Appendix of report: Vaccination of poultry with HVT-based H5 vaccine against highly pathogenic avian influenza (HPAI) H5N1 virus (clade 2.3.4.4b)

VAXXITEK HVT+IBD+H5 and VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccine

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1 Appendix Work Package 1

1.1 Appendix Table 1.1

*Vaccination program for test groups 2 (VAXXITEK HVT+IBD+H5), 3 (*VAXXITEK HVT+IBD+H5 and Volvac® B.E.S.T. AI+ND) *and 4 (control).*

Test group	Pathogen*	Day of submission	Way of submission
2,3	HVT + AI + IBD#	0	injection (sc)
2,3,4	IBV	0	spray
2,3,4	Coccidiosis	0	gel spray
2,3	Marek's disease (Rispens)	0	injection (sc)
4	Marek's disease + IBD	0	injection (sc)
2,3,4	SE/ST	7	drinking water
2,3,4	ND	14	spray
2,3,4	IBV	28	spray
2,3,4	SE/ST	42	drinking water
2,3,4	ND	49	spray
2,3,4	IBV	65	spray
2,3,4	ILT virus	84	eye drop
2,3,4	FP virus	84	wingweb
2,3,4	AMPV + IBV + ND + EDS	84	injection (im)
3	AI + ND	84	injection (sc)
2,3,4	IBV	105	spray and drinking water
2,3,4	SE/ST	112	drinking water
2,3,4	AEV**	126 (farm A)	drinking water
2,3,4	AEV**	132 (farm B)	drinking water

* Turkey herpesvirus (HVT), infectious bronchitis virus (IBV), infectious bursal disease virus (IBD), Salmonella *Enteritidis* (SE) Salmonella *Typhimurium* (ST), Newcastle Disease (ND), infectious laryngotracheitis virus (ILT virus), fowl pox virus (FP virus), avian metapneumovirus (AMPV), egg drop syndrome virus (EDS), and avian encephalitis virus (AEV).

** The AEV vaccination was planned to be administered at day 84, but due to a miscommunication, this has been performed at day 126 (farm A) and day 132 (farm B).

1.2 Appendix Tables 1.2 a, b and c

a) H5 HI-titers obtained in sera of the non AI-vaccinated chickens (Group 4) in the field using antigen A/Ch/Indonesia/7/03 EPI_ISL_11512.

Week	Location	Mean	Median	Min	Max	Range	SD	# of sera	% HI titer ≥3	% HI titer ≥4	% HI titer ≥5	% HI titer ≥6
5	Rearing	0.0	0	0	0	0	0.0	10	0	0	0	0
9	Rearing	1.0	1	1	1	0	0.0	10	0	0	0	0
14	Rearing	0.0	0	0	0	0	0.0	10	0	0	0	0
17	Rearing	0.0	0	0	0	0	0.0	10	0	0	0	0
22	A	0.0	0	0	0	0	0.0	10	0	0	0	0
22	B	0.1	0	0	1	1	0.3	9	0	0	0	0
26	A	0.0	0	0	0	0	0.0	10	0	0	0	0
26	B	0.0	0	0	0	0	0.0	9	0	0	0	0
30	A	0.0	0	0	0	0	0.0	10	0	0	0	0
31	B	0.0	0	0	0	0	0.0	10	0	0	0	0
35	A	0.0	0	0	0	0	0.0	10	0	0	0	0
35	B	0.0	0	0	0	0	0.0	10	0	0	0	0
39	A	0.0	0	0	0	0	0.0	9	0	0	0	0
39	B	0.0	0	0	0	0	0.0	8	0	0	0	0
43	A	0.2	0	0	2	2	0.6	10	0	0	0	0
44	B	0.0	0	0	0	0	0.0	10	0	0	0	0
47	A	0.0	0	0	0	0	0.0	10	0	0	0	0
48	B	0.0	0	0	0	0	0.0	10	0	0	0	0
52	A	0.0	0	0	0	0	0.0	10	0	0	0	0
52	B	0.0	0	0	0	0	0.0	10	0	0	0	0
56	A	0.0	0	0	0	0	0.0	10	0	0	0	0
56	B	0.0	0	0	0	0	0.0	10	0	0	0	0
60	A	0.0	0	0	0	0	0.0	10	0	0	0	0
61	B	0.0	0	0	0	0	0.0	10	0	0	0	0
64	A	0.0	0	0	0	0	0.0	9	0	0	0	0
65	B	0.1	0	0	1	1	0.3	10	0	0	0	0
69	A	0.0	0	0	0	0	0.0	9	0	0	0	0
69	B	0.0	0	0	0	0	0.0	10	0	0	0	0
73	A	0.0	0	0	0	0	0.0	10	0	0	0	0
74	B	0.0	0	0	0	0	0.0	10	0	0	0	0
77	A	0.0	0	0	0	0	0.0	10	0	0	0	0
78	B	0.0	0	0	0	0	0.0	10	0	0	0	0
82	A	0.0	0	0	0	0	0.0	10	0	0	0	0
82	B	0.0	0	0	0	0	0.0	10	0	0	0	0
84	A	0.0	0	0	0	0	0.0	10	0	0	0	0
85	B	0.0	0	0	0	0	0.0	10	0	0	0	0

b) H5 HI-titers obtained in sera of the chickens vaccinated with VAXXITEK HVT+IBD+H5 (Group 2) in the field using antigen A/Ch/Indonesia/7/03 EPI_ISL_11512.

Week	Location	Mean	Median	Min	Max	Range	SD	# of sera	% HI titer ≥3	% HI titer ≥4	% HI titer ≥5	% HI titer ≥6
5	Rearing	4.5	4	0	11	11	1.9	120	88	73	43	23
9	Rearing	6.1	6	1	11	10	2.1	120	98	94	80	54
14	Rearing	7.0	7	3	11	8	1.7	120	100	98	93	81
17	Rearing	6.7	7	2	11	9	1.4	117	99	99	95	83
22	A	6.5	7	0	11	11	1.7	120	99	95	88	74
22	B	7.2	7	3	11	8	1.5	118	100	99	98	90
26	A	6.0	6	3	10	7	1.4	120	100	97	88	67
26	B	7.0	7	3	11	8	1.6	120	100	99	94	84
30	A	6.4	6	3	11	8	1.8	120	100	93	90	70
31	B	6.5	6	0	11	11	2.0	120	98	96	87	68
35	A	6.5	7	2	11	9	1.7	120	99	96	88	74
35	B	6.3	6	0	11	11	1.6	120	99	98	89	73
39	A	7.0	7	2	11	9	2.0	120	99	98	93	80
39	B	7.1	7	3	11	8	1.8	120	100	99	94	82
43	A	6.8	7	3	11	8	1.4	120	100	99	95	84
44	B	6.6	7	3	11	8	1.5	120	100	99	93	76
47	A	7.4	7	2	11	9	2.1	120	99	97	94	84
48	B	7.0	7	3	11	8	1.8	119	100	99	94	76
52	A	7.5	8	3	11	8	1.8	120	100	99	94	89
52	B	7.4	7	0	11	11	2.1	120	99	99	96	80
56	A	7.2	7	2	11	9	2.2	120	99	96	90	79
56	B	7.4	7	0	11	11	2.2	120	99	98	93	79
60	A	6.2	6	3	11	8	1.6	120	100	94	85	66
61	B	6.9	7	3	11	8	1.7	120	100	99	91	79
64	A	6.3	6	2	11	9	1.8	116	97	93	83	73
65	B	6.7	7	0	11	11	1.9	118	99	98	87	75
69	A	7.2	7	2	11	9	2.0	120	99	98	91	83
69	B	6.9	7	2	11	9	1.9	117	98	97	91	78
73	A	7.6	8	4	11	7	1.6	120	100	100	98	90
74	B	6.9	7	0	10	10	1.5	120	99	98	95	84
77	A	7.5	8	2	11	9	2.0	120	99	98	94	86
78	B	7.5	7	4	11	7	1.8	120	100	100	96	88
82	A	7.4	8	2	11	9	1.9	120	98	98	97	84
82	B	7.5	7	0	11	11	1.8	120	99	99	96	88
84	A	7.9	8	2	11	9	2.0	120	99	99	95	86
85	B	7.8	8	0	11	11	2.1	120	99	98	96	85

c) H5 HI-titers obtained in sera of the chickens vaccinated with VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND (Group 3) in the field using antigen A/Ch/Indonesia/7/03 EPI_ISL_11512.

Week	Location	Mean	Median	Min	Max	Range	SD	# of sera	% HI titer ≥3	% HI titer ≥4	% HI titer ≥5	% HI titer ≥6
5	Rearing	4.6	4	1	11	10	1.9	119	93	72	45	23
9	Rearing	5.5	5	3	10	7	1.4	120	100	96	73	44
14	Rearing	7.5	8	4	11	7	1.5	120	100	100	98	89
17	Rearing	7.7	8	2	11	9	1.5	120	99	99	97	93
22	A	7.4	7	1	10	9	1.2	120	99	99	99	97
22	B	8.0	8	3	11	8	1.5	120	100	99	99	98
26	A	6.8	7	1	10	9	1.3	119	99	99	97	84
26	B	7.7	8	3	10	7	1.3	120	100	98	98	96
30	A	7.6	8	0	11	11	1.8	120	99	99	98	88
31	B	7.9	8	3	11	8	1.9	120	100	99	98	91
35	A	7.4	7	1	11	10	1.5	120	99	99	98	90
35	B	8.0	8	3	11	8	1.5	120	100	98	98	97
39	A	7.9	8	4	11	7	1.8	120	100	100	98	92
39	B	8.2	8	3	11	8	1.7	120	100	99	98	96
43	A	7.9	8	4	11	7	1.8	120	100	100	98	92
44	B	7.6	8	4	11	7	1.4	120	100	100	98	94
47	A	7.7	8	1	11	10	1.9	120	99	99	95	88
48	B	7.9	8	2	11	9	2.0	120	99	99	98	88
52	A	7.8	8	4	10	6	1.4	120	100	100	99	95
52	B	8.6	9	3	11	8	1.8	120	100	99	99	96
56	A	8.1	8	4	11	7	2.0	120	100	100	95	89
56	B	8.7	9	3	11	8	1.8	120	100	99	98	96
60	A	6.4	7	2	9	7	1.3	119	99	99	94	74
61	B	8.3	8	3	11	8	1.8	120	100	98	98	94
64	A	7.5	8	2	11	9	1.8	120	99	99	96	87
65	B	7.1	7	1	10	9	1.5	120	99	99	97	89
69	A	8.0	8	2	11	9	2.0	118	99	99	97	89
69	B	7.6	8	4	11	7	1.3	120	100	100	98	95
73	A	8.0	8	4	11	7	1.5	120	100	100	99	98
74	B	8.1	8	4	11	7	1.5	120	100	100	98	96
77	A	7.6	8	1	11	10	1.7	120	99	99	98	93
78	B	8.7	9	3	11	8	1.9	120	100	99	98	96
82	A	7.8	8	3	11	8	1.6	120	100	99	98	95
82	B	8.7	9	3	11	8	1.8	120	100	99	98	98
84	A	8.6	9	2	11	9	2.1	120	99	99	98	93
85	B	9.1	9	4	11	7	1.9	120	100	100	98	96

2 Appendix Work Package 2

2.1 Materials and Methods Transmission studies 3 and 4; inoculation was applied at 54 and 84 weeks of age respectively

2.1.1 Housing

All chickens were reared at commercial farm A & B, where the chickens with different vaccination strategies were kept separately from each other. Detailed information on housing in the field has been provided in the previous sections.

One week pre-challenge, a subgroup of chickens from both commercial farms of each test group was transported to the animal facilities of WBVR in Lelystad. For the first two experiments, upon arrival at WBVR, all chickens were randomly divided in their corresponding groups and received a wing tag for identification. The chickens from Farm A and Farm B were housed separately throughout the studies. For the third and fourth study, chickens were grouped based on their HI antibody titer. For the first week, chickens were housed under BSL2 conditions and from the day of challenge onwards, the chickens were housed under BSL3 conditions.

Housing during the studies were identical throughout all studies, except for the placement of laying boxes in studies 2, 3 and 4 in the pen throughout the entire study.

2.1.2 Chickens and Vaccinations

Detailed information about the chickens and vaccinations that the chickens received can be found in chapter 2.2.2 and 2.2.3 of the report and Appendix Table 1.1.

2.1.3 Inoculum

The same virus stock was used to infect the chickens in all transmission studies and is the same as used in our previous studies [1-5]. 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 specified 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 Log_2 7.72 (heterologous HI-titer). This prior information is relevant to understand why vaccinated chicken with a relatively high titer to vaccine antigen (homologous HI-titer) are still not protected against transmission of the challenge virus. The low titer group mentioned below would most likely be well protected against transmission of viruses antigenically similar to the vaccine antigen.

2.1.4 Study Design Transmission Studies

The study design of the transmission study is schematically presented in Appendix Figure 2.1. One week pre-challenge (-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

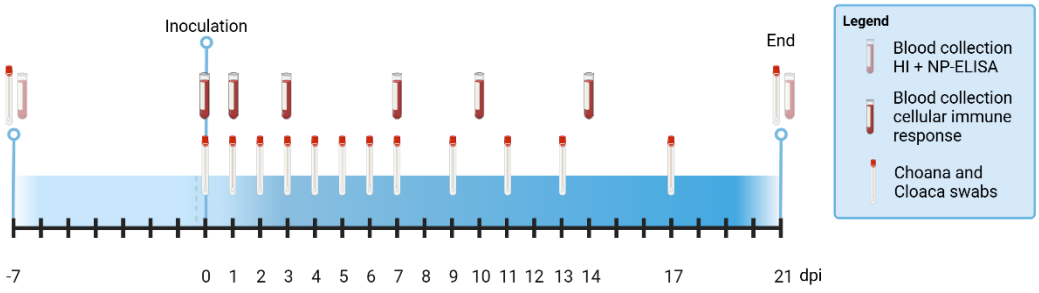
chickens of the non AI-vaccinated (control) group. Upon arrival at WBVR, the chickens were grouped (inoculated vs contacts) based on their homologous HI antibody titer and received a wing tag for identification. Chickens that originated from the different commercial farms were not mixed, so groups A 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.

Blood was collected on -7 and 0 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 (-7 dpi only). 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 6 vaccinated groups (replicates A & B, with each 3 vaccination regimes) 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 (Appendix Figure 2.1). 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 (see Work Package 3). At the end of the transmission study, all remaining chickens were euthanized under sedation and blood was collected for antibody detection in blood serum (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.



Appendix Figure 2.1 *Schematic overview of sample collection time points in the third and fourth transmission studies. HI: Hemagglutination Inhibition assay. Inoculation at 0 dpi was performed with 10^6 EID_{50}/ml HPAI H5N1 virus per chicken. In the transmission study at 24 weeks of age, the choanal and cloacal swabs were collected at -1 dpi, instead of 0 dpi.*

2.1.5 Nucleoprotein (NP) ELISA (in house WBVR)

To demonstrate the absence of prior exposure of the chickens to avian influenza and to confirm their eligibility for inclusion in the transmission studies, the NP-ELISA (in house WBVR) was performed. This 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 [6]. 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.

2.1.6 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 (-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 vaccinated chickens were tested against the HPAI H5N1 inoculum virus (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.

2.1.7 M-PCR (Matrix-gene Realtime Reverse Transcription Polymerase Chain Reaction, abbreviated to M-PCR)

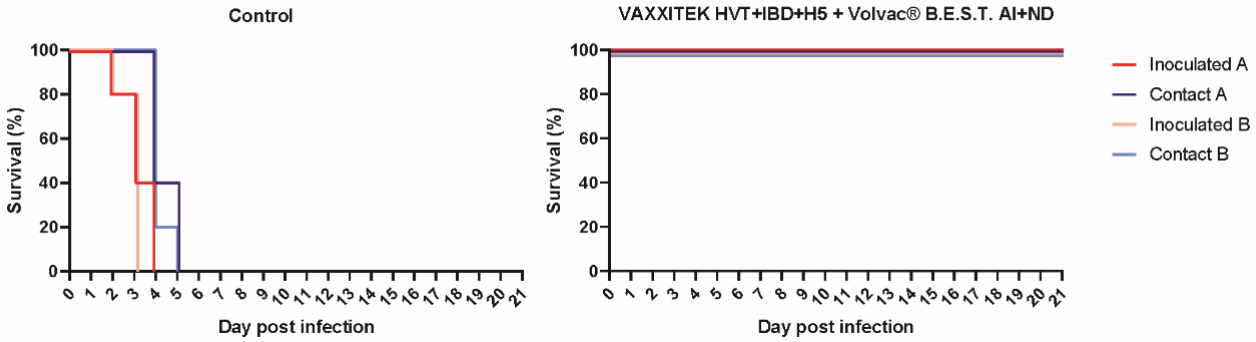
After sampling, the swabs were immediately placed in 2 ml Tryptose Phosphate Buffer (TBP) and frozen at -80°C until processing. After thawing the swabs, RNA was isolated using the MagNA Pure 96, and the RNA was tested in the PCR that detects the M-gene of influenza (M-PCR), as previously described [7]. 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 for this virus is around a titer of Log $10^{1.7}$ $eqEID_{50}$/ml, so values <Log $10^{1.7}$ $eqEID_{50}$/ml were considered negative.

2.2 Results Transmission studies 3 and 4; inoculation was applied at 54 and 84 weeks of age respectively

2.2.1 Survival

2.2.1.1 Transmission study 3, inoculation at 54 weeks of age

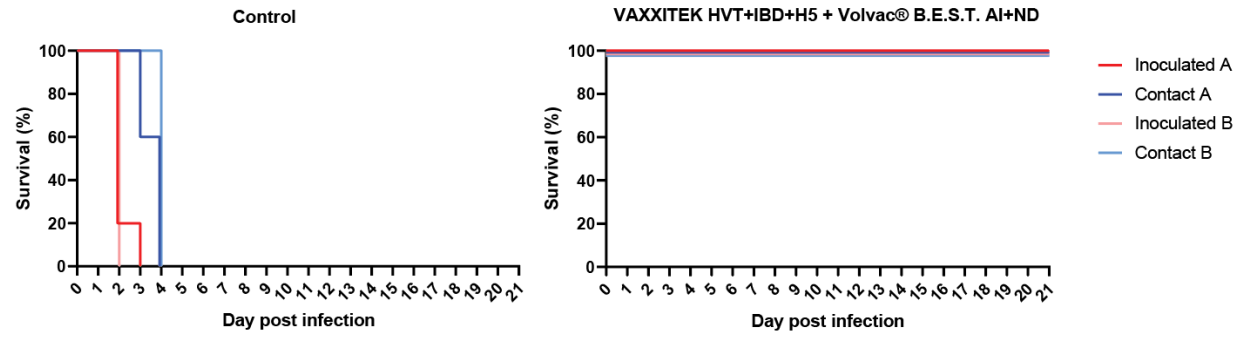
To assess the effectiveness of the vaccine in reducing disease and clinical signs, the time of death or reaching the humane endpoint was recorded for each chicken. The mortality that occurred in the groups is depicted in survival curves (Appendix Figure 2.2). In control group A, 1/5 inoculated chickens died at 2 dpi, 2 died at 3 dpi, and the remaining two died at 4 dpi. 3/5 contact chickens died at 4 dpi (2/3 humane endpoint) and the remaining 2 contact chickens died at 5 dpi. In control group B, 1/5 inoculated chickens died at 2 dpi, and the remaining 4/4 inoculated chickens died at 3 dpi. Four contact chickens died at 4 dpi (1/4 humane endpoint) and the remaining contact chicken died at 5 dpi (humane endpoint). Clinical signs in the non AI-vaccinated control groups were moderate to severe depression at most 24 hour prior death or humane endpoint.
In the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND groups no mortality was observed.



Appendix Figure 2.2 *Survival curve of non AI-vaccinated control and vaccinated groups when challenge was applied at 54 weeks of age. 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.*

2.2.1.2 Transmission study 4, inoculation at 84 weeks of age

The mortality that occurred in the groups is depicted in survival curves (Appendix Figure 2.3). In non AI-vaccinated control group A, 4/5 inoculated chickens died at 2 dpi (one reached the humane endpoint), and the remaining chicken died at 3 dpi. Two contact chickens died at 4 dpi (1/2 humane endpoint) and the remaining 3 contact chickens died at 4 dpi (2/3 humane endpoint). In non AI-vaccinated control group B, all 5/5 inoculated chickens died at 2 dpi (all found dead). All 5/5 contact chickens died at 5 dpi (1/5 humane endpoint). Clinical signs in the non AI-vaccinated control groups were moderate to severe depression at most 24-hour prior death or humane endpoint.
In the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND groups no mortality was observed.



Appendix Figure 2.3 *Survival curve of non AI-vaccinated control and vaccinated groups when challenge was applied at 84 weeks of age. 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.*

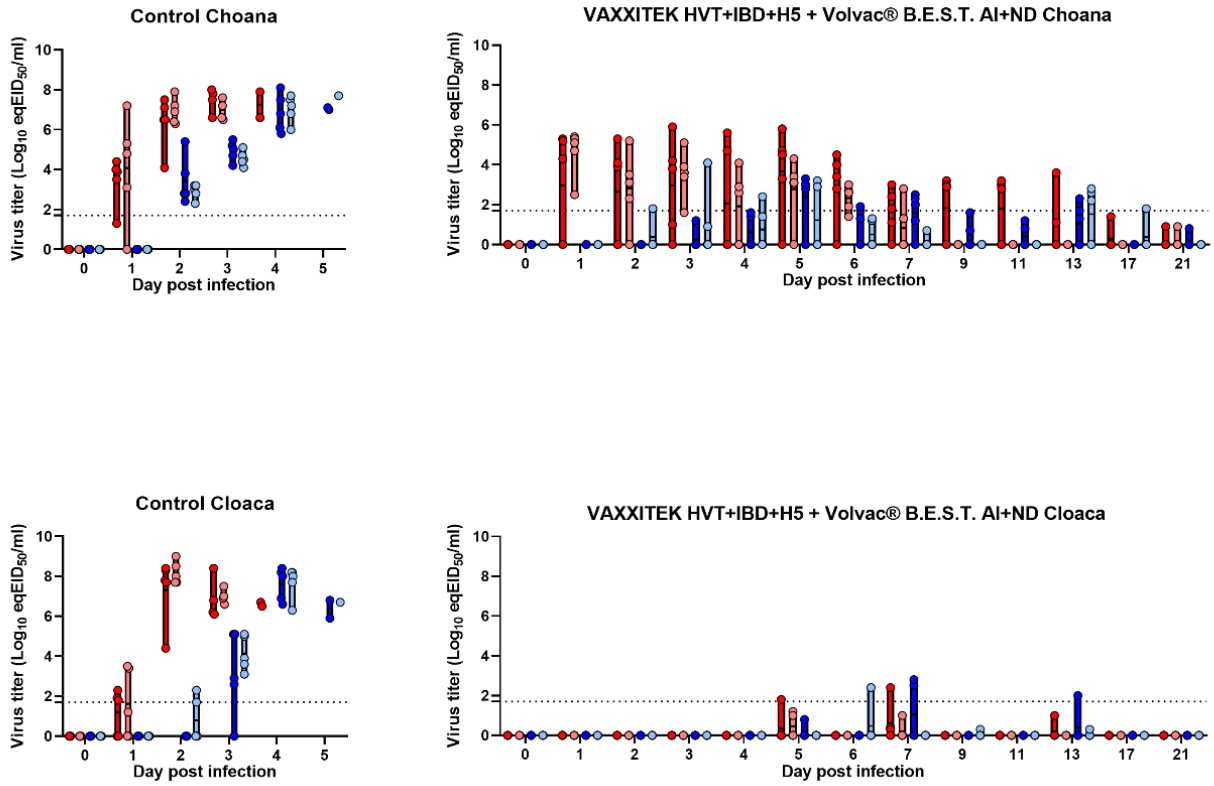
2.2.2 Virus shedding

2.2.2.1 Transmission study 3, inoculation at 54 weeks of age

The viral shedding from each chicken in the transmission studies was estimated by taking choanal and cloacal swabs to determine viral RNA quantities by the M-PCR. The obtained equivalent titers are depicted in Appendix Figure 2.4. 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 Appendix Figure 2.4).

In non AI-vaccinated control groups A and B, all (2x 10/10) chickens were scored positive for virus shedding. Inoculated chickens were shedding through the choana and cloaca from 1 until 4 dpi group A, until 3 dpi group B (time of death). The contact chickens were shedding through the choana and cloaca from 2 until 5 dpi (time of death) (Appendix Figure 2.4, left side).

In the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND group A, 4/5 inoculated and 2/5 contact chickens were considered positive for viral shedding. In group B, all 5/5 inoculated, and 2/5 contact chickens were considered positive for viral shedding. No positive results for ≥2 days were obtained in cloacal swabs.

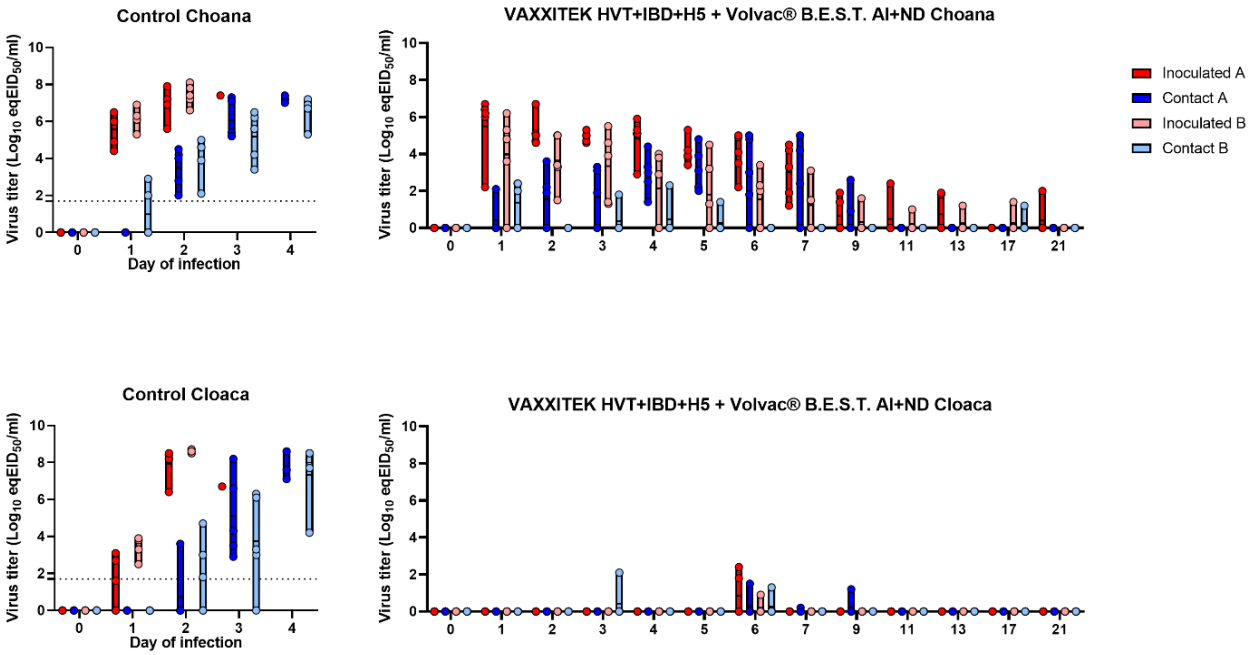


Appendix Figure 2.4 *The titer of virus excretion from the inoculated chickens (red) and contact chickens (blue) detected in choanal and cloacal swabs during the third transmission study where inoculation was applied at 54 weeks of age. For each group, subgroups A and B are shown separately. The detection 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.*

2.2.2.2 Transmission study 4, inoculation at 84 weeks of age

In non AI-vaccinated control groups A and B, all (2x 10/10) chickens were scored positive for virus shedding. Inoculated chickens were shedding through the choana and cloaca from 1 until 3 dpi group A, until 2 dpi in group B (time of death). The contact chickens were shedding through the choana and cloaca from 2 until 4 dpi (time of death) (Appendix Figure 2.5, left side).

In the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND group A, all 5/5 inoculated and 5/5 contact chickens were considered positive for viral shedding. In group B, 4/5 inoculated, and 0/5 contact chickens were considered positive for viral shedding. No positive results for ≥2 days were obtained in cloacal swabs.

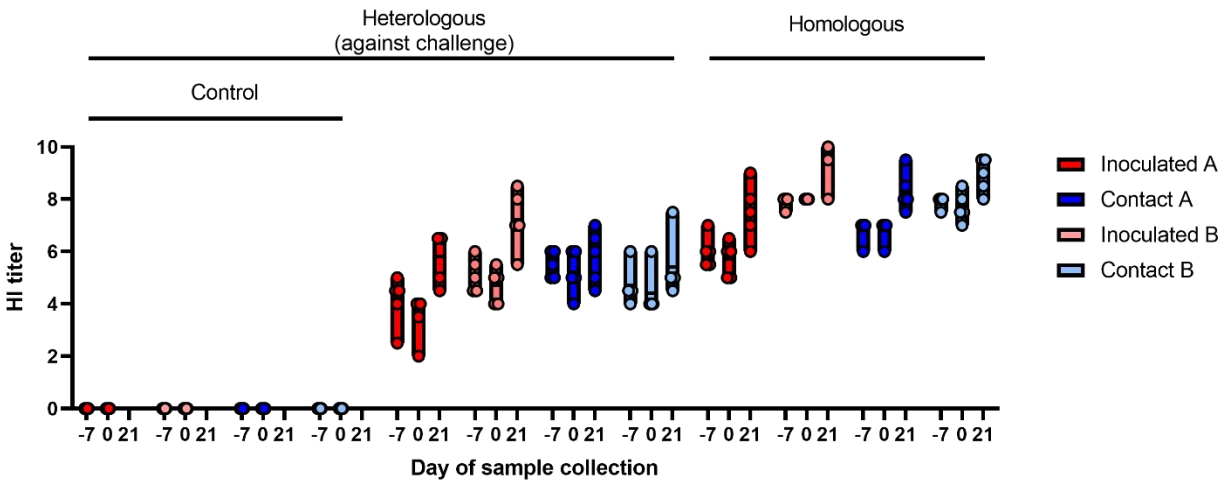


Appendix Figure 2.5 *The titer of virus excretion from the inoculated chickens (red) and contact chickens (blue) detected in choanal and cloacal swabs during the third transmission study where inoculation was applied at 84 weeks of age. For each group, subgroups A and B are shown separately. The detection 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.*

2.2.3 Humoral Immune Response

2.2.3.1 NP-ELISA and HI titers prior inoculation Transmission study 3 (inoculation at 54 weeks of age)

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 challenge) and homologous (against an antigen closely related to the H5 of the vaccine) titer after vaccination (Appendix Figure 2.6).



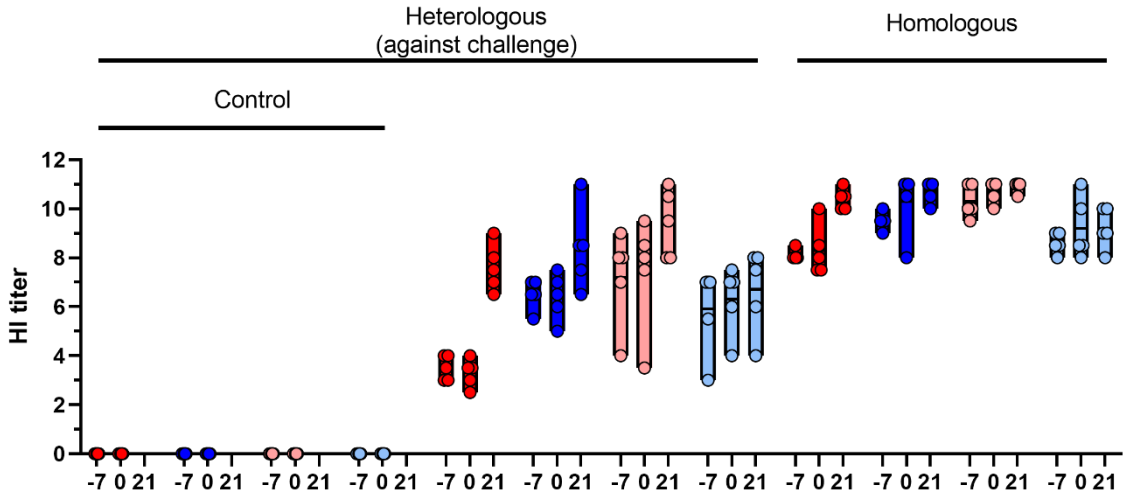
Appendix Figure 2.6 *The HI titer (Log_2) of the inoculated and contact chickens of the different groups in Transmission study 3, inoculation at 54 weeks of age. The blood serum collected before inoculation (-7 dpi, 0 dpi) and after inoculation (21 dpi) were tested in the HI against an antigen that is highly related to the H5 of the prime vaccine (homologous) and the current HPAI H5N1 inoculated virus (heterologous). Each dot is an individual chicken.*

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

On -7 dpi, prior inoculation all 40 chickens had a homologous HI titer. All vaccinated chickens had a heterologous HI-titer prior challenge as well. In Appendix Figure 2.6 all individual chickens are shown (individual circles) to demonstrate variation of HI-titers prior inoculation.

2.2.3.2 NP-ELISA and HI titers prior inoculation Transmission study 4 (inoculation at 84 weeks of age)

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 challenge) and homologous (against an antigen closely related to the H5 of the vaccine) titer after vaccination (Appendix Figure 2.7).



Appendix Figure 2.7 *The HI titer (Log_2) of the inoculated and contact chickens of the different groups in Transmission study 3, inoculation at 84 weeks of age. The blood serum collected before inoculation (-7 dpi, 0 dpi) and after inoculation (21 dpi) were tested in the HI against an antigen that is highly related to the H5 of the prime vaccine (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.

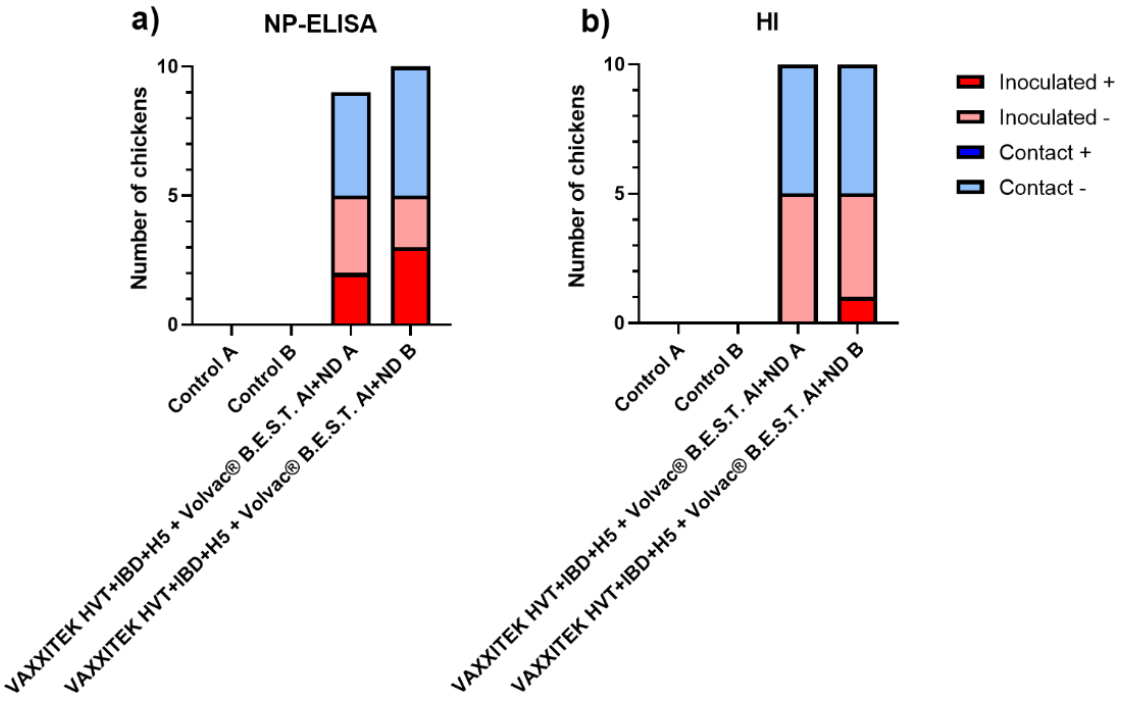
On -7 dpi, prior inoculation all 40 vaccinated chickens had a homologous and heterologous HI titer. In Appendix Figure 2.7, all individual chickens are shown (individual circles) to demonstrate variation of HI-titers prior inoculation.

2.2.3.3 NP-ELISA and HI titers post-inoculation Transmission study 3 (54 weeks of age inoculation)

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 non AI-vaccinated control groups died before the end of the study, therefore no serological tests could be performed.
In the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND groups, 2/5 and 0/5 inoculated chickens of groups A and B were positive in NP-ELISA respectively at 21 dpi. In the serum of the contact chickens 3/5 and 0/4 in group A and B respectively, the result of the NP-ELISA was positive (Appendix Figure 2.8a).



Appendix Figure 2.8 *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. One blood samples of a contact chicken in group A was insufficient in volume to perform the test.*

Hemagglutination Inhibition (HI) Assay
The blood serum collected at 21 dpi (end point of study?) was also tested in the HI assay. A chicken was scored positive for HI when an increased heterologous HI titer of $\log_2$ ≥3 was obtained. None of the chickens of the non AI-vaccinated control groups survived the study, so no blood serum could be obtained.

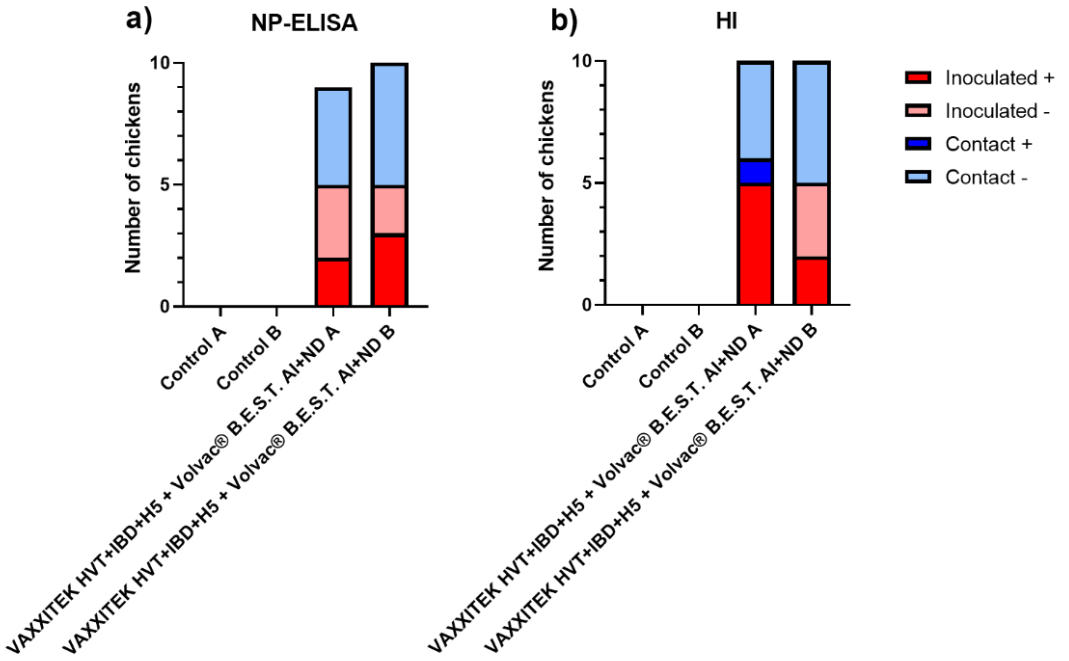
In the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND groups, 0/5 of the inoculated and 0/5 and 1/5 of the contact chickens in groups A and B respectively, an increased HI titer ≥3 was obtained compared to -7 dpi (Appendix Figure 2.8b). In Appendix Figure 2.6, HI titers of all (survived) individual chickens are shown (individual circles) to demonstrate variation of HI-titers post-inoculation.

2.2.3.4 NP-ELISA and HI titers post-inoculation Transmission study 4 (84 weeks of age inoculation)

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 non AI-vaccinated control groups died before the end of the study, therefore no serological tests could be performed.

In the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND groups, 4/5 and 3/5 inoculated chickens of groups A and B were positive in NP-ELISA respectively at 21 dpi. In the serum of the contact chickens 3/5 and 0/5 in group A and B respectively, the result of the NP-ELISA was positive (Appendix Figure 2.9a).

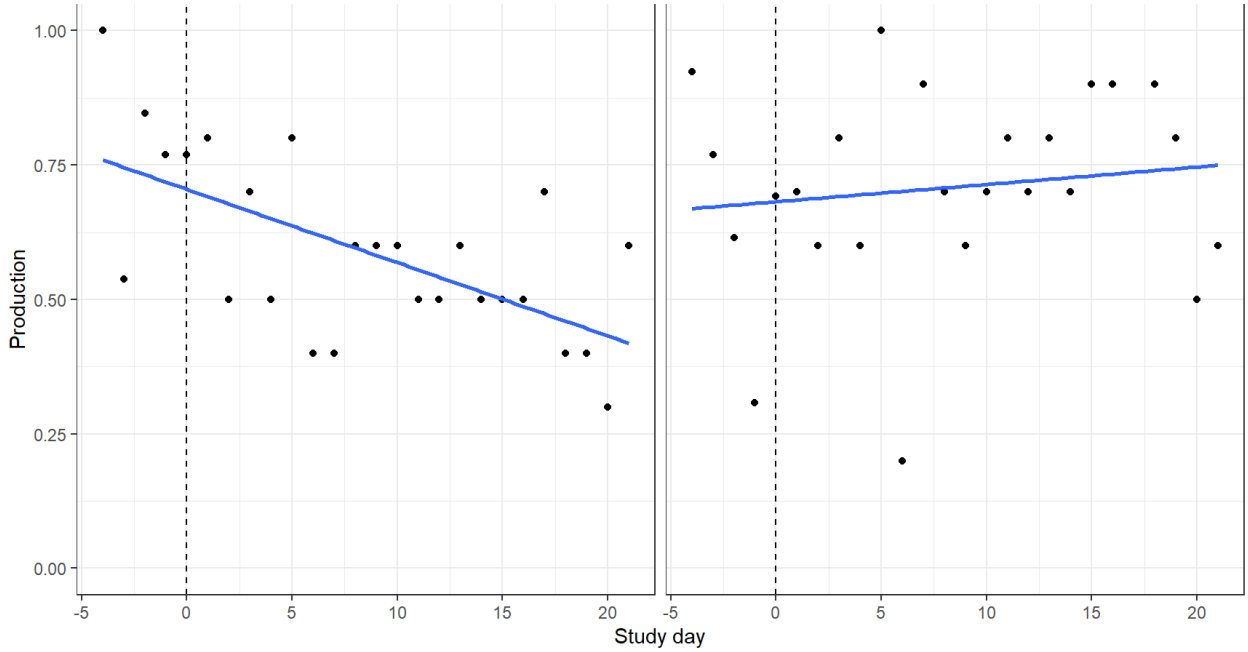


Appendix Figure 2.9 *The number of chickens that were positive in serology tests performed on the blood collected on the last day of the study (21 dpi) compared to collection before inoculation (-7 dpi). a) The number of chickens that obtained a positive or negative result in the NP-ELISA and b) in the HI-test. Red indicates inoculated chickens, blue for contact chickens. Bright color is positive (+), transparent is negative (-) result in the tests.*

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 $\log_2$ ≥3 was obtained. None of the chickens of the non AI-vaccinated control groups survived the study, so no blood serum could be obtained.

In the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND groups, 5/5 and 2/5 of the inoculated and 1/5 and 0/5 of the contact chickens in groups A and B respectively, an increased HI titer ≥3 was obtained compared to -7 dpi (Figure 6b). In Appendix Figure 2.7, HI titers of all (survived) individual chickens are shown (individual circles) to demonstrate variation of HI-titers post-inoculation.

2.2.4 Egg production in Transmission study 4, Inoculation at 84 weeks of age



Appendix Figure 2.10 *Daily egg production recorded during the experiment performed when layers were 84 weeks old. Each panel represents each transmission study group (A (left) or B(right)) consisting of 5 inoculated chickens and 5 contact chickens. Dashed vertical line (study day 0) indicates the day challenge was applied to the inoculated chickens. Blue line indicate the trend in mean production during the experiment.*

2.2.5 Virus Transmission: Calculation Of The Reproduction Number (R)

For the first two experiments, upon arrival at WBVR, all chickens were randomly divided in their corresponding groups (to be inoculated with challenge virus, or be a contact). For the third and fourth study, chickens were grouped based on their homologous HI antibody titer. If one would calculate the transmission parameters as if the groups were divided at random the results are demonstrated in Appendix Table 2.1. Hence estimates for these experiments should be considered with caution, as this is based on the method assuming homogeneous populations. The different transmission parameters where the non-randomized groups were taken into account are presented in the main report (Chapter 3).

Appendix Table 2.1: *Transmission parameters calculated 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. For completeness, all ages of layers are included.*

Transmission study	Age of the layers at moment of inoculation	Beta (±SD) (A&B combined)	Infectious period (days) (±SD) (A&B combined)	R-value (±SD) (A&B combined)
2	24 weeks	2.06 (1.39-2.94)	3.8 (1.4-6.4)	0.55 (0.16-1.62)
3	54 weeks	N/A	N/A	<1
4	84 weeks	0.17(0.04-0.45)	0.9 (0.6-1.2)	0.2 (0.1-0.4)

2.3 Daily results of transmission studies

Appendix Tables 2.3.1 t/m 2.3.4 *Daily results of transmission studies of Work Package 2. HI-titers obtained pre-challenge (day -7) are indicated by High (≥7) or Low (<7). Results obtained by M-PCR of choana and cloaca swabs are indicated by - / +, where viral titers <Log $10^{1.7}$ $eqEID_{50}$/ml are considered negative (-). Serology is indicated by neg or pos. The results are positive if a positive NP-ELISA result (after 21 days) and/or showed an increase of ≥3 log2 in the heterologous HI-titer. Study day 0 was day of challenge. If nothing is indicated, no samples were collected of that chicken at this timepoint. Cells indicated in red indicate chickens that were directly inoculated, in blue, chickens that were contacts in the same pen. Grey indicates no sample collected, as chicken was no longer in the study.*

2.3.1 Transmission study 1, inoculation at 8 weeks of age.

subgroup*	**Homologous HI-titer**	**Swabs Choana / Cloaca**															**Serology**
	day -7	day -7	day 0	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 9	day 10	day 11	day 12	day 13	day 17	day 21	day 21
Vaccinated A	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg
	Low	- / -	- / -	+ / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg
Vaccinated B	Low	- / -	- / -	+ / -	- / -	+ / -	+ / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / nt		- / nt	nt / nt	nt / nt	neg

	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / nt		- / nt	nt / nt	nt / nt	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / nt		- / nt	nt / nt	nt / nt	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / nt		- / nt	nt / nt	nt / nt	neg
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / nt		- / nt	nt / nt	nt / nt	neg
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / nt		- / nt	nt / nt	nt / nt	neg
Control group A	Low	- / -	- / -	+ / -	+ / +	+ / +											
	Low	- / -	- / -	+ / -	+ / +												
	Low	- / -	- / -	+ / -	+ / +	+ / +											
	Low	- / -	- / -	+ / -	+ / +	+ / +											
	Low	- / -	- / -	+ / -	+ / -	+ / +	- / -	- / -	- / -	- / -	- / -	- / -		- / -	- / -	- / -	neg
	Low	- / -	- / -	- / -	- / -	- / +	+ / -	+ / +	+ / -	+ / +							
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+ / +						
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / -	- / -	- / -	neg
	Low	- / -	- / -	- / -	- / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -		- / -	- / -	- / -	neg
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / -	- / -	- / -	neg
Control group B	Low	- / -	- / -	+ / -	+ / +	+ / +											
	Low	- / -	- / -	+ / -	+ / +	/											
	Low	- / -	- / -	+ / -	+ / +	+ / +											
	Low	- / -	- / -	+ / -	+ / +	/											
	Low	- / -	- / -	+ / -	+ / +	+ / +											
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / +	+ / +							
	Low	- / -	- / -	- / -	- / -	+ / -	+ / -	- / -	+ / -	+ / +							
	Low	- / -	- / -	- / -	- / -	+ / -	- / -	+ / -	- / +	+ / +							
	Low	- / -	- / -	- / -	- / -	+ / -	+ / +	+ / +									
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+ / -	+ / +	+ / +					

- Vaccinated is VAXXITEK HVT+IBD+H5 applied at day of hatch.

2.3.2 Transmission study 2, inoculation at 24 weeks of age.

subgroup*	Homologous HI-titer	Swabs Choana / Cloaca															Serology
	day -7	day -7	day 0	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 9	day 10	day 11	day 12	day 13	day 17	day 21	day 21
Vaccinated A	Low	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -		+ / +		- / -	+ / -	- / -	**pos**
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+ / -		+ / -		- / -	+ / -	- / -	**pos**
	High	- / -	- / -	+ / -	+ / -	+ / -	- / +	- / -	- / -	+ / -	+ / -		+ / -		+ / -	- / -	- / -	**pos**
	Low	- / -	- / -	- / -	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / +		+ / -		+ / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -	- / +		+ / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	- / -	- / -	+ / +	- / -	- / -	+ / -	+ / -	+ / +		+ / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	- / -	- / -	+ / -	+ / -	- / -	- / -	+ / -	+ / -		+ / -		+ / -	+ / -	- / -	**pos**
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / -		+ / -		- / -	- / -	- / -	**pos**
	Low	- / -	- / -	- / -	- / -	- / -	+ / +	+ / -	+ / +	+ / +	+ / +	+ / +						nd
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+ / -		+ / +	+ / -				nd
Vaccinated B	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -		+ / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -	- / -	+ / +		- / -		- / -	- / -	- / -	**pos**
	Low	- / -	- / -	+ / -	+ / -	+ / -	+ / +	+ / +	+ / -	+ / -	+ / +		+ / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / +	- / -	- / -	- / -	+ / -		- / -		- / -	+ / -	- / -	nd
	Low	- / -	- / -	+ / -	+ / -	+ / -	+ / +	+ / +	- / +	+ / -	+ / -		+ / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	- / -	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / +		+ / -		+ / -	- / -	- / -	**pos**
	Low	- / -	- / -	- / -	- / -	+ / -	+ / -	- / -	- / -	+ / -	+ / -		+ / -		- / -	- / -	- / -	**pos**
	Low	- / -	- / -	- / -	- / -	+ / -	- / -	+ / -	- / -	+ / -	+ / -		- / +		- / -	- / -	- / -	**pos**
	Low	- / -	- / -	- / -	- / -	+ / +	- / -	- / -	- / -	+ / -	+ / -		+ / -		+ / -	+ / -	- / -	**pos**
	Low	- / -	- / -	- / -	- / -	- / -	+ / -	- / -	- / -	+ / -	+ / +		+ / -		+ / -	- / -	- / -	**pos**
Vaccinated A	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -		- / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / +	- / +		- / +		- / -	- / +	- / -	**pos**
	Low	- / -	- / -	+ / -	- / -	- / -	- / -	- / -	- / -	+ / -	- / -		- / -		- / -	- / -	- / -	neg
	Low	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -	- / -		- / -		- / -	- / -	- / -	**pos**

	High	- / -	- / -	- / -	- / -	+ / -	+ / -	+ / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -	**pos**
	High	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+ / -	- / -	+ / -	- / -	- / -	- / -	- / -	**pos**
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	neg
	High	- / -	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -	+ / -	- / -	- / -	- / -	- / -	**pos**
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	**pos**
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	neg
Vaccinated B	High	- / -	- / -	+ / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -	- / -	- / -	- / -	- / -	**pos**
	Low	- / -	- / -	- / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	neg
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	**pos**
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	**pos**
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	neg
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	neg
Control group A	High	- / -	- / -	+ / +	+ / +	+ / +										
	High	- / -	- / -	- / -	+ / +	+ / +	+ / +									
	High	- / -	- / -	+ / -	+ / +	+ / +	+ / +									
	High	- / -	- / -	+ / -	+ / +	+ / +	+ / +									
	High	- / -	- / -	+ / -	+ / +	+ / +	+ / +									
	High	- / -	- / -	- / -	+ / +	+ / +	+ / +	+ / +								
	High	- / -	- / -	- / -	+ / -	+ / +	+ / +	+ / +								
	High	- / -	- / -	- / -	+ / +	+ / +	+ / +	+ / +								
	High	- / -	- / -	- / -	+ / -	+ / -	+ / +	+ / +								
	High	- / -	- / -	- / -	+ / -	+ / -	+ / +	+ / +								
Control group B	High	- / -	- / -	+ / -	+ / +	+ / +										

	High	- / -	- / -	+ / -	+ / +					
	High	- / -	- / -	+ / +	+ / +	+ / +				
	High	- / -	- / -	+ / -	+ / +					
	High	- / -	- / -	+ / -	+ / +					
	High	- / -	- / -	- / -	+ / +	+ / +	+ / +	+ / +		
	High	- / -	- / -	- / -	+ / +	+ / +	+ / +	+ / +		
	High	- / -	- / -	- / -	+ / -	+ / +	+ / +	+ / +		
	High	- / -	- / -	- / -	+ / +	+ / +	+ / +			
	High	- / -	- / -	- / -	+ / +	+ / +	+ / +			

Vaccinated is VAXXITEK HVT+IBD+H5 applied at day of hatch.

Vaccinated is VAXXITEK HVT+IBD+H5 applied at day of hatch and Volvac® B.E.S.T. AI+ND applied at approximately 12 weeks of age.

2.3.3 Transmission study 3, inoculation at 54 weeks of age.

subgroup*	Homologous HI-titer	Swabs Choana / Cloaca															Serology
	day -7	day -7	day 0	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 9	day 10	day 11	day 12	day 13	day 17	day 21	day 21
Vaccinated A	Low	- / -	- / -	+ / -	+ / -	- / -	- / -	+ / -	+ / -	- / +	+ / -		+ / -		+ / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -		+ / -		- / -	- / -	- / -	**pos**
	Low	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -		+ / -		- / -	- / -	- / -	neg
	Low	- / -	- / -	- / -	- / -	+ / -	- / -	+ / -	+ / -	+ / -	- / -		- / -		- / -	- / -	- / -	neg
	Low	- / -	- / -	- / -	- / -	- / -	- / -	- / +	- / -	- / -	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	- / -	+ / -	- / -		- / -		- / +	- / -	- / -	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	- / -	- / +	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+ / -	+ / +	- / -		- / -		+ / -	- / -	- / -	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+ / -	+ / -	- / -		- / -		- / -	- / -	- / -	neg
Vaccinated B	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -	- / -		- / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	- / -	+ / -	+ / -	+ / -	- / -	- / -	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	+ / -	+ / -	- / -	- / -	+ / -	+ / -	+ / -	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	+ / -	+ / -	+ / -	- / -	- / -	+ / -	- / -	- / -		- / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -	- / -		- / -		- / -	- / -	- / -	**pos**
	Low	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	- / -	- / -	- / -		- / -		+ / -	- / -	- / -	neg
	High	- / -	- / -	- / -	- / -	+ / -	- / -	- / -	- / -	- / -	- / -		- / -		+ / -	+ / -	- / -	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	- / -	- / -	- / -	+ / -	+ / -	- / +	- / -	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	- / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -		- / -		+ / -	- / -	- / -	neg
Control group A	Low	- / -	- / -	+ / +	+ / +	+ / +												
	Low	- / -	- / -	+ / +	+ / +	+ / +												
	Low	- / -	- / -	+ / -	+ / +	+ / +	+ / +											

	Low	- / -	- / -	+ / +	+ / +			
	Low	- / -	- / -	- / -	+ / +	+ / +	+ / +	
	Low	- / -	- / -	- / -	+ / -	+ / -	+ / +	+ / +
	Low	- / -	- / -	- / -	+ / -	+ / +	+ / +	
	Low	- / -	- / -	- / -	+ / -	+ / +	+ / +	+ / +
	Low	- / -	- / -	- / -	+ / -	+ / +	+ / +	
	Low	- / -	- / -	- / -	+ / -	+ / +	+ / +	
Control group B	Low	- / -	- / -	+ / +	+ / +			
	Low	- / -	- / -	+ / -	+ / +	+ / +		
	Low	- / -	- / -	- / -	+ / +	+ / +		
	Low	- / -	- / -	+ / -	+ / +	+ / +		
	Low	- / -	- / -	+ / +	+ / +	+ / +		
	Low	- / -	- / -	- / -	+ / -	+ / +	+ / +	
	Low	- / -	- / -	- / -	+ / -	+ / +	+ / +	
	Low	- / -	- / -	- / -	+ / +	+ / +	+ / +	
	Low	- / -	- / -	- / -	+ / -	+ / +	+ / +	+ / +
	Low	- / -	- / -	- / -	+ / +	+ / +	+ / +	

Vaccinated is VAXXITEK HVT+IBD+H5 applied at day of hatch and Volvac® B.E.S.T. AI+ND applied at approximately 12 weeks of age.

2.3.4 Transmission study 4, inoculation at 84 weeks of age.

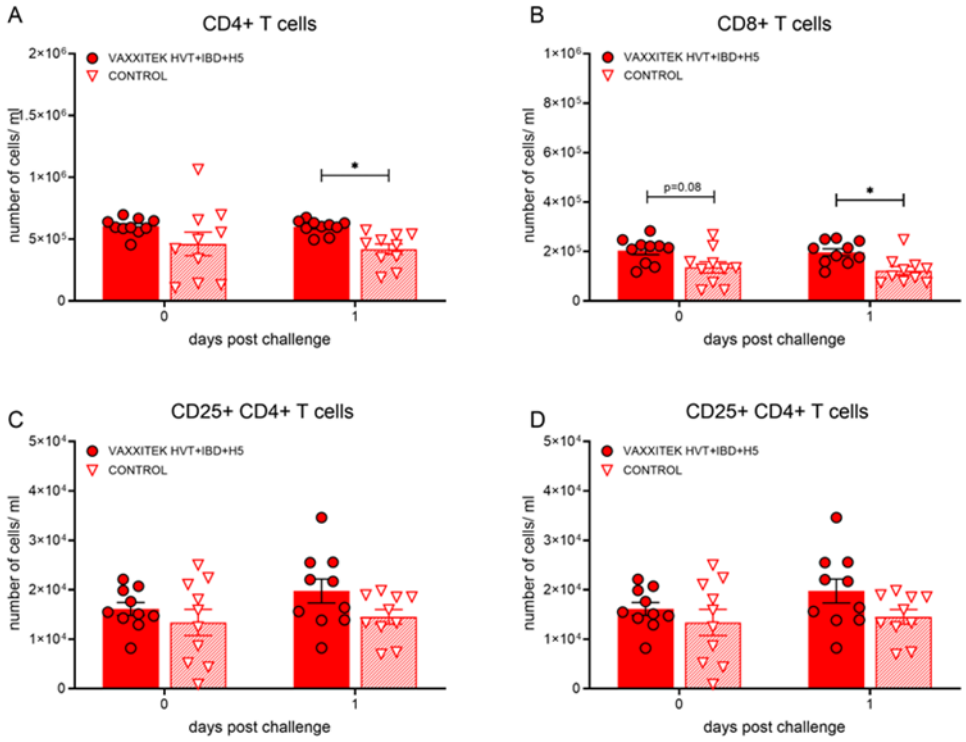
subgroup*	Homologous HI-titer	Swabs Choana / Cloaca															Serology
	day -7	day -7	day 0	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 9	day 10	day 11	day 12	day 13	day 17	day 21	day 21
Vaccinated A	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / +	+ / -	- / -		- / -		+ / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -	- / -		- / -		- / -	- / -	+ / -	**pos**
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / +	+ / -	- / -		- / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -		- / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -		+ / -		+ / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	- / -	- / -	- / -	+ / -	+ / -	- / -	- / -	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -		- / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	- / -	- / -	- / -	- / -	+ / -	+ / -	+ / -	- / -		- / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -		- / -		- / -	- / -	- / -	**pos**
Vaccinated B	High	- / -	- / -	+ / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -	- / -		- / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	- / -	+ / -	+ / -	- / -		- / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	- / -	- / -		- / -		- / -	- / -	- / -	**pos**
	High	- / -	- / -	+ / -	- / -	- / -	+ / -	- / -	- / -	- / -	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	+ / -	- / -	- / +	- / -	- / -	- / -	- / -	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -		- / -		- / -	- / -	- / -	neg
	High	- / -	- / -	+ / -	- / -	+ / -	- / -	- / -	- / -	- / -	- / -		- / -		- / -	- / -	- / -	neg
Control group A	Low	- / -	- / -	+ / +	+ / +													
	Low	- / -	- / -	+ / -	+ / +	+ / +												
	Low	- / -	- / -	+ / -	+ / +													

	Low	- / -	- / -	+ / +	+ / +	
	Low	- / -	- / -	+ / -	+ / +	
	Low	- / -	- / -	- / -	+ / -	+ / +	+ / +
	Low	- / -	- / -	- / -	+ / -	+ / +	+ / +
	Low	- / -	- / -	- / -	+ / -	+ / +	
	Low	- / -	- / -	- / -	+ / -	+ / +	+ / +
	Low	- / -	- / -	- / -	+ / +	+ / +	
Control group B	Low	- / -	- / -	+ / +	+ / +	
	Low	- / -	- / -	+ / +	+ / +	
	Low	- / -	- / -	+ / +	+ / +	
	Low	- / -	- / -	+ / +	+ / +	
	Low	- / -	- / -	+ / +	+ / +	
	Low	- / -	- / -	- / -	+ / +	+ / +	+ / +
	Low	- / -	- / -	+ / -	+ / +	+ / +	+ / +
	Low	- / -	- / -	+ / -	+ / -	+ / +	+ / +
	Low	- / -	- / -	- / -	+ / -	+ / -	+ / +
	Low	- / -	- / -	- / -	+ / +	+ / +	+ / +

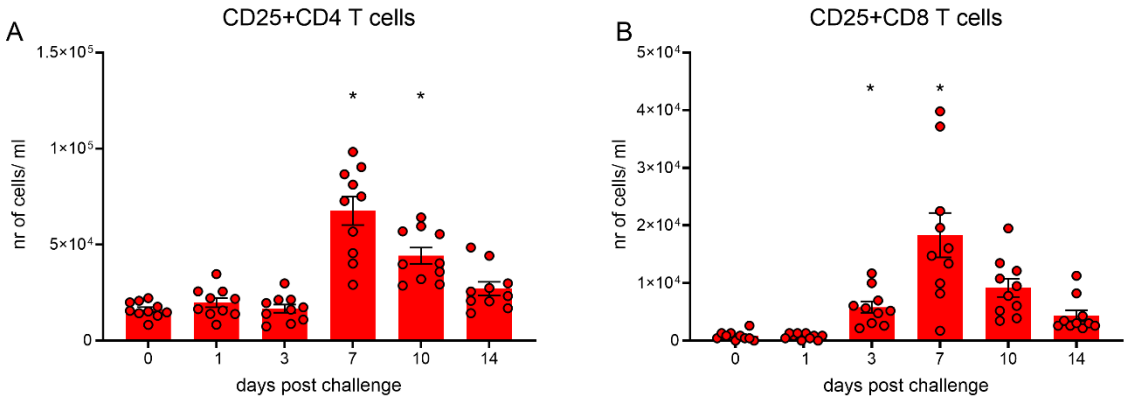
Vaccinated is VAXXITEK HVT+IBD+H5 applied at day of hatch and Volvac® B.E.S.T. AI+ND applied at approximately 12 weeks of age.

3 Appendix Work Package 3

Transmission study 1, inoculation at 8 weeks of age

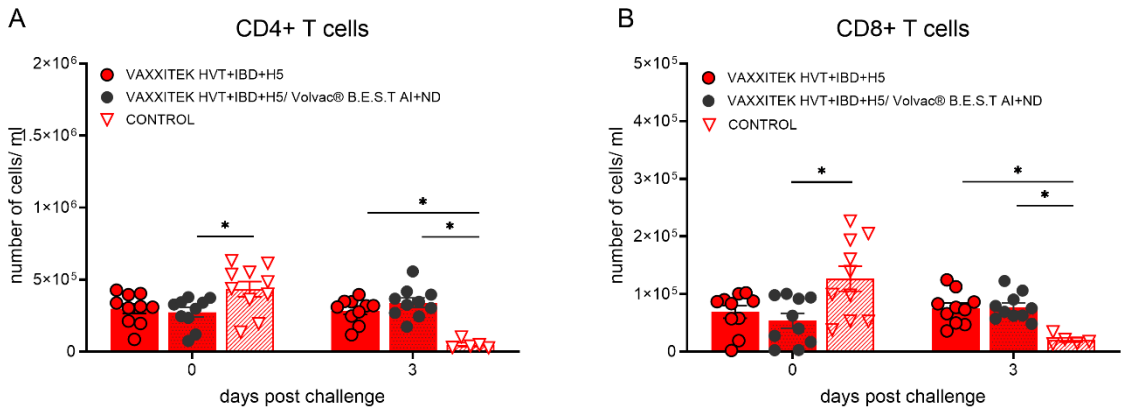


Appendix figure 3.1 *Absolute numbers of T cells in the blood of vaccinated and non AI-vaccinated controls. Absolute numbers of CD4+ T cells (A), CD8+ T cells (B) CD25+ CD4+T cells (C) and CD25+CD8+ T cells (D) and were quantified in the blood of vaccinated chickens and chickens in the non AI-vaccinated control group at 0 and 1 dpi. Each dot/triangle represents an individual chicken. Mean ± SEM is shown. Significant differences (p<0.05) are indicated (*)*

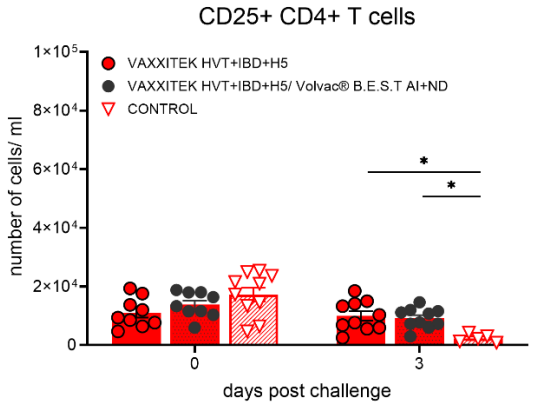


Appendix Figure 3.2 *Absolute numbers of activated T cells in the blood at different timepoints post challenge. At several timepoints post challenge, absolute numbers of CD25+ CD4+ T cells (A) and CD25+ CD4+T cells (B) were quantified in the blood of vaccinated chickens. Mean ± SEM of 10 chickens is shown. Each dot represents an individual chicken. Significant differences compared to day 0 (p<0.05) are indicated (*).*

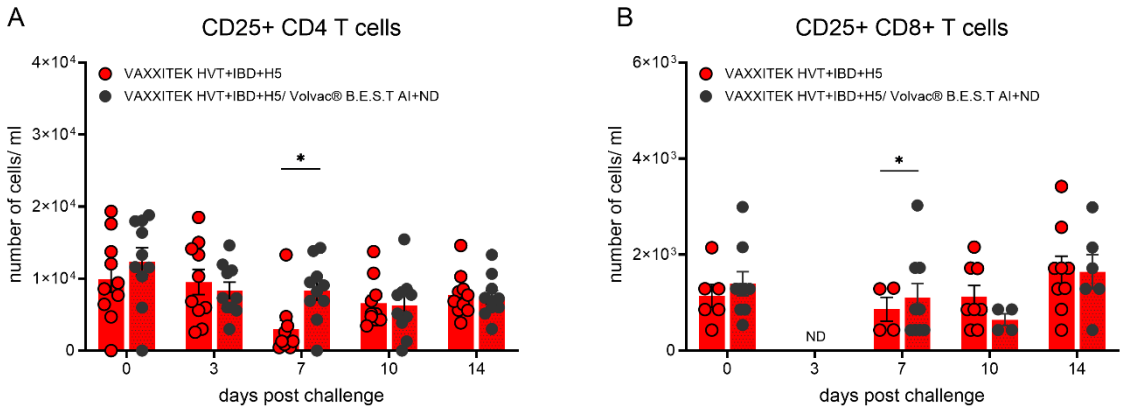
Transmission study 2, inoculation at 24 weeks of age



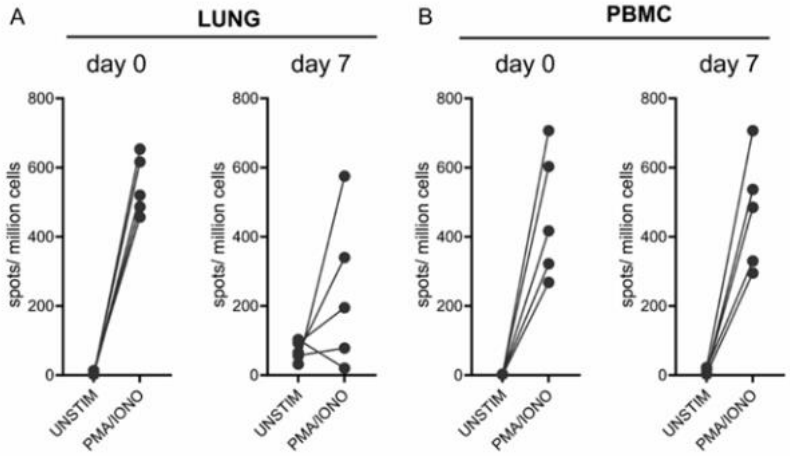
Appendix Figure 3.3 *Absolute numbers of T cells in the blood of vaccinated and non AI-vaccinated controls. Absolute numbers of CD4+ T cells (A) and CD8+ T cells (B) were quantified in the blood of VAXXITEK HVT+IBD+H5 vaccinated chickens, VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens and chickens in the non AI-vaccinated control group at 0 and 3 dpi. Each dot/triangle represents an individual chicken. Mean ± SEM is shown. Significant differences (p<0.05) are indicated (*).*



Appendix Figure 3.4 *Absolute numbers of CD25+CD4+ T cells in the blood of vaccinated and non AI-vaccinated controls. Absolute numbers of CD25+CD4+ T cells were quantified in the blood of VAXXITEK HVT+IBD+H5 vaccinated chickens, VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens and chickens in the non AI-vaccinated control group at 0 and 3 dpi. Each dot/triangle represents an individual chicken. Mean ± SEM is shown. Significant differences (p<0.05) are indicated (*)*

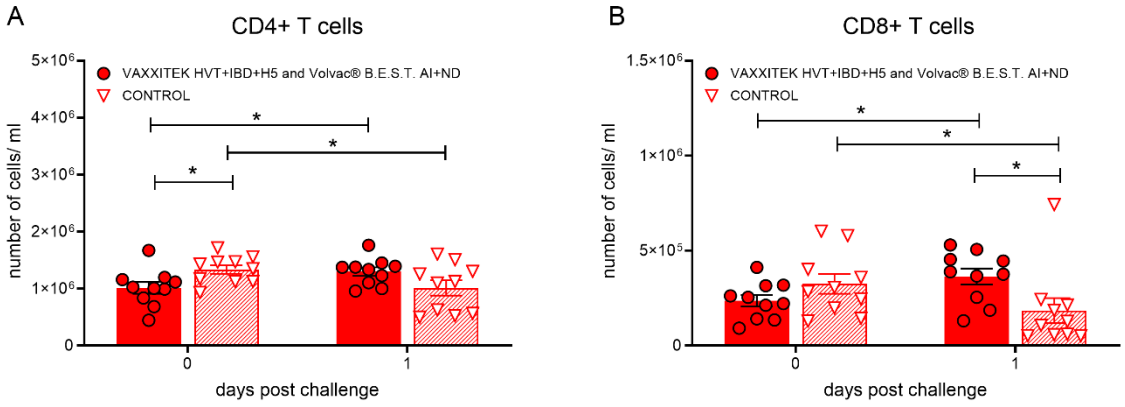


Appendix Figure 3.5 *Absolute numbers of activated T cells in the blood at different timepoints post challenge. At several timepoints post challenge, absolute numbers of CD25+ CD4+ T cells (A) and CD25+ CD4+T cells (B) were quantified in the blood of VAXXITEK HVT+IBD+H5 vaccinated chickens and VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens. Mean ± SEM of 10 chickens is shown. Each dot represents an individual chicken. Significant differences compared to day 0 (p<0.05) are indicated (*). Due to technical issues with the flow cytometer, no data are available at 1 day post challenge and the number of CD25+CD8+ T cells at day 3 post challenge was too low to be quantified.*



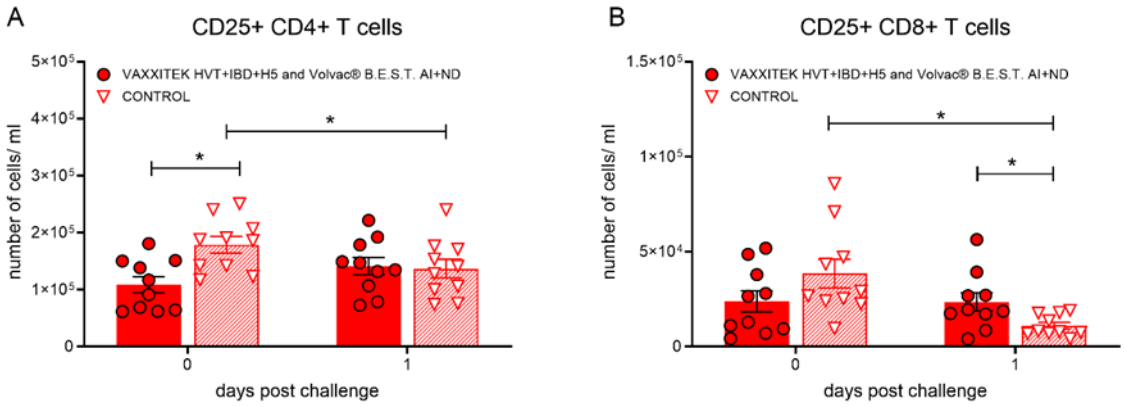
Appendix Figure 3.6 *Number of IFNγ producing cells isolated from lungs and blood (PBMC) of five VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND chickens sacrificed before (0 dpi) and after challenge (shown for 7 dpi). Results were obtained by ELIspot analysis*

Transmission study 3, inoculation at 54 weeks of age



Appendix Figure 3.7 *Absolute numbers of T cells in the blood of vaccinated and non AI-vaccinated controls. Absolute numbers of CD4+ T cells (A) and CD8+ T cells (B) were quantified in the blood of vaccinated and chickens and in the non AI-vaccinated control group at 0 and 1 day post challenge. Each dot/triangle represents*

an individual chicken. Mean ± SEM is shown (Groups A & B are combined). Significant differences (p<0.05) are indicated (*).



Appendix Figure 3.8 *Absolute numbers of activated T cells in the blood of vaccinated and non AI-vaccinated controls. Absolute numbers of CD25+ CD4+ T cells (A) and CD25+CD8+ T cells (B) were quantified in the blood of vaccinated and chickens in the non AI-vaccinated control group at 0 and 1 day post challenge. Each dot/triangle represents an individual chicken. Mean ± SEM is shown. Significant differences (p<0.05) are indicated (*).*

4 Appendix Work Package 4

Appendix Table 4.1 *Sensitivity analyses for different cut-off for high versus low titer chickens. Overview of outcomes after vaccination and for different sampling intervals. Within-farm are outcomes after introduction on to a farm. Neglible spread and outbreak are the percentages of introduction with negligible spread (≤0.1% of farm population infected) or extended spread (>0.1% of farm population infected) detected. Detection time is the time between virus introduction and detection. Infected chickens are the sum of infected dead and live chickens at the moment of detection. For eggs by infectious chickens outcome '>=1' is percentage of runs in which from a farm at least one egg produced by an infectious chicken was transported and Median number are median number of eggs transported from a farm.' Between-farm are outcomes after introduction into a random farm in a densely populated poultry area (DPPA) or sparsely populated poultry area (SPPA). Outcomes are percentage of runs at which primary case was first to be detected and fraction of runs with at least one secondary case.*

		Cut-off > 5							Cut-off > 7						
		Detection			Infected chickens at detection (75%-range)		Eggs laid by infectious chickens and transported		Detection				Infected chickens at detection (75%-range)	Eggs laid by infectious chickens and transported	
		Negligible spread	Outbreak	Detection time (75% - range)	Dead	Live	≥1 transported	Median(75 % range)	Negligible spread	Outbreak	Detection time (75% - range)	Dead	Live	≥1 transported	Median (75% range)
No AI-vaccination		0%	100%	11 (10-12)	396 (294-521)	5125 (3890-6369)	97%	244 (131 -403)	0%	100%	11 (10-12)	396 (294-521)	5125 (3890-6369)	97%	244 (131 -403)
VAXXITEK HVT + IBD + H5															
Surveillance interval	2	3%	98%	14 (10-19)	1 (1-1)	2 (1-5)	71%	4 (2-10)	2%	100%	8 (6-10)	1 (1-2)	2 (1-48)	73%	3 (2-13)
	7	3%	98%	16 (11-23)	1 (1-2)	2 (1-5)	71%	4 (2-11)	1%	100%	10 (8-13)	3 (1-7)	2 (1-155)	75%	7 (2-37)
	14	2%	98%	20 (15-26)	1 (1-2)	2 (1-5)	72%	4 (2-12)	1%	100%	15 (10-18)	9 (2-49)	2 (1-371)	76%	11 (2-156)
	30	1%	96%	28 (21-39)	2 (1-7)	2 (1-6)	72%	4 (2-15)	0%	100%	19 (14-24)	126 (6-739)	2 (1- 4307)	77%	27 (2 – 3184)
	Passive surveillance only	0%	0%	NA	NA	NA	NA	NA	0%	84%	24 (20-280)	893 (729 - 1164)	2 (1 – 27118)	77%	4340 (2- 11725)
VAXXITEK HVT + IBD + H5 + Volvac ® + B.E.S.T. + AI + ND															
Surveillance interval	2	4%	99%	22 (17 – 30)	1 (1-1)	1 (1-4)	71%	4 (2-9)	3%	100%	9 (7-13)	1 (1-1)	1 (1-4)	68%	3 (2-8)
	7	4%	95%	22 (21-31)	1 (1-1)	1 (1-5)	72%	4 (2-9)	2%	100%	11 (9-15)	1 (1-2)	1 (1-4)	69%	3 (2-11)

14	2%	93%	29 (19-33)	1 (1-1)	1 (1-5)	72%	4 (2-10)	2%	99%	16 (11-20)	2 (1-7)	1 (1-4)	70%	3 (2-17)
30	1%	64%	37 (33-40)	1 (1-2)	1 (1-5)	72%	4 (2-10)	1%	99%	22 (16-31)	4 (1-43)	1 (1-4)	70%	3 (2-23)
Passive surveillance only	0%	0%	NA	NA	NA	NA	NA	0%	22%	26 (22-32)	996 (2-2035)	1 (1-4)	70%	3 (2- 100)

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