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

First report, 8-weeks post vaccination with VAXXITEK HVT+IBD+H5

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Wageningen Bioveterinary Research Report

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1 Abstract

This progress report describes the first transmission study in a series of four studies. This long-term study aims to determine whether vaccination under field circumstances can protect layer flocks long term against HPAI H5N1 virus (clade 2.3.4.4b) and reducing within flock transmission (R<1). This transmission study was performed at 8-weeks post vaccination with layer hens which were housed under field circumstances. In addition to the main output, which is assessment of the level of transmission, other variables such as chicken survival, virus shedding, humoral and cellular immune responses were studied to assess the effectiveness of vaccination with VAXXITEK HVT+IBD+H5 vaccine in poultry against HPAI H5N1 virus infection. The estimated R for the control group was 1.3 (95% CI 0.58–2.87) and transmission in the VAXXITEK HVT+IBD+H5 could not be quantified as none of the inoculated chickens became infected.

For the analysis, a chicken is considered infected when the following parameters applied:

- 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
- If the chicken survived the challenge, additional parameters were:
    - 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 used in our previous study [1].

In the control groups 9/10 inoculated chickens and 7/10 contact chickens met these criteria and were considered infected. The non-infected inoculated chicken in control group A, was positive for virus shedding, but had no positive serological response.
After vaccination with the VAXXITEK HVT+IBD+H5 vaccine, none of the chickens became infected (although 2/10 inoculated chickens were positive for virus shedding). No positive serological results were obtained.
In addition, prior to inoculation, the vaccinated chickens had a significantly higher absolute numbers of T cell (subsets) in the blood. The absolute number of activated T cells in the blood peaked at 7 days post inoculation.

2 Introduction

With the highly pathogenic avian influenza (HPAI) virus year-round in the Netherlands, actions are needed to protect poultry from this highly pathogenic virus and mitigate the zoonotic potential. Vaccination is one of the additional measures for protection of chickens against avian influenza (AI). In this Public-Private-Partnership, institutes work together to investigate the potential of using HVT (herpesvirus of turkey)-based vaccine vectors expressing the hemagglutinin (HA) of HPAI under field conditions. Various parameters will be assessed to determine the effectiveness of a vaccine, which can be quantified in a transmission study. The most important objective is to determine if vaccination sufficiently prevents virus transmission (virus spread) between chickens in a flock, so that the within flock reproduction number R is lower than 1. A vaccine that is only able to reduce clinical signs without adequate reduction of virus transmission is not considered an effective vaccine in the context of disease control, especially not for (potential) zoonotic infections.

In a previous study, the efficiency of four vaccines was tested in 8-week old Lohmann Brown Classic laying hen pullets that were vaccinated and housed in laboratory facilities[1]. Two of the vaccines, HVT-H5 vaccine Vectormune® from CEVA (VECTORMUNE® AI) and HVT-H5 COBRA vaccine from BIAH (VAXXITEK HVT+IBD+H5), resulted in significantly lower than 1 estimates ($R<1$) for both vaccines, which was significantly lower than in the unvaccinated control groups. In addition, vaccination with both HVT-H5 vaccines was 100% effective in reducing disease and mortality after inoculation with HPAI H5N1 virus. Furthermore, the number of chickens shedding virus and the amount of virus shed by either one of the HVT-H5 vaccinated inoculated chickens was significantly less compared to the non-vaccinated challenged control group.

From this, we concluded that both HVT-based vaccines were effective in preventing transmission and clinical signs under laboratory conditions 8-weeks post vaccination. However, circumstances on commercial farms are not identical to the conditions in laboratory facilities. Furthermore, information on the duration of immunity and effectiveness when used in addition to other vaccinations is still unknown. In this study, commercial poultry were housed under field conditions and vaccinated following a standard vaccination scheme against several pathogens together with the VAXXITEK HVT+IBD+H5 vaccine to determine the level of protection after a HPAI H5N1 virus challenge. This study in layer flocks is an important part of the strategy towards a more sustainable approach to control avian influenza virus, as an avian influenza outbreak in layer poultry farms has a major welfare impact due to preventive measures, such as keeping chickens inside during high risk periods.

This progress report describes the first transmission study in a series of four studies. This long-term study is to determine whether vaccination under field circumstances can protect layer flocks long term against HPAI H5N1 virus (clade 2.3.4.4b) reducing within flock transmission($R<1$). This first transmission study was performed in laying hen pullets (vaccinated as day old chicks), at 8 weeks post vaccination. These laying hen pullets were housed under field conditions until challenge. In addition to the main output which is assessment of transmission, other variables such as survival, virus shedding and humoral and cellular immune responses were studied.

3 Material and Methods

3.1 Permits and Funding

The animal study was conducted in accordance with the guidelines of 2010/63/EU. The animal study was approved by the Central Committee for Animal Experiments (CCD) (permit application AVD40100202215972; experiment 2021.D-0036.004). 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".

Two transmission studies will be conducted within the BO-43-111-083 project and will be executed at 8-weeks and approximately 24-weeks post vaccination. These chickens are permitted by the study of Royal GD "Immunologische respons in kippen na AI-H5-vaccinaties onder praktijkomstandigheden, AVD42600202316719-1". The other two transmission studies will be conducted within the PPP project entitled "Vaccinatie van pluimvee tegen HPAI H5 vogelgriepvirus, Aanvraagnummer. LWV 22103" in the remaining laying period.

3.2 Housing

Detailed information on housing can be found in "Progress report for: PPP project Vaccination of poultry with HPAI H5". In short, the chickens were housed on a rearing farm (Commercial farm A) in 4 separate units within the same house. A total of 2399 chickens were included in the study, 4665 chicken of the same hatch were placed with the negative control group for commercial purposes. At the location of Farm A, in a house next to the rearing house, an additional 650 brown layers were present (hatched on 12 July 2022). Free range was not permitted in the study protocol. Flocks were housed and managed under standard conditions for laying hen pullets, using a standard lighting and temperature schedule, access to perches and *ad lib* provision of a commercial feed and water. All husbandry conditions were in compliance with the Dutch implementation of EU legislation for management of farmed animals.
Each unit was equipped with a partial elevated slatted floor and a concrete floor with a wood shaving bedding. The rearing management and monitoring of overall health and welfare was under supervision of a technician of the hatchery and the veterinary practitioner of the farm, next to a monthly health check performed by Royal GD. No health problems of the pullets were observed until day of transport to WBVR.

At the age of 7-weeks a subgroup of the chickens was transported to the animal facilities of WBVR in Lelystad. Here, the chickens were housed under BSL2 conditions for the first week. From 8-weeks of age and post-vaccination onwards, the chickens were inoculated and housed under vBSL3 conditions. The different groups of chickens (AI vaccinated and non AI vaccinated (control group)) were housed in identical pens which were separated with solid walls so that the chickens from different groups could not have direct contact. The pens had a floor area of ≥2 m^2, and the floor was covered with sawdust. The barns simulated a natural day-night rhythm through artificial lighting (13 hours light; 11 hours dark). The housing and care of the chickens were tailored to the specific needs of their age [2]. Throughout the entire study, the chickens had a perch and a piece of burlap as cage enrichment. The chickens had, as in Commercial farm No. 1, unlimited access to water and rearing layer feed. In this study, an attempt was made to make the contact structure and density as comparable as possible to field conditions, both maximum of 9 hens per m^2.

3.3 Chickens

Detailed information on the chickens can be found in "Progress report for: PPP project Vaccination of poultry with HPAI H5". In short, this study was conducted with Novogen Brown Light laying hen pullets. The parent chickens of the chickens in this study received standard vaccinations against Marek's disease on the day of hatch using a combination HVT with Rispens vaccines. In addition to various other standard vaccinations during the rearing period, the parent birds were only repeatedly vaccinated against Infectious Bronchitis virus (IBV) and Newcastle Disease virus during the production period (none of which were vector vaccines).

3.4 Vaccinations

Detailed information on vaccinations can be found in "Progress report for: PPP project Vaccination of poultry with HPAI H5". In short, The day-old chicks were derived from a commercial Dutch hatchery. The chickens were all vaccinated against IBV (MA5 and 4/91) and coccidiosis at the hatchery according to the Summary of Product Characteristics (SPC) of the vaccines. The Marek's (Rispens) vaccination of control group was also performed at the hatchery using a fully automatic vaccinator. The AI vaccinated test group was vaccinated against Marek's disease (Rispens) and Avian VAXXITEK HVT+IBD+H5[3-5] at Royal GD in Deventer. This was executed by an experienced vaccinator using semi-automatic equipment derived from the hatchery and under supervision of the experts of the vaccine producer. The test vaccine was administered only at day of hatch. After vaccination, the chickens were transported to the commercial farm.

3.5 Inoculum

The inoculum used to infect the chickens at 8-weeks of age, is an 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 was determined at the time of detection and can be found in the GISAID Database under the number EPI_ISL_6101848. It concerns A/chicken/Netherlands/21038165-006010/2021_H5N1_PB2_2021-11-07_LUTJEGAST. This was the same as used in our previous study: "Transmissiestudie met vier vaccins tegen H5N1 hoogpathogeen vogelgriepvirus (clade 2.3.4.4b)" [1]. 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 chickens was performed by the same (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.

3.6 Study Design

The study design of the transmission study is schematically presented in Figure 1. At 7-weeks of age (day -7), 22 chickens vaccinated with VAXXITEK HVT+IBD+H5 were delivered to WBVR together with 22 chickens of the control group. Upon delivery to WBVR, the control group and vaccinated chickens were each randomly divided in two groups (A or B, each consisting of 5 inoculated, 5 contact and 1 surplus chicken), and received colored wing tags for identification. Blood was collected on day -7 to determine the antibody titer (humoral immune response) using a Hemagglutination Inhibition (HI) assay. 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 day 0, the day of inoculation with HPAI H5N1 clade 2.3.4.4b, all surplus chickens were euthanized. 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. 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. 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. First contact chickens were swabbed followed by inoculated chickens to prevent infection by handling of the chickens. In the second week, swabs were taken every other day (day 9, 11 and 13), and in the third week, swabs were taken at two timepoints (day 17 and 21). Blood from the wing vein was collected to examine the cellular immune response of the inoculated chickens at days 0, 1, 3, 7, 10 and 14. At the end of the transmission study, all chickens were euthanized under sedation and blood was collected for antibody detection.

Throughout the study, daily inspection and care of the chickens were conducted. 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. Chickens were euthanized when they reached the humane endpoint.

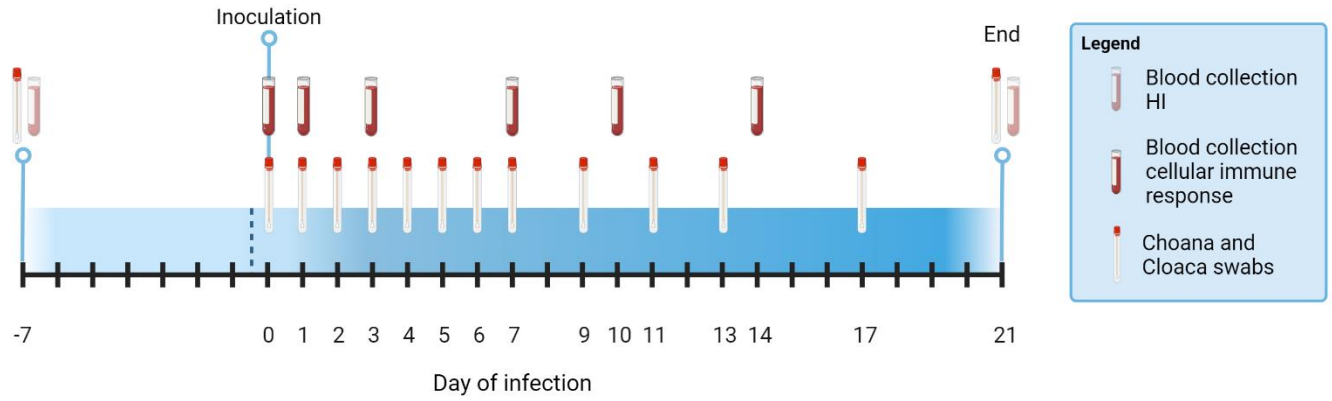

Figure 1: *Schematic overview of sample collection time points. HI: Hemagglutination Inhibition assay. Inoculation was performed with 10^6 EID_{50}/ml HPAI H5N1 virus per chicken.*

3.7 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. 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 method has been previously described [6]. The NP-ELISA was used at two different timepoints in this study: at day -7 (upon arrival at WBVR) and at day 21 (end of the study).

3.8 Hemagglutination Inhibition (HI) assay

Antibody responses after vaccination can be quantified in the Hemagglutination Inhibition (HI) assay. 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 (day -7) and at the end of the study (day 21) were tested in the HI. The HI is performed using different antigens (viruses).

First, the sera from the vaccination group were tested with an antigen closely related to the H5 of the vaccine (homologous antigen): A/Ch/Indonesia/7/03 EPI_ISL_11512. Additionally, all sera were tested against the HPAI H5N1 inoculum (heterologous antigen). The COBRA H5 sequence is 92.91% identical to the HA of the inoculum (heterologous) and 98.23% identical to the HA gene of Indonesia (Homologous) (not taking into

account the multi-basic cleavage site that is deleted in COBRA). All tests were performed in duplicate, and the results of the two tests were averaged for analysis.

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

To determine virus shedding, choanal and cloacal swabs were taken. During collection, the swabs were immediately placed in 2 ml TBP and frozen at -80°C until processing. After thawing, 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 [7]. 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, values <Log $10^{1.7}$ eqEID$_{50}$/ml were considered negative.

3.10 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 day 0, 1, 3, 7, 10 and 14. Whole blood was fixed in TransFix® reagent and stained using BD truecount tubes as previously described [8]. 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. In 2 chickens in the control group, T cells were not recognized by the anti-CD3 antibody. In these birds 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 was used to identify the T cells. This strategy does not allow analysis of γδ T cells, therefore γδ T cells were determined in 8 out of 10 chickens from the control group.
After the staining, samples were fixed with 4% paraformaldehyde and resuspended in FACSbuffer 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.

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

3.11 Statistical analysis

3.11.1 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 bird per day;
2) the infectious period (T) which is the average period of days an infected bird 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 bird.

For the estimation of 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. These data were analyzed using a generalized linear model (GLM) with a binomial error distribution and a complementary log-log link as described by [9]. Based on the previous transmission study [1] and the observations on the inoculated chickens, we considered a one day latent period (time from becoming infected to becoming contagious).

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 the data (judged by the model with lowest AIC) was a lognormal distribution.

The reproduction number R was estimated as the product of β and T. The 95% confidence intervals for R0 were derived by Monte Carlo (MC) simulations (1000 replications) assigning to β and T lognormal distributions, using the parameters from the GLM and the survival regression model respectively.

3.11.2 Whole blood staining

Statistical differences of the whole blood staining 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 between the groups were analysed using Mann-Whitney U tests. Differences in time were determined using a Friedman test followed by Dunn's multiple comparison testing. A p-value of <0.05 was considered statistically significant.

4 Results

4.1 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 group.

For the analysis, a chicken is considered infected when the following parameters applied:

- 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
- If the chicken survived the challenge, additional parameters were:
    - 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 used in our previous study [1].

In the control group, all inoculated chickens (n=10), from subgroups A (n=5) and B (n=5) shed virus from day 1 onward. In control group A, virus was transmitted to 2 of the contact chickens and in group B to 5 contact chickens. The estimated R for the control group was 1.3 (95% CI 0.58–2.87), the transmission rate parameter (β) was 0.47 (0.21-0.87) and the infectious period was 2.79 (1.61-4.82) days (table 2).

Table 2 shows the total number of infected chickens per group. In control groups A and B, 4/5 and 5/5 inoculated chickens and 2/5 and 5/5 contact chickens, respectively, meet these criteria and were considered infected. The one non-infected inoculated chicken that survived in control group A was positive for virus shedding, but had no positive result in the NP-ELISA and did not show a ≥3 increase in heterologous HI titer. The three non-infected contact chickens of control group A were not positive for virus shedding for ≥2 days and had no serological response (table 2).
None of the VAXXITEK HVT+IBD+H5 vaccinated chickens in both groups (A and B) became infected after inoculation.

Table 2: *Transmission parameters and number of chickens infected based on three parameters: 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. If the chicken survived the challenge, additional parameters were: a positive NP-ELISA result (after 21 days) and/or showed an increase of ≥3 $\log_2$ in the heterologous HI-titer. SD= standard deviation. a, one chicken was negative by definition, but was shedding virus for ≥2 days.*

Treatment	Inoculated infected	Inoculated not infected	Contact infected	Contact not infected	Beta (±SD) (A&B combined)	Infectious period (days) (±SD) (A&B combined)	R-value (±SD) (A&B combined)
Control A	4	1	2	3	0.47 (0.21-0.87)	2.79 (1.16 – 4.82)	1.3 (0.58-2.87)
Control B	5	0	5	0			
VAXXITEK HVT+IBD+H5 A	0[a]	5	0	5			
VAXXITEK HVT+IBD+H5 B	0[a]	5	0	5			

4.2 Survival and protection against clinical signs after inoculation

To assess the effectiveness of the vaccines 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, 4/5 inoculated chickens died within 3 days after inoculation, and one inoculated chicken survived for the remainder of the study. Two contact chickens died at day 7 and day 10 respectively. The remaining three contact chickens survived until the end of the study and did not show any clinical sign, and were not scored positive for infection during the study. In Control group B, all inoculated chickens died within 3 days after inoculation. From day 5 onward, mortality of the contact chickens was observed, and ultimately, all chickens died between day 5 and day 11 of the study (Figure 2).
All chickens in the VAXXITEK HVT+IBD+H5 groups remained healthy and survived the challenge.

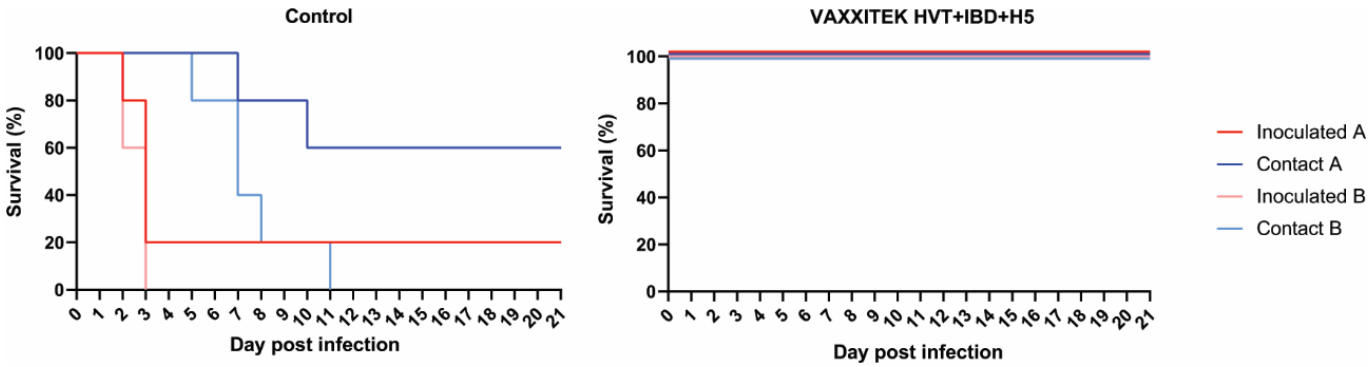

Figure 2: *Survival curve of control and vaccinated groups. Groups A and B are shown in one graph, where group A is clear line, group B is transparent line. Inoculated chickens are shown in red, contact chickens are shown in blue.*

4.3 Virus shedding

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

In the control groups, virus shedding through the choana already started at day 1 until day 3 for the inoculated chickens. The contact chickens first started shedding through the choana from day 3 until day 11 (figure 3, blue bars/dots). Values at days 13 and 21 were below the threshold.
In the cloaca, shedding of the inoculated chickens was detected on day 2 and day3, whereas the contact chickens shed virus from day 3 until day 11 (figure 3).
In control group A, all (5/5) inoculated chickens and 2/5 contact chickens were scored positive for virus shedding. In control group B, all inoculated (5/5) and contact chickens (5/5) were positive for virus shedding (figure 4).

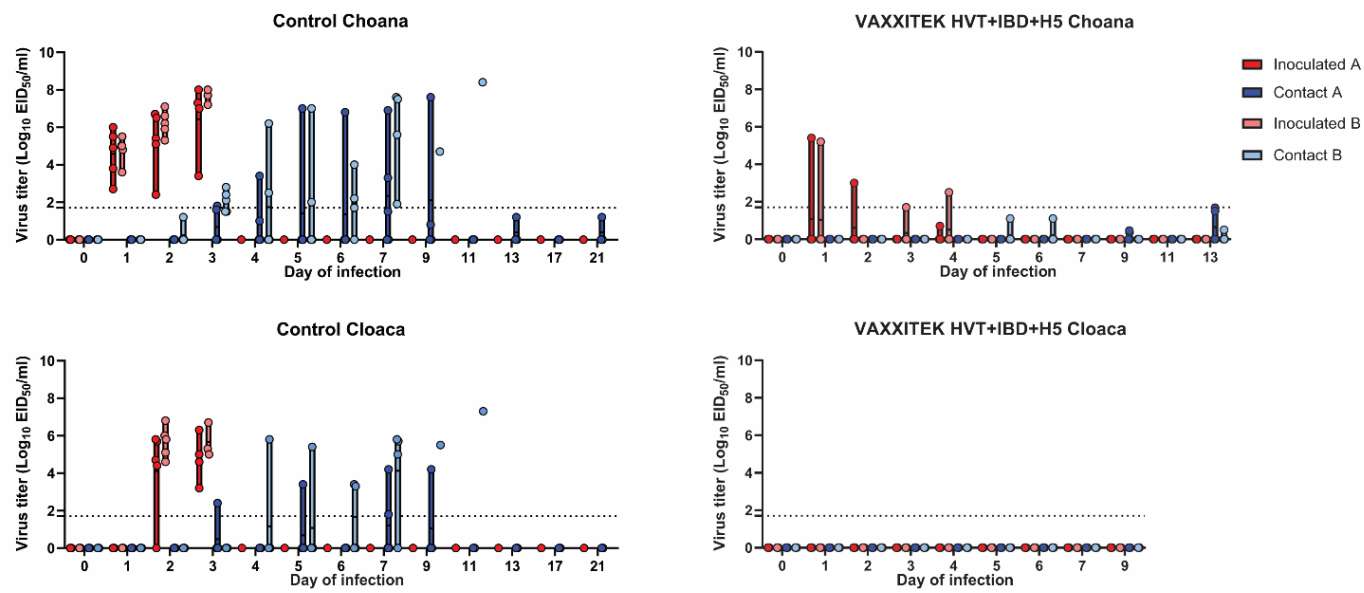

Figure 3: *The titer of virus excretion from the inoculated chickens (red) and contact chickens (blue) measured in choanal and cloacal swabs. For each group, subgroups A and B are shown separately. The detection limit of the PCR is 1.7 (*Log $10^{1.7}$ $eqEID_{50}$/ml*) (dotted line), and titers* < Log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

In VAXXITEK HVT+IBD+H5 groups A and B 1/5 inoculated chickens shed virus for 2 days and 3 days through the choana, respectively (figure 3). None of the contact chickens shed virus through the choana (0/10). No shedding was detected at any of the timepoints through the cloaca (0/20). In each of the VAXXITEK HVT+IBD+H5 groups A and B, 1/5 inoculated chicken was considered positive for shedding virus, but none of the contact chickens were positive for shedding the virus (0/10) (Figure 4).

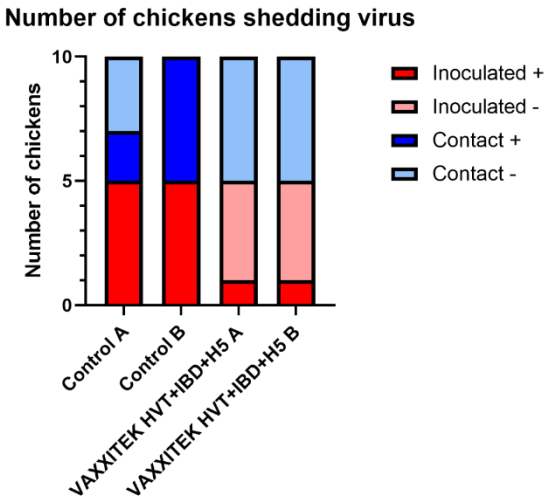

Figure 4: *The number of chickens per group for which ≥2 days virus shedding with a titer of* ≥Log $10^{1.7}$ $eqEID_{50}$/ml *was measured during the study. Red indicates inoculated chickens, blue for contact chickens. Bright color is positive for virus shedding (+), transparent negative for virus shedding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

The total amount of virus excreted (Area under the curve; AUC) and the peak of virus excretion were determined from the chickens that shed virus for ≥2 days with a minimum titer of ≥Log $10^{1.7}$ $eqEID_{50}$/ml (Table 3). Looking at the amount of virus excretion in the control group, the mean AUC and mean peak in the choana was Log $10^{6.27}$ $eqEID_{50}$/ml and Log $10^{6.18}$ $eqEID_{50}$/ml respectively. In the cloaca the mean AUC and mean peak were Log $10^{4.17}$ $eqEID_{50}$/ml and Log $10^{4.12}$ $eqEID_{50}$/ml respectively.
In the VAXXITEK HVT+IBD+H5 vaccine group two chickens shed virus through the choana, with mean AUC and mean peak shedding of Log $10^{5.31}$ $eqEID_{50}$ and Log $10^{5.31}$ $eqEID_{50}$/ml respectively.

Table 3: *The total amount of virus excreted (Area under the curve; AUC) and the peak of virus excretion of the positive animals.*

Group	Positive/total number of chickens	Swab	Mean AUC Log10 eqEID$_{50}$/ml (SD)	Mean peak Log 10 eqEID$_{50}$/ml (SD)
Control group (A&B)	20/20	Choana	6.27 (2.20)	6.18 (2.26)
		Cloaca	4.17 (2.34)	4.12 (2.30)
VAXXITEK HVT+IBD+H5 (A&B)	2/20	Choana	5.31 (0.11)	5.31 (0.11)
		Cloaca	-	-

4.4 Humoral immune Response

4.4.1 HI titers and NP-ELISA prior inoculation

From the blood collected from the chickens at day -7, the absence of antibodies against avian influenza virus were demonstrated in the NP-ELISA in all chickens before inoculation. In addition, this blood serum was tested using Hemagglutination Inhibition (HI) assay to determine the heterologous and homologous H5-antibody titer after vaccination. In Figure 5 and appendix 1, the HI titers are separately demonstrated for all the inoculated and contact chickens. None of the chickens in the control group had a positive HI result, demonstrating the absence of antibodies against H5-protein, prior to inoculation.

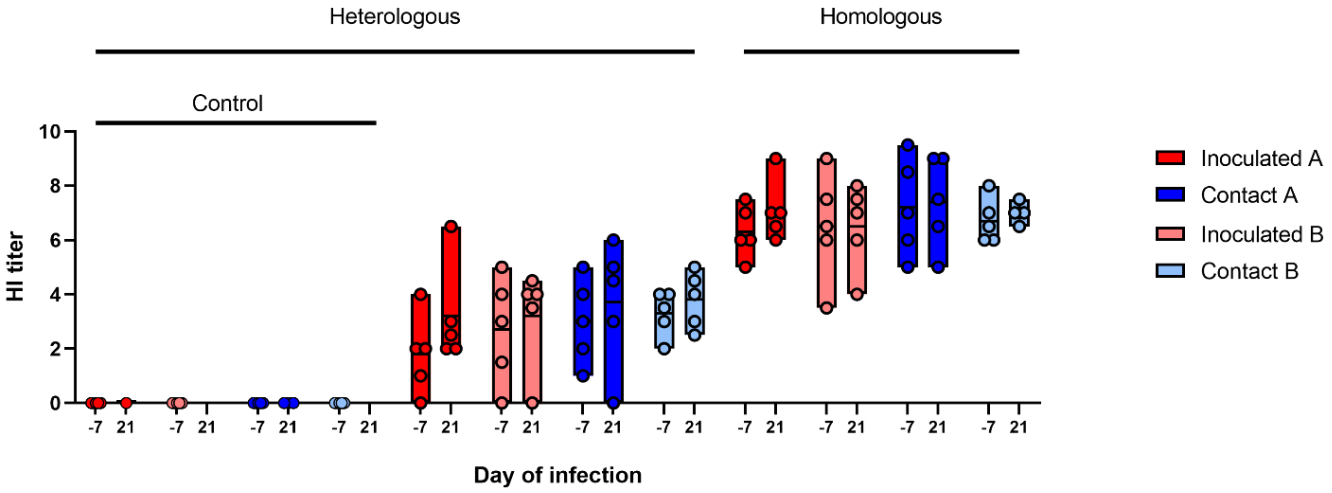

Figure 5: *The HI titer (Log$_2$) of the inoculated and contact chickens of the different groups. The blood serum collected before inoculation (day -7) and after inoculation (day 21) were tested in the HI against an antigen that is highly related to the vaccine virus (homologous) and the current HPAI H5N1 inoculated virus (heterologous). Each dot is an individual chicken.*

On day -7, prior inoculation, the mean average heterologous HI titer of inoculated vaccinated chickens was Log$_2$ 1.80 group A (4/5, HI titer), Log$_2$ 2.70 VAXXITEK HVT+IBD+H5 B (5/5, HI titer) and for the contact chickens on average Log$_2$ 3.00 VAXXITEK HVT+IBD+H5 A (4/5, HI titer) and Log$_2$ 2.75 VAXXITEK HVT+IBD+H5 B (5/5, HI titer). All 20 chickens in the VAXXITEK HVT+IBD+H5 vaccinated group had a homologous HI titers. The mean titers were Log$_2$ 6.30 and Log$_2$ 6.50 in group A and B, respectively. The mean titer in contact group A was Log$_2$ 7.20 and in contract group B Log$_2$ 5.58.

4.4.2 HI titers and NP-ELISA post inoculation

4.4.2.1 NP-ELISA post inoculation

On the last day of the study, day 21, blood was collected from all chickens that survived, and the serum was tested in the NP-ELISA. These results provide information on the number of chickens that produced antibodies in response to the inoculation (Figure 6a). None of the chickens in control group A showed a positive result in the NP-ELISA. All chickens in control group B died before the end of the study, therefore no serological tests could be performed. In the VAXXITEK HVT+IBD+H5 groups, all of the inoculated and contact chickens tested negative in the NP-ELISA.

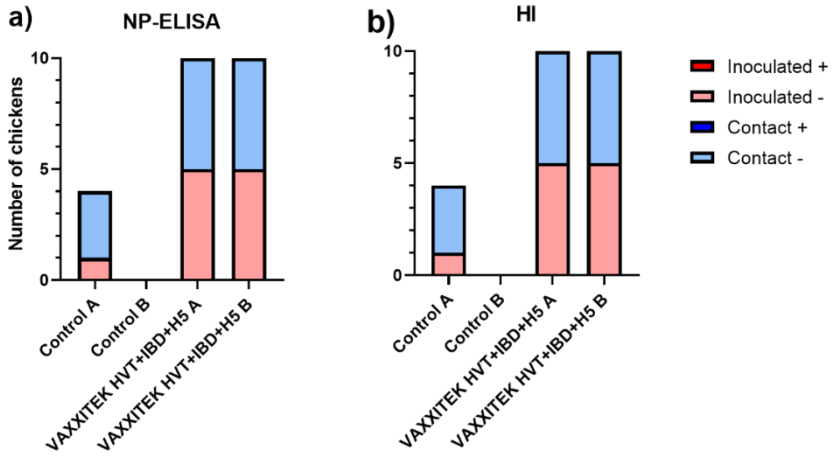

Figure 6: *The number of chickens that are positive on the serology performed on the blood collected on the last day of the study (day 21). a) The results of the NP-ELISA. b) The results of the HI-test. The positive chickens have an increased log_2 HI titer of ≥3 in the blood on day 21 compared to day -7. Red indicates inoculated chickens, blue for contact chickens. Bright color is positive (+), transparent is negative (-).*

4.4.2.2 Hemagglutination Inhibition (HI) Assay

The blood collected at day 21 was also tested in the HI assay. None of the survived chickens in the control groups had a HI-titer after challenge (Figure 6b, appendix 2). A chicken was scored positive for HI when an increased heterologous HI titer of log_2 ≥3 was obtained.

On day 21, post inoculation, the mean heterologous HI titer of inoculated chickens in VAXXITEK HVT+IBD+H5 group A was Log_2 3.20 (5/5, HI titer), and in group B Log_2 3.20 (4/5, HI titer) and the mean titer in the contact chickens of group A was Log_2 3.70 (4/5, HI titer) and group B Log_2 3.17 (5/5, HI titer). All 20 chickens in the VAXXITEK HVT+IBD+H5 vaccinated group had a homologous HI titer. The mean average homologous titer of the inoculated chickens in group A was Log_2 7.10 (5/5, HI titer), group B Log_2 6.50 (5/5, HI titer), and for the contact chickens in group A Log_2 7.40 (5/5, HI titer) and group B Log_2 5.83 (5/5, HI titer). For none of the chickens an increased heterologous HI titer ≥3 was obtained on day 21 compared to day -7 (Figure 5 and 6b).

4.5 Cellular immune response

4.5.1 Absolute numbers of T cells in the blood of vaccinated chickens compared to control group

Before (day 0) and post inoculation (day 1) of the study, the number of T cells in the blood of VAXXITEK HVT+IBD+H5 vaccinated chickens was compared to the number of T cells in the blood of chickens in the control group.
Before inoculation, chickens that received the VAXXITEK HVT+IBD+H5 vaccine tended to have a numerical higher amount of T cells (Figure 7A), specifically CD4 T cells (Figure 7B), CD7 T cells (Figure 7C) and γδ T cells (Figure 7D) at day 0, although this difference was not significant. However, at day 1, a significantly higher number of T cells, and numbers of T cell subsets were observed in AI vaccinated chickens compared to the control group (Figure 7D). At other timepoints, no significant differences were observed as only one of the control chickens survived (data not shown). These data indicate that vaccination with VAXXITEK HVT+IBD+H5 affects the number of T cells, as well as the number of the T cell subsets 1 day post inoculation.

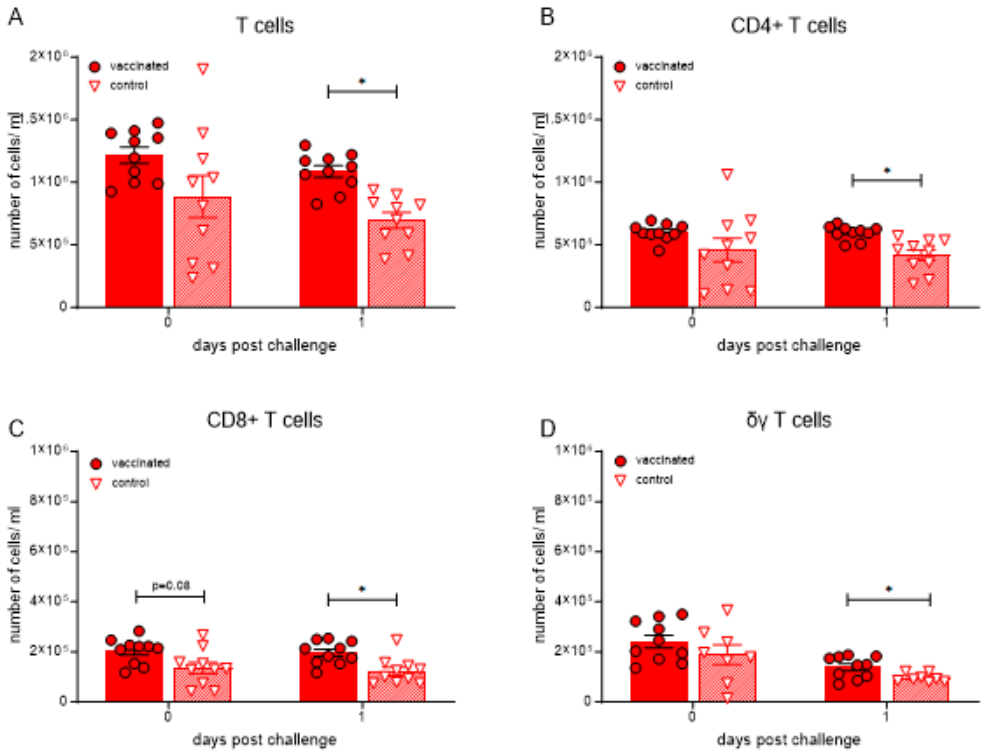

Figure 7: *Absolute numbers of T cells in the blood of vaccinated and control group chickens. Absolute numbers of total T cells (A), CD4 T cells (B), CD8 T cells (C) and γδ T cells (D) were quantified in the blood of vaccinated and chickens in the control group. Mean ± SEM of 10 chickens is shown (Groups A & B are combined). Each dot/triangle represents an individual chicken. Significant differences compared to day 0 (p<0.05) are indicated (*).*

4.5.2 Number of T cells over time in vaccinated chickens after inoculation

To investigate whether inoculation would affect the total number of T cells in the blood of VAXXITEK HVT+IBD+H5 vaccinated birds, the number of T cells, as well as numbers of CD4 T cells, CD8 T cells and γδ T cells was quantified. In general the inoculation did not result in an increase in the number of total T cells (Figure 8A). Also numbers of CD4 T cells (Figure 8B), CD8 T cells (Figure 8C) and γδ T cells (Figure 8D) did not show a significant change over time. Only at day 1, the number of γδ T cells was significantly lower and at day 10, numbers of total T cells and CD4 T cells were significantly lower when compared to day 0.

A comparison of the amount of T cells between the different subsets (Figure 8A-D) showed that CD4+ T cells comprise the majority of T cells, while numbers of CD8+ T cells and γδ T cells were similar but lower compared to the amount of CD4 T cells.

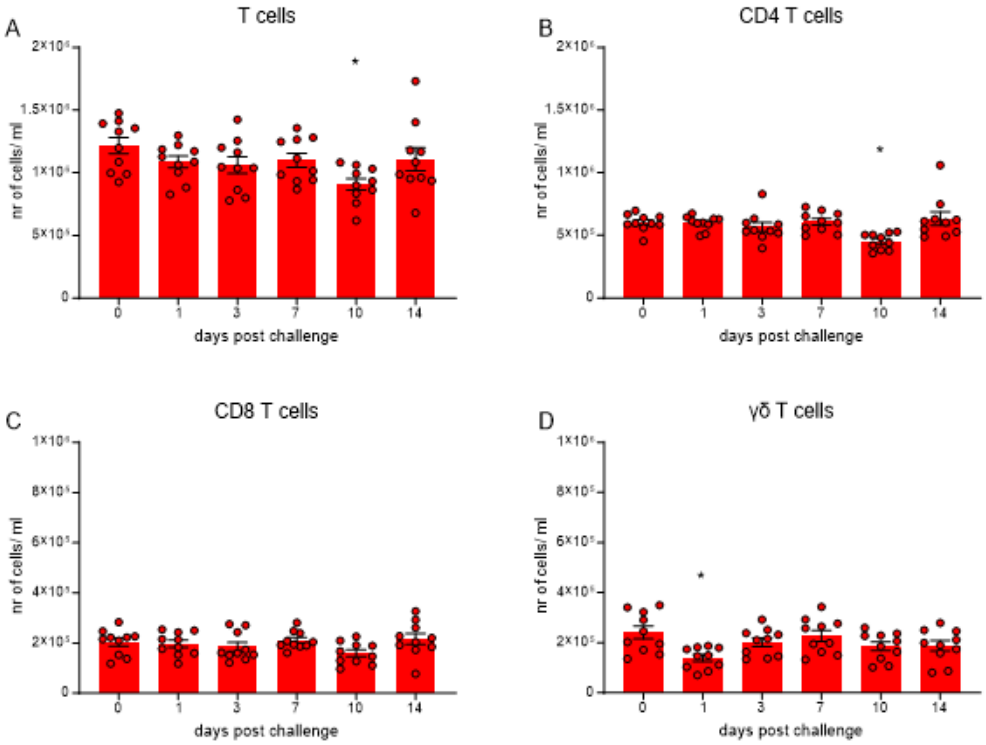

Figure 8: *Absolute numbers of T cells in the blood at different timepoints post inoculation. At several timepoints post inoculation, absolute numbers of total T cells (A), CD4 T cells (B), CD8 T cells (C) and γδ T cells (D) were quantified in the blood of vaccinated chickens. Mean ± SEM of 10 chickens is shown (Groups A & B are combined). Each dot represents an individual chicken. Significant differences compared to day 0 ($p<0.05$) are indicated (*).*

4.5.3 Number of activated T cells over time in vaccinated chickens

Next, the effect of the inoculation on the number of activated T cells in the blood was assessed by quantifying the number of CD25+ T cells. CD25 is the α chain of the high-affinity IL-2 receptor, expressed on the cell surface and known to be upregulated upon activation [10].
Interestingly, a peak in the amount of all CD25+ T cell subsets was observed at day 7 compared to day 0 (Figure 9). In addition, significant increases were observed at day 3 for CD25+CD8 T cells and CD25+γδ T cells (Figure 9C-D) and at day 10 for total CD25+ T cells, CD25+CD4 T cells and CD25+γδ T cells (Figure 9A, B and D). At day 14, numbers reached similar levels as before inoculation.

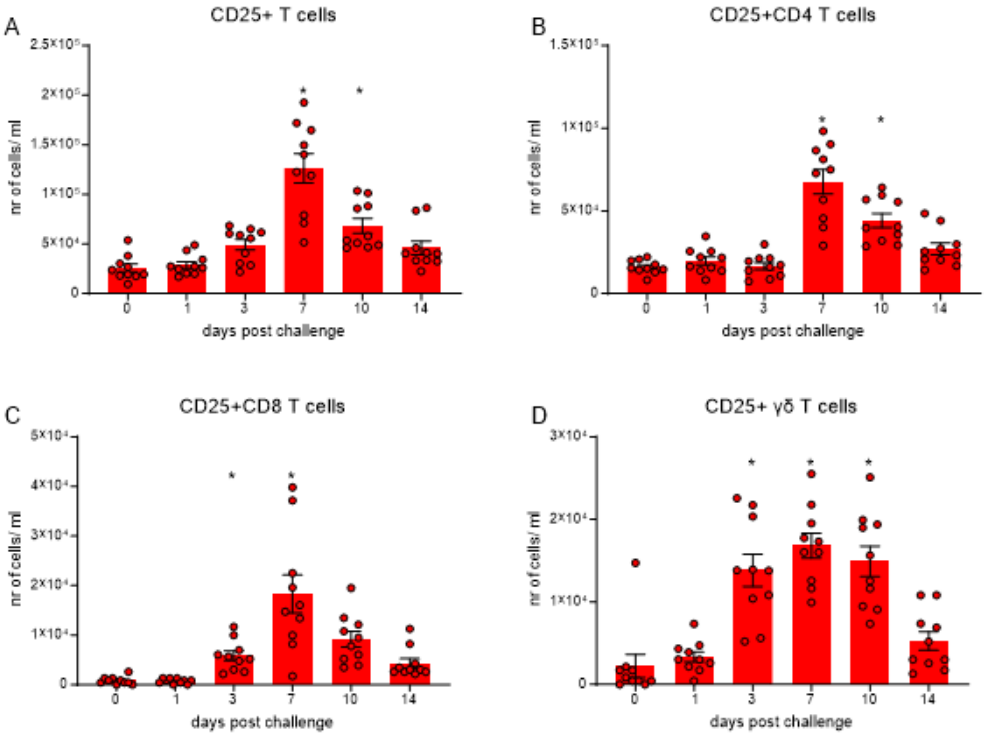

Figure 9: *Absolute numbers of activated T cells in the blood at different timepoints post inoculation. At several timepoints post inoculation, absolute numbers of CD25+ T cells (A), CD25+CD4 T cells (B), CD25+CD8 T cells (C) and CD25+γδ T cells (D) were quantified in the blood of vaccinated chickens. Mean ± SEM of 10 chickens is shown (Groups A & B are combined). Each dot represents an individual chicken. Significant differences compared to day 0 (p<0.05) are indicated (*).*

5 Discussion

This progress report describes the first transmission study in a series of four studies. This longitudinal study aims to determine whether vaccination under field circumstances can protect layer flocks for the duration of a production period against HPAI H5N1 virus (clade 2.3.4.4b) sustained transmission (R<1). This transmission study was performed at 8-weeks post vaccination with laying hen pullets which were housed under field conditions until challenge. In addition to the main output which was the assessment of transmission, other variables such as chicken survival, virus shedding and humoral and cellular immune responses were studied. The estimated R for the control group was 1.3 (95% CI 0.58–2.87), and transmission in VAXXITEK HVT+IBD+H5 could not be quantified because none of the inoculated chickens became infected.

In the previous study [1], all inoculated chickens in the control group became infected and died, however, in this study, one chicken in the control group shed virus for three consecutive days, survived but was seronegative at the end of the experiment. The estimated R in the control group in this experiment was lower, although not significant, than that estimated R previously [1]. Differences between these studies are unlikely explained by the inoculum, as the titer of the inoculum was confirmed before and after inoculation and the inoculation was performed similarly to the previous study by qualified personnel. However, in the previous study [1], the Lohmann Brown Classic layer hen chicks were housed under laboratory conditions after vaccination until they were challenged. In the present study, chickens from a different breed (Novogen Brown Light laying hen pullets) were housed in the field and only one week prior to inoculation transported to the lab facilities. In the lab facilities, housing conditions were as comparable to the field as possible.
Note that for the specific isolate (HPAI H5N1 virus clade 2.3.3.4b) used for inoculation in the previous and current study transmission characteristics are yet to be fully understood. This study contributes to improve our quantitative knowledge on transmission of this virus.

We could not assess transmission in the vaccinated group. Looking at the other parameters we measured in this study, vaccinated chickens were protected against challenge by inoculation with the HPAI H5N1 virus, since based on our definition of infection (positive for virus shedding ≥Log $10^{1.7}$ $eqEID_{50}$/ml in PCR for ≥2 days), has a positive NP-ELISA result and showed an increase of $\log_2$ ≥3 in the HI-titer, none of the vaccinated chickens became infected. This definition is consistent with the definition used in our previous study [1].
Consequently, no mortality was observed, whereas mortality in the control groups were 9/10 for the inoculated chickens and 7/10 contact chickens.

Although we did consider all vaccinated and subsequently inoculated chickens not infected (based on our three parameters), 2 of the inoculated chickens were positive for shedding between for 2 or more days (2-3 days). Viral RNA was only detected in the choana swabs and none of the cloaca swabs were positive for viral RNA. Because of the small number of birds that were positive for shedding, comparisons with the control group is not informative. Also, none of the sera obtained in this study gave a positive result in NP-ELISA and none of the chickens had an increase in heterologous HI titer ≥3 against the challenge virus. Thus there is uncertainty whether these birds were effectively infected and were shedding viable virus.

Finally, the number of T cells and T cell subsets in vaccinated chickens was significantly higher at day 1 after inoculation compared to the control group. We have not detected an increase in the absolute numbers of T cells in the blood of vaccinated and inoculated chickens. Numbers of activated T cells, both CD4 and CD8 and γδ T cells were significantly increased upon inoculation with a peak at day 7 post inoculation.

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Appendix 1 HI titers

Appendix 1: *The HI titer (Log_2) of the inoculated and contact chickens of the different groups before (day -7) and after inoculation (day 21). The blood serum was tested in the HI against an antigen that is highly related to the vaccine virus (homologous) and the current HPAI H5N1 (heterologous).*

Group		Inoculated/Contact	Antigen	Mean (Log_2)	SD	Day of collection
Control	A	Inoculated	Heterologous	0	0	-7
Control	B	Inoculated	Heterologous	0	0	-7
Control	A	Contact	Heterologous	0	0	-7
Control	B	Contact	Heterologous	0	0	-7
VAXXITEK HVT+IBD+H5	A	Inoculated	Heterologous	1,8	1,48	-7
VAXXITEK HVT+IBD+H5	B	Inoculated	Heterologous	2,7	1,99	-7
VAXXITEK HVT+IBD+H5	A	Contact	Heterologous	3	1,58	-7
VAXXITEK HVT+IBD+H5	B	Contact	Heterologous	2,75	0,84	-7
VAXXITEK HVT+IBD+H5	A	Inoculated	Homologous	6,3	0,97	-7
VAXXITEK HVT+IBD+H5	B	Inoculated	Homologous	6,5	2,03	-7
VAXXITEK HVT+IBD+H5	A	Contact	Homologous	7,2	1,82	-7
VAXXITEK HVT+IBD+H5	B	Contact	Homologous	5,58	0,84	-7
Control	A	Inoculated	Heterologous	0	0	21
Control	B	Inoculated	Heterologous	0	0	21
Control	A	Contact	Heterologous	0	0	21
Control	B	Contact	Heterologous	0	0	21
VAXXITEK HVT+IBD+H5	A	Inoculated	Heterologous	3,2	1,89	21
VAXXITEK HVT+IBD+H5	B	Inoculated	Heterologous	3,2	1,82	21
VAXXITEK HVT+IBD+H5	A	Contact	Heterologous	3,7	2,33	21
VAXXITEK HVT+IBD+H5	B	Contact	Heterologous	3,17	1,04	21
VAXXITEK HVT+IBD+H5	A	Inoculated	Homologous	7,1	1,14	21
VAXXITEK HVT+IBD+H5	B	Inoculated	Homologous	6,5	1,58	21
VAXXITEK HVT+IBD+H5	A	Contact	Homologous	7,4	1,71	21
VAXXITEK HVT+IBD+H5	B	Contact	Homologous	5,83	0,35	21

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