Progress report: Transmission study testing HVT-H5 vaccine against highly pathogenic avian influenza (HPAI) H5N1 virus (clade 2.3.4.4b)

Second report, 24-weeks post vaccination VAXXITEK HVT+IBD+H5 and VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccine

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Wageningen Bioveterinary Research Report

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1 Abstract

This report describes the results obtained in the second transmission study in a series of four. These transmission experiments are part of a longitudinal study which aims to determine whether vaccination of laying hen flocks under field conditions can provide long-term protection against HPAI H5N1 virus (clade 2.3.4.4b), especially against virus transmission (within-flock reproduction number R<1) measured under experimental conditions. In the first transmission study at 8 weeks post-vaccination with HVT-vector vaccine VAXXITEK HVT+IBD+H5 [2] the pullets were protected against clinical signs and transmission following challenge. For this second study a (random) selection of chickens, around time of peak egg production, were transported to high containment experimental facilities for the challenge with HPAI H5N1 virus (clade 2.3.4.4b). Up to that moment, the chickens were vaccinated and housed under field conditions.
The layers were challenged with HPAI H5N1 virus (clade 2.3.4.4b) 24 weeks post-vaccination with the vector vaccine VAXXITEK HVT+IBD+H5 (and 12 weeks post-booster vaccination with Volvac® B.E.S.T. AI+ND). Transmission from inoculated (challenged by inoculation) to contact chickens was determined, as well as survival of the chickens, virus shedding and humoral and cellular immune responses for 21 days post-inoculation (dpi).

The key findings in this study were:

- In the non-AI vaccinated control groups all chickens (20/20 inoculated and contact) became infected after challenge, and the estimated reproduction number was significantly >1, namely R (95% Confidence Interval) = 15.4 (6.0 – 33.0). In the VAXXITEK HVT+IBD+H5 vaccinated group, R was substantially reduced, namely R = 7.9 (3.2 – 16.3) or R = 2.8 (1.1 – 5.7) depending on the estimation method. Only in the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND group R-values <1 were estimated, namely R = 0.6 (0.2 – 2.2) or R = 0.3 (0.1 – 1.0) depending on the estimation method.
- Whereas 100% mortality by 5 dpi occurred in inoculated and contact-infected chickens in the non-vaccinated control groups, mortality was significantly reduced to 10% in the vaccinated groups. When chickens received a booster vaccination at 12 weeks of age, no mortality was observed, highlighting protection against clinical signs and death.
- Vaccinated chickens excreted reduced amount of virus when compared to chickens in the control groups.
- The VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND group, excreted reduced amount of virus when compared to chickens in the control groups particularly through the cloaca compared to the control groups.
- Serological responses post-inoculation indicated that the majority of vaccinated chickens developed antibodies in response to challenge (based on results of NP-ELISA and HI titers at 21 dpi).
- The total number of T cells demonstrated a tendency to increase after challenge; however, no significant differences were observed in number of T cells when compared to 0 dpi. At 7 dpi, the number of CD25+ T cells (activated T cells) was significantly higher in chickens vaccinated with VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND when compared to the VAXXITEK HVT+IBD+H5 vaccinated chickens. Additionally, immune cells from VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens produced IFNγ in response to the challenge virus, suggesting a strong anti-viral immune response.

The response of the vaccinated chickens, at 24 weeks post-vaccination with VAXXITEK HVT+IBD+H5 and 12 weeks after booster vaccination with VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND, demonstrated enhanced survival, and stimulated humoral and cellular immune responses against challenge with HPAI H5N1 virus (clade 2.3.4.4b) compared to non-AI vaccinated controls. Only the group that received a booster vaccination at 12 weeks of age, had a R-value < 1. However, the large confidence intervals around the R estimates, stress the need for careful conclusions based on the data of this study alone. In vaccinated flocks, transmission (R) is influenced by the proportion of chickens expressing low and high levels of immunity. In experimental setting, a small proportion of chickens with low neutralizing antibody titers can significantly

increase transmission (R>1) due to higher infectivity. Since the distribution of antibody titers in the field and how these change over time are not available yet, more detailed insights into vaccine effectiveness require integration of all field data and transmission studies. Therefore, it is not yet possible to draw conclusions on protection against sustained transmission without the additional data from later stages of the field- and third and fourth transmission studies.

At the end of the longitudinal study, the additional transmission experiment data and HI titer distribution in the field flock over time will enable a more accurate quantification of transmission and predictions of the duration of protection over time.

In the final report, the combined data will be presented to support conclusions on effectiveness of a large-scale single dose application of this vaccine to stop sustained transmission, and hence on its potential as preventive measure to control HPAI for the whole of the production cycle.

2 Introduction

In the most recent outbreak with highly pathogenic avian influenza (HPAI) virus in the Netherlands, infections were reported year-round and actions are needed to protect poultry from this virulent virus and mitigate its zoonotic potential. Vaccination is one of the measures for protecting chickens against avian influenza (AI). In this ongoing Public-Private-Partnership, various institutes are collaborating to investigate the potential of using HVT (herpesvirus of turkey)-based vaccine vectors expressing the hemagglutinin protein (HA, H5-subtype) of HPAI under field conditions in a longitudinal study. Various parameters will be assessed to determine the effectiveness of the vaccine, combining field measurements with four transmission studies. The most important objective is to determine if vaccination sufficiently reduces virus transmission (virus spread) between chickens in a flock, so that the within flock reproduction number R is lower than 1 ($R<1$). A vaccine that only reduces clinical signs without adequately reducing or preventing virus transmission is not considered an effective vaccine in the context of this study.

In the first transmission study of the longitudinal study, the effectiveness of VAXXITEK HVT+IBD+H5 from Boehringer Ingelheim Animal Health (BIAH) [2] was tested in 8-week-old commercial laying hen pullets. These chickens were vaccinated against AI at hatch and housed in a commercial rearing farm (commercial farm A), following a standard vaccination scheme against several pathogens [2]. The control group did not receive any AI vaccine but did receive all standard vaccinations. The results demonstrated that 8 weeks post-vaccination, the challenge did not result in virus replication in the inoculated chickens, whereas in the control groups, inoculation led to virus replication and transmission from inoculated to contact chickens.

In this second transmission study the effectiveness of vaccination was assessed 24 weeks post-vaccination in laying hens aged 24 weeks. At 12 weeks of age, one of the treatment groups, in which the chickens were vaccinated with VAXXITEK HVT+IBD+H5 at day of hatch, received a booster vaccination with Volvac® B.E.S.T. AI+ND, so for this group challenge was performed 12 weeks post-booster vaccination. At this age, laying hens are stepping up to the peak of egg production. The immune system of laying hens at this age can show changes induced by egg production [3], which makes it relevant to study the response to challenge with AI virus at this age. The results of the transmission study, especially the reduction of the transmission parameter R, are crucial for achieving a sustainable approach to controlling avian influenza in poultry. Effective vaccination could complement biosecurity practices, improving animal welfare and reducing the need for preventive confinement and culling during outbreaks.

3 Transmission study

3.1 Material and Methods

3.1.1 Permits and Funding

The animal study was conducted in accordance with the guidelines of 2010/63/EU [4]. The animal study was approved by the Central Committee for Animal Experiments (CCD) (permit application AVD40100202215972; experiment 2021.D-0036.005). The HVT-based Influenza vaccines are Genetically Modified Organisms (GMOs). Therefore, permits were obtained from the 'Bureau GGO' for conducting the animal study and for the analysis of samples in the laboratory (IG 22-080, IG 22-081, IG 22-097).

This study was funded by the Public-Private-Partnership (PPP) entitled "Vaccinatie van pluimvee tegen HPAI H5 vogelgriepvirus, aanvraagnummer: LWV 22103". The PPP will make use of knowledge and materials from two other studies that are separately funded, named "Veldproef AI-vaccinatie. Projectnumber 5082181" and "Eerste proef test effectiviteit van vaccins tegen vogelgriep. Number: BO-43-111-083".

The first two transmission studies were conducted within the BO-43-111-083 project, where the first was executed at 8 weeks [2] and this second study at approximately 24 weeks post-vaccination. The chickens that arrived at Wageningen Bioveterinary Research (WBVR), are a subset of the chickens that are housed in the field and part of the study of Royal GD "Veldproef AI-vaccinatie. Projectnumber 5082181".

3.1.2 Housing

All chickens were reared at commercial farm A, where the chickens with different vaccination strategies were kept separately from each other. Detailed information on housing in the field can be found in "Progress report of PPP project Vaccination of poultry with HVT-based H5 vaccine" [6].

In the field study, at 19 weeks of age, approximately half of the chickens per test group were relocated from commercial rearing farm A to a layer production facility (Commercial farm B) and housed, under field conditions, in one house in separated groups. The chickens that remained at commercial farm A stayed in the original house in the original units, which were equipped for the collection of the eggs. At farm B, an additional layer production flock was also present (not in the same house). The chickens were fed with commercial feed from different feed mills. The feed matched the standard requirements of chickens of their age. The water supply was provided *ad libitum*. Overall health and mortality were recorded, on a daily basis, and at both production locations standard biosecurity rules applied.

At the age of 23 weeks, a subgroup of the chickens from both commercial farms were transported to the animal facilities of WBVR in Lelystad. Upon arrival at WBVR, all chickens were randomly divided for each study and received a wing tag for identification. The chickens from Farm A and Farm B were housed separately throughout the studies ,except for the ELIspot study, where 12 chickens from Farm A and 13 chickens from Farm B were housed in 1 pen. For the first week, chickens were housed under BSL2 conditions and from 24 weeks of age onwards, the chickens were challenged and housed under BSL3 conditions.

Housing during the study was identical to our previous study [2], except for the placement of laying boxes in the pen throughout this entire study.

3.1.3 Chickens and Vaccinations

Detailed information about the chickens and vaccinations that the chickens received can be found in "Progress report of PPP project Vaccination of poultry with HVT-based H5 vaccine" [6]. At 12 weeks of age, one test group received a booster vaccination with Volvac® B.E.S.T. AI+ND through a subcutaneous injection in the neck by an experienced vaccination team following to instructions of the manufacturer.

3.1.4 Inoculum

The same virus stock was used to infect the chickens at 24 weeks of age as in our previous studies [2, 7]. It concerns a HPAI H5N1 clade 2.3.4.4b virus detected and isolated in 2021 from a laying hen farm in the Netherlands. The complete genome sequence of the A/chicken/Netherlands/21038165-006010/2021_H5N1_PB2_2021-11-07_LUTJEGAST virus used for the inoculum was determined and can be found in the GISAID Database under the number EPI_ISL_6101848. The virus was obtained by cultivating the virus in two passages in 9-11 day-old specific pathogen-free (SPF) embryonated eggs.
The virus was titrated in triplicate to determine the average egg infectious dose (EID_{50}). For inoculation, the virus was diluted in sterile Tryptose Phosphate Broth (TBP) 95% to a dilution of 10^7 EID_{50}/ml inoculum. The inoculation of all designated chickens was performed by qualified personnel. Afterwards the remaining inoculum was titrated in the lab, which confirmed the intended titer of the inoculum.
The antigenic distance of the VAXXITEK HVT+IBD+H5 vaccine to the challenge virus was estimated using the HI response against 36 chicken sera (from a cross table including two other viruses) to be 7.72. (For additional information regarding the antigenic distance see chapter 7).

3.1.5 Study Design Transmission Study

The study design of the transmission study, is schematically presented in Figure 1. At 23 weeks of age (-7 dpi), 22 chickens which were vaccinated with VAXXITEK HVT+IBD+H5 and 22 chickens vaccinated with VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND were delivered to WBVR together with 22 chickens of the non AI vaccinated (control) group. Upon arrival at WBVR, the chickens were randomly divided and received a wing tag for identification. Randomization did not mix chickens that originated from the different commercial farms, so groups A housed chickens that came from commercial farm A and groups B housed chickens that came from commercial farm B. Each group (A or B), consisted of 5 inoculated, 5 contact and 1 surplus chickens. The chickens that were vaccinated with VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND were placed in a separate house, due to legal requirements for minimal floor area per chicken [4].

Blood was collected on -7 dpi to determine the antibody titer (humoral immune response) using a Hemagglutination Inhibition (HI) assay and additionally the blood serum was tested using NP-ELISA. In addition, choanal and cloacal swabs were taken to demonstrate the absence of avian influenza virus. This was followed by one week of acclimatization.

On 0 dpi, the day of inoculation with HPAI H5N1 clade 2.3.4.4b, the surplus chickens of the vaccinated groups A & B and control groups were euthanized under sedation.

The inoculation was performed by applying 0.1 ml of the virus intra-choanally, so that each chicken received 10^6 EID_{50} HPAI H5N1 virus. Contact chickens were temporarily separated from the inoculated chickens so that the contact chickens could not become infected with the virus through exposure to the inoculum. After 8 hours, the contact chickens were placed in their original pens together with the inoculated chickens and stayed together for the remainder of the study. Swabs from the choana and cloaca of all chickens were collected daily in the first week to determine virus shedding (Figure 1). In the second week, swabs were taken every other day (9, 11 and 13 dpi), and in the third week, swabs were taken at two timepoints (17 and 21 dpi). At each sampling, contact chickens were swabbed first followed by inoculated chickens to avoid infection from handling the chickens. Blood from the wing vein was collected to examine the cellular immune response of the inoculated chickens at 0, 1, 3, 7, 10 and 14 dpi. (For additional information regarding the cellular immune response see chapter 7). At the end of the transmission study, all remaining chickens were euthanized under sedation and blood was collected for antibody detection (NP-ELISA and HI).

Throughout the study, daily inspection and care of the chickens were conducted by qualified personnel. In case mild to severe clinical signs resulting from infection were observed during an inspection, an additional inspection was carried out on the same day. Chickens were euthanized when they reached the humane endpoint. All clinical signs were documented. (For additional information regarding humane endpoints see chapter 7).

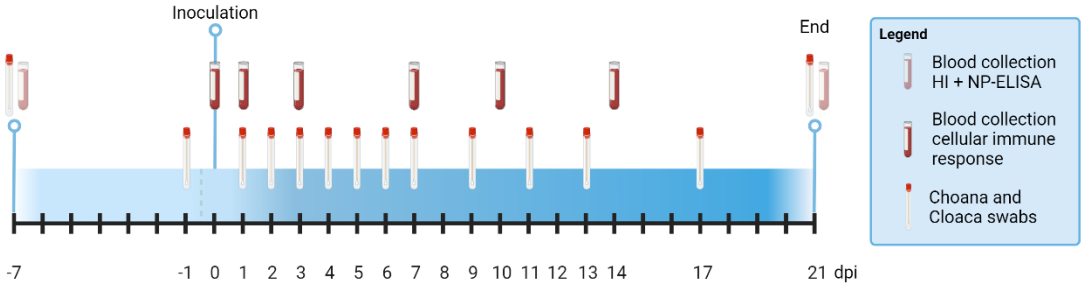

Figure 1: *Schematic overview of sample collection time points in this second transmission study. HI: Hemagglutination Inhibition assay. Inoculation at 0 dpi was performed with 10^6 EID_{50}/ml HPAI H5N1 virus per chicken.*

3.1.6 NP-ELISA

The NP-ELISA is an in-house enzyme-linked immunosorbent assay (ELISA) from WBVR that detects antibodies against avian influenza viruses in blood serum and has been previously described[8]. The NP-ELISA detects antibodies targeting the Nucleocapsid Protein (NP) of avian influenza virus. Therefore, when antibodies are detected with the NP-ELISA, it is a response to the inoculum, as the vaccines only encode the viral Hemagglutinin (HA) gene. The NP-ELISA was used at two different timepoints in this study: at -7 dpi (upon arrival at WBVR) and at 21 dpi (end of the study). A value above 50% blocking in the NP-ELISA is considered as a positive result.

3.1.7 Hemagglutination Inhibition (HI) Assay

Antibody responses after vaccination can be quantified in the Hemagglutination Inhibition (HI) assay. For additional information regarding terminology of Hemagglutination Inhibition (HI) Assay see chapter 7. The HI assay utilizes the hemagglutinating properties of the AI-virus, which causes red blood cells to clump. If the antibodies in the serum bind to the virus in the test, clumping of red blood cells is prevented. By testing the serum in a dilution series, the amount of HA-specific antibodies (titer) in the blood can be determined. The method is described in the 'Terrestrial Manual' of the World Organization for Animal Health (WOAH)'. All sera collected before inoculation (-7 dpi) and at the end of the study (21 dpi) were tested in the HI. The HI is performed using different antigens (viruses).

First, sera from the vaccination groups were tested against the HPAI H5N1 inoculum (heterologous antigen). Additionally, all sera were tested against an antigen closely related to the H5 of the primary vaccine (homologous antigen): A/Ch/Indonesia/7/03 EPI_ISL_11512.
The computationally optimized broadly reactive antigen (COBRA) H5 sequence of the VAXXITEK HVT+IBD+H5 vaccine is 92.91% identical to the HA gene of the inoculum and 98.23% identical to the HA gene of Indonesia (not taking into account the multi-basic cleavage site that is deleted in COBRA). All tests were performed as duplicates, and the results of the two tests were averaged for analysis.

3.1.8 M-PCR (M-gene Polymerase Chain Reaction)

After sampling, the swabs were immediately placed in 2 ml Tryptose Phosphate Buffer (TBP) and frozen at -80°C until processing. After thawing of the swabs, RNA was isolated using the MagNA Pure 96, and the RNA was tested in the PCR that detects the M-gene of influenza (M-PCR), as previously described [9]. In each PCR run, a standard curve made with virus was included to quantify the amount of virus and thus determine the

titer of the virus detected in a tested sample. Since the detection limit of the PCR is around a titer of Log $10^{1.7}$ $eqEID_{50}$/ml, so values <Log $10^{1.7}$ $eqEID_{50}$/ml were considered negative.

3.1.9 Whole Blood Staining to Determine Absolute Lymphocyte Counts

To determine absolute counts of several lymphocyte subsets after inoculation, blood samples of all inoculated chickens were collected in a 3K-EDTA tube at 0, 1[1], 3, 7, 10 and 14 dpi. Whole blood was fixed in TransFix® reagent and stained using BD truecount tubes as previously described [10]. The antibody mix (Table 1) consisted of the pan leukocyte marker mouse-anti-chicken-CD45-PE, the T cell recognizing antibodies mouse-anti-chicken-CD3-PB, mouse-anti-chicken-CD8α-AF700, mouse-anti-chicken-TCR-1-FITC, mouse-anti-chicken-CD4-PECy7 and the in-house conjugated activation marker mouse-anti-chicken CD25-APC.

Table 1: *An overview of the monoclonal antibodies and their target that were used in this study. All were obtained from Southern Biotech.*

Target	Antibody	Clone	Isotype
Leukocytes	Mouse-anti-chicken-CD45-PE	LT-40	IgM
Total T cell	Mouse-anti-chicken-CD3-PB	CT-3	IgG1
T helper cell	Mouse-anti-chicken CD4-PECy7	CT-4	IgG1
Cytotoxic T cell	Mouse-anti-chicken CD8α-AF700	CT-8	IgG1
Gamma delta T cell	Mouse-anti-chicken γδ-FITC	TCR-1	IgG1
Activated T cell	Mouse-anti-chicken-APC	AV142	IgG1
Alpha-beta 1 T cell	Mouse Anti-Chicken TCRαβ/Vβ1	TCR-2	IgG1
Alpha-beta 2 T cell	Mouse Anti-Chicken TCRαβ/Vβ2-FITC	TCR-3	IgG1

In one chicken in the VAXXITEK HVT+IBD+H5 group and 2 chickens in the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND group, T cells were not recognized by the anti-CD3 antibody. Blood of these chickens was stained using a combination of the T-cell receptor recognizing antibodies mouse-anti-chicken-αβ1-FITC, mouse-anti-chicken-αβ2-FITC and mouse-anti-chicken-γδ-FITC to identify the T cells. This strategy does not allow analysis of γδ T cells, therefore γδ T cells were determined in 9 out of 10 and 8 out 10 chickens respectively.

After staining, samples were fixed with 4% paraformaldehyde and resuspended in FACS-buffer before measuring using a FACS DIVA Flowcytometer (BD Biosciences) and 10,000 beads were recorded per sample. Analysis was performed using the software program FlowJo 10.10.0 (Tree star Inc, Ashland, OR, USA) and absolute cell counts were calculated. The number of events in the gates for CD4, CD8 and γδ T cells was too low to continue with further analysis of the CD25 expression at 3 and 7 dpi, due to technical problems with the flowcytometer.

3.1.10 Statistical analysis

3.1.10.1 Assessment of transmission

The following transmission parameters were quantified:
1) the transmission rate parameter (β), which is the average number of contact infections caused by a typical (average) infectious chicken per day;
2) the infectious period (T) which is the average period (in days) an infected chicken is counted as infectious for the estimation of the transmission rate parameter;
3) the reproduction number (R), which is the average number of individuals infected by a typical infectious chicken.

For the estimation of Beta (β), daily data on infection and transmission were collected in the form of the number of chickens Infectious (I), Susceptible (S), and new Cases (C) within a Time interval (Δt) of one day.

[1] At day 1, the overall number of T cells was too low to perform any additional analysis of subsets and activation markers.

These data were analyzed using a generalized linear model (GLM) with a binomial error distribution and a complementary log-log link as described by [11]. Based on the previous transmission studies [2, 7] and the observations on the inoculated chickens, we considered a one day latent period (time from becoming infected to becoming infectious).

The length of the infectious period T was quantified by performing a parametric survival analysis where different distributions were assessed. The distribution that best fitted this data (judged by the model with lowest AIC) was a Weibull distribution.

For the estimation of the infectious period of the vaccinated chickens we assessed two assumptions:

- The first assumption considers any PCR positive results (≥1.7 eqEID50) as indication of infectiousness. Hence, the infectious period PCR is the number of days from the first to the last obtained positive PCR result (Infectious period PCR).
- The second assumption is based on predicting the concentration of virus titer ($TCID_{50}$) as indicator of infectiousness (Infectious period Virus). This prediction was made because it is expected, particularly in vaccinated birds, that influenza virions loose infectivity faster than RNA integrity, resulting in positive results late post-infection when no live viruses might be present. This assumption is likely to result in estimated shorter infectious periods, as it was also observed for other diseases ([12]). This prediction was made following a model developed by [13].
    - This model predicts the concentration of infectious virus in samples from vaccinated-infected chickens, based on information of the day post-infection (dpi), the type of swab (choana or cloaca) and the estimated equivalent virus titers ($eqTCID_{50}/2ml$) following the equation:
- $TCID_{50}/2ml = -0.14 + 0.98 * eqTCID_{50}/2ml - 0.49 * swab - 0.07 * dpi$

    - Here we assumed that $eqEID_{50}$ are the same as $eqTCID_{50}$. Any predicted $TCID_{50}$ > 0.5 was considered as indication of presence of infectious virus.

The above assumptions for the estimation of Infectious period and corresponding R-values were assessed, as in this transmission study, different to the previous study, some vaccinated chickens were positive in M-PCR for long periods of time (several days, to longer than a week). Previous experience [13] indicates that in vaccinated infected chickens, M-PCR positive results longer than 6 days do no longer correlate with the presence of active virus. The results for both assumptions are shown for completeness. Interpretation needs to be done considering the assumptions made for the analysis and limitations of the study approach.
The reproduction number R was estimated using to methods: 1) the final size method [14] and 2) as the product of β and T. The 95% confidence intervals for R were derived by Monte Carlo (MC) simulations (1000 replications) assigning to β a lognormal distributions and T a Weibull distribution, using the parameters from the GLM and the survival regression model respectively.

3.1.10.2 Whole blood staining

Statistical differences were calculated using GraphPad prism version 10.1.2. Non-parametric statistical tests were used when the assumption of normally distributed data were not met. Differences in numbers of T cells between the groups were analysed using Kruskal-Wallis and Mann-Whitney U tests. Differences in T cell numbers over time were determined using a Friedman test followed by Dunn's multiple comparison testing. A p-value <0.05 was considered significant.

3.1.11 Definition of infection in the context of this study

For the analysis, a chicken is considered infected when the following criteria apply:

- Virus shedding: when virus was detected for 2 days or longer (≥2 days) with a minimum equivalent titer of ≥Log $10^{1.7}$ $eqEID_{50}$/ml by PCR in swabs collected from either choana or cloaca, and
- the chicken died or alternatively when the chicken survived the challenge, it had:
    - a positive NP-ELISA result (after 21 days) and/or
    - showed an increase of ≥3 $\log_2$ in the heterologous HI-titer.

This definition is consistent with the definition of an infected chicken used in our previous studies [2, 7]. (For additional information regarding this definition see chapter 7).

3.2 Results Transmission Study

3.2.1 Virus Transmission: Calculation Of The Reproduction Number (R) And Number Of Infected Chickens

The main objective of this transmission study was to investigate the vaccine effectiveness in reducing and/or preventing virus transmission, by determining whether R was <1 in the vaccinated groups.

In the control group, all inoculated chickens (10/10), from each of the subgroups A (n=5) and B (n=5) were infected based on the definition (Material and Methods 4.1.11) and shed virus from 1 dpi onward. In both control groups (A and B), virus was transmitted to all contact chickens, as all contact chickens became positive in M-PCR for ≥2 days. The estimated R-value (PCR and Virus) for the control group was 15.4 (95% CI 6–32.99) and the calculated R-final size was >1.52, the transmission rate parameter (β) was 5 (95% CI 2.39-9.46) and the infectious period was 3.2 (95% CI 1.1-5.3) days (Table 2). (For additional information regarding terminology of transmission parameters see chapter 7).

In VAXXITEK HVT+IBD+H5 group A, 5/5 inoculated and 5/5 contact chickens, and in group B 4/5 and all 5/5 contact chickens became infected. In VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND group A, 4/5 inoculated and 2/5 contact chickens, and in group B 4/5 and 0/5 contact chickens became infected (Table 2). The number of and reasons why chickens were not considered infected (based on our definition) are indicated in table 2. The transmission parameters (transmission rate parameter (β), Infectious period and R-value) were calculated and are presented in Table 2.

Table 2: *Transmission parameters and number of chickens infected. Infectious period and R-values were estimated using different assumptions about infectiousness (based on: PCR results and prediction of virus presence). CI= Confidence Interval. [a] positive for viral shedding (M-PCR on swabs), negative in serological response. [b] negative for viral shedding (M-PCR on swabs) and negative in serological response. [C] negative for viral shedding (M-PCR on swabs), positive in serological response. * Significant difference compared to control group.*

Group	**Inoculated infected**	**Inoculated not infected**	**Contact infected**	**Contact not infected**	**Beta (β) (95% CI)**	**Infectious period PCR in days (95% CI)**	**R-value PCR (95% CI)**	**Infectious period Virus in days (95% CI) [13]**	**R-value Virus (95% CI) [13]**	**R-value Final size (95% CI) [14]**
Control A	5	0	5	0	5.00 (2.39-9.46)	3.20 (1.10-5.30)	15.40 (6.00-32.99)			(> 1.52)
Control B	5	0	5	0						
VAXXITEK HVT+IBD+H5 A	5	0	5	0	0.80 * (0.40-1.43)	10.10 (3.60-17.00)	7.92 (3.17-16.29)	3.50 * (1.30-5.90)	2.76* (1.12-5.67)	(> 1.52)
VAXXITEK HVT+IBD+H5 B	4	1[a]	5	0						
VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND A	4	1[a]	2	3[2b, 1c]	0.08 * (0.01-0.24)	8.10 (2.80-13.60)	0.59 * (0.15-2.16)	3.80 (1.40-6.30)	0.28* (0.07-1.01)	0.39 (0.05-1.77)
VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND B	4	1[b]	0	5[4b, 1c]						

3.2.2 Survival And Protection Against Clinical Signs After Inoculation

To assess the effectiveness of the vaccine in reducing disease and clinical signs, the time of death or reaching the humane endpoint was recorded for each chicken. The mortality that occurred in the groups is depicted in survival curves (Figure 2). In control group A, 1/5 inoculated chickens died at 3 dpi, and the remaining 4/5 inoculated chickens died 4 dpi (1 humane endpoint, 3 found death). All contact chickens died at 5 dpi (2 humane endpoint, 3 found death). In control group B, 3/5 inoculated chickens died at 2 dpi, and the remaining 2/5 inoculated chickens died at 3 dpi. Two contact chickens died at 4 dpi (1/2 humane endpoint) and the remaining 3 contact chickens died at 5 dpi. Clinical signs in the control groups were moderate to severe depression at most 24 hour prior death or humane endpoint.
In the VAXXITEK HVT+IBD+H5 groups mortality was observed for 2/5 contact chickens in group B at 10 dpi (found death) and 12 (reached humane endpoint) respectively. In the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated groups no mortality was observed. The remaining chickens all survived the study without demonstration of clinical signs.

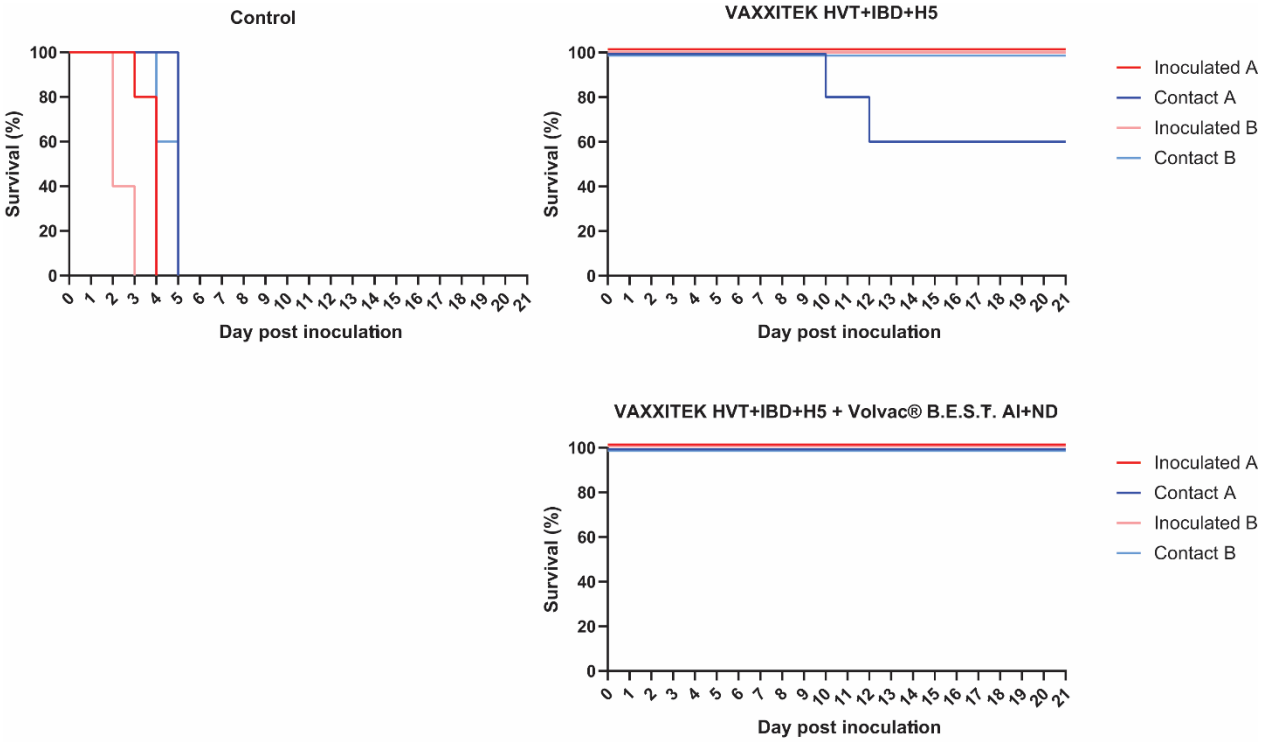

Figure 2: *Survival curve of control and vaccinated groups. Groups A and B are shown in one graph, where group A is clear line, group B is transparent line. Inoculated chickens are shown in red, contact chickens are shown in blue.*

3.2.3 Virus shedding

The viral shedding from each chicken in the transmission study was estimated by taking choanal and cloacal swabs to determine viral RNA quantities by the M-PCR. The obtained equivalent titers are depicted in Figure 3. A chicken is considered positive for virus shedding when the viral RNA was detected for 2 days or longer (≥2 days) with a minimum equivalent titer of ≥Log $10^{1.7}$ eqEID_{50}/ml by PCR in swabs collected from either choana or cloaca (above dashed line in Figure 3).

In control groups A and B, all (2x 10/10) chickens were scored positive for virus shedding (Figure 4).
Inoculated chickens were shedding through the choana and cloaca from 1 until 4 dpi (time of death). The contact chickens were shedding through the choana and cloaca from 2 until 5 dpi (time of death) (Figure 3, left side).

In both VAXXITEK HVT+IBD+H5 group A and B, all (10/10) chickens were considered positive for viral shedding (Figure 4).

In VAXXITEK HVT+IBD+H5 group A, in 5/5 inoculated and 5/5 contact chickens viral RNA was detected for ≥2 days through the choana (Figure 3). In addition, viral RNA was detected in none (0/5) inoculated chickens for ≥2 days through the cloaca, however in 2/5 contact chickens cloacal swabs were positive for ≥2 days. One of these contact chickens was still shedding virus through both the choana and cloaca at time of death (at 10 dpi).
In VAXXITEK HVT+IBD+H5 group B, 5/5 inoculated and 5/5 contact chickens viral RNA was detected for ≥2 days through the choana (Figure 3). Furthermore, viral RNA was detected in 2/5 inoculated and 0/5 contact in swabs from the cloaca for ≥2 days.

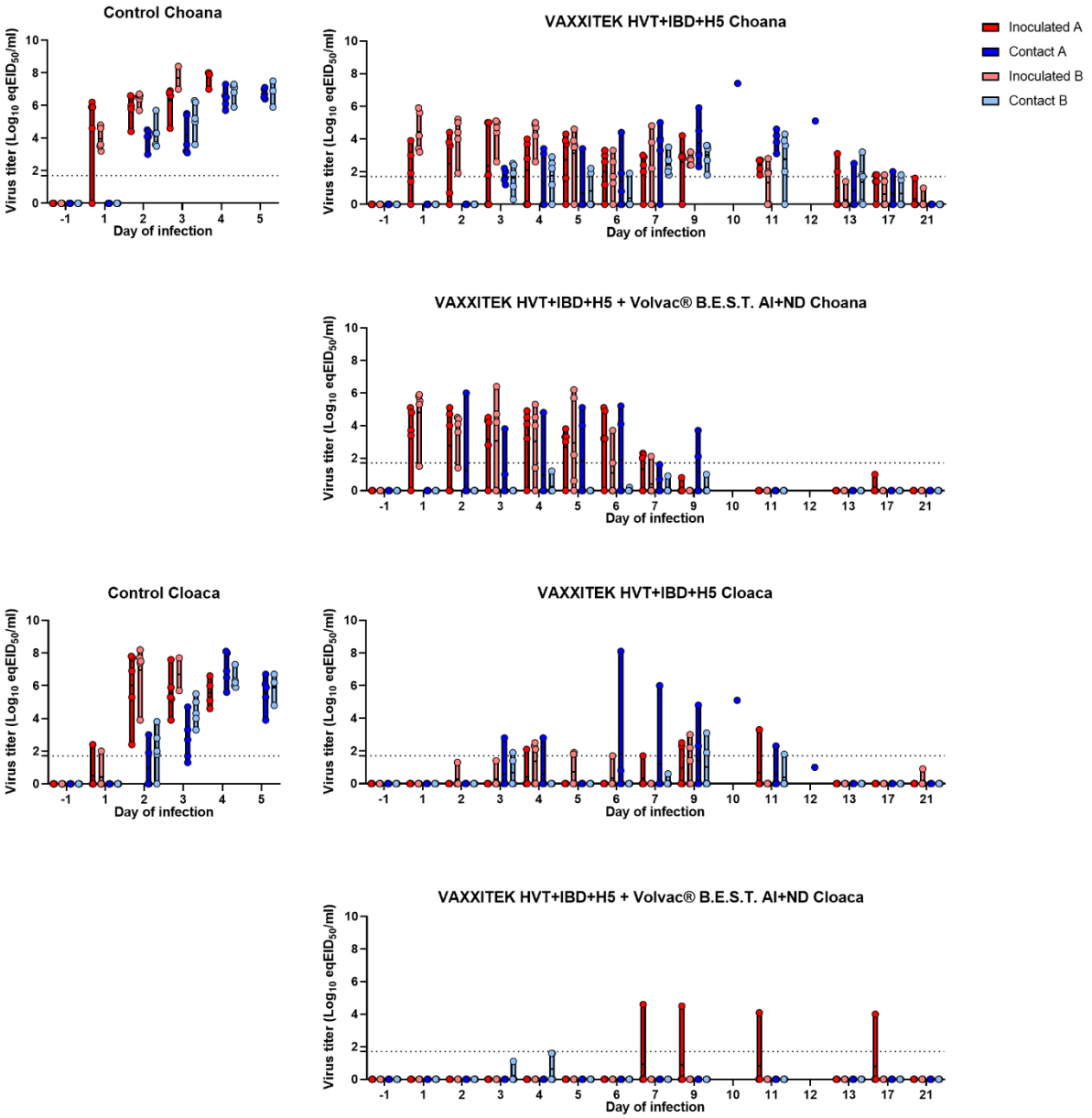

Figure 3: *The titer of virus excretion from the inoculated chickens (red) and contact chickens (blue) detected in choanal and cloacal swabs. For each group, subgroups A and B are shown separately. The detection limit of the PCR is 1.7 (*Log $10^{1.7}$ eqEID$_{50}$/ml*) (dashed line), and viral titers <Log $10^{1.7}$ eqEID$_{50}$/ml are considered negative. Each dot is an individual chicken.*

In VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND group A; 7/10 and in group B; 4/10 chickens were considered positive for viral shedding (Figure 4).
In VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND group A, viral RNA was detected in 5/5 inoculated and 2/5 contact chickens for ≥2 days through the choana (Figure 3). In 2/5 inoculated chickens M-PCR positive results were obtained for ≥2 days through the cloaca, and for none (0/5) of the contact chickens.
In VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND group B, 4/5 inoculated and none (0/5) contact chickens were positive for viral RNA detection for ≥2 days through the choana (Figure 3). No viral shedding was detected through the cloaca in this group.

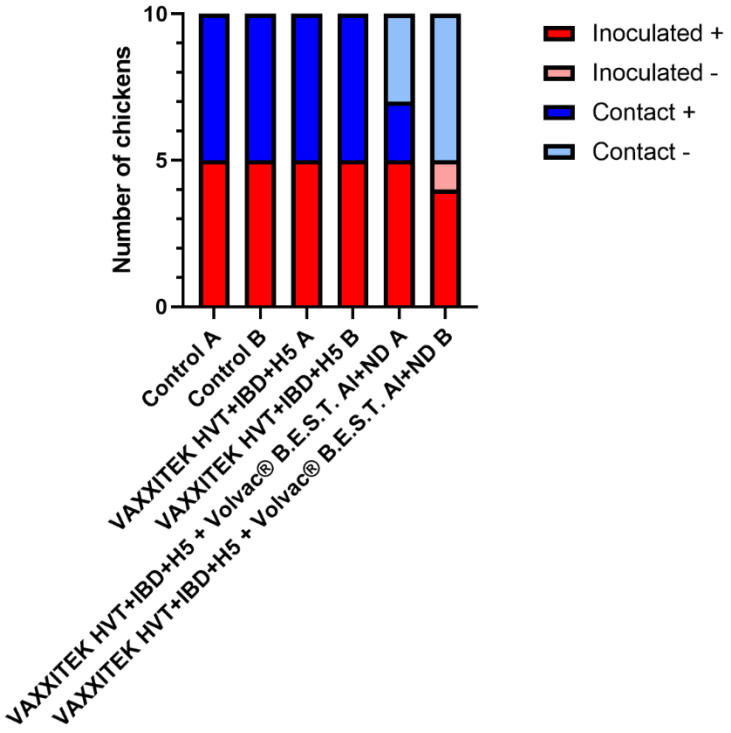

Figure 4: *The number of chickens per group for which ≥2 days virus shedding with a titer of ≥Log $10^{1.7}$ $eqEID_{50}$/ml was measured during the study. Red indicates inoculated chickens, blue for contact chickens. Bright color is positive for virus shedding (+), transparent color is negative for virus shedding (-).*

The total amount of excreted virus genome (Area under the curve; AUC) during the course of the infection was determined for the chickens considered infected (Table 3). Looking at the amount of virus genome excretion in the control group (A and B), the mean AUC in the choana was Log $10^{7.17}$ $eqEID_{50}$ and in the cloaca Log $10^{7.00}$ $eqEID_{50}$. No differences in mean AUC between inoculated and contact chickens were observed.

In the VAXXITEK HVT+IBD+H5 groups (A and B) the infected chickens shed virus with an estimated mean AUC and Log $10^{4.69}$ $eqEID_{50}$ and Log $10^{2.31}$ $eqEID_{50}$ through the choana and cloaca respectively.
In the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND groups (A and B) the infected chickens shed virus with an estimated mean AUC was Log $10^{5.56}$ $eqEID_{50}$ and Log $10^{0.49}$ $eqEID_{50}$ through the choana and cloaca respectively.

Table 3: *The total amount of virus excreted (Area under the curve; AUC) of the chickens that became infected after challenge. SD: Standard deviation. [a] Only two contact animals were PCR positive. These are the AUC estimates for these two contacts.*

Group	Infected/total number of chickens	Swab	Mean AUC Log10 $eqEID_{50}$ (SD)	Inoculated or contact	Mean AUC Log10 $eqEID_{50}$ (SD)
Control group (A&B)	20/20	Choana	7.17 (0.64)	Inoculated	7.26 (0.83)
				Contact	7.07 (0.38)
		Cloaca	7.00 (1.01)	Inoculated	7.09 (1.28)
				Contact	6.92 (0.71)
VAXXITEK HVT+IBD+H5 (A&B)	19/20	Choana	4.69 (1.12)	Inoculated	4.98 (1.03)
				Contact	4.42 (1.19)
		Cloaca	2.31 (1.73)	Inoculated	2.26 (0.95)
				Contact	2.36 (2.27)
VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND (A&B)	10/20	Choana	5.56 (0.67)	Inoculated	5.63 (0.60)
				Contact	5.29 (1.19)[a]
		Cloaca	0.49 (1.56)	Inoculated	0.62 (1.75)
				Contact	0.0

3.2.4 Humoral Immune Response

3.2.4.1 NP-ELISA and HI titers prior inoculation

In the blood collected from the chickens at -7 dpi, the absence of antibodies in the serum against avian influenza virus was demonstrated in the NP-ELISA for all chickens. In addition, this blood serum was tested using Hemagglutination Inhibition (HI) assay to determine the heterologous (against HPAI H5N1 inoculum) and homologous (against an antigen closely related to the H5 of the primary vaccine) H5-antibody titer after vaccination. In Figure 5 and Appendix 1, the (mean) HI titers and standard deviations are separately demonstrated for all the inoculated and contact chickens.

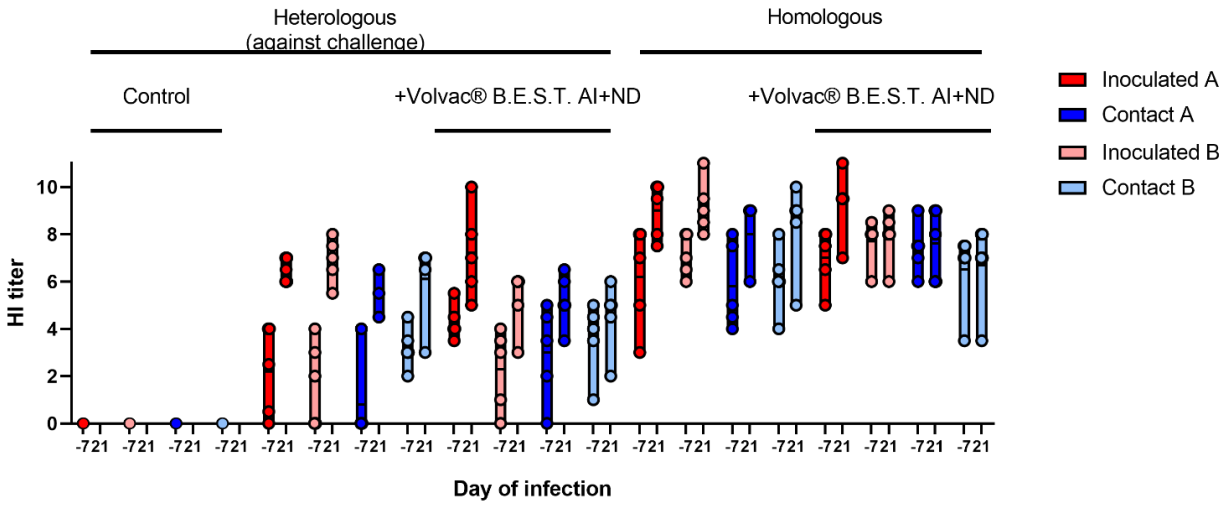

Figure 5: *The HI titer (Log_2) of the inoculated and contact chickens of the different groups. The blood serum collected before inoculation (-7 dpi) and after inoculation (21 dpi) were tested in the HI against an antigen that is highly related to the vaccine virus (homologous) and the current HPAI H5N1 inoculated virus (heterologous). Each dot is an individual chicken.*

None of the chickens in the control group had a positive HI result, demonstrating the absence of antibodies against H5-protein prior to inoculation.

On -7 dpi, prior inoculation all 40 chickens had a homologous HI titer. In the VAXXITEK HVT+IBD+H5 group A, 4/5 inoculated and 1/5 contact chickens had a heterologous HI titer and in group B, 2/5 and 5/5 inoculated and contact chickens respectively. In the groups that received the additional Volvac® B.E.S.T. AI+ND vaccine at 12 weeks, in group A, 5/5 inoculated and 4/5 contact chickens had a heterologous HI titer and in group B, 4/5 and 5/5 inoculated and contact chickens respectively. In figure 5 all individual chickens are shown (individual circles) to demonstrate variation of HI-titers prior inoculation.

3.2.5 NP-ELISA and HI titers post-inoculation

3.2.5.1 NP-ELISA

On the last day of the study, 21 dpi, blood was collected from all chickens that survived the transmission study, and the serum was tested in the NP-ELISA. These results provided information on the number of chickens that produced antibodies in response to the inoculation/ exposure to the virus.

All chickens in the control groups died before the end of the study, therefore no serological tests could be performed.

In the VAXXITEK HVT+IBD+H5 vaccinated groups A and B, 5/5 and 4/5 of the inoculated chickens and 3/3 and 5/5 contact chickens were positive in NP-ELISA respectively at 21 dpi.
In chickens vaccinated with VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND, 3/5 and 3/5 inoculated chickens of groups A and B were positive in NP-ELISA respectively. In the serum of the contact chickens 2/5 and 1/5 in group A and B respectively, the result of the NP-ELISA was positive (Figure 6a).

3.2.5.2 Hemagglutination Inhibition (HI) Assay

The blood serum collected at 21 dpi was also tested in the HI assay. A chicken was scored positive for HI when an increased heterologous HI titer of $\log_2 \geq 3$ was obtained. None of the chickens of the control groups survived the study, so no blood serum could be obtained.

In the VAXXITEK HVT+IBD+H5 vaccinated group, 4/5 and 4/5 and 2/3 and 3/5 of the contact chickens in groups A and B respectively, an increased HI titer ≥3 was obtained compared to -7 dpi.

In the groups that received the additional Volvac® B.E.S.T. AI+ND vaccine at 12 weeks, 3/5 and 2/5 of the inoculated chickens and 2/5 and 0/5 of the contact chickens in groups A and B respectively, an increased HI titer ≥3 compared to -7 dpi (Figure 6b). Averages of the HI titers per group in appendix 1. In figure 5, HI titers of all (survived) individual chickens are shown (individual circles) to demonstrate variation of HI-titers post-inoculation.

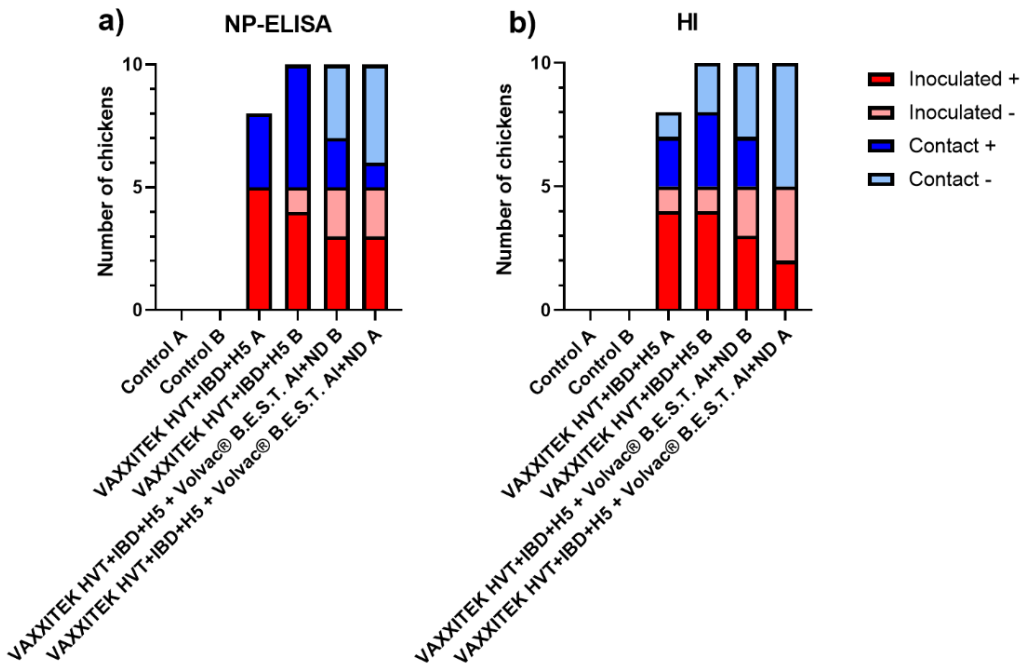

Figure 6: *The number of chickens that were positive in serology tests performed on the blood collected on the last day of the study (21 dpi) compared to collection before inoculation (-7 dpi). a) The number of chickens that obtained a positive or negative result in the NP-ELISA and b) in the HI-test. Red indicates inoculated chickens, blue for contact chickens. Bright color is positive (+), transparent is negative (-) result in the tests.*

3.2.6 Cellular Immune Response

3.2.6.1 Absolute numbers of T cells over time in the blood of vaccinated chickens

The total number of T cells, as well as number of CD4 T cells, CD8 T cells and γδ T cells were quantified over time after challenge with HPAI H5N1 clade 2.3.4.4b in the blood of VAXXITEK HVT+IBD+H5 and VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens. Although the number of total T cells tended to increase upon challenge, no significant differences compared to 0 dpi were observed for the number of total T cells not the number of CD4+, CD8+ or γδ T cells, at all timepoints after challenge (Figure 7). A comparison between the groups showed that at 7dpi the number of total T cells (Figure 7A) , CD4 T cells (Figure 7B), CD8 T cells (Figure 7C) and γδ T cells (Figure 7D) was significantly lower compared in VAXXITEK HVT+IBD+H5 vaccinated group compared to the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND group.

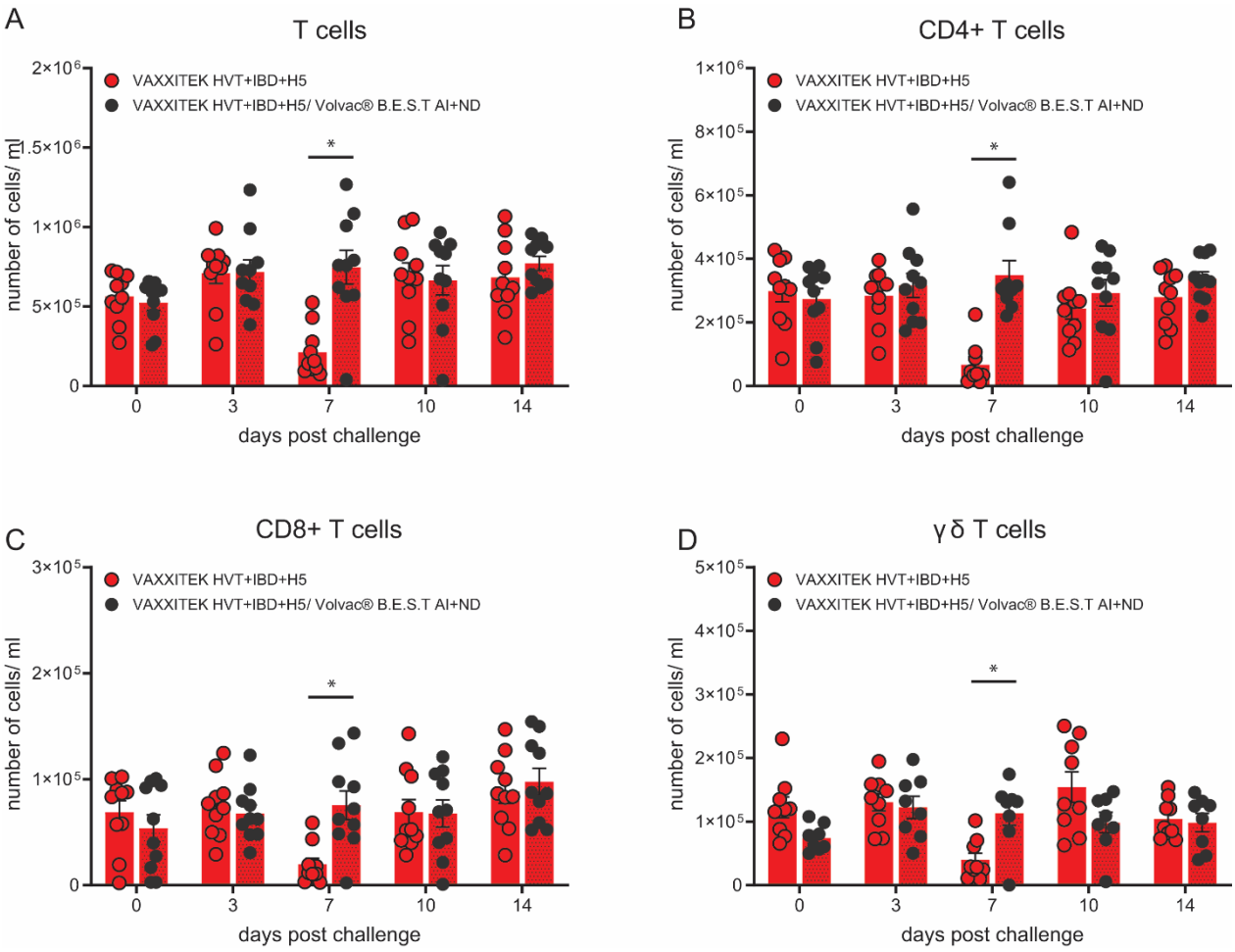

Figure 7: *Absolute numbers of T cells and T cell subsets in the blood of vaccinated chickens at several timepoints post-challenge. Absolute number of total T cells (A), CD4 T cells (B), CD8 T cells (C) and γδ T cells (D) was quantified in the blood of vaccinated chickens. Mean ± SEM of 10 vaccinated chickens is shown except for the γδ T cells were the results of 9 (VAXXITEK HVT+IBD+H5) and 8 (VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND) chickens are presented. Each dot represents an individual chicken. No significant differences compared to 0 dpi were observed. * Significant differences between the groups are indicated (p<0.05).*

3.2.6.2 Number of activated T cells over time in vaccinated chickens

Next, the effect of the HPAI H5N1 clade 2.3.4.4b challenge on the number of activated T cells in the blood of vaccinated chickens was assessed by quantifying the number of CD25+ T cells. CD25 is known to be upregulated upon activation and thus a marker of T cell activation. No significant differences compared to 0 dpi were observed. At 7 dpi, the number of CD25+ T cells tended to be higher compared to 0 dpi in the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens. This was not observed in the VAXXITEK HVT+IBD+H5 vaccinated chickens resulting in a significant higher number of CD25+ T cells in the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens compared to VAXXITEK HVT+IBD+H5 vaccinated chickens (Figure 8A). Due to the low number of CD8+ T cells and γδ T cells, analysis of CD25 expression in these subsets was not possible.

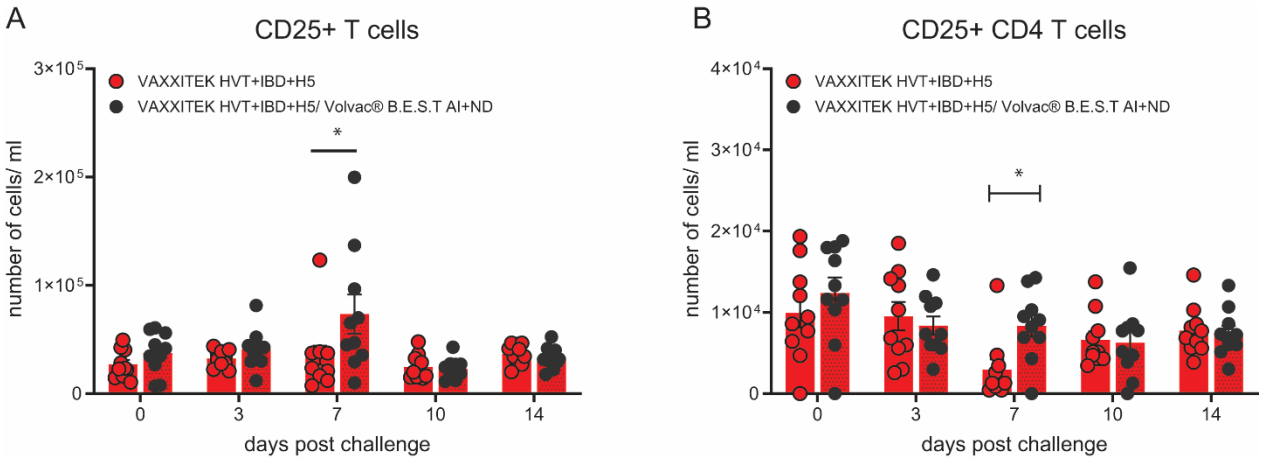

Figure 8: *Absolute number of activated T cells in the blood at different timepoints post-challenge with HPAI H5N1 clade 2.3.4.4b virus. Absolute numbers of CD25+ T cells (A) and CD25+CD4 T cells (B) were quantified in the blood of vaccinated chickens. Mean ± SEM of 10 chickens is shown. Each dot represents an individual chicken. No significant differences compared to 0 dpi were observed. * Significant differences between the groups are indicated (p<0.05).*

4 ELIspot study

4.1 Introduction

In addition to the transmission study, a parallel study was performed to determine whether a challenge with H5N1 clade 2.3.4.4b virus would result in IFNγ producing cells in vaccinated chickens. Additionally, in the final report, the results of the ELIspot will be compared to calibrate the results of the whole blood staining to investigate the relation between the number of CD25+ T cells and the number of IFNγ producing cells, two different readouts of T cell activation. For this study, chickens were vaccinated with VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND since animals that received multiple vaccinations, often exhibit a stronger and more robust immune response compared to single vaccinated animals. Therefore, in addition to the chickens in the transmission study, 25 VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens were challenged with H5N1 clade 2.3.4.4b virus, and blood and lung tissue samples were collected upon necropsy at designated timepoints. To determine the cellular immune reaction after inoculation with HPAI, the production of cytokine IFNγ and analysis of T cell CD25 expression and proliferation, was determined after activation. An ELIspot was performed with isolated lung cells and peripheral blood mononuclear cells (PBMC's), isolated from the obtained blood and tissue sample.

4.2 Material and Methods

All material and methods are identical to chapters 3.1.1-4.1.4 and 3.1.6-3.1.9, described in the transmission study except for study design (chapter 3.1.5, see chapter 4.2.1) and ELIspot protocol (see chapter 4.2.2 and 4.2.3).

4.2.1 Study Design ELIspot

The study design of the ELIspot study, is schematically presented in Figure 9. At 23 weeks of age (-7 dpi), a total of 25 chickens vaccinated with VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND were delivered to WBVR. Twelve chickens originated from commercial farm A and thirteen chickens originated from commercial farm B. Upon arrival at WBVR, the chickens were randomly given a wing tag with number for identification and timepoint for necropsy. These chickens were housed in one pen, in the same house that also housed the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND groups of the transmission study.

Blood was collected on -7 dpi to determine the antibody titer (humoral immune response) using a HI assay and NP-ELISA. In addition, choanal and cloacal swabs were taken to demonstrate the absence of avian influenza virus. This was followed by one week of acclimatization.
On 0 dpi, the day of inoculation, 20 chickens were inoculated with HPAI H5N1 clade 2.3.4.4b. The inoculation was performed by applying 0.1 ml of the virus intra-choanally, so that each chicken received 10^6 EID_{50} HPAI H5N1 virus.
Swabs (choana and cloaca) were taken from the chickens that were sacrificed at that timepoint and blood (collected in EDTA-tubes and Heparin-tubes) lung tissue was collected at necropsy. Per timepoint, 5 chickens were sacrificed, at 0 (prior inoculation) and 7 dpi three chickens originated from Commercial farm A, and 2 originated from commercial farm B, and vice versa for 3, 10 and 14 dpi.

Throughout the study, daily inspection and care of the chickens was conducted by qualified personnel. In case mild to severe clinical signs resulting from infection were observed during an inspection, an additional inspection was carried out on the same day. All clinical signs were documented.

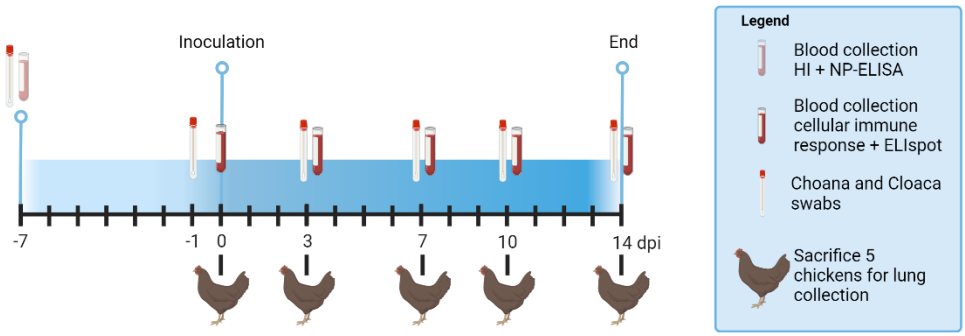

Figure 11: *Schematic overview of sample collection time points in ELIspot study. HI: Hemagglutination Inhibition assay. Inoculation was performed with 10^6 EID_{50}/ml HPAI H5N1 virus per chicken. Swabs collected at 3, 7, 10 and 14 dpi of the 5 sacrificed chickens only.*

4.2.2 Isolation of cells

Lung tissue was dissected into small fragments and enzymatically digested in RPMI medium containing collagenase A from *Clostridium histolyticum* and DNAse I from bovine pancreas (Roche Applied Science, Almere, the Netherlands) for 30 minutes at 37°C. The resulting suspension was then passed through a 70 µM cell strainer (Beckton Dickinson (BD), Franklin Lakes, NJ, USA). Heparin blood was diluted in PBS. Viable cells from lung and blood were isolated via Ficoll-Paque density gradient centrifugation. Cells were washed in PBS and resuspended in complete medium, composed of RPMI medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamax (Gibco BRL, United Kingdom).

4.2.3 IFNγ ELIspot Analysis

IFNγ ELIspot was performed as previously described[16]. IFNγ ELIspot analysis was conducted using 96-well Mulitiscreen® HTS plates Merck; MAIPS4510) that were incubated with 70% ethanol for 1 minute at room temperature and washed with H2O and PBS. Next, wells were coated with mouse anti-chicken IFNγ (2.5 µg/well in PBS; chicken matched antibody pair, Thermofisher) and incubated overnight at 4°C. After washing with complete medium, the plates were blocked for 1 hour with complete medium at 41°C, 5% CO_2. Next, cells were seeded at 2×10^5 cells/well in triplicate and incubated for 24 hours at 41°C, 5% CO_2. As a positive control for the capacity of cells to produce IFNγ a combination of 50 ng/ml phorbol myristrate acetate (PMA) and 500 ng/ml Ionomycin was included (Sigma-Aldrich). Plates were washed with PBS supplemented with 0.05% Tween-20 (PBS-Tween) and incubated with anti-chicken IFNγ biotin (1 µg/well in PBS, chicken matched antibody pair) for 1 hour at room temperature. Plates were washed 5 times with PBS-Tween and incubated with poly-HRP (0.2 µg/well; Fisher) for 1 hour at room temperature. Plates were washed again 5 times with PBS-Tween and TMB was added (50 µl/well, TMB for ELIspot, Mabtech). After spots became visible, plates were washed with tap water and airdried. Spot analysis was performed using the AELVIS automated spot analyzer (Sanquin).

4.3 Results ELIspot

4.3.1 Pre-challenge serology and post-inoculation clinical signs

From the blood collected from the chickens in the ELIspot study at -7 dpi, the absence of antibodies in the serum against avian influenza virus was demonstrated in the NP-ELISA for all chickens (data not shown). In addition, this blood serum was tested using Hemagglutination Inhibition (HI) assay to determine the heterologous and homologous H5-antibody titer after vaccination with the average homologous HI-titer: $\log_2$ 6.96 (SD 0.89) and average heterologous HI-titer: $\log_2$ 3.16 (SD 1.54). All chickens survived until their designated timepoint, without demonstration of clinical signs.

Swabs (choanal and cloacal) were taken of all chickens prior inoculation, and no viral RNA was detected (data not shown). Prior necropsy, choanal and cloacal swabs were obtained from the sacrificed chickens. We did not detect viral RNA by M-PCR in any of the swabs obtained from the cloaca at any timepoint. In the swabs obtained from the choana, only at 3 dpi (5/5, average titer $log_{10}^{3.66}$ $eqEID_{50}$/ml, SD 1.3) and 7 dpi (1/5, with titer of $log_{10}^{2.2}$ $eqEID_{50}$/m) positive results were obtained by M-PCR.

4.3.2 ELIspot analysis lung cells and PBMC's

In addition to surface expression of CD25 and proliferation, memory T cell also produce the cytokine IFNγ upon activation. To determine whether challenge with HPAI H5N1 clade 2.3.4.4b virus would result in IFNγ producing cells, an ELIspot analysis was performed with cells isolated from lung and blood of VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens. As shown in Figure 10, restimulation with PMA/Ionomycin resulted in an increase in IFNγ producing cells both isolated from the lung (Figure 10A) and from the blood (PBMC) (Figure 10B). This experimental stimulation demonstrates that the isolated cells are indeed capable of IFNγ production(shown for 7dpi, performed at every timepoint).

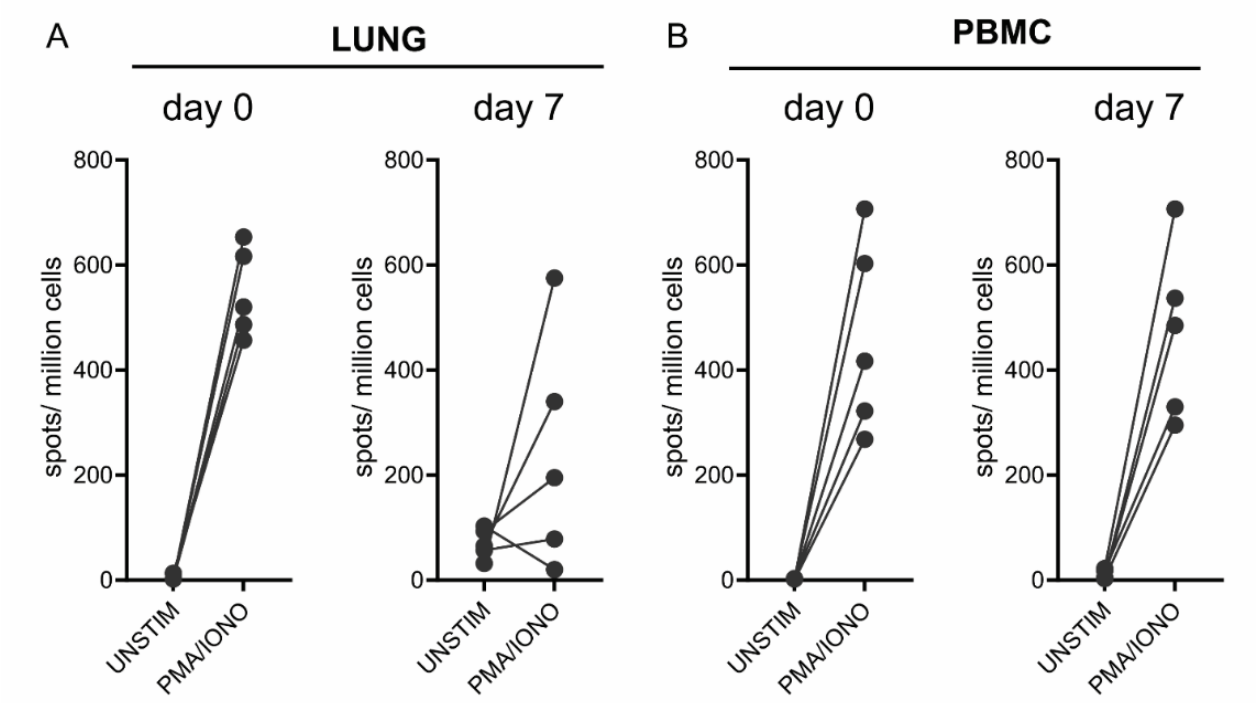

Figure 10: *Number of IFNγ producing cells isolated from lungs and blood (PBMC) of five VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND chickens sacrificed before (0 dpi) and after challenge (shown for 7 dpi). Results were obtained by ELIspot analysis.*

Next the number of IFNγ producing cells upon inoculation was determined. Figure 11 shows the number of IFNγ producing cells without experimental restimulation. This reflects the *in vivo* stimulation; the presence of virus will activate the cells thus induce the production of IFNγ. At 3, 7, 10 and 14 dpi an increase in IFNγ producing cells was observed in lung (Figure 11A) and in the blood (Figure 11B) compared to non-inoculated chickens (results demonstrated at 0dpi). The number of IFNγ producing cells was higher in cells isolated from lungs compared to cells isolated from the blood.

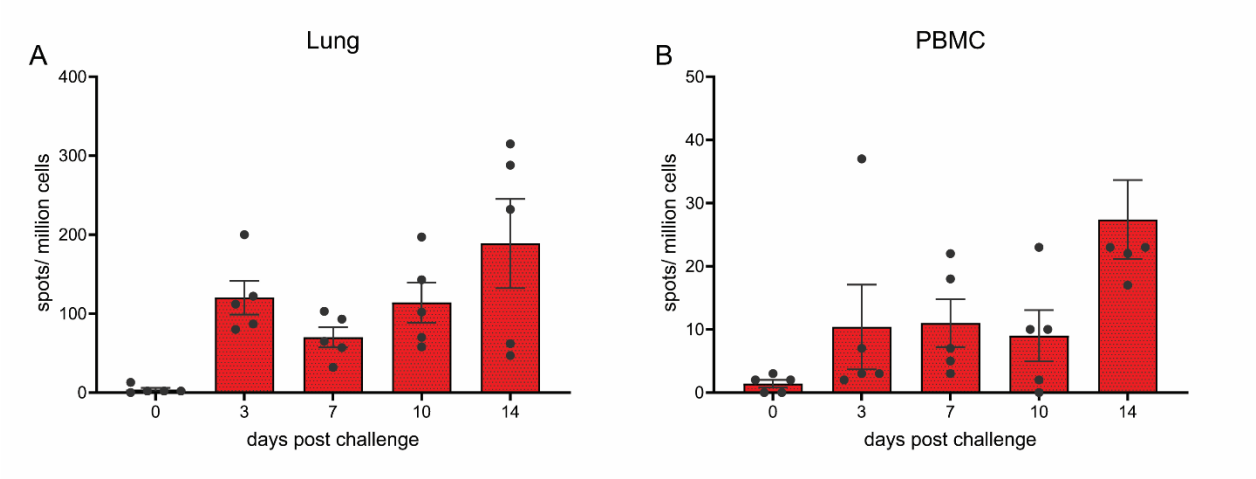

Figure 11: *Number of IFNγ producing cells isolated from lungs and blood (PBMC) of five VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens sacrificed before (0 dpi) and post-challenge. Results were obtained in unstimulated cells by ELIspot analysis. Mean ± SEM of 5 chickens is shown. Each dot represents an individual chicken.*

5 Discussion

The overall goal of this longitudinal study is to determine whether vaccination of laying hen flocks under field conditions can provide long-term protection against HPAI H5N1 virus (clade 2.3.4.4b), especially against virus transmission (within-flock transmission R<1) measured under experimental conditions. This progress report summarizes the results of the second out of four transmission studies, providing data to reach the overall goal.

Laying hen pullets were vaccinated at day of hatch, with one group receiving a booster vaccination at 12 weeks of age, and were reared under field conditions until 23 weeks of age. The effectiveness of these vaccines was assessed at 24 weeks of age, when laying hens are stepping up to the peak of egg production. The results demonstrate that the estimated transmission parameter R at this timepoint for vaccinated chickens was substantially reduced compared to the non-AI vaccinated control group, however not <1. Only in the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND R-values <1 were estimated.

Drawing conclusions on protection against sustained transmission requires additional data from later stages of the field- and third and fourth transmission studies. In vaccinated flocks, the transmission rate (R) is influenced by the proportion of chickens expressing low and high levels of immunity, typically indicated by HI antibody levels [17]. For these vaccines, the HI antibody levels necessary to classify immune response levels (e.g. HI-titer) as low or high against transmission have not yet been determined.
In our previous studies, a pilot ([7], 2023) and the first transmission study [2], chickens were inoculated at 8 weeks of age (8 weeks post-vaccination) and the groups (control and vaccinated) were considered as a homogeneous and representative subset of chickens, with regard to levels of infectivity that were housed in the field. Then, we assumed minimal variation in HI titers and uniform transmission characteristics, such as susceptibility and infectiousness. However, previous research suggests that immunity levels and transmission characteristics are not necessarily uniform among vaccinated chickens, especially when the antigenic distance from the challenge virus is large [17]. This could have an impact when assessing transmission experimentally. Given the small number of animals used (for one cross-sectional study), a small proportion of chickens with low neutralizing antibody titers can significantly increase transmission (R>1) due to higher infectivity [17]. Therefore, at the end of the longitudinal study, the additional transmission experiment data and HI titer distribution in the field flock over time will enable a more accurate quantification of transmission and predictions of the duration of protection over time. In the final report, the combined data will be presented to support conclusions on effectiveness of a large-scale single dose application of this vaccine to stop sustained transmission, and hence on its potential as preventive measure to control HPAI for the whole of the production cycle.

In this study, where the challenge was conducted at 24 weeks of age, greater variation in HI antibody titers was expected compared to 8 weeks post-vaccination. Figure 5 demonstrates all HI antibody titers in each group for each chicken prior inoculation (bars labeled with -7). Chickens were randomly selected in the field and randomly assigned inoculated or contact chicken upon arrival, however chickens with low antibody levels, could influence the transmission dynamics within the group. The distribution of (homologous) HI-titers prior inoculation is an important indication regarding circulating antibodies in the blood of chickens induced by vaccination. For the remaining two transmission studies, HI-titers will be evaluated pre-inoculation to avoid accidental allocation of chickens over the groups (inoculated and contact) with an exceptionally heterogenous distribution of HI-titers.

In this transmission study, few chickens reached their humane endpoint (HEP), as they demonstrated severe clinical signs and were humanely euthanized prior to spontaneous death to prevent additional suffering. Removing chickens that shed a high load of virus can reduce the overall virus transmission within the study group, potentially leading to an underestimation of the virus's natural dynamics. In the control groups a total of 4/20 chickens reached the HEP and were removed from the study. In group A, three chickens reached HEP (one inoculated chicken at 4 dpi and two contacts at 5 dpi) and one contact chicken in group B at 4 dpi. At the timepoints these chickens were humanely euthanized, all other chickens of the control groups were found

death, or were already shedding virus with high titers ≥5.7 Log_{10} $eqEID_{50}$/ml. Therefore, we assume it is unlikely that in the control groups transmission parameter R was underestimated.
Also in the vaccinated groups we think the likeliness of underestimation of transmission parameter R is small. Only in VAXXITEK HVT+IBD+H5 group A one contact chicken reached the HEP (10 dpi) and was euthanized, while all other chickens in this group already shed virus with titers between 2 and 5 Log_{10} $eqEID_{50}$/ml.

The whole blood analysis demonstrated that although the number of total T cells tended to increase upon challenge no significant differences were observed for the T cell subsets in both vaccinated groups compared to 0 dpi. Interestingly, the number of activated T cells at 7 dpi tended to be higher compared to 0 dpi in the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens. This was not observed in the VAXXITEK HVT+IBD+H5 vaccinated chickens resulting in a significant higher number of CD25+ cells in VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens compared to VAXXITEK HVT+IBD+H5 vaccinated chickens at 7 dpi.

The IFNγ ELIspot analysis showed that IFNγ producing cells are readily detected in the lungs and in the blood from 3 dpi onward. This indicates that immune cells have been activated by the challenge virus, resulting in the production of the cytokine IFNγ which play a central role in activation of the immune system and has an anti-viral effect [18].

This progress report solely includes data obtained in the second transmission and ELIspot study, and does not include a comparative analysis of the results obtained from chickens still in the field and the previous transmission study performed at 8 weeks of age. The final report will contain all obtained field data and all data obtained in the four transmission studies. Where applicable, we will then include comparative and combined analysis of the data obtained to reach the overall goal of this longitudinal field study; to determine whether vaccination of laying hen flocks under field conditions can provide long-term protection (and its expected duration) against HPAI H5N1 virus (clade 2.3.4.4b), especially against virus transmission (within-flock transmission $R<1$) measured under experimental conditions.

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7 Terminology

The transmission parameters R, the infectious period, and the infection rate beta (β) are critical components in understanding and modeling the dynamics of infectious diseases. The effective reproduction number R_e or R_t, is the average number of secondary infections induced by a single infected individual at a given time, taking into account the current state of the population, including those who are no longer susceptible (due to immunity, vaccination, or other factors). Therefore, R reflects the current transmissibility in the context of the actual population and ongoing control measures. The infectious period; the duration an infected individual can transmit the disease to others, directly influences the number of contacts during which transmission can occur, thereby impacting the overall epidemic trajectory. The infection transmission parameter, beta (β), represents the per-contact probability of transmission times the contact rate per unit of time, thus it is rate parameter i.e. the expected number of infections per unit of time and is pivotal in quantifying how quickly an infection spreads through the population. The transmission rate parameter times the infectious period is the basic reproduction ratio R_0, the average number of new cases cause by a typical (average) infectious individual in a completely susceptible population. From all this it follows that $R_e=(S/N)R_0$ where S/N the fraction susceptible individuals. Together, these parameters are essential for designing effective control strategies, predicting outbreak scenarios, and implementing interventions to mitigate the impact of infectious diseases. Understanding the interplay between R, the infectious period, and beta (β) is therefore crucial for the development of robust epidemiological models and the formulation of evidence-based policy decisions.

Definition of infection in the context of this study:
For the analysis, a chicken is considered infected when the following criteria apply:

- Virus shedding: when virus was detected for 2 days or longer (≥2 days) with a minimum equivalent titer of ≥Log $10^{1.7}$ $eqEID_{50}$/ml by PCR in swabs collected from either choana or cloaca, and
- the chicken died or alternatively when the chicken survived the challenge, it had:
    - a positive NP-ELISA result (after 21 days) and/or
    - showed an increase of ≥3 $\log_2$ in the heterologous HI-titer.

This definition is consistent with the definition of an infected chicken used in our previous studies [2, 7].

Defining an infected chicken by being both positive for virus shedding and antibody response is essential.
Virus shedding; the release of the virus from the host into the environment, indicates active replication and the chicken's potential to transmit the virus to others. This measure alone, however, may not provide a complete picture of the infection dynamics or the host's immune response. The presence of an antibody response is a crucial complement to virus shedding as it signifies the host's adaptive immune system has recognized and responded to the challenge virus. Antibodies, particularly those detectable by assays such as hemagglutination inhibition (HI) or ELISA, indicate past or ongoing exposure and provide evidence that the immune system has mounted a defence. These dual criteria of virus shedding and antibody response provide a more comprehensive and accurate characterization of infection, enhancing the understanding of disease dynamics, the effectiveness of vaccination strategies.

Humane endpoints are in animal experiments pre-determined criteria that signal when an animal should be humanely euthanized or otherwise removed from the study to prevent unnecessary suffering. These endpoints are designed to minimize the pain and distress experienced by the animals, aligning with ethical considerations and regulatory requirements. Implementing humane endpoints can affect the transmission parameters in studies of infectious diseases by potentially altering the natural progression and observation period of the disease. This might lead to underestimation or overestimation of transmission rates, as animals may be removed from the study before the full course of infection is observed. Consequently, researchers need to carefully design experiments to balance ethical considerations with the accuracy of transmission data.

Hemagglutination inhibition (HI) titers pre-challenge are a critical metric in the evaluation of immunological responses and the efficacy of vaccines against viral infections. HI titers measure the presence and level of specific neutralising antibodies capable of inhibiting the hemagglutination process, where viruses agglutinate red blood cells. Higher pre-challenge HI titers indicate a stronger pre-existing immunity, which is predictive of an individual's ability to mount an effective defence against viral exposure. In the context of vaccine studies, assessing HI titers before exposure to the pathogen provides essential data on the protective threshold needed to prevent infection. Moreover, understanding the correlation between pre-challenge HI titers and clinical protection helps in establishing immune correlates of protection, which are pivotal for regulatory approvals and public health decision-making.

Antigenic distance refers to the measure of difference between the immune responses elicited by different viral proteins, specifically of the vaccine antigen in relation to the circulating field virus (represented in the transmission experiments by the challenge virus). This concept is crucial in understanding how well an immune response generated by a prior infection or vaccination might protect against a new strain of the virus. A greater antigenic distance indicates more significant differences in the immune response to the different viral proteins, suggesting that the immune system may not recognize or effectively respond to the new challenge virus. Conversely, a smaller antigenic distance implies that the immune response to the original virus or vaccine is likely to provide better cross-protection against the challenge virus.

The cellular immune response, particularly the quantification of T cells, activated T cells, and the production of interferon-gamma IFNγ, plays a pivotal role in the body's defense against infections and in the evaluation of vaccine efficacy. T cells, especially CD4+ and CD8+ subsets, are essential for orchestrating the immune response through the direct killing of infected cells and the support of antibody production. T cell activation reflects the readiness of these cells to respond to pathogens and can be determined by various readouts, including proliferation (increased T cell numbers), surface expression of markers such as CD25, and the production of cytokines like IFNγ., Actually the production of IFNγ by these activated T cells is a crucial indicator of a robust immune response, as IFNγ is instrumental in enhancing the antimicrobial activity of macrophages and in promoting the overall coordination of the immune response. Monitoring the levels of T cells, their activation, and IFNγ production provides comprehensive insights into the effectiveness of immune responses elicited by infections or vaccines. This information is vital for understanding the mechanisms of protection, guiding the design of more effective vaccines. Consequently, the assessment of these cellular immune parameters is indispensable for advancing immunological research and improving public health interventions.

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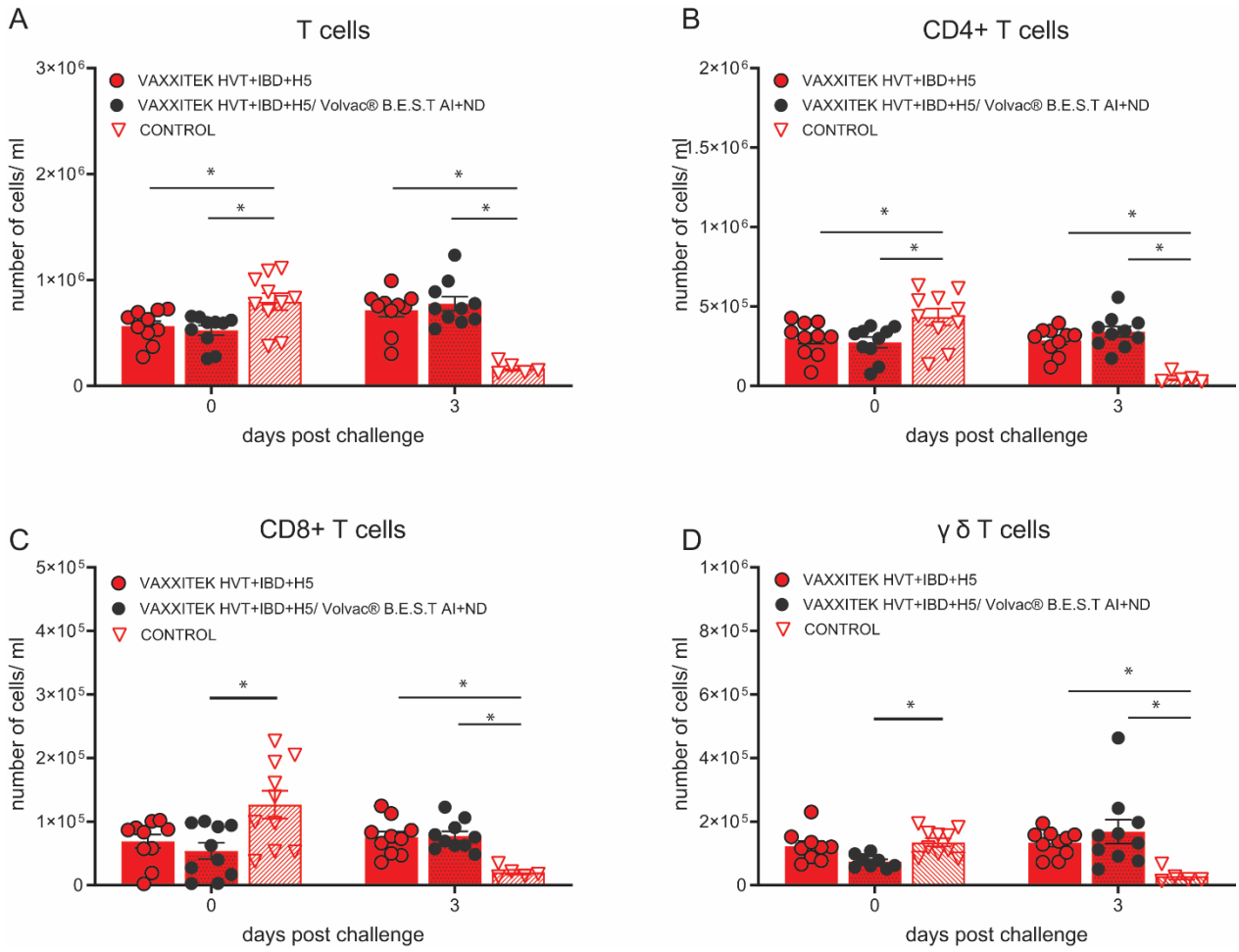
Appendix 1: HI titers Transmission study

Appendix table 1: *The mean HI titer (Log_2) of the inoculated and contact chickens of the different groups. The blood serum collected before inoculation (-7 dpi) and after inoculation (21 dpi) were tested in the HI against an antigen that is highly related to the vaccine virus (homologous) and the HPAI H5N1 challenge virus (heterologous). SD: Standard deviation.*

Group		Inoculated or Contact	Antigen	-7 dpi Mean (SD)	21 dpi Mean (SD)
Control	A	Inoculated	Heterologous	0	ND
	B	Inoculated	Heterologous	ND	ND
	A	Contact	Heterologous	0	ND
	B	Contact	Heterologous	ND	ND
VAXXITEK HVT+IBD+H5	A	Inoculated	Heterologous	2.20 (1.89)	6.50 (0.50)
	B	Inoculated	Heterologous	1.80 (1.79)	6.90 (0.96)
	A	Contact	Heterologous	0.80 (1.79)	5.50 (1.00)
	B	Contact	Heterologous	3.20 (0.91)	6.10 (1.75)
	A	Inoculated	Homologous	6.20 (2.17)	9.00 (1.17)
	B	Inoculated	Homologous	7.10 (0.89)	9.20 (1.15)
	A	Contact	Homologous	5.80 (1.82)	8.00 (1.73)
	B	Contact	Homologous	6.10 (1.43)	8.30 (1.92)
VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND	A	Inoculated	Heterologous	4.30 (0.76)	7.20 (1.72)
	B	Inoculated	Heterologous	2.30 (1.72)	5.20 (1.3)
	A	Contact	Heterologous	3.00 (2.03)	5.20 (1.15)
	B	Contact	Heterologous	3.60 (1.56)	4.50 (1.5)
	A	Inoculated	Homologous	7.00 (1.27)	9.30 (1.44)
	B	Inoculated	Homologous	7.70 (0.97)	7.90 (1.14)
	A	Contact	Homologous	7.40 (1.08)	7.60 (1.52)
	B	Contact	Homologous	6.50 (1.7)	6.70 (1.86)

Appendix 2: Absolute numbers of T cells in the blood of vaccinated and non AI vaccinated chickens

Before inoculation (0 dpi) and at 3 dpi, the number of T cells in the blood of VAXXITEK HVT+IBD+H5 vaccinated and VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens was compared with the number of T cells in the blood of chickens in the control group. Both the total number of T cells, as well as a number of T cell subsets were assessed (Appendix Figure 1). Before challenge (0 dpi), the number of T cells (A), CD4 T cells (B), CD8 T cells (C) and γδ T cells (D) were significantly higher in blood obtained from chickens in the control groups compared to chickens that received a vaccination (VAXXITEK HVT+IBD+H5 and VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND). At 3 dpi, the number of T cells (A), CD4 T cells (B), CD8 T cells (C) and γδ T cells (D) was significantly lower in chickens in the control groups compared to vaccinated chickens (VAXXITEK HVT+IBD+H5 and VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND).



Appendix Figure 1: *Absolute numbers of T cells in the blood of chickens in vaccinated and control groups. Before (0 dpi) and at 3 dpi, absolute number of total T cells (A), CD4 T cells (B), CD8 T cells (C) and γδ T cells (D) was quantified in the blood of VAXXITEK HVT+IBD+H5 vaccinated and VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens. Mean ± SEM of 10 chickens is shown, except for the γδ T cells where the results of 9 (VAXXITEK HVT+IBD+H5) and 8 (VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND) vaccinated chickens are shown. At 3 dpi results of the five chickens that were alive in the control groups are shown. Each dot represents an individual chicken. * Significant differences between the groups are indicated ($p<0.05$).*

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Wageningen Bioveterinary Research
Report

The mission of Wageningen University & Research is "To explore the potential of nature to improve the quality of life". Under the banner Wageningen University & Research, Wageningen University and the specialised research institutes of the Wageningen Research Foundation have joined forces in contributing to finding solutions to important questions in the domain of healthy food and living environment. With its roughly 30 branches, 7,600 employees (6,700 fte) and 13,100 students and over 150,000 participants to WUR's Life Long Learning, Wageningen University & Research is one of the leading organisations in its domain. The unique Wageningen approach lies in its integrated approach to issues and the collaboration between different disciplines..