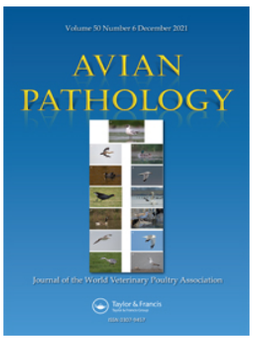

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ORIGINAL ARTICLE

In ovo application of a live infectious bursal disease vaccine to commercial broilers confers proper immunity

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ABSTRACT

Infectious bursal disease (IBD) is an economically important disease of young chickens caused by the Avibirnavirus infectious bursal disease virus (IBDV). Besides biosecurity, vaccination is the most important measure for IBDV control. Sufficient levels of maternally derived antibodies (MDA) protect against early challenge and also interfere with the take of live conventional vaccines. Recently, the field surveys conducted in four countries, published by Ashash, U., Noach, C., Perelman, B., Costello, C., Sansalone, P., Brazil, T. & Raviv, Z. [(2019). *In ovo* and day of hatch application of a live infectious bursal disease virus vaccine to commercial broilers. *Avian Diseases*, 63, 713–720] using the MB-1 vaccine strain by *in ovo* application or sub-cutaneous route at the day of hatch seem to conflict with the rule that very early application of a conventional live vaccine in birds with significant levels of MDA has very little chance of a successful immune response. An *in ovo* vaccination-challenge controlled experiment with MB-1 vaccine was performed using commercial broilers with high levels of MDA against IBDV and a vvIBDV challenge at 22 or 36 days of age. Clinical signs, bursa-bodyweight ratios, histology, serology, RT–PCR, Sanger- and deep sequencing were used to study the efficacy and safety of the *in ovo*-applied MB1 vaccine in comparison to an established immuno-complex vaccine. The study findings confirmed that the *in ovo* application of the live MB-1 vaccine in commercial broilers was successful and induced full protection against a vvIBDV challenge at 22 and 36 days of age, demonstrated by the bursa lesion score and qPCR and IBDV genotyping. Comparable to the field studies, a delayed viral replication of 2–3 weeks, following the *in ovo* administration of the MB1 vaccine, was observed.

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Infectious bursal disease virus; live conventional IBDV vaccine strain; MB-1; in ovo; protection; vvIBDV

Introduction

Since its first description as a clinical entity in the fall of 1957 (Cosgrove, 1962), infectious bursal disease (IBD) still presents a significant challenge to the global poultry industry, and the search for better control measures continues. The aetiological agent is the infectious bursal disease virus (IBDV), a highly contagious and highly stable Avibirnavirus in the family *Birnaviridae* (Eterradossi & Saif, 2020). There are two serotypes of IBD viruses, of which only serotype 1 is pathogenic. The viruses of serotype 1 target IgM-bearing immature B-lymphocytes and directly and indirectly activate macrophages and T-lymphocytes, respectively (Ingrao *et al.*, 2013). Although turkeys, ducks, guinea fowl, pheasants, and ostriches may be infected, clinical disease occurs solely in young chickens (OIE, 2018). Other factors that influence the outcome of infection are the age of infection, the type of chicken, the pathogenicity of the strain, immune status, and co-infections. Infection can manifest as asymptomatic, long-lasting immunosuppressive disease in very young chickens (infection up to 3 weeks of age) or severe symptomatic disease in older birds. The highest susceptibility to acute IBD occurs in chickens between 3 and 6 weeks of age; infections after 10 weeks of age are often subclinical. In general, light breeds such as layers show more clinical signs than heavy breeds (Van den Berg & Meulemans, 1991). Three pathotypic strains of IBDV have been described: "variant", "classical", and "very virulent (vv)" (OIE, 2018). Variant IBDVs induce few if any clinical signs with no mortality and marked bursal lesions; classical IBDVs induce moderate mortality with typical signs and lesions, whereas vvIBDVs induce severe mortality with typical signs and lesions. The descriptive nomenclature for IBDV is far from clear-cut. For example, not all viruses identified as vvIBDV by genotyping are highly pathogenic; some have reassorted genome segments that result in lower virulence.

Furthermore, variant viruses are not an antigenically homogeneous group and the term "classic virus" has been used interchangeably to describe antigenic and pathogenic types of IBDV. For this reason, a new nomenclature that includes a genotyping system that can easily be applied worldwide was recently proposed (Jackwood *et al.*, 2018; Islam *et al.*, 2021). The immunosuppression associated with IBDV infection

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may have very significant economic implications because of vaccination failure, susceptibility to opportunistic pathogens, and overall loss of performance (Eterradossi & Saif, 2020).

IBDV transmission is horizontal, and the virus is highly resilient in poultry environments (Sanchez *et al.*, 2005). Besides biosecurity, vaccination is the most important measure for IBDV control. Sufficient levels of maternally derived antibodies (MDA) protect against early challenges and also against the take of live vaccines. Application of a conventional live vaccine (or field strain) to a flock at an age that the MDA levels against IBDV are still too high for the vaccine to overcome, will result in neutralization of the vaccine resulting in no or a delayed immune response (Hitchner, 1971; Winterfield & Thacker, 1978; Lucio & Hitchner, 1979; Skeeles *et al.*, 1979; Winterfield *et al.*, 1980; Wyeth, 1980; Wood *et al.*, 1981; Wyeth *et al.*, 1981; Naqi *et al.*, 1983; Van den Berg & Meulemans, 1991; Goddard *et al.*, 1994; Kouwenhoven & Van den Bosch, 1994; De Wit & van Loon, 1998; Alam *et al.*, 2002; Al-Natour *et al.*, 2004; Hair-Bejo *et al.*, 2004; Rautenschlein *et al.*, 2005; Block *et al.*, 2007; Jackwood, 2011).

To avoid the risk of vaccine neutralization, two new concepts of vaccines for IBDV have been introduced successfully in the last few decades: genetically engineered viral vector vaccines and immune-complex vaccines. Genetically engineered viral vectors were developed based on herpesvirus of turkeys (HVT) that expresses the VP2 surface protein of IBDV. These commercially available vaccines can be administered *in ovo* or on the day of hatch (DOH) since their replication and expression of the VP2 protein are not hindered or only minimally hindered by maternal immunity to IBDV (Darteil *et al.*, 1995; Bublot *et al.*, 2007; Perozo *et al.*, 2009).

Immune complex vaccines (Icx) are also applied *in ovo* or at DOH by parenteral application independent of the presence and level of MDA (Whitfill *et al.*, 1995). In an immune-complex vaccine, the virions are covered with specific IBDV IgY antibodies which results, in a yet unknown mechanism, in a delay of the vaccine replication in SPF birds and to a more significant extent in MDA-positive birds (Jeurissen *et al.*, 1998; Ivan *et al.*, 2005; De Wit *et al.*, 2018).

Application of a conventional live vaccine in SPF birds by the *in ovo* route or on the DOH has been shown to be effective (Gagic *et al.*, 1999; Rautenschlein & Haase, 2005). Recently, Ashash *et al.* (2019) reported the results of *in ovo* and subcutaneous application at the DOH of a conventional live IBD vaccine strain, MB-1, to commercial broilers in four large-scale field trials. These field trials were conducted in the Republic of South Africa, Israel, Brazil, and Argentina. They demonstrated the relative safety, humoral immune response, and production performances of pre- and post-hatch MB-1 vs. conventional live and Icx IBD-vaccinated broilers. A delayed viral replication following *in ovo* and DOH parenteral administration of the live MB-1 vaccine strain, similar to the Icx-vaccinated birds, was observed.

These field observations seem to conflict with many papers (see above) and field experiences that the very early application of live conventional vaccines in birds with significant levels of MDA has very little chance of a successful immune response. For this reason, and to obtain more data, an *in ovo* vaccination-challenge experiment was performed under controlled, experimental conditions using commercial broilers with high levels of MDA against IBDV, and a vvIBDV challenge at 22 and 36 days of age. The design of the study was according to the requirements of the European Pharmacopeia for testing the efficacy of live IBDV vaccines. Clinical signs, bursa-bodyweight ratio, histology, serology, RT–PCR, and Sanger- and deep sequencing were used to study the efficacy and safety of the *in ovo*-applied MB-1 vaccine compared to an established Icx vaccine.

Materials and methods

Experimental design

The design of the study is shown in Table 1. The experiment was conducted in the animal facility of Royal GD (Deventer, The Netherlands). A total of 250 commercial broiler hatching eggs (Ross® 308) were incubated for 18 days in the same setter (Petersime®, Zullte, Belgium). The eggs were randomly divided into three groups (MB-1, Icx, and mock), individually marked and subsequently vaccinated accordingly with MB-1, Transmune (Icx vaccine), or Ringers lactate by the *in ovo* route. Each group of eggs was transferred to hatch in separate hatchers (Petersime®). On the DOH, the chicks were placed in negative pressure high-efficiency particle air (HEPA)-filtered isolators. Eighteen day-old chicks of the mock group were bled for the determination of the level of MDA against IBDV.

Groups 1 (MB-1), 3 (Transmune), and 5 (mock) were challenged with the vvIBDV D6948 strain via the eye-drop route at 22 days of age (DOA), groups 2 (MB-1), 4 (Transmune), and 6 (mock) were challenged with D6948 via the same route at 36 DOA. Blood samples for IBDV ELISA were collected from the different groups at 7, 14, 22, 28, and 36 DOA. At 5 days post-challenge (dpc) 10 birds per group were euthanized, and bursa tissue was collected for RT–PCR and sequencing. At 10 dpc the remaining birds of each group were euthanized sampled for bursa-bodyweight ratio (BBWR) and bursal lesion score (BLS). Throughout the study, all experimental groups

Table 1. Study design.

Group	Vaccine	Total number of birds	Number of birds sampled for IBDV ELISA at days						Challenge at 22 days of age			Challenge at 36 days of age		
			0	7	14	21	28	36	vvIBDV challenge virus	Number of birds		Challenge virus	Number of birds	
										RT–PCR at 5 dpc	BBWR[a]/BLS[b] At 10 dpc		RT-PCR at 5 dpc[c]	BBWR/BLS at 10 dpc
Hatch	–		18											
1	MB-1	33			33	33			D6948	10	20			
2	MB-1	33			33	33	33	33				D6948	10	20
3	Transmune	22			22	22			D6948	10	10			
4	Transmune	22			22	22	22	22				D6948	10	10
5	Mock	22		10	22	22			D6948	10	10			
6	Mock	22		10	22	22	22	22				D6948	10	10

[a]Bursa-bodyweight ratio × 1000.
[b]Bursa lesion score.
[c]Days post-challenge.

were examined daily for clinical signs, and cull birds and mortality were submitted for pathological examination.

Birds

Hatching eggs from a healthy, well-performing Ross® 308 broiler breeder flock were purchased from a commercial hatchery. The broiler breeders had been vaccinated against IBDV, using a live and inactivated vaccine.

IBDV vaccines and application

The IBDV MB-1 vaccine strain was derived from the IBDV M.B. vaccine strain previously described by Lazarus *et al.* (2008). Transmune™ is a commercial IBDV Icx vaccine from the W2512 IBDV strain (Kelemen *et al.*, 2000). Both vaccines were prepared in accordance with the manufacturer's instructions, containing 1 dose per 0.05 ml suspension, in the animal facility laboratory at GD. Both vaccines and the mock group's Ringer's Lactate control were applied manually in a volume of 0.05 ml by *in ovo* injection to the embryo amniotic sac with a 23G × 1 (0.6 × 25 mm) needle and 1 ml syringe.

Challenge virus and application

The IBDV challenge was performed with the vvIBDV D6948 strain (provided by Royal GD) that was initially isolated in 1989 and was purified by five limited-dilution passages in embryonated eggs (Boot *et al.*, 2000; Maas *et al.*, 2001). The challenge virus was applied by the eye-drop route at 22 or 36 DOA with $10^{3.7}$ EID_{50} and $10^{3.0}$ EID_{50}, respectively, divided over two drops per bird (one drop in each eye).

Serology

Individual blood samples were collected in serum-gel-tubes following the experimental design schedule (Table 1). The individual antibody titres for IBDV were assessed with the IDEXX IBD Ab Test (IDEXX Laboratories, Inc., Westbrook, ME, USA) following the manufacturer's instructions.

Bursa-bodyweight ratio

At 32 and 46 DOA, the assigned birds were euthanized, weighed, necropsied, and the bursas were removed and weighed. The BBWR was calculated to determine the relative mass increase or decrease of the bursa. The ratio was established as follows: weight of BF(g) × 1000 / body weight(g) (Olesen *et al.*, 2018).

Histopathological bursal analysis

After the bursas were removed and weighed at 32 and 46 DOA, the bursal tissues were fixed in 10% neutral buffered formalin, processed, and stained with haematoxylin and eosin (H&E). The bursas were microscopically evaluated by the same certified pathologist and scored in the range of 0 for a normal bursa to 5 for a severely affected bursa following the European Pharmacopeia monograph 01/2008:0587. The average bursal lesion score (BLS) per group was calculated by dividing the sum of bursa scores by the number of analysed bursas (Olesen *et al.*, 2018).

IBDV RT–PCR and sequencing

Genomic RNA was isolated from bursa homogenates, using the High Pure Viral Nucleic Acid kit (Roche Applied Science, Penzberg, Germany), following the manufacturer's guidelines. The reverse transcriptase PCR was performed using the LightCycler RNA Amplification SYBR Green I kit (Roche Applied Science). The following primers were used: forward, 5′-GGT AGC CAC ATC TGA CAG-3′ (Boot *et al.*, 1999); and reverse, 5′-CGC TCG AAG TTR CTC ACC C-3′ (Islam *et al.*, 2001). The RT–PCR was performed on a LightCycler 480 (Roche Applied Science) under the following conditions: reverse transcription

reaction for 30 min at 52°C; denaturation for 30 s at 95°C, followed by 40 cycles with 5 s at 95°C, 10 s at 57°C, and 30 s at 72°C. Positive, negative, and extraction controls were included. The Sanger sequencing of the 540-bp long RT–PCR product was conducted with the described forward and reverse primers at BaseClear (Leiden, The Netherlands). Consensus sequences were constructed using MEGA 6.0 (Tamura *et al.*, 2013) and aligned using BioNumerics 7.5 (Applied Maths). The consensus sequence of the amplified fragment was 540 bp long (positions 710-1248 of VP2), according to Bayliss *et al.* (1990). The obtained partial VP2 gene sequences were aligned with the MB-1, W2512, and D6948 strain sequences.

Strain differentiation via deep sequencing

To further verify the study's bursal IBDV strain infection, aliquots from all the 60 individual bursa IBDV VP2 RT-rtPCR products were collected in six separate experimental group pools (1–6) and submitted for deep sequencing. Deep sequencing was conducted with the Illumina MiSeq platform at the BaseClear sequencing facility (BaseClear B.V., The Netherlands). Libraries were constructed by PCR product fragmentation and ligation with the Illumina barcodes and adapters, utilizing the KAPA Hyper Plus rapid ligation kit (KAPA Biosystems by Roche Diagnostics, Basel, Switzerland) following the manufacturer's instructions. The library quality was assessed with the Bioanalyzer System (Agilent, Santa Clara, CA, USA). Subsequently, libraries with sufficient quality were sequenced with the MISeq PE300 sequencing device (Illumina, San Diego, CA, USA). Illumina reads were trimmed and assembled using the CLC Genomics Workbench program (Qiagen, Hilden, Germany). Reference assembly was performed with the GenBank accession number NC_004178 sequence by the following parameters: match score 1, mismatch cost 2, insertion cost 3, deletion cost 3, length fraction 0.5, and similarity fraction 0.8. Low-frequency variant detection was performed on the assembled contigs, using the following parameters: required significance 0.25%, ignored broken pairs, minimum coverage 8. The obtained partial VP2 gene sequences were aligned and compared with the MB-1, W2512, and D6948 strains.

Ethical statement

The experiment was approved by the Animal Welfare Body, according to Dutch law on experimental birds (Wet op de Dierproeven).

Statistical analysis

Mean antibody titres were expressed as geometric mean titres (GMT). Statistical differences between mean antibody titres were determined using a one-way ANOVA model in a random design and multiple comparisons were made by the Tukey Kramer HSD *t*-test. The statistical analyses of the BLS and BBRW data were done using a two-way ANOVA model in a random design.

Results

Back titration of the challenge virus

The D6948 challenge virus, applied at 22 or 36 DOA, was titrated immediately after the application using embryonated SPF eggs. The titres were $\log_{10}$ 3.7 EID_{50}/chicken and $\log_{10}$ 3.0 EID_{50}/chicken for days 22 and 36, respectively.

Development and clinical signs

All groups demonstrated adequate average body weights (BW) 10 dpc. Before challenge, five birds were observed with non-vaccine-related clinical signs, and one of these birds, from group 2, was found dead on the challenge day (22 DOA). In general, post-challenge clinical signs were mild. In group 5 (mock-vaccinated, challenged at day 22), two birds showed ruffled feathers and such depression post-challenge that they had to be euthanized as they reached the humane endpoints (Table 2). A *post-mortem* examination and testing confirmed the IBDV infection. In group 4, all birds showed ruffled feathers and a delayed feed intake at 4 dpc at 22 days of age. At 5 dpc, all birds of group 4 were recovered. The birds of group 4 that were challenged at D36 showed a delayed feed intake at 5 dpc, and one of the birds showed ruffled feathers. All birds were recovered the day after.

Serology

The GMT of the IBDV ELISA titres per group and age are shown in Table 3. The GMT of 3741 for the day-old hatch mates shows that the MDA level was quite typical for Dutch broilers. The decay of the MDA was as expected (De Wit, 1998), as shown by the GMTs of all groups at 7 and 14 days of age, no statistical differences were observed between the groups. At 22 days of age, all birds of the non-vaccinated groups 5 and 6 and the Transmune vaccinated group 4 were negative in the ELISA. Fourteen to 24% of the birds of groups 1, 2, and 3 had seroconverted. At 28 DOA, 66% of birds of group 2 were seropositive compared to 9% and 0% for groups 4 and 6, respectively. At 36 DOA, 91% of birds of group 2 were seropositive compared to 0% for groups 4 and 6. Group 2 (MB-

Table 2. Mortality, mean bursa-bodyweight ratios (×1000), and mean bursal lesion score after vvIBDV D6948 challenge of MB-1, Transmune or mock-vaccinated broilers by the *in ovo* route.

Group	Vaccine (*in ovo*)	D6948 challenge day	Number of birds	Mortality post-challenge	Mean BBWR[A] at 10 dpc	Mean BLS[B] at 10 dpc[C]
1	MB-1	22	20	0%	0.66[a]*	3.2[b]
2	MB-1	36	20	0%	0.71[a]	2.5[c]
3	Transmune	22	10	0%	0.66[a]	2.8[bc]
4	Transmune	36	10	0%	0.69[a]	4.8[a]
5	Mock	22	10	20%	0.74[a]	4.6[a]
6	Mock	36	10	0%	0.69[a]	4.5[a]

[A]Bursa-bodyweight ratio × 1000.
[B]Bursal lesion score.
[C]Days post-challenge.
*Statistical significance per challenge age X vaccine interaction (groups not sharing the same superscript letter are significantly different).

Table 3. Mean IBDV antibody response (GMT) post-vaccination.

Group	Vaccine (*in ovo*)	D6948 challenge day	Number of birds	Geometric mean titres (GMT) of ELISA IBDV antibodies (percentage of positive sera) at days of age					
				0	7	14	22	28	36
Hatch mates	–		18	3741 (100%)					
1	MB-1	22	33			174[a] (22%)	133[ab] (16%)		
2	MB-1	36	33			213[a] (12%)	270[a] (24%)	1402[a] (66%)	2884[a] (91%)
3	Transmune	22	22			123[a] (14%)	99[ab] (14%)	–	–
4	Transmune	36	22			230[a] (23%)	51[ab] (0%)	101[b] (9%)	38[b] (0%)
5	Mock	22	22		1113 (100%)	277[a] (27%)	15[b] (0%)	–	–
6	Mock	36	22			315[a] (32%)	14[b] (0%)	5[b] (0%)	13[b] (0%)

Note: Different superscript letters indicate significant differences between groups ($P < 0.05$, *t*-test)

1) had significantly higher titres, at 28 and 36 DOA, than groups 4 (Transmune) and 6 (mock).

Bursa to bodyweight ratio

The mean BBWR scores at 10 dpc at 22 or 36 DOA are summarized in Table 2. The mean BBWR scores of the six groups ranged from 0.66 to –0.74 with no significant differences ($P = 0.8914$) .

Bursal lesion scores

The mean BLS scores at 10 days post-challenge at 22 or 36 days of age are summarized in Table 2. Table 4 shows the distribution of the individual BLS scores per group. The mean BLS scores of groups 1, 2, and 3 were significantly ($P < 0.0001$) lower than the BLS means of groups 4, 5, and 6. There were no statistically significant differences ($P > 0.05$) among groups 4, 5, and 6. The mean BLS of group 1 was significantly higher ($P < 0.001$) than the later challenged group 2, while the scores of the former challenged group 3 did not differ considerably from groups 1 and 2.

Bursal IBDV RT–PCR and strain differentiation

The individual bursa RT–PCR and Sanger sequencing results and deep sequencing results of the pooled samples per group, all collected at 5 dpc at 22 or 36 DOA, are summarized in Table 5.

As expected, only vvIBDV D6948 strain sequences could be detected by Sanger and deep sequencing in the bursas of the mock-vaccinated birds of groups 5 and 6 at 5 dpc. The mean Ct-values of about 20 indicated high amounts of RNA of D6948.

In the 10 bursas of group 1 (MB-1 vaccinated by *in ovo*, challenged at 22 days of age, sampled at 27 days of age), Sanger sequencing showed the presence of MB-1 in all 10 bursas. The deep sequencing results of the pooled sample confirmed the presence of MB-1 sequences, no D6948 sequences were detected.

Table 4. Overview of the histopathological bursal lesion scores (BLS) per group.

Group	Vaccine (*in ovo*)	D6948 challenge day	Number of birds	Number of birds per bursal lesion score at 10 days post-challenge					
				0	1	2	3	4	5
1	MB-1	22	20	–	–	1	14	5	–
2	MB-1	36	20	–	4	7	5	4	–
3	Transmune	22	10	–	1	–	9	–	–
4	Transmune	36	10	–	–	–	–	2	8
5	Mock	22	8	–	–	–	–	3	5
6	Mock	36	10	–	–	–	–	5	5

Table 5. Bursal IBDV RT-rtPCR and Strain Differentiation/Genotyping at 5 days post-challenge with D6948 of MB-1, Transmune, or mock-vaccinated groups (10 birds per group).

Group	Vaccine (*in ovo*)	D6948 challenge day	RT-PCR mean Ct value	Individual Sanger sequencing	Deep sequencing (pooled sample)	
					Number of contigs	Percentage
1	MB-1	22	23.2	10x MB-1	954	100% MB-1
2	MB-1	36	29.5	8x MB-1, 2x undetermined	668	100% MB-1
3	Transmune	22	24.1	9x W2512, 1x W2512/D6948	799	97% W2512, 3% D6948
4	Transmune	36	22.3	10x D6948	1392	100% D6948
5	Mock	22	19.2	10x D6948	664	100% D6948
6	Mock	36	21.1	10x D6948	1320	100% D6948

In the 10 bursas of group 2 (MB-1 vaccinated by *in ovo*, challenged at 36 days of age, sampled at 41 days of age), Sanger sequencing showed the presence of MB-1 in eight bursas. In two bursas, RT–PCR was positive for IBDV, but cDNA was too low for a successful Sanger sequencing. Both samples were subsequently submitted for Ilumina sequencing, which showed the presence of MB-1 in one sample; the second sample was of insufficient quality; therefore, these were excluded from Illumina sequencing. The mean Ct value of the RT–PCR was 29.5, significantly lower than the mean Ct value of 23.2 in group 1, indicating a decrease of vaccine-related RNA at day 41 compared to day 27. The deep sequencing results of the pooled sample of group 2 confirmed the presence of MB-1 sequences; no D6948 sequences were detected. In the 10 bursas of group 3 (Transmune-vaccinated by *in ovo*, challenged at 22 DOA, sampled at 27 DOA), Sanger sequencing showed the presence of W2512 in nine bursas. In one bursa, Sanger sequencing showed a mixed sequence of W2512 and D6948. The deep sequencing results of the pooled sample of group 3 confirmed the presence of W2512 (97%) and D6948 (3%) sequences.

In the 10 bursas of group 4 (Transmune-vaccinated by *in ovo*, challenged at 36 days of age, sampled at 41 days of age), Sanger sequencing showed the presence of D6948 in all 10 bursas. The deep sequencing results of the pooled sample confirmed the presence of D6948 sequences; no vaccine-related sequences were detected.

Discussion

The immunization of chickens is the principal method used for the prevention of IBD in poultry production. The adequate immunization of breeder flocks and the transfer of maternal immunity to protect the offspring from early immunosuppressive infection is highly significant (Eterradossi & Saif, 2020). The importance of maternal immunity could not be overemphasized; yet, MDA levels decay during the first few weeks of the chick's life, and the induction of active immunization is essential. Various live attenuated IBDV strains can induce active immunity in young chicks (Muller *et al.*, 2012). However, the neutralization of live IBD vaccines by MDA is a significant obstacle (Hitchner, 1971; Winterfield & Thacker, 1978; Lucio & Hitchner, 1979; Skeeles *et al.*, 1979; Winterfield *et al.*, 1980; Wyeth, 1980; Wood *et al.*, 1981; Wyeth *et al.*, 1981; Naqi *et al.*, 1983; Van den Berg & Meulemans, 1991; Goddard *et al.*, 1994; Kouwenhoven & Van den Bosch, 1994; De Wit & van Loon, 1998; Alam *et al.*, 2002; Al-Natour *et al.*, 2004; Hair-Bejo *et al.*, 2004; Rautenschlein *et al.*, 2005; Block *et al.*, 2007; Jackwood, 2011; Muller *et al.*, 2012). To overcome the interference to live IBD vaccine uptake by MDA, calculations of the optimal application age have been proposed (De Wit, 1998; Block *et al.*, 2007).

Recently, Ashash *et al.* (2019) reported the positive results of *in ovo* and sub-cutaneous application on the day of hatch of a conventional live IBD vaccine strain (MB-1) to commercial broilers in the field. In these four field studies, MB-1 genome was detected in 33% to 100% of the sampled bursas at 24 days of age and in 83% to 100% in the bursas collected at 28 days of age. In these four flocks, seroconversions to IBDV were detected at 28 days of age, supporting the findings by the RT–PCR and sequencing. These observations seem to conflict with many papers (see above) and field experiences that very early application of live conventional vaccines in birds with significant levels of MDA has very little chance of a successful immune response. For this reason and to obtain more data, an *in ovo* vaccination-challenge experiment was performed under controlled, experimental conditions using commercial broilers with intermediate to high levels of MDA against IBDV and a vvIBDV challenge strain. Clinical signs, bursa-bodyweight ratio, histology, serology, RT–PCR, and Sanger- and deep sequencing were used to study the efficacy and safety of the *in ovo*-applied MB-1 vaccine compared to an established immuno-complex vaccine (Transmune).

The GMT of the IBDV antibodies of the hatchmates was 3741, showing a representative level of MDA of the birds used in this study. As expected for MDA titres, all non-vaccinated birds were negative in the IBDV antibody ELISA at 22 days of age (Table 3) (De Wit, 1998). The challenge by eye-drop at 22 days and 36 days of age with $10^{3.7}$ EID_{50} and $10^{3.0}$ EID_{50}, respectively, of the very virulent IBDV strain D6948 was successful, as shown by the high

amounts of D6948 genome in the bursas at 5 dpc, and the high BLS scores of 4 or 5 at 10 dpc of groups 5 and 6 (Table 4). The mortality rate in these groups 5 and 6, using the modern fast-growing broilers (such as Ross 308), was not as high as is to be expected in white SPF layers. In young white SPF layers, mortality close to 100% is expected post-challenge with vvIBDV strain D6948 (unpublished data, Royal GD).

None of the birds vaccinated with MB-1 (groups 1 and 2) showed clinical signs post-challenge at D22 or D36 related to the challenge virus. At D22, 20% of the MB-1 vaccinated birds (16% in group 1 and 24% in group 2) had already seroconverted (ELISA positive), indicating that the MB-1 vaccine most likely had started replicating in these birds about 1 week earlier (De Wit *et al.*, 2001). The Sanger and deep sequencing results at D27, 5 dpc with D6948, showed only MB-1 sequences. This complete lack of D6948 sequences showed that all birds already had a high level of protection against replication of the field strain at day 22, either by full immunity or viral interference (Jackwood, 2011). The sequencing results at 5 dpc at D36 confirmed the high level of protection against challenge. The mean Ct value of 29.5 at D41 was significantly higher than the mean Ct value of 23.2 at D27, showing that the clearance of the vaccine strain was on-going. The average BBWR scores of 0.66 and 0.71 for group 1 and group 2 were comparable to the BBWR scores of the other four groups. The mean BLS at 10 dpc of group 1 (3.2) and group 2 (2.5) were comparable to that of group 3 (2.8) and significantly lower than the scores of groups 4, 5, and 6 ($P < 0.05$). The BLS of groups 1, 2, and 3 were at the level expected for intermediate vaccines after application in SPF birds (Jungback & Nutolo, 2001). High bursal lesion scores in this study were highly correlated with the detection of replication of the vvIBDV strain in the bursa.

The results of both Transmune-vaccinated groups 3 and 4 were quite different from each other despite the similarity in the vaccine application (batch, dosage, person, observer, source of hatching eggs, and coding) and hatcher. In group 4, challenged at day 36, no response to the vaccine was detected by serology or RT–PCR and sequencing. At 5 dpc, only D6948 sequences were detected, and the mean BLS at 10 dpc was 4.8, similar to the scores of the mock-vaccinated groups post-challenge.

The results of Transmune-vaccinated group 3 were quite similar to group 1 No statistical differences were detected. Fourteen per cent of the birds had seroconverted at D22 and at 5 dpc, in nine out of 10 birds only vaccine-related W2512 sequences could be detected. In bird 10, a mixture of vaccine and challenge virus could be detected. The BBWR and mean BLS were also similar to the scores of group 1.

The results of this controlled vaccination-challenge experiment support the post-vaccination findings in the field reported by Ashash *et al.* (2019) and show the development of protective immunity to a vvIBDV challenge after the *in ovo* application of conventional live IBD vaccine MB-1 to commercial broilers. The results showed that the *in ovo* applied MB-1 vaccine was not neutralized by the high level of maternal antibodies and its replication had been delayed for about 2–3 weeks by an unknown mechanism. This delay was comparable to that of the Transmune vaccine. One might speculate that the way of application (*in ovo* or sub-cutaneous) might play a role in the lack of neutralization by the MDA and delayed replication of MB-1.

To summarize, the findings of this controlled vaccination-challenge study using commercial broilers confirm the field observations after *in ovo* and sub-cutaneous vaccination on the day of hatch using live MB-1 vaccine, as reported by Ashash *et al.* (2019) and showed complete protection against a vvIBDV challenge at 22 or 36 days of age. Comparable to the Icx vaccine, a delay of about 2–3 weeks was noticed for the replication of the vaccine. The mode of action of *in ovo* IBD vaccine remains to be elucidated. It may reveal an alternative IBDV *immunogenic* mechanism following *in ovo* and parenteral exposures compared to mucosal applications.

Disclosure statement

No potential conflict of interest was reported by the authors.

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