherichia coli septicemia

K. B. Goonewardene,[*] S. Popowich,[*] S. Gebhardt,[†] T. Gunawardana,[*] A. Gupta,[*] S. Kurukulasuriya,[*] R. Karunarathna,[*] M. Liu,[*] B. Chow-Lockerbie,[*] L. Ayalew,[*] K. A. Ahmed,[*] Houman Kamali,[*,¶] S. K. Tikoo,[‡] M. Foldvari,[§] P. Willson,[#] J. Boire,[†] K. Roberts,[†] N. Ambrose,[‖] C. Simonson,[¶] and S. Gomis[*,1]

*Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4, Canada; [†]RMD Engineering Inc., Saskatoon, SK S7K 3J7, Canada; [‡]Vaccinology and Immunotherapy, School of Public Health, University of Saskatchewan, Saskatoon, Saskatchewan 7N 5E3, Canada; [§]School of Pharmacy, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada; [#]Canadian Centre for Health and Safety in Agriculture, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E5, Canada; [‖]Sunrise Farms, Surrey, British Columbia V3W 1C9, Canada; and [¶]Department of Mechanical Engineering, College of Engineering, Saskatoon, Saskatchewan S7N 5A9, Canada

ABSTRACT Synthetic DNA containing CpG motifs (**CpG-ODN**) are potent innate immune stimulators in neonatal and adult broiler chickens against bacterial septicemia. We have recently demonstrated that intrapulmonary (**IPL**) delivery of CpG-ODN as microdroplets under laboratory conditions can protect neonatal chickens against lethal *Escherichia coli* septicemia. The objectives of this study were to develop a commercial-scale poultry nebulizer (**CSPN**) that can deliver CpG-ODN as microdroplets in neonatal broiler chicks in the hatcheries and study the efficacy of CSPN in inducing immune-protective effects under different environmental conditions in 2 geographical locations in Canada. Three field experiments were conducted in commercial poultry hatcheries during different seasons of the year in Saskatchewan and British Columbia, Canada. Neonatal broiler chicks (n = 8,000/experiment) received CpG-ODN by the IPL route in the CSPN chamber for 30 min, and control chicks received distilled water (**DW**) for 30 min. Broiler chicks (CpG-ODN—240 chicks/experiment and DW—40 chicks/experiment) were randomly sampled from all locations of the CSPN after nebulization and challenged with a lethal dose of *E. coli* to examine the CpG-ODN nebulization induced protection. We found a significant level ($P < 0.05$) of protection in broiler chicks against *E. coli* challenge, suggesting that the newly built CSPN successfully delivered CpG-ODN via the IPL route. We found that when the CSPN was maintained at humidex 28°C or below and relative humidity (**RH**) between 40 and 60%, neonatal birds were significantly ($P < 0.05$) protected against *E. coli* septicemia after IPL delivery of CpG-ODN. By contrast, protection in chicks was adversely affected when the CSPN was maintained at the humidex of 29°C or higher and RH of 70%. Overall, the present study successfully built a CSPN for CpG-ODN delivery in chicks at the hatchery and revealed that the temperature, humidity, and humidex were critical parameters in CSPN for efficient delivery of CpG-ODN.

Key words: hatchery, commercial-scale poultry nebulizer, immunoprotection, neonatal broiler

2021 Poultry Science 100:100934
https://doi.org/10.1016/j.psj.2020.12.031

INTRODUCTION

Bacterial infections during the first week of neonatal life cause substantial economic losses and animal welfare issues in the poultry industry. Among bacterial infections, *Escherichia coli* infection in neonatal chicks causes a variety of disease syndromes, including yolk-sac

Received May 21, 2020.
Accepted December 16, 2020.

[1]Corresponding author: susantha.gomis@usask.ca

infection, omphalitis, respiratory tract infection, and septicemia (Nolan et al., 2013). *Escherichia coli* infections in neonatal poultry are characterized by acute septicemia resulting in death, and subacute infection resulting in pericarditis, airsacculitis, and perihepatitis (Lutful Kabir, 2010; Nolan et al., 2013). Many *E. coli* isolates commonly associated with commercial broiler chickens belong to serogroups O1, O2, and O78 (Gomis et al., 2001; Ewers et al., 2004). Some *Salmonella* species cause yolk-sac infections and septicemia in neonatal chicks triggering increased first-week mortality resulting in significant economic losses to the poultry industry (Yassin et al., 2009; Kemmett et al., 2014). Antibiotics in feed have long been used in the poultry industry to prevent neonatal bacterial infections and improve animal welfare. Owing to the growing concerns of the emergence of antibiotic-resistant bacteria, the Chicken Farmers of Canada agreed to eliminate the prophylactic use of antibiotics, and urgently need suitable alternatives to antibiotics. Our laboratory provided several lines of evidence that synthetic DNA containing CpG motifs (**CpG-ODN**) alone can be a promising alternative to antibiotics against *E. coli* and *Salmonella* Typhimurium infections in chickens (Gomis et al., 2004; Taghavi et al., 2008; Gunawardana et al., 2019, 2020). We have recently reported that intrapulmonary (**IPL**) delivery of CpG-ODN as aerosolized microdroplets can protect neonatal broiler chicks against lethal *E. coli* septicemia under laboratory conditions (Goonewardene et al., 2017, 2020).

In veterinary medicine, the aerosol route has been used to administer vaccines and therapeutics to livestock animals, including chickens (Rundfeldt et al., 2013; Calderon-Nieva et al., 2017). In commercial poultry hatcheries, several vaccines against viral infections such as infectious bronchitis and Newcastle disease (Abdul-Cader et al., 2018) as well as against *E. coli* (Poulvac *E. coli*) (Sadeyen et al., 2015) are delivered to day-old chicks as coarse sprays. Aerosolized particles sprayed as coarse droplets of 5 to 10 μm diameter usually are trapped in the upper respiratory tract and do not reach into the deeper lung tissue (Miller, 1984; Tell et al., 2006). To reach the deeper lung, including secondary bronchi and air capillaries (Tell et al., 2006, 2012) in the area that contains bronchi-associated lymphoid tissues (Reese et al., 2006), the particles should be aerosolized as microdroplets of 1 to 3 μm diameter. A study conducted in pigeons using fluorescent microspheres demonstrated that particles less than 6 μm were distributed throughout the respiratory tract including deeper lung tissues while larger particles were deposited in the upper airway (Tell et al., 2006). Our study reported that the immune-protective function of CpG-ODN through intrapulmonary route is only effective when the CpG-ODN are aerosolized as microdroplets (particle size of 0.5–5 μm) using a compressor nebulizer (705-470) unit (AMG Medical Inc; Montreal, QC, Canada) (Goonewardene et al., 2017). However, the poultry industry lacks equipment that can deliver CpG-ODN microdroplets through the IPL route for large-scale applications in the hatchery.

Therefore, the objectives of this study were first to develop a commercial-scale poultry nebulizer (**CSPN**) that can deliver CpG-ODN through IPL route at hatch in broiler chicks. Next, *E. coli* challenge experiments were conducted to evaluate the efficacy of CSPN in facilitating CpG-ODN-induced protection in neonatal broiler chickens under different environmental conditions in Canada.

MATERIALS AND METHODS

Capacity and Features of Commercial-Scale Poultry Nebulizer

The CSPN was designed and manufactured to deliver CpG-ODN by the IPL route to a batch of 8,000 neonatal chicks during each run (Figure 1). The CSPN, comprising a nebulizer unit, an air conditioning unit, fans, and a chick enclosure, was designed to deliver a calculated amount of CpG-ODN while maintaining temperature (**T**), humidex, and relative humidity (**RH**) and monitoring carbon dioxide ($\mathbf{CO_2}$) concentration in the chick enclosure. The chick enclosure had a volume of 7.55 m^3 and received conditioned air from the nebulizer unit of the CSPN (where CpG-ODN was aerosolized). Before the air entered the nebulizer unit, it was conditioned by the air conditioning, and fan units (where the T, airflow rate, RH, and humidex were controlled). T and RH sensors were installed at the air inlet of the chick enclosure. A CO_2 sensor was installed at the air outlet of the chamber. The environmental parameters (T and RH) were monitored every second within the enclosure.

The nebulizer consisted of an ultrasonic array system, capable of delivering CpG-ODN at a particle size of 0.5 to 5 μm. An electric field applied across piezoelectric ceramic plate generated high-frequency ultrasonic waves to convert the CpG-ODN solution into aerosol droplets (Figure 2). The nebulizer system was located close to the air inlet of the chamber. Between each batch of broiler chicks, the CSPN was purged with fresh outside air to remove CO_2. The CSPN was designed and manufactured at RMD Engineering Inc. Saskatoon SK, Canada.

Broiler chicks were placed in the chick enclosure of the CSPN in chick baskets containing 102-104 chicks and stacked as columns, each containing 10 baskets (9 baskets with chicks and one empty basket on top). Eight stacks of chick baskets were loaded in the chick enclosure of CSPN for each nebulization test. An average of 70 mL CpG-ODN at a concentration of 12 mg/mL was nebulized for 30 min during each run.

Sampling of Chicks for E. coli Challenge

After nebulization, broiler chicks (n = 240/experimental trial) from the top, middle, and bottom baskets from each of the 8 stacks of chick baskets were collected to study the uniformity of dispersion of CpG-ODN (Figure 3). Broiler chicks were individually tagged to

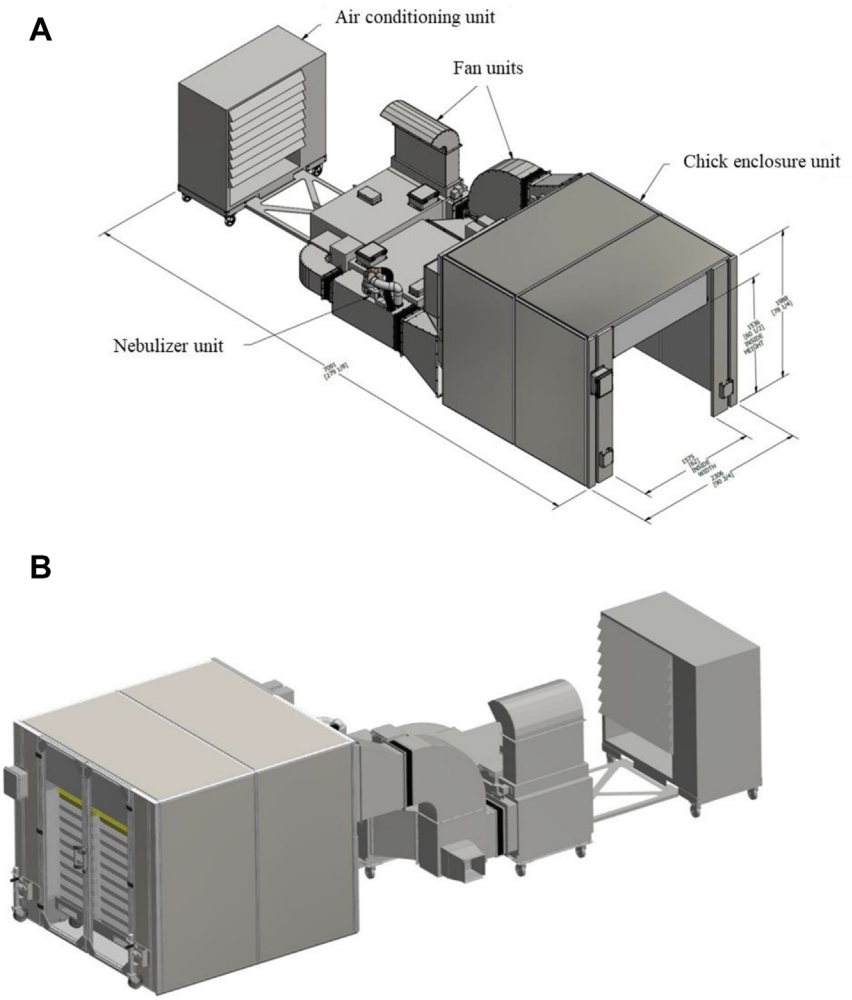

Figure 1. Conceptual design of the CSPN (commercial-scale poultry nebulizer). (A) Left view of the CSPN, and (B) right view of the CSPN.

record the basket of origin. A total of 10 broiler chicks per basket were collected from the 24 baskets labeled 1 to 24 in Figure 3. Chicks from the 24 baskets were divided into 6 separate groups in accordance with the color coding in Figure 3. The chicks from 4 baskets of an area were combined to form each group (n = 40, 10 birds from each basket), where baskets 1-4 formed group 1 (top left), baskets 5-8 formed group 2 (top right), baskets 9-12 formed group 3 (middle left), baskets 13-16 formed group 4 (middle right), baskets 17-20 formed group 5 (bottom left), and baskets 21-24 formed group 6 (bottom right). In addition, a group of birds (n = 40) that was exposed to nebulized (aerosolized) distilled water (**DW**) was included as group 7. The selected and tagged broiler chicks were transported from the respective hatchery to Animal Care Unit, Western College of Veterinary Medicine, University of Saskatchewan, Canada. The immune-protection study using the *E. coli* challenge was carried out to evaluate the efficacy and uniformity of the aerosol that was delivered to the birds by CSPN.

Ethics Statement

The animal experiment was approved by the University Committee on Animal Care and Supply Animal Research Ethics Board at the University of Saskatchewan (Animal use protocol number 20070008) and conducted following the guidelines of the Canadian Council on Animal Care.

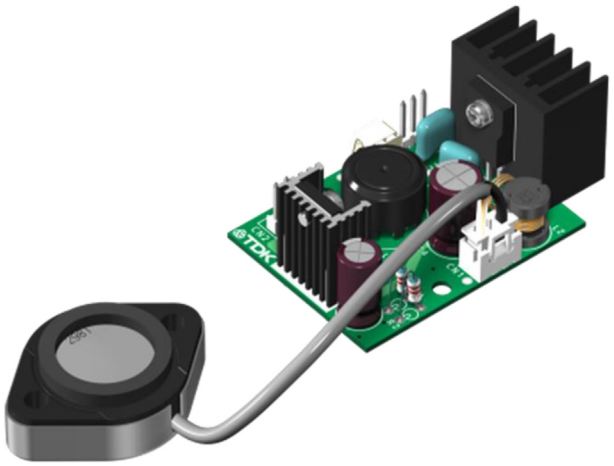

Figure 2. Ultrasonic-type nebulizer. An individual ultrasonic type nebulizer that consisted of a piezoelectric ceramic plate with the ability to generate high-frequency ultrasonic waves to convert the CpG-ODN solution into aerosol droplets. An array of these was used. Abbreviation: CpG-ODN, cytosine phosphodiester guanine oligodeoxynucleotide.

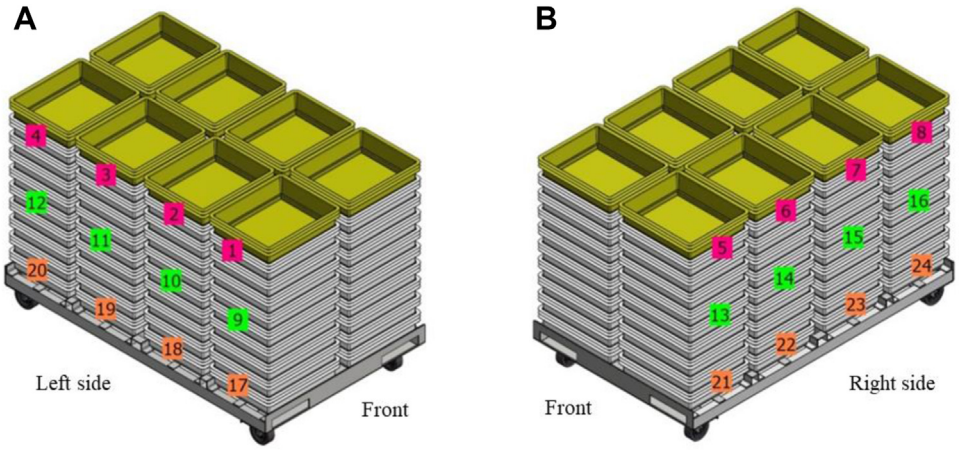

Figure 3. Schematic representing the stacks of broiler chick baskets and sampling scheme after nebulization. Day-old broiler chicks were sampled from individual chick baskets (n = 10) numbered 1 to 24 from the top, middle, and bottom and left and right side stacks for lethal *Escherichia coli* challenge after CpG-ODN delivery by the IPL (intrapulmonary) route in the chick enclosure of CSPN (commercial-scale poultry nebulizer). (A) Left view of the stacks of broiler chick baskets, and (B) Right view of the stacks of broiler chick baskets.

Animal Housing

Water and commercial broiler feed were provided ad libitum during the experimental observation period of 7 d. Broiler chicks were raised at 32°C for the first week of life, and after that, the temperature was decreased 0.5°C per day until a room temperature of 27.5°C was reached. The light was provided for 24 h/day for 0 to 2 d after hatch. Darkness was introduced at 3 d after hatch with 1 h of dark added daily until 4 h of darkness was achieved.

Escherichia coli Challenge

The challenge strain used was a field isolate of *E. coli* from a turkey with septicemia. Preparation of the *E. coli* challenge was carried out as described previously (Gomis et al., 2004; Goonewardene et al., 2017). This *E. coli* was nonhemolytic, serogroup O2, serum-resistant, produced aerobactin, with type 1 pili and a K1 capsule. Briefly, one colony of *E. coli* was taken and mixed in 100 mL of Luria broth (Difco LB broth, Miller, Becton Dickinson and Company; Sparks, MD). It was incubated at 37°C for 16 to 18 h, shaking at 150 rpm. A serial dilution of the diluted culture was plated in duplicates on 5% Columbia sheep blood agar plates, incubated for 18 h at 37°C, and the colonies were counted to confirm the *E. coli* challenge dose.

The *E. coli* challenge procedure was conducted as previously described (Goonewardene et al., 2017). Briefly, at day 2 after CpG-ODN treatment, birds were challenged with either 1×10^5 or 1×10^6 colony-forming units of *E. coli* by the subcutaneous route in the neck. Two doses of *E. coli* were given to birds to simulate field conditions because all birds in a commercial poultry barn are not exposed to a consistent dose of *E. coli*. Birds were evaluated 3 times daily at the critical stage (first 3 d after challenge) and twice thereafter for 8 to 10 d after challenge. Each bird was observed for clinical signs and a daily clinical score was assigned: 0 = normal; 0.5 = slightly abnormal appearance, slow to move; 1 = depressed, reluctant to move; 1.5 = reluctant to move, may take a drink and peck some; 2 = unable to stand or reach for food or water; and 3 = found dead. Birds that received a clinical score of 2 were euthanized by cervical dislocation. At the end of the trial, each bird was given a cumulative clinical score (**CCS**; the sum of daily clinical scores as previously described) (Gomis et al., 2004; Goonewardene et al., 2017). Chicks that were found dead or euthanized were necropsied immediately. On 8 to 10 d after challenge, the remaining birds were euthanized by cervical dislocation. Bacterial swabs were taken from the air sacs of dead and euthanized birds and cultured on 5% Columbia sheep blood agar in accordance with the quadrant streaking technique, and the same swabs were streaked on to a MacConkey agar plate as well. A semiquantitative estimate of *E. coli* isolation was conducted in accordance with the growth on blood agar. Growth on these plates was recorded on a scale from 0 to 4+, where 0 = no growth; few = less than 5 colonies; 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 (Hoeprich, 1972). Data from 3 individual experiments are described in this article to demonstrate the importance of RH and humidex in the chick enclosure when broiler chicks were nebulized with CpG-ODN using the CSPN. Experiment 1 was conducted in Saskatchewan, whereas experiments 2 and 3 were conducted in British Columbia, Canada.

CpG-ODN

The CpG-ODN (2007) sequence was 5′ – TCGTCGTTGTCGTTTTGTCGTT – 3′ and was free of endotoxin and produced with a phosphorothioate backbone (Operon Biotechnologies Inc., Huntsville, AL). The CpG-ODN was dissolved in sterile DW at a concentration of 12 mg/mL.

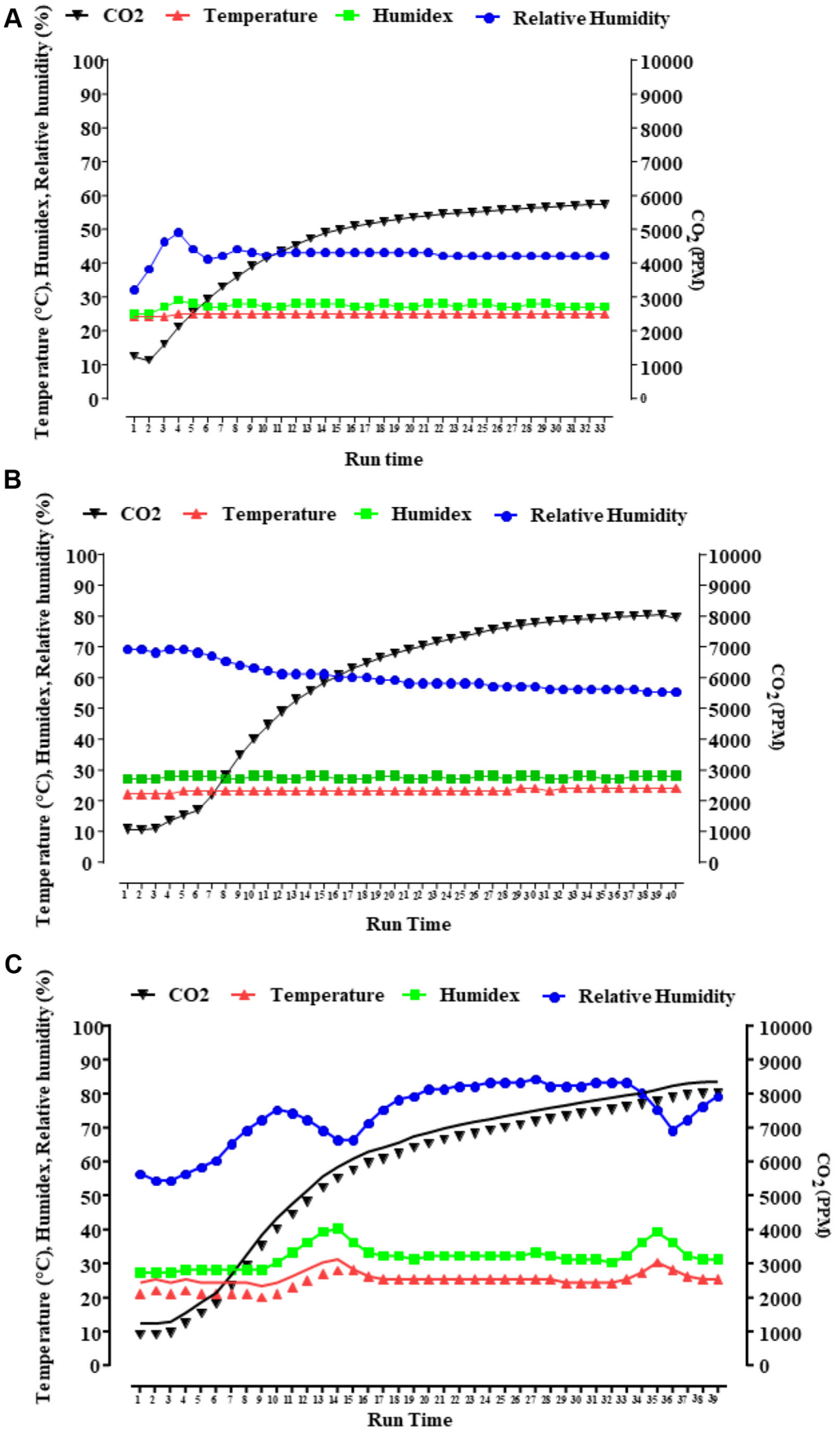

Figure 4. Temperature, humidity, humidex, and CO_2 level in the CSPN (commercial-scale poultry nebulizer) from experiment 1 (A), experiment 2 (B), and experiment 3 (C). Experiment 1 was conducted in Saskatchewan while experiments 2 and 3 were conducted in British Columbia. Humidex was set to 28, whereas relative humidity (RH) was set between 40 and 60% in experiments 1 and 2. RH was set to >60% in experiment 3.

Statistical Analysis

Clinical scores of broiler chicks after the *E. coli* challenge were assigned as previously described (Goonewardene et al., 2017). The significance of differences among groups in survival, bacteriological scoring, and CCS was analyzed and graphically presented using Prism (Prism 6.0, GraphPad Software Inc; San Diego, CA) with a significance level of $P < 0.05$. The significance among groups in survival patterns and median survival times were analyzed using the log-rank test. Significance of differences in CCS among groups was tested using Kruskal Wallis nonparametric analysis of variance when 3 or more groups were compared (i.e., to compare the effect of the location of the chick in the enclosure) or Mann Whitney nonparametric test when 2 groups were compared (i.e., to compare the exposure to aerosolized CpG-ODN with exposure to aerosolized DW).

RESULTS

Efficacy of the CSPN

Experiment 1 was conducted in Saskatchewan, Canada, during the winter of 2017. The outdoor conditions during the experiment were −3°C and 76% RH, giving a humidity ratio of 2.2 g/kg and a dew point temperature of −6.2°C. The command module of the CSPN was set to maintain humidex of 28 and temperature of 24°C at the beginning of experiment 1. The air conditions in the chick enclosure of CSPN were maintained at 25.5°C and 42% RH giving a humidex of 27.6 and humidity ratio of 8.3 g/kg (Figure 4A). The CO_2 concentration at the start of the test was 1,000 ppm and increased to 4,085 ppm at the end of 30 min nebulization period. The CO_2 concentration increases because the air in the chamber was recirculated; there was no fresh outdoor air supplied during the tests. Experiment 2 was conducted in British Columbia, Canada, during the summer of 2017. The outdoor conditions during the experiment were 14°C and 81% RH, giving a humidity ratio of 8.1 g/kg and a dew point temperature of 10.8°C. The command module of the CSPN was set to maintain humidex of 28 and temperature of 22°C at the beginning of experiment 2. The temperature inside the chick enclosure of CSPN was maintained at 23.7°C, RH was maintained at 61%, and humidex was maintained at 28 with a humidity ratio of 10.7 g/kg (Figure 4B). CO_2 level reached to 5,794 ppm at the end of the 30 min nebulization. Experiment 3 was conducted in British Colombia, Canada, during the winter of 2018, where the outdoor conditions were 5.5°C and 99% RH, giving a humidity ratio of 5.6 g/kg and a dew point temperature of −5.4°C. The command module of the CSPN was set to maintain humidex of 29 and temperature of 23°C at the beginning of experiment 3. The temperature of the CSPN was maintained at 22.9°C, RH was maintained at 71%, and humidex was 29.3, humidity ratio = 12.07 g/kg (Figure 4C). CO_2 level reached to 8,000 ppm at the end of 30 min period of nebulization. Airflow was maintained at 1,100 CFM in all 3 experiments. The climatic conditions of the chick enclosure of CSPN were summarized in Table 1.

Efficacy of Intrapulmonary Delivery of CpG-ODN by CSPN

In experiment 1, CpG-ODN nebulized chicks collected from different locations of CSPN were divided into 6 groups, group 1 (top left), group 2 (top right), group 3 (middle left), group 4 (middle right), group 5 (bottom left), and group 6 (bottom right) to conduct the *E. coli* challenge experiments to examine if CpG-ODN aerosol as generated by CSPN was equally distributed in CSPN nebulization chamber. We found no significant difference in the survival (Figure 5A) and CCS (Figure 6A) among the chicks of 6 groups representing the various areas of the CSPN. These results demonstrated that the CpG-ODN aerosol, as generated by CSPN, was equally distributed in multiple corners of the CSPN chamber. Therefore, after that, we combined data of all 6 groups to compare the CpG-ODN group with the DW group in our further experiments for the comparison.

The combined 6 groups of broiler chicks that were administered CpG-ODN by the IPL route in the chamber of the CSPN during experiment 1 had significantly higher survival ($P < 0.05$) than the group of broiler chicks that received DW (Figure 5B). Chicks administered CpG-ODN showed an average survival of 69%, whereas the group given DW had a survival of 43% after the *E. coli* challenge. Intrapulmonary CpG-ODN-administered chicks had a significantly lower CCS score than the group that received DW (Figure 6B). The same group of chicks demonstrated lower bacterial load in the thoracic cavity than the group that received DW (Figure 7A).

In experiment 2, the chicks that received CpG-ODN by the IPL route in the chamber of CSPN had significantly higher survival ($P < 0.05$) than the group of birds that were administered DW (Figure 5C). Chicks administered CpG-ODN by the IPL route had 51.7% survival after the *E. coli* challenge. By contrast, the group that received DW by the IPL route had 25% survival. Intrapulmonary CpG-ODN-administered birds had a significantly lower CCS score than the DW control (Figure 6C). The birds that were administered CpG-ODN by the IPL route had a lower bacterial load than the DW control group (Figure 7B).

No significant protection ($P > 0.05$) was seen in experiment 3 between groups that received CpG-ODN or DW by the IPL route (Figure 5D). No significant difference was noted in CCS between groups of broiler chicks that received CpG-ODN or DW by the IPL route (Figure 6D). There was no difference between bacterial loads in groups of birds that received CpG-ODN or DW by the IPL route (Figure 7C).

Table 1. Temperature, relative humidity, humidex, humidity ratio, and CO_2 in the chick enclosure of the CSPN for individual experiment.

Experiment	T ± SD	RH ± SD	Humidex ± SD	W ± SD	CO_2 start (ppm)	CO_2 end (ppm)
1	25.5 ± 0.3	42 ± 2	27.6 ± 0.8	8.3 ± 0.6	1,250	5,700
2	23.7 ± 0.6	61 ± 5	28.0 ± 0.5	10.7 ± 0.6	1,000	8,000
3	22.9 ± 1.8	71 ± 9	29.3 ± 3.39	12.1 ± 2.0	1,000	8,000

Abbreviations: CSPN, commercial-scale poultry nebulizer; T temperature; RH, relative humidity; W, humidity ratio; CO_2 in the chick enclosure of the CSPN.

DISCUSSION

To the best of our knowledge, this is the first study that conducted field testing of a CSPN for IPL delivery of CpG-ODN to induce protective immunity against bacterial infections during the first week of neonatal life. We designed CSPN with a large chamber that can hold up to 8,000 chicks during each run and can aerosolize CpG-ODN as microdroplets (size 0.5–3 μm) to nebulize newly hatched broiler chicks. The CSPN has a built-in integrated climate control system designed and fabricated to assist with the regulation of the climate inside the chamber to ensure efficient delivery of CpG-ODN while maintaining the bird's comfort and welfare. This system was designed to operate without interfering with the pace and efficiency of routine operations of a commercial hatchery. We performed several field experiments to evaluate the efficacy of CSPN to aerosolize CpG-ODN, and its ability to maintain optimum temperature, CO_2, humidity inside the nebulizer chamber to ensure bird's welfare and comfort. The field experiments were carried out in 2 commercial broiler hatcheries in the winter months in Saskatchewan and the winter and summer months in British Colombia, Canada, to test the efficacy of CSPN under different environmental conditions. As a readout of efficient intrapulmonary delivery of CpG-ODN by CSPN, we have used our well-established *E. coli* animal challenge model (Goonewardene et al., 2017) to test the immune-protective effect of CpG-ODN after IPL delivery using CSPN.

The ambient environmental conditions in the CSPN chick enclosure had a significant impact on aerosol dynamics as well as the inhalation efficacy of chicks. Our experiments identified that humidex of 28 and RH below 60% is critical for nebulization of broiler chicks to induce CpG-OD-mediated protective immunity against bacterial infections and lethal septicemia. We found that

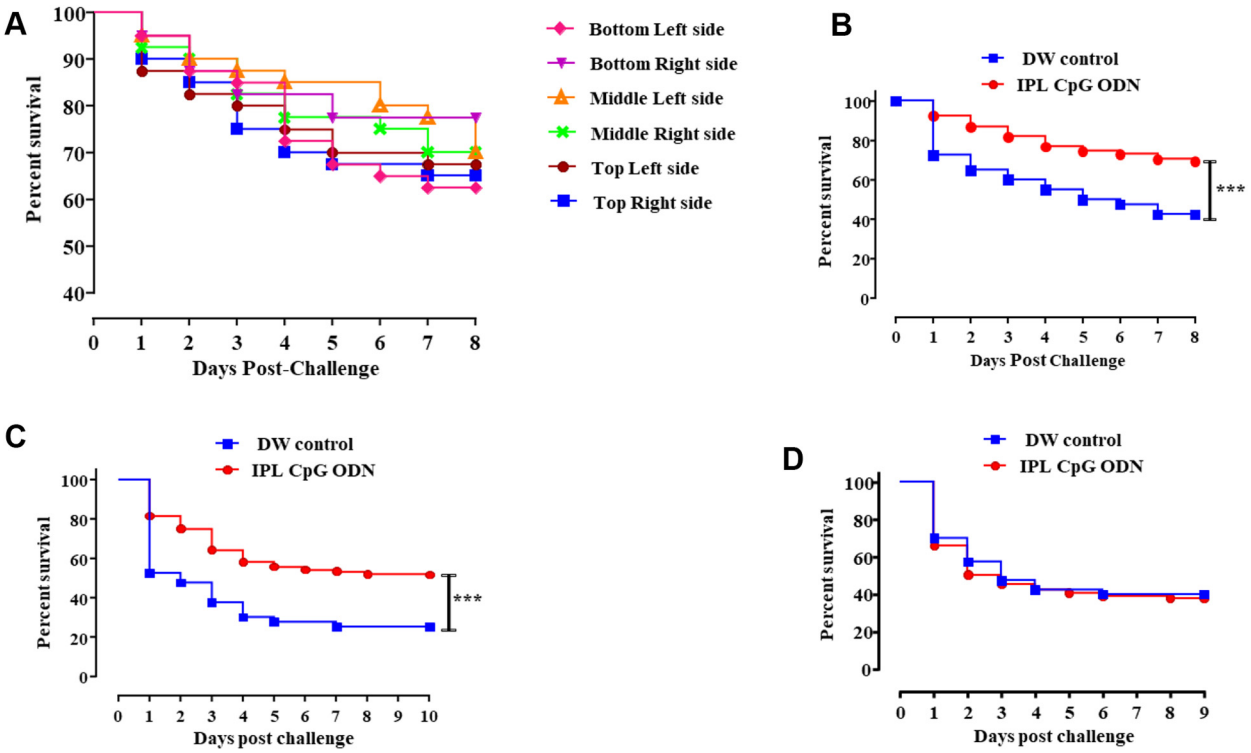

Figure 5. Survival of birds after lethal *Escherichia coli* challenge from experiment 1 (A, B), experiment 2 (C) and experiment 3 (D). In experiment 1, CpG-ODN groups were compared among themselves for the chicks' survival after the IPL (intrapulmonary) CPG-ODN delivery. The survival percentages were not significantly different among the 6 CpG-ODN groups (n = 40/groups). The 6 CpG-ODN groups were combined in further experiments to compare with the DW (distilled water) group. The birds (n = 240) administered with CpG-ODN via the intrapulmonary (IPL) route showed significantly better survival ($P < 0.05$) compared to the DW control (n = 40) in experiments 1 (B) and 2 (C). No significant protection was seen between the DW control and IPL CpG-ODN groups ($P > 0.05$) in experiment 3 (D).

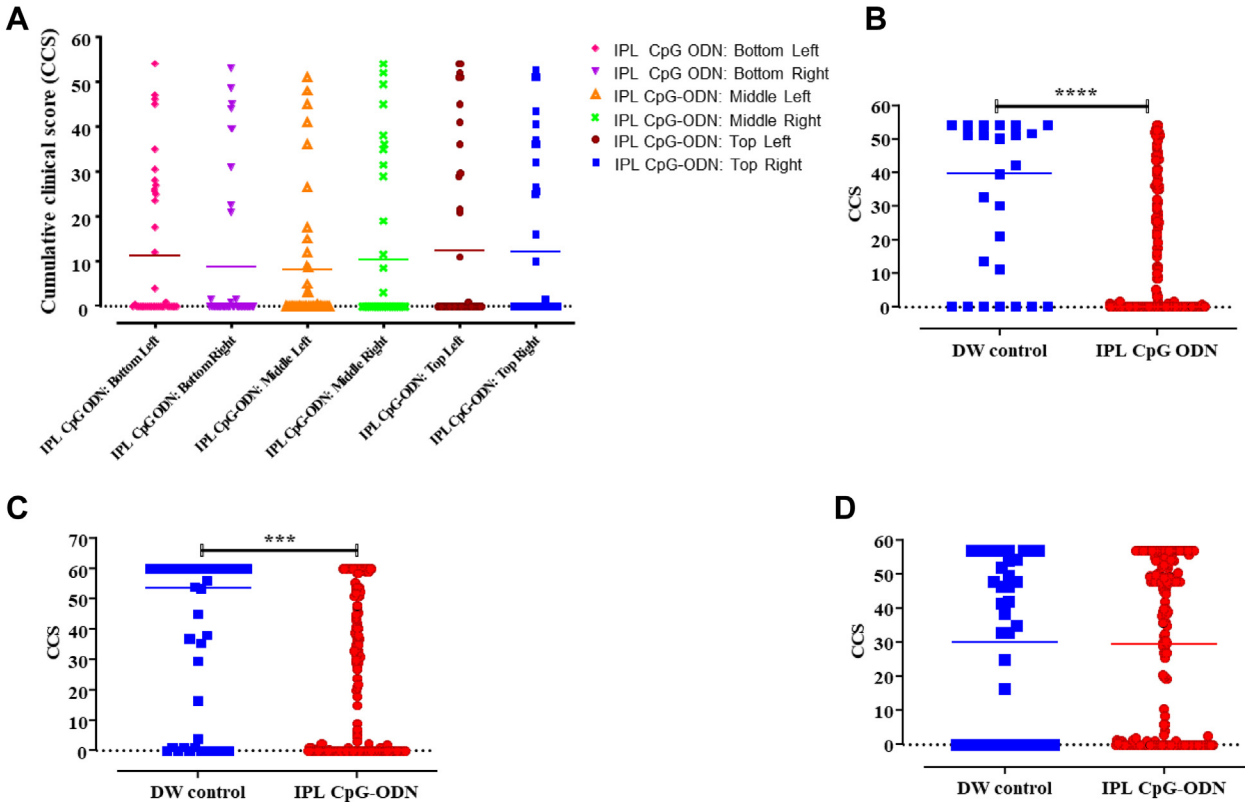

Figure 6. Cumulative clinical score (CCS) of broiler chicks after lethal *Escherichia coli* challenge. (A) In experiment 1, nebulized chicks were collected from the top, middle, and bottom baskets from each of the 8 stacks of chick baskets and were grouped accordingly. These CpG-ODN groups were compared among themselves for the clinical scores after the IPL (intrapulmonary) CPG-ODN delivery. The CCS were not significantly different among the 6 CpG-ODN groups (n = 40/groups). The 6 CpG-ODN groups were combined in further experiments to compare with the DW (distilled water) group (B–D). Birds that received CpG-ODN by the IPL route had a significantly low CCS ($P < 0.05$) compared with birds that received DW in experiment 1 (B) and experiment 2 (C). D. In experiment 3, birds that received CpG-ODN or DW by the IPL route did not have a significant difference in CCS ($P > 0.05$).

when RH was increased above 60% and humidex above 28 in the chick enclosure, CSPN was inefficient in inducing CpG-ODN-mediated immune protection in chicks. Humidity ratio (**W**) is a powerful variable that represents the potential for moisture transfer between the air in the CSPN enclosure and the lungs of the birds (ASHRAE, 2017). Moisture transfer rate in the lungs would be proportional to the difference between the W of the air in the CSPN enclosure and the W of the air exhaled by the chicks. The W of the air exhaled by the chicks is likely constant in most cases for healthy chicks. Thus, the W of the air in the CSPN may have the most significant effect on the function of the lungs and thereby, CpG-ODN efficacy. Our third experiment displayed the highest W in the air inside the CSPN chamber and was associated with no protection from CpG-ODN against *E. coli*. If the W of the air entering into the lung is too high, the respiration air will not dry the lung optimally, and some parts of the lung would become flooded. This could reduce the air exchange in the lung and concurrently reduce the absorption of CpG-ODN. As a result, protection against *E. coli* septicemia did not occur after CpG-ODN nebulization in experiment 3.

Another critical parameter that requires constant monitoring is the levels of CO_2 in the CSPN chamber to which birds are exposed during the nebulization process. It has been demonstrated that 6- to 8-week-old chickens exposed to 50,000 ppm of CO_2 for 1 h showed difficulty in breathing (Anderson et al., 1966). Studies have reported that newly hatched chicks when exposed to 750,000-900,000 ppm CO_2 lost posture and motion (Gurung et al., 2018) and lost consciousness upon exposure to 900,000 ppm CO_2 in the air (Raj and Whittington, 1995). During our experiments, CSPN maintained CO_2 levels between 1,000 and 8,000 ppm inside the chamber, and the chicks did not show any signs of discomfort during the nebulization process, indicating that the CSPN was able to maintain CO_2 levels inside the chamber at a safe level.

Synthetic CpG-ODN mimic bacterial DNA and act as danger signals to stimulate immune cells (Krieg, 2002). We have previously demonstrated that CpG-ODN administration via the subcutaneous and intramuscular routes can protect neonatal chicks against *E. coli* and *Salmonella* Typhimurium septicemia (Gomis et al., 2003, 2004; Taghavi et al., 2008). Furthermore, we demonstrated that *in ovo* delivery of CpG-ODN induces antimicrobial immunity not only by upregulating cytokines but also through the enrichment of various immunological niches (Gunawardana et al., 2019). Although *in ovo* delivery is a feasible industry technique, antimicrobial protective effects last only for 3 d after hatch

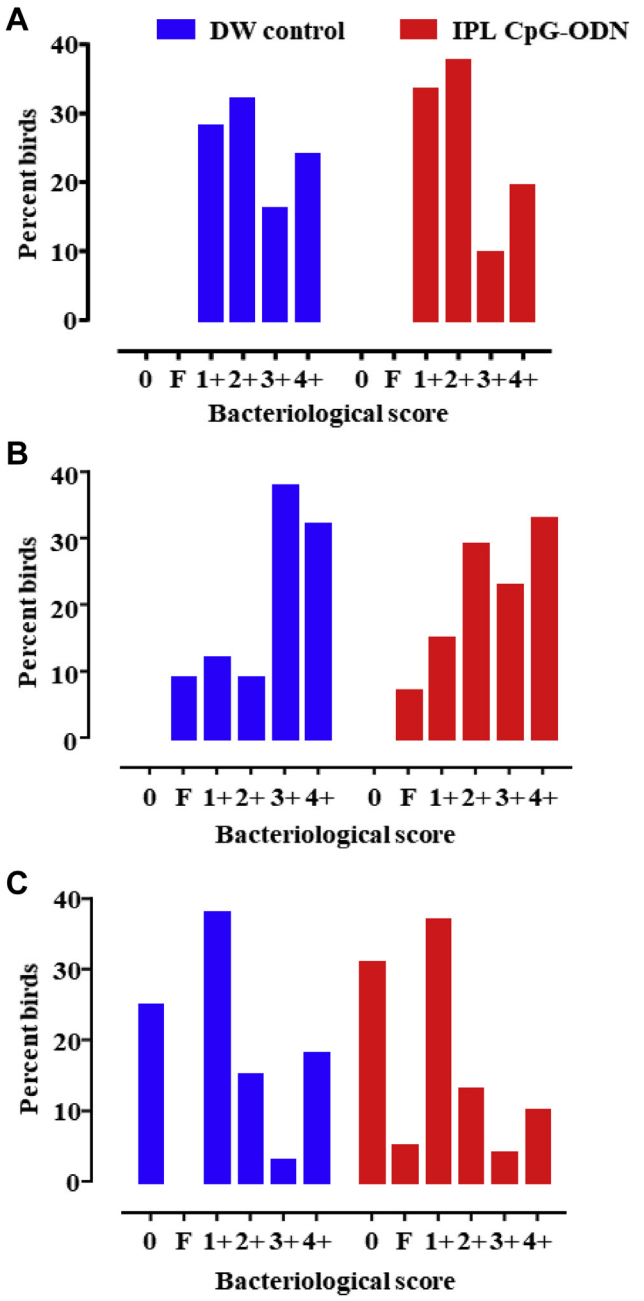

Figure 7. Bacterial score of broiler chicks after a lethal *Escherichia coli* challenge. Birds that received CpG-ODN by the IPL (intrapulmonary) route in CSPN in experiment 1 (A) and experiment 2 (B) tended to have lower bacterial loads than the birds that received DW (distilled water). This tendency was not as apparent in experiment 3 (C) where the bacterial loads appear similarly in the birds administered CpG-ODN in the CSPN (commercial-scale poultry nebulizer) and the DW control.

after *in ovo* delivery of CpG-ODN (Taghavi et al., 2008; Gunawardana et al., 2014). We have recently demonstrated that IPL delivery of CpG-ODN using a small-scale nebulizer can protect neonatal chicks against *E. coli* septicemia as early as 6 h and up to 5 d after treatment under laboratory conditions (Goonewardene et al., 2017). In this study, we conducted field trials of our newly developed CSPN in different seasons of Saskatchewan and British Colombia, Canada, where climatic conditions are considerably different. Our data from experimental trials 1 and 2 indicate that IPL delivery of CpG-ODN through our newly developed CSPN-induced protective immunity against *E. coli* in neonatal broiler chickens. The protective effect is also evidenced by a substantial reduction of clinical signs and bacterial loads in broiler chicks that received CpG-ODN by the IPL route using CSPN under field conditions. These data are also supported by our previous studies that demonstrated reduced clinical signs and bacterial loads in chicks treated with CpG-ODN by different routes (Gomis et al., 2003, 2004; Taghavi et al., 2008; Goonewardene et al., 2017).

In summary, the present study successfully built and field-tested CSPN for CpG-ODN delivery to induce antimicrobial immunity in neonatal broiler chicks and provided data on critical parameters, such as temperature, humidity, and humidex that are required for an efficient CpG-ODN nebulization. In the present study, although we nebulized birds with CpG-ODN for 30 min using the CSPN, we could potentially reduce this duration by 50% in the future, as we have demonstrated using our laboratory-scale nebulizer (Goonewardene et al., 2017). We believe that the CSPN can be a very useful tool not only for the CpG-ODN delivery but also for other drugs or medications that require a large-scale IPL delivery of aerosolized microdroplets.

ACKNOWLEDGMENTS

The authors greatly appreciate and are thankful to the commercial broiler hatcheries and growers for their help and support during the field trials. The authors greatly appreciate the support from Prairie Pride Chick Sales Ltd, Grandora, Saskatchewan, Canada and Fraser Valley Chick Sales Ltd. Abbotsford, British Columbia, Canada, for all of their help and support during the field trials. Special thanks to Klaas Korthius of Homeland Farms Ltd, Chilliwack British Columbia, Canada, for his assistance and encouragement. Finally, credits to the animal care technicians at the Western College of Veterinary Medicine, Canada for their service and helping hands. Funding for this part of the project was provided by the Western Economic Diversification Canada (13187), Natural Sciences and Engineering Research Council of Canada (CRDPJ 478086-14), Alberta Livestock and Meat Agency (2015F031 R), Canadian Poultry Research Council (AMN081) and Chicken Farmers of Saskatchewan (345544).

DISCLOSURES

Authors declare no conflict of interest.

REFERENCES

Abdul-Cader, M. S., V. Palomino-Tapia, A. Amarasinghe, H. Ahmed-Hassan, U. De Silva Senapathi, and M. F. Abdul-Careem. 2018. Hatchery Vaccination against poultry viral diseases: potential Mechanisms and Limitations. Viral Immunol. New Rochelle 31:23–33.

Anderson, D. P., C. W. Beard, and R. P. Hanson. 1966. The Influence of inhalation of carbon dioxide on chickens, including resistance to infection with Newcastle disease Virus. Avian Dis. 10:216–224.

ASHRAE. 2017. Pages 1–7 in ASHRAE 2017 Fundamentals Hand Book. ASHRAE, Atlanta, GA.

Calderon-Nieva, D., K. B. Goonewardene, S. Gomis, and M. Foldvari. 2017. Veterinary vaccine nanotechnology: pulmonary and nasal delivery in livestock animals. Drug Deliv. Transl. Res. 7:558–570.

Ewers, C., T. Janßen, S. Kießling, H.-C. Philipp, and L. H. Wieler. 2004. Molecular epidemiology of avian pathogenic Escherichia coli (APEC) isolated from colisepticemia in poultry. Vet. Microbiol. 104:91–101.

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

Gomis, S. M., C. Riddell, A. A. Potter, and B. J. Allan. 2001. Phenotypic and genotypic characterization of virulence factors of Escherichia coli isolated from broiler chickens with simultaneous occurrence of cellulitis and other colibacillosis lesions. Can J. Vet. Res. 65:1–6.

Goonewardene, K., K. A. Ahmed, T. Gunawardana, S. Popowich, S. Kurukulasuriya, R. Karunarathna, A. Gupta, L. E. Ayalew, B. Lockerbie, M. Foldvari, S. Tikoo, P. Willson, and S. Gomis. 2020. Mucosal delivery of CpG-ODN mimicking bacterial DNA via the intrapulmonary route induces systemic antimicrobial immune responses in neonatal chicks. Sci. Rep. 10:5343.

Gunawardana, T., K. A. Ahmed, K. Goonewardene, S. Popowich, S. Kurukulasuriya, R. Karunarathana, L. E. Ayalew, A. Gupta, B. Lockerbie, M. Foldvari, S. K. Tikoo, P. Willson, and S. Gomis. 2020. CpG-ODN induces a dose-Dependent enrichment of immunological niches in the Spleen and lungs of neonatal chicks that Correlates with the protective immunity against Escherichia coli. J. Immunol. Res. 2020:2704728.

Goonewardene, K., S. Popowich, T. Gunwardana, A. Gupta, S. Kurukulasuriya, R. Karunarathna, B. Chow-Lockerbie, K. A. Ahmed, S. K. Tikoo, M. Foldvari, P. Willson, and S. Gomis. 2017. Intrapulmonary delivery of CPG-ODN microdroplets Provides protection against Escherichia coli septicemia in neonatal broiler chickens. Avian Dis. 61:503–511.

Gunawardana, T., K. A. Ahmed, K. Goonewardene, S. Popowich, S. Kurukulasuriya, R. Karunarathna, A. Gupta, B. Lockerbie, M. Foldvari, S. K. Tikoo, P. Willson, and S. Gomis. 2019. Synthetic CpG-ODN rapidly enriches immune compartments in neonatal chicks to induce protective immunity against bacterial infections. Scientific Rep. 9:341.

Gunawardana, T., M. Foldvari, T. Zachar, S. Popowich, B. Chow-Lockerbie, M. V. Ivanova, S. Tikoo, S. Kurukulasuriya, P. Willson, and S. Gomis. 2014. Protection of neonatal broiler chickens following in ovo delivery of Oligodeoxynucleotides containing CpG motifs (CpG-ODN) Formulated with carbon Nanotubes or Liposomes. Avian Dis. 59:31–37.

Gurung, S., D. White, G. Archer, D. Zhao, Y. Farnell, J. A. Byrd, E. D. Peebles, and M. Farnell. 2018. Evaluation of Alternative Euthanasia Methods of Neonatal Chickens. Animals (Basel) 8:37.

Hoeprich, P. D. 1972. Infectious Diseases: A Guide to the Understanding and Management of Infectious Processes. Medical Dept., Harper & Row; Hagerstown, MD.

Kemmett, K., N. J. Williams, G. Chaloner, S. Humphrey, P. Wigley, and T. Humphrey. 2014. The contribution of systemic Escherichia coli infection to the early mortalities of commercial broiler chickens. Avian Pathol. 43:37–42.

Krieg, A. M. 2002. CpG motifs in bacterial DNA and their immune effects. Annu. Rev. Immunol. 20:709–760.

Lutful Kabir, S. M. 2010. Avian colibacillosis and Salmonellosis: a closer Look at epidemiology, Pathogenesis, Diagnosis, control and Public Health concerns. Int. J. Environ. Res. Public Health 7:89–114.

Miller, T. A. 1984. Nebulization for avian respiratory disease. Mod. Vet. Pract. 65:309–311.

Nolan, L. K., H. J. Barnes, J.-P. Vaillancourt, T. Abdul-Aziz, and C. M. Logue. 2013. Colibacillosis. Pages 751–805 in Diseases of Poultry. 13th ed. John Wiley & Sons, Ltd., Hoboken, NJ.

Raj, A. B., and P. E. Whittington. 1995. Euthanasia of day-old chicks with carbon dioxide and argon. Vet. Rec. 136:292–294.

Reese, S., G. Dalamani, and B. Kaspers. 2006. The avian lung-associated immune system: a review. Vet. Res. 37:311–324.

Rundfeldt, C., E. Wyska, H. Steckel, A. Witkowski, G. Jeżewska-Witkowska, and P. Wlaź. 2013. A model for treating avian aspergillosis: serum and lung tissue kinetics for Japanese quail (Coturnix japonica) following single and multiple aerosol exposures of a nanoparticulate itraconazole suspension. Med. Mycol. 51:800–810.

Sadeyen, J.-R., Z. Wu, H. Davies, P. M. van Diemen, A. Milicic, R. M. La Ragione, P. Kaiser, M. P. Stevens, and F. Dziva. 2015. Immune responses associated with homologous protection conferred by commercial vaccines for control of avian pathogenic Escherichia coli in turkeys. Vet. Res. 46:5.

Taghavi, A., B. Allan, G. Mutwiri, A. Van Kessel, P. Willson, L. Babiuk, A. Potter, and S. Gomis. 2008. Protection of neonatal broiler chicks against Salmonella Typhimurium septicemia by DNA containing CpG motifs. Avian Dis. 52:398–406.

Tell, L. A., K. Stephens, S. V. Teague, K. E. Pinkerton, and O. G. Raabe. 2012. Study of nebulization delivery of aerosolized fluorescent microspheres to the avian respiratory tract. Avian Dis. 56:381–386.

Tell, L. A., S. Smiley-Jewell, D. Hinds, K. E. Stephens, S. V. Teague, C. G. Plopper, and K. E. Pinkerton. 2006. An aerosolized fluorescent microsphere technique for evaluating particle deposition in the avian respiratory tract. Avian Dis. 50:238–244.

Yassin, H., A. G. J. Velthuis, M. Boerjan, and J. van Riel. 2009. Field study on broilers′ first-week mortality. Poult. Sci. 88:798–804.