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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Table of contents

Summary **7**

Nederlandse samenvatting **9**

1 Introduction **11**

1.1 Global HPAI landscape since 2020 and its impact on poultry production 11
1.2 Study overview 13
1.2.1 Timeline of the longitudinal field trial 13

2 Field Observations (Work Package 1) **15**

2.1 Key Findings 15
2.2 Field situation 16
2.2.1 Housing and locations 16
2.2.2 Chickens 16
2.2.3 Vaccines and vaccinations 16
2.3 Results Field Observations 17
2.3.1 Clinical inspection 17
2.3.2 Humoral immunity 17
2.3.3 M-PCR (M-gene Realtime Reverse transcription Polymerase Chain Reaction) 19
2.3.4 Mortality 19
2.3.5 Egg production 20
2.3.6 Cellular immune response 21

3 Transmission studies (Work Package 2) **27**

3.1 Key Findings 27
3.2 Aim 28
3.3 Results: Transmission parameters (Combining results of the 4 transmission studies performed at 8, 24, 54 and 84 weeks of age) 28
3.3.1 Transmission of highly pathogenic avian influenza (HPAI) H5N1 virus in non AI-vaccinated flocks 28
3.3.2 Effect of vaccination on transmission (β) 29
3.3.3 Protection Against Clinical Signs and Mortality 30
3.3.4 Effect of vaccination on levels of viral genome shedding 31
3.3.5 Predicting Re in the field 31
3.3.6 Individual results of transmission studies where challenge was performed at 54 and 84 weeks of age 32

4 Cellular immunity in Transmission studies (Work Package 3) **33**

4.1 Key Findings 33
4.2 Aim 33
4.3 Results Cellular Immunity In All Transmission Studies 34
4.3.1 Transmission study 1, inoculation at 8 weeks of age 34
4.3.2 Transmission study 2, inoculation at 24 weeks of age 34
4.3.3 Transmission study 3, inoculation at 54 weeks of age 35
4.3.4 Transmission study 4, inoculation at 84 weeks of age 36
4.4 Discussion 38

5 Safety of Poultry Products **39**

5.1 Key Findings 39
5.2 Aim 39

5.2.1 Study Design Safety of Poultry products 39
5.3 Results Safety of Poultry Products 40
5.3.1 NP-ELISA and HI titers prior inoculation 40
5.3.2 Detection of viral RNA in poultry products 40
5.3.3 Detection of viral RNA in eggs collected from the laying box in the pen. 41

6 Recommendations for Surveillance Program (Work Package 4) 43

6.1 Key Findings 43
6.2 Aim 44
6.3 Results Recommendations for Surveillance Program 45
6.3.1 Final size 45
6.3.2 Detection of outbreaks 46
6.3.3 Infected chickens and eggs produced by infectious chickens 48
6.3.4 Between-farms 49
6.3.5 Sensitivity to the cut-off value for high titer 49
6.4 Summarizing conclusion 50
6.5 Recommendations 51

7 Materials and Methods 52

7.1 Field Observations (Work Package 1) 52
7.1.1 Clinical inspection 52
7.1.2 Blood sampling 52
7.1.3 Mortality 53
7.1.4 Egg production 53
7.1.5 Humoral immunity 53
7.1.6 M-RRT-PCR (Matrix-gene Realtime Reverse Transcription Polymerase Chain Reaction, abbreviated to M-PCR) 53
7.1.7 Cellular immunity 54
7.2 Transmission studies (Work package 2) 55
7.2.1 Definition of infection in the context of the transmission studies within this longitudinal field study 55
7.2.2 Statistical analysis, Assessment of Transmission 55
7.2.3 Predicting the expected Re in vaccinated flocks 57
7.3 Cellular immunity Transmission studies (Work Package 3) 58
7.3.1 Whole Blood Staining to Determine Absolute Lymphocyte Counts 58
7.3.2 Isolation of lung and blood cells 58
7.3.3 IFNγ ELIspot Analysis 58
7.3.4 Statistical analysis 59
7.4 Safety of Poultry products 59
7.4.1 Preparation of samples prior RNA isolation 59
7.4.2 Virus isolation 59
7.5 Recommendations for Surveillance Program (Work Package 4) 59
7.5.1 Within farm model 59
7.5.2 Between farm model 61
7.5.3 Outcome measures 61
7.5.4 Input parameters and surveillance scenarios. 62
7.5.5 Input into the between-farm model 63

Terminology 65

References 68

Acknowledgements 71

Funding and Permits 72

Summary

This longitudinal field study on two commercial layer farms in the Netherlands provides comprehensive evidence on the **safety, effectiveness, and population-level impact of vaccination against highly pathogenic avian influenza (HPAI) H5** throughout the full production cycle of laying hens. All chicks received standard Dutch hatchery vaccinations, while test groups additionally received a **subcutaneous avian influenza (AI) vaccination at day of hatch. Throughout the rearing period, chicks received the standard Dutch vaccination program as prescribed by the veterinary advisors of the rearing company, and in one test group, a heterologous to the prime booster vaccination was provided (at approx. 12 weeks of age).** Chickens were monitored under field conditions from hatch through end of lay, with detailed assessment of immunity, health, mortality, production, virus transmission, poultry product safety, and surveillance performance.

Vaccine safety and flock health. (Chapter 2)
AI vaccination was well tolerated: **no local vaccine reactions** were observed, and monthly clinical inspections revealed no major abnormalities. Vaccine related **mortality in vaccinated flocks remained low** over the rearing period (~1%) and production period (4.4-7.6%), and well within expected ranges for healthy brown layer flocks during the production period. The **non AI-vaccinated control group on farm A showed higher cumulative mortality** (12.4%), attributable to non AI causes (e.g. *E. coli* septicaemia, feather pecking, red mite infestation, and a single fright-related event). There was no indication that AI vaccination negatively affected flock health, welfare, or overall performance.

Immune responses and DIVA performance.
Vaccinated chickens developed robust and durable humoral immunity. Mean homologous H5 HI titers ranged from approximately **$\log_2$ 4.5 at 5 weeks of age to $\log_2$ 7.9–9.1** (VAXXITEK HVT+IBD+H5 and VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND) **at 85 weeks**, with similar trends on both farms. Antibody levels fluctuated over time, with **temporary dips around 30–35 weeks and 60–65 weeks** in chickens that received only a VAXXITEK HVT+IBD+H5 vaccination, whereas **prime-heterologous booster-vaccinated groups maintained consistently high titers throughout the production cycle**.
DIVA surveillance using NP-ELISA (IDEXX) was highly specific: **8,630 serum samples** were tested, of which **99.8% were NP-ELISA negative**. The small fraction of positives (0.2%, n=17) fell within the expected false-positive range and showed no evidence of field infection. Vaccinated and non AI-vaccinated groups had comparable NP-ELISA negativity rates (99.95 and 99.91% vs 99.81% respectively).

Absence of field infection.
Extensive PCR surveillance, as well as the serological results mentioned above, supported AI freedom of both vaccinated and non AI-vaccinated groups during the field study. **All 196 pooled tracheal and cloacal swabs** collected from mortality cases tested **negative by M-gene RRT-PCR**. This indicates that there was no evidence of silent circulation of avian influenza field infections during the study period.

The effect of vaccination on the cellular immune response.
The whole blood staining assay developed in this study can be used to monitor vaccine-induced changes in T-cell activation in blood. In field situations, including this field study, where multiple vaccines are administered, the assay cannot attribute responses to a single vaccine. However, under controlled experimental conditions the use of this assay showed that vaccinated chickens mounted **a rapid and strong T cell response within 3-7 days post challenge** which is reflected by increased numbers of activated CD4+ and CD8+ T cells as well as the presence of IFNγ positive cells in the blood. This response is consistent with a vaccine-induced memory cell response originating from vaccination at day of hatch.

Transmission, mortality, production effects and poultry product safety. (Chapters 3 and 5)
Experimental transmission studies (containing two duplo groups A and B) demonstrated strong l**ife stage - dependent transmission (pullets versus layers)**in the non AI-vaccinated control group: the estimated

basic reproduction number (R_0) was **1.6 (95% CI 0.6–3.1)** in 8-week-old pullets and **6.4 (2.5–12.0)** in adult layers. This means that one infected adult layer could infect on average more than 6 other birds in a fully susceptible flock. This means that one infected adult layer could infect on average more than 6 other birds in a fully susceptible flock. This difference between pullets and adult layers was due to a significant difference in infection rate (β). No significant differences in transmission rates were observed among layers of different ages. Vaccination reduced transmission, virus shedding, and mortality, particularly in chickens with **homologous HI titers ≥ $\log_2$ 7**. Vaccination reduced mortality to **0.0% (0.0-10.3%)** in chickens vaccinated with VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND, compared to non AI-vaccinated controls (mortality: **100% (94-100%**)**.** Among vaccinated chickens, no significant difference in mortality was observed despite having high or low HI-titers.

Egg production in group B remained stable during experimental challenge at **84 weeks of age**, with no detectable production losses in vaccinated groups before and after challenge. In contrast, group A showed a drop in egg production, declining from 0.8 pre-challenge to 0.5 post-challenge. Under field conditions, vaccinated flocks reached a normal peak of lay despite subclinical avian metapneumovirus (AMPV) infection, whereas reduced egg production was observed only in non AI-vaccinated chickens of farm A (Chapter 2.2.7). In experimentally infected chickens, viral RNA (of the challenge virus) was detected in choanal and/or cloacal swabs in 10 of 12 chickens vaccinated with VAXXITEK HVT+IBD+H5, with feather positivity in 6 of 12 chickens. Infectious virus was isolated (3 days post challenge) only from a chicken lacking HI antibodies (pre-challenge), indicating systemic infection in the absence of detectable humoral immunity. Egg contents were mostly negative for viral RNA detection. Occasional positivity detected on eggshells or in a single egg white, along with limited eggshell contamination, was consistent with **external contamination rather than internal infection. This indicates that the risk to egg safety and poultry products is minimal.**

Population-level impact and surveillance implications. (Chapter 6)
Epidemiological modeling, integrating field and experimental data, demonstrated that vaccination substantially reduced both outbreak probability and outbreak size. The predicted **effective reproduction number (R_e)** fell **below 1 during approximately 25-69% of the production period** (4 and 11/16 monthly samplings done for Flocks A and B respectively) only in the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND groups. This was also dependent on the antibody cut-off used, and was **reduced from 6.4 to a median of ~1.5-1.7** during the remaining weeks (Chapter 3). This R value above 1 means transmission is still possible, but the substantial reduction indicates that spread progresses more slowly and affects fewer chickens before detection. For the flocks without booster vaccination, the level of induced antibody responses were insufficient to drop the predicted R_e below 1 for the duration of the production period. **As a result, vaccination reduced the probability of outbreaks to 10-28% vs 96% without vaccination** and reduced the median epidemic size to **1–2 farms instead of a median of 14 farms if vaccination is not applied**. However, because vaccination lowers mortality, **passive surveillance alone detected only 0.0-19.2% of outbreaks** in vaccinated flocks. This reduced visibility of disease highlights **the need for structured active surveillance**, including regular testing of dead chickens, to ensure timely detection and rapid response.

Overall conclusion.
This longitudinal field study demonstrates that AI vaccination in layer chickens is **safe, compatible with commercial production vaccination schedules**, provides robust clinical protection, safeguards poultry products, and reduces within flock transmission, although it does not consistently reach the R<1 threshold required to effectively halt transmission within a flock. Although an R>1 indicates a theoretical potential for sustained transmission in a AI-vaccinated flock**, effective surveillance enables early detection and rapid intervention**. The significant reduction in R in vaccinated flocks in combination with enhanced surveillance is, however, sufficient to **prevent most of the onward transmission**, within the flock, poultry flocks, and humans. As a result, onward transmission can be halted despite an underlying R greater than 1. **In conclusion, while vaccination alone may not fully stop within-flock transmission under all circumstances, the combined strategy of additional vaccination and surveillance on top of existing biosecurity measures, is a robust, evidence-based, strategy for effective long term control of avian influenza.**

Nederlandse samenvatting

Deze meerjarige veldstudie, uitgevoerd op twee commerciële leghennenbedrijven in Nederland, laat zien dat vaccinatie tegen vogelgriep (H5) een belangrijke bijdrage kan leveren aan de beheersing van de infectie en daarmee de ziekte door vogelgriep op leghennenbedrijven. In de studie werden twee koppels leghennen, onder praktijkomstandigheden gevolgd, vanaf het uitkomen uit het ei tot het einde van de legperiode. Het hoofddoel was om te bepalen in hoeverre Het hoofddoel was om te bepalen in hoeverre vaccinatie (zowel enkelvoudige als in combinatie met een heterologe booster) de transmissie (verspreiding) van het vogelgriepvirus binnen en tussen koppels kan beperken.

Op verschillende leeftijden werden groepjes kippen overgebracht naar de faciliteiten van WBVR om de effectiviteit van vaccinatie tegen transmissie te kunnen bepalen. Hierbij werd de helft van de kippen geïnfecteerd met het vogelgriepvirus (H5), waarna de mate van verspreiding van dat virus binnen deze groepen werd gevolgd. Zonder vaccinatie bleek één besmette volwassen leghen gemiddeld meer dan zes andere kippen te kunnen infecteren. Bij de gevaccineerde volwassen leghennen ging de transmissie van het virus tussen de kippen langzamer en hing de mate van vermindering sterk af van het antistofniveau (hoeveelheid afweerstoffen in het bloed) in de groep. Groepen waarin vrijwel alle dieren hoge antistofniveaus hadden, vertoonden duidelijk minder transmissie dan de groepen waar meer leghennen lagere antistofniveaus hadden. Met andere woorden: niet alleen vaccineren is belangrijk, maar vooral het bereiken en behouden van voldoende uniform hoge antistofniveaus binnen een koppel.

Om de antistofniveaus van de leghennen in het veld te volgen, werden zij met een grote steekproef maandelijks bemonsterd. De gemiddelde antistofniveaus namen toe gedurende de opfok en bereikten hoge niveaus gedurende de productieperiode. Daarbij was er een duidelijk verschil tussen vaccinatiestrategieën: koppels met alleen een basisvaccinatie vertoonden meer fluctuatie in antistofniveaus, terwijl koppels met een booster gedurende de hele productiecyclus consistenter hoge antistofniveaus behielden.

Op basis van de gecombineerde gegevens uit de transmissiestudies en veldmetingen zijn berekeningen gemaakt op koppelniveau. Deze laten zien dat vaccinatie zowel de kans op een uitbraak als de omvang ervan sterk vermindert. Zonder vaccinatie is de kans op een uitbraak van het vogelgriepvirus na introductie zeer groot (96%), terwijl deze met vaccinatie daalt tot ongeveer 10-28% (afhankelijk van het toedienen van een booster).

Een aandachtspunt bij vaccinatie is dat verminderde ziekte en sterfte kunnen leiden tot minder zichtbare uitbraken. Zonder actieve monitoring zou naar schatting slechts 0 tot 19.2% van de uitbraken worden gedetecteerd op bedrijven waar de kippen zijn gevaccineerd. Met een passend surveillancesysteem kan echter minimaal 93.7%van de uitbraken worden opgespoord in gevaccineerde koppels.
De vaccinatie bleek veilig voor de dieren. Er werden geen zichtbare bijwerkingen waargenomen, de gezondheid van de koppels bleef stabiel en de sterfte bleef binnen normale waarden. Ook de eiproductie bereikte een normaal niveau. Er zijn dus geen negatieve effecten van de vaccinatie(s) op de productieprestaties en het dierenwelzijn waargenomen.

Uit meer dan 8.600 bloedtesten bleek bovendien dat veldinfecties bij gevaccineerde koppels goed detecteerbaar blijven met de huidige bloedtest omdat deze niet positief wordt door de vaccinatie zelf: 99,8% van de monsters testte negatief (binnen het verwachte fout-positieve bereik). Dit wijst op de afwezigheid van viruscirculatie, wat werd bevestigd door negatieve PCR-resultaten van gepoolde monsters van gestorven dieren.

Wat betreft voedselveiligheid werden slechts incidenteel PCR-positieve monsters op de eierschaal en in weefsels gevonden in het transmissie-experiment (op 24 weken leeftijd), zonder dat infectieus virus kon worden geïsoleerd. Infectieus virus werd uitsluitend geïsoleerd uit monsters van één individuele leghen zonder aantoonbare antistoffen voorafgaand aan infectie.

Samenvattend laat deze studie zien dat vaccinatie tegen vogelgriep bij leghennen effectief bijdraagt aan het verminderen van virus transmissie en sterfte bij leghennen. In combinatie met adequate surveillance is vaccinatie daarmee een aanvullende strategie om vogelgriep te beheersen.

1 Introduction

1.1 Global HPAI landscape since 2020 and its impact on poultry production

Since 2020, highly pathogenic avian influenza (HPAI) has caused a large and prolonged epizootic in poultry populations, driven primarily by the evolution and global dissemination of H5 A/goose/Guangdong/1/1996 (Gs/Gd) related H5Nx viruses belonging to clade 2.3.4.4b [1]. The current panzootic has persisted for multiple years, spread intercontinentally and exhibited endemic-like persistence in several regions. The scale of losses has been unprecedented: more than 200 million domestic birds have died or been culled worldwide between 2020 and 2025 [2], severely disrupting poultry production systems and supply chains. Persistent circulation of these H5Nx HPAI viruses in migratory wild bird populations continuously places enormous pressure on the global poultry sector, a cornerstone of affordable animal protein.

Infection of gallinaceous species (example chicken and Turkeys) can result in near 100% mortality. For layers and breeders, this means catastrophic losses in egg production, hatching capacity, and genetic resources. Additional indirect consequences, such as, stamping-out policies, implemented to contain infection, combined with movement restrictions, have caused economic losses estimated in the billions of euros annually, affecting producers, integrators, and associated industries such as feed, transport, and processing [2]. Prolonged production downtimes and depopulations reduce flock turnover and compromise long-term viability. Trade disruptions have been profound: major poultry exporters faced temporary bans from importing countries following outbreaks, destabilizing international markets and leading to surges in poultry and egg prices in affected regions.

The sustained circulation of Gs/GD-related HPAI viruses (first human cases detected in Hong Kong in the 1990's) has also forced major structural adjustments in poultry production worldwide, but particularly in high-density regions of Europe, Asia, and North America. Producers have invested heavily in enhanced biosecurity prompting some to relocate production to lower-risk areas. However, these measures alone have proven insufficient, as outbreaks continue to occur in commercial poultry.

The detection of novel reassortant genotypes across multiple European regions illustrates the dynamic genetic landscape and the potential for altered host range or viral fitness [3]. Although current assessments characterize the risk to the general human population as low, sporadic spillovers to mammals and documented zoonotic infections highlight the ongoing threat of adaptation toward more efficient mammalian transmission [4]. This evolving risk landscape demands integrated surveillance, comprehensive genomic monitoring, and a One Health framework to anticipate and mitigate potential zoonotic and pandemic outcomes [5]. Altogether, the ongoing nature of this panzootic has renewed international interest in vaccination as a complementary strategy to reduce transmission to, within and between poultry farms.

This study aims to demonstrate the effectiveness of HPAI vaccines in reducing infection, limiting onward transmission between chickens, and mitigating disease during repeatedly introduced HPAI outbreaks, to support the Netherlands' ambition to implement vaccination. This study consists of a series of experimental studies which were designed to inform policy and improving HPAI outbreak control strategies.
The first study of this series evaluated four vaccine formulations in 8-week old Lohmann Brown Classic laying hen pullets vaccinated and housed under controlled laboratory facilities [6]. This highly standardized approach minimized environmental and management-related confounders, enabling a clear assessment of vaccine-induced protection. Chickens vaccinated with HVT-H5 vaccine from CEVA (VECTORMUNE® AI) or HVT-H5 COBRA vaccine from BIAH (VAXXITEK HVT+IBD+H5) showed a significantly reduced transmission of H5N1 clade 2.3.4.4b virus compared to non AI-vaccinated controls with reproduction ratio estimates below 1 ($R<1$), indicating the potential to halt virus circulation within a flock. Both HVT-H5 vaccines provided complete protection against clinical disease and mortality, and substantially reduced both the number of virus-shedding chickens and the quantity of virus shed, yielding critical data for selecting these candidates for further evaluation.

Building on these findings, the selected vaccines were evaluated in field settings to assess their ability to induce immunity under commercial housing conditions, where chickens are naturally exposed to environmental stressors and diverse microbial challenges. This approach is essential, as previous studies in Indonesia and the Netherlands have shown that immunity induced by inactivated vaccines can be substantially lower in the field than under controlled laboratory conditions [7, 8]. In the present study, chickens were vaccinated and housed under field conditions, from approximately 19 weeks of age at two commercial farms. At predefined time points, subsets of chickens were transferred to animal facilities, where standardized transmission experiments were conducted to enable a controlled assessment of vaccine effectiveness on infection, onward transmission, and disease outcomes (see paragraph 1.2 for details of the study design).

Two progress reports of this longitudinal study have been published [9, 10] and [11, 12]. In the first progress report, Germeraad et al. (2024) described the effectiveness of the vaccines tested at 8 weeks post-vaccination using chickens that were vaccinated and raised on a commercial farm, before being included in a transmission study in the controlled animal facilities of WBVR. In that transmission study, none of the vaccinated chickens became infected, while non AI-vaccinated controls exhibited efficient transmission. These findings highlighted the strong protective effect of vaccination during the rearing phase when tested at 8 weeks post vaccination and demonstrated that real-world environmental factors did not compromise the effectiveness of the vaccines at 8 weeks of age.

Following, a second transmission study was conducted [10, 11] involving a second subset of the chickens that had been maintained under field conditions for 23 weeks, and were challenged at 24 weeks of age (in animal facilities of WBVR), corresponding to the onset of the peak laying period. Vaccinated layers exhibited substantially reduced virus shedding and mortality compared to non AI-vaccinated controls, and the estimated R-value in the small subset of vaccinated chickens tested, ranged between 2.76 (confidence interval 1.12-5.67) in the VAXXITEK HVT-IBD-H5 and 0.28 (0.07-1.01) in VAXXITEK HVT-IBD-H5 + Volvac® B.E.S.T. AI+ND vaccinated groups, whereas non AI-vaccinated chickens in the control groups exhibited an estimated R-value of approximately 15 (6.00-32.99). The wide confidence intervals around these R estimates underscore the need for cautious interpretations of these results, and it was not yet possible to draw firm conclusions regarding protection against sustained transmission. Nonetheless, these results indicate that vaccination can significantly mitigate transmission, even when immunity may have waned over time. In this final report all results obtained within the scope of this study are presented, including two additional transmission studies performed with additional subsets of chickens from the field, now transported to the animal facilities at 53, and 83 weeks of age, and challenged at 54 and 84 weeks of age.

1.2 Study overview

This final report of the longitudinal field study further expands the evidence base by monitoring vaccinated commercial flocks throughout the entire production cycle. This study incorporates repeated serological sampling and observational data on the distribution of antibody titers in these flocks over time enabling a comprehensive assessment of immune persistence, inter-individual variability, and operational feasibility of implementing HPAI vaccines in the currently applied Dutch vaccination programs. In addition, transmission studies under controlled conditions enabled collection of critical parameters, such as transmission parameters between individuals with different antibody titers, serological responses to challenge, and levels of protection following challenge. Unlike previous controlled studies, this study captures real-world heterogeneity in vaccine responses. This variation is shaped by factors such as immune waning, reactivation of the vector virus, antigenic distance between vaccines and the challenge virus, host genetics, management practices, concurrent infections, and environmental stress. Capturing such variability is essential for understanding flock immunity dynamics and for designing vaccination strategies that are effective at scale.

1.2.1 Timeline of the longitudinal field trial

In September 2023, the eggs were hatched at a commercial hatchery, and the regular vaccinations were given (if compatible with the AI-vaccination, details in chapter 2.2.3 and Appendix Table 1.1). The vaccination against avian influenza virus (VAXXITEK HVT-IBD-H5) was applied at the Royal GD test facilities on the same day. Chicks were housed in separate groups from that point onward and transported to a single combined rearing and production farm (referred as rearing farm/farm A) and raised entirely under field conditions (chapter 2.2.1). The schematic representation of the entire study is visualized in Figure 1.1.

Throughout the rearing period, chicks received the standard Dutch vaccination program (details, chapter 2.2.3, and Appendix Table 1.1). At 12 weeks of age (day 84), one of the two AI vaccinated groups received a heterologous to the prime booster vaccination with Volvac® B.E.S.T. AI+ND.

At 19 weeks of age, approximately half of the chickens per test group were relocated to another layer production facility (farm B). The chickens were housed there (Farm B) and in farm A until the end of the study (chapter 2.2.1).

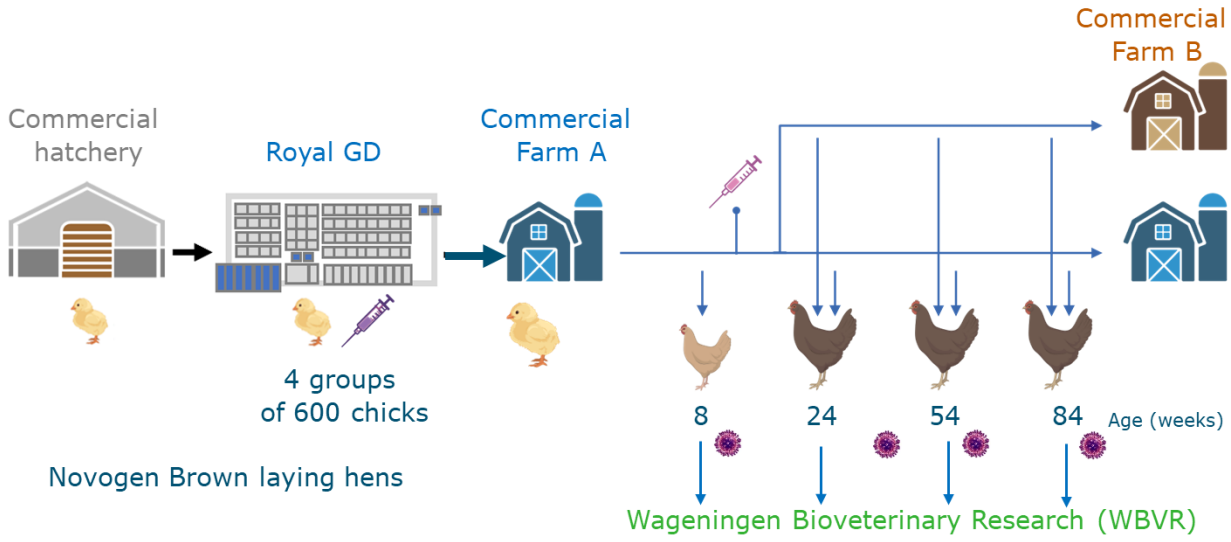

Figure 1.1 *Study overview; Overview of locations: hatchery (grey), Royal GD (white), rearing farm/farm A (blue) and farm B (brown). The arrows indicate the age of the chickens at the moment the challenge was applied to a subset of the chickens from rearing farm/farm A and farm B at WBVR. The syringes indicate the moment of vaccination with VAXXITEK HVT+IBD+H5 (at Royal GD) and a heterologous to the prime booster with Volvac® B.E.S.T. AI+ND (at farm A).*

This longitudinal field study is a collaboration between different research institutes in the Netherlands, and the work was divided in separate Work Packages. Work Package 1 included all observations and analysis on samples collected in the field phase, at the different farms. Here egg production, morbidity and mortality were monitored, blood samples* and swabs were collected. The collected samples were analyzed in the research facilities of the Royal GD. The results are reported in chapter 2.

*some blood samples were analyzed in Work Package 3.

In Work Packages 2, the efficacy of vaccination against transmission of HPAI, at different ages of the production cycle, was experimentally evaluated. Subsets of randomly selected vaccinated and non AI-vaccinated chickens were relocated from the field, to the animal facilities of Wageningen Bioveterinary Research (WBVR) at four timepoints (7, 23, 53 and 83 weeks of age). Whitin these transmission studies chickens were challenged with an HPAI H5N1 clade 2.3.4.4b virus (see experimental methods in Appendix chapter 2.1). The challenge virus was detected and isolated in 2021 from a laying hen farm in the Netherlands (details in Appendix chapter 2.1). The experimental set-up and statistical analysis were a cooperation between Wageningen Bioveterinary Research and Wageningen University (Infectious Disease Epidemiology, IDE).

In Work Packages 3, immunological parameters were analyzed by Wageningen University & Research (Cell Biology and Immunology, CBI) . Some of the blood samples collected in Work Packages 1 were analyzed to determine the number of T cells, a specific type of immune cells, by whole blood staining. Blood samples were taken from 10 chickens per study group to assess numbers of activated T cells on day 3, 7, 10 and 14 after vaccination with VAXXITEK HVT+IBD+H5 and on day 7 and 14 after vaccination with Volvac® B.E.S.T. (Details in paragraph 2.3.6).
Furthermore, samples were collected of the challenged chickens in the four transmission studies and were analyzed to determine cellular immunological parameters post challenge. During the second transmission study (inoculation at 24 weeks of age), different methods (ELISPOT and whole blood staining) were compared to demonstrate the development of a cellular immune response. This comparison was performed by testing 25 chickens.

Work Package 4, a collaboration between Utrecht University, WBVR and Wageningen University (Infectious Disease Epidemiology, IDE), integrated data from Work Packages 1, 2 and 3 to estimate and simulate scenarios related to the implementation of vaccination against HPAI and its potential effects on outbreaks in poultry farms in the Netherlands. Outcomes of interest included expected transmission of avian influenza viruses within and between farms, as well as implications for risk-based monitoring and surveillance programs.

2 Field Observations (Work Package 1)

2.1 Key Findings

Safety and health: No local vaccine reactions were observed, and no major clinical abnormalities occurred on either farm. Subclinical avian metapneumovirus (AMPV) and *Mycoplasma synoviae* (Ms) infections were detected, with no clinically relevant impact.

Vaccine-induced immunity: Vaccinated chickens developed robust and sustained H5 HI antibody responses from 5 weeks of age onwards, with mean titers increasing to $\log_2$ 7.9 (VAXXITEK HVT+IBD+H5) and $\log_2$ 9.1 (VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND) by the end of the study. No H5 HI antibodies were detected in non AI-vaccinated chickens.

DIVA performance and AI freedom: NP-ELISA (IDEXX) testing of 8,630 sera showed 99.8% negativity, with the proportion of positives within the expected false-positive range (Monitoring data Royal GD). All pooled tracheal and cloacal swabs from dead chickens were M-PCR negative for avian influenza virus. Together, these results provide evidence of the absence of avian influenza field infection in the groups during the study period.

Production performance: Despite AMPV infection during the transition to lay, all vaccinated groups reached a normal peak of egg production. Reduced egg production was observed only in the non AI-vaccinated group on farm A due to early loss of productivity in some low-ranking chickens.

Mortality: Cumulative mortality was within the expected range in the vaccinated groups and higher in the non AI-vaccinated group on farm A (Table 2.2). Elevated mortality on farm A in the non AI-vaccinated group was attributed to multiple causes including *E. coli* septicaemia, feather pecking, cannibalism, red mite infestation, and an isolated fright-related piling event.

Cellular Immunity:
The newly developed whole blood staining assay can be used to monitor changes in activated CD4+ and CD8+ T cells in the blood upon vaccination. Activated CD4+ and CD8+ were readily detected in all chickens and fluctuated in time. No clear differences were observed between the AI-vaccinated and the non AI-vaccinated groups. This assay measures activation across the entire T-cell population, but it does not allow attribution of the observed activation to a specific vaccine in situations where multiple vaccinations are applied, such as a field study.

Overall conclusion: Vaccination was safe, induced strong and persistent humoral immunity, supported effective DIVA surveillance, and was associated with normal production performance.

2.2 Field situation

2.2.1 Housing and locations

The chickens were housed at two Dutch commercial poultry holding locations (housing of the chickens, and collection of samples). The collected samples were tested at the research facility of Royal GD (GD) in Deventer (Humoral immunity and M-PCR for monitoring, paragraphs 2.3.2-2.3.3) and at Wageningen University & Research (CBI) in Wageningen (Cellular immunity paragraph 2.3.6).

The eggs were hatched in a commercial hatchery on September 15th 2023. The day-old chicks (D0) were all placed at a single combined rearing and production farm (Farm A) in the province of Overijssel and raised entirely under field conditions. The chickens were initially all housed in one barn, separated in four test groups of 599 or 600 chickens per test group. Each group was housed indoors on a combined slatted and litter floor with wood shaving bedding, an automated feeding system and a nipple drinking system. At farm A, commercial layer hens were present in an adjoining house. Flock information regarding mortality was recorded by the poultry farmer and handed to GD weekly. Water and feed intake were not recorded.

At weeks 7, 23, 53 and 83 of age, a subset of the chickens was relocated, under controlled conditions, to the animal facility of Wageningen Bioveterinary Research (WBVR). At WBVR, HPAI transmission studies were conducted in the BSL3 facilities ([9-12] and Appendix Work Package 2).

At 19 weeks of age, at least 215 chickens per test group were relocated to the second layer production facility (farm B) in the province of Flevoland. At this farm, another layer production flock was also present. The 19-week-old pullets were housed, under field conditions, in one house in four separate groups. The chickens were housed similar to farm A, but on a full litter floor bed. The rest of the pullets remained on farm A, in the same house which was equipped for the collection of eggs.

The chickens at the rearing farm A were housed in an open window house with roof top windows for natural daylight. Additionally, LED lights were present. From the day of arrival onwards at rearing farm A the pullets received 23 hours of light (timer-controlled) which, together with the light intensity, was gradually reduced from day 3 to day 49 (7 weeks of age) to 10 hours. From 18 weeks – to 22 weeks of age the light period, together with the light intensity, gradually increased to 14 hours at both farms. The chickens on farms A and B were fed with commercial feed from different feed mills. The feed of both feed mills matched the standard requirements for chickens of their age. The water supply was *ad libitum*. At both farms standard biosecurity rules were applied. The density during the rearing period was less than 18 chickens per m^2. During the layer period, the density was lower than the legal maximum of 9 chickens per m^2 on both farms.

2.2.2 Chickens

The chickens were commercial Novogen Brown Light layer chickens, offspring of a commercial layer reproduction flock. The parents were vaccinated according to the standard vaccination program for layer reproduction, including a combined HVT and Rispens vaccination against Marek's disease. The commercial day-old chickens used in this study were therefore expected to have maternally derived antibodies (MDA) against HVT.

2.2.3 Vaccines and vaccinations

VAXXITEK HVT+IBD+H5 (manufactured by Boehringer Ingelheim Animal Health) was applied at day of hatch (day 0) with a subcutaneous injection. The cryo-preserved cell-associated recombinant turkey herpesvirus vector vaccine contains an H5 insert (rHVT-H5) in the FC126 strain of HVT. For safety reasons, the High Pathogenic (HP) cleavage site of the COBRA (Computationally Optimized Broadly Reactive Antigen) generated HA gene is changed to a low pathogenic motif. The vector vaccine was applicated subcutaneously in the upper third of the neck. The VAXXITEK HVT+IBD+H5 vaccination and the Marek vaccination in the test groups were conducted at the Royal GD test facility using semi-automatic equipment (Innoject Pro) by an experienced vaccinator.

One test group (Group 3), received a heterologous to the prime booster vaccination at 12 weeks of age (D84) with Volvac® B.E.S.T. AI+ND (manufactured by Boehringer Ingelheim Animal Health) through a subcutaneous injection in the neck by an experienced vaccination team. All vaccinations were performed according to the instructions of the manufacturers. (Table 2.1 and Appendix Table 1.1 for complete vaccination schedule per test group).

Table 2.1 *Test groups based on the different AI H5 vaccinations.*

Test group	Vaccination (day 0)	Vaccination (12 weeks)
1	VECTORMUNE® AI	None
2	VAXXITEK HVT+IBD+H5	None
3	VAXXITEK HVT+IBD+H5	Volvac® B.E.S.T. AI+ND
4 (non AI-vaccinated)	None	None

All chicks were (gel) spray vaccinated at day of hatch with a combination of two live infectious bronchitis virus (IBV) vaccines and against coccidiosis with fully automatic equipment. During the rearing period, chickens were vaccinated according to the standard vaccination program of the veterinary advisors of the rearing company.

2.3 Results Field Observations

2.3.1 Clinical inspection

2.3.1.1 Palpation of the location of injection

None of the palpated pullets showed a local vaccine reaction after both AI vaccinations (performed 30 days post prime vaccination, and twice at 14 and 34 days post booster application).

2.3.1.2 Clinical inspection and further testing

During the monthly clinical inspections by the research team of Royal GD, no significant abnormalities were detected except for sporadic feather pecking, and the presence of poultry red blood mite (*Dermanyssus gallinae)* on farm A.

At 18 weeks of age (farm A) and 19 weeks of age (farm B), an infection with avian metapneumovirus (AMPV) was detected using RT-PCR on tracheal swabs. No clinical signs were observed in both flocks except for a small delay in onset of egg production and a temporary discoloration of eggs. At the same time, a subclinical infection with *Mycoplasma synoviae* (Ms) was detected using PCR on tracheal swabs.

All ND HI-titers were in the range that is expected according to the vaccination scheme and the Dutch law. No antibodies against *Mycoplasma gallisepticum* were detected at the end of the rearing period and at the end of the study. No infection with *Salmonella spp*. was detected on either farm.

2.3.2 Humoral immunity

2.3.2.1 Homologous HI titers in the field (both rearing and production phase)

Figures 2.1 and 2.2 present the boxplots of the homologous HI titers per farm and per test group of the monthly collected 120 serum samples from each AI vaccinated test group and 10 samples of the non AI-vaccinated control group using an antigen closely related to the H5 of the vaccine 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).

Additional details can be found in Appendix Tables 1.2 a, b and c, which demonstrate the mean and median HI titer, range, standard deviation and percentage of sera with an homologous HI titer of ≥3, ≥4, ≥5, and ≥6.

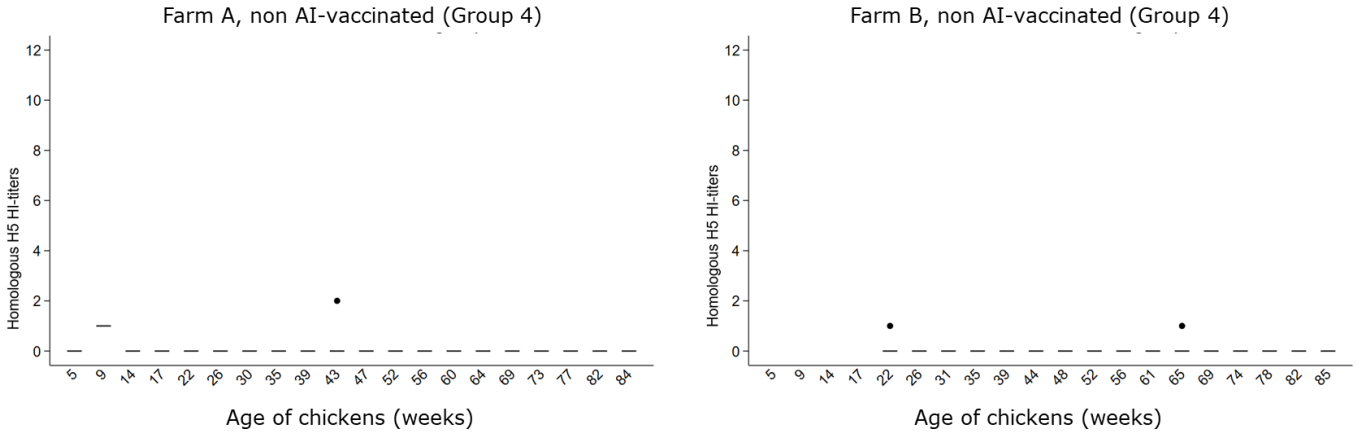

Figure 2.1 *Boxplot demonstrating the H5 HI-titers obtained in sera of non AI-vaccinated chickens (Group 4) in Farm A (left) and Farm B (right) using antigen A/Ch/Indonesia/7/03 EPI_ISL_11512 (Homologous antigen of VAXXITEK HVT+IBD+H5). Per timepoint approximately 10 samples were tested. (additional details in Appendix Table 1.2).*

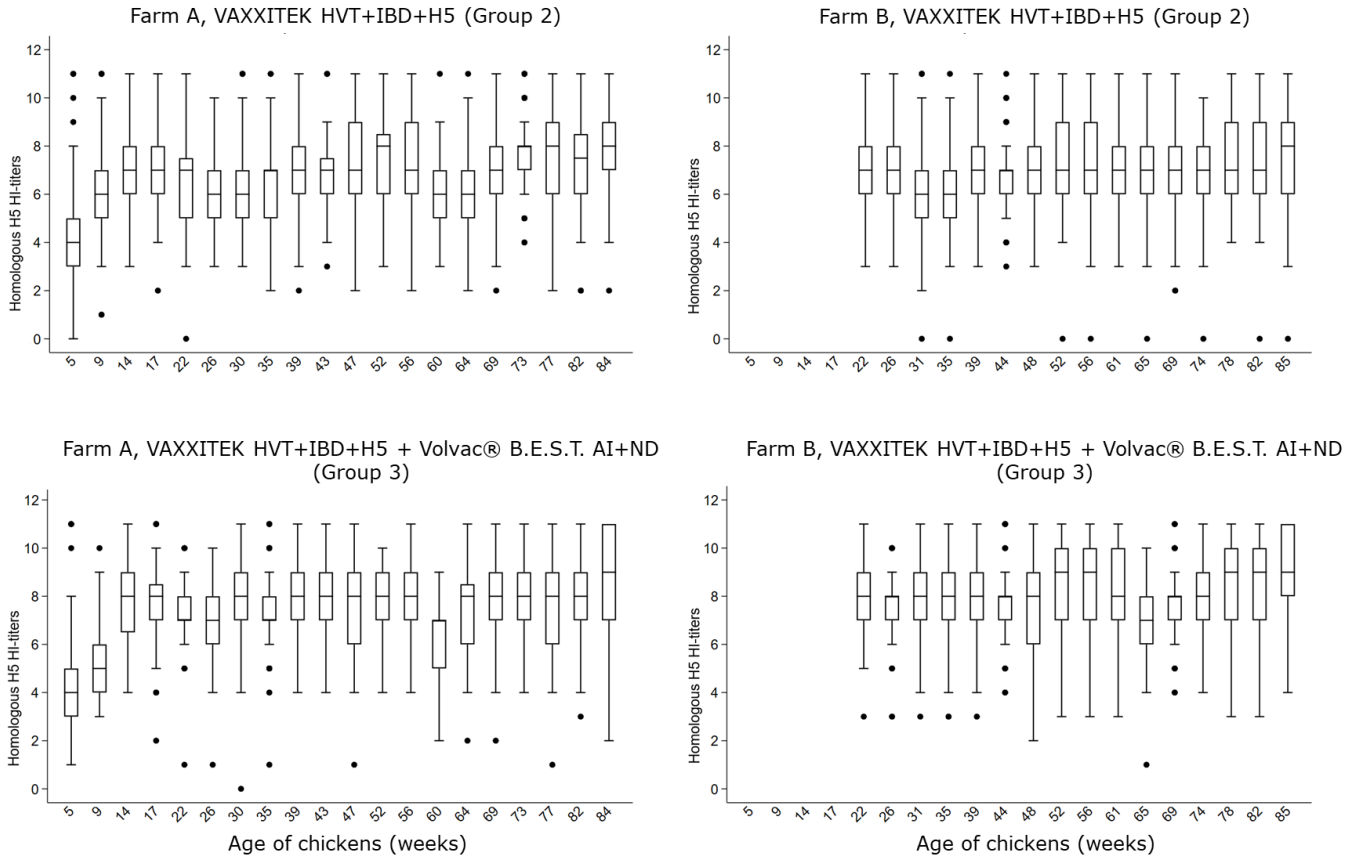

Figure 2.2 *Boxplot demonstrating the H5 HI-titers obtained in sera of the chickens vaccinated with VAXXITEK HVT+IBD+H5 (Group 2) in Farm A (top left) and Farm B (top right). Titers of the chickens vaccinated with VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND (Group 3) in Farm A (low left) and Farm B (low right). All samples were tested using antigen A/Ch/Indonesia/7/03 EPI_ISL_11512 (Homologous antigen). Per timepoint approximately 120 samples were tested. (additional details in Appendix Table 1.2).*

2.3.2.2 NP-ELISA test (IDEXX, Multispecies AIV antibody ELISA)

During the study, 60 blood samples were collected on a monthly basis per test group to demonstrate the absence of antibodies against the NP protein with the ELISA. In total, 8630 sera were tested by ELISA from which 99.80% were negative, 0.2% (17 sera) were ELISA positive. Of these 17 sera, 12 were also positive in the H5 HI-test performed by WBVR using H5 antigen (A/Ch/Neth/14002541/2014 and A/Ch/Neth/13015884/13). The percentage ELISA-negative sera from the vaccinated groups and from the non AI-vaccinated control group was comparable (99.95 and 99.91% and 99.81% respectively). These percentages are within the expected range of false positive rate using this method (Monitoring data Royal GD).

From both test groups 2 (VAXXITEK HVT+IBD+H5), 2153 sera were tested by ELISA from which 2152 were negative (99.95%). The serum that reacted in the ELISA (n=1 at Week 21) was also H5 HI-test positive.
From both test groups 3 (VAXXITEK HVT+IBD+H5 and Volvac® B.E.S.T. AI+ND) 2160 sera were tested by ELISA from which 2158 were negative (99.91%). From sera that reacted in the ELISA (n=2 at Week 47) one was also H5 HI-test positive

2.3.3 M-PCR (M-gene Realtime Reverse transcription Polymerase Chain Reaction)

During the entire field study, all mortality if present (with a maximum of 10 chickens per week per farm) was collected and stored at the farm at -20°C. Every week, dead chickens were transported to the postmortem room of GD for sampling the cloacas and tracheas by swabbing. All tracheal swabs and all cloacal swabs were pooled separately into 2 pools (maximum of 10 swabs per sample). In total, 196 pools of tracheal swabs (n=98) and cloacal swabs (n=98) were tested at Royal GD with the Real-time reverse transcriptase (RRT) PCR for the detection of the M-gene of AI virus (M-PCR). All results were negative.

2.3.4 Mortality

The cumulative mortality (in percentage) during the rearing period (Week 0-18) and production period is shown in Table 2.2. These percentages exclude chickens that have been removed for T cell research and the different transmission studies but include chickens euthanized for animal welfare reasons, for example because of severe lameness. For each farm, the mortality during the production period is subdivided into 4 periods: Week 19-35, Week 36-52, Week 53-69, and Week 70-85.

The total mortality rate for Groups 2 and 3 on both Farms A and B and Group 4 on Farm B were within the expected range of healthy brown layer flocks. Group 4 on Farm A showed an increased level of mortality. Most of the increased mortality was caused by septicaemia due to *E coli*, feather pecking and cannibalism from week 60 onwards. Earlier in this group, a severe infestation of *Dermanyssys gallinae* had been detected (Week 51) and extra mortality occurred in this group in week 31 when a number of chickens flew into a pile during a fright reaction.

Table 2.2 *Cumulative mortality during rearing and production period for both vaccinated test groups (Group 2 =VAXXITEK HVT+IBD+H5, Group 3 =VAXXITEK HVT+IBD+H5 and Volvac® B.E.S.T. AI+ND) and chickens of the non AI-vaccinated control group (Group 4) in both Farm A and Farm B.*

	VAXXITEK HVT+IBD+H5 (Group 2)		VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND (Group 3)		Non AI-vaccinated (Group 4)	
Period	**A**	**B**	**A**	**B**	**A**	**B**
Rearing (Week 0-18)	1.0		1.3		2.9	
Production period						
Week 19-35	1.9	1.9	0.3	0.9	2.3[C]	2.0
Week 36-52	0.6	1.9	0.3	0.9	1.2[D]	1.2
Week 53-69	0.6	0.9	0.6	1.8	2.5	0.4
Week 70-85	4.4[E]	0.0	5.8[E]	0.9	6.4[E]	1.2
Total (Week 19-85)	7.6	4.7	7.1	4.4	12.4	4.8

[C] Including 0.9% mortality due to chickens flying into a pile during a fright reaction

[D] Increased mortality due to severe infestation of *Dermanyssus gallinae*

[E] Increased mortality due to *E. coli* septicaemia due to feather picking and cannibalism

2.3.5 Egg production

During the transition of the rearing period into the production period, a field infection occurred with AMPV. On both farms, the chickens in all vaccinated groups reached the normal peak of egg production (Figures 2.3). At farm A, some chickens in the non AI-vaccinated group that were low in the pecking order lost their productive state at an early age, without reaching the humane endpoints. Since all groups consisted of relatively small numbers of chickens, these non-producing chickens reduced the overall egg production of this group by a few percent.

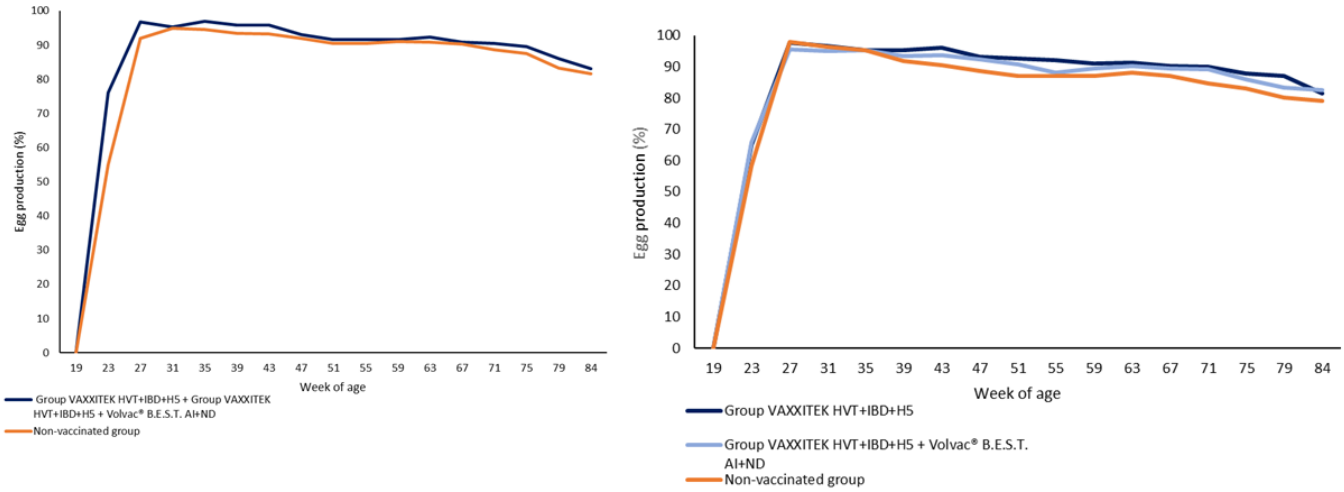

Figure 2.3 *Egg production in percentage of the vaccinated chickens and non* AI*-vaccinated chickens on Farm A (left) Farm B (right) from start of the production period till the end of the study. It was not possible to give a separate percentage between Group VAXXITEK HVT+IBD+H5 and Group VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND on Farm A.*

2.3.6 Cellular immune response

To determine possible changes in the number of T cells in the blood upon vaccination, blood was collected at 3, 7, 10 and 14 days post vaccination with Vaxxitek HVT+IBD+H5. Moreover, blood was collected every month to monitor possible long term effects on T cells in the blood and at 7 and 14 days after the vaccination with Vaxxitek HVT+IBD+H5 in presence of absence of the heterologous to the prime booster vaccination with Volvac® B.E.S.T ND+AI. Total numbers of T cells were gated by selecting the CD45+ CD3+ cells and within this population CD4+ and CD8+ T cells were determined. In addition, numbers of CD25+CD4+ T cells and CD8+CD25+ T cells were determined, since surface expression of CD25 indicates T cell activation.

Table 2.3 *Numbers of T cells in the blood of VAXXITEK HVT+IBD+H5 vaccinated versus chickens of the non AI-vaccinated control group at 7 days post vaccination. Significant differences are indicated in bold and marked with [1].*

	VAXXITEK HVT+IBD+H5 (Group 2)				**Non AI-vaccinated (Group 4)**				
No. of T cells/ ml	**Mean**	**Median**	**Min.**	**Max.**	**Mean**	**Median**	**Min.**	**Max.**	**p-value**
CD8+	86,902	87,875	63,036	128,806	73,236	59,012	51,6	126,557	**0.09**
CD4+	292,938	313,096	41,016	441,38	236,049	249,82	104,51	405,779	0.21
CD25+CD8+	47,447	43,688	35,107	66,628	40,658	38,562	26,18	62,836	0.23
CD25+CD4+	166,905	178,011	35,404	265	141,429	149,981	75,68	230,104	0.36

[1]Total numbers of T cells were gated by selecting the CD45+ CD3+ cells. Data from both groups of Vaxxitek HVT+IBD+H5 vaccinated are combined.

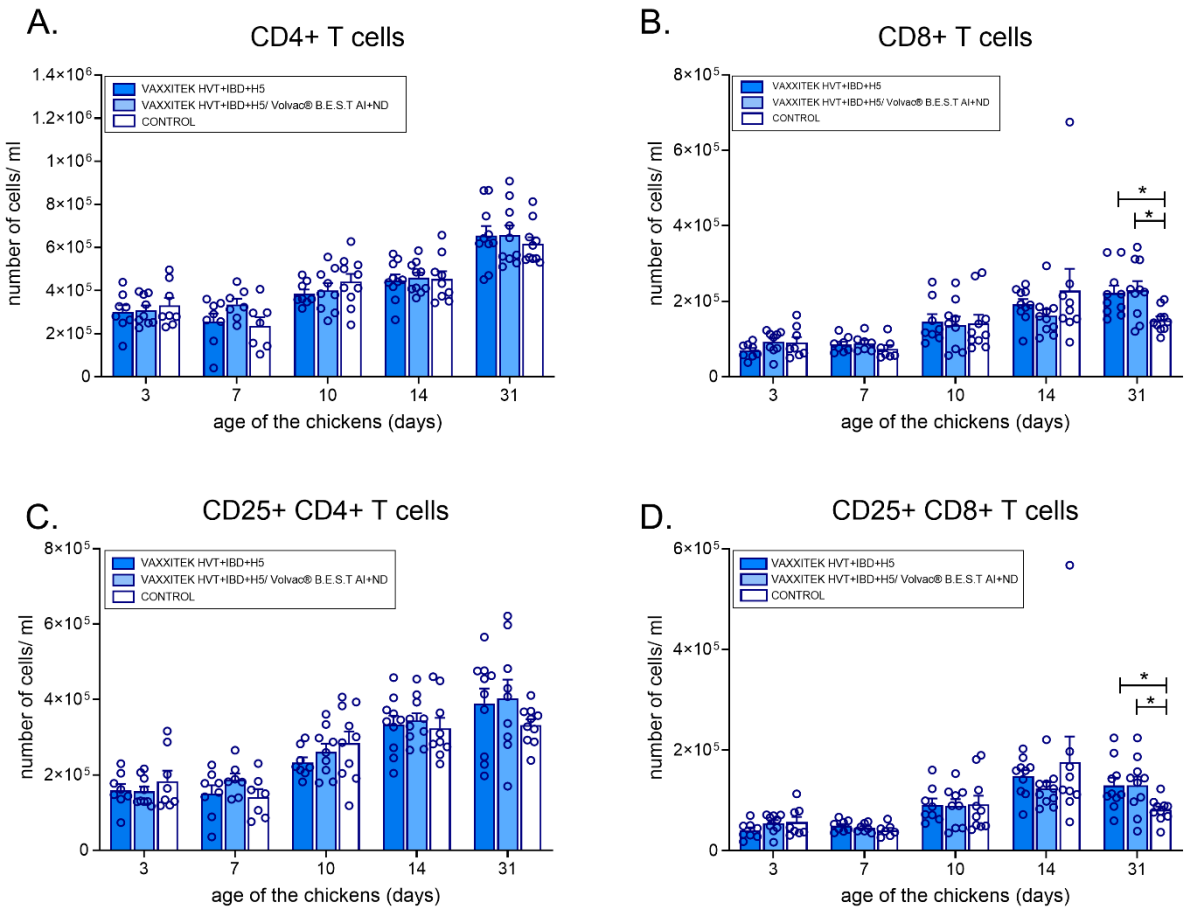

Figure 2.4 *Absolute numbers of T cells in the blood in the first weeks post primary vaccination. In the first weeks post vaccination, absolute numbers of CD4+ T cells (A), and CD8+ T cells (B) were quantified in the blood of Vaxxitek HVT+IBD+H5 vaccinated chickens and non AI-vaccinated controls. In addition, numbers*

of (activated) CD25+CD4+ T cells (C) and CD25+CD8+T cells (D) were determined. Mean + SEM of 10 chickens per group is shown. Each dot represents an individual chicken. Significant differences between vaccinated and non AI-vaccinated controls (p<0.05) are indicated (*).

First the primary response to the Vaxxitek HVT+IBD+H5 vaccine was studied, by comparing the total numbers of T cells and the number of activated T cells in the first 2 weeks post vaccination between the Vaxxitek HVT+IBD+H5 groups compared to the non AI-vaccinated control group. At day 7 after vaccination, numbers of CD8+ and CD4+ T cells (Figure 2.4, Table 2.3) as well as numbers of activated CD4+ and CD8+ T cells (Figure 2.4, Table 2.3) were approximately 1.2 times higher in the Vaxxitek HVT+IBD+H5 vaccinated chickens compared to the non AI-vaccinated controls. The number of activated CD8+ T cells in chickens that received Vaxxitek HVT+IBD+H5 vaccine tended to be higher compared to non AI-vaccinated controls (86,902 versus 73,236 cells/µl, p=0.09). This suggests the induction that vaccine specific CD8+ and CD4+T cells may have been induced upon vaccination with Vaxxitek HVT+IBD+H5.

Next, the effect of the booster vaccination with Volvac® B.E.S.T. ND+AI at day 84 was determined, by comparing numbers of total CD4+ and CD8+ T cells, as well as numbers of activated CD4+ and CD8+ T cells in the 4 weeks post booster vaccination.

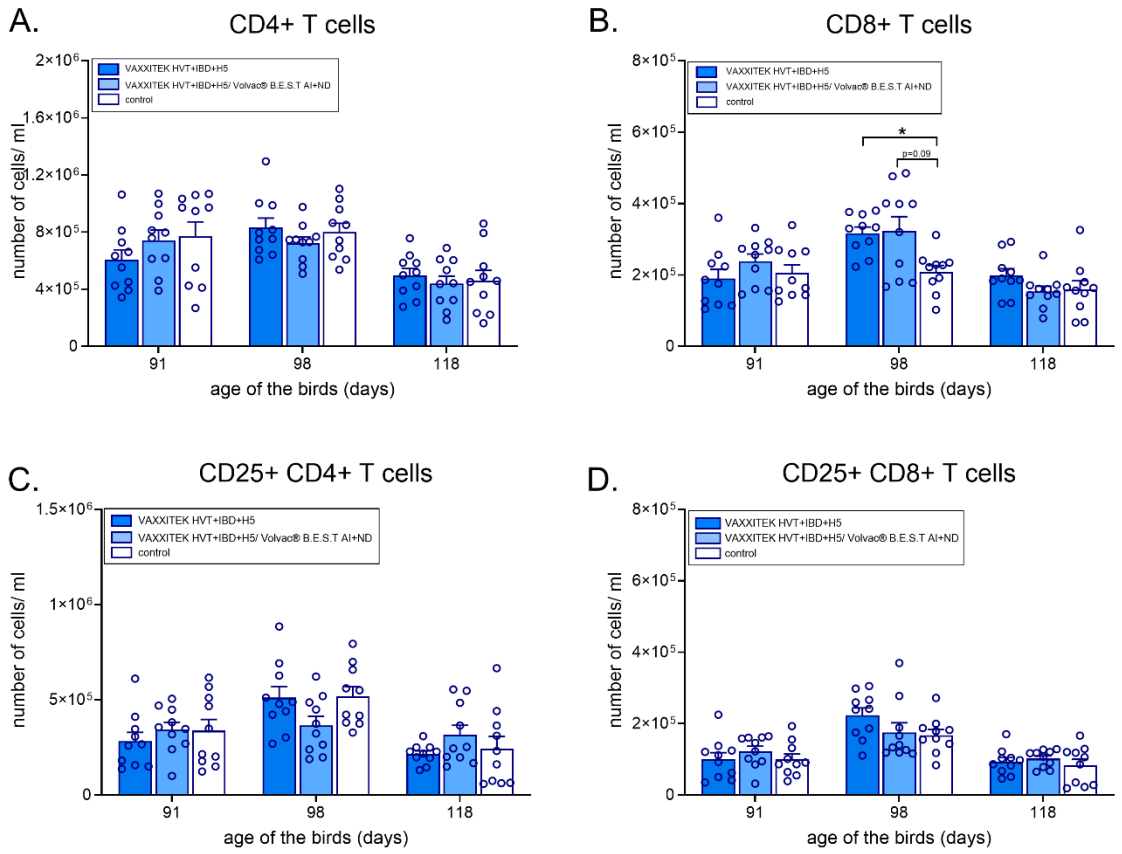

Figure 2.5 *Absolute numbers of T cells in the blood in the first weeks post booster vaccination. Absolute numbers of CD4+ T cells (A), and CD8+ T cells (B) were quantified in the blood of chickens that received a booster vaccination with Volvac® B.E.S.T ND+AI in comparison with chickens that only received the primary vaccination and non AI-vaccinated controls that did not receive any AI vaccine. In addition, numbers of (activated) CD25+CD4+ T cells (C) and CD25+CD8+T cells (D) were determined. Mean + SEM of 10 chickens per group is shown. Each dot represents an individual chicken. Significant differences between vaccinated and non AI-vaccinated controls (p<0.05) are indicated (*).*

No differences were observed in T cell numbers between chickens that received only the primary vaccination with Vaxxitek HVT+IBD+H5 and chickens that received Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI (Figure 2). At day 98, numbers of CD8+ T cells were significantly higher in the Vaxxitek HVT+IBD+H5 group compare the non AI-vaccinated control group. A similar trend was observed when comparing numbers of CD8+

T cells between Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens and non AI-vaccinated controls.

Next, numbers of CD4+ T cells, CD8+T cells, CD25+CD4+ T cells and and CD25+CD8+ T cells were monitored for a period of 587 days. During this period, the chickens received multiple vaccinations and may have been exposed to field infections.

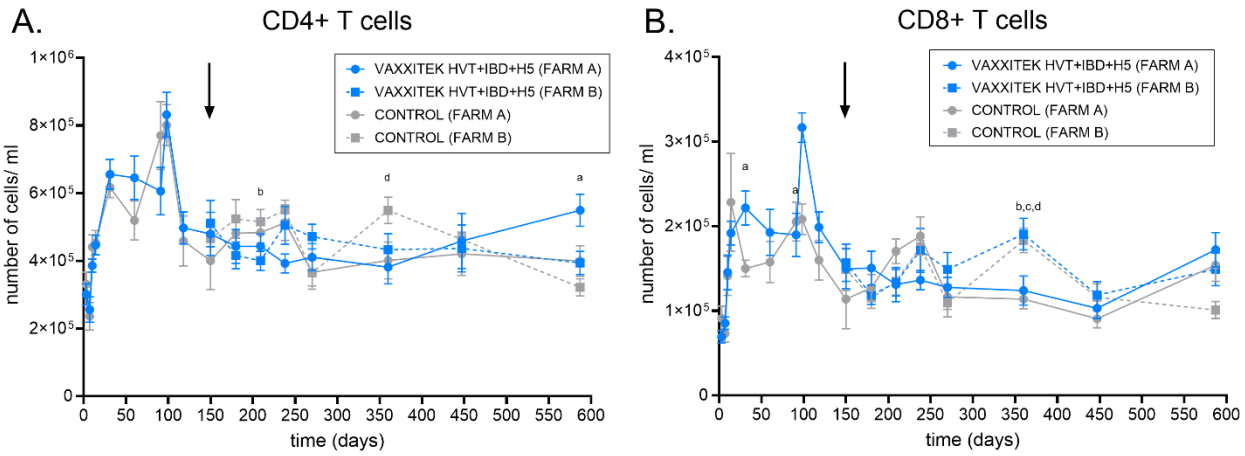

Figure 2.6 *Absolute numbers of T cells in the blood over time post vaccination. Upon vaccination, absolute numbers of CD4+ T cells (A), and CD8+ T cells (B) were quantified in the blood of Vaxxitek HVT+IBD+H5 vaccinated chickens and non AI-vaccinated controls during a period of 587 days. At day 150, half of the chickens were transported to another farm. This timepoint is indicated by the arrow, and from day 150 onwards 4 groups are shown divided over two farms. Mean + SEM of 10 chickens per group is depicted. Significant differences between Vaxxitek HVT+IBD+H5 vaccinated and non AI-vaccinated controls ($p<0.05$) are indicated; a indicates a significant difference between Vaxxitek HVT+IBD+H5 vaccinated and non AI-vaccinated controls on farm A, b indicates a significant difference between Vaxxitek HVT+IBD+H5 vaccinated and non AI-vaccinated controls on farm B, c indicates a significant difference between Vaxxitek HVT+IBD+H5 vaccinated chickens on farm A versus farm B and d represents a significant difference between non AI-vaccinated controls on farm A versus farm B.*

During the first 80 days the number of CD8+ cells tended to be higher in chickens that were also vaccinated with the Vaxxitek HVT+IBD+H5 vaccine with significantly higher numbers of CD8+ T cells in the Vaxxitek HVT+IBD+H5 vaccinated chickens at day 60 post vaccination compared to chickens in the non AI-vaccinated control group (Figure 2.7B). At day 150, half of the chickens were transported to another farm, and T cell numbers in both groups were monitored throughout the study. In general, numbers of CD4+ and CD8+ T cells varied over time, but were not significantly different between groups or farms, except for 350 days post vaccination where differences in especially CD8+ T cell numbers were observed between farms in the Vaxxitek HVT+IBD+H5 vaccinated chickens and the chickens from the non AI-vaccinated control group.

Similar results were observed in chickens that were vaccinated with Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI. Vaccinated chickens had more CD8+ T cells compared to non AI-vaccinated controls until day 80. In time, numbers of CD4+ and CD8+ T cells varied and were occasionally different between farms in both the vaccinated and the non AI-vaccinated control groups.

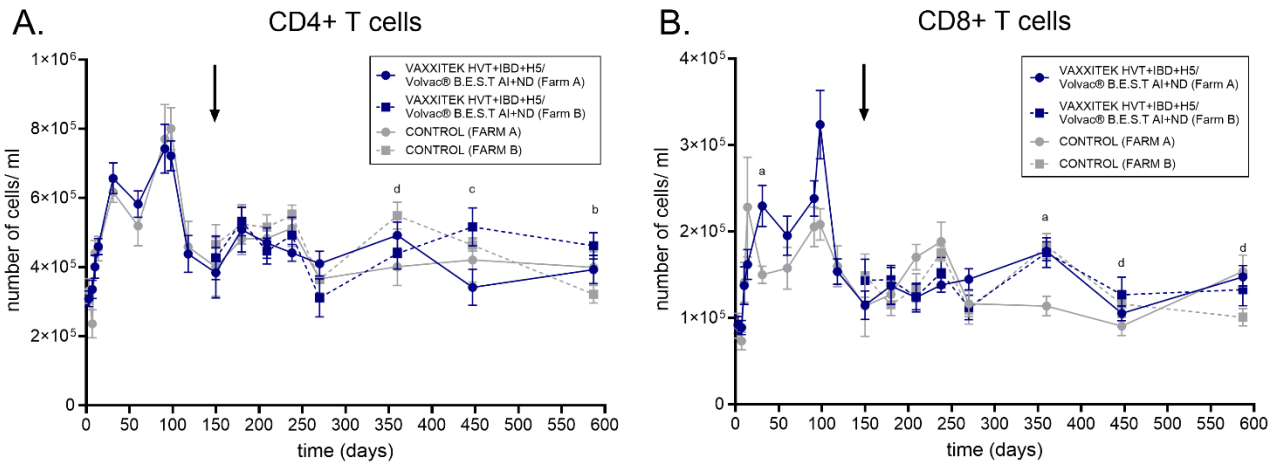

Figure 2.7 *Absolute numbers of T cells in the blood over time post vaccination in Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens. Upon vaccination, absolute numbers of CD4+ T cells (A), and CD8+ T cells (B) were quantified in the blood of* between Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens *and non AI-vaccinated controls during a period of 587 days. At day 150, half of the chickens were transported to another farm. This timepoint is indicated by the arrow, and from day 150 onwards 4 groups are shown divided over two farms. Mean + SEM of 10 chickens per group is depicted. Significant differences between Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens vaccinated and non AI-vaccinated controls ($p<0.05$) are indicated; a indicates a significant difference between Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens vaccinated and non AI-vaccinated controls on farm A, b indicates a significant difference between Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens vaccinated and non AI-vaccinated controls on farm B, c indicates a significant difference between Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens vaccinated chickens on farm A versus farm B and d represents a significant difference between non AI-vaccinated controls on farm A versus farm B.*

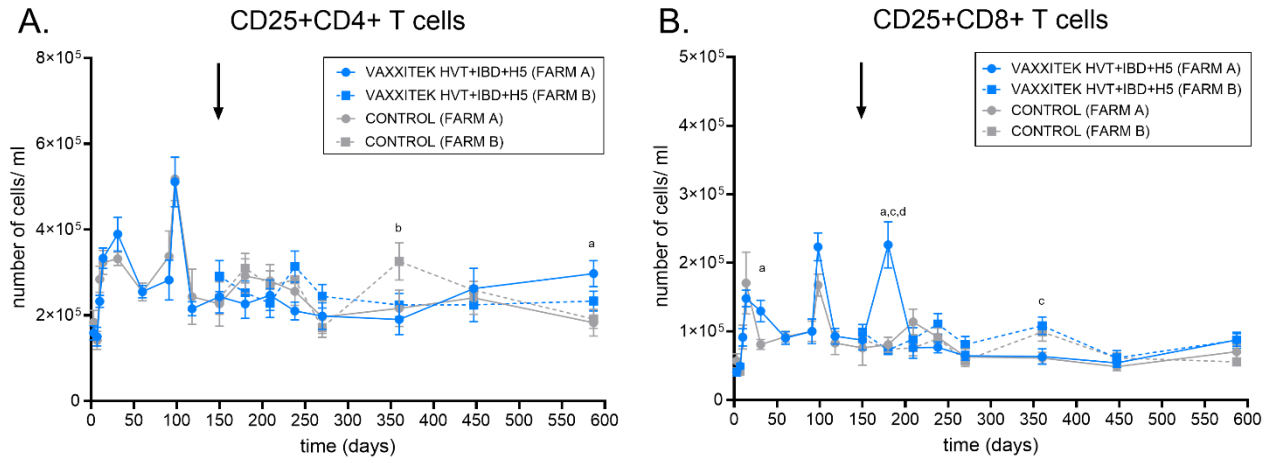

Figure 2.8 *Absolute numbers of activated T cells in the blood over time post vaccination with Vaxxitek HVT+IBD+H5. Upon vaccination, absolute numbers of CD4+ T cells (A), and CD8+ T cells (B) were quantified in the blood of Vaxxitek HVT+IBD+H5 vaccinated chickens and non AI-vaccinated controls during a period of 587 days. At day 150, half of the chickens were transported to another farm. This timepoint is indicated by the arrow, and from day 150 onwards 4 groups are shown divided over two farms. Mean + SEM of 10 chickens per group is depicted. Significant differences between Vaxxitek HVT+IBD+H5 vaccinated and non AI-vaccinated controls ($p<0.05$) are indicated; a indicates a significant difference between Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens vaccinated and non AI-vaccinated controls on farm A, b indicates a significant difference between Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens*

vaccinated and non AI-vaccinated controls on farm B, c indicates a significant difference between between Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens vaccinated chickens on farm A versus farm B and d represents a significant difference between non AI-vaccinated controls on farm A versus farm B.

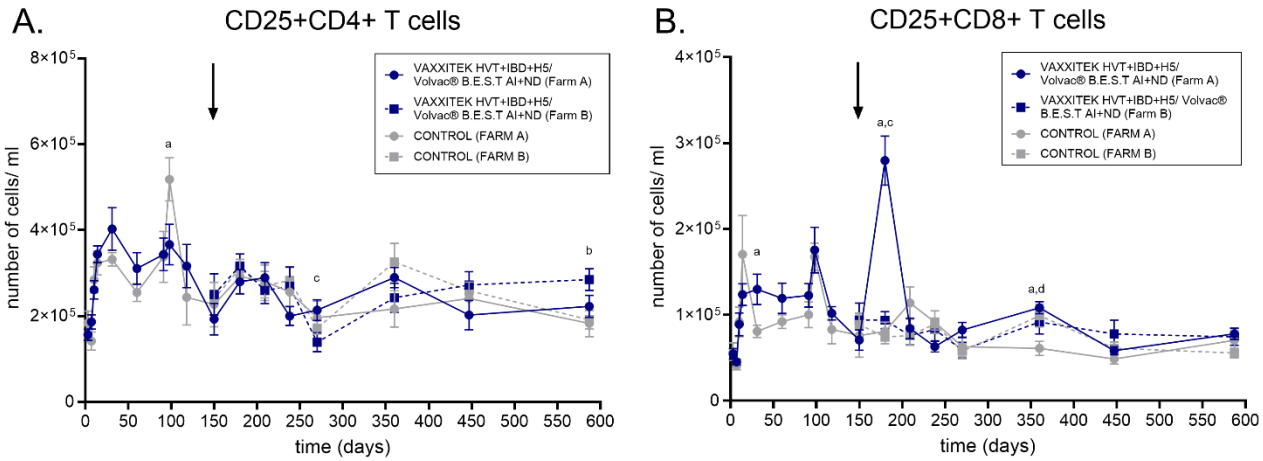

Figure 2.9 *Absolute numbers of activated T cells in the blood over time post vaccination in Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens. Upon vaccination, absolute numbers of CD4+ T cells (A), and CD8+ T cells (B) were quantified in the blood of between Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens chickens and non AI-vaccinated controls during a period of 587 days. At day 150, half of the chickens were transported to another farm. This timepoint is indicated by the arrow, and from day 150 onwards 4 groups are shown divided over two farms. Mean + SEM of 10 chickens per group is depicted. Significant differences between Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens vaccinated and non AI-vaccinated controls ($p<0.05$) are indicated; a indicates a significant difference between Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens vaccinated and non AI-vaccinated controls on farm A, b indicates a significant difference between Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens vaccinated and non AI-vaccinated controls on farm B, c indicates a significant difference between between Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI vaccinated chickens vaccinated chickens on farm A versus farm B and d represents a significant difference between non AI-vaccinated controls on farm A versus farm B.*

Also the number of activated CD8+ T cells was higher in chickens that received the Vaxxitek HVT+IBD+H5 vaccine (Figure 2.8) or the Vaxxitek HVT+IBD+H5 together with Volvac® B.E.S.T ND+AI (Figure 2.9) at 60 dpv in chickens (Figure 2.9). After 60 dpv, numbers of activated CD8+ T cells varied over time (Figure 2.8B, 2.9B). No significant differences were detected between the different groups or farms throughout most of the study period with the exception of the higher number of CD4+ T cells in the non AI-vaccinated control group at farm A at 350 dpv and the higher number of CD8+ T cells in the Vaxxitek HVT+IBD+H5 vaccinated chickens at day 180.

Next, possible relations between the number of activated CD4+T cells and HI- titers were analysed (Table 2.4) since CD4+ T cells are known to play a supportive role in the production of antibodies. Occasionally (at 2 timepoints in the Vaxxitek HVT+IBD+H5 vaccinated chickens and 1 timepoint in the Vaxxitek HVT+IBD+H5 + Volvac® B.E.S.T ND+AI) a weak but significant correlation between activated CD4+ T cells and HI- titers was observed. At all other timepoints, no correlation was observed. Thus, in general we did not observe a positive relation between numbers of activated CD4+ T cells and HI- titers in this study.

Table 2.4 *Relationship between activated CD4⁺ T cells and HI-titers in Vaxxitek HVT+IBD+H5 (Group 2) and Vaxxitek HVT+IBD+H5+ Volvac® B.E.S.T ND+AI (Group 3) vaccinated chickens.*

	Vaxxitek HVT+IBD+H5 (Group 2)		***Vaxxitek HVT+IBD+H5+ Volvac® B.E.S.T. ND+AI (Group 3)***	
Days post vaccination	**Correlation HI- titers and CD25+CD4+ T cells (Spearman R)**	**p value (*=p<0.05)**	**Correlation HI-titers and CD25+CD4+ T cells (Spearman R)**	**p value (*=p<0.05)**
31	0.358	0.308	0.076	0.833
60	-0.104	0.776	-0.182	0.615
91	-0.308	0.391	-0.368	0.292
118	0.263	0.491	0.381	0.283
150	-0.232	0.326	0.233	0.323
180	0.390	0.089	0.156	0.510
209	-0.244	0.299	0.040	0.868
238	0.007	0.977	-0.051	0.830
270	0.467	**0.038***	-0.047	0.846
360	-0.154	0.516	-0.448	**0.047***
447	-0.039	0.878	0.128	0.590
587	0.511	**0.020***	0.415	0.069

3 Transmission studies (Work Package 2)

3.1 Key Findings

Life stage -dependent transmission (pullets versus layers): HPAI H5N1 transmission was significantly higher in non AI-vaccinated layer chickens compared to 8-week-old non AI-vaccinated pullets (because of significant difference in Infection rate (β), $p < 0.001$). No differences were observed among layers tested at 24, 54 and 84 weeks of age. Estimated R_0 was 1.6 (95% CI 0.6–3.1) in pullets and 6.4 (95% CI 2.5–12.0) in layers, indicating substantially higher outbreak potential in adult layers.

Effect of (booster) vaccination on transmission: In layers (tested at 24, 54 and 84 weeks of age), higher homologous HI antibody titers ($\geq \log_2 6$ ("high titers")) significantly reduced transmission. The higher the proportion of layers in the group/flock with these high titers, the higher the chance is that the R_e is below 1.

Protection and mortality: Vaccination significantly reduced mortality in both duplo groups that were included in the transmission studies compared with non AI-vaccinated controls (100% (94-100%)), and among booster vaccinated chickens to as low as 0.0% (0.0-10.3%). No significant difference in mortality was observed despite their HI-titers (either high or low homologous HI-titer pre-challenge).

Viral genome excretion: Total viral genome shedding was reduced in vaccinated chickens. Choanal shedding in the non AI-vaccinated group was 7.0 (SD 0.6) and was reduced to 4.7 (SD 0.8) in the VAXXITEK HVT+IBD+H5 vaccinated chickens that had 'high' HI-titers or 5.0 (SD 1.2) in VAXXITEK HVT+IBD+H5 vaccinated chickens with 'low' HI titers. In the chickens vaccinated with both VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND, shedding through the choana was reduced to 5.5 (SD 0.8) in chickens with 'high' HI-titers or 5.2 (SD 0.4) in chickens with 'low' HI-titers.

Egg production: During the transmission study at 84 weeks of age, egg production remained stable in VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens before and after challenge in duplo group B. In duplo group A, a drop in egg production was observed (0.8 pre-challenge to 0.5 post-challenge).

Field-level impact: Combining data from both the longitudinal monitoring of antibody titers in the flocks kept in the field (Figure 2.1) and the transmission experiments, allows assessment of the expected level of protection any time during the totality of the production period (from week 19 to end of production).

- Antibody levels in the field fluctuated during the production period. In the booster vaccinated flocks these antibody levels were high enough to confer sufficient protection (predicted effective reproduction number ($R_e < 1$) between 25% (4/16 monthly samplings done for Flock A) and 69% (11/16 monthly samplings done for Flock B) during the production period. For the flocks without booster vaccination, the level of induced antibody responses were insufficient to drop R_e bellow 1 for the duration of the production period (demonstrated in Figure 3.1).
- Particularly for the booster group, even when the predicted R_e remained ≥1 during the production period, vaccination substantially reduced R_e (from 6.4 to ~1.5-1.7), thereby markedly lowering the likelihood of outbreaks.

3.2 Aim

Quantitative analysis of transmission parameters is essential for understanding the epidemiological impact of infectious disease interventions under real-world conditions. In field studies, estimates of key parameters such as susceptibility, infectiousness, and the reproduction ratio provide critical insight into whether an intervention, like vaccination, is likely to reduce virus spread at the population (flock) level. However, in order to perform field studies, such as clinical trials in countries such as the Netherlands, where infections of HPAI needs to be quickly eradicated in order to keep a disease free status, along with other study design limitations, limits the ability to quantify the effect of vaccination on transmission under field conditions.

To address these challenges, we implemented a "hybrid" approach in which groups of vaccinated and non AI-vaccinated chickens were reared under field conditions while the efficacy of vaccination against transmission was assessed through controlled laboratory challenge experiments (in dulpo groups (A+B) which included 5 inoclulated (challenged) chickens and 5 contact chickens). Under field conditions vaccine safety and vaccine induced immune responses were monitored throughout the chickens' growing and production cycle (chapter 2). The transmission experiments involved selecting small subgroups of both vaccinated and non AI-vaccinated chickens from the field and challenging them under laboratory controlled conditions in which exposure dose, timing, and contact structure are standardized. This allowed for a direct quantification of vaccine-induced effects on infection, shedding, and onward transmission which could then be extrapolated to field conditions. Integrating the field and laboratory data into epidemiological models strengthens the inference on intervention effectiveness and enhances their predictive value for disease control and risk assessment.

3.3 Results: Transmission parameters (Combining results of the 4 transmission studies performed at 8, 24, 54 and 84 weeks of age)

In the 4 transmission experiments, the effectiveness of vaccination was assessed when the chickens were of different ages. The VAXXITEK HVT+IBD+H5 only group was included in the experiments performed at both 8 and 24-weeks of age, and the group that received an additional, heterologous to the prime Volvac® B.E.S.T. AI+ND, booster was included in the experiments performed 24, 54 and 84 weeks of age.

3.3.1 Transmission of highly pathogenic avian influenza (HPAI) H5N1 virus in non AI-vaccinated flocks

The statistical analysis showed a significant difference ($p<0.001$) in the transmission rate parameter (β) between non AI-vaccinated 8-week-old pullets and layer chickens (older than 23 weeks of age). No significant differences in β were observed between layers of different ages (when tested at 24-, 54-, or 84 weeks of age). There were no differences in the length of the infectious period between pullets and layer chickens. The estimated basic reproduction number R_0 (95% confidence interval (CI)) was 1.6 (0.6 – 3.1) in pullets and 6.4 (2.5 – 12.0) in layers (Table 3.1).

Table 3.1 *Transmission of HPAI H5N1 virus in non AI-vaccinated pullets and layer chickens. Infection rate (β), infectious period (T), effective reproduction number (R0). Significant differences ($p < 0.001$) between layers and pullets, are marked in bold and with *.*

Age	Number of groups (5 inoculated + 5 contacts)	Transmission parameters β (95% CI)	T (95% CI)	R_0 (95% CI)
Layers (>23 weeks)	6	**2.1 (1.3 - 3.2)***	3.2 (1.4 – 4.9)	6.4 (2.5 – 12.0)
Pullets (8 weeks)	4	0.5 (0.3 - 0.8)	3.1 (1.3 – 4.7)	1.6 (0.6– 3.1)

3.3.2 Effect of vaccination on transmission (β)

The model for heterogeneous populations was used for this analysis. Because of the significant differences in transmission (β) between pullets and layers, this analysis was done for layers only. Hence, the results of this analysis only apply to the production phase. In this analysis, non AI-vaccinated chickens were included as part of the chicken population with low level of immunity (low antibody titers). This was done to improve the power of the analysis.

Transmission was quantified for groups of chickens with "low" or "high" levels of homologous antibody titers, dichotomized using HI cut-off values (in $\log_2$) of <5, <6, <7 or <8. The analysis was done fitting generalized linear model (GLM) and the best model was selected based on the models' corrected Akaike Information Criterion (AICc), with models with lower AICc preferred over those with higher AICc values. The model with the lowest AICc, was the model using an HI cut-off value <7 (Table 3.2) and this model was used for estimation of β. At this cut-off any chicken with an HI titer <7 was considered to have a low level of immunity (Low).

Table 3.2 *Results of the GLM models[a] for the estimation of the transmission parameters (β). These models were those for an homologous HI cut-off value <7. The parameter values are reported in their log-transformed form.*

Parameter	Mean estimated	Std error	p. value
Infectiousness based on predicted viable virus			
Intercept	0.753	0.173	
S_h (Susceptibility)	-0.005	0.540	0.993
I_f (Infectiousness)	-2.97	0.644	0.000
Infectiousness based on RT PCR			
Intercept	0.251	0.178	
S_h (Susceptibility)	-0.987	0.470	0.083
I_f (Infectiousness)	-2.70	0.544	0.000

[a]Presented models are those fitted to data prepared a chicken was assumed infectious based on the prediction of shedding viable virus or presence of virus genome (RT PCR)

In Table 3.3 the results of the estimated transmission parameters for the different HI cut-off valuesare presented.

These parameters can be used to estimate the expected effective R (R_e) in the field (Figure 3.1) based on information of the fraction of chickens with high homologous HI-titers in the flock (Appendix tables 1.2 b and c).

As for the pullets, the estimated parameters in vaccinated chickens, were reported within the report describing the first experiment [9, 12]

Table 3.3 *Transmission parameters quantified for different cut-off levels to discriminate between chickens with low and high antibody titers following vaccination with the VAXXITEK HVT+IBD+H5 and additional Volvac® B.E.S.T. AI+ND booster.*

HI cut-off	Level of Protection	β (day^{-1}) (95% CI)	Infectious period T (in days) (95% CI)	R (95% CI)	AICc*
Infection period based on predicted viable virus					
<5	Low	2.13 (1.45 – 3.01)	4.4 (1.6 – 7.5)	9.4 (3.1 – 18.2)	142.7
	High	0.21 (0.12 – 0.36)	3.4 (1.1 –5.8)	0.7 (0.2 – 1.6)	
<6	Low	2.07 (1.42 – 2.90)	4.3 (1.6 – 7.3)	8.8 (3.0 – 16.9)	140.9
	High	0.17 (0.09 – 0.32)	3.3 (1.2 – 5.6)	0.5 (0.2 – 2.3)	
<7	Low	2.12 (1.47 – 2.96)	3.7 (1.3 – 6.4)	7.8 (2.5 – 15.2)	136.0
	High	0.11 (0.05 – 0.23)	3.4 (1.2 – 5.9)	0.4 (0.1 – 1.0)	
< 8	Low	1.63 (1.13 – 2.29)	3.5 (1.2 – 6.1)	5.7 (1.8 – 11.2)	149.1
	High	0.06 (0.02 – 0.18)	3.4 (1.2 – 6.0)	0.2 (0.0 – 0.7)	
Infectious period based on RT PCR					
<5	Low	2.13 (1.45 – 3.01)	9.6 (2.7 – 18.4)	20.5 (5.5 – 43.5)	151.8
	High	0.13 (0.08 – 0.23)	8.2 (2.3 – 15.3)	1.1 (0.3 – 2.6)	
<6	Low	2.12 (1.44 – 3.00)	8.7 (2.4 – 16.4)	18.5 (4.8 – 39.2)	149.5
	High	0.11 (0.06 – 0.20)	8.1 (2.3 – 15.6)	0.9 (0.2 – 2.3)	
<7	Low	2.06 (1.41 – 2.90)	9.3 (2.6 – 17.7)	19.2 (5.1 – 40.5)	148.2
	High	0.06 (0.03 – 0.13)	7.7 (2.3 – 14.8)	0.5 (0.1 – 1.3)	
< 8	Low	1.29 (0.87 – 1.83))	10.1 (3.4 – 17.8)	13.0 (4.0 – 25.7)	176.0
	High	0.02 (0.01 – 0.08)	7.0 (2.3 – 12.2)	0.2 (0.0 – 0.6)	

* AICc is the Akaike Information Criterion corrected for the generalized linear models used for the estimation of the transmission rate parameter (β) Beta.

3.3.3 Protection Against Clinical Signs and Mortality

Here we explored the relationship between the levels of antibodies and disease. Based on the analysis of transmission, we selected, for this analysis, an homologous HI cut-off of (log_2) 7 to discriminate between chickens with "low" levels of protection (HI <7) and chickens with "high" levels of protection. Overall, vaccination, induced either "High" or "Low" level of HI titers, which resulted in a significant reduction in mortality when compared with the non AI-vaccinated controls ($p < 0.001$). Among vaccinated chickens, there was no significant differences in mortality between chickens with High and Low titers ($p = 0.17$) (Table 3.4).

Table 3.4 *Combined mortality observed in all three transmission experiments (only adult layers) where chickens layers were grouped as non AI-vaccinated, and if vaccinated grouped as Low levels of antibody titers ($log_2HI < 7$) or High ($log_2HI \geq 7$).*

HI Titer	Number infected	Number dead	Mortality (%)	Lower Confidence Limit (%)	Upper Confidence Limit (%)
Low	14	1	7.1	0.2	33.9
High	34	0	0.0	0.0	10.3
non AI-vaccinated	60	60	100	94.0	100

During the transmission study performed at 84 weeks of age, egg production was also monitored. During this experiment all chickens had HI titers ≥7. Appendix Figure 2.10 shows the daily recorded proportion of eggs produced/collected per test group before, during and after the day of challenge (day 0).

In group A, all five inoculated chickens and 3/5 contacts became infected, the median level of production before challenge was 0.8 and production dropped to a median of 0.5 after challenge. In group B, 3/5 inoculated chickens became infected and no transmission to the contacts was observed. No changes in the median egg production were observed, with the median egg production being 0.69 before and 0.7 after challenge (Appendix Figure 2.10).

Mortality in vaccinated and non AI-vaccinated pullets (8 wk of age) has been previously reported [9, 12]

3.3.4 Effect of vaccination on levels of viral genome shedding

The total amount of shed virus genome (Area under the curve; AUC) during the course of the infection in all transmission studies was determined (Table 3.5). Viral genome shedding was significantly lower ($p < 0.05$) in vaccinated chickens, regardless of their titer, compared with non AI-vaccinated controls for both choana and cloaca swabs. No differences in shedding were identified among vaccinated infected chickens. However, a significant lower ($p < 0.001$) number of infected chickens (n = 14) booster vaccinated (VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND) shed viral genome via the cloaca (n = 4) compared with the Choana (n = 14).

Table 3.5 *The total amount of viral RNA excreted (Area under the curve; AUC) of the chickens that became infected after virus inoculation (challenge) at 24, 54 and 84 weeks of age. SD: Standard deviation.*

Group (Total number of chickens exposed)*	**HI titer**	**Swab**	**Number shedding/total Infected**	**Mean AUC Log10 eqEID$_{50}$ (SD)**
non AI-vaccinated (60)		Choana	60/60	7.0 (0.6)
		Cloaca	60/60	7.3 (1.0)
VAXXITEK HVT+IBD+H5 (20)	High	Choana	8/8	4.7 (0.8)
		Cloaca	6/8	2.6 (0.5)
	Low	Choana	11/11	5.0 (1.2)
		Cloaca	10/11	3.1 (1.8)
VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND (A&B) (60)	High	Choana	14/14	5.5 (0.8)
		Cloaca	4/14	2.5 (1.9)
	Low	Choana	3/3	5.2 (0.4)
		Cloaca	1/3	2.6**

* Chickens were exposed either by inoculation (30) or direct contact (30) during the transmission experiments, where each group consisted of 5 inoculated chickens and 5 contacts.

** This observation was not included in statistical comparisons.

3.3.5 Predicting Re in the field

Using the transmission parameters estimated experimentally and field data on the fraction of chickens in the flock with high-titers, we predicted the effective reproduction number R_e throughout the production cycle (Figure 3.1). The critical fraction of chickens with a high-titer, such that R_e is below 1, was never reached for the flocks that did not received a heterologous to the prime booster vaccination. The median predicted R_e in these flocks were 2.2 (HI cut-off ≥ 6) or 3.2 (HI cut-off ≥7). As for the flocks receiving booster vaccination (VAXXITEK HVT + IBD + H5 + Volvac ® B.E.S.T. AI + ND), these flocks reached expected $R_e < 1$ between 25% (4/16 monthly samplings done for Flock A) and 69% (11/16 monthly samplings done for Flock B) of the time when considering an HI cut-off ≥ 6. Expected R_e <1 were not reached when considering an HI cut-off ≥ 7. Extrapolating to the whole production period, one could expect that the flock is protected against outbreaks (using ≥ 6) between 16 and 45 weeks of the total production cycle of around 65 weeks. For the weeks when $R_e \geq 1$, R_e is substantially reduced compared to the non AI-vaccinated control group (from 6.4 to a median R_e of 1.5 (for HI ≥6) or 1.7 (HI ≥7)) reducing thereby the probability of outbreaks (calculations thereof in Chapter 6).

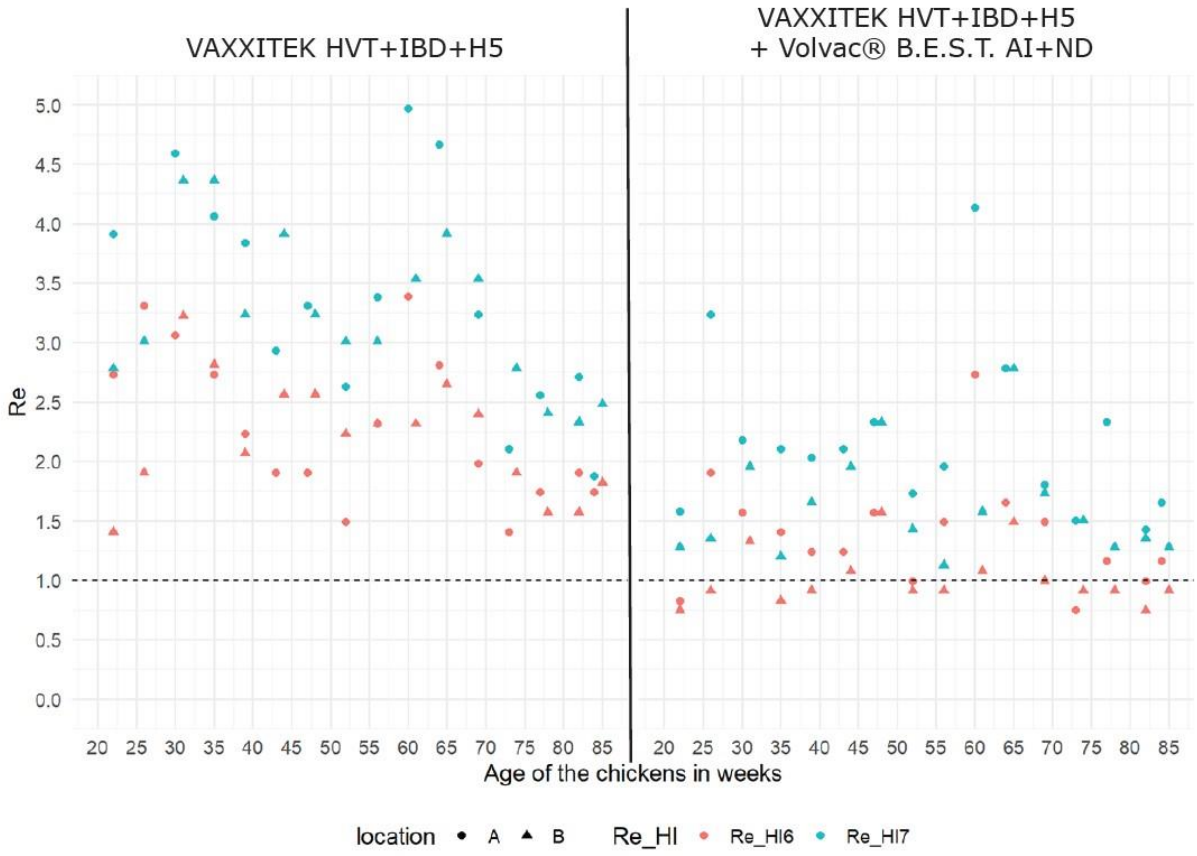

Figure 3.1 *The effective reproduction number (R_e) estimated based on the fraction of chickens in the experimental groups (kept in the field) having high HI titers at each sampling time throughout the production cycle. These estimates were done for the models (with the lowest AICs) considering log_2 HI titers: HI ≥6 (red) or HI ≥ 7 (blue) as cut-off values. The left panel shows the expected R_e for the experimental groups without booster vaccination whilst the right panel show these R_e values for the flocks receiving a heterologous to the prime booster vaccination. Dashed line indicates threshold value R_e =1.*

3.3.6 Individual results of transmission studies where challenge was performed at 54 and 84 weeks of age

In Appendix Work Package 2 the materials and methods and results of the last two transmission studies that were performed within this longitudinal study can be found, including tables that give an overview of the daily process of infection for all transmission studies performed in this longitudinal study (Appendix tables 2.3.1-2.3.4).

4 Cellular immunity in Transmission studies (Work Package 3)

4.1 Key Findings

The whole blood staining assay: (developed in this study) is a reliable tool to monitor changes in T-cell activation under controlled experimental conditions.
Vaccine induced cellular immune responses: VAXXITEK HVT+IBD+H5 and VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccination induced a rapid and strong T cell response within 3–7 days post-challenge. In the first days post-challenge, vaccinated chickens demonstrated **higher numbers of activated CD4+ and CD8+ T cells** in the blood compared to non AI-vaccinated chickens. This response was characterized by **increased numbers of CD25+ T cells** and the presence of **IFNγ-positive cells in the lungs and in the blood**. The observed immune profile is consistent with a vaccine-induced **memory T-cell response established by vaccination at day of hatch**.

4.2 Aim

Poultry vaccines protect chickens from infectious diseases, but the type of immune responses (e.g. neutralizing antibodies or cellular responses) that lead to protection, the so-called correlates-of-protection, are often unknown. During the development of new vaccines, the ability to induce protection is a critical parameter. Antibody titers (humoral immunity) in serum often correlate with protection, however this is not always the case based on supporting evidence from field studies [13]. However there are strong indications that also the cellular immunity, e.g. T cells play an important role in the protection against several avian pathogens [14].

The immune system defends the body through innate and adaptive mechanisms. The innate immune system is present from birth, is non-specific and is activated within hours upon an encounter with a pathogen, while the adaptive immune response is pathogen-specific and takes more time to develop. Typically, the T cell response peaks between 7 and 15 days upon the initial exposure to the pathogen [15, 16]. Immunological memory is established following the primary immune response. This means that upon a second encounter with the same pathogen —similar to a previous infection or vaccination— the immune response develops more rapid and is more effective.

The adaptive immune system consists of B cells, that produce antibodies, and T cells. T cells are divided into CD4+ or CD8+ T cells, which have different functions during an immune response. CD8+ T cells, or cytotoxic T cells, are involved in killing of infected cells, thus limiting the infection. CD4+ T cells differentiate into helper T cells that support other immune cells in exerting their function. For example, CD4+ T cells help B cells to produce high quality antibodies and assist CD8+ T cells in killing infected cells. Both CD4+ and CD8+ T cells can develop into memory cells that are quickly reactivated if the body encounters the same pathogen again. These memory cells play a key role in long-term immune protection.
Activation of T cells, whether through vaccination or infection, leads to the expression of specific proteins, including CD25 (the α-chain of the high-affinity IL-2 receptor). Thus, the presence of $CD25^+$ T cells is a measurement of T cell activation [17]. Following activation, T cells begin to proliferate, resulting in an increased number of T cells. Additionally, activated T cells produce cytokines such as IFNγ, which play a central role in activation of other immune cells and exerts antiviral effects [18]. Thus, upregulation of activation markers such as CD25 in the blood which is followed by increased proliferation as well as increased production of IFNγ can be used to measure T cell activation.

The aim of this study was to investigate the impact of a challenge with a highly pathogenic avian influenza virus on the T cell response induced by vaccination. Additionally, CD25 expression on T cells in the blood was

measured and compared to IFNγ production to assess whether CD25 expression on T cells can serve as a reliable marker for monitoring T cell activation during future vaccination and challenge studies.

Application of VAXXITEK HVT+IBD+H5 is expected to generate memory T cells. Upon vaccination with VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND as booster more memory T cells are expected, and memory T cells may be present for a longer time. These memory T cells which will be reactivated upon challenge with the HPAI H5N1 clade 2.3.4.4b, which will lead to an increase in $CD25^+$ T cells in the blood during the first week post challenge. The increase in $CD25^+$ cells will coincide with an overall increase in circulating T cell numbers and will be followed by enhanced IFNγ production.

4.3 Results Cellular Immunity In All Transmission Studies

4.3.1 Transmission study 1, inoculation at 8 weeks of age

These data indicate that 8 weeks post vaccination, a challenge with HPAI H5N1 clade 2.3.4.4b induced a T cell response in the chickens that received VAXXITEK HVT+IBD+H5, reflected by an increase in the number of activated (CD25+) CD4+ and CD8+ T cells [9] [12] (Appendix Work Package 3).

4.3.2 Transmission study 2, inoculation at 24 weeks of age

Before challenge with HPAI H5N1 clade 2.3.4.4b, significantly higher numbers of CD4+ T cells and CD8+T cells were observed in the non AI-vaccinated control group compared to the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens.

No major changes in the number of CD25+CD4+ and CD25+CD8+ T cells were observed in time except at day 7 where numbers of CD25+CD4+ and CD25+CD8+ T cells were lower in the VAXXITEK HVT+IBD+H5 vaccinated chickens compared to chicken that also received the booster vaccine. Due to technical issues associated with this experiment, strong conclusions should therefore be avoided (Appendix Work Package 3).

In addition to surface expression of CD25 and proliferation, memory T cell also produce the cytokine IFNγ upon activation. To determine whether challenge with HPAI H5N1 clade 2.3.4.4b virus would result in IFNγ producing cells, an ELISpot analysis was performed with cells isolated from lung and blood of VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens. At 3, 7, 10 and 14 dpc an increase in IFNγ producing cells was observed in lung (Figure 4.1A) and in the blood (Figure 4.1B) compared to non-inoculated chickens (results demonstrated at 0 dpi). The number of IFNγ producing cells was higher in cells isolated from lungs compared to cells isolated from the blood, and a correlation was observed between the number of IFNγ producing cells in the lung and the number of IFNγ producing cells in the blood (R=0.51, p=0.01)

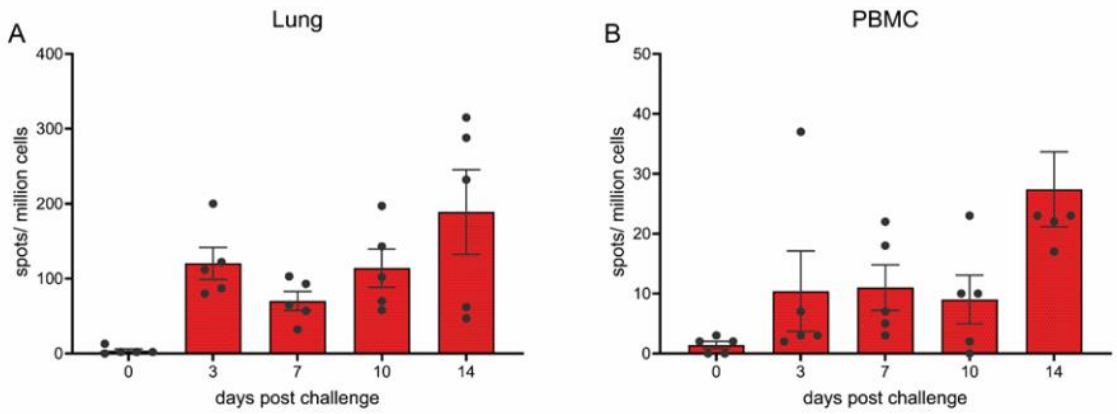

Figure 4.1 *Number of IFNγ producing cells isolated from lungs and blood (PBMC) of five VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens sacrificed before (0 dpi) and post challenge. Results were obtained in unstimulated cells by ELIspot analysis. Mean ± SEM of 5 chickens is shown. Each dot represents an individual chicken*

4.3.3 Transmission study 3, inoculation at 54 weeks of age

The third transmission experiment was performed at 54 weeks of age. In this study, again numbers of CD4+ and CD8+ T cells as well as numbers of activated (CD25+) CD4+ and CD8+ T cells were quantified in whole blood of VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens and the non AI-vaccinated control group. Moreover, also the production of IFNγ by lymphocytes in the blood was determined.

Between day 0 and day 1 post challenge, numbers of CD4+ T cells and CD8+ T cells significantly increased in the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens, while numbers of CD4+ T cells and CD8+ T cells in the non AI-vaccinated control group decreased. This resulted in a significant difference in numbers of CD4+ T cells and CD8+ T cells between both groups at 1 day post challenge (Appendix Figure 3.1). Also numbers of CD25+ CD4+ T cells and CD25+CD8+ T cells decreased in the non AI-vaccinated controls between day 0 and day 1, resulting in a significantly higher number of CD25+CD4+ T cells and CD25+CD8+ T cells in the vaccinated chickens compared to the non AI-vaccinated control group at 1 day post challenge (Appendix Figure 3.2).

Analysis of the total numbers of CD4+ T cells and CD8+ T cells showed a biphasic pattern with a significant increase in numbers of both CD4+T cells and CD8+ T cells at day 3 post challenge compared to day 0. At 7 days post challenge, numbers of CD4+ and CD8+ T cells reached similar levels compared day 0, and increased again at day 10 post challenge (Figure 4.2).

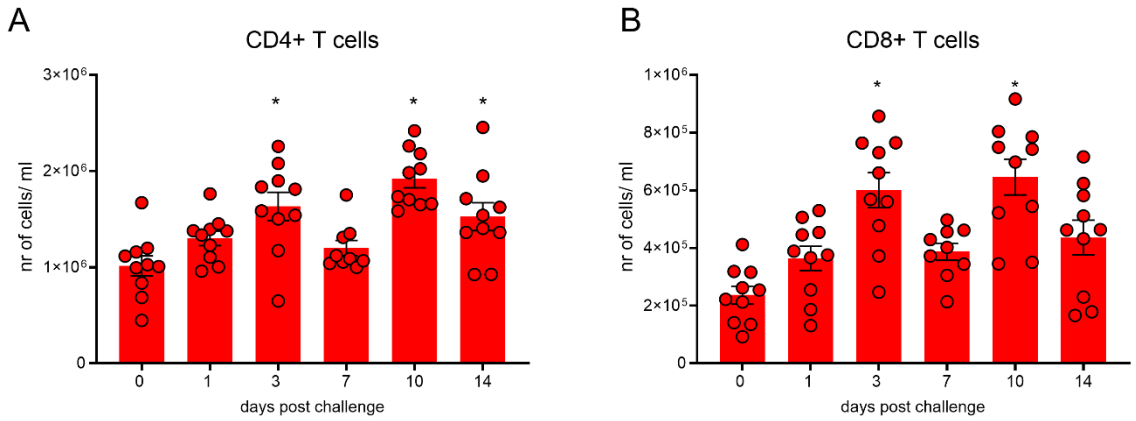

Figure 4.2 *Absolute numbers of T cells in the blood of vaccinated chickens. Absolute numbers of CD4+ T cells (A) and CD8+ T cells (B) were quantified in the blood of infected chickens at multiple time points post challenge. Each dot/triangle represents an individual chicken. Mean ± SEM is shown. Significant differences compared to day 0 ($p<0.05$) are indicated (*).*

CD8+ T cells demonstrated the same biphasic pattern as was observed for CD4+ T cells (Figure 4.2B).
Numbers of CD25+ CD4+ T cells showed a similar pattern as was observed for the number of CD4+ T cells and were significantly higher at day 3, day 10 and day 14 post challenge, with lowest numbers at 7 days post challenge (Figure 4.3A). Numbers of CD25+CD8+ T tend to decrease until day 7 post challenge, and low numbers of CD25+CD8+ T cells in the blood were observed from 7 days post challenge onwards (Figure 4.3B).

To make a comparison and to assess whether CD25 expression on T cells can serve as a reliable marker for monitoring T cell activation during future vaccination and transmission studies, IFNγ producing cells were readily detected (Figure 4.4A). After a numerical decrease between day 0 and day 3 post challenge, the number of IFNγ producing cells started to increase from day 3 post challenge onwards until day 10 post challenge. This indicates that in VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens IFNγ producing cells are induced upon challenge. When comparing the IFNγ producing cells in the blood with the number of activated CD8+ T cells in the same chickens (Figure 4.4B), no clear relation was observed in this experiment.

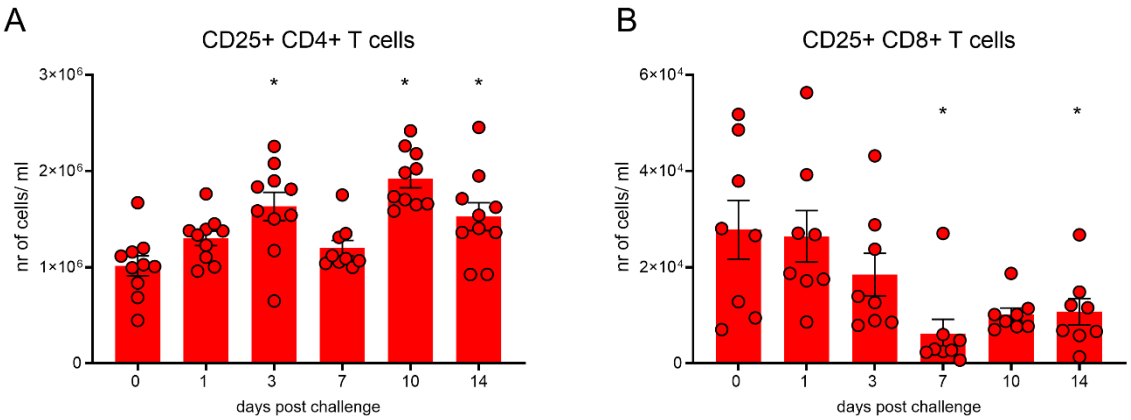

Figure 4.3 *Absolute numbers of activated T cells in the blood of vaccinated chickens. Absolute numbers of CD25+ CD4+ T cells (A) and CD25+ CD8+ T cells (B) were quantified in the blood of infected chickens at multiple time points post challenge. Each dot represents an individual chicken. Mean ± SEM is shown. Significant differences compared to day 0 (p<0.05) are indicated (*).*

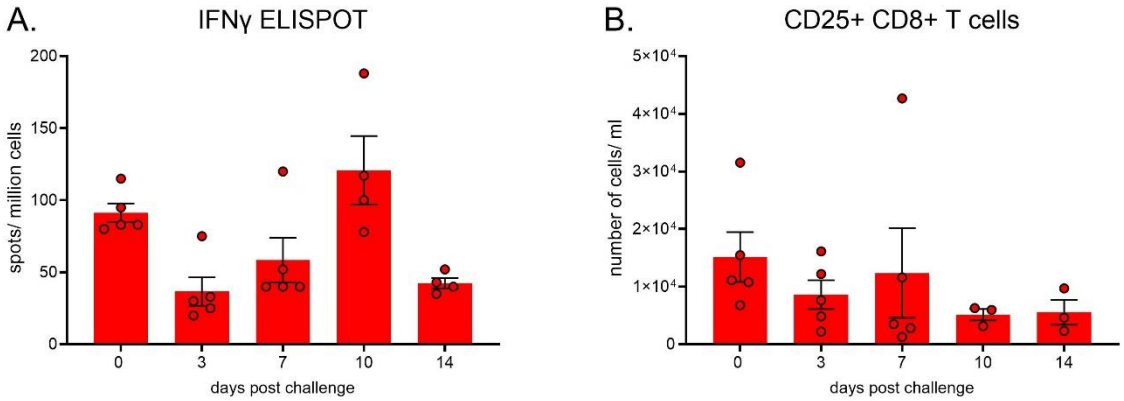

Figure 4.4 *T cell responses in the blood at several timepoints post challenge. At several timepoint post challenge lymphocytes were isolated from the blood and T cell activation was assessed by measuring the number of IFNγ producing cells (A) and the number of CD25+CD8+ T cells (B). Mean +- SEM is shown. Each dot represents an individual chicken.*

In this transmission experiment, the choanal viral load increased until 1 day post challenge, and after this rapidly decreased to low levels from day 10 onwards (Appendix Work Package 2).
These data indicate that at 54 weeks post vaccination, a challenge with HPAI H5N1 clade 2.3.4.4b still affects the T cells in the chickens that received VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND. This is reflected by the difference in T cell numbers 1 day post challenge in the vaccinated versus the non AI-vaccinated control group, as well as the increase in activated CD4+ T cell post challenge.

4.3.4 Transmission study 4, inoculation at 84 weeks of age

At day 0, numbers of CD4+ T cells and CD8+ T cells were similar between the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens and chickens in the non AI-vaccinated control group (Appendix Figure). At day 1 post challenge, numbers of CD4+ T cells and CD8+ T cells were numerically higher in the vaccinated chickens compared to the non AI-vaccinated control group, but this was not significant. At day 0, the non AI-vaccinated control group had a higher number of CD25+CD4+ T cells and CD25+CD8+ T cells compared to the vaccinated chickens. Challenge resulted in an increase in the number of CD25+CD8+ T cells, but only in the vaccinated chickens. (Appendix Work Package 2).

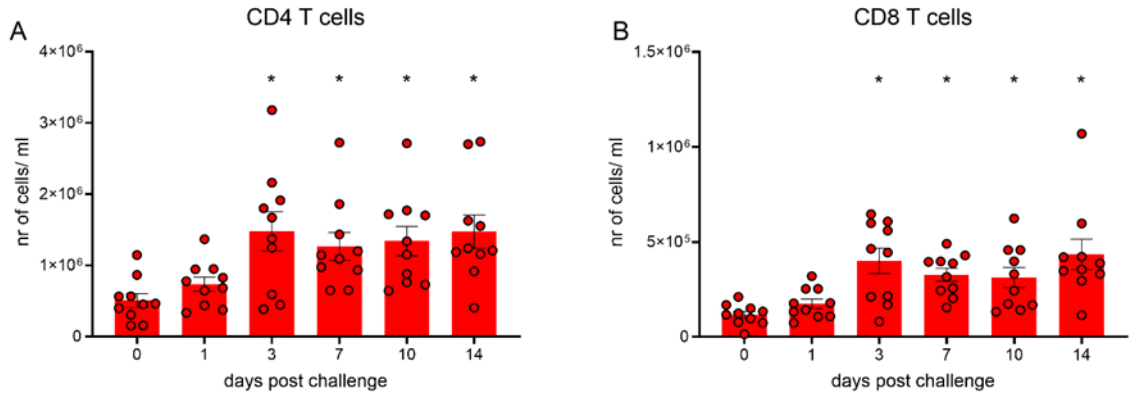

Figure 4.5 *Absolute numbers of T cells in the blood of vaccinated chickens. Absolute numbers of CD4+ T cells (A) and CD8+ T cells (B) were quantified in the blood of vaccinated chickens at multiple time points post challenge. Each dot represents an individual chicken. Mean ± SEM is shown. Significant differences compared to day 0 ($p<0.05$) are indicated (*).*

Upon challenge, numbers of CD4+ T cells and CD8+ T cells stared to increase and were significantly higher from 3 dpc onwards compared to day 0 (Figure 4.5).

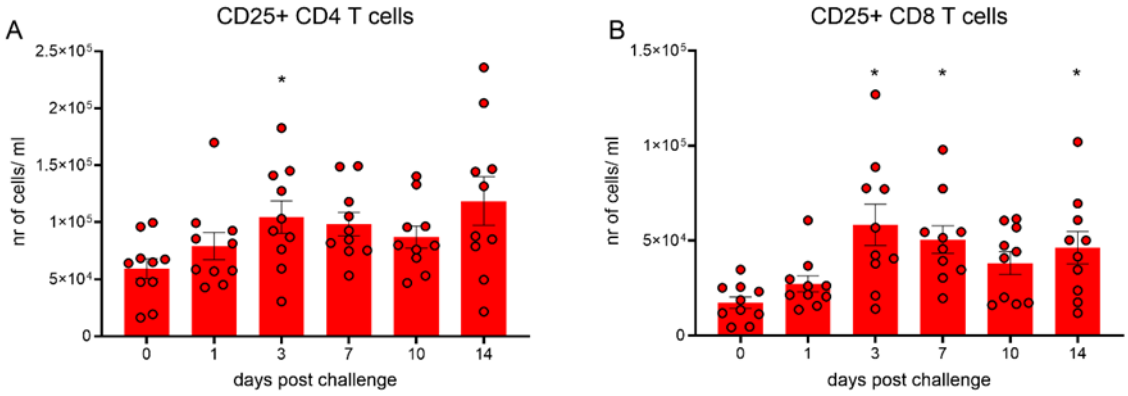

Figure 4.6 *Absolute numbers of activated T cells in the blood of vaccinated chickens. Absolute numbers of CD25+ CD4+ T cells (A) and CD25+ CD8+ T cells (B) were quantified in the blood of vaccinated chickens at multiple time points post challenge. Each dot/triangle represents an individual chicken. Mean ± SEM is shown (Groups A & B are combined). Significant differences compared to day 0 ($p<0.05$) are indicated (*).*

As is shown in Figure 4.5, also the numbers of activated T cells changed upon challenge. This is most pronounced for the number of CD25+CD8+ T cells (Figure 4.6B). Upon challenge, the number of CD25+CD8+ T cells started to increase, resulting in significantly more CD25+CD8+ T cells at 3 and 7 dpc. After this peak, numbers of CD25+CD8+ T cells decreased at day 10 dpc, and significantly higher numbers compared to day 0 were observed again at 14 dpc. Numbers of CD25+CD4+ T cells changed in a similar way, but other than at 3 dpc the changes compared to day 0 were not significant.

In this experiment, the viral load in the choana started to decline from 3 dpc onwards, and became undetectable from 10dpc onwards.

This transmission experiment demonstrates that a challenge with HPAI H5N1 clade 2.3.4.4b at 84 weeks post-vaccination induces a T cell response in chickens that were vaccinated with VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND, as evidenced by an increase in activated $CD8^+$ T cells—and to a lesser extent, $CD4^+$ T cells—in the blood. The differences between vaccinated and non AI-vaccinated control chickens were less pronounced at 1 day post challenge compared to the experiment performed at 54 weeks. However, by days 3 and 7 post challenge, there was a significant increase in activated T cells in the blood. Interestingly, the number of total

and activated CD8+ T cells followed a biphasic pattern: a temporary decline was observed at day 10 post challenge, followed by a subsequent increase thereafter.

4.4 Discussion

The aim of this work was to investigate the impact of a challenge with a highly pathogenic avian influenza virus on the T cell immune response that developed in response to vaccination.
In all experiments, vaccinated chickens had more activated T cells in the blood immediately post challenge compared to non AI-vaccinated chickens. This was observed for chickens that received VAXXITEK HVT+IBD+H5 (experiment 1 and experiment 2), or VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND (experiment 2-4) observed in chickens that received VAXXITEK HVT+IBD+H5 (experiment 1 and experiment 2), or VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND (experiment 2-4). This effect was strongest in transmission experiments 1, 3 and 4. In these studies, a clear peak in the number of activated CD4+ T cells and CD8+ T cells was observed at 3 dpc and 7 dpc. Normally, when the immune response encounters a novel pathogen for the first time, the T cell response peaks around 10 days post infection. Here, a peak at earlier timepoints was observed, which suggests that vaccination induced memory cells that have been activated by the challenge. Memory cells will respond more rapidly, and have a high proliferative potential.

In transmission experiments 2, 3 and 4, the choanal viral load in the chickens that received VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND started to decline from 3 dpc onwards. Increased numbers of activated T cells were also observed from 3 dpc onwards in these experiments.
Whether higher numbers of activated CD4+ or CD8+ T cells can be correlated to lower virus levels was hard to determine in the series of transmission experiments. In these transmission experiments, the amount of virus shed by the chicken through the upper respiratory tract (choana) was determined, a key indicator of transmission potential which was the main focus of the current experiments. However, the viral load in the lungs better reflects the severity of the respiratory disease caused by the virus, and the viral load in the blood indicates how widely the virus has spread within the chicken's body. Viral load data from the blood were not available, comparing levels of activated T cells with choanal viral load did not show a clear relationship.
Another aim of the study was to assess whether the T-cell activation marker CD25 could be a useful and practical way to monitor T-cell responses in future vaccination and challenge and/or transmission experiments. The performed experiments demonstrate that CD25 expression can be readily determined in a whole blood staining, and that the expression of CD25 indeed changes upon challenge. This opens up new possibilities for future studies, since for this whole blood assay the blood can be collected and stored for up-to 1 week. This is in sharp contrast to other T cell readouts such as the production of IFNγ which require freshly isolated cells.
Although CD25+ expression and IFNγ production are both signs of T cell activation, they don't necessarily occur at the same moment. Upregulation of CD25 is one of the earliest signs of activation. Next cells start to proliferate and start to produce IFNγ. It is important to keep in mind that T cell activation does not occur in the blood itself, T-cell activation happens in tissues like the lungs, so activated T cells that are observed in the blood moved there from elsewhere.

To conclude, VAXXITEK HVT+IBD+H5 and VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND vaccinated chickens were able to mount a strong T cell response upon challenge with HPAI H5N1 clade 2.3.4.4b within 3-7 days post challenge which is reflected by increased numbers of CD25+ T cells as well as the presence of IFNγ positive cells in the lungs and the blood. The timing and speed is consistent with a memory cell response, induced by vaccination at day of hatch.

5 Safety of Poultry Products

5.1 Key Findings

Baseline status: At study start, chickens were AI virus–negative by M-PCR and largely NP-ELISA (in house WBVR) negative; two NP-ELISA–positive chickens were excluded by necropsy at day 0.

Pre-existing immunity: Most chickens had measurable HI antibodies prior to inoculation (14 out of the 15).

Clinical outcome: After inoculation, 11/12 chickens remained clinically healthy; one chicken with low or absent HI titers died at 6 dpi, suggesting insufficient protective immunity.

Viral shedding and replication: Viral RNA was detected in choanal and/or cloacal swabs in 10/12 chickens, with feather positivity in 6/12 chickens; infectious virus was isolated only from the chicken lacking HI antibodies, demonstrating systemic infection in the absence of detectable immunity.

Food safety: Viral RNA was largely absent from poultry products and egg contents; limited eggshell and single egg white positivity showed low genomic viral loads, consistent with external contamination rather than productive infection or infectious virus presence in eggs.

5.2 Aim

To determine viral presence in poultry products that may be consumed or come in contact with consumers a parallel study was conducted at 24 weeks of age. Therefore, additional prime only vaccinated chickens were challenged with H5N1 clade 2.3.4.4b virus, and samples from different poultry products (chicken breast, heart and wing feathers) upon necropsy and eggs from the laying box in the pen were collected. Detection of viral RNA was performed on all sampled poultry products.

5.2.1 Study Design Safety of Poultry products

The study design of the poultry product study is schematically presented in Figure 5.1. A total of 13 chickens vaccinated with VAXXITEK HVT+IBD+H5 were delivered to WBVR at 23 weeks of age. Upon arrival at WBVR, the chickens were randomly divided and given a wing tag for identification and therefore the predetermined timepoint for necropsy. Six chickens originated from Farm A and seven chickens originated from Farm B (of which 1 chicken served as surplus). These chickens were housed together in one pen in the barn that also housed the VAXXITEK HVT+IBD+H5 + Volvac® B.E.S.T. AI+ND groups of the transmission study.
To detect the antibodies (humoral immune response) at -7 dpi, blood serum was collected and tested in HI assay and NP-ELISA. Choanal and cloacal swabs were taken to demonstrate the absence of avian influenza virus using M-PCR.

After one week of acclimatization, all chickens were inoculated with HPAI H5N1 clade 2.3.4.4b. The inoculation was performed by applying 0.1 ml of the virus intra-choanal, so that each chicken received 10^6 EID_{50} HPAI H5N1 virus. At four timepoints, 0 dpi (pre inoculation) and 3, 5 and 7 dpi, necropsy was performed on 4 chickens[1], two from each commercial farm. Choana and cloaca swabs were only taken from the chickens designated for necropsy. After euthanasia, heart and breast tissue and wing feathers were collected. Four eggs were collected from the pen and were swabbed on the egg shell, and a sample of the egg white of each egg was taken.

[1] At day 0 dpi, a total of 3 chickens were sacrificed (n=1 surplus chicken this study + n=2 surplus chickens of the transmission study groups [11]

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. All clinical signs were documented.

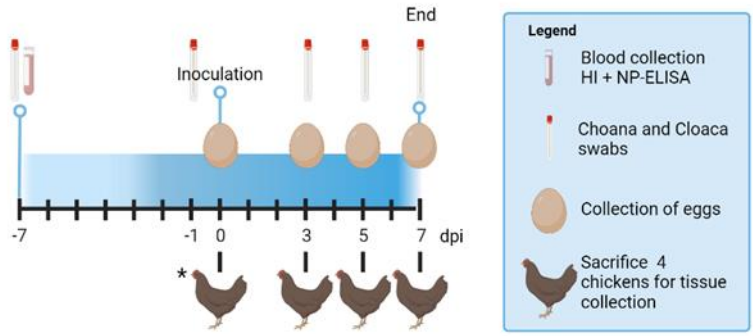

Figure 5.1 *Schematic overview of sample collection time points in safety of poultry products study. Inoculation was performed with 10^6 EID_{50}/ml HPAI H5N1 virus per chicken. Prior euthanasia, choana and cloaca swabs were taken. Per timepoint, 4 eggs were collected from the pen. Upon necropsy, heart, breast and wing feathers were collected from each chicken. HI: Hemagglutination Inhibition assay. * Total of 3 chickens were sacrificed (n=1 surplus chicken this study + n=2 surplus chickens of the transmission study groups).*

5.3 Results Safety of Poultry Products

5.3.1 NP-ELISA and HI titers prior inoculation

At the start of the study, the absence of avian influenza virus and presence of antibodies against avian influenza virus were demonstrated by M-PCR on choanal and cloacal swabs and by NP-ELISA (in house WBVR) on blood serum samples taken upon arrival. However, 2/15 chickens, chicken 1 and 3 in Table 5.1, had a positive NP-ELISA result therefore these chickens were included for necropsy at day 0.

The HI titers prior inoculation were measured for all chickens (Table 5.1). The mean homologous HI-titer was $\log_2$ 6.2 (SD 2.13, 13/15 chickens) and mean heterologous HI-titer was $\log_2$ 3.1 (SD 1.86, 14/15 chickens). One chicken had no HI-titers (Table 5.1, chicken number 7) and was included in the group designated for necropsy at 3 dpi.

5.3.2 Detection of viral RNA in poultry products

After inoculation, 11/12 chickens remained healthy, no clinical signs were noted and survived until their designated timepoint. In total, 10/12 chickens shed virus in choana and/or cloaca at the moment of necropsy (or found death). One chicken was found death in the pen at 6 dpi, without demonstrating clinical signs prior death. From this chicken (number 12 in Table 5.1), swabs of the choana and cloaca were obtained (both positive in M-PCR), but no necropsy was performed. This chicken (number 12) had a low homologous HI titer (5.0) and no heterologous HI-titer was obtained (0,0). These low HI-titers may explain why this chicken did not survive the challenge, as protective antibody levels were low.

From 6/12 wing feather samples positive M-PCR results were obtained, which were collected at 3, 5 and 7 dpi (Table 5.1). The feather sample of chicken number 4 was included for virus isolation but the results to detect infectious virus were inconclusive.
From only 1/11 tested samples of both breast and heart positive M-PCR results were obtained. These positive samples were collected from chicken number 7, which had no detectable HI-titers pre-challenge (table 5.1). Breast, heart and feather samples of chicken number 7 were included in virus isolation and infectious virus

could be isolated. This demonstrates that the absence of detectable HI antibodies does allow active viral replication and systemic dissemination, and that chicken number 7 became infected.

Table 5.1 *Results of the safety of poultry products samples. NP-ELISA results and HI-titers obtained in the blood serum collected at -7dpi. The detection limit of the M-PCR is 1.7 (Log $10^{1.7}$ $eqEID_{50}$/ml) and titers < Log $10^{1.7}$ $eqEID_{50}$/ml are considered negative. Bold: positive M-PCR results.* tissues that were included for virus isolation (≥Log $10^{3.6}$ $eqEID_{50}$/ml (correspond to ct-value in M-PCR ≤33)).*

Chicken number	**Necropsy day**	**NP-ELISA** (WBVR)	**HI titer (-7 dpi)**		**Swabs**		Breast	Heart	Wing feather
			Homologous	Heterologous	Choana	Cloaca			
1	0	**+**	8,5	5,0	0,0	0,0	0,0	0,0	0,0
2	0	-	9,0	7,0	0,0	0,0	0,0	0,0	0,0
3	0	**+**	6,0	3,5	0,0	0,0	0,0	0,0	0,0
4	3	-	5,5	3,0	0,0	0,0	0,0	0,0	**3,6***
5	3	-	6,0	2,5	**5,6**	0,0	1,3	0,0	0,9
6	3	-	8,5	3,0	**3,7**	0,0	0,0	0,0	**2,4**
7	3	-	0,0	0,0	**7,1**	**6,1**	**6,0***	**7,9***	**5,3***
8	5	-	5,5	2,5	**3,6**	0,0	0,0	0,0	**2,3**
9	5	-	5,0	3,0	**2,8**	0,0	0,0	0,0	1,6
10	5	-	6,5	1,0	**3,2**	0,0	0,0	0,0	1,6
11	5	-	7,0	3,0	**3,3**	0,0	0,0	0,0	0,0
12	6	-	5,0	0,0	**7,2**	**4,7**	NT	NT	NT
13	7	-	7,0	4,0	**2,7**	0,0	0,0	0,0	**2,2**
14	7	-	7,5	5,0	1,5	**3,8**	0,0	0,0	0,0
15	7	-	6,0	4,0	0,0	0,0	1.4	0,0	**1,9**

5.3.3 Detection of viral RNA in eggs collected from the laying box in the pen.

Each timepoint, 4 eggs were collected from the laying box and swabs from the egg shell were obtained. Samples of the egg white of the corresponding egg were tested directly after collection using the M-PCR to detect viral RNA. The results demonstrate the absence of viral RNA in all egg white samples, except for 1 sample collected at 5 dpi. Swabs obtained from the egg shell after inoculation of the chickens were positive for M-PCR in 11/12 samples (Table 5.2, bold). One can speculate the genomic viral RNA on the outside of the eggs came from chickens that were shedding virus through choana and/or cloaca and may have been in contact with the outside of the egg prior the collection timepoints (indicated in Table 5.1).
None of these positive samples were included for virus isolation, as obtained titers in M-PCR were below the cut-off value (cut-off value for virus isolation; titer in M-PCR ≥3.6 (corresponding to ct-value ≤33 in M-PCR)).

Table 5.2 *Viral RNA detection in the egg white and swabs obtained from the outside of collected eggs using the M-PCR. Log 10 titers are shown and the detection limit of the PCR is 1.7 (Log $10^{1.7}$ eqEID50/ml), so titers < Log $10^{1.7}$ eqEID50/ml are considered negative. Bold: M-PCR positive samples.*

Sample	Collection (dpi)	Egg number			
		1	**2**	**3**	**4**
Egg white	0	0,0	0,0	0,0	0,0
	3	0,0	0,0	0,0	0,0
	5	0,0	0,0	**2,3**	0,0
	7	0,0	0,0	0,0	0,0
Egg outside	0	0,0	0,0	0,0	0,0
	3	**2,9**	**3,4**	**3,2**	**2,3**
	5	0,0	**1,8**	**2,6**	**2,3**
	7	**2,4**	**3,0**	**2,9**	**2,8**

6 Recommendations for Surveillance Program (Work Package 4)

6.1 Key Findings

Vaccination substantially reduces HPAI outbreaks and onward spread

- Most virus introductions in vaccinated flocks result in negligible spread within a flock (72–90% vs 4% without vaccination).
- When outbreaks do occur, they are smaller and infect fewer chickens.

Passive surveillance alone is insufficient in vaccinated flocks

- Reduced mortality and clinical signs reduce detection to 0.0–19.2% of outbreaks in vaccinated flocks through passive surveillance, compared to 100% in non AI-vaccinated flocks.

Active surveillance is essential for reliable outbreak detection

- Sampling dead chickens can detect >93–97% of outbreaks, even with sampling intervals up to 30 days.
- Sampling live chickens is markedly less effective (20.6–48.5% detection).

Detection is delayed by vaccination but can be mitigated

- Detection times are longer and more variable in vaccinated flocks.
- Active surveillance every 2–7 days achieves detection times comparable to passive detection in non AI-vaccinated flocks.

Vaccination reduces risk beyond the farm

- Far fewer secondary farms become infected; epidemics are typically limited to 1–2 farms, even in high-density areas.
- Active surveillance increases the chance that the index farm is detected first.

Reduced transmission via infected chickens and eggs

- Vaccinated flocks have fewer infected chickens at detection.
- Fewer farms ship eggs from infected chickens, and those farms, that do so, ship fewer eggs produced by infected chickens.

Vaccine regimen matters

- Adding a heterologous to the prime booster further lowers outbreak probability and impact, though it slightly reduces passive detectability.
- Outcomes remain superior to no vaccination across all surveillance intervals.

Effectiveness depends on immune response and virus strain

- Results are sensitive to the fraction of chickens with high HI-antibody titers and strain match (antigenic distance) of the vaccine.
- 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. Even with this antigenic difference, **the vaccination consistently outperforms no AI-vaccination.**
- Virus strains with a larger antigenic distance to the vaccine, may reduce vaccine effectiveness but are more likely to be detected via passive surveillance.

Overall conclusion

- Vaccination with either VAXXITEK HVT + IBD + H5 alone or combined with additional Volvac ® B.E.S.T. AI + ND vaccine reduces outbreak frequency, spread, number of infectious chickens, and contaminated egg production—though the combined regimen performs better within farms. As vaccination may delay detection, sampling dead chickens (bucket sampling) at different frequencies can ensure timely outbreak detection.

6.2 Aim

The basic reproduction number, R_0, as explained in Chapter 3, is a central parameter for quantifying transmission and is defined as the average number of secondary infections caused by one typical infected animal in a fully susceptible population. If one infected chicken infects more than one other chicken on average ($R_0 > 1$), an outbreak can grow (but may fade out by chance); if it infects fewer than one ($R_0 < 1$), the infection will always fade out. This means that an introduction of HPAI in a farm may result in a large exponential growing outbreak if R_0 within the poultry flock is above one. In the current situation, these outbreaks are detected with passive surveillance due to reporting of farmers when clinical signs or mortality is observed. The current surveillance is efficient as shown by previous studies for non AI-vaccinated flocks [19, 20]. However, in the context of vaccination this may change, for the worse or the better, due to reduced transmission, mortality, and morbidity in vaccinated chickens.

Vaccination will induce an immune response, which can be characterized (humoral immune response) by the level (titer) of neutralizing or hemagglutinating antibodies [21] expressed by a vaccinated chicken in time post vaccination. In a vaccinated flock, there is variation in the levels of antibodies expressed by each chicken, which for the purpose of this study, were characterized in high and low HI-levels by setting a predetermined antibody cut-off level fitted to the analysis of the transmission experiments with vaccinated chickens (Chapter 3). Thereby, we obtained a measure of how well the flock (local population) is protected: i.e. the fraction of chickens with a high titer of homologous antibodies determines the effective reproduction number (Re) of the vaccinated flock. Throughout this chapter, the term high and low titer will be used for vaccinated chickens with an antibody titer below (< cut-off) or equal to or above (≥ cut-off) this set value. For our baseline scenario we use a cut-off of 6 (see Chapter 3) but also performed a sensitivity analysis for 5 and 7 (see Appendix Work Package 4).

Vaccinated chickens were protected against clinical signs and mortality, with the latter being significantly reduced in vaccinated chickens (vaccinated with VAXXITEK HVT + IBD + H5 + Volvac ® B.E.S.T. AI + ND) compared to non AI-vaccinated ones (at most 7.1% instead of 100% mortality, see Chapter 3 and 6.3.2.2). It has been shown before for HPAI H5N1 [22] and again in this project (Chapter 3), that vaccinated chickens, with a high titer, have a reduced transmission rate per day. The transmission rate per day and the infectious period together determine the reproduction number. The reduction in transmission rate is so much higher than the increase in infectious period that overall the reproduction number decreases in these chickens (chapter 3). In the analysis of the different surveillance programs, we used the transmission rate parameters from the experiments performed in chapter 3.

In general, when the reproduction number is above 1 (one), the distribution of the number of chickens affected at the end of an outbreak is two-peaked ('bimodal'): either almost no chickens are infected or nearly the whole flock infected, i.e. there is one peak near 0% of chickens infected and one near 100% of chickens infected. The probability of an outbreak being in either one of the peaks can be determined analytically for a closed population with two types of individuals, i.e. chickens with a high and low titer [23, 24]. When vaccination is successful, a larger fraction of the chickens will shift towards the peak near 0% [23, 24]. For this analysis, we assumed that any outbreak where the total number of infected chickens at the end of the outbreak is more than 0.1% of the population, will be called an outbreak. When equal or less than 0.1% of the flock is infected at the end of the outbreak, this will be called an introduction with negligible spread. This classification is based on the scenario of an uncontrolled situation, i.e. without detection of the outbreak and without culling of an affected farm. Introductions with negligible spread are assumed not to transmit to other farms.

As demonstrated in chapter 3, vaccination will reduce the transmission between chickens, which will result in more introductions leading to negligible spread and thus decrease the vulnerability of farms for outbreaks. However, because morbidity and mortality are reduced due to vaccination (chapter 3), some outbreaks that would normally be detected through passive surveillance may now go unnoticed.

In this chapter, the overall aim was to quantify the expected effects of vaccinating commercial layer chickens against highly pathogenic avian influenza virus on the spread of the virus within and between farms and use that to develop a surveillance program for the detection of infected vaccinated flocks.
To quantify the expected effects of vaccination on transmission at the farm level, we developed a within flock transmission model to assess the effect of vaccination on the prevention of large outbreaks and to design and evaluate the following surveillance strategies:

1) passive surveillance based on mortality.
2) passive surveillance and active surveillance by sampling of live chickens (EU regulation).
3) passive surveillance and active surveillance by sampling dead chickens (bucket sampling) at different frequencies (i.e. interval at which a single farm is sampled).

For each surveillance strategy, we further estimated the time of detection of an outbreak and both the number of expected infectious chickens and number of transported infected eggs by the time of detection.

Finally, we quantified the effect of vaccination at the country level by combining the within-farm outcomes regarding surveillance strategy and expected detection times with the between-farm spread. The effect is quantified by **the proportion of outbreaks with at least one secondary infected farm(s)** after introduction and the probability of **finding the index case before a secondary infected farm** is detected.

6.3 Results Recommendations for Surveillance Program

6.3.1 Final size

The final size is the fraction of the total population infected during the entire course of the outbreak if it is not controlled. When this is less than 0.1%, we call this an introduction with negligible spread. In non AI-vaccinated flocks only 4% of introductions have negligible spread, while for vaccinated flocks in the production phase 72% of introductions after vaccination with VAXXITEK HVT + IBD + H5 and 90% of introduction with applying VAXXITEK HVT + IBD + H5 + Volvac ® B.E.S.T. AI + ND result in negligible spread. Additionally, without control measures, the outbreaks in non AI-vaccinated farms results in most of the chickens being infected while for vaccinated flocks outbreaks occur with smaller final sizes.

6.3.2 Detection of outbreaks

Table 6.1 *Overview of outcomes after vaccination and for different sampling intervals. Within-farm are outcomes after introduction on to a farm. Negligible 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 given as percentages of runs. The outcomes were percentage of the primary farm was first to be detected and percentage with at least one secondary cased.*

		Within-farm							**Between-farm**					
		Detection			**Median number of infected chickens at detection (75%-range)**		**Eggs laid by infectious chickens and transported**		**Introduction in DPPA**				**Introduction in SPPA**	
		Negligible spread	**Outbreak**	**Median detection time(75%-range)**	**Dead**	**Live**	**≥1transported**	**Median number (75%-range)**	**Primary first**	**>1 inf. farm**	**Primary first**	**>1 inf. farm**	**Primary first**	**>1 inf. farm**
No AI-vaccination		0%	100%	11 (10-12)	396 (294-521)	5125 (3890-6369)	97%	244 (131-403)	94.2	87.5	-	-	99.1	22.2
VAXXITEK HVT + IBD + H5									DPPA		All Layers		DPPA	
Active surveillance interval (days)	2	2.4%	99.6%	11 (8-15)	1 (1-1)	2 (1-19)	71%	3 (2-12)						
	7	1.8%	99.6%	13 (10-17)	2 (1-4)	2 (1-29)	72%	4 (2-22)						
	14	1.1%	99.6%	18 (14-21)	3 (1-12)	2 (1-47)	72%	4 (2-46)						
	30	0.5%	99.6%	25 (18-32)	15 (2-246)	2 (1-84)	72%	4 (2-123)						
Passive surveillance only		0.0%	19.2%	29 (28-32)	571 (736-2370)	2 (1-29123)	73%	5 (2-45766)						

VAXXITEK HVT + IBD + H5 + Volvac ® + B.E.S.T. + AI + ND								DPPA		All Layers		DPPA	
Active surveillance interval (days)	2	2.3%	97.9%	13 (9-18)	1 (1-1)	1 (1-4)	68%	3 (2-6)	95.3	13.1	95.7	13.1	99.1	7.8
	7	1.8%	97.9%	16 (12-20)	1 (1-2)	1 (1-4)	68%	3 (2-7)	95.0	14.9	95.1	14.4	99.4	8.8
	14	1.1%	97.9%	20 (17-25)	2 (1-7)	1 (1-4)	68%	3 (2-7)	92.5	20.6	92.9	18.7	98.9	7.6
	30	0.7%	93.7%	28 (18-37)	3 (1-35)	1 (1-4)	68%	3 (2-7)	89.3	22.4	90.7	24.1	99.5	6.3
Passive surveillance only		0.0%	0.0%	-	437 (1-765)	1 (1-4)	68%	3 (2-8)	66.1	47.3	81.0	46.0	99.6	6.7

* Between-farm is only assessed for VAXXITEK HVT + IBD + H5 + Volvac ® + B.E.S.T. + AI + ND

6.3.2.1 Detection of introductions with negligible spread

Introductions with negligible spread (i.e. < 0.1% of the flock) are not detected in the current surveillance system. These introductions are considered of no relevance for onwards spread to humans or other farms. In the simulations with no vaccination, the only introductions with negligible spread were those where the initially infected chicken died before further transmission. Active surveillance, including sampling of dead chickens followed by confirmation testing, with a frequency of 2 days will detect 2.4% of introductions with negligible spread. The number of infected chickens after introduction is on average larger (median 10 to 15) when the introduction is detected in active surveillance than those that are not detected (median 2).

6.3.2.2 Detection of outbreaks

Introductions leading to outbreaks will be detected with high probability in non AI-vaccinated farms. For non AI-vaccinated farms, in the simulations 100 out of 100 outbreaks were detected in passive surveillance.
When vaccinating without applying the booster (VAXXITEK HVT + IBD + H5), only 19.2% of outbreaks in the production phase will be detected by passive surveillance. This is due to a decreased mortality in the vaccinated flocks. In the simulations, 20% of vaccinated chickens with a low titer and only 1% of the chickens with a high titer die of the infection. This may be an overestimation of the effect on reducing clinical signs, because it only considers mortality and infected chickens may still show other clinical signs, therefore this is a worst-case scenario and a higher percentage of detection by passive surveillance could occur. Implementing active surveillance of dead chickens, a minimum of 99.6% of outbreaks is detected during the production phase, when the sampling interval is 30 days. Surveying live chickens will result in detection in only 48.5% of outbreaks in the production phase.

When a heterologous to the prime booster was applied (VAXXITEK HVT + IBD + H5 + Volvac ® + B.E.S.T + AI + ND), none of the simulations in the production phase reached the thresholds for passive detection. When implementing active surveillance of dead chickens, a minimum of 93.7% of outbreaks will be detected during the production phase, when the sampling interval is 30 days. Surveying live chickens will result in detection in only 20.6% of outbreaks in the production phase, because the number of live infected chickens remains low in most simulated outbreaks.

6.3.2.3 Detection times

The median detection times are higher and more variable for vaccinated flocks than non AI-vaccinated flocks for all surveillance frequencies (Table 6.1). Depending on the proportion high titers in the flock (see difference preproduction vs production and different vaccines), the median detection times are similar to passive detection in non AI-vaccinated flock when active surveillance occurs every 2 to 7 days.

6.3.3 Infected chickens and eggs produced by infectious chickens

The number of infected chickens and eggs produced are measures of the risk associated with introductions into a farm. The median cumulative number of infected chickens is lower under all active surveillance programs. Using passive surveillance only, the median number of infected chickens at detection (assumption; infected layers will have a 50% reduction in production, and 10% of the eggs laid by infected layers are not suitable as table eggs) is higher than in non AI-vaccinated farms due to the reduced mortality in the vaccinated farms (Table 6.1).
For the number of transported eggs produced by infected chickens, vaccination will lead to a reduction of the median number of eggs produced by infected chickens being transported but the variation is much larger even with a surveillance interval of 14 days (Table 6.1). The number of farms that transport at least one egg produced by an infected chicken is lower for vaccinated than non AI-vaccinated flocks (Table 6.1). The seemingly discrepancy between the number of live infected chickens and the number of shipped eggs is due to the delay in shipping eggs, such that it is less than expected. In these simulations, eggs laid by infected chickens were calculated. In chapter 5 we have demonstrated that genetic material of the virus can be detected on the egg shell, however, no infectious virus could be isolated.

6.3.4 Between-farms

For the effects of between-farm transmission, three measures were considered

1) number of affected farms in an epidemic after a random introduction leading to an outbreak on the farm, and
2) whether the index farm is the first to be detected, and
3) the proportion of simulation runs in which more than one farm was infected.

The first measure will be described below. The second and third measures are shown in Table 6.1.

In the baseline scenario without vaccination the number of affected farms was generally low when the index farm was in a in sparsely populated poultry area (median 1, maximum 36). In contrast, when the index farm was in a densely populated area epidemic sizes were substantially larger, with a 25% percentile of 4, a median of 14 and a maximum of 61 infected farms. Recent introductions in densely populated areas may have led to only a few secondary infected farms, where most introductions did not (Bird flu at poultry farms, updates 2025/2026 | WUR, 26january2026). This suggest that on average the spread between farms is overestimated in the outcomes of the model scenarios (both with and without vaccination).

When vaccination was applied, epidemic sizes were markedly reduced, with median of 1 farm and 75% percentile 2 infected farms including the index farm. The maximum number of infected farms in the simulations was 37 when only layer farms DPPAs were vaccinated and the index farm was also located in in such a DPPA. When introduction occurred in a sparsely populated poultry areas (SPPA) under the same vaccination strategy maximum epidemic sizes were smaller, reaching up to 30 infected farms in the absence of active surveillance. The likelihood that a secondary infection was detected before the index farm was primarily determined by the location of the initial introduction. In SPPAs, the probability that the primary farm was detected first exceeded 98% across all sampling intervals. In DPPAs, the index farm was first detected in 66.1% and 81.0% respectively for vaccination of all or only laying farms om DPPAs. Implementing active surveillance every 2 days increases the probability of detecting the primary farm first to 95.3% and 95.7% for vaccination all or only laying farms in DPPAs respectively. Finally, introductions in SPPA's without vaccination already resulted in a high percentage of simulation runs in which the primary farm was detected first. Vaccination does not considerably increase this probability, but it reduces the likelihood of onward transmission to additional farms (Table 6.1).

6.3.5 Sensitivity to the cut-off value for high titer

In the analysis we used a cut-off of $\log_2$ HI-titer of ≥6 to dichotomize between high and low titer chickens. The two classes had a transmission rate as determined in Chapter 3. Changing this cut-off changes the estimates for the time during which the fraction high titer chickens are above the critical value. For a cut-off of 5, the transmission rate of high titer chickens estimated to be higher, resulting a higher reproduction number and thus higher critical fraction. Also, the detection times increase, because more chickens are protected against dying and will thus not end up in the sample. Median detection times can increase to over 20 days even when sampling every 2 days. For cut-off of 7 the fraction of chickens with a high titer decreases substantially and therefore more often drop below the critical fraction. For cut-off value 7 the detection time decreases as outbreak evolve faster and thus more infected chickens end up in the sample. The detection times will then be around 15 days even when sampling only every 14 days. However, the consequences of an outbreak measured by the number of infected chickens and eggs produced by infectious chickens that were transported from the farm remains lower than in the non AI-vaccinated scenario (Appendix Work Package 4).

6.4 Summarizing conclusion

For introductions leading to outbreaks (>0.1% of the farm) the detection by passive surveillance only is limited 19% of the outbreaks in vaccinated flocks, while this will detect all outbreaks in non AI-vaccinated flocks. Introductions with negligible spread will not lead to exposure of other farms or humans, and such introductions will not be detected by passive surveillance, but with a low probability when adding active surveillance by sampling dead chickens every 2 days. Adding active surveillance with vaccination VAXXITEK HVT + IBD + H5 will detect outbreaks with 99.9% to 99.6% for 2 and 30 day sampling intervals respectively, and for applying VAXXITEK HVT + IBD + H5 + Volvac ® + B.E.S.T. + AI + ND is 99.9% to 93.7% for 2 and 30 day sampling intervals respectively. These results are in line with Nielsen et al. (2024), although Nielsen et al. (2024) use a different modelling approach. The time until detection also depends on the sampling interval with similar median time until detection ranging from 11 days to 27 days for 2 to 30 day sampling interval respectively. Sampling intervals of 2 to 7 days will have a similar median detection time as passive surveillance in non AI-vaccinated farms, but the variation is higher. This holds whether using VAXXITEK HVT + IBD + H5 with or without Volvac ® + B.E.S.T. + AI + ND

At farm level the consequences of vaccination are measured by the number of eggs produced by infectious chickens transported from the farm and number of infected chickens at detection. The fraction of vaccinated farms, that transported at least one infectious egg before detection, is lower in all active surveillance sampling intervals than that for non AI-vaccinated farms. Additionally, those farms that have transported eggs from infectious chickens will have transported fewer eggs from infectious chickens than non AI-vaccinated farms. It should be noted that eggs from infectious chickens not necessarily need to be contaminated by the virus (see Chapter 5). Also, the numbers of infected chickens in vaccinated flocks are less at the moment of detection for every active sampling interval. Applying VAXXITEK HVT + IBD + H5 + Volvac ® + B.E.S.T. + AI + ND reduces the consequences as measured by numbers of infected chickens and transported eggs from infectious chickens.

In the absence of active surveillance, introductions are more likely to be detected in the primary case farm without vaccination, but the number of introductions leading to secondary farms being infected is always reduced by vaccination. This holds for both introductions in densely populated poultry areas (DPPA) and in a sparsely populated poultry area (SPPA). Surveillance further decreases the probability of onward spread before the primary farm is detected. The extend of spread is limited both when the introduction is in low or high densely populated areas. Compared to the current situation, the number of secondary farms being infected following an outbreak in a farm is thus expected to be lower in any vaccination scheme, independent of sampling frequency.

Efficacy of the vaccine to introductions of the new field strains may be different from the transmission experiments (Chapter 3). Virus strains with a larger antigenic distance to the vaccine, may spread better in a vaccinated flock. In case the circulating virus strain is antigenically more distant, this will probably result in more clinical signs in vaccinated chickens. This means that the passive surveillance will pick up more of these outbreaks. If the antigenic distance of a circulating field virus to the vaccine is less, this will probably result in better effects (examples; reduced transmission, less clinical signs and mortality).

In the model, it is assumed that farmers will report at a certain threshold or for a ratio of mortality in two days compared to the previous week. This may be an underestimation of the reporting by farmers in the current situation, where farmers are known to report as soon as they observe small increases in mortality (Daily mortality data from outbreak farms, NVWA). The current passive detection has thus most likely shorter detection times than assumed in this model. However, the effect of vaccination can be a decrease in symptoms hampering this early detection and behaviour of farmers may change if they believe their farm is protected.
In conclusion, vaccination will decrease the number of outbreaks and onwards spread to other farms. Also the number of infectious chickens during an outbreak and the number of eggs produced by infected chickens and transported from the farm is lower for vaccinated farms. Vaccination may lead to longer detection times, therefore active sampling is required to detect outbreaks with certainty, and to further decrease the impact of HPAI introductions into farms.
In conclusion, vaccination with either VAXXITEK HVT + IBD + H5 + Volvac ® + B.E.S.T. + AI + ND or VAXXITEK HVT + IBD + H5 only will decrease the number of outbreaks and onwards spread to other farms. Also the number of infectious chickens during an outbreak and the number of eggs produced by infected chickens and

transported from the farm is lower for vaccinated farms. VAXXITEK HVT + IBD + H5 + Volvac ® + B.E.S.T. + AI + ND is expected to perform better in terms of within-farm outcomes that VAXXITEK HVT + IBD + H5. For both, vaccination may lead to longer detection times, therefore active sampling is required to detect outbreaks with certainty, and to further decrease the impact of HPAI introductions into farms.

6.5 Recommendations

A sampling interval of less than 30 days will ensure limited outbreaks sizes and detection times, but any vaccination combined with active surveillance will reduce the impact of avian influenza in layer flocks on the number of infected chickens and eggs produced by these infected chickens, fraction of missed outbreaks, and further spread. Applying VAXXITEK HVT + IBD + H5 + Volvac ® + B.E.S.T. + AI + ND increases the time in which the reproduction number is below the threshold (Figure 3.1) and reduces the impact of introductions (Table 6.1). Implementation of a sampling scheme requires a monitoring and evaluation framework to address assessment of the accuracy of the model predictions. The model predictions inherently rely on laboratory estimates and idealized non-existing situations and therefore gaining empirical evidence can allow for relaxing of sampling or showing the need to intensify sampling.

Recommendations for a monitoring and evaluation plan based on the models and used assumptions should encompass:

- An evaluation of the detection time after implementation of vaccination e.g. using back-calculation methods [19] based on mortality records, to update or consolidate monitoring frequency.
- Monitoring of the effectiveness of the vaccine to the strain circulating in the field.
- Monitoring changes in practices and behaviours influencing the detection and spread of avian influenza outbreaks underlying the model results, such as the current time until detection and biosecurity leading affecting spread.

7 Materials and Methods

7.1 Field Observations (Work Package 1)

7.1.1 Clinical inspection

In addition to the daily monitoring of the general health by the poultry farmer, a clinical health inspection was performed by the research team of Royal GD, on a monthly basis. This visual inspection focused on the noticeable alterations of the individual chickens, the activity and uniformity of the flock, the quality of the droppings and the litter, the presence of respiratory, locomotion and nervous signs and the climate situation. The location of the injection in the pullets was inspected at 31 days of age (n=60) in test group 2 (VAXXITEK HVT+IBD+H5) and, for test group 3 (VAXXITEK HVT+IBD+H5 and Volvac® B.E.S.T. AI+ND), at 98 and 118 days of age (n=60) to check for vaccination reaction like cellulitis, cysts or myositis.

To get an impression of potential ongoing field infections present on the farm during the rearing period, tracheal swabs were collected at 14 to 18 weeks of age and analyzed by PCR for *Mycoplasma gallisepticum*, *Mycoplasma synoviae* (both field strain and MSH vaccine), avian metapneumovirus, *Avibacterium paragallinarum*, infectious bronchitis virus and infectious laryngotracheitis virus (both field strain and vaccine-like strains).

During the study, the chickens were tested ten times for the presence of *Salmonella* spp using culture after enrichment in accordance with applicable Dutch/EU surveillance requirements. At the end of the rearing period (week 17) and at the end of the study (approx. 84 weeks of age), the chickens were tested for the level of hemagglutination inhibition antibody titers for Newcastle Disease (ND) and the presence of antibodies against *Mycoplasma gallisepticum* by rapid plate agglutination test according to Dutch law.

7.1.2 Blood sampling

Throughout the entire study at multiple time points blood samples were collected from randomly selected chickens, by puncture of the wing vein, from the test groups (Figure 7.1) on a monthly basis (30 days). During each sampling, 120 samples per vaccinated test group and 60 samples of the non AI-vaccinated control group were collected. Blood serum was used to determine individual HI antibody titers against the vaccine antigen. Per sampling time, 10 additional whole blood samples were collected to determine parameters of cellular immunity. Additional timepoints for whole blood analysis were at 3, 7, 10, and 14 days post HVT AI vaccination (at day 0) and 7 and 14 days post AI booster vaccination (at day 84). From week 43/44 onwards, whole blood samples were collected on weeks 52, 64/65 and at the end of the study at 84/85 weeks of age.

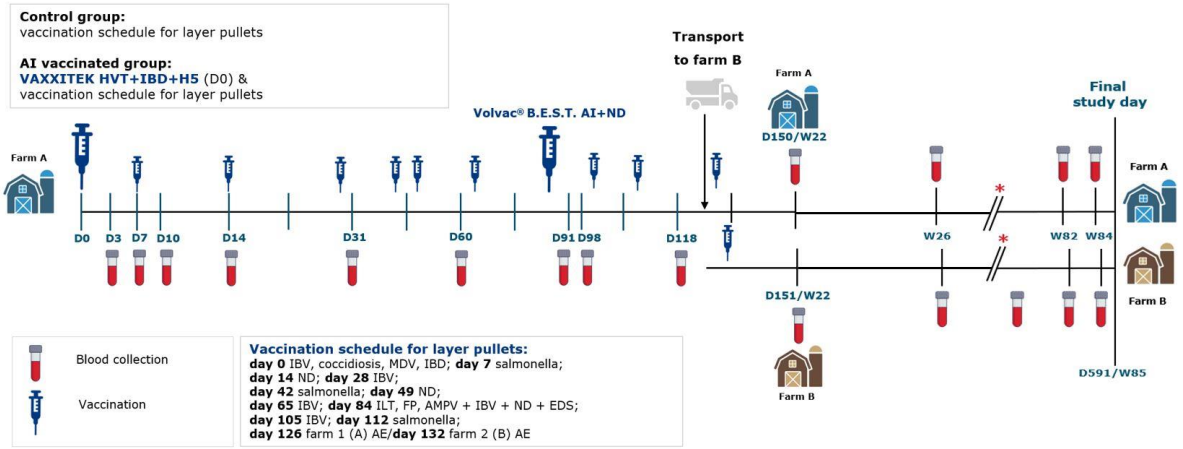

Figure 7.1 *Overview of the blood sampling moments and the time of application of the different vaccinations within the field study. Volvac® B.E.S.T. AI+ND was applied to test group 3 only.*

7.1.3 Mortality

On a weekly basis, based on the registration by the farmer, the cumulative mortality rates were calculated for the individual test groups. Dead chickens were collected by the farmers during their daily management and stored in a freezer (-20°C). After the first thirty days of rearing, the stored carcasses were directly presented to the rendering company for disposal, in line with the Genetically Modified Organisms (GMO) permit. Beyond day 30, dead chickens were stored in the freezer by the farmer and weekly transported to the Royal GD facilities in order to be sampled and tested for the presence of AI virus by M-PCR. After testing, carcasses were presented to the rendering company for disposal.

7.1.4 Egg production

On a weekly basis, from the start of the production period, eggs were collected at both farms and transported to Royal GD for counting and destruction of the eggs in line with the GMO permit.

7.1.5 Humoral immunity

Of the collected blood samples, blood serum was collected and this serum was tested at the Royal GD laboratory within three days after sampling. Tests performed were hemagglutination inhibition (HI) test and the multispecies AI antibodies Nucleoprotein (NP) ELISA (IDEXX).

7.1.5.1 Homologous H5 hemagglutination inhibition (HI) test

Monthly, the homologous HI test was performed on 120 serum samples from each AI vaccinated test group and 10 samples of the non AI-vaccinated control group 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).

The HI tests were conducted according to the standard World Organization for Animal Health (WOAH) protocol, using 8 hemagglutination units (HAU) per well. A serum was considered positive for H5 specific antibodies when HI titers of 3 or higher (≥3) were obtained. The distribution of the individual titers in the entire flock was estimated using the sample of 120.

7.1.5.2 NP-ELISA (multispecies AI antibodies NP-ELISA (IDEXX))

In this study, the DIVA test for distinguishing infected from vaccinated animals was performed by testing blood sera collected from chickens in the field using this NP-ELISA, as the presence of antibodies against the Np protein indicates a field infection. Monthly, the NP-ELISA test (IDEXX, Multispecies AIV antibody ELISA) was performed on 60 serum samples from each AI vaccinated test group and the non AI-vaccinated control group. Sera with 'Not negative' NP-ELISA results were sent to the national reference laboratory for avian influenza and Newcastle disease at WBVR for confirmation. This is in accordance with the procedures followed in the Dutch national monitoring program for AI in poultry.

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

In case of AI vaccination, a laboratory surveillance should be implemented to detect the occurrence of infection with HPAI field virus in the vaccinated flocks according to Commission Delegated Regulation (EU) 2023/361 of 28 November 2022. Therefore, all mortality (with a maximum of 10 chickens per week) was collected and sampled once a week, and pooled tracheal and cloacal swabs were tested separately (maximum of 10 swabs per sample). In accordance with the GMO permit, collection of dead chickens started from day 30 onwards. The pools were tested at Royal GD with the Real-time reverse transcriptase (RRT) PCR for the detection of the M-gene of AI virus (M-PCR) based on the technique described by Ward et al [25]. RNA was isolated using a MagMax Pathogen RNA/DNA kit (Life Technologies). Detection limit was determined at around $10^{-0.7}$ EID_{50}/0.2mL.

7.1.7 Cellular immunity

7.1.7.1 Absolute lymphocyte counts

At multiple timepoints after vaccination, 1 ml blood was collected in 3K-EDTA tubes to determine absolute counts of several lymphocyte subsets [26] in blood of chickens that were vaccinated as described in paragraph 2.2.3.

Whole blood was stained using BD Trucount™ Tubes (BD Biosciences, San Jose, USA) according to the manufacturer's instructions and analyzed by flow cytometry. Firstly, 200 µl of EDTA-blood was mixed thoroughly with 40 µl Transfix® (Sanbio) and subsequently fixed blood was 1:50 diluted in PBA (PBS containing 0.5% bovine serum albumin and 0.1% sodium azide). Next, 20 µl antibody mix was transferred into a BD Trucount Tube followed by 50 µl diluted fixed blood and incubated for 15 minutes in the dark at room temperature.

The antibody mix (Table 7.1) 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 some chickens, T cells were not recognized by the anti-CD3 antibody. In these chickens 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 T cells. This strategy does not allow analysis of gamma delta (γδ) T cells, therefore γδ T cells were determined in 6 to 10 chickens in the VAXXITEK HVT+IBD+H5 group (Group 2), in 6 to 10 chickens in the VAXXITEK HVT+IBD+H5 boosted group (Group 3) and 5 to 10 in the non AI-vaccinated chickens (Group 4), depending on the timepoint. At the earliest timepoints, this alternative strategy was not available yet, therefore 8 chickens are shown at day 3, 7 and 10 days post vaccination in the VAXXITEK HVT+IBD+H5 group (Group 2); 9, 7 and 8 chickens are shown at respectively day 3, 7 and 10 days post vaccination in the chickens that are boosted with VAXXITEK HVT+IBD+H5 (Group 3) and in the non AI-vaccinated control group (Group 4).

After the staining, 450 µl PBA was added and flowcytometry was performed. Samples were measured using a CYTOFLEX LX (Beckman Coulter) 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 7.1 *An overview of the monoclonal antibodies and their target that were used in this study. Antibodies were obtained from SouthernBiotech, Birmingham, USA.*

Target	Antibody (all mouse-anti-chicken)	Clone	Isotype
Leukocytes	CD45-PE	LT-40	IgM
Total T cells (CD3+)	CD3-PB	CT-3	IgG1
T helper cells (CD4+)	CD4-PECy7	CT-4	IgG1
Cytotoxic T cells (CD8+)	CD8α-AF700	CT-8	IgG1
Gamma delta T cells (γδ)	γδ-FITC	TCR-1	IgG1
Activated T cells (CD25+)	APC	AV142	IgG1
Alpha-beta1 T cells	TCRαβ/Vβ1	TCR-2	IgG1
Alpha-beta 2 T cells	TCRαβ/Vβ2-FITC	TCR-3	IgG1

7.1.7.2 Statistical analysis

Statistical differences were calculated using GraphPad prism version 10.1.2. Differences between the groups were analysed using Mann-Whitney U tests. A p-value of <0.05 was considered statistically significant.

7.2 Transmission studies (Work package 2)

7.2.1 Definition of infection in the context of the transmission studies within this longitudinal field study

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

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

This definition is consistent with the definition of an infected chicken used in our previous studies [6, 9-12]

7.2.2 Statistical analysis, Assessment of Transmission

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

To estimate the transmission rate parameters β's two types of generalized linear models (GLM) were used: (1) GLM for homogeneous population and (2) GLM for heterogeneous populations.

GLM for homogeneous populations

For this model, it is assumed that all individuals are equally susceptible (before infection) and equally infectious (after infection). Therefore, this model was used to quantify β for the non AI-vaccinated (controls) chickens, which were used in the four different experiments (age of chickens: 8, 24, 54 and 84 weeks), and for the quantification of β of vaccinated groups from experiments performed at weeks 8 and 24 of age. In these experiments, vaccinated chickens were randomly assigned to each 5X5 group and donors chickens, within each group, randomly selected for inoculation. Here again we took the simplifying assumption that vaccinated chickens are equally infectious and susceptible regardless of the levels of their antibody response. This approach was not followed for experiments performed at ages 54 and 84 weeks (See below).

Estimation of β was based on daily infection and transmission data, prepared as described in previous reports[9-12], assuming a one-day latent period (time from infection to becoming infectious). Data were analyzed using a GLM with a binomial error distribution and a complementary log-log link, specified as [7].

$$cloglog\left(\frac{C_t}{S_t}\right) = \log(\beta) + \log\left(\frac{I_t}{N_t}\Delta t\right) \quad (1)$$

Where $log\left(\frac{I_t}{N_t}\Delta t\right)$ is the offset and $\log(\beta)$is the model intercept (regression coefficient).

The length of the infectious period T was estimated using parametric survival analysis, testing different distributions. The best model was selected based on the lowest AIC and the agreement between the model's median time estimate with the observed average number of infectious days. The Weibull distribution provided the best fit, as determined by the lowest AIC. Once β and T were quantified, the reproduction number R was estimated as

$$R = \beta T \quad (2)$$

Confidence intervals for R were generated using Monte Carlo sampling. Random values for B were drawn from a lognormal distribution with mean and standard deviation estimated from the GLMs. Similarly, random values

for T were drawn from a Weibull distribution using the shape and scale parameters estimated from the survival regression analysis.

GLM for heterogeneous populations

Following vaccination, antibody titers vary among individuals, with some chickens developing high titers and others low titers. Chickens with higher titers are likely to be better protected against transmission than those with lower titers. As a result, vaccination produces heterogeneity in the level of protection within a flock, which is critical when evaluating vaccine efficacy at the population level. To account for this, we quantified transmission by estimating R, incorporating the effect of the immune response (homologous HI titer) on transmission. To this end, we used a model described by Sitaras et al [22]. Briefly, in each 5 × 5 test group, chickens were classified as having either high (h) or low (l) immunity according to their homologous HI titer, using cutoff values of <5, <6, <7 and <8 (cutoff ≤4 could not be evaluated because too few chickens met this criterion). For each cutoff value, we estimated β and T separately for the chickens with low and high HI titers. This resulted, per cut off, in the estimation of two parameters for T: T_h and T_l; and four parameters for β: β_{hh}; β_{hl}; β_{lh}; β_{ll}. The later four parameters represent respectively: transmission from high-titer (h) infectious chickens to h recipients, from h infectious chickens to low-titer (l) recipients, from l infectious chickens to a h recipients, and from l infectious chickens to l recipients.

For this analysis, the data was used from experiments performed with chickens at 24, 54 and 84 weeks of age. Estimation of β was based on daily infection and transmission data, prepared as described by Sitaras et al[22]. And assuming a one-day latent period (based on observations on inoculated chickens). Data were analyzed using a GLM with a binomial error distribution and a complementary log-log link, specified as [22]:

$$cloglog\left(\frac{C_{l\ or\ h,t}}{S_{l\ or\ h,t}}\right) = C_0 + C_1\,S_h + C_2\,F_h + Log(\frac{I_{tot}\,\Delta t}{N}) \quad (3)$$

In this model S_h is an indicator variable equal to 1 when susceptible chickens have a high-titer and 0 otherwise; F_h is the fraction of h infectious chickens; I_{tot} Is the total number of infectious chickens ($l + h$); N is the total number of chickens; and Δt is the time interval of analysis. Parameters $C_0, C_1\ and\ C_2$ are fitted in the analysis, with C_1 representing the effect of vaccination on susceptibility and C_2 representing the effect of vaccination on infectiousness. These parameters were then used to derive the different βs as follow:

$$\beta_{hh} = Exp[C0 + C1 + C2]$$

$$\beta_{lh} = Exp[C0 + C1]$$

$$\beta_{hl} = Exp[C0 + C2]$$

$$\beta_{ll} = Exp[C0]$$

The length of the infectious period T was estimated using parametric survival analysis, testing different distributions. The best model was selected based on the lowest AIC and the agreement between the model's median time estimate with the observed average number of infectious days. The Weibull distribution provided the best fit.

For the estimation of β and T of the vaccinated chickens we assessed for two different assumptions:

- The first assumption considers any PCR positive results (≥1.7 eq EID_{50}) as infected and infectious. Hence, the infectious period PCR is the number of days from the first to the last obtained positive PCR result (Infectious period PCR).
- The second assumption is based on the predicted concentration of virus titer ($TCID_{50}$) considering every virus positive ($TCID_{50}$ > 0.5) individual as infected and infectious (Infectious period Virus). This prediction was made because it is expected, particularly in vaccinated chickens, that influenza virions loose infectivity faster than RNA integrity, resulting in positive results late post-infection when no live viruses might be present. This assumption is likely to result in estimated shorter infectious periods, as it was also observed for other diseases [7, 27]. This prediction was made following a model developed by [28].

- This model predicts the concentration of infectious virus in samples from vaccinated-infected chickens, based on information of the day post-infection (dpi), the type of swab (choana or cloaca) and the estimated equivalent virus titers ($eqTCID_{50}/2ml$) following the equation:

- $TCID_{50}/2ml = -0.14 + 0.98 * eqTCID_{50}/2ml - 0.49 * swab - 0.07 * dpi$ (4)

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

The above assumptions for the estimation of Infectious period and corresponding R-values were assessed, as in these transmission studies, some vaccinated chickens were positive in M-PCR for long periods of time (several days, to longer than a week). Previous experience [27] indicates that in vaccinated infected chickens, M-PCR positive results longer than 6 days no longer correlate with the presence of viable virus. The results for both assumptions are shown for completeness. Interpretation needs to be done considering the assumptions made for the analysis and limitations of the study approach.

Once the parameters β and T were estimated, the effective reproduction number R_e was estimated as follow:

$Re = F_h \beta_{hh} T_h + (1 - F_h) \beta_{ll} T_l$ (5)

Based on these equation, the required critical fraction of chickens with high titers at which $Re < 1$, and therefore, no major outbreaks should occur, can be determined as follow:

$F_h \geq \frac{1 - R_l}{R_h - R_l}$ (6)

For both models (homogenous and heterogeneous), the 95% confidence intervals for R were derived by Monte Carlo (MC) simulations (10000 replications) assigning to β a lognormal distributions and T a Weibull distribution, using the parameters from the GLM and the survival regression models respectively.

7.2.3 Predicting the expected Re in vaccinated flocks

The humoral responses (homologous HI titers) monitored in the field throughout the course of the production period (Chapter 2, Figures 2.1 and 2.2 and Appendix Table 1.2) were used to determine the fraction of chickens in the flock considered to have high titers F_h. This information was then used in combination with experimentally estimated transmission parameters to quantify the expected Re (equation 5) throughout the course of the flock's production period.

7.3 Cellular immunity Transmission studies (Work Package 3)

7.3.1 Whole Blood Staining to Determine Absolute Lymphocyte Counts

To determine absolute counts of several lymphocyte subsets after inoculation, blood samples of inoculated chickens in the transmission studies were collected in a 3K-EDTA tube at 0, 1, 3, 7, 10 and 14 dpi (Appendix Figure 2.1). Whole blood was fixed in TransFix® reagent and stained using BD truecount tubes as previously described [9-12, 26]. The antibody mix (Table 7.1) 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 some chickens, T cells were not recognized by the anti-CD3 antibody. Blood of these chickens was stained using a combination of the T-cell receptor recognizing antibodies mouse-anti-chicken-αβ1-FITC, mouse-anti-chicken-αβ2-FITC and mouse-anti-chicken-γδ-FITC to identify the T cells[2]. After staining, samples were fixed with 4% paraformaldehyde and resuspended in FACS-buffer before measuring using a FACS DIVA Flowcytometer (BD Biosciences) in transmission studies 1 and 2, or the Cytek Aurora Cytek® Biosciences in transmission studies 3 and 4. In all cases 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. CD4+ and CD8+ T cells were selected by gating on the CD3+ CD45+ cells. In transmission study 2 the number of events in the gates for CD4 and CD8 T cells was too low to continue with further analysis of the CD25 expression at 3 and 7 dpc, due to technical problems with the flowcytometer.

7.3.2 Isolation of lung and blood cells

Lung tissue was collected and dissected into small fragments aided by enzymatic digestion in RPMI medium containing collagenase A from Clostridium histolyticum and DNAse I from bovine pancreas (Roche Applied Science, Almere, the Netherlands) for 30 minutes at 37°C. The resulting suspension was then passed through a 70 µM cell strainer (Beckton Dickinson (BD), Franklin Lakes, NJ, USA). Heparin blood was diluted (1:1) in PBS. Viable cells from lung and blood were isolated via Ficoll-Paque density gradient centrifugation. Cells were washed in PBS and resuspended in complete medium, composed of RPMI medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamax (Gibco BRL, United Kingdom).

7.3.3 IFNγ ELIspot Analysis

IFNγ ELIspot was performed as previously described [15, 29]. IFNγ ELIspot analysis was conducted using 96-well Mulitiscreen® HTS plates Merck; MAIPS4510) that were incubated with 70% ethanol for 1 minute at room temperature and washed with H_2O and PBS. Next, wells were coated with mouse anti-chicken IFNγ (2.5 µg/well in PBS; chicken matched antibody pair, Thermofisher) and incubated overnight at 4°C. After washing with complete medium, the plates were blocked for 1 hour with complete medium at 41°C, 5% CO_2. Next, cells were seeded at 2×10^5 cells/well in triplicate and incubated for 24 hours at 41°C, 5% CO_2. As a positive control for the capacity of cells to produce IFNγ a combination of 50 ng/ml phorbol myristrate acetate (PMA) and 500 ng/ml Ionomycin was included (Sigma-Aldrich). Plates were washed with PBS supplemented with 0.05% Tween-20 (PBS-Tween) and incubated with anti-chicken IFNγ biotin (1 µg/well in PBS, chicken matched antibody pair) for 1 hour at room temperature. Plates were washed 5 times with PBS-Tween and incubated with poly-HRP (0.2 µg/well; Fisher) for 1 hour at room temperature. Plates were washed 5 times with PBS-Tween and TMB was added (50 µl/well, TMB for ELIspot, Mabtech). After spots became visible, plates were washed with tap water and air-dried. Spot analysis was performed using the AELVIS automated spot analyzer (Sanquin).

7.3.4 Statistical analysis

Statistical differences were calculated using GraphPad prism version 10.5.0. Non-parametric statistical tests were used when the assumption of normal distributed data was not met. Differences in the number of T cells between the groups were analyzed using Mann-Whitney U tests. Differences in T cell numbers over time were determined using a Friedman test followed by Dunn's multiple comparison test. Comparisons between day 0 and day 1 responses in the control group were determined using a Wilcoxon matched-pairs signed rank test. Correlations between viral load, numbers of (activated) T cells and IFNγ producing cells were where calculated using Spearman's rank correlations. A p-value <0.05 was considered significant.

7.4 Safety of Poultry products

Material and methods were identical to the methods described in Appendix Work Package 2, 2.1.1-2.1.7 (except paragraph 2.1.4). Material and methods specific for this chapter are described below.

7.4.1 Preparation of samples prior RNA isolation

After sampling, swabs from choana, cloaca and egg shell were immediately placed in 2 ml Tryptose Phosphate Buffer (TBP) and stored at -80°C until processing. Wing feathers collected at necropsy were collected and stored at -80°C until processing. During processing, two feather shafts were pooled, shortened to approximately 0,5cm in length and were dissected lengthwise. Thereafter, the feather shafts were placed in 300µl sterile PBS, vortexed (10sec) and incubated for 45min at room temperature before RNA isolation.
Breast and heart samples were placed in 1.5ml PBS immediately after collection and homogenized for 45sec (6m/s) and centrifuged (5min, 950g) to collect the supernatant. The supernatant was diluted 3x (breast) or 2x (heart) prior RNA isolation. Egg white samples were collected, and diluted 4x in sterile PBS prior RNA isolation.

Because M-PCR positive samples from breast and heart tissue supernatant and diluted egg white may be tested in the virus isolation, these samples were stored in the refrigerator until the results of the M-PCR were obtained.

7.4.2 Virus isolation

M-PCR positive samples with titers $\geq Log10^{3.6}$ $eqEID_{50}$/ml (corresponding to Ct-value in M-PCR ≤ 33) were included for virus isolation. Homogenates from the tissues were incubated for 1 hour with 1% penicillin and 1% gentamicin at room temperature. The homogenates were filtered and inoculated in nine-day-old specified-pathogen-free (SPF) embryonated chicken eggs, as described previously. Allantois fluid was harvested from the deceased eggs and tested in the Haemagglutination assay using chicken erythrocytes.

7.5 Recommendations for Surveillance Program (Work Package 4)

7.5.1 Within farm model

7.5.1.1 **Infection module**

The model is a two-type S-L-I-R model with an Erlang-distributed (i.e. a gamma-distribution with an integer shape parameter) latent and infectious period. We use a stochastic variant and simulate the outcome with discrete variables (number of chickens in different states) and discrete time steps, at which the number of new infections, recoveries and deaths are drawn. Infections occur based on the number of infectious and susceptible individuals and the per capita transmission rate of the infectious individuals. The end of the infectious period is determined by random transitions through I states resulting in a random drawn infectious period for each individual from an Erlang distribution. The length of the infectious period is based on whether the chicken has a high vaccine titer or not. non AI-vaccinated chickens die at the end of the infectious period, while vaccinated chickens have a probability of dying depending on whether they have a high titer (1% mortality) or low titer (20% mortality).

In the simulations, the program keeps track of the number of exposed, infectious, and recovered chickens separated between high and low titer. Additionally, it records the number of dead chickens and the number of eggs produced and shipped of the farm.

7.5.1.2 **Detection module**

Based on the number of chickens in different states in the simulated outbreaks the detection module is run.
For detection two possible routes exist passive or active surveillance.
Detection occurs either through **passive detection** based on two criteria:

- the daily mortality for two consecutive days is 3x higher than mean daily mortality of the previous week.
- 2-day mortality is greater than or equal to 0.5% and confirmation testing of live chickens [1].

Active surveillance based on dead chickens occurs by the following protocol (Figure 7.2):
At regular intervals (2, 7,14 or 30 days) chickens died in the last 2 days are collected and tested.
Detectable chickens are dead chickens that have an active infection at the moment of dying or died from other causes after recovery (e.g. were seropositive). In terms of the model compartments this will be the L, I and R compartments.
One day after a positive test in the dead chickens a confirmation is done by:

- All dead chickens in the last day
- A sample of 20 live chickens showing symptoms (representative clinical cases). In modelling terms are the chickens in compartments L and I. Here we assume that also infected vaccinated chickens will show some symptoms.
- The test (M-PCR for swab samples) specificity and sensitivity are assumed to be 100%. Hence if an infected chicken dies it will always be found.

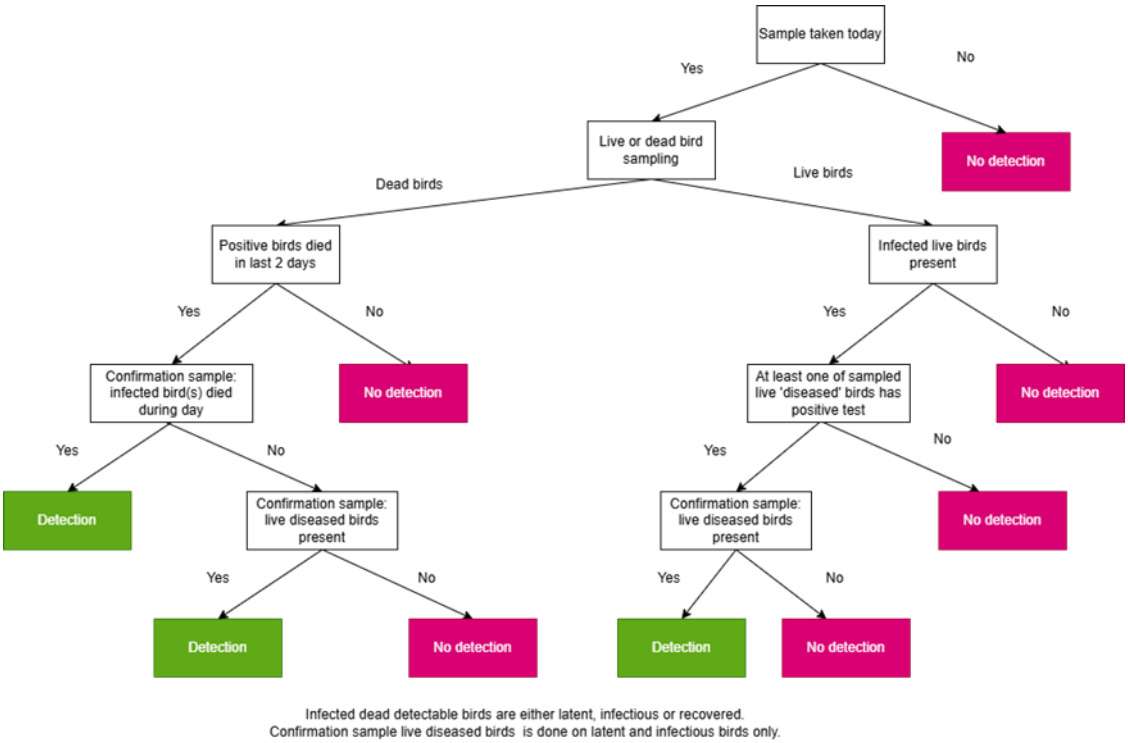

Figure 7.2 *Decision tree for active surveillance. Confirmation test is done one day after first detection.*

Active detection based on live chickens occurs by the following protocol (Figure 7.2)
At regular interval of 30 days a sample of 60 live chickens are collected, where apparently sick chickens (most likely to be infected) are sampled and if not sufficient apparently sick chickens are present additional chickens are added.
One day after a positive test in the dead chickens a confirmation is done by:
All dead chickens in the last day

A sample of 20 live chickens showing symptoms (representative clinical cases). In modelling terms are the chickens in compartments L and I. Here we assume that also infected vaccinated chickens will show some symptoms.
The test specificity (M-PCR for swab samples) and sensitivity are assumed to be 100%.

7.5.1.3 **Initial values**

The within-farm model is initialized assuming introduction of the virus during the production cycle at the same age as during the sampling moments in the field study (Chapter 2). This reflects an introduction uniformly distributed over the production cycle of laying hens. The fraction of chickens with a high titer is the observed value at the sampling moment. Because the outbreak durations are expected to be less than 90 days, which is in the range of the 2-3 sample moments, we do not consider changes of the proportion of high titer chickens during a simulation. The initial introduction occurs in a randomly drawn chicken from the total flock, and the probability of an introduction in a high titer chicken is thus equal to the fraction of high titer chickens in the population. Sampling moments before the production phase are not included in the analyses, as the transmission parameters in pullets has different values and the change during the preproduction phase is not well characterized (Chapter 3).
All runs were done for an average farm size of 46 000 based on RVO data (https://www.rvo.nl/). Work by the EFSA panel AHAW has shown that the size (within the ranges here) does not affect the results substantially[20].

7.5.2 Between farm model

Delayed detection in farms will increase the infectious period of these farms. To assess the combined effect of prolonged detection times on the one hand and protection against outbreaks on the other, a spatial explicit individual based farm-to-farm model was used (Chanchaidechachai et al., submitted , [30]). Individual attributes of the farm were location, size, species, farm type (species, breeder or not, out/indoor access), and vaccination status.
The transmission rate between farms is determined by the distance of that farm, the farm type of the susceptible farm, the size of the farms, and the vaccination status of the farm. For this analysis only the farm type "commercial layer farms" are vaccinated. Vaccinated farms have a reduced susceptibility equal to the probability of an outbreak (i.e. 1 in non AI-vaccinated farms).
The infectious period of a farm is determined by the detection time distribution of farms based on species, farm type, farm size and vaccination status. The model is run for 1000 times.

7.5.3 Outcome measures

7.5.3.1 **Introductions leading to outbreaks.**

In the analyses, only exposed farms are considered. With this is meant that an introduction has taken place by infecting one of the chickens in a farm. Whether this introduction leads to an outbreak depends on the reproduction number in the flock. Introductions with negligible spread can be considered as less relevant, because the probability of further spread, development of new variants or infection of other chickens (including humans) is negligible. Therefore, the probability of detecting introductions with negligible spread is determined from the simulations, as increased surveillance may increase the detection of otherwise irrelevant introductions. It is stressed that the division between large outbreaks and outbreaks with negligible spread is at 0.1% of the population size of a farm. In our simulations this means a maximum of 46 infected chickens at the end of the outbreak.

7.5.3.2 **Time between introduction and detection**

The time between introduction and detection is the moment between the introduction and the first positive sample or first moment of reporting. Only those times are counted for which a positive confirmation is given, but then the moment of first report is indicated. For example, if active sampling finds a positive sample at day 5 past introduction and this is confirmed on day 6, the detection time is 6 days.

7.5.3.3 **Infected chickens and eggs produced by infected chickens.**

The main consequences of an introduction are those that are related to further spread between-farms and transmission to other species. The number of eggs produced by infectious chickens and transported before

detection of the farm is an outcome defining public health risk. The cumulative number of infectious chickens between introduction and detection is an indication of the potential for transmission to other farms, wildlife populations and humans in contact with the chickens.

7.5.3.4 **Number of infected farms at detection and probability of secondary infected farms**

For the between-farm transmission, three measures were considered 1) number of affected farms in an epidemic after a random introduction leading to an outbreak on the farm, and 2) whether the index farm is the first to be detected, and 3) the proportion of simulation runs in which more than one farm was infected. The first and third measure indicate the impact of vaccination on a national level following an introduction and outbreak in a farm. The second measure indicates a change in the risk of spreading between farms before detection on an introduction that leads to an outbreak.

7.5.3.5 **Software and scripts**

All analyses were done in R (R version 4.4.2 'Pile of Leaves') with user defined simulations. The codes used for the analysis can be found in https://github.com/UtrechtUniversity/HPAI_vaccination.

7.5.4 Input parameters and surveillance scenarios.

7.5.4.1 **Input to the within-farm model, Transmission parameters and proportion of chickens with high titer**

7.5.4.1.1 Transmission parameters & proportion high titer

From the transmission experiments based on chickens from the field study (Chapter 2), we assumed that chickens with a log HI-titer of at least 6 are high-titer chickens with reduced transmission, while chickens with a log-titer less than 6 are considered low-titer chickens. Only estimates of the transmission parameters for VAXXITEK HVT + IBD + H5 were available (Chapter 3) and these were used in both groups in which VAXXITEK HVT + IBD + H5 and VAXXITEK HVT + IBD + H5 + Volvac ® + B.E.S.T. + AI + ND is applied (Chapter 3). For the baseline scenario we used the overall estimate of all experiments for low titer chickens. We used the parameter values determined by the data based on the prediction of the presence of viable virus(for details see Chapter 3) This assumption results in the set of values used in the simulations for transmission coefficients (β's) provided in Table 6.2. The β 's are constant over the simulations and equal for each chicken given its titer (high or low).

As infectious period distribution, we used the mean from the transmission experiments and refitted an Erlang distribution to the observed variance. A shape parameter in this Erlang distribution of 2 proved the best fit. The infectious period for each individual chicken was a randomly drawn value from the infectious period distribution.

Table 6.2 *Transmission parameters for chickens with low or high titers in vaccinated and for all chickens in non AI-vaccinated groups (control groups). The β is the per capita daily transmission rate (day^{-1}), is the mean infectious period in days, k is the shape parameter of the infectious period distribution, and R the reproduction number.*

	Low titer				**High titer**			
	β_{low}	T_{inf}	**k**	R_{low}	β_{high}	T_{inf}	**k**	R_{high}
VAXXITEK HVT + IBD + H5	2.07	4.29	2	8.88	0.17	3.35	2	0.57
Non AI-vaccinated	2.04	3.89	2	7.91				

The field experiment (Chapter 2) provided values of the proportion of chickens with a high titer. The data below show the change over time of the proportion of high titer chickens in the field experiments.

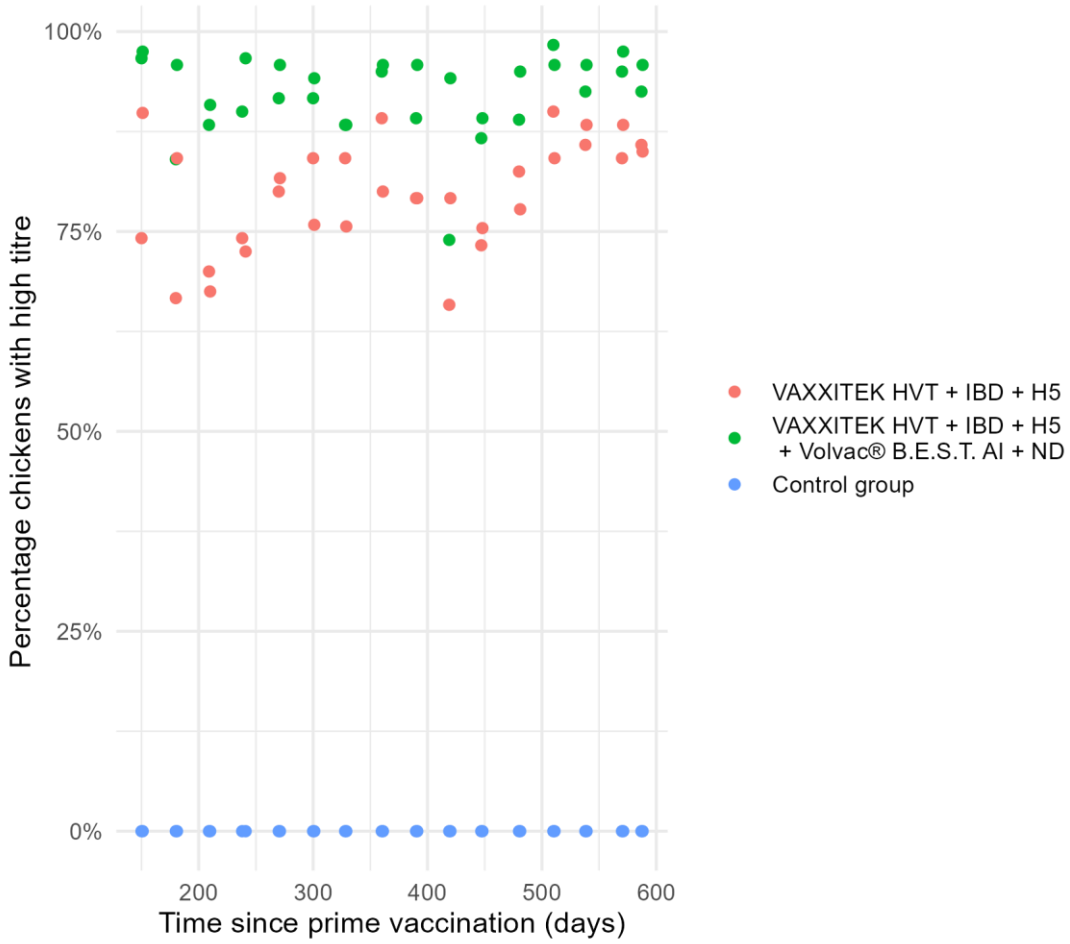

Figure 7.3 *Percentage of chickens with a high titer ($log_2HI \geq 6$) over time.*

7.5.4.2 Mortality and production curve

Here, a selection of the consequences of an outbreak were simulated: number of infected chickens, number of chickens that die due to the infection and the number of eggs produced and transported off the premises by an infected chicken before detection of the infection.

The effect of vaccination on mortality was assumed at 0.1% mortality in high titer chickens and 20% in low titer chickens at the end of the infectious period. In non AI-vaccinated flocks 100% mortality in chickens was assumed .

For the number of eggs produced by a chicken, an egg production curve reported by the breeding company based on the age of the chicken was used https://lohmann-breeders.com/outstanding-performances-lohmann-layers-above-the-standards/ [31]. The period before 120 days of age (±17 weeks) was considered the preproduction phase. The number of eggs for infectious chickens is discounted by a reduction in egg-laying (50% reduction) and disfigurement of eggs (10%).

7.5.5 Input into the between-farm model

The between-farm model parameterization is fully based on Chanchaidechachai et al (submitted). The main input in the between-farm model are individual farm characteristics; location (geographical coordinates), farm size, and farm type obtained from RVO for 2024. For the between-farm transmission parameter, the parameterization used the parameters from Hagenaars et al. [32] and the odds ratios of introduction for farm types from Gonzales et al. [33]. The parameters were then calibrated such that the distribution of the size and duration of epidemics matched those simulated by Hagenaars et al. [32]. For vaccinated farms, the susceptibility was multiplied by the fraction of large outbreaks. The infectious period of a farm was either equal

to the parameterization of Chanchaidechachai et al. in prep. for non AI-vaccinated farms. For vaccinated farms, the distribution of detection was derived from the within-farm model and the different surveillance intervals. The model was initialized with two options for compulsory vaccination of farms: 1) vaccination of all commercial layer farms in a densely populated poultry area, defined as those commercial layer farms with a between-farm reproduction number above 0.75, and 2) vaccination of all commercial layer farms. The initial farm was picked randomly drawn from a densely populated poultry area or at random outside that area. The model was run 1000 for each scenario.

Terminology

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

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

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

This definition is consistent with the definition of an infected chicken used in our previous studies [9-12].
Defining an infected chicken by being both positive for virus shedding and antibody response is essential.
Virus shedding; the release of the virus from the host into the environment, indicates active replication and the chicken's potential to transmit the virus to others. This measure alone, however, may not provide a complete picture of the infection dynamics or the host's immune response. The presence of an antibody response is a crucial complement to virus shedding as it signifies the host's adaptive immune system has recognized and responded to the challenge virus. Antibodies, particularly those detectable by assays such as hemagglutination inhibition (HI) or ELISA, indicate past or ongoing exposure and provide evidence that the immune system has mounted a defense. These dual criteria of virus shedding and antibody response provide a more comprehensive and accurate characterization of infection, enhancing the understanding of disease dynamics, the effectiveness of vaccination strategies.

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

Hemagglutination inhibition (HI) titers pre-challenge are a critical metric in the evaluation of immunological responses and the efficacy of vaccines against viral infections. HI titers measure the presence

and level of specific neutralizing antibodies [21] capable of inhibiting the hemagglutination process, where viruses agglutinate red blood cells. Higher pre-challenge HI titers indicate a stronger pre-existing immunity, which is predictive of an individual's ability to mount an effective defense against viral exposure. In the context of vaccine studies, assessing HI titers before exposure to the pathogen provides essential data on the protective threshold needed to prevent infection. Moreover, understanding the correlation between pre-challenge HI titers and clinical protection helps in establishing immune correlates of protection, which are pivotal for regulatory approvals and public health decision-making. In this study we quantified the relation between the distribution of neutralizing antibodies in the population to the transmission in that (vaccinated) population.

Antigenic distance refers to the measure of difference between the immune responses elicited by different viral proteins, specifically of the vaccine antigen in relation to the circulating field virus (represented in the transmission experiments by the challenge virus). This concept is crucial in understanding how well an immune response generated by a prior infection or vaccination might protect against a new strain of the virus. A greater antigenic distance indicates more significant differences in the immune response to the different viral proteins, suggesting that the immune system may not recognize or effectively respond to the new challenge virus. Conversely, a smaller antigenic distance implies that the immune response to the original virus or vaccine is likely to provide better cross-protection against the challenge virus. Basically, it is the difference in the expected titer against the field (challenge) virus compared **to** the titer against the vaccine virus, with both titers expressed as Log_2(titer), also referred to as the fold difference.

Humoral immune response: Humoral immunity is the component of the adaptive immune system responsible for generating soluble immune factors—primarily antibodies—that circulate in the bloodstream and other extracellular fluids to neutralize pathogens. When a virus enters the body, antigen-specific B lymphocytes recognize viral epitopes and, upon activation, differentiate into plasma cells that secrete antibodies with high affinity for the pathogen. These antibodies can neutralize viruses by preventing their attachment and entry into host cells, or they can opsonize viral particles, facilitating their clearance by phagocytic immune cells. Functionally, humoral immunity serves as a systemic defense mechanism capable of targeting extracellular pathogens throughout the body. It plays a critical role in long-term protection, particularly against pathogens previously encountered through natural infection or vaccination, owing to the formation of memory B cells that enable rapid and robust responses upon re-exposure.

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

Innate immunity, constitutes the body's immediate, nonspecific defense system that provides the first barrier against invading pathogens. It includes physical and chemical barriers—such as the skin, mucosal surfaces, and acidic environments—as well as cellular components like macrophages, neutrophils, dendritic cells, and natural killer cells. These innate immune elements recognize conserved molecular patterns common to broad classes of microbes through pattern-recognition receptors, enabling rapid detection and elimination of potential threats. Unlike adaptive immunity, the innate response does not rely on antigen specificity or memory but acts within minutes to hours to contain or neutralize pathogens. This early response limits pathogen replication and facilitates the activation and orchestration of subsequent adaptive immune responses.

Adaptive immunity, is the highly specialized branch of the immune system characterized by antigen specificity, immunological memory, and the capacity to mount progressively more efficient responses upon repeated exposure to the same pathogen. During a primary encounter, antigen-presenting cells process and present pathogen-derived molecules to naïve T and B lymphocytes, initiating their activation, clonal expansion, and differentiation into effector and memory subsets. Effector cells mediate pathogen clearance through mechanisms such as cytotoxic T-cell–mediated killing of infected cells and antibody production by plasma cells. Following resolution of the infection, long-lived memory lymphocytes persist and enable a rapid, robust, and antigen-specific response upon re-exposure. Vaccination leverages this principle by presenting antigens in a controlled manner to prime the adaptive immune system without causing disease, thereby establishing protective immunological memory.

Correlates of protection are measurable signs or reactions in the body that demonstrate a chicken is likely protected from a disease. For example, after a vaccine or an infection, the body may produce antibodies or activate certain immune cells. Scientists look for specific levels of these immune markers to understand whether an individual is well shielded from getting sick again. These markers help researchers to evaluate vaccines and predict immunity without having to wait for individuals to actually encounter the disease. For protection against transmission of the infection as is the main goal in this study, immune parameters with a particular distribution in the flock can be seen as a population correlate of protection. In this study a flock with a high enough fraction of the chickens with a HI titer (neutralizing antibodies) above threshold will be protected against transmission (R_e <1).

Outbreaks (in the context of this study): An outbreak happens when a disease affects a large number of chickens on a farm. Outbreaks can lead to further spread of the virus to other farms, as well as to humans, other animals, and contaminated products. The primary goal during an outbreak is to act quickly to prevent further transmission so the disease does not affect other animals and/or humans, and stop the disease from infecting other premises, which could escalate in a regional or even national epidemic.

Introduction with negligible spread (in the context of this study): An introduction with negligible spread is defined as an introduction of the virus infecting at least one chicken on a farm, but in which the total number of infected chickens remains small. In the context of this study we set a limit of 0.1% of the population of the farm. Introductions with negligible spread are considered self-limiting (the infection goes extinct by chance) without causing increased risk of further spread to other premises or other animals and/or humans. Introductions with negligible spread typically go unnoticed and do not pose any public or veterinary health risk.

Passive surveillance (in the context of this study) is a system where a potential infection in poultry farms would be noted by poultry farmers, who will alert public health agencies and samples will be collected for initial diagnosis. It's called "passive" because the public health agencies do not actively go out and search for cases; it simply receives whatever information is voluntarily submitted. This method is affordable and easy to maintain, which is why it's widely used. Often mortality is an informative parameter.

Active surveillance (in the context of this study) is a more hands-on approach used by public health agencies to find out which farms may be infected and to reduce the time between introduction and detection of the infection. Instead of waiting notification by poultry farmers, public health agencies actively collect samples at set time intervals. It involves regular sample collections and targeted testing of avian influenza viruses. In the context of HPAI and as used in this study, at moment of sample collection, all chickens that have died within the preceding two days are collected and tested. If a positive result is obtained from any dead chicken, a confirmation step is carried out the following day.

This confirmation includes:
- Testing all chickens that died within the previous 24 hours.
- Sampling 20 live chickens exhibiting clinical signs, representing symptomatic cases.

In our analysis, it is assumed that vaccinated but infected chickens also display detectable symptoms, and are positive in diagnostic tests.

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The first two transmission studies (challenge at 8 and 24 weeks of age) were previously reported [9-12] and conducted within the BO-43-111-083 project. The remaining two transmission studies (challenge at 54 and 84 weeks of age) were conducted within Work Package 2 of the PPP project (Appendix Work Package 2).

Permits
Royal GD; Work Package 1: The study was carried out according to the legal regulations as stipulated in the 'Wet op de dierproeven' (Wod) and 'Dierproevenbesluit'. The animal study was approved by the Central Committee for Animal Experiments (CCD) (permit application AVD42600202316719). The HVT AI vaccines include a genetically modified organism (GMO). Therefore, permits were obtained from the 'Bureau GGO' for conducting the animal study (IM-MV 23-005).

Wageningen Bioveterinary Research (WBVR); Work Package 2: The transmission studies were conducted in accordance with the guidelines of 2010/63/EU [34]. These animal studies were approved by the Central Committee for Animal Experiments (CCD) (permit application AVD40100202215972; experiments 2021.D-0036.004, 2021.D-0036.005, 2021.D-0036.006 and 2021.D-0036.007). 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). The chickens used in the studies at Wageningen Bioveterinary Research (WBVR), are a subset of the chickens that are housed in the field and part of the study of Royal GD "Uitvoering wetenschappelijke veldstudie vaccin HPAI". Projectnumber 5082181".

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