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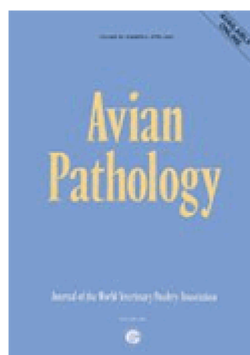

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A field study on the significance of vaccination against infectious bursal disease virus (IBDV) at the optimal time point in broiler flocks positive for maternally derived IBDV antibodies

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A field study on the significance of vaccination against infectious bursal disease virus (IBDV) at the optimal time point in broiler flocks with maternally derived IBDV antibodies

Hermann Block,[1] Karen Meyer-Block,[1] Dierk E. Rebeski,[2] Heike Scharr,[2] Sjaak de Wit,[3] Karl Rohn[4], and Silke Rautenschlein[5]*

[1]Poultry Practice Meyer-Block, Uelsen, Germany; [2]Lohmann Animal Health, Cuxhaven, Germany; [3]GD Deventer, The Netherlands; [4]Institute for Biometry, Epidemiology and Information Processing; University of Veterinary Medicine, [5]Clinic for Poultry, University of Veterinary Medicine Hannover, Germany

Short title: IBDV vaccination under field conditions

*Corresponding Author:
Silke Rautenschlein
Address: Clinic for Poultry ,University of Veterinary Medicine Hannover, Bünteweg 17, 30559 Hannover, Germany
Tel.: 49-511-953-8763
Fax: 49-511-953-8580

Email: Silke.Rautenschlein@tiho-hannover.de

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Note to publisher:
There are also alterations needed to the Figures, which I cannot manage!
In Figure 1 – titer needs changing to titre and nummer to number
Titer- titre also needed on Figure 2

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Abstract

The right strategy for infectious bursal disease (IBD) control and its success rate under field conditions depends on hygiene management, IBD field pressure, level and variation in maternally derived IBD antibody (MDA) levels, and the IBD-vaccine strains to be used. Usually, standard vaccination programmes are used, which are not always adapted to the specific conditions on the farm and to the strain of chicken. Employing the 'Deventer formula' may help to estimate the optimal time for vaccination for a specific flock based on the MDA level, its variation, the genetic background of the chicken, and the IBD vaccine strain. Two field studies with 16 or 20 commercial broiler flocks were conducted, applying an intermediate IBD vaccine before, at the best, and after the estimated optimal vaccination time estimated by the 'Deventer formula'. These studies showed that flocks which had been IBD-vaccinated between one day before, at, or up to three days after the estimated optimal time point, developed detectable humoral immunity up to 14 days post vaccination. If birds had been vaccinated more than one day before the calculated optimal vaccination date, the humoral immune response was delayed or non-detectable until slaughter. The induction of humoral immunity correlated with the incidence of bursa lesions and IBDV-detection by RT-PCR. As indicated in this study, under field conditions bursa lesions may develop later than predicted based on experimental experiences. The late

incidence of bursa lesions after vaccination may be confused with field virus-induced lesions, in which case sequencing may offer a valuable tool for differentiation.

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Introduction

Immunosuppressive diseases are of constant concern for poultry producers worldwide. Infectious bursal disease virus (IBDV) is one of the most important immunosuppressive agents in modern poultry production. Depending on the virulence of the IBDV strain, age at the time of infection, presence of IBDV antibodies and the genetic background of the infected chicken, infection with IBDV may induce a temporary or permanent destruction of the bursa cloacalis and other lymphoid tissues (Lukert & Saif, 2003). Destruction of B cells and macrophages, and their functions contribute to IBDV-induced immunosuppression (Sharma *et al.*, 2000; Khatri *et al.*, 2005).

Besides hygiene strategies, the current infectious bursal disease (IBD) control methods involve passive and active immunisation (Fussell, 1998). It has been shown that the timing of IBD vaccine administration in broiler progeny is pivotal (Ather, 1993; van den Berg *et al.*, 2000). The optimal vaccination time depends upon the maternally derived antibody (MDA) level of the broiler chicks, the vaccine strain to be used, its breakthrough titre, and the field pressure (de Wit, 1998, 2001, de Wit & van Loon, 1998). A high variation in MDA levels between birds can make it advisable to vaccinate a broiler flock twice to induce homogeneous protection in birds (McIlroy *et al.*, 1992).

Vaccination in the presence of IBDV antibody levels above the breakthrough titre of the vaccine will lead to a significant delay of IBDV replication and the induction of immunity, as shown by recent laboratory investigations (McCarty *et al.*, 2005; Rautenschlein *et al.*, 2005). Other experimental studies have shown that IBD vaccine virus may even be completely neutralized by maternally derived antibodies

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(van den Berg & Meulemans, 1991; Tsukamoto *et al.,* 1995; Alam *et al.*, 2002; Hair-Bejo *et al.*, 2004; Moraes *et al.*, 2005). In order to have chickens protected against IBDV field challenge, it is crucial to determine the optimal timing for IBD vaccine delivery (Tsukamoto *et al.*, 1995). The optimal timing is often predicted based on serological data following detection of IBDV MDA by an ELISA system during the first week post hatch (Kouwenhoven & van den Bos, 1992; 1994). The "Deventer formula" was developed to estimate the optimal vaccination time point based on the half life time of the MDA, the age of the chicken at sampling, genetic background, breakthrough titre of the vaccine, and the requested percentage of the flock having antibody levels below the breakthrough titre of the vaccine at the time of administration (de Wit, 1998, 2001). So far, hardly any studies have been available comparing the outcome of IBDV vaccination at the optimal, before the optimal and after the optimal time point under field conditions in commercial broiler flocks. One recent study indicates that the estimation of the optimal vaccination time in the field, which was based on ELISA antibody titres, may lead to too early vaccination (de Herdt *et al.*, 2005). The technical performance of ELISA systems may vary between manufacturers and laboratories and may affect the interpretation of the maternal antibody levels (de Wit, 2001; de Wit *et al.,* 2007).

Two field studies including 16 and 20 broiler flocks were conducted comparing the IBDV vaccine response in broiler flocks with variable MDA levels that were vaccinated before, at, or after the estimated optimal time point for a single IBDV vaccination. Besides the induction of humoral immunity, we also looked at the development of IBDV-induced bursa lesions and detected and characterized the IBDV genome in bursa samples of vaccinated flocks. The overall performance of the flock was evaluated. To our knowledge this is the first study comparing the IBDV vaccine

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response in broilers with different levels of maternally derived antibodies under field conditions.

Materials and Methods

Chickens. Overall, 36 commercial broiler flocks with 20,000 to 140,000 chickens per farm (14,000-48,000 broilers/house) in the North-Western region of Lower Saxony in Germany participated in the two field trials. These are representative farms for this region, which had already established a vaccination regime against IBDV and were under regular veterinary control. Broilers were raised under controlled conditions based on national animal welfare regulations. The broilers belonged to the hybrid lines Cobb 500, Ross 308 and Ross 508. Besides vaccination against IBDV, birds were also vaccinated by standard procedures against infectious bronchitis by spray at day of hatch and Newcastle disease at 7 days by drinking water. The broiler breeder flocks had been vaccinated against IBDV with an intermediate live vaccine; some breeders had also been boosted with an inactivated IBDV vaccine between 16 and 17 weeks of age (see Table 2 for data on Trial 1; in Trial 2 only flock 18 had been boosted with an inactivated IBDV vaccine).

Vaccine. A commercially available intermediate IBDV vaccine strain was used. As previous laboratory studies indicated, this vaccine induces bursa lesions around 3 days post inoculation in specific pathogen free (SPF) layer-type and antibody free broiler chickens (Jung, 2007). Recovery from bursa lesions would be expected to take place around 10 days post vaccination of antibody free birds (Jung, 2007). One dose consisted of at least 10^3 median egg-infectious doses (EID_{50}) per bird. Vaccination

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was performed based on the manufacturer's recommendations by drinking water. The break through titre of this intermediate vaccine in the IDEXX enzyme linked immunosorbent assay (ELISA) is 1: 125. This ELISA titre corresponds to the neutralizing maternal antibody levels at the optimal time of vaccination (Lucio & Hitchner, 1979; Skeeles *et al*., 1979; Lukert & Saif, 2003). Because the IDEXX ELISA is very commonly used in the field, in the present study, we refer to the break through IDEXX ELISA titre.

Serology. IBDV antibodies were detected in collected serum samples using a commercially available ELISA kit (FlockChek® IBD ELISA, IDEXX, Germany) and titres were calculated as described by the manufacturer.

Deventer formula. The optimal vaccination time was determined by the Deventer formula (de Wit, 1998; 2001):

Vaccination age = {($\log_2$ IBDV antibody ELISA titre of the bird (%)-$\log_2$ breakthrough titre of the vaccine) x t ½} + age at sampling + correcting value 0-4

In which

Bird (%): titre of the bird (at sampling) that represents a certain percentage of the flock (in this study: 75 %) that is desired to be susceptible to the vaccine at the time of the application

Breakthrough: breakthrough (ELISA) titre of the vaccine to be used (for this vaccine in the ELISA system used, a titre of 125)

t1/2: half life time of the antibodies (ELISA titre) in the type of chickens that were sampled (broilers: 3 days)

Age at sampling: age of the birds at sampling

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Correcting value 0-4: extra days when the sampling was done at 0-4

days post hatch.

Histology. Bursae cloacalis were collected *post mortem* and fixed in 10% phosphate buffered formalin, paraffin embedded, cut and stained with haematoxylin and eosin. Bursa lesion scores were determined microscopically and compared between groups (Muskett *et al*., 1979). The scoring system was as follows: *score 0*: no lesions; *score 1*: 1-25 % of follicles show lymphoid depletion (less then 50 % depletion per follicle), accumulation of heterophils; *score 2*: 26-50 % of follicles show almost complete lymphoid cell depletion (more than 75 % depletion per follicle), necrosis and accumulation of heterophils; *score 3*: 51-75 % of follicles show almost complete depletion of bursa follicles with necrosis and heterophils; *score 4*: 76 – 100 % of follicles show almost complete depletion of bursa follicles with necrosis and heterophils, hyperplasia and cysts may be observed; *score 5*: 100 % of follicles show almost complete depletion of bursa follicles with loss of bursa architecture, and fibrosis.

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Detection of IBDV by RT-PCR and sequencing of the VP2-region. Five bursal samples per flock were pooled for the detection of IBDV by RT-PCR. Total RNA was isolated using the TriPure Isolation Reagent (Roche) following the guidelines of the manufacturer. RNA isolation was followed by a RT-PCR reaction with the RNA-Amplification Kit SYBR Green I (Roche). The following primers were used: forward: 5’-GGT AGC CAC ATC TGA CAG-3’ (Boot *et al*., 1999); reverse 5’-CGC TCG AAG TTR CTC ACC C-3’ (Islam *et al*., 2001). The RT-PCR was performed at the following conditions: RT-reaction for 30 min at 52°C; denaturation for 30 sec at 95°C,

40 cycles with 5 sec at 95°C, 10 sec at 57°C, and 30 sec at 72°C. The RT-PCR was followed by a melting curve analysis. Sequencing of the RT-PCR product of 540 bp was conducted with the described forward and reverse primer at BaseClear (Leiden, NL).

Reisolation of IBDV. Pools of bursal homogenate were inoculated into 10-day-old SPF embryonated chicken eggs following standard procedures (Rosenberger *et al*., 1998). At 5 days post inoculation, the allantoic fluid was harvested and RT-PCR was performed as described above to detect the IBDV genome.

Production parameters. The following parameters were collected and evaluated to compare the production values between flocks:

Total animal loss ($loss_{total}$) = animal loss (%) during the production period + condemnation at slaughter (%).

DWG (daily weight gain) = final weight at slaughter (g) / fattening days.

FCR (feed conversion rate) =feed conversion / final total live weight

Production index = {100- $loss_{total}$ (%)} x DWG / FCR x 10

Experimental protocol. *Trial 1*: Sixteen broiler farms were selected as described. Serum samples were collected from 30 randomly chosen birds/flock between 3 and 12 days of age, and at a second time between 2 to 5 days before vaccination, to detect maternal IBDV antibodies, to estimate and confirm the optimal time for vaccination by the Deventer formula (Table 1). At the estimated optimal time, birds were vaccinated by drinking water with one dose of the intermediate IBDV vaccine

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following the manufacturer's instructions. At day of vaccination, and in weekly intervals until slaughter, all flocks were observed for clinical disease, mortality and performance. At the same times except the day of vaccination, 30 serum samples were collected randomly for IBDV antibody detection, and five randomly chosen broilers were killed to determine bursal lesions. Bursal samples were taken for IBDV detection by RT-PCR and histology. During the fattening period all dead birds were examined *post mortem* to determine the cause of death.

Trial 2: Twenty broiler flocks were selected as described above. Unlike in Trial 1, in Trial 2 broiler flocks were vaccinated based on the recommendation of the breeder companies or the hatcheries. Serum samples (n = 24/flock) that had been taken between the 3rd and 7th day post hatch were used for the retrospective estimation of the optimal IBD vaccination time using the Deventer Formula. Based on the difference between the actual time of vaccination and the retrospectively estimated optimal time, the broiler flocks were divided into three groups: birds vaccinated 8 to 1 day before the estimated optimal time for vaccination (group 1); birds vaccinated at the optimal time (group 2); and birds vaccinated up to 6 days after the optimal time (group 3; Table 3). Vaccination was conducted as described in Trial 1. Further serum samples (n = 30) were collected at the day of vaccination, and in weekly intervals until slaughter. Clinical observations and production parameters were obtained weekly. Mortality rates were determined and all dead birds were examined to identify the cause of death.

Statistical analysis. All collected data were included in a descriptive analysis. The log-normal distribution of model residuals of IBDV antibody titres was confirmed by visual assessment of normal probability plots and by the Shapiro-Wilk Test. For these

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parameters logarithm to the base 2 transformation was performed prior to analysis; the description is shown as box-plots of original data. Bursal lesion scores as ordinal scaled data are neither normally nor log-normally distributed, because of that nonparametric methods were applied.

Differences in IBDV antibody development between flocks depending on vaccination before, at, and after the optimal time point were compared. Two-way analysis of variance was used with the flock as an independent effect and time-points of vaccination between flocks as repeated measurements with Tukey's post-hoc test for multiple pair wise comparisons. Significance was defined as $P \leq 0.05$.

Analyses were carried out with the statistical software SAS, version 9.1 (SAS Institute, Cary, NC). For the analysis of the linear model, the procedure GLM (General Linear Model) was used. As nonparametric methods we used Kruskal-Wallis and Wilcoxon two sample test for independent samples.

Results

Estimation of the optimal vaccination time. In field Trial 1, 30 serum samples/flock were investigated for IBDV MDA by ELISA within the first two weeks post hatch. Based on the Deventer formula the optimal vaccination time for these broiler flocks in this trial was between 17 and 23 days post hatch (Table 1). IBDV antibody titres in 30 serum samples/flock taken at 2-5 days before the estimated optimal vaccination time confirmed the estimated optimal vaccination time (Table 2). All flocks showed the expected drop in MDA levels (Table 2).

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In Trial 2, all broiler flocks were vaccinated based on the recommendation of the breeder companies or the hatcheries. Serum samples were taken between the 3rd and 7th day post hatch for the retrospective estimation of the optimal IBD vaccination time. Based on the difference between the actual time of vaccination and the retrospectively estimated optimal time, the broiler flocks were divided into three groups (Table 3): four flocks that had been vaccinated between 8 to 1 day before the estimated optimal time for vaccination (group 1); five flocks that had been vaccinated at the estimated optimal time (group 2); and 11 flocks that had been vaccinated between one and six days after the optimal time (group 3). Antibody detection in Trial 2 at the day of vaccination revealed that birds in group 1 had a significantly higher antibody level (titre range 25-6590) than birds of groups 2 (titre range 6-3126) or 3 (titre range 2-2680) (data not shown; $P < 0.05$). In flock 9, which was vaccinated eight days before the optimal vaccination time, 92% of the serum samples had IBDV antibody levels above the estimated cut-off value of 125 in the ELISA system used (Table 3). With the exception of flock 1, all the other flocks in group 2 had MDA levels above the cut-off value in less than 25% of the tested birds/flock (Table 3). In group 3, which was vaccinated after the optimal vaccination time, at the time of vaccination 0-17% of the tested birds had MDA levels above the cut-off value. Forty-five percent of the tested flocks of group 3 had only birds with antibody levels below the cut-off value (Table 3).

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Induction of humoral immunity after IBDV-vaccination. In Trial 1, all broiler flocks showed seroconversion before slaughter (Figures 1a, 1b and 1c). Only one flock (flock 13) showed a significant increase in IBDV ELISA antibodies at 7 days post vaccination (DPV; Figure 1a) in comparison to antibody levels detected 4-7 days

before vaccination ($P < 0.05$; data not shown). At 14 DPV, flocks 1, 4, 6, 7, 10, and 14 had comparable antibody levels to their IBDV antibody titres at 7 DPV (Figure 1a,b). ($P > 0.05$). Flocks 8, 12 and 16 had significantly higher antibody levels at 14 DPV in comparison to flocks 2, 3, 5, 9, 11, 14 and 15 ($P < 0.05$). With the exception of flock 13, all other flocks showed a significant increase in IBDV antibody levels at 21 DPV in comparison to titres at 7 DPV ($P < 0.05$).

In Trial 2, only three flocks of group 3 (flocks 14, 17, 20, vaccinated at 5 or 6 days after the estimated optimal time) showed a significant increase in IBDV antibodies at 7 DPV in comparison to the day of vaccination ($P < 0.05$), while no significant seroconversion was detected in any of the other 17 flocks at this time (Figure 2a,b). At 14 DPV, all flocks of groups 2 and 3 showed a significant increase in IBDV antibodies in comparison to antibody levels of the same flock at day of vaccination (Figure 2a,c). In group 1 only birds that had been vaccinated 1 day before the optimal vaccination time showed a significant increase in IBDV antibody production at 14 DPV in comparison to 7 DPV (Figure 2b,c; $P < 0.05$). At 21 DPV, all flocks except flock 9 (group 1), which had been vaccinated 8 days before the optimal vaccination time point, had seroconverted.

Induction of histological bursa lesions. In Trial 1, five bursae per flock were evaluated histologically for IBDV specific lesions at different times before and after vaccination. None of the investigated flocks showed bursa lesions at the time of vaccination (data not shown). At 7 DPV, 14 of 16 flocks had detectable bursa lesions (Figure 3). In flock 12 and 13 bursa lesion scores were detected in three out of five, and four out of five chickens, respectively. At 14 DPV, all vaccinated flocks had developed lesions with an average bursa lesion score of < 2 for flocks 4 and 14;

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scores of 2 to 3 for flocks 1, 11, 15, and 16; and scores of 3.1- 4.2 for flocks 2, 3, 5, 6, 7, 8, 9, 10, 12, and 13.

Detection of IBDV in bursal tissue. In Trial 1, no IBDV was detected by RT-PCR in bursal tissue at the day of vaccination (data not shown). As indicated in Figure 3, some of the flocks already had IBDV positive bursal samples at 7 DPV. By 14 DPV, all flocks were confirmed as IBDV positive. In flock 5, 8, and 9, IBDV was only detected at the indicated times by RT-PCR after previous propagation of the virus in chicken embryos. By 21 DPV 58% of the investigated flocks were still positive for IBDV by RT-PCR (Figure 3). Sequencing of the VP2-region of IBDV, which had been amplified from bursal samples of the investigated flocks by RT-PCR, revealed that the IBDV strain detected was solely the administered IBDV vaccine strain (data not shown). The sequence homology between the predicted amino-acid sequences of the RT-PCR products and the original vaccine strain was 100%. After propagation of the bursal samples in embryonated eggs (samples of flocks 5, 8, 9) and subsequent sequencing of the RT-PCR products we detected a sequence homology to the original vaccine stain of 99.7%. Amino-acid variations were detectable at position 316 (Arg → Lys) and 325 (Ile → Met).

Health status, *post-mortem* results and production parameters of the broiler flocks. None of the 16 broiler flocks of Trial 1 showed any clinical disease during the growing period. Total animal loss ranged between 2.2 and 6.2% in the different flocks. *Post mortem* examination of the dead birds and the randomly selected five birds per flock, which had been killed before and at 7, 14, and 21 days post vaccination, revealed sporadic diagnosis of pericarditis (flock 1 and 7, in one of five

birds each), or polyserositis (flock 11, one of five birds). Mild catarrhal enteritis was detected in one of five broilers of each of the following flocks: 1, 2, 4, 9, and 15. *Eimeria acervulina* was detected in the gut of two of five birds each of flocks 2, 9, and 13 between the 25th and 29th days post hatch. No IBDV-specific macroscopical bursa lesions such as gelatination, haemorrhages or necrosis were seen.

The production indexes varied between 286 and 352 (Trial 1). On average the fattening period lasted 40 days with a daily weight gain between 51.88 and 61.95 g/day. The feed conversion rate ranged between 1.608-1.766.

In Trial 2, none of the 24 broiler flocks showed any clinical disease during the growing period. The total animal loss ranged between 1.9 and 5.6% in the different flocks. Examination of the dead birds and the randomly selected five birds per flock at the different times during the field trial revealed sporadic diagnosis of lesions in the respiratory or digestive tract. At 7 or 14 DPV, one or two of five investigated birds of flocks 1, 7, 9, 11, 14, 15, and 19 showed mild catarrhal enteritis. Aerosacculitis was detected in two of five birds of flocks 2 and 12 at 14 DPV. *E. acervulina* was detected in the gut of birds from flocks 20 and 11 at 14 or 21 DPV, respectively. No IBDV-specific macroscopical bursa lesions such as gelatination, haemorrhages or necrosis were seen.

The production indexes were between 267 and 326 (Trial 2). On average the fattening period lasted 41 days with a daily weight gain between 49.2 and 57.9 g/day. The feed conversion rate ranged between 1.609 and 1.859.

Discussion

This is the first study to demonstrate the importance of keeping to the optimal

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time for IBDV vaccination under field conditions in order to achieve a detectable immune response. Previous observations in the field and experimental studies under laboratory conditions had indicated that high MDA at the time of IBDV vaccination may interfere with the vaccine response and neutralise the vaccine virus (van den Berg & Meulemans, 1991; Tsukamoto *et al.*, 1995; van den Berg, 2000; Alam *et al.*, 2002; Hair-Bejo *et al.*, 2004; Moraes *et al.*, 2005). But no information has been available so far from controlled field conditions about the real influence of MDA on the outcome of the IBDV vaccine response, accompanied by consecutive monitoring of the flocks. In two large field trials we determined the effect of MDA on the vaccine response of broilers to one intermediate IBDV strain. In the first trial the vaccine response was investigated in broiler flocks vaccinated at the optimal vaccination time estimated with the Deventer formula (de Wit, 1998; 2001). All vaccinated flocks of Trial 1 seroconverted within 14 days of IBDV vaccination. IBDV antibody development correlated with the incidence of IBDV-induced bursal lesions and IBDV detection by RT-PCR. These observations were confirmed in the second trial. Furthermore, the vaccine response of optimally vaccinated birds was compared in the second trial with the vaccine response of birds which had been vaccinated earlier or later than the optimal vaccination time. Our study clearly confirms previous laboratory investigations that high levels of MDA interfere with a homogeneous IBDV vaccine response, and delay or even prevent the induction of humoral immunity (van den Berg & Meulemans, 1991; Goddard *et al.*, 1994; Tsukamoto *et al.*, 1995; Alam *et al.*, 2002; Hair-Bejo *et al.*, 2004; Jung, 2006). A delayed or prevented immune response may subsequently lead to broilers being susceptible to IBDV field challenge (van den Berg *et al.*, 1991; Kouwenhoven & van den Bos, 1992; de Wit & van Loon, 1998). It is not clear from this study what would be the effect on the

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protective immunity of the immunized flock of vaccination at different days ‘ too early’ compared to the optimal calculated time. Consecutive challenge studies of broilers from each flock at different days post vaccination would have been necessary to provide information regarding this point. But certainly our study shows that the Deventer formula provides a useful tool to estimate the optimal vaccination time in order to induce detectable humoral immunity in a timely manner. Future studies are needed to determine how flexible the optimal vaccination day will be under field conditions. In our study it was shown that the IBDV antibody levels at 21 DPV were comparable between chickens vaccinated at the optimal, and one day before the optimal, time. Further investigated is necessary to determine the effect of ‘too early’ vaccination on the outcome of the vaccine response.

The different genetic backgrounds of the broiler flocks (Ross and Cobb) seem not to have had an influence on the outcome of the IBDV vaccine response (data not shown). Previous laboratory studies have confirmed that the genetic background of the chicken may not influence the pathogenesis of an intermediate IBDV strain (Jung, 2006), whereas the immune responses to virulent strains may vary significantly between chicken lines (Ruby *et al*., 2006). Furthermore, the vaccination scheme of the parent flocks under field conditions did not significantly influence the outcome of the vaccine response of the progeny as seen in Trial 1. Based on the variation in MDA levels between progeny of parents which had been vaccinated only with one dose of an IBDV live strain, we may speculate that some of the parent flocks may have gone through a field challenge resulting in unexpected high levels of circulating antibodies. These antibody levels were comparable to titres of parents which had been boosted with an inactivated IBDV vaccine (Table 2). The heterogenicity of the MDA levels in

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the progeny broiler flocks varied between flocks but the levels where still homogeneous enough to justify only a single vaccination (de Wit, 2001).

As demonstrated in field trial 1, the induction of humoral immunity clearly correlated with the induction of bursal lesions and IBDV replication, as shown previously under experimental conditions (Rautenschlein *et al.*, 2005). If birds were vaccinated at the optimal time, all vaccinated flocks developed IBDV antibodies as well as bursal lesions up to 14 DPV. These studies show that bursal lesions may develop later than would be expected from studies in SPF layer-type chickens (Tanimura & Sharma, 1998; Kim *et al.*, 1999). This is possibly due to residual levels of MDA (McCarty *et al.*, 2005; Rautenschlein *et al.*, 2005). Furthermore, the bursal lesion scores were unexpectedly high. Most investigated flocks had severe average lesion scores of over 3.3 at one of the investigated times. Interestingly, we did not observe any regeneration of bursal tissue, as would be expected after vaccination with this strain or other intermediate strains, based on laboratory studies (Kim *et al.*, 1999; Alam *et al.,* 2002; Rautenschlein & Haase, 2005; Jung, 2006). Under field conditions, circulating IBDV vaccine virus may lead to deviating observations in comparison to experimental laboratory studies. Furthermore, slight variation of MDA levels at the time of vaccination may also contribute to an 'extended' period of severe lesions after vaccination on the basis of the whole flock.

The observed high bursal lesion scores in Trial 1, which for many flocks reached its peak between 14 and 21 DPV, may easily be confused with field virus-induced lesions (Ezeokoli *et al.*, 1990; van den Berg *et al.*, 2000). But sequencing of the VP2 region of IBDV, which was detected by RT-PCR in the bursal samples, confirmed solely the detection of the vaccine strain in all 16 flocks. No field virus was detected during these trials. This may be due to low field pressure of virulent IBDV

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strains or to good protection and displacement of the field virus. Experimental studies with this vaccine strain in broilers indicated that it protected vaccinated broilers against challenge with very virulent IBDV (Jung, 2006). Boilers, which had been vaccinated with this intermediate strain were protected against morbidity, mortality and the development of severe bursa lesions (score >3) in comparison to non-vaccinated broilers, in which 100 % of the birds challenged with very virulent IBDV had developed lesion scores of > 3 (Jung, 2006).

Despite the high bursa lesion scores and the possibility of at least a temporary immunosuppression due to the IBDV vaccination (Kim *et al*., 1999; Kim & Sharma, 2000; Sharma *et al*., 2000; Rautenschlein *et al*., 2007), the performance of the broiler flocks was not affected. All flocks had production indices which coincided with field experiences. The observation of pathological lesions in only some of the birds examined *post mortem* was related to bacterial or parasitic infections but not to IBD, which indicates that some individual birds became sick without affecting the entire flock.

IBD vaccine virus was detected in Trial 1 in 93 % and 58 % of the broiler flocks at 14 and 21 DPV respectively. One flock was positive by RT-PCR only at 7 DPV. Since the sample size was small and the bursae were pooled from each flock for IBDV detection, we may not have detected all positive flocks at the indicated times due to the limitations of the detection method (Smiley *et al*., 1999). The histological bursa lesions of chickens which had been IBDV-negative by RT-PCR, indicate that virus replication had taken place in all investigated flocks.

Overall, our study demonstrated the importance of the estimation of the optimal vaccination time for IBDV vaccination under field conditions. The Deventer formula was shown to be a useful tool for the estimation of the optimal vaccination

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time. We demonstrated that under field conditions, vaccine induced bursa lesions may be detected later and may be more severe, as would be expected from laboratory investigations with SPF chickens. Previous experimental studies with MDA-positive commercial broilers using other intermediate IBDV strains support this observation (Rautenschlein *et al*., 2005). It is possible that re-circulating vaccine virus may interfere with the clearance of the vaccine virus from the flock. This may especially be true in flocks with heterogeneous MDA levels that allow infections of the vaccine virus to take place in a staggered manner. These vaccine-induced bursa lesions may easily be confused with field virus challenge. Sequencing of the detected virus, however allows differentiation between field and vaccine strains.

Finally, it needs to be remembered that only one intermediate IBDV vaccine strain was tested in this field studies. Because this vaccine strain may differ in its virulence and other characteristics from other intermediate IBDV vaccine strains, our observations cannot necessarily be extrapolated to other intermediate IBD vaccines.

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Figure legends

Figure 1: *IBDV antibody development after vaccination at the optimal time point (Trial 1). Serum samples were collected at 7 (a), 14 (b), and 21 (c) days post vaccination and tested for IBDV antibodies by ELISA. n = 30/flock. The ELISA data are presented in the box & whisker diagram: the short line within the grey rectangular box represents the median of the variables; the upper and lower boarder of the rectangular box represent the 25 and 75 quartiles, respectively; the whiskers indicate highest and lowest value; outliers are indicated by +.*

Figure 2: *IBDV antibody development after vaccination before (days -8, -3, -1), at (day 0), and after the optimal time point (days 1 to 6) (Trial 2). Serum samples were collected at the day of vaccination (a), 7 (b), 14 (c), and 21 (d) days post vaccination and tested for IBDV antibodies by ELISA; at 21days post vaccination, flocks vaccinated at day 2 and 6 after the optimal time had been slaughtered so no data are available for these flocks at this time. 30 serum samples/flock; the number of flocks per day varied: day -8, -1, +1, +5: n = 1; day -3, +2, +3, +6: n = 2; day + 4: n= 3; day 0: n= 5. The ELISA data are presented in the box & whisker diagram: the short line within the grey rectangular box represents the median of the variables; the upper and lower boarder of the rectangular box represent the 25 and 75 quartiles, respectively; the whiskers indicate highest and lowest value; outliers are indicated by +.*

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Figure 3: *Development of histological bursa lesions and detection of IBDV by RT-PCR after vaccination of broiler flocks with an intermediate IBDV strain (Trial 1). Flocks 3, 8, 9, and 16 were slaughtered before 21 days post vaccination and no data were available for this time. dpv = days post IBDV vaccination. n = 5 bursae per flock were investigated. + = detection of IBDV by RT-PCR from pooled bursa tissue (n = 5/pool); - = no detection of IBDV by RT-PCR. IBDV detection in flocks 5, 8, 9 was possible after propagation of IBDV in embryonated chicken eggs followed by RT-PCR.*

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Table 1. *Estimation of the optimal IBD vaccination time in Trial 1*

Broiler flock number	First serum collection at days post hatch[a]	Flock range of IBD MDA[b] levels at time of sampling (% birds ELISA antibody positive)[c]	Estimated optimal vaccination day
1	3	1149-11346 (100)	21
2	6	202-6327 (97)	21
3	6	42-5131 (93)	21
4	7	679-7129 (100)	21
5	7	206-2892 (80)	17
6	7	303-5606 (93)	21
7	7	949-4960 (100)	21
8	7	458-4001 (100)	21
9	7	455-6970 (100)	22
10	8	186-4905 (93)	21
11	7	1112-6595 (100)	22
12	6	49-3233 (87)	17
13	6	1107-8688 (100)	22
14	7	129-5324 (73)	17
15	12[d]	108-1585 (83)	20
16	8	462-7133 (100)	23

[a] serum samples were collected at the indicated times between 3 and 12 days post hatch and tested for IBDV antibodies by ELISA.
[b] MDA = maternally derived antibodies.
[c] 30 serum samples collected/flock.
[d] no samples were available from earlier days post hatch.

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Table 2. *Antibody decline between first and second serum sampling of flocks before the actual day of vaccination (Trial 1)*

Broiler flock number	Parent flock vaccinated live/inactivated (age in weeks)	Mean MDA[a] ELISA antibody level at age of first sampling [b]		Mean MDA ELISA antibody level at age of second sampling	
		Day bled	ELISA titre ± SD	Day bled	ELISA titre ± SD
9	+/+ [c] (28)	7	4023 ± 1721	17	270 ± 167
2	+/+ (31)	6	3194 ±1536	18	959 ± 598
3	+/+ (31)	6	2782 ± 1421	18	926 ± 646
11	+/+ (35)	7	3690 ± 1173	17	413 ± 219
10	+/+ (37)	8	2218 ± 1356	16	266 ± 223
1	+/+ (38)	3	6143 ± 1993	17	592 ± 434
4	+/+ (52)	7	2791 ± 1332	18	355 ± 241
6	+/-[d] (31)	7	2128 ± 1423	17	228 ± 170
12	+/- (38)	6	1211 ± 837	ND [e]	ND
13	+/- (39)	6	4441 ± 1642	17	199 ± 131
8	+/- (41)	7	2072 ± 901	18	448 ± 466
14	+/- (44)	7	1122 ± 1260	ND	ND
7	+/- (52)	7	2711 ± 804	18	256 ± 181
5	+/- (53)	7	1048 ± 712	15	146 ± 142
15	+/- (54)	12	794 ± 385	18	172 ± 110
16	+/- (55)	8	3262 ± 1394	18	433 ± 366

Serum samples were collected at the indicated times and tested for IBDV antibodies by ELISA. The second serum samples were collected 2 to 5 days before the estimated optimal vaccination day.

[a] MDA - maternally derived antibodies.
[b]30 samples/flock.
[c]+/+ flock was vaccinated by drinking water with a live IBDV vaccine and boosted with an inactivated IBDV vaccine by injection.
[d] +/- flock was vaccinated by drinking water with a live IBDV vaccine.
[e] ND -no second serum samples were available from these flocks.

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Table 3. *Retrospective determination of the optimal IBD vaccination time and grouping of the flocks based on actual vaccination before, at, or after the optimal time (Trial 2)*

Assigned groups and broiler flock numbers[a]	Estimated optimal vaccination time in days post hatch	Actual day of vaccination (% birds with MDA[b] titre above the ELISA cut-off at day of vaccination)	Difference in days between optimal and actual vaccination time
Group 1 vaccinated before the optimal day			
9	22	14 (92)	-8
3	24	21 (54)	-3
4	24	21 (54)	-3
12	22	21 (50)	-1
Group 2 vaccinated at the optimal day			
1	18	18 (54)	0
5	21	21 (4)	0
6	19	19 (13)	0
8	20	20 (21)	0
10	20	20 (0)	0
Group 3 vaccinated after the optimal day			
15	21	22 (8)	+1
18	21	23 (17)	+2
19	21	23 (13)	+2
2	18	21 (8)	+3
11	21	24 (17)	+3
7	14	18 (0)	+4
13	20	24 (0)	+4
16	18	22 (0)	+4
14	17	22 (0)	+5
17	17	23 (13)	+6
20	17	23 (0)	+6

[a] 30 serum samples per flock.
[b] MDA = maternally derived antibodies.

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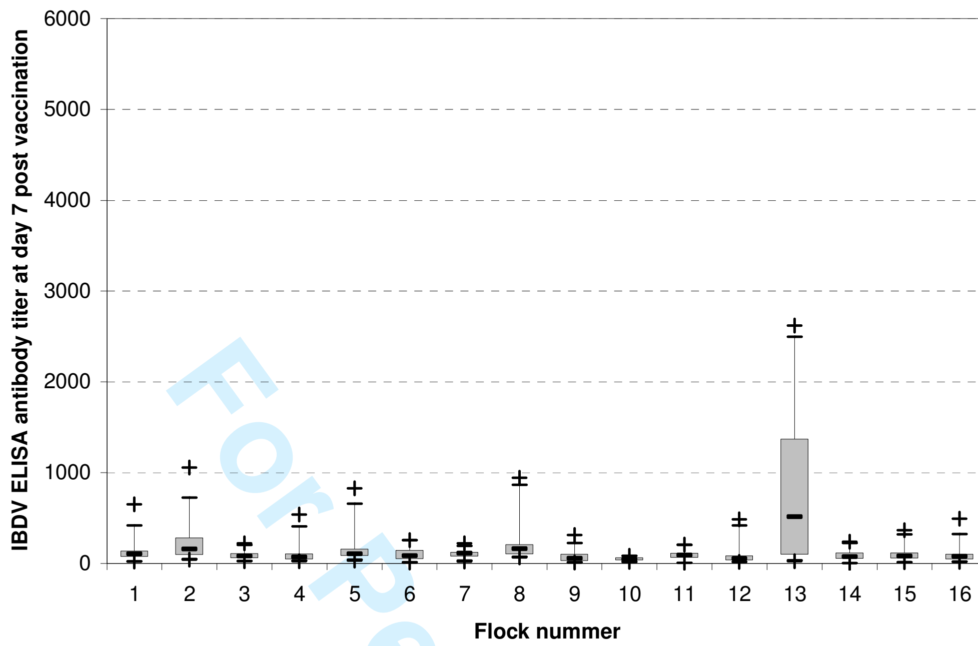

Figure 1a

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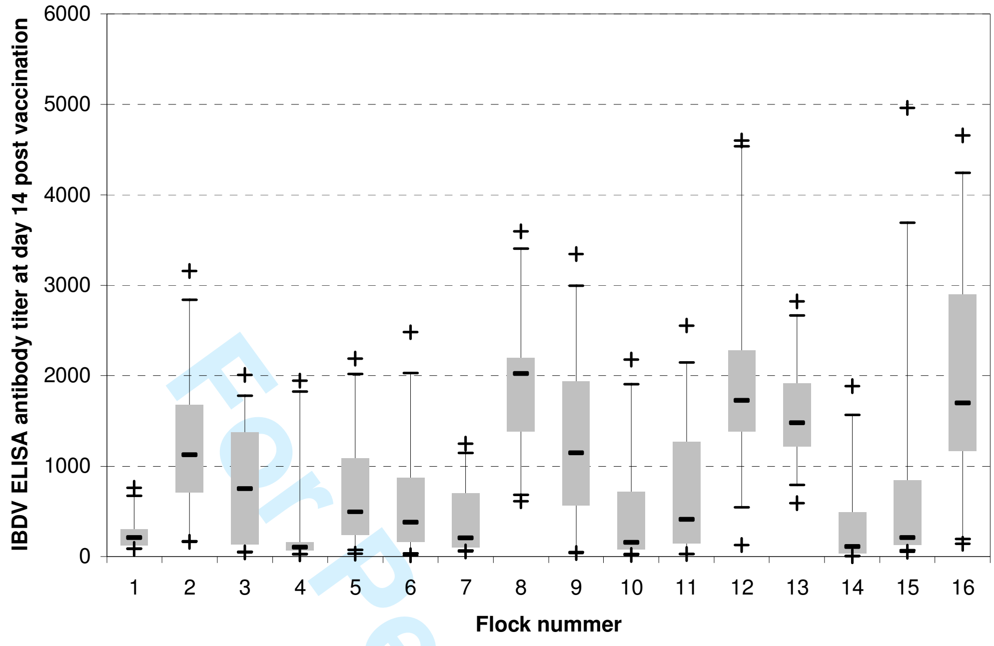

Figure 1b

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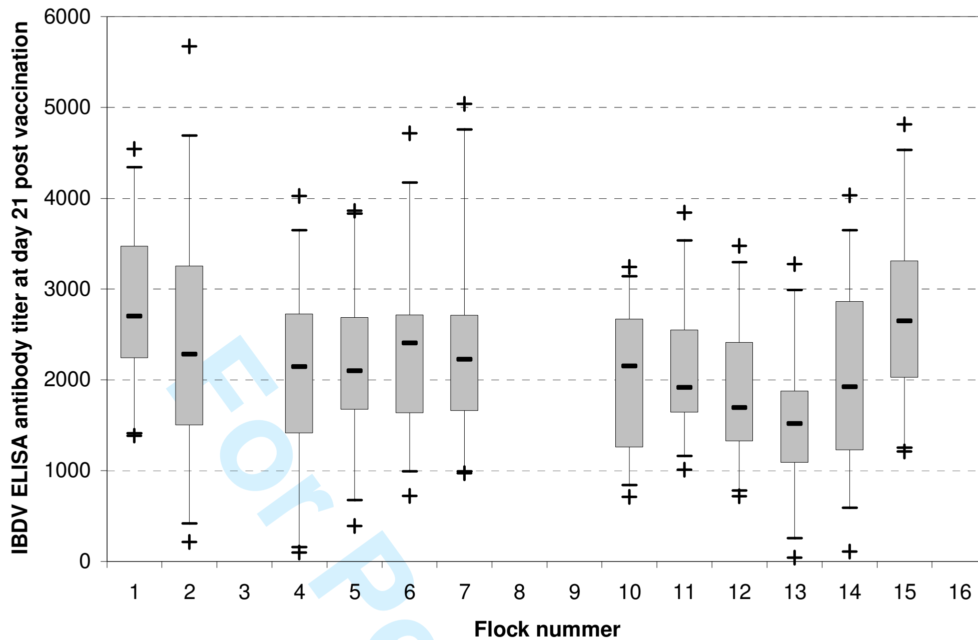

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Figure 1c:

Figure 1: IBDV-antibody development after vaccination at the optimal time point (Trial 1). Serum samples were collected at 7 (a), 14 (b), and 21 (c) days post vaccination and tested for IBDV antibodies by ELISA. n = 30/flock. The ELISA data are presented in the box & whisker diagram: the short line within the grey rectangular box represents the median of the variables; the upper and lower boarder of the rectangular box represent the 25 and 75 quartiles, respectively; the whiskers indicate highest and lowest value; outliers are indicated by a +.

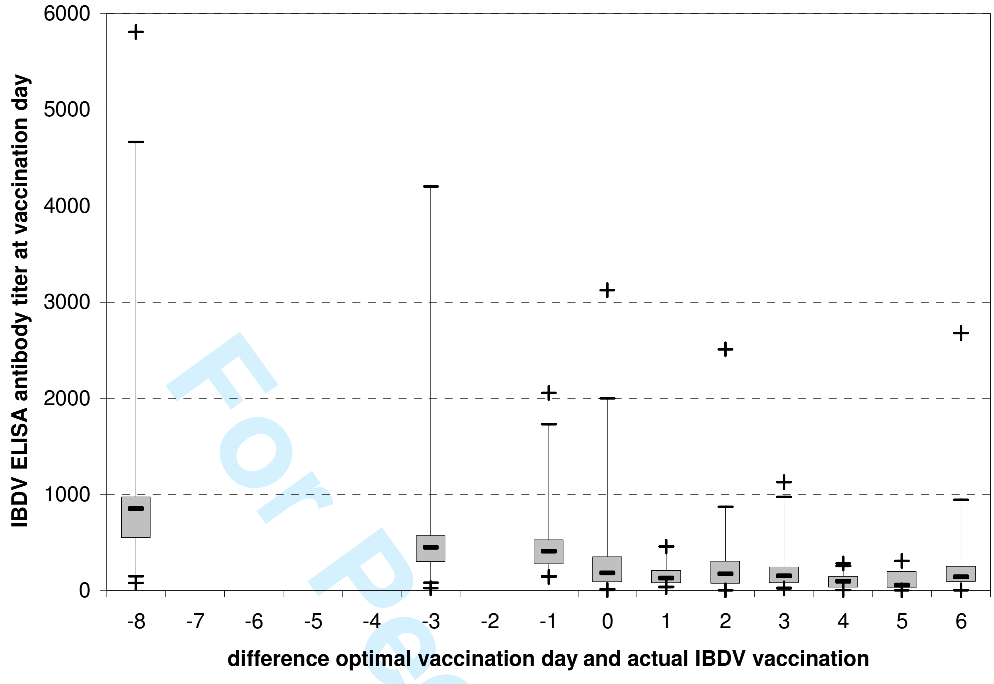

Figure 2a

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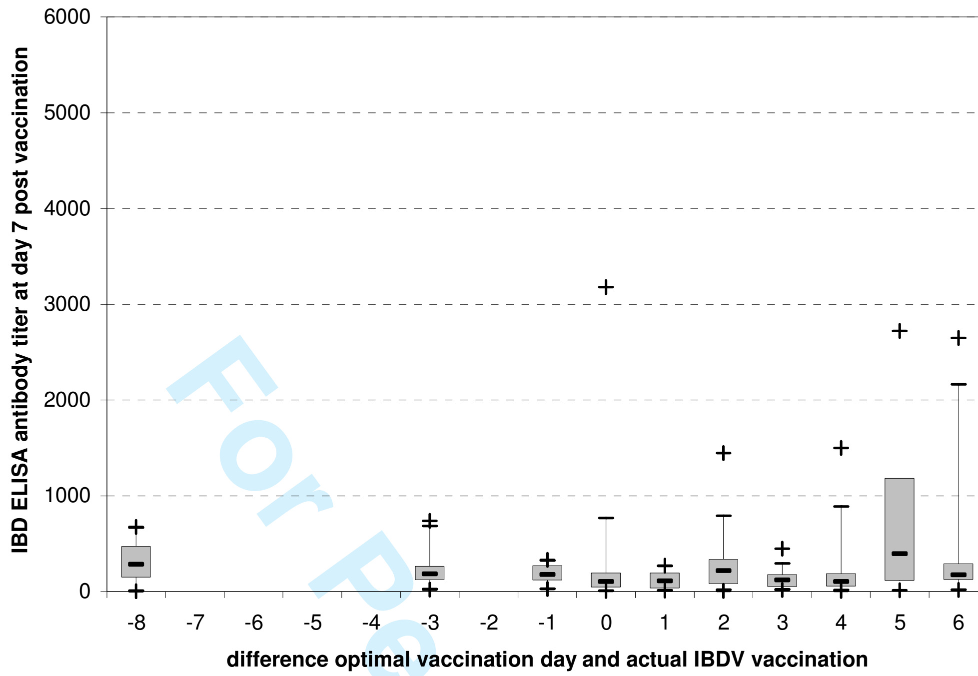

Figure 2b

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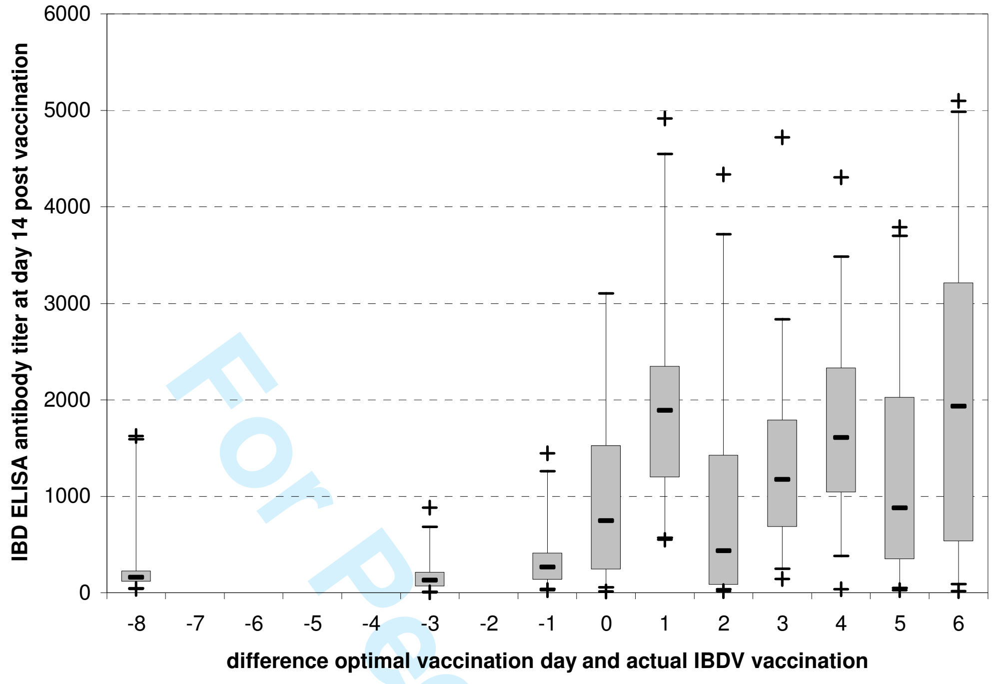

Figure 2c

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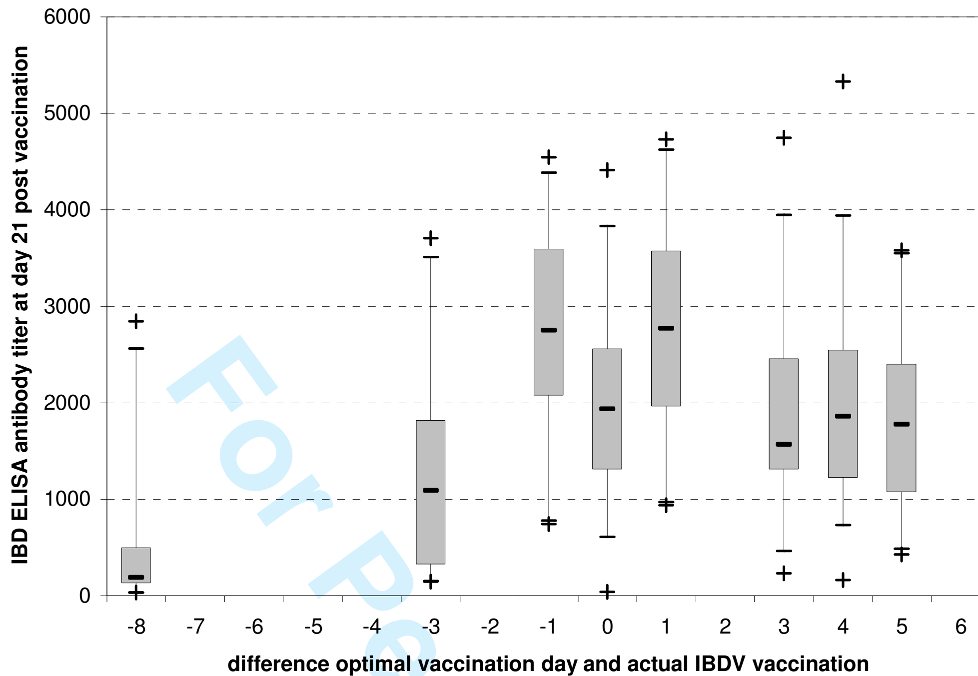

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Figure 2d

Figure 2: IBDV-antibody development after vaccination before (days -8, -3, -1), at (day 0), and after the optimal time point (days 1 to 6) (Trial 2). Serum samples were collected at the day of vaccination (a), 7 (b), 14 (c), and 21 (d) days post vaccination and tested for IBDV antibodies by ELISA; at 21 days post vaccination, flocks vaccinated at day 2 and 6 after the optimal time had been slaughtered, no data are available for these flocks at this day point. 30 serum samples/flock; the number of flocks per day point varied: day -8, -1, +1, +5: n = 1; day -3, +2, +3, +6: n = 2; day + 4: n= 3; day 0: n= 5. The ELISA data are presented in the box & whisker diagram: the short line within the grey rectangular box represents the median of the variables; the upper and lower boarder of the rectangular box represent the 25 and 75 quartiles, respectively; the whiskers indicate highest and lowest value; outliers are indicated by a +.

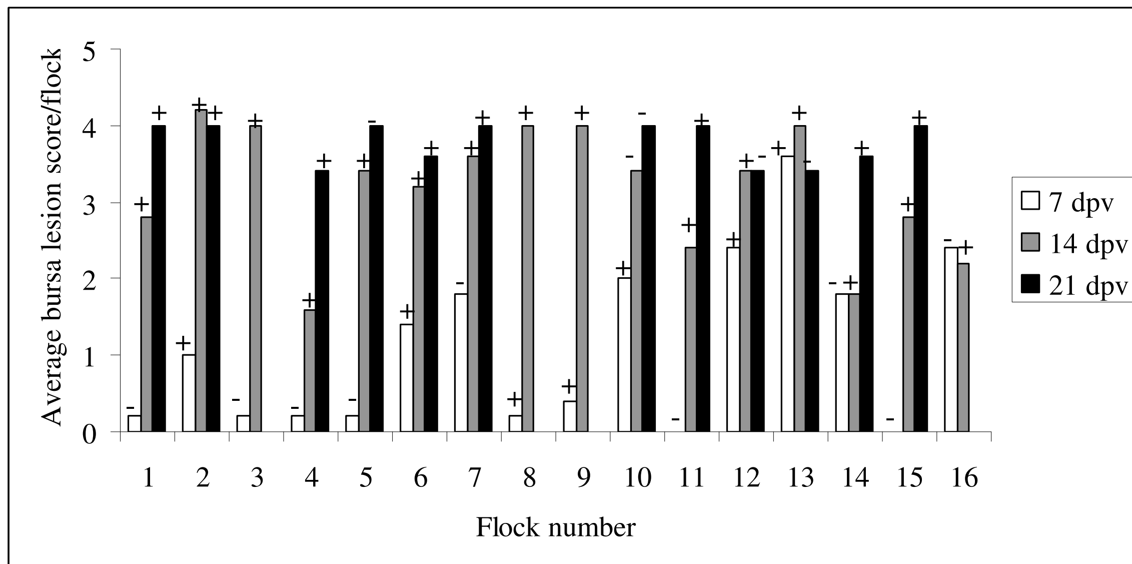

Figure 3. *Development of histological bursa lesions and detection of IBDV by RT-PCR after vaccination of broiler flocks with an intermediate IBDV strain (Trial 1). Flocks 3, 8, 9, and 16 were slaughtered before 21 days post vaccination, and no data were available for this time point. dpv = days post IBDV vaccination. n = 5 bursae per flock were investigated. + = detection of IBDV by RT-PCR from pooled bursa tissue (n = 5/pool); - = no detection of IBDV by RT-PCR. IBDV detection in flocks 5, 8, 9 was possible after propagation of IBDV in embryonated chicken eggs and consecutive RT-PCR.*

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