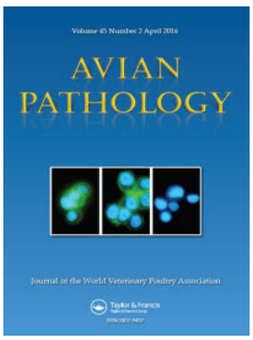

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ORIGINAL ARTICLE

Heterologous live infectious bronchitis virus vaccination in day-old commercial broiler chicks: clinical signs, ciliary health, immune responses and protection against variant infectious bronchitis viruses

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ABSTRACT

Groups of one-day-old broiler chicks were vaccinated via the oculo-nasal route with different live infectious bronchitis virus (IBV) vaccines: Massachusetts (Mass), 793B, D274 or Arkansas (Ark). Clinical signs and gross lesions were evaluated. Five chicks from each group were humanely killed at intervals and their tracheas collected for ciliary activity assessment and for the detection of CD4+, CD8+ and IgA-bearing B cells by immunohistochemistry (IHC). Blood samples were collected at intervals for the detection of anti-IBV antibodies. At 21 days post-vaccination (dpv), protection conferred by different vaccination regimes against virulent M41, QX and 793B was assessed. All vaccination programmes were able to induce high levels of CD4+, CD8+ and IgA-bearing B cells in the trachea. Significantly higher levels of CD4+ and CD8+ expression were observed in the $Mass_2 + 793B_2$-vaccinated group compared to the other groups (subscripts indicate different manufacturers). Protection studies showed that the group of chicks vaccinated with $Mass_2 + 793B_2$ produced 92% ciliary protection against QX challenge; compared to 53%, 68% and 73% ciliary protection against the same challenge virus by $Mass_1 + D274$, $Mass_1 + 793B_1$ and $Mass_3 + Ark$, respectively. All vaccination programmes produced more than 85% ciliary protection against M41 and 793B challenges. It appears that the variable levels of protection provided by different heterologous live IBV vaccinations are dependent on the levels of local tracheal immunity induced by the respective vaccine combination. The $Mass_2 + 793B_2$ group showed the worst clinical signs, higher mortality and severe lesions following vaccination, but had the highest tracheal immune responses and demonstrated the best protection against all three challenge viruses.

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IBV; live vaccine; vaccination; immunity; protection; broiler chicks

Introduction

Infectious bronchitis (IB) is controlled by the administration of live attenuated infectious bronchitis virus (IBV) vaccines and it has been suggested that mucosal immunity plays an important role for effective protection against this virus (Gomez & Raggi, 1974). The development of local immunity may rely on the direct interaction between elements of the mucosal immune system and IBV itself (Toro *et al.*, 1997; Guo *et al.*, 2008). Previous studies have reported the development of humoral immune responses following live IBV vaccination (Cook *et al.*, 1999; Terregino *et al.*, 2008). However, conflicting studies demonstrate that humoral responses have a low correlation with protection against IBV infection (Raggi & Lee, 1965; Roh *et al.*, 2013). Other studies suggested the importance of local and cell-mediated immunity in successful elimination or prevention of IBV infection (Dhinakar Raj & Jones, 1997; Liu *et al.*, 2012; Gurjar *et al.*, 2013). Being a main target organ of IBV, the trachea's cellular and local immunity has been the focus of many studies (Nakamura *et al.*, 1991; Dhinakar Raj & Jones, 1996a; Kotani *et al.*, 2000). It has been shown that following an IBV vaccination, the trachea's IgA and CD8+ T cell responses are potentially good indicators of protection against the virus (Okino *et al.*, 2013). Local anti-IBV antibodies, particularly from IgA and cytotoxic T cells, have been shown as crucial elements in terms of restricting or eliminating IBV (Gillette, 1981; Collisson *et al.*, 2000; Mondal & Naqi, 2001). It has been demonstrated that chickens' lachrymal IgA fluid levels are associated with resistance against IBV infection (Toro & Fernandez, 1994). However, little information is available regarding the evaluation of cellular and local immune responses elicited by different live IBV vaccines, especially when given in strategic vaccination programmes.

It is well recognized that many serotypes or genotypes of IBVs are circulating in poultry flocks globally. Massachusetts 41 (M41) and 793B serotypes have spread worldwide and commercial vaccines are available against both serotypes (Bijlenga *et al.*, 2004; Jones, 2010). Despite vaccination efforts, novel field IBVs continue to emerge in many parts of the world (de Wit *et al.*, 2011a), including the persistence of some antigenic variants that makes prevention of

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IBV infections very challenging. One such variant is QX, which was first isolated in China in 1996 from birds with proventriculitis (Wang *et al.*, 1998), and later reported in Europe (Beato *et al.*, 2005; Worthington *et al.*, 2008; Ganapathy *et al.*, 2012), Middle East (Amin *et al.*, 2012) and Africa (Toffan *et al.*, 2011).

Using combinations of different live IBV vaccines has been shown to induce a wider protection against several heterologous virulent IBV strains (Cook *et al.*, 1999; Gelb *et al.*, 2005). The phenomenon of IBV cross-protection has been recognized before (Hofstad, 1981; Cook *et al.*, 1999) and has been attributed to the host immune response towards several IBV epitopes, especially the S1 subunit (Cavanagh *et al.*, 1997). Despite the stronger and wider protection induced by heterologous vaccinations, the underlying immune mechanism remains unknown.

In this study, using the tracheal ciliary activity scoring method, we evaluated the tracheal health of chicks with IBV-maternal antibodies following dual vaccinations with a number of live IBV vaccine viruses belonging to serotypes of Mass, D274, 793B or Ark. The cellular and local immune responses in trachea were assessed using immunohistochemistry (IHC). Following vaccination, effects on the decline in maternal antibodies and subsequent humoral immune responses were evaluated using enzyme-linked immunosorbent assay (ELISA). The protection conferred by the different vaccination programmes against virulent M41, QX and 793B was also assessed. Following challenge, protection against respiratory signs and ciliostasis was examined.

Materials and methods

Chicks and ethical statement

Day-old commercial broiler chicks with maternally derived IBV antibodies (MDA) were obtained from a commercial hatchery. Chicks were kept in an isolation unit (University of Liverpool) and reared on deep litter (wood-shavings) with water and feed provided *ad libitum*. No antibiotics were used either in the feed or water throughout the study. All experimental procedures were undertaken after approval of the University of Liverpool ethical review committee and according to the UK legislation on the use of birds for experiments, as permitted under the project license PPL 112 40/3723.

IBV vaccines

Commercially available live IBV vaccine viruses were used. They belong to monovalent Mass (H120 or Ma5) or 793B (e.g. 4/91 or CR88) serotypes, and combined vaccines of Mass + D274 and Mass + Ark. For this study, the Mass vaccines are referred to as $Mass_1$ and $Mass_2$, and the 793B vaccines are referred to as $793B_1$ and $793B_2$. These monovalent vaccines were produced by different manufacturers. Two mixtures of $Mass_1 + 793B_1$ and $Mass_2 + 793B_2$ was prepared as previously described (Awad *et al.*, 2015a, 2015b). In addition, readily combined live vaccines of $Mass_1$ + D274 and $Mass_3$ + Ark were also used. All vaccines were prepared prior to administration to provide the dosages per chick as recommended by the manufacturers.

IBV challenge strains

Virulent IBV challenge viruses belonging to three different serotypes were used. M41 has been maintained in our laboratory for several years (Dhinakar Raj & Jones, 1996b). The QX (KG3P) strain was first isolated from the proventriculus of a flock of broilers in England (Ganapathy *et al.*, 2012). The 793B (KG12/11) strain was isolated from caecal tonsils of a flock of layers suffering a drop in egg production (Ganapathy, unpublished). All viruses were grown in embryonated chicken eggs and titrated in trachea organ cultures as previously described (Cook *et al.*, 1976). Titres were expressed as median ciliostatic doses (CD_{50}) and calculated as previously described (Reed & Muench, 1938). Through polymerase chain reaction (PCR), it was found that the inocula were free of Newcastle disease virus (Aldous & Alexander, 2001), avian influenza virus (Abdelwhab *et al.*, 2011), avian metapneumovirus (Awad *et al.*, 2014), infectious laryngotracheitis virus (Diallo *et al.*, 2010), infectious bursal disease virus (Purvis *et al.*, 2006) and fowl adenovirus (Raue & Hess, 1998). The inocula were also free of bacterial contamination when tested using blood and MacConkey agars, with no mycoplasmas detected either by culture or PCR.

Experimental design

A total of 275-day-old chicks were randomly divided into 5 groups and kept in separate isolation units, with 55 chicks per group. Each chick in each of the groups was inoculated via the ocular (50 µl) and nasal (50 µl) routes with one of the following, $Mass_1$ + D274 (Group 1), $Mass_1 + 793B_1$ (Group 2), $Mass_2 + 793B_2$ (Group 3), $Mass_3$ + Ark (Group 4) and sterile water (SW) (Group 5, control). Dosages were given as recommended by the respective manufacturers. Following vaccination, chicks were observed daily for clinical signs as previously described (Grgic *et al.*, 2008). Coughing, head shaking and depression of short duration were considered mild signs, whereas gasping, coughing and depression, accompanied by ruffled feathers were scored as severe signs. Mortality and lesions at post-mortem were recorded.

Evaluation of tracheal health of chicks following live IBV vaccinations

At 3, 6, 10, 14, 18 and 25 days post vaccination (dpv), five chicks from each group were humanely killed by

wing vein injection of sodium pentobarbital (Rhone Merieux, Ireland). Tracheas were removed from each chick and processed for ciliary and percentage protection assessment as previously described (Cook *et al.*, 1999). Ciliary activity was scored as follows: all cilia beating in each ring = 0, 75% cilia beating = 1, 50% cilia beating = 2, 25% cilia beating = 3 and 0% cilia beating = 4. A ciliary activity score of 4 indicates 100% ciliostasis. For each bird, out of the 10 rings examined, the maximum possible ciliary score is 40, indicating no cilia beating in all 10 rings. The mean ciliary score for each bird was calculated and percentage protection for each group was calculated using a formula described by others (Cook *et al.*, 1999): [1 − (mean ciliostasis score for vaccinated/challenge group)/mean ciliostasis score for corresponding challenge controls] × 100.

Evaluation of tracheal immunity induced by live IBV vaccines

During necropsy, pieces of the trachea were collected at 3, 6, 10, 14, 18 and 25 dpv from five chicks in each group for IHC. Tracheal pieces were immediately placed in aluminium foil cups containing cryo embedding compound medium (Solmedia Laboratory, Shrewsbury, UK), and frozen in liquid nitrogen (−190°C). Following sectioning on a cryostat, specific monoclonal antibodies were used to identify CD4+, CD8+ or IgA-bearing B cells (Southern Biotech, Birmingham, AL, USA). IHC staining and calculation of average number of positive cells per 400× microscopic field were carried out as previously described (Rautenschlein *et al.*, 2011; Awad *et al.*, 2015a; Chhabra *et al.*, 2015).

Measuring of maternal or humoral antibody levels following live IBV vaccination

IBV antibodies were detected using a commercial ELISA kit (Biochek, Gouda, The Netherlands) following the manufacturer's instructions. Serum was collected prior to vaccination and then at 3, 6, 10, 14, 18 and 25 dpv from eight chicks per group to establish mean antibody titres.

Assessment of protection induced by vaccination against virulent IBVs

At 21 days of age, five chicks were taken from each group and challenged oculonasally with $10^{5.00}$ CD_{50}/ml virulent IBV M41 per dose of 0.1 ml. The same number from each group was challenged with $10^{5.00}$ CD_{50}/ml virulent IBV QX per dose of 0.1 ml and a further five birds were challenged with $10^{5.00}$ CD_{50}/ml virulent IBV 793B per dose of 0.1 ml via the same route. The remaining chicks in each group were left unchallenged in control groups. Following challenge, all birds were observed daily for clinical signs attributable to IBV infection. Five days post challenge (dpc), the ciliary activity of tracheal explants was examined in both the challenged and unchallenged chicks. As described above, the ciliostasis test was performed to examine tracheal health. Percentage protection against respective challenge virus was calculated as outlined by Cook *et al.* (1999).

Statistical analysis

Statistical analysis of cellular, local and humoral antibody response data was conducted using one-way analysis of variance, followed by Tukey's test to examine differences between pairs of means. Differences were considered to be significant when $P \leq 0.05$. All analysis was conducted using GraphPad Prism, 6.0.1 (GraphPad Software, La Jolla, CA, USA, http://www.graphpad.com/scientific-software/prism/).

Results

Clinical signs following vaccination

Control birds (Group 5) remained free of clinical signs throughout the experiment. Birds that received $Mass_1$ combined with either D274 (Group 1) or $793B_1$ (Group 2) vaccines showed mild clinical signs starting at 5 dpv, which then subsided by 14 dpv. Birds that received the combined $Mass_2$ + $793B_2$ vaccine (Group 3) showed mild clinical signs from 2 dpv, starting with coughing and sneezing. At 4 dpv some of the chicks were showing depression, ruffled feathers and coughing, which continued up to 10 dpv, thereafter, the chicks showed mild respiratory signs and all signs ultimately disappeared at 14 dpv. Birds that received the combined $Mass_3$ + Ark vaccine (Group 4) showed signs of mild respiratory distress from 2 dpv which continued up to 10 dpv (Figure 1).

Two birds died in Group 1 at 8 and 14 dpv, respectively, and 1 bird died in Group 2 at 14 dpv. No gross

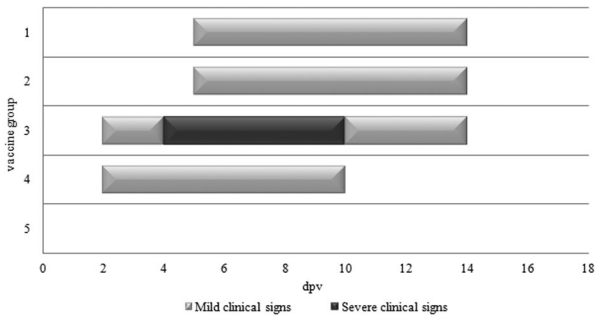

Figure 1. Onset and duration of clinical signs within each of the five vaccine groups. Group 1 = $Mass_1$ + D274, Group 2 = $Mass_1$ + $793B_1$, Group 3 = $Mass_2$ + $793B_2$, Group 4 = $Mass_3$ + Ark and Group 5 = SW.

lesions were observed in these chicks during post-mortem examination. In Group 3, three birds died at 6, 9 and 10 dpv. Tracheal congestion, pale and enlarged kidneys, fibrinous pericarditis, fibrinous perihepatitis, airsacculitis and peritonitis were found in these chicks. No deaths were recorded in Group 4 or 5.

Tracheal health of chicks following live IBV vaccinations

Results of the ciliary activity assessment are presented in Figure 2. The ciliary activity of the control birds was not affected throughout the experimental duration. Both, $Mass_1$ + D274 and $Mass_1$ + $793B_1$ groups, showed similar onset of ciliary inhibition up to 6 dpv but peak damages occurred at 10 and 14 dpv, respectively. In the $Mass_2$ + $793B_2$ group, there was quick onset of the ciliary damages which rapidly peaked at 10 dpv, with almost full recovery by 14 dpv. $Mass_3$ + Ark showed mild ciliary damage peaking at about 10 dpv and almost full recovery by 18 dpv. By 25 dpv, the tracheas showed greater than 90% ciliary activity in all vaccinated groups.

Tracheal immunity induced by live IBV vaccinations

All vaccine viruses induced measurable levels of CD4+, CD8+ (Table 1) and IgA-bearing B (Table 2) cells in the trachea of vaccinated birds compared to unvaccinated birds. CD4+ and CD8+ cell counts varied between each vaccinated group throughout the sampling time. In all vaccinated groups the expression levels of CD4+ increased from 3 dpv, peaked at 6 dpv and then decreased after 10 dpv (Table 1). A significantly higher expression of CD4+ cells was found at 3 and 10 dpv for Groups 3 and 4 compared to other vaccinated groups. By 25 dpv, no significant differences were seen between vaccinated and control groups.

For CD8+ cell counts, no significant differences were observed at 3 dpv between vaccinated and control groups. In the vaccinated groups, the average number

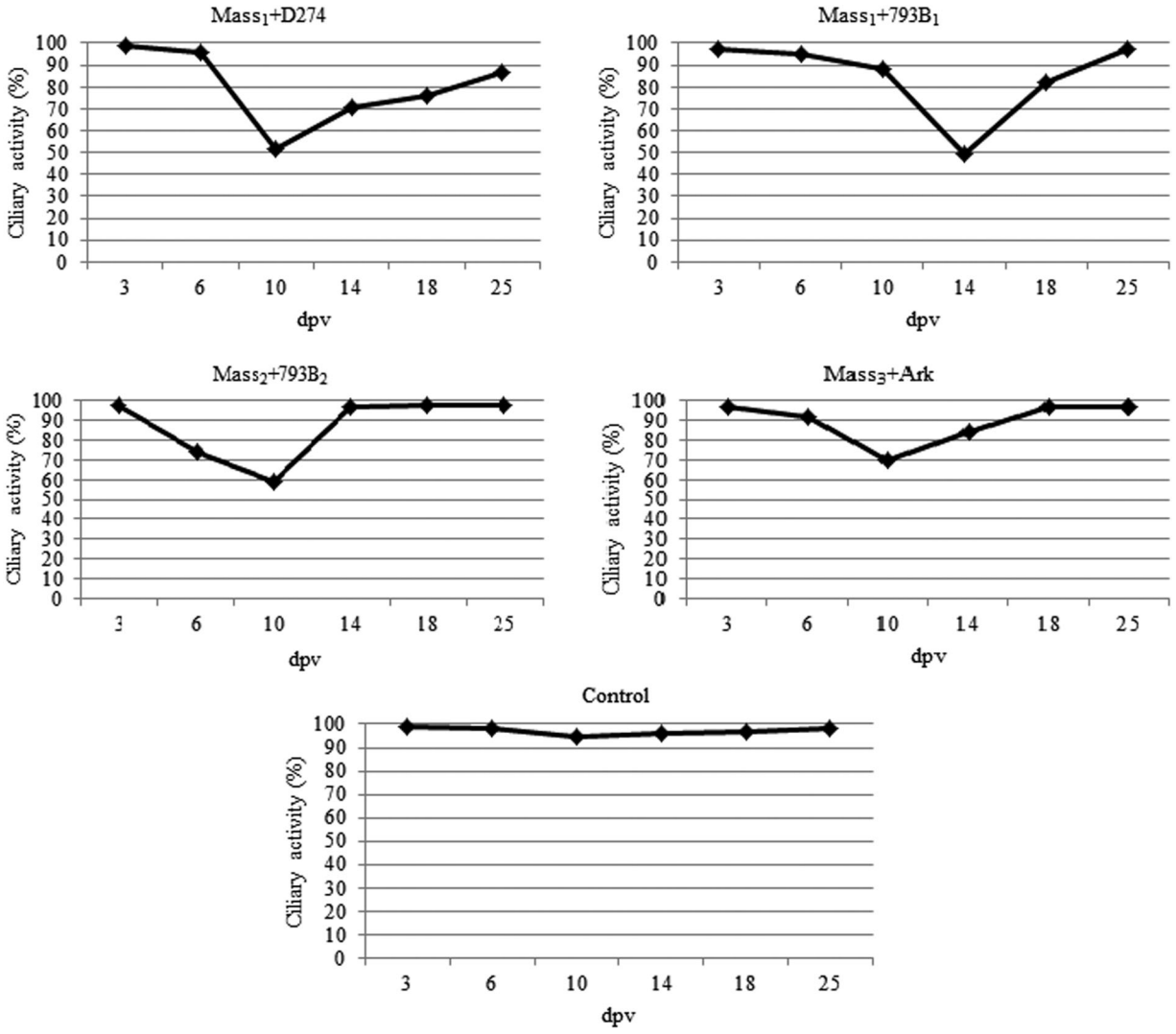

Figure 2. Comparison of ciliary activity in the chicks that received different IBV vaccination programmes. Group 1 = $Mass_1$ + D274, Group 2 = $Mass_1$ + $793B_1$, Group 3 = $Mass_2$ + $793B_2$, Group 4 = $Mass_3$ + Ark and Group 5 = SW.

of CD8+ cells increased after 6 dpv and peaked by 14–18 dpv (Table 1). However, no significant differences were observed between any of the vaccinated groups. The average number of CD8+ cells subsided by 25 dpv. The decline of CD4+ cells corresponded with an increase of CD8+ cells.

All vaccinated groups demonstrated significantly higher IgA-bearing B cell counts when compared to the control group at all sampling points. The IgA-bearing B cell levels peaked at 10 dpv in all the vaccinated groups (Table 2).

Humoral antibody response induced by live IBV vaccinations

The mean ELISA (±SD) antibody titre in the chicks at day-old was 5702 (±324). Mean titres of each group following vaccination are shown in Table 3. At three dpv, Group 3 ($Mass_2 + 793B_2$) had a significant reduction in antibody titre ($P < 0.05$) when compared to the other groups. At 6 dpv, no significant differences were seen between the vaccinated and control groups. Antibody titre levels in all groups declined further and dropped below the cut-off point by 10 dpv. Despite an increase in antibody titres in all four vaccinated groups at 18 dpv, all groups remained below detectable levels until the end of the experiment (Table 3).

Ciliary protection induced by homologous or heterologous vaccination against virulent IBVs

Following challenge with virulent M41, QX and 793B strains, clinical signs such as head shaking, sneezing, tracheal râles and coughing were observed in the unvaccinated-challenged group. No clinical signs were observed in all vaccinated-challenged groups.

Challenge by M41, QX or 793B caused severe ciliostasis in unvaccinated-challenged birds (Table 4). Ciliary scores showed that the vaccination programmes gave an excellent protection (>85% protection) against M41 and 793B. Group 3 ($Mass_2 + 793B_2$) was the only group protected against QX, whereas the rest of the groups provided partial protection. The unvaccinated/vaccinated-unchallenged groups showed almost 100% ciliary activity.

Discussion

In this study, following simultaneous application of live IBV vaccine viruses, the chick tracheal ciliary activity was assessed as a reflection of their health. To our knowledge, this is the first study to report on the impact of live heterologous IBV vaccine viruses on the tracheal ciliary activity in young IBV-maternal antibody positive broiler chicks. Between the vaccinated groups, the pattern of damage to the ciliary activities differed. For example, in Group 2 ($Mass_1$ +

Table 1. Immunohistochemical detection of CD4+ and CD8+ cells in the trachea of chickens that received different heterologous IBV vaccinations.

	CD4+					CD8+				
Vaccine groups	3 dpv	6 dpv	10 dpv	14 dpv	18 dpv	25 dpv	3 dpv	6 dpv	10 dpv	14 dpv	18 dpv	25 dpv
1	35 ± 0.3^{A}	79 ± 8.7^{A}	34 ± 3.7^{A}	58 ± 0.6^{B}	37 ± 10^{AB}	13 ± 0.2	16 ± 4.5	14 ± 1.3^{B}	43 ± 7.1^{A}	80 ± 10^{A}	51 ± 0.3^{A}	21 ± 12
2	50 ± 4.5^{A}	70 ± 5.1^{A}	29 ± 3.7^{A}	44 ± 0.2^{AB}	52 ± 16^{B}	25 ± 0.6	25 ± 0.6	21 ± 0.2^{A}	49 ± 7.1^{A}	57 ± 0.4^{A}	59 ± 0.4^{A}	25 ± 11
3	79 ± 0.6^{B}	93 ± 7.5^{A}	81 ± 0.6^{B}	35 ± 0.3^{AB}	17 ± 0.3^{A}	15 ± 0.4	24 ± 0.8	19 ± 2.4^{AB}	87 ± 18^{A}	77 ± 2.9^{A}	58 ± 2.7^{A}	18 ± 0.2
4	76 ± 0.6^{B}	70 ± 0.7^{A}	62 ± 5.6^{B}	68 ± 21^{B}	27 ± 0.7^{A}	14 ± 0.3	28 ± 10	29 ± 2.4^{A}	12 ± 0.7^{B}	68 ± 21^{A}	56 ± 0.6^{A}	12 ± 0.7
5	8 ± 0.1^{C}	13 ± 0.2^{B}	10 ± 0.1^{C}	6.0 ± 0.5^{C}	8.0 ± 0.2^{C}	4.0 ± 0.1	6.0 ± 0.3	9.2 ± 0.2^{B}	7.0 ± 0.1^{B}	5.6 ± 0.6^{B}	8.2 ± 0.2^{B}	12 ± 0.1

Notes: Data are expressed as mean values ± standard error of the mean (SEM). Significant differences within each column (dpv) are labelled with either A, B, AB or C. Groups with no significant difference between them are labelled with the same letter, whereas groups with a significant difference are labelled with a different letter ($P < 0.05$). Time points with no significant differences are not labelled. Group 1 = $Mass_1 + 793B_1$, Group 2 = $Mass_1 + D274$, Group 3 = $Mass_2 + 793B_2$, Group 4 = $Mass_3$ + Ark and Group 5 = SW.

Table 2. Immunohistochemical detection of IgA-bearing B cells in the trachea of chickens that received different IBV vaccination programmes.

	dpv					
Vaccine groups	3	6	10	14	18[a]	25[a]
1	58 ± 0.9^{A}	50 ± 0.9^{A}	72 ± 21^{A}	59 ± 20^{A}	ND	ND
2	52 ± 0.8^{A}	60 ± 10^{A}	96 ± 15^{AB}	70 ± 13^{AB}	ND	ND
3	40 ± 13^{A}	68 ± 19^{A}	122 ± 15^{B}	102 ± 22^{B}	ND	ND
4	42 ± 14^{A}	70 ± 15^{A}	88 ± 14^{AB}	78 ± 0.5^{AB}	ND	ND
5	5.0 ± 0.1^{B}	4.0 ± 0.5^{A}	10 ± 0.4^{C}	10 ± 0.4^{C}	ND	ND

Notes: Data are expressed as mean values ± SEM. Significant differences within each column (dpv) are labelled with either A, B, AB or C. Groups with no significant difference between them are labelled with the same letter, whereas groups with a significant difference are labelled with a different letter ($P < 0.05$). Time points with no significant differences are not labelled. Group 1 = $Mass_1$ + D274, Group 2 = $Mass_1$ + $793B_1$, Group 3 = $Mass_2$ + $793B_2$, Group 4 = $Mass_3$ + Ark and Group 5 = SW.
[a]ND: not done for 18 or 25 dpv.

Table 3. Mean anti-IBV ELISA antibody titres in the chicks that received different IBV vaccination programmes.

	dpv					
Group	3	6	10	14	18	25
1	2324 ± 254^{A}	2108 ± 380	440 ± 94^{A}	328 ± 77^{A}	406 ± 72	391 ± 77^{AB}
2	2374 ± 334^{A}	1788 ± 355	748 ± 101^{AB}	291 ± 41^{A}	404 ± 178	639 ± 97^{A}
3	1846 ± 199^{B}	1911 ± 208	501 ± 49^{AB}	216 ± 47^{A}	238 ± 56	512 ± 47^{A}
4	3094 ± 379^{A}	2049 ± 205	881 ± 75^{B}	578 ± 96^{B}	461 ± 57	444 ± 41^{A}
5	2253 ± 392^{A}	1728 ± 183	695 ± 107^{AB}	290 ± 28^{A}	241 ± 29	128 ± 21^{B}

Notes: Data are expressed as mean values ± SEM. Significant differences within each column (dpv) are labelled with either A, B, AB or C. Groups with no significant difference between them are labelled with the same letter, whereas groups with a significant titre difference are labelled with a different letter ($P < 0.05$). Time points with no significant differences are not labelled. Cut-off point titre = 834. Group 1 = $Mass_1$ + D274, Group 2 = $Mass_1$ + $793B_1$, Group 3 = $Mass_2$ + $793B_2$, Group 4 = $Mass_3$ + Ark and Group 5 = SW.

Table 4. Ciliary protection induced by IBV vaccination programmes against virulent IBV challenges at 21 dpv.

	Protection scores (ciliostasis test)		
Vaccine group	M41	QX	793B
1	90	53	90
2	96	68	93
3	90	92	93
4	98	73	85
5	0	15	0

Notes: Group 1 = $Mass_1$ + D274, Group 2 = $Mass_1$ + $793B_1$, Group 3 = $Mass_2$ + $793B_2$, Group 4 = $Mass_3$ + Ark and Group 5 = SW. Protection score = 1 − the mean score for vaccinated and challenged group/mean score for challenge control group × 100; the higher the score, the better the protection.

$793B_1$), the tracheal health decreased gradually to the lowest level by 14 dpv and then slowly came to full recovery by 25 dpv. This compares to Group 3 ($Mass_2$ + $793B_2$) where the ciliary health declined quickly to reach the lowest percentage by 10 dpv but showed a fast recovery thereafter. Even though $Mass_1$ and $Mass_2$ or $793B_1$ and $793B_2$ vaccines belong to the Mass and 793B serotypes, respectively, when they were used in combination, they showed a high variation in their effects on tracheal health. Differences in the virulence of vaccine strains may have played a role in the degree and pattern of tracheal damage. Cubillos and others have reported that in unvaccinated chicks challenged with four IBV isolates, the tracheal damage in terms of ciliary activity differed between them (Cubillos *et al.*, 1991). In another study, the severity of the ciliostasis caused by virulent 793B strain proved to be mild, while the effect of M41 was more severe (Benyeda *et al.*, 2009). Our results emphasize the variable virulence of the vaccine viruses when they are co-administered and the ability of some of the combinations to cause more tracheal damage than others. Differential effects of these live IBV vaccines or vaccination regimes on tracheal health should be considered in designing vaccination programmes using attenuated live respiratory virus vaccines, including Newcastle disease virus and avian metapneumovirus.

Relatively little research exists regarding cellular and local immune responses induced by IBV vaccination. To further our understanding, we evaluated CD4+, CD8+ and IgA-bearing B cell expression in the trachea following vaccination regimes used in this study. The presence of CD4+ and CD8+ cells in large numbers in IBV vaccinated or infected birds has a protective role against viral infections (Kotani *et al.*, 2000). We report the detection of both types of T cells as early as 3 dpv, which then peaked by 6 (CD4+) and 14 (CD8+) dpv. These findings are similar to those reported by Kotani *et al.* (2000) who identified that CD4+ and CD8+ cell numbers peaked at 5 days following infection, but they used a virulent IBV strain. This study reveals that CD4+ cells were recruited into the trachea earlier than CD8+. This observation was in accordance with a previous study using a nephropathogenic IBV strain, where on day 5 post-infection, CD4+ outnumbered CD8+ cells (Janse *et al.*, 1994). In contrast, it has been observed that CD8+ cells were recruited into the trachea earlier than CD4+ cells after infection with virulent 793B (Dhinakar Raj & Jones, 1996a) or live attenuated IBV vaccine (Chhabra *et al.*, 2015). It is not clear if the strain and virulence of the viruses could have contributed to this variation.

It was noted that compared to other groups, a stronger cellular immunity was observed in the groups given $Mass_2 + 793B_2$ (Group 3) and $Mass_3 + Ark$ (Group 4). It is likely that increases in the intensity of local immune responses in these groups are related to the virulence of the vaccine viruses. Group 3 was the only group with higher chick mortality and severe lesions, reflecting stronger effects of this vaccine combination in chicks. Despite these disadvantages, the best protection against all challenge viruses used in our study was achieved in this group, demonstrating a stronger induction of immunity with this heterologous vaccination. Nakamura *et al.* (1991) observed IgA cells in the trachea from 7 to 12 days following infection with virulent IBV M41. We observed in all vaccinated groups that IgA-bearing B cells in the trachea appeared at 3 dpv and peaked at 10 dpv. In this study, the highest level of IgA-bearing B cells was observed in the group given $Mass_2 + 793B_2$, the group with the most severe clinical signs and lesions. Nakamura *et al.* (1991) reported an increase in the number of these cells at the tracheal site as a result of greater tracheal damage (Nakamura *et al.*, 1991).

In this study, following live IBV vaccination in IBV MDA-positive day-old broiler chicks, no significant increases in serum antibody titres were found. It has been well documented that low or undetectable antibody titres in young chicks following IBV vaccination could be attributed to interference of active antibody production by IBV MDA (Raggi & Lee, 1965; Davelaar & Kouwenhoven, 1977). Based on our results, it seems that low levels of humoral antibody titres are not associated with protection against IBVs. In a previous study, vaccination with live H120 conferred protection against homologous challenge, although it induced low IBV antibody levels (Meir *et al.*, 2012). Inefficient induction of humoral antibody by live attenuated IBV vaccines has been demonstrated before (Cook *et al.*, 1991; Roh *et al.*, 2013). Our findings provide further support that the resistance against virulent IBVs was due to the cellular and local immunity. We also evaluated the protection conferred by the different vaccination programmes against virulent M41, QX and 793B. Strong protection was induced by all the vaccination programmes against both M41 and 793B challenge viruses. In addition, birds vaccinated with $Mass_1 + D274$ (Group 1) or $Mass_3 + Ark$ (Group 4), vaccine antigens that poorly relate to the challenge antigen, induced high ciliary protection against 793B challenge. Immunization with a bivalent vaccine containing Mass and Ark-type strains provides cross-protection against many field strains (Gelb *et al.*, 1991; Martin *et al.*, 2007), including 793B (Jones, 2010).

The vaccine programme of $Mass_2 + 793B_2$ (Group 3) provided excellent protection against the heterologous challenged virus QX and also protected against M41 and 793B. It was previously proposed that vaccination with a live Mass-type vaccine at one day of age followed by a 793B vaccine two weeks later provided good protection against many heterologous virulent IBV viruses (Cook *et al.*, 1999; Terregino *et al.*, 2008; de Wit *et al.*, 2011b). This study, for the first time, shows that the effectiveness of a vaccination programme is associated with the degree of cellular and local immune responses at tracheal level. Group 3 ($Mass_2 + 793B_2$) interestingly achieved excellent protection against M41, QX and 793B, though this is the group that had high rapid onset of ciliary damage, and mortality with severe lesions despite high induction of tracheal CD4+, CD8+ and IgA-bearing B cells. Therefore, it appears that the significantly higher cellular and local tracheal immunity in this group might have contributed to the protection. In selecting the appropriate live IBV vaccine combinations, poultry health advisors need to give careful consideration to the characteristics of the live vaccines, potential clinical and pathological consequences, levels of cellular and local immunity induced, and the protection efficacies against conventional and variant IBVs.

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