The effect of diatomaceous earth in live, attenuated infectious bronchitis vaccine, immune responses, and protection against challenge

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ABSTRACT Live virus vaccines are commonly used in poultry production, particularly in broilers. Massive application and generation of a protective local mucosal and humoral immunity with no adverse effects is the main goal for this strategy. Live virus vaccines can be improved by adding adjuvants to boost mucosal innate and adaptive responses. In a previous study we showed that diatomaceous earth (DE) can be used as adjuvant in inactivated vaccines. The aim of this study was to test DE as adjuvant in an Ark-DPI live infectious bronchitis virus (IBV) vaccine after ocular or spray application. Titrating the virus alone or after addition of DE showed that DE had no detrimental effect on the vaccine virus. However, adding DE to the vaccine did not induce higher IgG titers in the serum and IgA titers in tears. It also did not affect the frequency of CD4$^+$ T cells, CD8$^+$ T cells and monocytes/macrophages in the blood and the spleen determined by flow cytometry. In addition, protection generated against IBV homologous challenges, measured by viral load in tears, respiratory signs and histopathology in tracheas, did not vary when DE was present in the vaccine formulation. Finally, we confirmed through our observations that Ark vaccines administered by hatchery spray cabinet elicit weaker immune responses and protection against an IBV homologous challenge compared to the same vaccine delivered via ocular route.

Key words: Diatomaceous earth, vaccines, adjuvants, IBV, immune responses

INTRODUCTION

Infectious bronchitis virus (**IBV**) is a Gamma coronavirus and the causal agent of IB. This disease causes significant economic losses in the poultry industry worldwide. To date, vaccination is the most widely used method to control IBV. Live, attenuated vaccines are the most commonly used in commercial poultry, particularly in broiler chickens. These vaccines are usually applied massively, through drinking water or spray and/or individually via the eye drop route. The main objective of the strategy is the generation of a protective local mucosal and humoral immunity. Secondary effects of live, attenuated IBV vaccination, such as mild respiratory signs as well as reduced egg production and eggshell quality in layers (Bwala et al., 2012), are a common outcome of their use. Due to the dozens of recognized serotypes and even more genotypes, IBV vaccination is challenging. The constant surveillance of field strains and the use of homologous vaccines is the only strategy to avoid failed protection against heterologous strains (Capua et al., 1999; Cavanagh, 2003; Cook et al., 2012). Therefore, improving live virus vaccines by adding components, such as adjuvants to boost mucosal innate and adaptive responses, is worth investigating.

Adjuvants have been used for decades in vaccine formulation. Oil-based emulsions and aluminium derivatives are routinely used in poultry inactivated vaccines to increase their immunogenicity and improve antigen delivery (Spickler and Roth, 2003). However, the use of adjuvants substantially increases the price of inactivated vaccines (Meeusen et al., 2007). Experimental utilization of adjuvants in live, attenuated vaccines for poultry has been previously investigated. Mucosal immune responses and protection of chickens against Newcastle disease virus (**NDV**) challenge after live attenuated vaccination of NDV were improved by using chitosan nanoparticles as adjuvant (Zhao et al., 2012). In addition, better antibody responses and protection against challenge in chickens was demonstrated when a live IBV vaccine was mixed with various types of adjuvants compared with a non-adjuvanted vaccine (Deville et al., 2012). Kjaerup and co-workers tested mannose-binding lectin ligands such as mannan, chitosan, and fructo-oligosaccharide as adjuvants in live IBV vaccines with mixed success (Kjaerup et al., 2014). In this study, we investigated the use of diatomaceous earth (**DE**) as

an inexpensive and readily available adjuvant to boost local mucosal and adaptive immune responses generated by an IBV live, attenuated vaccine.

DE is a natural siliceous sediment of fossilized remains of microalgae called diatoms (Dolatabadi and de la Guardia, 2011). The surface of DE is covered with numerous porous silica capsules, increasing its total surface and allowing its use as a drug-delivery carrier (Aw et al., 2013; Wang et al., 2013). In addition, silica, the main component of DE, has been reported to have higher colloid stability than aluminium hydroxide and to act as an adjuvant (Lincopan et al., 2009). We have previously demonstrated that DE possess adjuvant capabilities comparable to aluminium hydroxide when used to formulate NDV inactivated vaccines (Nazmi et al., 2017).

In this study, we investigated the use of DE as an inexpensive and readily available adjuvant to boost local mucosal and adaptive immune responses generated by an IBV live, attenuated vaccine.

MATERIALS AND METHODS

IBV Vaccine and Challenge Virus

A commercial live, attenuated IBV Ark Type live virus MILDVAC®-ARK vaccine (Merck, Omaha, NE) was reconstituted in sterile phosphate-buffered saline (**PBS**) following the manufacturer's instructions. The vaccine was titrated in 10-day-old, embryonated, specific-pathogen-free (**SPF**) eggs (Charles River, Franklin, CT) according to routine procedures (Villegas, 2008). For the challenge, we used a virulent IBV Ark-DPI strain kindly provided by Dr. Haroldo Toro, Auburn University, Auburn, AL (Gallardo et al., 2011).

IBV Vaccine Viability After Mixed with DE

To test the effect of DE on IBV ArkDPI vaccine virus viability, the vaccine was reconstituted in tryptose phosphate broth (**TPB**) (Becton, Dickinson and Company, Sparks, MD). Half of the vaccine final volume was mixed with 20 mg of DE (Earthworks Health, Norfolk, NE) per ml. Viral titration was performed immediately in 10-day-old, embryonated SPF eggs as described in Villegas, 2008.

Chickens

Seventy-two chicks were hatched from SPF eggs and housed in wired cages inside BSL2 rooms at the Teaching and Res. Animal Care Services at the University of California, Davis. Feed and water were provided ad libitum. All procedures and animal care were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis (protocol #: 17863).

Table 1. Experimental design showing number of birds, viral titer, and DE dose in each experimental group.

Group	Number of birds	Titer per dose ($EID_{50\%}$)	DE (mg/dose)
Spray DE+IBV	13	$10^{3.7}$	2
Spray IBV	13	$10^{3.7}$	-
Spray DE	9	-	2
Ocular DE+IBV	13	$10^{3.7}$	2
Ocular IBV	15	$10^{3.7}$	-
Ocular DE	9	-	2

DE: diatomaceous earth, IBV: infectious bronchitis virus, $EID_{50\%}$: embryo infectious dose 50%.

Experimental Design

At 3 d of age, SPF chickens were divided into 6 groups (Table 1). The first 3 groups were vaccinated with DE+IBV, IBV alone or DE alone, using a standard hatchery spray cabinet (MSD, Gainesville, GA). The other 3 groups were vaccinated intraocularly with DE+IBV, IBV alone, or DE alone. The final vaccine titer was $10^{3.7}$-embryo infectious dose 50% ($EID_{50\%}$) per dose per bird, regardless of the route of delivery, and was chosen as a lower dose that would not elicit an optimal response without an adjuvant. The amount of DE in each dose was 2 mg.

Twenty-one days post-vaccination (**dpv**), at 24 d of age, birds from all groups were challenged with a homologous IBV Ark-DPI strain via the oculo-nasal route. The challenge virus dose titer was $2 \times 10^{5.8}$ EID_{50} per bird. Chicks were weighed weekly starting at 3 d of age (0 dpv) and until 24 d of age (21 dpv).

Serum IgG Antibody

Blood was collected from all birds via wing vein puncture at 7, 14, 21 dpv and 6 d post-infection (**dpi**). IBV-specific IgG titers in the sera were assessed using a commercial IBV enzyme-linked immunosorbent assay (**ELISA**) (IDEXX, Westbrook, ME) according to the manufacturer's instructions. All samples were tested in duplicate. Samples with titers higher than 396 were considered positive, according to the manufacturer specifications.

Tear IgA Antibody

Tears were collected from each chicken at 14 and 21 dpv and 6 dpi. Tears were diluted 1:5 in sample diluent of ELISA kits and tested in duplicate. IBV specific IgA was measured using a commercial IgG ELISA kit (IDEXX) with substitution of the conjugated anti-chicken IgG antibody for a HRP conjugated anti-chicken IgA antibody (Gallus Immunotech, Cary, NC). The conjugated IgA antibody was used at a 1:1000 dilution. Results were reported as optical density (OD) measured at 650 nm wavelength using a spectrophotometer (BioTek, Winoosk, VA).

Flow Cytometry

At 11 dpv and 6 dpi, blood was collected in a heparinized tube from 2 to 3 birds per group for isolation of peripheral blood mononuclear cells (**PBMC**). The same birds were euthanized and the spleen was removed. A single cell suspension was obtained from the spleens by mechanical disruption as described (van Ginkel et al., 2008). Blood or spleen suspensions were layered over a histopaque 1077 gradient (Sigma-Aldrich, St. Louis, MO) at room temperature and centrifuged. The interface was removed and washed. Live cells were counted by trypan blue exclusion. Cells were adjusted to 1×10^6 per mL and stained with mouse anti-chicken monocyte/macrophage-FITC, CD4-PE, and CD8α-Alexa Fluor 647 conjugated antibodies (Southern Biotech, Birmingham, AL) to detect monocytes/macrophages, CD4 T cells and CD8α T cells, respectively. After staining, cells were washed and fixed in 4% paraformaldehyde. Immune cell populations were detected using a Becton Dickinson FACScan (Franklin Lake, NJ).

Viral Load

IBV RNA was isolated from tears obtained 7 dpv and 6 dpi using the QIAamp Viral RNA Mini Kit in a QIAcube (Qiagen, Redwood, CA), following the manufacturer's instructions. Viral load was assessed by RT-qPCR as described in (de Quadros, 2011), using the QuantiTect Multiplex PCR Master Mix (Qiagen). In a 20 μL reaction, 5 μL of isolated RNA was added to 15 μL of master mix (10 μL 2× buffer, 0.2 μL enzyme mix, 1 μL primer mix, and 3.8 nuclease-free water). The primer mix contained primers HmqF2 (ATACTCCTAACTAATG-GTCAACAATG) and HmqR (GGCAAGTGGTCTG-GTTCAC) at a concentration of 10 pmol/μL. These primers amplify a 134 base-pair fragment of the IBV M gene. In addition, we used the probe HmQs (CAAGCCACTGACCCTCACAATAA) modified with 6–FAM and Black Hole Quencher at a concentration of 5 pmol/μL. The thermal profile consisted of an initial denaturation step of 15 min at 95°C and 45 cycles of denaturation for 60 seconds at 94°C, annealing for 30 seconds at 50°C and extension for 30 seconds at 60°C. Fluorescent data were collected after annealing and reported as Cq values.

Respiratory Sounds

Each chicken was evaluated for respiratory sounds at 4, 5, and 6 dpi as we previously described (Toro et al., 2012). In brief, severity scores were recorded as (0) no signs, (1) mild nasal rales or upper respiratory tract sounds, (2) moderate tracheal rales, or (3) severe respiratory sounds audible from a 20-cm distance. An index was calculated based on the severity of the signs.

Respiratory index = ($\sum$respiratory scores per group / number of birds per group × 3) ×100, where 3 is the highest respiratory score.

Histopathology and Histomorphometry

At 6 dpi, birds were euthanized, tracheas were collected, and slides were prepared and stained with haematoxylin and eosin for histopathological analysis. Tracheal histomorphometry was performed on trachea sections at 40× magnification measuring mucosal thickness using the NIH Image J software. In addition, tracheal deciliation was assessed on a scale from 1 to 4 where 1 represented normal, 2 focal, 3 multifocal, and 4 diffuse cilia loss as described by Jackwood et al. (2015).

Statistical Analysis

Data was statistically analyzed using Prism Graphpad 5. Body weights were analyzed by two-way analysis of variance (**ANOVA**) followed by Tukey test for group comparisons. All other measurements were analyzed using Kruskal-Wallis test, followed by Dunn test for group comparisons. Significance was determined at $P < 0.05$. Flow cytometry data was analyzed using FlowJo software (Ashland, OR).

RESULTS

Vaccine Virus Viability after Mixed with DE

Viral titers of the vaccine viruses with and without the addition of 20 mg/mL DE were measured in order to detect the effect of DE on virus viability. The titer of the vaccine virus reconstituted in TPB was $10^{5.38}$ EID$_{50\%}$ per milliliter compared to $10^{5.77}$ EID$_{50\%}$ per milliliter for the virus reconstituted in TPB with DE under the same conditions.

Chicken Body Weight and Mortality

No significant differences ($P > 0.05$) were detected in live body weights between the different chicken groups at 0, 7, and 14 dpv (data not shown). At 21 dpv, the ocular and spray IBV groups showed lower body weights compared with ocular and spray DE and DE-IBV groups.

Two chickens, one from the ocular IBV+DE and the second from the ocular IBV group, died due to causes not related to the experiment.

Serum IgG Antibodies

All IBV-vaccinated groups (spray DE+IBV, spray IBV, ocular DE+IBV, and ocular IBV) showed a slight increase in IgG titers compared to Spray DE and ocular DE groups at 7, 14, and 21 dpv (Table 2). Six d after the challenge, both ocular DE+IBV and ocular

Table 2. Serum IBV IgG titers measured by ELISA after IBV vaccination and challenge in the different experimental groups. Data are shown as mean $\pm$ standard error.

	Days post-vaccination			Days post infection
Groups	7	14	21	6
Spray DE+IBV	38.5 ± 6.4^a	28.6 ± 7.7^{ns}	29.5 ± 8.7^{ns}	$78.7 \pm 26.1^{a,b,c}$
Spray IBV	35.1 ± 8.2^a	36.5 ± 26.5^{ns}	35.7 ± 16.1^{ns}	$21.3 \pm 7.1^{a,b,c}$
Spray DE	0 ± 0^b	0 ± 0^{ns}	6.6 ± 4.2^{ns}	$19.8 \pm 11.5^{b,c}$
Ocular DE+IBV	$18.1 \pm 9.5^{a,b}$	44.2 ± 15.2^{ns}	29 ± 9.9^{ns}	$317.6 \pm 87.9^{a,b}$
Ocular IBV	46.6 ± 9.3^a	28.2 ± 6.8^{ns}	60.4 ± 33.7^{ns}	357.9 ± 76.6^a
Ocular DE	0 ± 0^b	0 ± 0^{ns}	8 ± 4.9^{ns}	0 ± 0^c

Lowercase superscripts indicate significant differences between groups ($P < 0.05$).
[ns]Not statistically significant.

Table 3. Tear IBV IgA optical density (OD) measured by ELISA after IBV vaccination and challenge in the different experimental groups. Data are shown as net OD (net OD = OD of sample – OD of blank). Tears were diluted 1:5 with sample diluent prior being assayed. OD was measured at 650 nm. Data are shown as mean $\pm$ standard error.

	Days post-vaccination		Days post infection
Groups	14	21	6
Spray DE+IBV	0.0012 ± 0.0004^b	0.0088 ± 0.004^{ns}	0.0234 ± 0.0059^{ns}
Spray IBV	$0.0044 \pm 0.0022^{a,b}$	0.0116 ± 0.0014^{ns}	0.018 ± 0.0052^{ns}
Spray DE	0.0024 ± 0.0013^b	0.0096 ± 0.0024^{ns}	0.037 ± 0.0105^{ns}
Ocular DE+IBV	$0.0474 \pm 0.0149^{a,b}$	0.019 ± 0.0055^{ns}	0.0523 ± 0.03^{ns}
Ocular IBV	0.0396 ± 0.0127^a	0.0322 ± 0.0209^{ns}	0.0601 ± 0.0193^{ns}
Ocular DE	$0.003 \pm 0.0007^{a,b}$	0.0092 ± 0.0004^{ns}	0.0216 ± 0.0038^{ns}

Lower case superscripts indicate significant differences between groups ($P < 0.05$).
[ns]Not statistically significant.

Table 4. Percentage of $CD4^+$ T cells, $CD8^+$ T cells, and monocytes/macrophages of the total isolated cells from chicken blood and spleen at 11 dpv.

	Blood[1]			Spleen		
Groups	% $CD4^+$ T cells	% $CD8^+$ T cells	% Monocytes	% $CD4^+$ T cells	% $CD8^+$ T cells	% Macrophages
Spray DE+IBV	8.4	4.8	0.3	9.4 ± 1^{ns}	7.7 ± 1^{ns}	5 ± 1.6^{ns}
Spray IBV	4.6	3.4	0.2	9.2 ± 0.7^{ns}	4.9 ± 0.9^{ns}	4.1 ± 0.4^{ns}
Spray DE	5.6	3.7	0.2	11.5 ± 4.4^{ns}	3.9 ± 1^{ns}	5.8 ± 1.4^{ns}
Ocular DE+IBV	3.6	2.1	0.2	8.7 ± 1.2^{ns}	6.1 ± 1.6^{ns}	4.5 ± 0.5^{ns}
Ocular IBV	6.7	2.1	0.2	9.4 ± 2^{ns}	8 ± 2.4^{ns}	6.4 ± 0.5^{ns}
Ocular DE	3.4	1.3	0.1	9.1 ± 1.1^{ns}	6 ± 0.4^{ns}	5.7 ± 1.6^{ns}

[1]samples were pooled.
[ns]Not statistically significant.
Data are shown as mean $\pm$ standard error.

IBV groups showed higher IgG responses ($P < 0.05$) than spray vaccinated and DE only groups (Table 2).

Tear IgA Antibodies

Similar to the serum IgG responses, the ocular DE+IBV and ocular IBV only groups exhibited higher tear IgA responses at all time points (Table 3). At 14 dpv, the ocular DE+IBV group showed the highest net IgA response (OD: 0.047) followed by the ocular IBV group (OD: 0.040). After the challenge, the intraocularly vaccinated groups continued to show the highest IgA values (0.052 and 0.060, respectively), even though differences were not significant (Table 3).

Flow Cytometry

Flow cytometric analysis of PBMCs showed that the spray DE+IBV group had the highest frequen-cies of $CD4^+$ and $CD8^+$ T cells, as well as monocytes at 11 dpv (Table 4). In contrast, ocular DE vaccinated chickens had the lowest $CD4^+$ and $CD8^+$ T cells frequencies. There were no statistical differences between the groups in the frequencies of $CD4^+$ T cells, $CD8^+$ T cells and macrophages at 11 dpv in the spleen. The spray DE+IBV group had higher percentages of cell populations ($CD4^+$, $CD8^+$ T cells and macrophages) than the spray IBV group, while the opposite was observed between the groups that had been intraocularly vaccinated with DE+IBV or IBV only.

Table 5 illustrates the percentage of $CD4^+$ T cells, $CD8^+$ T cells and monocytes/macrophages in blood and spleen at 6 d following the challenge. There were no significant changes in the frequency of cells in blood and splenic tissues between groups.

Table 5. Percentage of CD4$^+$ T cells, CD8$^+$ T cells, and monocytes/macrophages of the total isolated cells from chicken blood and spleen at 6 dpi.

Groups	Blood			Spleen		
	% CD4$^+$ T cells	% CD8$^+$ T cells	% Monocytes	% CD4$^+$ T cells	% CD8$^+$ T cells	% Macrophages
Spray DE+IBV	15.6 ± 6^{ns}	3.4 ± 1^{ns}	5.4 ± 1.7^{ns}	16.1 ± 2.1^{ns}	21.8 ± 4.6^{ns}	3.3 ± 0.4^{ns}
Spray IBV	12.2 ± 1.5^{ns}	3.3 ± 0.6^{ns}	3.7 ± 0.7^{ns}	11.5 ± 1.5^{ns}	13.1 ± 1.6^{ns}	1.7 ± 0.5^{ns}
Spray DE	8.8 ± 0.6^{ns}	2 ± 0.4^{ns}	3.2 ± 0.3^{ns}	15.6 ± 2.2^{ns}	17.3 ± 2.9^{ns}	2.3 ± 0.6^{ns}
Ocular DE+IBV	6.5 ± 1.4^{ns}	1.8 ± 0.4^{ns}	1.7 ± 0.2^{ns}	18.2 ± 3.4^{ns}	21 ± 5^{ns}	3 ± 0.6^{ns}
Ocular IBV	8.1 ± 1^{ns}	2.9 ± 0.8^{ns}	1.7 ± 0.3^{ns}	9.3 ± 1.7^{ns}	8.1 ± 1.9^{ns}	2.5 ± 0.5^{ns}
Ocular DE	8.9 ± 0.8^{ns}	2.4 ± 0.1^{ns}	2.7 ± 0.4^{ns}	14 ± 2.9^{ns}	12.5 ± 3.1^{ns}	2.2 ± 0.2^{ns}

Data are shown as mean $\pm$ standard error.
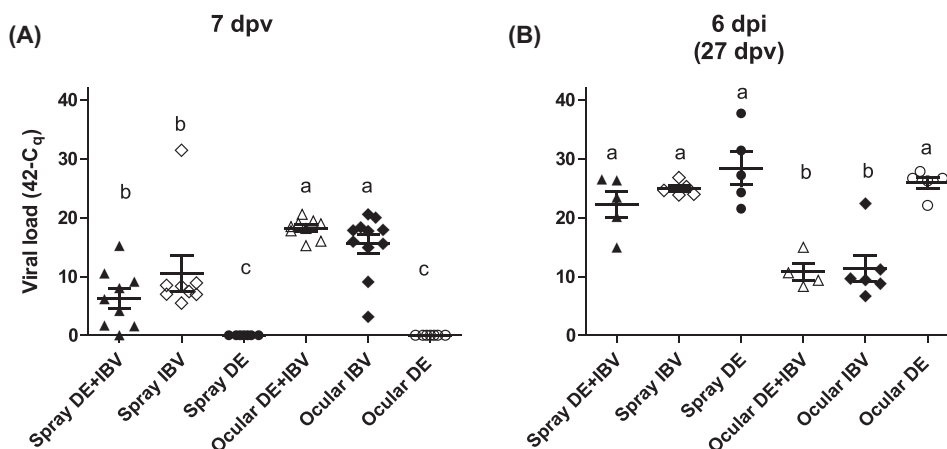
^{ns}Not statistically significant.

Figure 1. IBV viral load in tears detected by RT-qPCR. A: viral load at 7 dpv (10 d of age); B: viral load at 6 dpi (27 dpv, 30 d of age). Error bars represent the standard error. Letters represent significant differences among groups.

IBV Viral Load

Seven d after the vaccination, the IBV viral load in the DE+IBV and IBV ocular groups was higher ($P < 0.05$) than in the spray IBV and DE-IBV vaccinated groups (Figure 1A). Six d after the challenge (6 dpi), the DE+IBV and IBV ocular vaccinated groups displayed lower IBV loads in tears ($P < 0.05$) compared to the spray IBV vaccinated and control groups (Figure 1B). There were no significant viral load differences between DE+IBV groups and IBV vaccinated groups.

Figure 2. Respiratory signs index at 4, 5, and 6 dpi. Low case letters represent significant differences among groups. Respiratory index was calculated using the formula $R = (\sum \text{respiratory scores per group} / \text{number of birds per group} \times 3) \times 100$.

Respiratory Sounds

Spray DE and ocular DE groups showed the highest respiratory index on all d after the IBV challenge. In contrast, the ocular IBV group had no respiratory sounds at all times. Ocular DE+IBV showed the highest level of respiratory sounds of all the IBV vaccinated groups with a reduction at 6 dpi (Figure 2). The spray DE+IBV group had a higher respiratory index than the spray IBV group at 4 dpi. Respiratory sounds decreased at 5 dpi, disappearing at 6 dpi. Spray DE+IBV showed decreased respiratory signs from 4 to 6 dpi, whereas spray IBV group showed increasing signs.
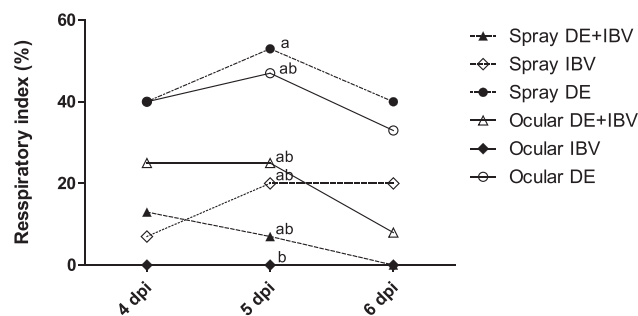
Histopathology

Six d after the homologous IBV challenge, differences among groups were not significant. The addition of DE as adjuvant to the vaccine slightly increased mucosal thickness and deciliation score in both spray and ocular vaccinated groups (Table 6).

DISCUSSION

In this study, we formulated an experimental Ark-DPI live vaccine using DE as an adjuvant to improve the antigen delivery in the mucosa enhancing the mu-

Table 6. Mean tracheal mucosal thickness and deciliation scores at 6 dpi in the different experimental groups.

	Spray DE+IBV	Spray IBV	Spray DE	Ocular DE+IBV	Ocular IBV	Ocular DE
Mucosal thickness[1]	751 ± 72^{ns}	628 ± 93^{ns}	718 ± 117^{ns}	571 ± 79^{ns}	541 ± 116^{ns}	891 ± 83^{ns}
Deciliation score[2]	3.8 ± 0.2^{ns}	3.4 ± 0.4^{ns}	4.0 ± 0.0^{ns}	2.5 ± 0.3^{ns}	2.0 ± 0.6^{ns}	4.0 ± 0.0^{ns}

[1]Mucosal thickness was measured using ImageJ at 40× magnification image.
[2]Deciliation score was assessed using a scale from 1 to 4 where 1 (normal), 2 (focal), 3 (multifocal), and 4 (diffuse).
[ns]Not statistically significant.
Data are shown as mean ± standard error.

cosal immune responses against IBV. First, we tested whether mixing DE with IBV live vaccine could affect the viral viability. DE did not affect the overall viral titer or the ability of the virus to replicate in embryonated chicken eggs. Unpublished experiments performed in our lab showed similar results when a lentogenic NDV vaccine was blended into a DE solution. These results demonstrate the innocuity of DE for these 2 live viruses.

During the in-vivo study, we tested whether DE had any detrimental effect on live body weight following vaccination. No effects on body weight between chicken groups were detected at 7 and 14 d. Similar results were obtained when we used DE as an adjuvant in an experimental NDV inactivated vaccine (Nazmi et al., 2017). We saw differences in body weight between the groups at d 21; however, groups with heavier body weights contained higher male-to-female ratio. Most likely these differences in weight were not related to the vaccination treatment.

We characterized the effect of adding DE to the IBV vaccine on different immunological parameters. The IBV-IgG titers in serum of all birds after vaccination were below the threshold specified in the commercial ELISA. This result can be attributed to a low experimental vaccine dose of $10^{3.67}$ $EID_{50\%}$, however the RT-qPCR results showed that birds took the vaccine. After a homologous viral challenge, we saw a better response in birds inoculated intraocularly than in spray vaccinated groups.

The ocular DE+IBV group showed the highest IBV-specific IgA responses in tears at 14 dpv followed by the ocular IBV group, while the spray-vaccinated groups had lower titers (Table 3). While most of these results showed to be non-statistically significant the Ocular IBV IgA values showed to be statistically higher than spray DE+IBV and Spray DE. After the viral challenge, no differences were detected between the groups. The better efficacy of the ocular vaccination, compared with the spray cabinet, was shown by the higher increase of IgG and IgA titers in the groups vaccinated by that route after the challenge. Other studies also found that ocular vaccination of broiler chickens with IBV Ark gave sufficient protection compared to vaccination with a hatchery spray cabinet (Roh et al., 2013; Roh et al., 2015).

The flow cytometric analysis did not show differences in the frequencies of $CD4^+$ T cells, $CD8^+$ T cells and monocytes/macrophages in blood and spleen at 11 dpv

and 6 dpi. Considering that the route of vaccination and challenge was mucosal, it is possible that immune cells were predominantly recruited at local mucosal sites rather than at systemic organs. For instance, the frequency of effector T cells was significantly increased in Harderian glands at 11 dpv of an ocular IBV vaccine, but not in spleen (Gurjar et al., 2013). Intra-tracheal and intra-nasal infection with IBV recruited natural killer cells to lung at 1 dpi and to blood at 1 and 4 dpi (Vervelde et al., 2013). Macrophages also increased in trachea and lung at 1 dpi (Kameka et al., 2014). Collectively, selection of different tissues and time points might lead to a better understanding of the role of immune cells in IBV protection.

In view of the poor antibody responses after vaccination and in order to confirm vaccine take, we tested IBV viral load in tears by RT-qPCR at 7 dpv. We found that both spray-vaccinated groups had a lower viral load at 7 dpv than the groups that had been vaccinated intraocularly. This correlates with results by others in which they correlate higher vaccine load early after vaccination with adequate protection (Roh et al., 2013). A goal of an ideal vaccine is to decrease viral shedding after challenge. In this work we found that both ocular groups had lower viral loads in tears at 6 dpi. As the immunological results, this shows a better protection when the vaccine is applied intraocularly.

In order to assess the protection conferred by our experimental vaccines we assessed respiratory sounds after challenge. Only the ocular IBV group did not show respiratory signs confirming the effectiveness of this vaccination route in protecting chickens but also suggesting that the addition of DE to the live IBV Ark vaccine does not confer better protection to the homologous challenge. An interesting trend was found in the respiratory signs assessment; a decrease in respiratory signs in the Spray DE+IBV group from d 4 to 6 post infection. This trend correlates with increasing IgG antibodies. Unfortunately, no statistical significance was encountered.

The histopathology confirmed the respiratory sounds assessment. The groups mock vaccinated with DE alone either by spray cabinet or intraocularly showed higher mucosal thickness and deciliation scores, indicating the efficacy of the vaccination. The lack of significant differences might be due to the reduced number of birds per group.

In conclusion, there were few indications that adding DE to Ark-DPI live vaccines had any advantages in the

immune responses and protection against IBV homologous challenges under experimental conditions. Ark vaccines administered by hatchery spray cabinet elicit weaker immune responses and protection against an IBV homologous challenge compared to the same vaccine delivered via the ocular route.

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