Effect of vaccination on transmission of highly pathogenic avian influenza in birds

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Effect of vaccination on transmission of highly pathogenic avian influenza in birds

Het effect van vaccinatie op de transmissie van
hoog pathogene aviaire influenza bij vogels

(met een samenvatting in het Nederlands)

Proefschrift

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Foar Yme en Bauke

Contents

Chapter 1

General introduction

1.1 Avian influenza

1.1.1 Historical aspects, the virus, and the disease

Highly pathogenic avian influenza (HPAI) was first described in 1878 in Italy as "fowl plague", a virulent disease affecting chickens (Perroncito, 1878). Subsequently outbreaks of HPAI in 1894 and 1901 in Italy were reported, which later spread to Austria, Germany, Belgium and France (Lupiani and Reddy, 2008). During the 1920s the disease was also reported in the USA, Africa and the Far East (Alexander in Jordan et al., 2001). HPAI disappeared in Europe in the mid 1930s, and the last outbreak in the USA was described in 1929 (Lupiani and Reddy, 2008). No outbreaks were recorded for a few decades and it was not until 1959 that another HPAI outbreak was reported. A more extensive overview about the history of avian influenza can be found in the paper of Lupiani and Reddy (Lupiani and Reddy, 2008), and in the thesis by Catherine Rülke (Rülke, 2007, in German).

Nowadays HPAI is recognized as a disease of poultry caused by H5 or H7 avian influenza virus strains. Influenza viruses belong to the family of *Orthomyxoviridae*, which are enveloped viruses with segmented, negative sense, single strand RNA genomes. The *Orthomyxoviridae* family consists of five genera: *Influenzavirus A, B, and C*, *Thogotovirus,* and *Isavirus*. Only viruses of the *Influenzavirus A* genus are known to infect birds. Type A influenza viruses are divided into subtypes based on two surface proteins, hemagglutinin (HA) and neuraminidase (NA). At present 16 HA subtypes (H1-H16) and nine NA subtypes (N1-N9) are known. The majority of possible combinations of H and N subtypes has been isolated from birds (Alexander, 2007) (Table 1).

Often the first sign of HPAI in chickens and turkeys is the sudden onset of high mortality, which can approximate 100% within a few days. Clinical signs are cessation of egg laying, respiratory signs, rales, excessive lacrimation, sinusitis, edema of the head and face, subcutaneous hemorrhage with cyanosis of the skin, diarrhea and nervous signs. The clinical signs of HPAI are influenced by the strain of virus, the host species, the age of the host, concurrent infections, deficiency conditions and environmental factors (Jordan et al., 2001).

1.1.2 Pathogenicity of avian influenza viruses

Virulence of avian influenza viruses is mainly determined by the hemagglutinin protein (Webster et al., 1991). The hemagglutinin of avian influenza viruses needs post translational cleavage by proteases of the host before it is functional and virus particles are infectious. HPAI strains have multiple basic amino acids at the cleavage site of the hemagglutinin (Senne et al., 1996; Vey et al., 1992; Wood et al., 1993), enabling cleavage by ubiquitous intracellular proteases (Stienekegrober et al., 1992), and allowing the virus to spread systemically.

Table 1. Influenza A serotypes and their host species

HA subtype	Human	Swine	Equine	Avian
H1	N1	N1, N2		N1-9
H2	N2			N1-9
H3	N2	N2	N8	N1-9
H4				N1-9
H5				N1-9
H6				N1-9
H7			N7	N1-9
H8				N1-9
H9				N1-9
H10				N1-9
H11				N1-9
H12				N1-9
H13				N1-9
H14				N1-9
H15				N1-9
H16				N1-9

In contrast, the hemagglutinin of avian influenza viruses of low pathogenicity (LPAI) has a cleavage site that can be cleaved only by trypsin like proteases that are restricted to the respiratory and intestinal tract. Up to now, cleavage sites with multiple basic amino acids are only described for H5 and H7 viruses.

Phylogenic studies show that HPAI viruses do not exist as separate genetic lineages, but arise by mutation and selection after LPAI viruses have been introduced into poultry (Banks et al., 2000; Banks et al., 2001; Rohm et al., 1995). The mutation at the cleavage site may be the result of spontaneous duplication of purine triplets caused by a transcription fault by the polymerase complex, as a result of nucleotide substitution or as a result of recombination (Bowes et al., 2004; Suarez et al., 2004). Several outbreaks have been described during which both the low and high pathogenicity avian influenza viruses have been isolated: Pennsylvania (Bean et al., 1985; Kawaoka and Webster, 1985), Mexico (Garcia et al., 1996; Villareal-Chavez and Flores, 1998), Italy (Capua et al., 2000), Chile (Suarez et al., 2004), and Canada (Bowes et al., 2004). In some cases mutation seems to have taken place rapidly (at the primary site) after introduction, in others the LPAI virus has circulated in poultry for months before mutating.

Table 2 shows all reported epidemics of HPAI since 1959, and it appears that there is a

large variety in size. Some are restricted to one farm, while others cause an epidemic or even a panzootic. Tools to control and eradicate an epidemic of HPAI are zoo sanitary measures (stand still, implementation of restriction zones), culling of infected poultry, pre-emptive culling of poultry at risk, and vaccination.

1.1.3 Vaccination as a tool for prevention and control of HPAI outbreaks

At present, two different types of vaccine are available: inactivated vaccines based on adjuvanted whole virus and live recombinant vaccines. Inactivated vaccines are based on viruses from natural outbreaks or on viruses generated by reverse genetics. Usually these vaccines are formulated as an oil emulsion, which enhances the immunogenicity of the vaccine. Live recombinant vaccines are based on a vector virus expressing a protein of avian influenza, mostly the hemagglutinin (Capua, 2007). Examples of recombinant vaccines are fowl poxvirus (Taylor et al., 1988), infectious laryngotracheitis virus (Luschow et al., 2001), and Newcastle disease virus (Nakaya et al., 2001) expressing the H5 or H7 antigen of avian influenza. The fowlpox vectored vaccine and the Newcastle based vaccine are commercially available in some regions. A limitation for the use of vector based vaccines is the possible existing immunity to the vector. Currently the inactivated vaccines are by far the most used.

Although AI vaccines reduce the excretion of virus, virus shedding after vaccination is observed (Lee, Senne, and Suarez, 2004; Swayne et al., 1999; Swayne et al., 2000a; Swayne et al., 2000b), raising questions as to the effectiveness of vaccination in preventing transmission from bird to bird, and viral spread in the population. Such silent transmission is very undesirable, because it increases the risk of new outbreaks and poses a threat to humans. Therefore it is increasingly believed that vaccination programs should always be accompanied by active surveillance. This surveillance can be based on the use of unvaccinated sentinels, on virus detection in the vaccinated birds or on a serological DIVA test (Differentiating Infected from Vaccinated Animals) (CEC, 2006; Van Oirschot, 1999).

Different serological DIVA strategies for avian influenza can be applied: a heterologous neuraminidase strategy, a NS1 DIVA strategy and subunit vaccines (CEC, 2006; Suarez, 2005). The heterologous neuraminidase strategy is based on the presence of antibodies against the neuraminidase of the field strain that differs from the neuraminidase of the vaccine strain. A heterologous neuraminidase strategy is developed and applied as an indirect immunofluorescent antibody test (iIFAT) in the field in Italy for the N1 serotype (Capua et al., 2003), and later for the N3 serotype (Cattoli et al., 2006). The neuraminidase inhibition (NI) assay, can also be used as a DIVA test in case of a heterologous neuraminidase (Van Deusen et al., 1983; World Health Organization, 2002). The principle behind the NS1 DIVA strategy is that the NS1 protein is produced during virus replication, and is not incorporated into virus particles. Consequently, after

Table 2. Reported highly pathogenic avian influenza epidemics since 1959

HPAI virus	Subtype	Numbers of poultry involved
A/Chicken/Scotland/59	H5N1	1 small farm
A/Turkey/England/63	H7N3	29,000
A/Turkey/Ontario/7732/66	H5N9	8,000
A/Chicken/Victoria/76	H7N7	58,000
A/Chicken/Germany/79	H7N7	1 chicken farm, 1 goose farm
A/Turkey/England/199/79	H7N7	9,000
A/Chicken/Pennsylvania/1370/83	H5N2	17,000,000
A/Turkey/Ireland/1378/83	H5N8	307,000, mostly ducks
A/Chicken/Victoria/85	H7N7	240,000
A/Turkey/England/50–92/91	H5N1	8,000
A/Chicken/Victoria/1/92	H7N3	18,000
A/Chicken/Queensland/667-6/94	H7N3	22,000
A/Chicken/Mexico/8623-607/94	H5N2	Unknown - millions?
A/Chicken/Pakistan/447/94	H7N3	>6,000,000
A/Chicken/NSW/97	H7N4	160,000
A/Chicken/Hong Kong/97	H5N1	3,000,000
A/Chicken/Italy/330/97	H5N2	8,000
A/Turkey/Italy/99	H7N1	14,000,000
A/Chicken/Chile/2002	H7N3	700,000
A/Chicken/Netherlands/2003	H7N7	>25,000,000
A/Chicken/Eurasia-Africa/2003–2008	H5N1	Unknown -100s of millions
A/Chicken/Texas/2004	H5N2	6,600
A/Chicken/Canada-BC/2004	H7N3	16,000,000
A/Ostrich/South Africa/2004	H5N2	30,000
A/Chicken/North Korea/05	H7N7	219,000
A/Chicken/England/08	H7N7	25,000

Adapted from (Alexander, 2007).

vaccination with inactivated vaccines no NS1 is produced, and no antibody response to the NS1 protein will be evoked. Thus NS1 antibodies in vaccinated animals indicate a preceding infection with field virus (Birch-Machin et al., 1997; Ozaki et al., 2001; Tumpey et al., 2005). The use of subunit vaccines will not be discussed because this thesis concentrates on serological DIVA tests that can be used in combination with inactivated vaccines.

1.2 Aim and outline of this thesis

For decades HPAI was considered an exotic disease of poultry with a low incidence of epidemics. However, during the last 35 years the frequency and magnitude of HPAI epidemics have seemed to increase, and several very large epidemics of HPAI have occurred. Moreover, the H5N1 HPAI virus, that had its origin in South East Asia in 1996, now has become endemic in large parts the world (Figure 1). Next to the worldwide spread in poultry the H5N1 virus appeared to be capable of infecting and killing humans. The increasing magnitude of HPAI epidemics and the endemicy of H5N1 ask for alternative strategies for prevention and control of HPAI next to measures like culling of infected poultry flocks, pre-emptive culling, and the imposement of movement restrictions. Such an alternative is offered by vaccination, which has been applied during some HPAI epidemics (Capua, 2007). Over the last years, experimental studies have yielded important information about the effect of vaccination on disease symptoms and excretion (Swayne et al., 1999; Swayne et al., 2000b; Webster et al., 2006). However, little is known about the effect of vaccination on transmission, which is crucial when an epidemic has to be controlled.

The main goal of this thesis was to gain insight in the transmission parameters of HPAI viruses in birds, and in the effect of vaccination on transmission. To this end, transmission experiments with different HPAI strains in unvaccinated and vaccinated birds of different species were performed.

To better understand the origin of an HPAI outbreak from an LPAI outbreak the transmission of both strains from a well-known virus subtype (A/Chicken/Pennsylvania/83 H5N2) were compared (Chapter 2). Vaccination is used against HPAI as an intervention tool, but little is known about the effect of vaccination on transmission of HPAI. The effect of vaccination on the transmission dynamics of a HPAI H7N7 virus in chickens was studied, using two different vaccines and two different vaccine schedules (Chapter 3). The same vaccine and HPAI H7N7 virus was used in transmission experiments with golden pheasants (*Chrysolophus pictus*, family Phasianidae) and ringed teals (*Callonetta leucophrys*, family Anatidea), to compare the effect of vaccination in different bird species (Chapter 4). Domestic ducks play an important role in the

epidemiology of H5N1 outbreaks in poultry in Southeast Asia. A clear correlation is shown between H5N1 outbreaks in poultry and the presence of grazing ducks in Thailand and Vietnam (Gilbert et al., 2006; Gilbert et al., 2008). To prevent and control outbreaks of H5N1 in ducks different vaccines are used, varying in their genetic homology with the circulating H5N1 field strains. The effect of a genetically distant vaccine on the transmission of an H5N1 virus in Pekin ducks was studied in Chapter 5. Since many circumstances may lead to suboptimal vaccination, a vaccination strategy should always include a surveillance program to detect silent spread of virus. One of the ways to perform the monitoring is by using a serological DIVA test. Three different DIVA tests were validated to gain more insight in the performance of these tests (Chapter 6). In the last chapter of this thesis the results, implications, and critical elements of the work from the previous chapters are discussed (Chapter 7).

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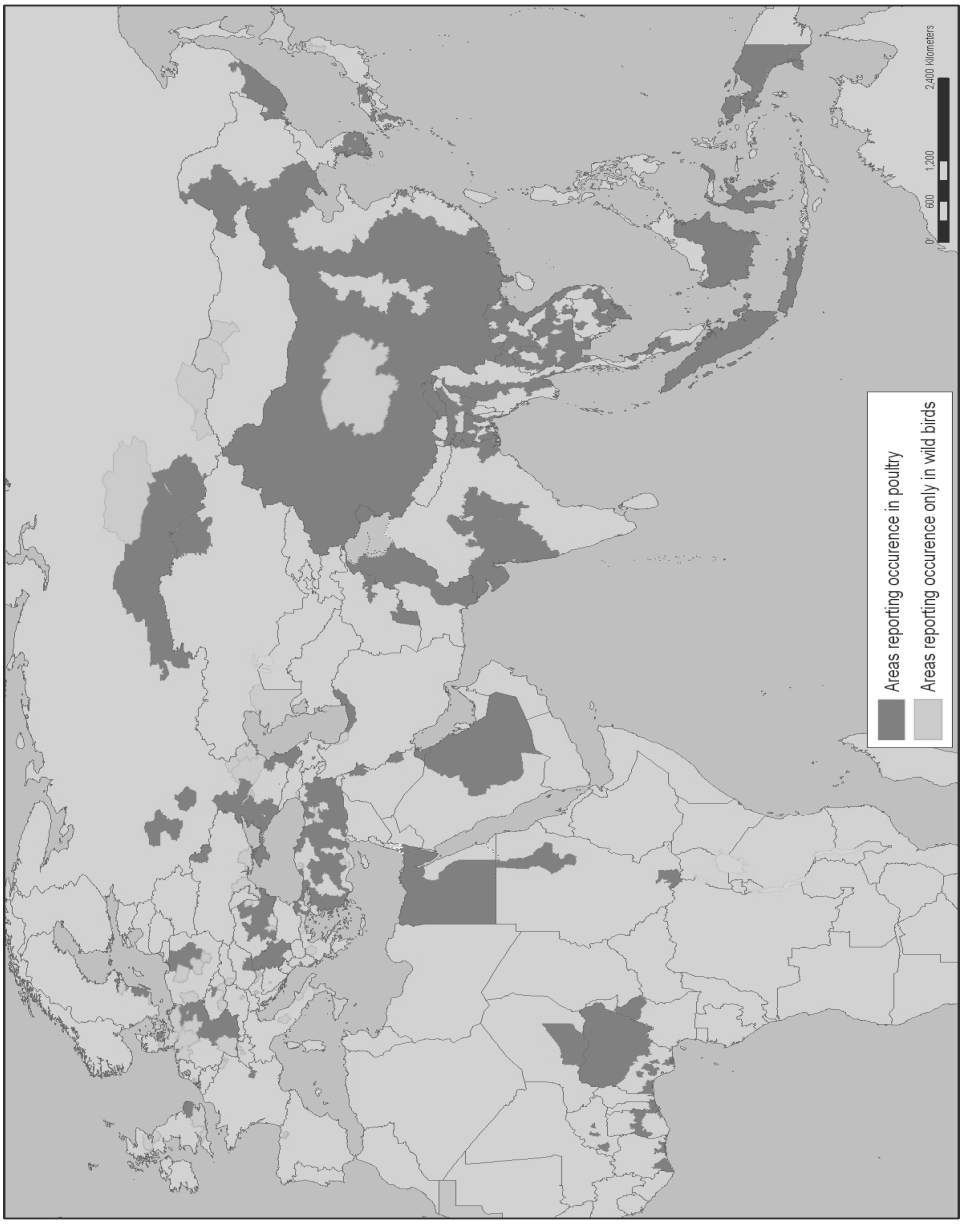

Figure 1. Areas reporting confirmed occurrence of H5N1 avian influenza in poultry and wild birds since 2003. Last updated 12 December 2008. Available at (http://gamapserver.who.int/mapLibrary/Files/Maps/Global_SubNat_H5N1inAnimalConfirmedCUMULATIVE_20081003.png) (accessed on 04 January 2009).

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Chapter 2

Comparison of the transmission characteristics of low and high pathogenicity avian influenza A virus (H5N2)

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Abstract

Low pathogenicity avian influenza A strains (LPAI) of the H5 and H7 type are noted for their ability to transform into highly pathogenic counterparts (HPAI). Here we compare the transmission characteristics in poultry of LPAI H5N2 (A/Chicken/Pennsylvania/83) and corresponding HPAI virus by means of transmission experiments. In the experiments, five inoculated animals are placed in one cage with five contact animals, and the infection chain is monitored by taking blood samples, and samples from the trachea and cloaca. The data are analyzed by final size methods and a generalized linear model. The results show that HPAI virus is more infectious and has a longer infectious period than LPAI. In fact, fully susceptible animals are invariably infected when confronted with HPAI virus and die within six days after infection. Animals previously infected with LPAI virus, on the other hand, survive an infection with HPAI virus or escape infection all together. This implies that a previous infection with LPAI virus effectively reduces susceptibility of the host to infection and decreases transmission of HPAI virus. We discuss the implications of these conclusions for the control and evolution of avian influenza viruses.

2.1 Introduction

The primary reservoir of influenza A viruses is generally considered to be in wild aquatic birds of the orders Anseriformes and Charadriiformes (Webster et al, 1992). In these species all major subtypes of the virus can be found, and the virus causes no or only limited mortality (Slemons et al., 1974; Kawaoka et al., 1988). A subset of the subtypes is found also in a variety of other avian and mammalian species. In these hosts some strains induce considerable mortality. The close relationships between strains found in different species suggest that interspecies transmission events are fairly common (e.g., Shu et al., 1994; Alexander and Brown, 2000; Lin et al., 2000; Suarez, 2000; Subbarao and Katz, 2000).

In poultry certain strains of influenza A are especially noted for their ability to cause high mortality. Indeed, the term ‘fowl plague’ is colloquially used for disease caused by these highly pathogenic avian influenza A strains (HPAI). In Hong Kong a large outbreak of a such a HPAI strain occurred in 1997. Besides leading to substantial economic losses, the virus also spread to at least 18 humans. This led to the suggestion that outbreaks of HPAI virus in poultry could constitute a ‘pandemic threat’ to the human population (De Jong et al., 1997).

Up to now, HPAI virus in poultry has been limited to strains of the H5 and H7 subtype. Eighteen outbreaks of HPAI virus have been recorded in chickens and turkeys since 1959 (Alexander, 2000; Capua et al., 2000). Virulence is mainly determined by the hemagglutinin protein (which defines the H type of the strain), although there are other determinants of virulence (Rott, 1980; Rott et al., 1976; Scholtissek et al., 1977; Perdue and Suarez, 2000). In order to attain efficient replication the hemagglutinin needs post translational cleavage by proteases of the host. HPAI strains have multiple basic amino acids at the cleavage site of the hemagglutinin (Vey et al., 1992; Wood et al., 1993; Senne et al., 1996), making cleavage by ubiquitous proteases possible and allowing the virus to spread systemically and cause high mortality.

It is known that outbreaks of HPAI virus can arise out of outbreaks of the corresponding LPAI virus (e.g., Capua et al., 2000; Bean et al., 1985). An example is the outbreak of LPAI in chickens in Pennsylvania in 1983. The outbreak started in April 1983 with a low pathogenicity virus that caused only limited mortality. In October of the same year the virus population had transformed into a state of high pathogenicity, causing over 80% mortality (Bean et al., 1985; Kawaoka and Webster, 1985). The most recent example of such a transformation event is the Italian outbreak of HPAI H7N1 in December 1999 that had started as an outbreak of LPAI H7N1 in April of the same year (Capua et al., 2000).

In this paper we are interested in a comparison of the differences between HPAI and LPAI virus in chickens. The questions that arise naturally are the following. Are animals infected with HPAI virus more infectious than animals infected with LPAI virus? How does the infectious period of HPAI virus compare to the infectious period of LPAI virus? Are animals previously infected with LPAI virus protected against infection with HPAI virus? Is HPAI virus able to spread in a population in which LPAI virus is circulating or has circulated before? The answers to these questions are of considerable interest since they form the basis for an improved understanding of the selective differences between LPAI and HPAI viruses and, in the long run, for the design of rational control strategies to minimize economic costs and the risk of interspecies transmission.

We tackle the aforementioned questions by means of transmission experiments. Transmission experiments form an invaluable tool to study the effect of a single factor (strain type, vaccination, host genotype) as they offer a controlled setting in which confounding variation due to other factors is kept to a minimum (De Jong and Kimman, 1994). In the field of avian influenza transmission experiments have been carried out before (Narayan et al., 1969; Alexander et al., 1978; Alexander et al., 1986; Westbury et al., 1979, 1981; Horimoto et al., 1995). However, these previous studies were qualitative and the conclusions were not based on a sound statistical analysis. Here we base the analysis on a well-founded epidemic model (the

susceptible-infected-removed or SIR model). As a consequence, all parameters have a clear-cut biological interpretation (susceptibility, infectiousness, infectious period), making it possible to ascribe differences between strains or treatments to specific, biologically interpretable parameters.

In the experiments, five intranasally and intratracheally inoculated chickens were put into one cage with five susceptible contact chickens, and the infection chain was monitored by regularly taking swabs from the trachea and the cloaca. The swabs were subjected to virus isolation procedures and PCR. In addition, blood samples were taken weekly to determine blood antibody levels.

The present study consists of three sets of experiments. First, we carried out four replicate experiments with a LPAI H5N2 isolated from an outbreak in Pennsylvania in 1983. These experiments yield information on the transmission characteristics of the low pathogenicity strain (i.e. infectiousness, infectious period, virulence). Second, two experiments with the corresponding HPAI virus were performed to determine the strain characteristics of HPAI H5N2 and, more importantly, to compare the LPAI and HPAI strains. Finally, we performed two experiments with HPAI H5N2, taking animals that previously had been infected with LPAI virus as contact animals to obtain insight in the impact of previous infection on the transmission of HPAI virus.

2.2 Materials and methods

2.2.1 Materials and experimental setup

All animal experiments were done in a high containment unit under BSL3+ conditions at the Institute for Animal Science and Health. The experiments comply with the Dutch law on animal experiments and were reviewed by an ethical committee.

Viruses

Two avian H5N2 strains were used, a low pathogenicity strain isolated from the index case in April 1983 (A/Chicken/Pennsylvania/21525/83), and a high pathogenicity strain isolated in October 1983 (A/Chicken/Pennsylvania/1370/83). Both strains were sent from the USDA (Ames, Iowa) to our laboratory in 1984. The strains were grown in allantoic fluid and stored at -70°C. We performed a standard intravenous pathogenicity test with both strains. The intravenous pathogenicity index (IVPI) of the LPAI strain was 0.0 implying low pathogenicity, while IVPI of the HPAI strain was 2.0 implying high pathogenicity. We checked for mutations in the hemagglutinin gene by sequencing the cleavage and glycosylation sites. No differences were found with published sequences (Kawaoka et al., 1984; Desphande et al., 1987).

Animals

Six week old specified pathogen free White Leghorn chickens were used. The animals were housed in cages. In each cage five chickens were inoculated at day 0. After 24 h, five susceptible contact animals were added. During the first seven days post infection tracheal and cloacal swabs were taken every day. From day 7 onwards swabs were taken twice a week. Blood samples were taken once a week.

2.2.2 Experimental procedures

Inoculation route and dose

Animals were inoculated both intranasaly and intratracheally with 0.1 ml diluted allantoic fluid containing 10^6 EID50 per ml.

Virus isolation and serology

Swabs were put in 2 ml 2.95% tryptose phosphate buffer with 5 x 10^3 IU of penicillin-sodium and 5 mg streptomycin per ml. The swabs were stored at –70°C until analyzed. Three embryonated chicken eggs incubated for nine days were injected with 0.2 ml of the solution per egg. The eggs were then incubated for another 72 h. A standard hemagglutination assay was carried out on the allantoic fluid of the eggs. The hemagglutinin inhibition (HI) test on the sera was done by standard methods.

RNA isolation and PCR

RNA isolation and reverse transcriptase PCR were done as described in Fouchier et al., 2001.

2.2.3 Statistical analyses

Final size analysis

In first instance, the analysis of the experiments is based on the final size of the experiments, i.e. the number of contact animals that have been infected when the infection chain has ended. The final sizes are used to obtain estimates of the (basic) reproduction ratio, i.e. the number of infections that would be caused by a single infected individual in a large population of susceptibles. A forte of final size methods is that they are robust (e.g., inclusion of a latent period does not alter the results) and that different assumptions on the distribution of the infectious period are easily incorporated. The methods are based on maximum likelihood estimation (MLE). That it is possible to use MLE hinges on the fact that final size distributions can be determined under a wide range of assumptions (Ball, 1986). We focus on two extreme scenarios, one in which the infectious period is exponentially distributed, and one in which the infectious period is of fixed duration. The estimator of the reproduction ratio is labeled by R_{MLE1} in case of the first scenario, and by R_{MLE2} in case of the second scenario. For the first two sets of experiments the analyses are based on virus isolation as well as on serology. In case of virus isolation an animal is

marked as infected if it is positive in either the tracheal or cloacal swab on at least one day. In case of serology, an animal is marked as positive if it has a HI titer or if it has died from the infection. For the third set of experiments no analysis is carried out on the serological data because all contact animals already had a high titer from a previous infection.

Generalized linear modeling

Although final size methods are flexible, they do not make use of all the available information. To take the time course of the experimental epidemics into account, we estimate the transmission parameter β of the stochastic SIR model by means of a Generalized Linear Model (McCullagh and Nelder, 1989; Becker, 1989). To this end the data in Figures 1-3 are first rendered into the format (S,i,C). Here S is the number of susceptible animals in a certain time period, i is the prevalence of infection (i.e. the average number of infectious animals divided by the total number of animals), and C represents the number of new infections that have appeared at the end of the time period. By standard reasoning we assume that the number of cases C arising in a day is binomially distributed with parameter $p_{\text{inf}} = 1 - e^{-\beta i}$ (the probability of infection) and binomial totals S:

$$C \sim Bin(S, 1 - e^{-\beta i}) . \qquad (1)$$

Notice that the above model entails the following assumptions: (*i*) all susceptible birds are equally susceptible; (*ii*) all infected birds are equally infectious; and (*iii*) each infected bird poses an independent risk of infection to each susceptible bird. These assumptions can be relaxed, but this seems wise only if the fit of the model is unsatisfactory or if a large amount of data is available. In the model (1), $\log(\beta)$ is estimated using a complementary log-log link function while taking $\log(i)$ as offset variable. The fit of the model is checked by inspection of the (residual) deviance that, under standard assumptions (McCullagh and Nelder, 1989, p. 118), is approximately χ^2-distributed. The analyses are carried out in GenStat 5.4. The infectious periods are directly observed. Hence, estimation of the infectious periods and corresponding confidence intervals is straightforward. The estimate of the reproduction ratio is given by the product of the estimates of the transmission parameter and infectious period: $\hat{R} = \hat{\beta}\hat{\gamma}$. Construction of the corresponding confidence intervals is based on the identity $Var(\beta\gamma) = (E\gamma)^2 Var\beta + E\beta^2 Var\gamma$ (Rao, 1973). Insertion of the estimated means and variances of β and γ into this formula yields an estimate for the variance of R, thereby allowing the construction of confidence intervals. Notice that as the model yields estimates of $\log(\beta)$ we need to take into account that (asymptotically) the estimator of β is lognormally distributed.

2.3 Results

2.3.1 The transmission characteristics of LPAI virus

Figure 1 summarizes the four replicate transmission experiments with LPAI virus (experiments 1-4). The estimates of the reproduction ratio R, based on the final size analyses, are given in Table 1. If we take virus isolation as indicative of infection R is estimated at 1.1. If, on the other hand, we take serology as an indicator of infection (so that we regard the positives in the virus isolation that are negative in the serological analysis as superficial infections that cannot spread further) R is estimated at 0.6. The limits of the confidence intervals of the reproduction ratio range from 0.2 to 2.4, and we cannot make strong statements as to whether $R>1$ or $R<1$.

Table 2 shows the analyses based on the time course of experiments 1-4. It appears that our inoculation route and dose resulted in a reliable infection (see Figures 1-3). In fact, in experiments 1-4 most of the inoculated animals were marked as positive (and hence as infectious) for 5 days, while the remainder of the inoculated animals were positive for 4 days. The mean of the infectious period of the inoculated animals is 4.8 days with a coefficient of variation (CV) of just 9%.

The mean of the infectious period of the contact infected animals is comparable to the infectious period of the inoculated animals (4.3 days versus 4.8 days). However, variation in the infectious period is considerably higher for the contact infected animals than for the inoculated animals (CV = 55% for contact infections versus CV = 9% for inoculated animals; $F_{(9,19)} = 27.9$, $p < 0.001$). This indicates contact infections are more variable than artificially induced infections. The infectiousness of infected animals is determined by the transmission parameter β. Loosely speaking, β represents the number of susceptible animals that would be infected in one day by a single infected animal in a large population of susceptible animals. If the cases arising at the end of a certain time period are assumed to have been infected in the time period under consideration (i.e. the latent period is at most one day), β is estimated at 0.22 (day^{-1}). The deviance is 32.5 (df = 25), so that there is no reason to suspect the fit of the model. We also considered a model with a latent period of one to two days (i.e. the new cases arising at the end of a time period had actually been infected at the beginning of the time period). For this model β is estimated at 0.24 (day^{-1}) with deviance of 24.9 (df = 22), also suggesting a satisfactory fit of the model.

The estimate of the reproduction ratio is given by the products of the estimates of β and γ. For the LPAI experiments the reproduction ratio is estimated at 0.95 or at 1.00. The confidence intervals as determined with the methods described in the Materials and Methods range from 0.0 to 2.4.

Experiment 1

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 10	Serology
I	+/+/-	+/+/-	+/+/-[1]	+/+/-[1]	-/+/-[1]	-/-/-[1]	-/-/-	-/-/-	+(5)
I	+/+/-	+/+/-	+/+/-[1]	+/+/-[1]	+/+/-[1]	-/-/-[1]	-/-/-	-/-/-	+(6)
I	+/+/-	+/+/-	+/+/-[1]	+/+/-[1]	+/+/-[1]	-/-/-[1]	-/-/-	-/-/-	+(6)
I	+/+/-	+/+/-	+/+/-[1]	+/+/-[1]	-/-/-[1]	-/-/-[1]	-/-/-	-/-/-	+(5)
I	+/+/-	+/+/-	+/+/-[1]	+/+/-[1]	-/-/-[1]	-/-/-[1]	-/-/-	-/-/-	+(3)
S	nd	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-
S	nd	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-
S	nd	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-
S	nd	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-
S	nd	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-

Experiment 2

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 10	Serology
I	+/+/-	+/+/-	+/+/-[1]	+/+/-[1]	+/+/-[1]	-/-/-[1]	-/-/-	-/-/-	+(4)
I	+/+/-	+/+/-	+/+/-[1]	+/+/-[1]	+/+/-[1]	-/-/-[1]	-/-/-	-/-/-	+(6)
I	+/+/-	+/+/-	+/+/-[1]	+/+/-[1]	+/-/-[1]	-/-/-[1]	-/-/-	-/-/-	+(5)
I	+/+/-	+/+/-	+/+/-[1]	+/+/-[1]	+/+/-[1]	-/-/-[1]	-/-/-	-/-/-	+(6)
I	+/+/-	+/+/-	+/+/-[1]	+/+/-[1]	+/+/-[1]	-/-/-[1]	-/-/-	-/-/-	+(6)
S	nd	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-
S	nd	-/-/-	-/-/-	-/-/-	+/-/-	+/-/-	+/-/-	+/-/-[2]	+(4)
S	nd	-/-/-	+/-/-	+/+/-	+/+/-	+/+/-	+/-/-	-/-/-	+(4)
S	nd	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-
S	nd	-/-/-	-/-/-	-/-/-	+/-/-	+/+/-	+/-/-	-/-/-	+(3)

Experiment 3

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 10	Serology
I	+/x/x	+/x/x	+/x/-	+/x/x	+/x/x	-/x/x	-/x/-	-/x/x	+(6)
I	+/x/x	+/x/x	+/x/-	+/x/x	+/x/x	-/x/x	-/x/-	-/x/x	+(6)
I	+/x/x	+/x/-	+/x/+	+/x/-	-/x/-	-/x/x	-/x/-	-/x/x	+(5)
I	+/x/x	+/x/x	+/x/-	+/x/x	-/x/x	-/x/x	-/x/-	-/x/x	+(6)
I	+/x/x	+/x/x	+/x/-	+/x/x	+/x/x	-/x/x	-/x/-	-/x/x	+(4)
S	nd	-/x/x	-/x/-	-/x/x	-/x/x	-/x/x	+/x/-	-/x/x	+(2)
S	nd	-/x/x	+/x/-	+/x/x	-/x/x	-/x/x	-/x/-	-/x/x	-
S	nd	-/x/x	+/x/-	+/x/x	+/x/x	+/x/x	+/x/-	-/x/x	+(6)
S	nd	-/x/x	-/x/-	-/x/x	+/x/x	+/x/x[3]	+/x/-	-/x/x	-
S	nd	-/x/x	+/x/-	+/x/x	+/x/x	+/x/x	+/x/-	-/x/x	+(4)

Experiment 4

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 10	Serology
I	+/x/x	+/x/x	+/x/-	+/x/x	+/x/x	-/x/x	-/x/-	-/x/x	+(6)
I	+/x/x	+/x/x	+/x/-	+/x/x	+/x/x	-/x/x	-/x/-	-/x/x	+(5)
I	+/x/x	+/x/x	+/x/-	+/x/x	+/x/x	-/x/x	-/x/-	-/x/x	+(5)
I	+/x/x	+/x/x	+/x/-	+/x/x	+/x/x	-/x/x	-/x/-	-/x/x	+(6)
I	+/x/x	+/x/x	+/x/-	+/x/x	+/x/x	-/x/x	-/x/-	-/x/x	+(6)
S	nd	-/x/x	-/x/-	-/x/x	-/x/x	-/x/x	-/x/-	-/x/x	-
S	nd	-/x/x	-/x/-	-/x/x	+/x/x	-/x/x	-/x/-	-/x/x	-
S	nd	-/x/x	-/x/-	-/x/x	-/x/x	+/x/x	+/x/-	-/x/x	-
S	nd	-/x/x	-/x/-	-/x/x	-/x/x	-/x/x	-/x/-	-/x/x	-
S	nd	-/x/x	-/x/-	-/x/x	-/x/x	-/x/x	-/x/-	-/x/x	-

Figure 1. Summary of the LPAI transmission experiments (A/Chicken/Pennsylvania/83 H5N2). Four replicate experiments are carried out. In each experiment five chickens are inoculated at day 0 (I). From day 1 onwards five contact animals (S) are placed in the cage with the inoculated chickens, and the infection chain is monitored. The data X/Y/Z represent the following: X/Y/Z=egg culture trachea/PCR trachea/egg culture cloaca. An animal is marked positive in the serology if it is positive in one of the samples (taken at day 7, 14, 21, and also at day 28 in experiments 1 and 2). Maximum 2log HI titers are given between brackets. x: no analysis was carried out on the sample. [1]cloacal swabs were also negative after second passage in embryonated chicken eggs. [2]no virus was isolated when tested at day 14. [3]the animal was marked as positive only after retesting.

2.3.2 The transmission characteristics of HPAI virus

Figure 2 shows the results of the two replicate experiments with HPAI virus using susceptible contact animals (experiments 5-6). Tables 1 and 2 present the outcome of the analyses. The mean infectious period of the 10 infected contact animals is 6.8 days (95% CI = 4.91; 8.69).

Estimation of the transmission parameter is based on the GLM described in the Materials and Methods section. If the latent period is at most one day, $\hat{\beta}$ is 0.8 (day^{-1}) (*95%* CI = 0.4; 1.5). However, the deviance of the model is high (23.6 with $df = 4$), implying that the model does not fit the data well. If, on the other hand, the latent period is assumed to be between one and two days, $\hat{\beta}$ is increased more than fivefold ($\hat{\beta}$ = 4.7 (day^{-1})), while the deviance is considerably lower (1.5 with $df = 2$). This implies the model with a latent period of one to two days fits the data better than the model with a latent period of at most one day.

If we take the lower bounds of both the confidence intervals of β and γ as indicative of the lower bound of R, we arrive at a value of 0.42 * 4.91 = 2.1. Since this is considerably higher than 1 we conclude that the reproduction ratio of HPAI virus is higher than 1.

Table 1. Estimation of the reproduction ratio, based on the final size of the experimental epidemics

Experiments	Virus isolation			Serology		
	Number infected	R_{MLE1}	R_{MLE2}	Number infected	R_{MLE1}	R_{MLE2}
1-4 (LPAI)	0,2,3,5	1.17 (0.47-2.39)	1.06 (0.47-1.89)	0,0,3,3	0.62 (0.20-1.52)	0.59 (0.21-1.27)
5-6 (HPAI)	5,5	∞ (1.30-∞)	∞ (1.33-∞)	5,5	∞ (1.30-∞)	∞ (1.33-∞)
7-8 (HPAI; contact animals with titer)	1,3	0.79 (0.20-2.68)	0.78 (0.21-2.02)	NA	NA	NA

The three sets of experiments are described in the main text. The final size analyses are based on virus isolation for all three sets of experiments, and on serology for the first two sets of experiments (see Figures 1-3). R_{MLE1} is the maximum likelihood estimator of R if the infectious period is exponentially distributed, while R_{MLE2} is the maximum likelihood estimator of R if the infectious period is of fixed duration. *95%* confidence intervals are given between brackets.

2.3.3 Comparison of the transmission characteristics of LPAI and HPAI virus

Let us first compare the excretion patterns of LPAI and HPAI virus in the trachea and cloaca. As Figure 1 shows (experiments 1 and 2), LPAI virus is only found once in the cloaca of the 60 positives in the trachea. Hence, we may conclude that an infection with LPAI virus does only sporadically lead to appreciable virus shedding in the gastro-intestinal tract. The picture is quite different for infections with HPAI virus, which does occur in high frequency in the cloaca (Figure 2). In the majority of cases, the cloacal swabs are positive if the tracheal swabs are positive. There are some exceptions to this rule, most notably on the first day of infection.

Let us now compare the LPAI and HPAI experiments on a more quantitative basis. A comparison of the infectious period of the LPAI contact infected animals with the HPAI contact infected animals shows that there is evidence that the infectious period in HPAI contact infected animals is significantly longer than the infectious period of LPAI contact infect animals (t = 2.28, df = 18, $p < 0.05$). Hence, the fact that HPAI efficiently exploits the host and that nine out

Experiment 5

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 10	Day 14	Serology
I	+/-	+/+	+/+	+/+	+/-	†				na
I	+/+	+/+	+/+	+/+	†					na
I	+/+	+/+	+/+	+/+	†					na
I	+/+	+/+	+/+	+/+	†					na
I	+/-	+/+	+/+	+/+	+/+	+/-	+/-	†		+(9)
S	nd	-/-	+/-	+/-	+/+	+/+	+/-	+/+	†	+(5)
S	nd	-/-	+/-	+/+	+/+	+/+	+/+	†		+(5)
S	nd	-/-	+/-	+/+	+/-	+/+	+/+	†		-
S	nd	-/-	+/-	+/+	-/+	+/+	+/+	-/+	†	+(4)
S	nd	-/-	-/-	+/-	+/-	-/-	-/-	-/-	-/-	+(2)

Experiment 6

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 10	Day 14	Serology
I	+/+	+/+	+/+	+/+	+/+	+/-	-/+	†		+(7)
I	+/+	+/+	+/+	†						na
I	+/-	+/+	+/+	+/+	+/-	+/-	+/-	†		+(3)
I	+/+	+/+	†							na
I	+/-	+/+	+/+	+/+	+/+	+/-	+/-	†		+(4)
S	nd	-/-	-/+	+/+	+/+	+/+	+/+	+/-	†	-
S	nd	-/-	+/-	+/+	+/-	+/+	+/+	†		-
S	nd	-/-	+/+	+/+	+/-	+/+	†			na
S	nd	-/-	+/-	+/+	+/+	+/+	+/+	-/+	†	-
S	nd	-/-	+/-	+/+	+/+	+/+	+/+	†		+(6)

Figure 2. Summary of the HPAI experiments (A/Chicken/Pennsylvania/83 H5N2). Two replicate experiments using fully susceptible contact animals (S) are carried out. The data X/Y represent the following: X/Y=egg culture trachea/egg culture cloaca. † indicates that the animal died. Notice that all susceptible contact animals were infected, and that only one of the infected animals survived the infection.

of ten HPAI contact infected animals die within two weeks after infection in effect does not reduce the infectious period.

In addition, there is evidence that the transmission parameter of HPAI virus is higher than that of LPAI virus. This is true if we assume that the period of latency is at most one day and compare the transmission parameters of LPAI virus and HPAI virus ($t' = 3.48$, $df \approx 6$, $p < 0.05$; (Welch, 1938)), or if we assume that the latent period is between one and two days ($t' = 3.07$, $df \approx 4$, $p < 0.05$).

Table 2. Estimation of the reproduction ratio, based on the time course of the experimental epidemics (see Figures 1-3)

Experiments	Number of contact infections	Infectious period $\hat{\gamma}$ (*day*)	Number of records (S,i,C)	Transmission parameter $\hat{\beta}$ (day^{-1})	Reproduction ratio $\hat{R} = \hat{\beta}\hat{\gamma}$
1-4 (LPAI)	10	4.25 (2.57-5.93)	26[1] 23[2]	0.22[1] (0.12-0.42) 0.24[2] (0.12-0.45)	0.95[1] (0.00-2.29) 1.00[2] (0.00-2.42)
5-6 (HPAI)	10	6.80 (4.91-8.69)	5[1] 3[2]	0.78[1] (0.42-1.47) 4.66[2] (2.09-10.36)	5.30[1] 31.7[2]
7-8 (HPAI; contact animals with titer)	4	1.00	13[1] 11[2]	0.17[1] (0.07-0.47) 0.24[2] (0.08-0.69)	0.17[1] 0.24[2]

The records (*S, i, C*) represent the number of susceptibles, the prevalence of infection, and the number of new cases. Estimates of the infectious period γ are based on the observed contact infections. The transmission parameter β of the stochastic SIR epidemic is estimated with a Generalized Linear Model. *95 %* confidence intervals are given between brackets. Not all confidence intervals are calculated because of the limited number of data. [1] the latent period is at most one day. [2]the latent period is between one and two days.

Summarizing, both the infectious period and transmission parameter of HPAI virus exceed the infectious period and transmission parameter of LPAI virus of LPAI virus. Hence, we conclude that the reproduction ratio of HPAI virus is higher than the reproduction ratio of LPAI virus.

2.3.4 Prior infection with LPAI virus provides effective protection against HPAI virus

Inspection of Figures 2 and 3 indicates that there are considerable differences between experiments 5 and 6 where fully susceptible contact animals were used, and experiments 7 and 8 where the contact animals had been infected before. Two features are noteworthy. First, only four of the contact animals are infected in experiments 7 and 8, while all ten contact animals are infected in experiments 5 and 6. Second, none of the four infected contact animals die in experiments 7 and 8, while nine out of ten infected contact animals die in experiments 5 and 6. Formal tests corroborate these findings: the infectious period, transmission rate, and pathogen

Experiment 7

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 10	Day 14	Serology
I	+/-	+/+	+/+	+/-	+/+	†				na
I	+/-	+/+	+/+	+/+	†					na
I	+/+	+/+	+/+	+/+	†					na
I	+/+	+/+	+/+	+/+	+/+	†				na
I	+/-	+/-	+/+	+/+	+/+	+/+	-/-	-/-	-/-	+(3)
S*	nd	-/-	-/-	-/-	-/-	-/-	-/-	x/x	x/x	6[1]
S*	nd	-/-	-/-	-/-	-/-	-/-	-/-	x/x	x/x	6[1]
S*	nd	-/-	-/-	-/-	-/-	-/-	-/-	x/x	x/x	5[1]
S*	nd	-/-	-/-	-/-	-/-	-/-	-/-	x/x	x/x	6[1]
S*	nd	-/-	-/-	+/-	-/-	-/-	-/-	x/x	x/x	4[1]

Experiment 8

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 10	Day 14	Serology
I	+/-	+/+	+/+	+/+	†					na
I	+/-	+/+	+/+	+/+	+/x	†				na
I	+/+	+/+	+/+	+/-	+/x	†				na
I	+/+	+/+	+/+	+/+	+/x	†				na
I	+/-	+/+	-/+	+/-	+/+	+/+	†			na
S*	nd	-/-	-/-	-/-	-/-	-/-	-/-	x/x	x/x	6[1]
S*	nd	-/-	-/-	-/-	-/-	-/-	-/-	x/x	x/x	5[1]
S*	nd	-/-	-/-	-/+	-/-	-/-	-/-	x/x	x/x	5[1]
S*	nd	-/-	-/-	-/+	-/-	-/-	-/-	x/x	x/x	6[1]
S*	nd	-/-	+/-	-/-	-/-	-/-	-/-	x/x	x/x	6[1]

Figure 3. Summary of the HPAI H5N2 experiments with contact animals that have a titer against LPAI H5N2 (denoted by S*). Two replicate experiments are carried out. The data X/Y represent the following: X/Y=egg culture trachea/egg culture cloaca. † indicates that the animal died. Notice that only four contact animals are infected. All four infected contact animals survived the infection. [1]2log HI titer at day 0.

induced host mortality are higher in experiments 7 and 8 than in experiments 5 and 6. We conclude that an infection with LPAI provides effective protection against infection and mortality caused by HPAI virus.

2.4 Discussion

2.4.1 The transmission characteristics of LPAI and HPAI virus

We have shown, on the basis of an explicit epidemic model, that fully susceptible birds infected with HPAI virus are more infectious than birds infected with LPAI virus, and that the infectious period induced by the HPAI virus is longer than the infectious period induced by LPAI virus. The latter finding is somewhat surprising as one could have argued that the fact that HPAI efficiently exploits the host and causes rapid death (within 5-10 days) would in effect result in a reduction of the infectious period.

Second, the transmission of HPAI virus is strongly reduced in a population where all animals previously went through an infection with LPAI virus. This can be taken as a *prima facie* evidence that in the field a primary infection with LPAI virus will effectively protect against a secondary infection with HPAI virus.

Third, birds infected by contact with HPAI virus shed virus in the trachea as well as in the cloaca, while birds infected by contact with LPAI virus shed virus in the trachea only (see also Bean et al., 1985). It is tempting to speculate that the observed differences in the transmission characteristics of LPAI virus and HPAI virus are a consequence of differences in excretion patterns of these particular LPAI and HPAI strains. To what extent these results hold for other combinations of low and high pathogenicity viruses (e.g., Mexico 1995 (H5N2) or Italy 1999 (H7N1)) is still an open question.

2.4.2 The value and limitations of experimental transmission studies

A forte of experimental transmission studies is that it is relatively easy to control for unwanted confounders, so that differences between treatment groups can directly be ascribed to differences in treatments. On the downside, results of transmission experiments are in principle not easily extrapolated to the situation in the field. In fact, in the field there are numerous confounding variables that may affect the transmission chain (breed used, age of the animals, concurrent infections, stocking density, immunological status, ambient temperature, feed status, etcetera). Hence, transmission studies are well suited to make comparisons between treatments, but care should be taken in extrapolation of the parameter estimates unless the experimental conditions closely match the real-life situation.

Nevertheless, in our opinion experimental transmission form an indispensable tool not only to test for differences between treatment groups, but also to obtain baseline estimates of epidemiologically relevant parameters. Ideally, transmission experiments such as described here are complemented by an assessment of the situation in the field (e.g., by carrying out a population study) thereby making it possible to extrapolate the results from the transmission

experiments to the field situation, and to interpret the results from population studies in terms of relevant mechanisms and covariates (strain type, breed, stocking density, etcetera).

2.4.3 Implications for the transmission dynamics of influenza A

The main value of the reproduction ratio is that it combines different aspects of the host-pathogen interaction into a single summary parameter that gives insight in the transmission dynamics of the pathogen. Consider, for instance, the following question. How many animals should have had an infection with LPAI virus to prevent an outbreak of HPAI virus? In the SIR model the critical fraction of the population that needs to be immune to obtain herd immunity, p_c, is related to the reproduction ratio R_{HPAI} through $p_c = 1 - 1/R_{HPAI}$ (Anderson, 1991). Assuming that our estimates of the reproduction ratio of HPAI virus (e.g., $\hat{R}_{HPAI} = 31.7$; Table 2) are indicative of the reproduction ratio in the field, this implies that the critical fraction of animals that need to be infected previously with LPAI virus to prevent an outbreak of HPAI virus is $1 - 1/31.7 = 0.97$.

In view of the large differences in the reproduction ratios of LPAI and HPAI virus it is unlikely that the critical infection fraction will be reached in the field. In fact, the fraction of susceptibles S^* that is left at the end of a major outbreak of LPAI virus is related to the reproduction ratio R_{LPAI} through a 'final size equation' (e.g., $\ln S^* = -R_{LPAI}(1-S^*)$ in case of an exponentially distributed infectious period (Ball, 1986)). Our estimates of the reproduction ratio of LPAI do not exceed 2.39 (Table 1), implying that at most 88% of the individuals will have protective immunity after an outbreak. This is considerably lower than the required 97% to prevent an outbreak of HPAI. The practical implication is that a major outbreak of LPAI virus is unlikely to provide herd immunity against a major outbreak of HPAI virus.

2.4.4 Evolution of avian influenza A viruses

Why do low pathogenicity influenza A strains in poultry that are quite capable to transform into highly pathogenic counterparts prevail in the field, and how can this fact be reconciled with the apparent selective advantage of highly pathogenic strains over strains of low pathogenicity? An intuitive explanation states that 'the main goal of parasitic microbes is not to multiply in their host as much as possible, but to ensure that they can still do so after years, decades, or even centuries' (De Jong et al., 2000, p. 218). Other authors have expressed similar ideas (e.g., Webster et al., 1992; Suarez, 2000). The argument, however, does not stand scrutiny as it invokes a group selectionist argument which is known to be flawed (Williams, 1966). In fact, the argument suggests that outbreaks of HPAI are artifacts that are a consequence of the virus not yet being well adapted to its host. It may even give a false sense of security as it suggests that -given

enough time- the pathogen population will eventually evolve so as to minimize the damaged inflicted upon the host.

In theory, one may still rescue the argument that the pathogen population will evolve to be able to persist on the longer time scale (Nunney, 1999). The conditions under which this may happen are, however, restrictive. First, it requires that the number of lineages that have become extinct because of overexploitation of the host is large, and second, the time scale on which the phenomenon could be effective is very long. It is therefore unlikely that selection has moulded influenza strains of poultry to a state where they exploit the host prudently. In fact, it is questionable whether such a state will ever be reached as highly pathogenic H5 and H7 viruses do not form unique lineages but share a recent common ancestor with nonpathogenic H5 and H7 viruses (Kawaoka et al., 1988; Rohm et al., 1995), and as interspecies transmission events are common (Suarez, 2000; Ito and Kawaoka, 2000). In view of the above reasoning it remains enigmatic why we do not observe outbreaks of highly pathogenic avian influenza A more often.

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Chapter 3

Quantification of the effect of vaccination on transmission of avian influenza (H7N7) in chickens

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Abstract

Recent outbreaks of highly pathogenic avian influenza (HPAI) viruses in poultry and their threatening zoonotic consequences emphasize the need for effective control measures. Although vaccination of poultry against avian influenza provides a potentially attractive control measure, little is known about the effect of vaccination on epidemiologically relevant parameters such as transmissibility and the infectious period. We used transmission experiments to study the effect of vaccination on the transmission characteristics of HPAI A/Chicken/Netherlands/03 H7N7 in chickens. In the experiments a number of infected and uninfected chickens is housed together and the infection chain is monitored by virus isolation and serology. Analysis is based on a stochastic SEIR (susceptible, latently infected, infectious, recovered) epidemic model. We found that vaccination is able to reduce the transmission level to such an extent that a major outbreak is prevented, important variables being the type of vaccine (H7N1 or H7N3) and the moment of challenge after vaccination. Two weeks after vaccination both vaccines completely block transmission. One week after vaccination, the H7N1 vaccine is better than the H7N3 vaccine at reducing spread of the H7N7 virus. We discuss the implications of these findings for the use of vaccination programmes in poultry and the value of transmission experiments in the process of choosing vaccines.

3.1 Introduction

Highly pathogenic avian influenza (HPAI) is a disease of poultry caused by H5 or H7 avian influenza A strains with mortality that ranges up to 100%. The number of outbreaks in the last few years has been unprecedented: Hong Kong (1997) (Claas et al., 1998), Italy (1999) (Capua and Marangon, 2000), Chile (2002) (Suarez et al., 2004), The Netherlands (2003) (Stegeman et al., 2004), Canada (2004) (Bowes et al., 2004) and the continuing outbreaks in South-East Asia (2003-2005) (Li et al., 2004). Aside from causing havoc in poultry it is becoming increasingly clear that certain HPAI viruses have the potential to directly cross the human-bird species barrier and may become a pandemic treat (De Jong et al., 1997; Fouchier et al., 2004; Li et al., 2004). To reduce the primary risk of human HPAI infection, it is crucial to prevent infection of poultry. Common methods to control outbreaks of HPAI are killing and destruction of infected poultry, pre-emptive culling, bio-security measures like stand still or movement control of transport of live poultry and poultry products, and vaccination.

By now, several vaccines have been developed against H5 and H7 influenza viruses in poultry. Vaccination can protect chickens from overt disease and mortality (Swayne et al., 2000b). Although AI vaccines reduce the replication of HPAI viruses in the respiratory and gastrointestinal tracts, virus shedding after vaccination is observed (Sawyne et al., 1999; Capua et al., 2002). This raises questions as to the effectiveness of vaccination in preventing transmission from animal to animal, and viral spread in the population is conceivable. Such silent transmission is very undesirable because it increases the risk of new outbreaks and poses a threat to humans. To overcome this problem sentinel chickens and DIVA (differentiating infected from vaccinated animals) vaccines have been used (Capua et al., 2002), but the best solution would be a vaccine that prevents transmission. An ideal vaccine against HPAI should reduce the spread of virus between animals in a flock and subsequently the spread of virus between flocks to such an extent that a major outbreak will not occur. Unfortunately, not much is known about the ability of AI vaccines to reduce transmission of HPAI viruses in chickens and the quantification of this reduction.

We studied the effect of vaccination on the spread of virus in a population of chickens using so-called transmission experiments. In a transmission experiment a number of infected chickens is housed together with a number of uninfected chickens, and the infection chain is monitored. Transmission experiments offer a way to look at the spread of virus under experimental conditions in a population of known composition. This makes it possible to quantify the effect of vaccination, on the transmission dynamics (De Jong and Kimman, 1994; Van der Goot et al., 2003). We focused on the transmission characteristics of A/Chicken/Netherlands/621557/03 H7N7 in chickens, using two different commercial inactivated oil-emulsion vaccines (H7N1 and H7N3) in different vaccination schemes. Our results show that vaccination does not only protect chickens against mortality and morbidity, but also reduces the spread of virus within a flock to such an extent that a major outbreak can be prevented.

3.2 Materials and Methods

3.2.1 Viruses

The virus used in this study was A/Chicken/Netherlands/621557/03 H7N7. This virus was isolated on the index farm of the outbreak in the Netherlands in 2003. The virus had an intravenous pathogenicity index (IVPI) of 2.93, as determined by the procedure described elsewhere (CEC, 1992). Briefly, ten chickens were injected intravenously with 0.1 ml of tenfold diluted allantoic fluid. Birds were examined at 24-hour intervals for ten days. At each observation

each chicken was recorded as normal (0), sick (1), severely sick (2) or dead (3). The index is calculated by adding up all scores and by dividing the total by 100. When the index is greater than 1.2 the avian influenza virus is considered as highly pathogenic.

3.2.2 Animals

All animal experiments were undertaken in a high containment unit under biosafety level (BSL) 3+ conditions at the Central Institute for Animal Disease Control Lelystad. The experiments comply with the Dutch law on animal experiments and were reviewed by an ethical committee. In all experiments six weeks old specific pathogen free (SPF) white leghorn chickens were used. The chickens were inoculated both intranasaly and intratracheally with 0.1 ml diluted allantoic fluid containing 10^6 median egg infectious dose (EID_{50}) per ml.

3.2.3 Vaccines

Two commercially available oil emulsion vaccines were used: a H7N1 (A/Chicken/Italy/99) vaccine and a H7N3 (A/Chicken/Pakistan/95) vaccine. The dosage was: 0.5 ml for the H7N1 vaccine and 0.3 ml for the H7N3 vaccine (as recommended by the manufacturer), it was administered in the leg-muscles. The HA antigen content was 45 μg/ml for the H7N1 vaccine and 13 μg/ml for the H7N3 vaccine (Figure 2 of the Supporting Information). The protein homology of the immunogenic part of the hemagglutinin (HA1) between the challenge strain H7N7 and the H7N1 vaccine was 98%, for the H7N3 vaccine 92%.

3.2.4 Transmission experiments

Group experiments were performed with unvaccinated chickens (exp. 1), with chickens challenged one week after vaccination with H7N1 (exp. 2) or H7N3 (exp. 3), and with chickens challenged two weeks after vaccination with H7N1 (exp. 4) or H7N3 (exp. 5). All group experiments were done in duplicate (see Table 1 for an overview). The design of the experiments was as follows: five chickens were placed in a cage (1.2 x 1.2 m.) and inoculated with virus; 24 hours later five contact chickens were added. The chickens were monitored by taking tracheal and cloacal swabs daily during the first ten days and twice a week for the next 11 days. A blood sample was taken weekly. The experiments were terminated three weeks after the challenge.

Pair experiments were performed with vaccinated inoculated chickens and unvaccinated contact chickens (see Table 1 for an overview). All pair experiments were done with four pairs of chickens. Chickens were challenged one week after vaccination with H7N1 (exp. 6) or H7N3 (exp. 7) or challenged two weeks after vaccination with H7N1 (exp. 8) or H7N3 (exp. 9). In each experiment a chicken was vaccinated and one or two weeks after vaccination was then challenged with H7N7 virus. The vaccinated inoculated chicken was placed in a cage and 24 hours later an

Table 1. Overview of the experiments.

Number	Type of experiment	Challenge after vaccination	Vaccine
1	group	unvaccinated	
2	group	one week	H7N1
3	group	one week	H7N3
4	group	two weeks	H7N1
5	group	two weeks	H7N3
6	pair	one week	H7N1
7	pair	one week	H7N3
8	pair	two weeks	H7N1
9	pair	two weeks	H7N3

All experiments were done in duplicate.

unvaccinated chicken was added. The chickens were monitored by taking tracheal and cloacal swabs daily during the first ten days and at day 14, and through a weekly blood sample. As soon as a contact chicken showed signs of illness the contact chicken was euthanized.

3.2.5 Vaccination response experiment

The serological response after vaccination was studied by the hemagglutination inhibition (HI) assay. In total 40 chickens were vaccinated with the H7N1 vaccine, and 40 with the H7N3 vaccine. The animals were bled from the wing vein at days 6, 8, 10, 12, 14, 17, 21, 24, 28, 31 and 35.

3.2.6 Virus isolation

Swabs were put in 2 ml 2.95% tryptose phosphate buffer with 5 x 10^3 IU of penicillin-sodium and 5 mg streptomycin per ml. The swabs were stored at -70°C until analyzed. Three embryonated chicken eggs incubated for 9 days were inoculated with 0.2 ml per egg. After 72 h the allantoic fluid was harvested. A hemagglutination assay (HA) was performed following standard procedures. When at least one of the eggs was positive in the HA the swab was considered to be positive.

3.2.7 Hemagglutination inhibition (HI) assay

This assay was performed by standard methods. Briefly, the test was performed in V-bottom 96-well microtiter plates with 8 hemagglutinating units of H7N7 challenge virus and 1% SPF

chicken erythrocytes.

3.2.8 Sequencing of the hemagglutinin

Before sequencing the antigen was extracted from the vaccines as described previously (Maas et al., 2003): two ml of the vaccine was mixed with 8 ml isopropylmyristate (Sigma). The mixture was centrifuged at 1000 x g for 10 minutes and the water phase was collected. Viral RNA was extracted by using the High Pure Viral Nucleic Acid kit (Roche). RT-PCR of the hemagglutinin was performed and the PCR products were sequenced. The protein sequences of the HA1 were compared using BLASTP 2.2.

3.2.9 Antigen content of the vaccines

The antigen was extracted from the vaccines as described in the previous section. A series of diluted BSA standard (Pierce) (600, 500, 400, 300 and 200 ng) and the vaccines (5, 4 and 2 µl) were run on a 12% denaturating Bis-Tris gel (NuPAGE, Invitrogen). The gel was stained for 60 minutes in SYPRO-Orange dye (Molecular Dynamics) in 7,5% (v/v) acetic acid, washed for 1 minute in 7,5% (v/v) acetic acid and scanned on a Storm 860 laser scanner (Molecular Dynamics) (Steinberg et al., 1996). Bands were quantified with ImageQuant 5.1 software (Molecular Dynamics).

3.2.10 Statistical analysis

The analysis of the transmission experiments is based on a stochastic SEIR epidemic model in which individuals are susceptible (S), latently infected (i.e. infected but not yet infectious) (E), infected and infectious (I), and recovered and immune or dead (R). Throughout, the analyses are aimed at estimation of the (basic) reproduction ratio (denoted by R). The reproduction ratio is defined as the mean number of infections that would be caused by a single infected individual in a large population of susceptible animals. If $R > 1$, an infected animal infects on average more than 1 susceptible animal, and a chain reaction of infections may occur. If $R < 1$, a prolonged chain reaction of infections is not possible, and the epidemic comes to a halt. In our context, the reproduction ratio is given by the product of the mean infectious period $E(T_I)$ (dimension: *time*) and the transmission rate parameter β (dimension: $time^{-1}$): $R = \beta E(T_I)$.

We used two different methods to estimate the reproduction ratio: *(i-)* final size methods and *(ii-)* a Generalized Linear Model (GLM). The appeal of final size methods is that they are flexible and robust (Ball, 1986, 1995). For instance, the final size does not depend on whether or not there is a period of latency, and different assumptions on the infectious period distribution are easily incorporated. On the other hand, final size methods do not make use of all the information,

and do not allow separate estimation of the transmission rate parameter and infectious period. For this purpose the Generalized Linear Model is appropriate (Van der Goot et al., 2003).

The final size of an experiment is given by the number of contact animals that has been infected when the infection chain has ended. Central to our analysis is the fact that final size distributions can be determined under a wide range of assumptions. Specifically, the probability $p(k)$ of an outbreak of size k in a population where initially s_0 uninfected and i_0 infected animals are present is determined recursively from the equation (Ball, 1986, 1995).

$$p(k)=\left(\frac{L[\beta(s_0-k)]}{s_0+i_0}\right)^{s_0+i_0}\left(\binom{s_0}{k}-\sum_{l=0}^{k-1}\binom{s_0-l}{k-l}\frac{p(l)}{\left(L[\beta(s_0-k)]/(s_0+i_0)\right)^{l+i_0}}\right), \qquad (1)$$

where $L[z]$ is the Laplace transform of the infectious period probability distribution. We focus on two extreme scenarios, one in which the infectious period is exponentially distributed, and one in which the infectious period is of fixed duration. If the infectious period is exponentially distributed L is given by

$$L[z]=\frac{1}{1+z/a}, \qquad (2)$$

while if the infectious period is of fixed duration L is given by

$$L[z]=e^{-z/a}. \qquad (3)$$

By rescaling the time-axis we may measure time in units of the expected infectious period (Ball, 1986). As a consequence, $E(T_I)=1$ *and* $R=\beta E(T_I)=\beta$. In other words we may, without loss of generality, take $\alpha=1$ and equate β with R in the final size equation (1).

With formulas for the final size at hand it is possible to obtain estimates of the reproduction ratio by Maximum Likelihood (Bailey, 1975; Becker, 1989). Estimates of the reproduction ratio based on a final size analysis are labelled by R_{fix} in case of an infectious period of fixed duration, and by R_{exp} in case of an exponentially distributed infectious period. Exact 95% confidence intervals are obtained by finding all possible r-values such that the hypothesis H_0: $R=r$ is not rejected, i.e. by finding all values of r with a p-value larger than 0.05 (Kroese and De Jong, 2001). In the same manner, exact tests of R against the threshold value 1 are performed (Kroese and De Jong, 2001). Furthermore, taking the difference in the number of contact infections between treatments as a natural test statistic, it is possible to make comparisons

between treatments based on R, i.e. to test whether $R_{vaccine} = R_{control}$. All calculations are carried out using the software package Mathematica 5.0.

To take the time course of the experimental epidemics into account two approaches are possible. First, one could take a Bayesian approach using MCMC methodology, based on the likelihood of the data under the SEIR model (Streftaris and Gibson, 2004). The Bayesian approach is flexible and allows one to include prior information in the estimation procedure. Here, however, we take an approach based on a generalized linear regression. This approach is appealing because of its conceptual simplicity. Specifically, assuming a fixed latent period we estimated the transmission parameter β of the SEIR model by means of a Generalized Linear Model (Becker, 1989; McCullagh and Nelder 1989; Van der Goot et al., 2003). To this end the data in Tables 2-4 are rendered into the format *(S(t), I(t), C(t), Δt)*, where *S(t)* is the number of susceptible chickens at the beginning of a time period of length Δt, *I(t)* represents the average number of infectious chickens in this time period, and *C(t)* represents the number of new infections that have appeared. As in our previous study (Van der Goot et al., 2003) we assume a latent period of two days. The total number of chickens that is alive is also relevant, and is denoted by *N(t)*. By standard reasoning (Becker, 1989; Van der Goot et al., 2003) we accept that the number of cases is binomially distributed with parameter

$$p_{\inf}(t,t+\Delta t)=1-\exp\left(-\beta \frac{I(t)}{N(t)}\Delta t\right) \tag{4}$$

(the probability of infection) and binomial totals *S(t)*:

$$C(t,t+\Delta t)\sim Bin\left(S(t), p_{\inf}(t,t+\Delta t)\right). \tag{5}$$

The model specified by eqns. (4) and (5) can be formulated as a GLM with a complementary log-log link function, taking $\log(I(t)/(N(t)))$ as offset variable. The intercept of this generalized regression estimates $\log(\beta)$. The analyses are carried out using the software package GenStat 6.0.

The infectious periods are directly observed from the infected contact animals. Hence, estimation of the infectious period and the construction of confidence interval is straightforward. An estimate of the reproduction ratio is given by the product of the estimates of the transmission parameter and infectious period (Van der Goot et al., 2003; Stegeman et al., 2004).

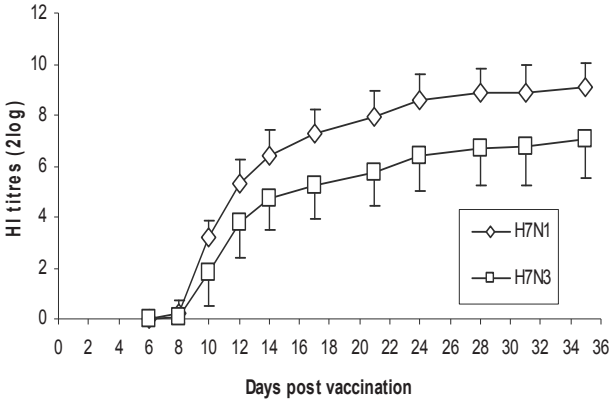

Figure 1. Serological response (HI titres) of chickens after vaccination. Groups of 40 chickens were vaccinated with H7N1 (◊)(0.5 ml/chicken i.m.) or with H7N3 (□)(0.3 ml/chicken i.m.).
The standard error is given by vertical bars.

3.3 Results

3.3.1 Kinetics of the antibody response

The antibody response in chickens vaccinated with H7N1 and H7N3 vaccine is given in Fig. 1 (and in the Supporting Information, Tables 6-7). Antibodies were detectable by the HI assay from day 8 post-vaccination. A significant difference between the geometric mean HI titres of the H7N1 and the H7N3 vaccinated groups developed: at day 35 the mean HI titre in the H7N1 vaccinated group is 2 2log steps higher than in the H7N3 vaccinated group. These differences in HI titres between the vaccines are also observed in the transmission experiments (Supporting Information, Tables 8-12).

3.3.2 Transmission experiments with unvaccinated chickens

To determine the transmission characteristics of H7N7 virus in a susceptible host population we performed transmission experiments with unvaccinated chickens (Exp.1). All inoculated as well as the contact chickens became positive by virus isolation (Table 2). However, while all inoculated chickens died within two to five days, two contact chickens in the first group and one contact chicken in the second group survived the infection. The estimate of the reproduction ratio based on the final size method with a fixed infectious period is $R_{fix}=\infty$ *(95% CI = 1.33-*∞*)* (Table 6). The estimate of the reproduction ratio based on the final size method with an exponentially

Table 2. Transmission of H7N7 in unvaccinated chickens.

	Day after challenge												
Chicken	1	2	3	4	5	6	7	8	9	10	14	16	21
I	+/+	+/+	†										
I	+/+	+/+	+/+	†									
I	+/+	+/+	+/+	†									
I	+/+	+/+	+/+	+/+	†								
I	+/+	+/+	+/+	†									
S	nd	+/-	+/-	+/+	+/+	†							
S	nd	-/-	+/-	+/+	+/+	+/+	†						
S	nd	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/-	-/-	-/-
S	nd	-/-	+/-	+/+	+/+	+/+	†						
S	nd	+/-	+/-	+/-	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/+	-/-
I	+/+	+/+	+/+	+/+	†								
I	+/-	+/+	+/+	+/+	†								
I	+/+	+/+	+/+	+/+	†								
I	+/-	+/+	+/+	+/+	†								
I	+/+	+/+	+/+	+/+	+/+	†							
S	nd	-/-	+/+	+/+	+/+	+/+	†						
S	nd	-/-	+/-	+/+	+/+	+/+	†						
S	nd	-/-	+/-	+/+	+/+	+/+	†						
S	nd	-/-	+/-	+/+	+/+	+/+	†						
S	nd	-/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/+	-/-

Chickens were challenged with 0.2 ml of diluted allantoic fluid containing 10^6 EID_{50} per ml (0.1ml intranasaly and 0.1 ml intratracheally) of A/Chicken/Netherlands/621557/03. I, inoculated chicken; S, contact chicken; †, chicken died; nd, not determined; EID_{50}, egg-infectious dose; +/+, positive tracheal swab/positive cloacal swab; +/-, positive tracheal swab/negative cloacal swab; -/+, negative tracheal swab/positive cloacal swab; -/-, negative tracheal swab/negative cloacal swab.

distributed infectious period is almost identical ($R_{exp}=\infty$ *(1.30-∞)*), while the GLM is in good agreement with this estimate (Table 6). These experiments confirm that the H7N7 virus spreads very easily in an unvaccinated population and can readily cause a major outbreak.

3.3.3 Transmission experiments with vaccinated chickens

Does vaccination reduce transmission of HPAI viruses? To answer this question experiments were done with chickens challenged one or two weeks after vaccination (Exp. 2-5). When challenged one week after vaccination with the H7N1 vaccine, all but one of the chickens in the inoculated groups became positive by virus isolation (Table 3). In the contact groups one and none of the five chickens became positive. The chickens showed no signs of illness. Analysis of these data shows that the estimate of the reproduction ratio based on the final size method is

Table 3. Transmission of H7N7 in vaccinated chickens, 7 days after vaccination

		Day after challenge										
Vaccine	Chicken	1	2	3	4	5	6	7	8	9	10	14
H7N1	I	+/-	-/+	+/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	nd/-
	I	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/+	-/+	-/+	nd/-
	I	+/-	+/-	+/-	+/+	-/+	-/+	-/+	-/+	-/+	-/-	nd/-
	I	+/-	+/+	-/+	-/+	-/-	-/+	-/-	-/+	-/-	-/-	nd/-
	I	+/-	-/-	-/+	-/+	-/+	-/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
	I	+/-	+/-	-/+	-/-	-/-	-/+	-/-	-/-	-/-	-/-	nd/-
	I	-/-	-/-	-/-	-/-	-/-	-	-/-	-/-	-/-	-/-	nd/-
	I	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/-	-/+	-/-	nd/-
	I	+/-	-/-	-/-	-/-	-/-	-	-/-	-/-	-/-	-/-	nd/-
	I	+/+	+/-	+/+	+/+	-/+	-/+	-/-	-/-	-/-	-/+	nd/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
H7N3	I	+/+	+/+	+/+	+/+	+/+	-/+	-/+	-/-	-/+	-/+	nd/-
	I	+/-	+/+	+/+	+/+	-/+	-/+	-/-	-/-	-/-	-/+	nd/-
	I	+/-	+/+	+/+	-/+	-/+	+/+	-/+	-/-	-/-	-/-	nd/-
	I	+/-	+/+	+/+	-/+	-/+	-/+	-/+	-/-	-/-	-/-	nd/-
	I	+/+	+/+	+/+	+/+	+/+	-/+	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/-	+/-	-/-	+/-	-/-	-/+	-/+	-/-	-/-	nd/-
	S	nd	-/-	+/-	-/+	+/+	-/+	-/+	-/+	-/+	-/-	nd/-
	S	nd	-/-	-/-	-/-	-/+	+/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
	I	+/-	+/-	-/+	-/+	-/+	-/-	-/-	-/-	-/-	-/-	nd/-
	I	+/-	+/+	-/+	+/+	-/+	-/+	-/-	-/-	-/-	-/-	nd/-
	I	+/+	+/+	+/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+
	I	+/+	+/+	+/+	-/+	-/+	-/+	-/+	-/+	-/-	-/-	nd/-
	I	+/-	+/+	-/+	+/+	-/+	-/+	-/+	-/-	-/-	-/-	nd/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	+/-	-/-	-/-	-/-	+/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/-

Chickens were challenged 7 days after vaccination with 0.2 ml of diluted allantoic fluid containing 10^6 EID_{50} per ml (0.1 ml intranasaly and 0.1 ml intratracheally) of A/Chicken/Netherlands/621557/03.
I, inoculated chicken; S, contact chicken; nd, not determined; EID_{50}, egg-infectious dose; +/+, positive tracheal swab/positive cloacal; swab; +/-, positive tracheal swab/negative cloacal swab; -/+, negative tracheal swab/positive cloacal swab; -/-, negative tracheal swab/negative cloacal swab; nd/-, tracheal swab not determined/negative cloacal swab.

$R_{fix} = 0.2$ (0.005-1.1), which is significantly below 1 ($p = 0.04$, Table 6). The results based on the final size method with an exponentially distributed infectious period ($R_{ex\ p} = 0.2$ (0.005-1.4)) and based on the GLM again are in good agreement with this estimate. When challenged one week after vaccination with the H7N3 vaccine all challenged chickens became positive. In the contact group five and one out of the five chickens became positive. These chickens also showed no signs of illness. When analysed, the estimate of the reproduction ratio based on the final size method is $R_{fix} = 1.4$ (0.4-2.9) (Table 6). The results from the final size method with an exponentially distributed infectious period ($R_{exp} = 1.7$ (0.4-4.3)) and the GLM yield similar results. The transmission characteristics were also studied when chickens were challenged two weeks after vaccination (Exp. 4-5). All inoculated and contact chickens in the H7N1 and H7N3 vaccinated groups remained negative in the tracheal and cloacal swabs (Table 4). In these experiments the SEIR model was not applied as no virus could be detected even from the inoculated chickens.

To decide whether transmission is significantly reduced by vaccination the reproduction ratios of the vaccinated groups and the unvaccinated groups were compared. A significant difference is found for the H7N1 vaccine ($p < 0.001$) one week after vaccination, but not for the H7N3 vaccine ($p = 0.1$) (Table 6). In the groups challenged two weeks after vaccination the SEIR model is not applied, but it is obvious that no transmission occurs.

3.3.4 Transmission from vaccinated to unvaccinated chickens

Do vaccinated chickens still excrete virus and pose a threat of infection to unvaccinated chickens? To answer this question we carried out so-called pair experiments with one inoculated vaccinated chicken and one unvaccinated contact chicken (Exp. 6-9; Table 1).

For both vaccines transmission was still observed if the experiments were carried out one week after vaccination (exp. 6-7; Supporting Information, Table S13). For the H7N1 vaccine all four inoculated animals became positive by virus isolation, and three out of four unvaccinated contact chickens were infected. For the H7N3 vaccine all the vaccinated as well as the unvaccinated chickens became positive by virus isolation. In both experiments, the contact animals became positive at day 3 or 4, indicating efficient transmission from the vaccinated to

Table 4. Transmission of H7N7 in vaccinated chickens, 14 days after vaccination.

		Day after challenge							
Vaccine	Chicken	1	2	3	4	5	6	7	8
H7N1*	I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-
H7N3*	I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-

Chickens were challenged 14 days after vaccination with 0.2 ml of diluted allantoic fluid containing 10^6 EID_{50} per ml (0.1 ml intranasaly and 0.1 ml intratracheally) of A/Chicken/Netherlands/621557/03. I, inoculated chicken; S, contact chicken; nd: not determined; EID_{50}, egg-infectious dose; -/-, negative tracheal swab/negative cloacal swab.

*Experiments were performed in duplicate. The results of the replicates were identical; therefore, only one replicate is shown.

unvaccinated chickens. The infected contact animals died from the infection, while the inoculated chickens showed no signs of illness and survived.

When the pair transmission experiments were carried out two weeks after vaccination (Exp. 8-9), none of the inoculated and contact chickens became positive by virus isolation, and all chickens survived. These experiments show that no transmission is possible from vaccinated to fully susceptible contact chickens two weeks after vaccination.

Table 5. Overview of the statistical analyses of the group experiments.

	Final Size	R_{fix} (95% CI)	p-value, H_0:R≥1	p-value, H_0:R_v=R_c*	Infectious Period (day) (95%Cl)	Transmission Parameter (day^{-1}) (95%Cl)	R_{GLM} (95%Cl)
Unvaccinated	5,5	∞ (1.3-∞)	1		6.3 (3.9-8.7) (n=10)	33 (n=2)	208
H7N1 (1 week)†	1,0	0.2 (0.005-1.1)	0.04	<0.001	1 (n=1)	0.030 (0.01-0.09) (n=18)	0.03
H7N3 (1 week)†	5,1	1.4 (0.4-2.9)	0.78	0.10	3.7 (0.7-6.7) (n=6)	0.30 (0.09-0.9) (n=12)	1.1 (0-3.1)
H7N1 (2 weeks)‡	0,0	na§	na§	na§	na§	na§	na§
H7N3 (2 weeks)‡	0,0	na§	na§	na§	na§	na§	na§

*, R_v= reproduction ratio amongst vaccinated chickens, R_c= reproduction ratio amongst unvaccinated chickens; †, Birds were challenged 7 days after vaccination; ‡, Birds were challenged 14 days after vaccination; §, No virus was detected from the inoculated or the contact chickens; therefore no statistical analysis was performed.

3.4 Discussion

Our transmission experiments demonstrate that vaccination not only protects chickens against disease symptoms and mortality, but is also an effective strategy to reduce transmission. Specifically, when challenged two weeks after vaccination transmission of the virus is completely halted so that a major outbreak can be prevented. It is important to realize, however, that it may take some time after vaccination until herd immunity is obtained. In our experiments, one week after vaccination with the H7N1 vaccine some transmission is still observed, but the reproduction ratio is already significantly below 1. This means that an introduction of the virus may cause a small number of secondary infections, but the virus probably cannot spread extensively. In contrast, we were unable to disproof the hypothesis that $R \geq 1$ for the H7N3 vaccine one week after vaccination ($p = 0.78$). This may indicate either that the H7N3 vaccine does not sufficiently reduce transmission one week after vaccination (i.e. R does exceed 1), but it could also indicate that the number of replicates is too small (i.e. the power of the experimental set-up was not sufficiently high). A (two-sided) test of the hypothesis $R_{H7N3} = R_{H7N1}$ against the alternative $R_{H7N3} \neq R_{H7N1}$ yields a p-value of $p = 0.10$, indicating that there is marginal evidence that the H7N1 vaccine performs better than the H7N3 vaccine. Summarizing, we have shown that vaccination can be an attractive tool to prevent outbreaks of highly pathogenic avian influenza

viruses in poultry, thereby achieving the aim of eliminating the source of human infections.

Whether transmission between two chickens occurs depends on the infectiousness of the infected chicken and the susceptibility of the uninfected chicken. The reproduction ratio is a composite measure which incorporates both factors (Diekmann and Heesterbeek, 2000). An indication for infectiousness is the amount of virus shed, which is reduced by vaccination (Swayne et al., 1999; Capua et al., 2002). This was confirmed by our experiments: when chickens were challenged two weeks after vaccination no transmission occurred, not even to unvaccinated chickens in close contact. This suggests that vaccination reduces infectiousness. The minimum infective dose can be used as a proxy for susceptibility. For example, in vaccinated turkeys it was higher than in unvaccinated turkeys (Capua et al., 2004). Our experiments confirm this finding: when the contact chickens were vaccinated (Exp. 2-3) the number of contact infections was lower than was expected from the experiments where the contact chickens were not vaccinated (Exp. 6-7). This decrease in transmission can be attributed to the decrease in susceptibility of the contact chickens, since the infectiousness in both experiments is the same. We conclude that vaccination reduces the infectiousness of infected chickens as well as the susceptibility of uninfected chickens.

The two vaccines differed in their effect on transmission. Two important factors that determine the effectiveness of a vaccine are: antigen content (Brugh et al., 1979; Swayne et al., 1999) and antigenic differences between the vaccine and the challenge virus (Swayne et al, 1999; Lee et al., 2004). In the H7N1 vaccine the HA antigen content per dose was higher than in the H7N3 vaccine (H7N1 22 μg/dose, H7N3 4 μg/dose). The other factor is the sequence similarity of the hemagglutinin, which is correlated with the reduction of virus shedding from the oropharynx or trachea (Swayne et al., 2000a, 2000b; Lee et al., 2004). The homology between the HA1 of the H7N1 vaccine and the H7N7 challenge strain is 98%, the homology between the H7N3 vaccine and the H7N7 is 92%. These differences in antigen content and sequence homology are in agreement with the observed differences between the two vaccines. To what extent antigen content and homology contribute to transmission reduction is at present an open question that merits further investigation.

Is there a relation between the immune response that develops after vaccination and the reduction of transmission? Antibodies against the hemagglutinin evoke the major protection against infection with HPAI (Webster et al., 1991). HI titres after vaccination with H7N1 and H7N3 are detectable from day 8 (Fig. 1). These results are in good agreement with HI titres from our transmission experiments (Supporting Information, Table 8). Remarkably, despite the low titres at day 8-10 after vaccination (day 1-3 after challenge) there is already a considerable reduction in transmission. This suggests that other immune mechanisms might contribute to protection, or that low HI titres are already effective in preventing infection.

The choice of a vaccine is an issue of major importance. In poultry, a number of vaccines are available for use against highly pathogenic H5 and H7 influenza A viruses. Given the choice, which of these vaccines should be used? In our opinion, besides safety aspects and side-effects, the most important requirement is that the vaccine is effective in preventing viral spread. As we have shown, transmission experiments are eminently suited to address this question. Our experiments indicate that vaccines against highly pathogenic H7N7 influenza virus can completely block transmission two weeks after vaccination. We conclude that vaccination of poultry can be an effective tool to prevent spread of highly pathogenic avian influenza viruses.

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Supporting information

Supporting information associated with this article can be found, in the online version, at www.pnas.org.cgi/doi/10.1073/pnas.0505098102. A selection (Table S13) is given below.

Table S13. Transmission of H7N7 from vaccinated to unvaccinated chickens

		Day after challenge										
Vaccine	Chicken	1	2	3	4	5	6	7	8	9	10	14
H7N1	I	+/-	-/-	-/-	nd/-	-/nd	-/-	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	-/-	nd/-	-/nd	-/-	-/-	-/-	-/-	-/-	-/-
	I	+/-	-/+	-/+	nd/+	-/nd	-/+	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	-/-	nd/+	+/nd	+/+	†				
	I	+/-	+/+	+/+	nd/+	+/nd	-/+	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	+/-	nd/+	+/nd	+/+	+/+	+/-	†		
	I	+/-	+/+	+/+	nd/+	-/nd	-/+	-/-	-/-	-/-	-/-	-/-
	S	nd		+/-	nd/-	-/nd	+/+	+/+	+/+	†		
H7N3	I	+/-	+/+	+/+	+/+	-/+	-/+	-/-	-/-	-/-	-/-	-/-
	S	nd	+/-	+/+	+/+	nd	+/+	†				
	I	+/-	+/+	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	+/-	+/+	nd	+/+	†				
	I	+/-	+/+	+/+	+/+	-/+	-/+	-/-	-/-	-/-	-/-	-/-
	S	nd	+/-	+/-	+/+	nd	+/+	†				
	I	+/-	+/+	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/-	-/-
	S	nd	-/-	+/-	+/+	nd	+/+	†				

Chickens were challenged 7 days after vaccination with 0.2 ml of diluted allantoic fluid containing 10^6 EID50/ml (0.1 ml intranasally and 0.1 ml intratracheally) of A/Chicken/Netherlands/621557/03. I, inoculated chicken (vaccinated); S, contact chicken (unvaccinated); nd, not determined; †, chicken died; EID_{50}, egg-infective dose; +/+, positive tracheal swab/positive cloacal swab; +/–, positive tracheal swab/negative cloacal swab; –/+ negative tracheal swab/positive cloacal swab; –/–, negative tracheal swab/negative cloacal swab; nd/+, tracheal swab not determined/positive cloacal swab; nd/–, tracheal swab not determined/negative cloacal swab.

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Chapter 4

Variable effect of vaccination against highly pathogenic avian influenza (H7N7) virus on disease and transmission in pheasants and teals

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Abstract

Highly pathogenic avian influenza viruses can affect many bird species, with disease symptoms ranging from severe morbidity and high mortality to mild transient illness. Much is known about infections in chickens, but for other captive birds the relations between disease symptoms, excretion patterns, and transmission, as well as the effect of vaccination on these relations are not clear. We report results from experimental transmission studies with a highly pathogenic H7N7 virus and two commonly kept bird species (ringed teals and golden pheasants). The results show that depending on the host species the virus can spread in unvaccinated birds with or without disease symptoms. Vaccination reduces disease symptoms markedly, but need not always reduce virus transmission. We discuss the implications for the control of highly pathogenic avian influenza.

4.1 Introduction

Highly pathogenic avian influenza (HPAI) is a disease of poultry and other captive birds caused by H5 or H7 avian influenza viruses, with mortality that ranges up to 100%. Natural and experimental infections have been documented in different species. The observed disease symptoms in different bird species vary from sub-clinical to severe illness and death (Wood et al., 1985; Alexander et al., 1986; Alexander, 2000; Perkins and Swayne, 2001, 2002, 2003; Ellis et al., 2004; Editorial, 2005).

Since the outbreak of HPAI H5N1 in Hong Kong in 1997 and of HPAI H7N7 in The Netherlands in 2003 it has been recognized that some HPAI viruses can infect humans (Claas et al., 1998; Subbarao et al., 1998; Fouchier et al., 2004). This implies that outbreaks of HPAI in birds can have major consequences for public health, and are not just a veterinary problem. A lot is known about HPAI infections in chickens and other poultry, but for other birds less information is available about the disease symptoms, excretion patterns, duration of infection and transmissibility of viruses. Since most non-commercial birds are kept in close contact with their owners measures should be taken to prevent infection of these birds. It is therefore important not only to investigate the nature of infections with HPAI virus in non-commercial captive birds, but also to study the potential impact of vaccination. Recently some experience has been gained in vaccination of species other than poultry. Vaccination of zoo birds has been practiced against H5N1 virus in Singapore (Oh et al., 2005), against H7N7 virus in The Netherlands (Philippa et al., 2005), and against H5N1 virus in several European countries (EFSA, 2007). It was shown

that different species vary in their seroresponse to vaccination, but the majority of bird species develop an antibody response after two vaccinations (Oh et al., 2005; Phillipa et al., 2005; EFSA, 2007). It is, however, by and large unknown how antibody titers correlate with protection against infection, disease, mortality, and transmission.

To study the effect of vaccination on infection, disease, and transmission of HPAI virus A/Chicken/Netherlands/621557/03 H7N7 in two species other than poultry we performed experimental transmission studies with ringed teals (*Callonetta leucophrys*, family Anatidae) and golden pheasants (*Chrysolophus pictus*, family Phasianidae). To quantify the effect of vaccination on transmission a number of infected birds are housed together with a number of uninfected birds, and the infection chain is monitored on a regular basis. Disease symptoms and excretion of virus are monitored daily while the antibody response is determined weekly. The infection chain data are analyzed by a stochastic SEIR model (Anderson and May, 1991). The aim of this model is to obtain estimates of the basic reproduction number (*R)*, which is defined as the number of secondary infections that would be caused by one primary infected individual in a large population of susceptible individuals. If the value of the reproduction number exceeds the threshold value of 1, an infected individual infects on average more than one susceptible individual in the early stages of an outbreak, and an epidemic may occur. If, however, the reproduction number is smaller than 1, an infected individual will on average infect less than one susceptible individual and an introduction of the virus cannot cause an epidemic.

The experiments were performed with ringed teals and golden pheasants, these species were chosen as representatives of the two commonly kept bird families Anatidea and Phasianidae. Infections of wild and domestic ducks with low pathogenic avian influenza (LPAI) virus and HPAI virus have been reported (Webster et al., 1992; Capua et al., 2001; Alexander, 2007). Ringed teals that were found dead in a waterfowl park with an outbreak of HPAI H5N1 were tested positive for H5N1 virus (Ellis et al., 2004). Several species of Phasianidae are susceptible to infections with LPAI virus and HPAI virus (Wood et al., 1985; Perkins and Swayne, 2001; Humberd et al., 2006). After infection with HPAI virus disease symptoms vary from mild transient illness (Wood et al., 1985) to 100% mortality (Perkins and Swayne, 2001). Pheasants that were infected with LPAI viruses of each of the 15 HA subtypes excreted virus for up to 45 days, and most of the subtypes spread to contact pheasants (Humberd et al., 2006).

Our transmission experiments show that ringed teals and golden pheasants react very differently to infection with HPAI H7N7 virus and to vaccination against HPAI H7N7 virus. We conclude that it may not be easy to make general statements on the effectiveness of vaccination in different bird species.

4.2 Materials and methods

4.2.1 Animals

Golden Pheasants (*Chrysolophus pictus*) en Ringed Teals (*Callonetta leucophrys*) from different breeders were housed together five weeks before the start of the experiment. The golden pheasants were 20 weeks and the ringed teals were 18 weeks of age at the start of the experiment, and the birds were of mixed sex. During the experiments groups of 10 birds were housed in a compartment of 1.5 x 1.6 m., the teals were kept on a grid floor with access to water for bathing, and the pheasants were kept on wood shavings. All animal experiments were performed in a high containment unit under BSL3+ conditions at the Central Institute for Animal Disease Control Lelystad. The experiments comply with the Dutch law on animal experiments and were reviewed by an ethical committee.

4.2.2 Virus

The influenza virus used in this study was A/Chicken/Netherlands/621557/03 H7N7 HPAI. The virus has an intravenous pathogenicity index (IVPI) of 2.93, according to a standard procedure (CEC, 2006). Briefly, ten chickens were injected intravenously with 0.1 ml of tenfold diluted allantoic fluid. Birds were examined at 24-hour intervals for ten days. At each observation each chicken was recorded normal (0), sick (1), severely sick (2) or dead (3). The index is calculated by adding up all scores and by dividing the total by 100. When the index is higher than 1.2 the avian influenza virus is considered highly pathogenic.

4.2.3 Vaccine

An inactivated oil emulsion H7N1 vaccine (A/Chicken/Italy/99) was used. A dosage of 0.5 ml was injected in the muscles of the leg. The protein homology of the immunogenic part of the hemagglutinin (HA1) of the challenge strain and the vaccine strain is 98% (Van der Goot et al., 2005).

4.2.4 Transmission experiments

The birds in the experiments were inoculated both intranasaly and intratracheally with 0.1 ml diluted allantoic fluid containing 10^6 median egg infectious dose (EID_{50}) of HPAI H7N7 virus per ml.

Experiments were performed with groups of unvaccinated birds and groups of vaccinated birds. The vaccinated birds were challenged two weeks after a single vaccination. Each experiment was done in duplicate. The design of the experiments was as follows: five birds were placed in a cage, and inoculated with virus. Subsequently, five contact birds were added 24 hours

later. The transmission chain was monitored by taking tracheal and cloacal swabs daily during the first ten days and twice a week for the next 14 days. A blood sample was taken once a week. Each day the birds were inspected visually and disease symptoms were recorded. The experiment was terminated 24 days after the challenge.

4.2.5 Virus isolation

Swabs were put in 2 ml 2.95% tryptose phosphate buffer with 5 x 10^3 IU of penicillin-sodium and 5 mg streptomycin per ml. The swabs were stored at -70°C until analyzed. Three embryonated 9-day-old chicken eggs were inoculated with 0.2 ml per egg. After 72 h the allantoic fluid was harvested and a hemagglutination assay was performed following standard procedures. When at least one of the eggs was positive in the hemagglutination assay the swab was considered to be positive.

4.2.6 Serology

All sera were incubated for 30 minutes at 56 ºC. For the hemagglutination inhibition (HI) test the sera were subsequently incubated with 20% chicken erythrocytes at 4 ºC overnight. The HI test was performed by standard methods, using 8 hemagglutinating units of the H7N7 challenge virus and 1% chicken erythrocytes in V-bottom 96-well microtiter plates. Titers are given as the 2log of the serum dilution. For the virus neutralisation (VN) assay serial dilutions of the sera were incubated for 1 h at 37°C with 100 $TCID_{50}$ of the H7N7 challenge virus. After 1 h a suspension of MDCK cells was added, and the plates were incubated for 2 days. After 2 days the cells were fixated, and incubated for 1 h with a HRPO-conjugated monoclonal antibody against the nucleoprotein of influenza A (Hb65, ATCC). Plates were washed, dried and examined microscopically. Titers are given as the 2log of the serum dilution.

The Spearman's rank correlation coefficient was calculated to assess the degree of correlation between the HI titers and VN titers (Conover, 1980).

4.2.7 Statistical analysis

The infection chain data are analyzed by a stochastic SEIR model (Anderson and May, 1991). The reproduction number is estimated by two different methods: a final size method and a Generalized Linear Model (GLM) (Becker, 1989; De Jong and Kimman, 1994; Velthuis et al., 2007). The final size method enables direct estimation of the reproduction number, while the GLM estimates the transmission rate parameter (β). In our context the reproduction number is given by the product of the transmission rate parameter and the mean infectious period. The final size method makes use of the number of contact birds that has been infected when the infection chain has ended, while the GLM takes the time course of the experimental epidemic into account.

The infectious periods are directly observed from the infected contact birds. In the analyses we assume a latent period of one day, and an exponentially distributed infectious period. For technical details we refer to Van der Goot et al., 2003, 2005. The analyses were carried out in GenStat 6.0. and Mathematica 5.2.

4.3 Results

4.3.1 Disease and transmission in unvaccinated pheasants and teals

Transmission experiments with unvaccinated birds were performed to study disease and transmission dynamics in an unvaccinated population, and to set a baseline against which the experiments with vaccinated birds can be compared. In the experiments with pheasants eight of the inoculated pheasants and four of the contact pheasants showed severe signs of illness (apathy, paralysis), and died (Table 1). The inoculated pheasants died five to ten days after inoculation, the contact pheasants died eight to nine days after being infected (Table 2). All inoculated pheasants became infected, and rapidly transmitted the virus to all contact pheasants (Table 2). The estimate of the reproduction number based on the final size method is $R > 1.5$ with 95% confidence. The transmission rate parameter calculated using the GLM is 2.8 (day^{-1}) (95% CI: 1.4-5.5), and the infectious period is 12.2 days (95% CI: 7.7-16.7) (Table 3). These results show that the reproduction number is larger than the threshold value 1 and imply that a major outbreak is possible in unvaccinated pheasants.

With respect to transmission the results of the experiments with unvaccinated teals were almost identical to the results of the pheasants. All inoculated teals became infected and quickly transmitted the virus to all contact birds (Table 4). However, with respect to morbidity and mortality the teals differed considerably from the pheasants. Only mild disease symptoms were observed in a minority of the teals. Four teals developed conjunctivitis (two inoculated birds and two contact birds), and no other disease symptoms were observed (Table 1). One of the teals was suffering from severe foot problems from day 5 onwards and died on day 12. The injuries were presumably caused by the grid floor. As with the experiments with the pheasants the estimate of the reproduction number based on the final size method is $R > 1.5$ with 95% confidence, the transmission rate parameter is > 2.7 (day^{-1}) with 95% confidence, and the infectious period is 10.4 days (95% CI: 7.6-13.2) (Table 3). In the unvaccinated teals the reproduction number is also larger than the threshold value 1, and this implies that a major outbreak is possible in unvaccinated teals.

Table 1. Disease symptoms and mortality

Experiment	Animal category[a]	Disease symptoms and mortality
Unvaccinated pheasants	Inoculated	Severe signs of illness[b] and dead (8)
	Contact	Severe signs of illness[b] and dead (4)
Unvaccinated teals	Inoculated	Conjunctivitis (2)
	Contact	Conjunctivitis (2)
Vaccinated pheasants	Inoculated	Sneezing (2)
	Contact	Conjunctivitis (2)
Vaccinated teals	Inoculated	No symptoms (10)
	Contact	No symptoms (10)

[a]number of birds in every category is 10. [b]apathy and paralysis.

4.3.2 Disease and transmission in vaccinated pheasants and teals

In the experiments with vaccinated pheasants the disease symptoms were in contrast with the symptoms of the unvaccinated groups, after vaccination hardly any disease symptoms were observed. Two of the inoculated pheasants were sneezing at day 5 and 6 after inoculation and two of the contact pheasants developed conjunctivitis, and all birds survived the infection (Table 1). Despite the mild symptoms all inoculated and all contact pheasants became infected (Table 5). Again the estimate of the reproduction number based on the final size method is $R > 1.5$ with 95% confidence. The transmission rate parameter is estimated at 1.0 (day^{-1}) (95% CI: 0.4-2.5), and the infectious period is 13.3 days (95% CI: 9.6-16.9) (Table 3).

In the experiments with vaccinated teals no disease symptoms were observed at all (Table 1). Moreover, vaccination of the teals had a major effect on transmission: nine of ten inoculated teals became infected but only during 1-3 days, and no contact infections were observed (Table 6). The estimate of the reproduction number based on the final size method is $R < 0.70$ with 95% confidence, which is significantly lower than the threshold value 1 ($p = 0.017$). The transmission rate parameter is estimated at < 0.5 (day^{-1}), with 95% confidence (Table 3). The infectious period cannot be calculated because there are no infected contact birds. There is a significant difference between the reproduction number of the unvaccinated and the vaccinated teals ($p < 0.001$). In the vaccinated teals the reproduction number is below the threshold value of 1, and this implies that an introduction of virus in vaccinated teals cannot cause an epidemic.

Table 2. Transmission of H7N7 in unvaccinated pheasants

Pheasant	D 1[a]	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10	D 14	D 17	D 21	D 24
I	+/+	+/+	+/+	+/+	+/+	+/+	†							
I	+/+	+/+	+/+	+/+	+/+	+/+	+/-	-/-	†					
I	+/+	+/+	+/+	+/+	†									
I	+/-	+/+	+/+	+/+	+/+	+/+	†							
I	+/+	+/+	+/+	+/+	+/+	+/+	†							
S	nd	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	†				
S	nd	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/-	-/-	-/-
S	nd	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/-	-/+	-/-
S	nd	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/-	-/-	-/-	-/-	-/-	-/-
S	nd	+/-	+/+	+/+	+/+	+/+	+/+	+/+	†					

Pheasant	D 1[a]	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10	D 14	D 17	D21	D 24
I	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/+	-/-	-/-
I	+/+	+/+	+/+	+/+	+/+	†								
I	+/-	+/+	+/+	+/+	+/+	+/+	†							
I	+/+	+/+	+/+	+/+	+/-	+/+	+/-	†						
I	-/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	+/+	+/+	-/-	-/-	-/-	-/-
S	nd	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/+	-/-	-/+
S	nd	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/-	-/-	-/-	-/-	-/-
S	nd	-/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/+	-/-	-/-
S	nd	-/-	+/+	+/+	+/+	+/+	+/+	+/-	+/-	†				
S	nd	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/-	†				

Pheasants were inoculated with A/Chicken/Netherlands/621557/03 H7N7 virus. I, inoculated bird; S, contact bird; x/y, tracheal swab/cloacal swab; nd, not determined; †, pheasant died. [a]day after challenge.

4.3.3 Serology

Hemagglutination inhibition (HI) titers were determined at regular intervals to study the dynamics of the antibody response after vaccination and/or infection (Figure 1). In the unvaccinated birds 95% of the teals and 100% of the pheasants already has a detectable HI titer 7 days after challenge. The mean HI titer at day 7 is 6.7 (95% CI: 5.7-7.8) for the inoculated teals and 7.9 for the inoculated pheasants. It should be noted that in the groups with pheasants only two inoculated birds were still alive at day 7 after inoculation. HI titers reach a maximum 14 days after challenge (teals 8.5 (95% CI: 7.8-9.1), pheasants 9.7), and decline from that day onwards (Figure 1). Vaccination titers come up later than post-infection titers, particularly in the pheasants. Seven days after vaccination none of the pheasants has a detectable HI titer, 14 days after vaccination the mean HI titer of the pheasants is 3.8 (95% CI: 2.9-4.8) (Figure 1). Seven days after vaccination 55% of the teals has an HI titer with a mean of 1.6 (95% CI: 0.7-2.4), and

Table 3. Overview of the statistical analyses

Treatment group	Final size	*R* (Final size)	H_0:$R \geq 1$	H_0:R_v=R_c[a]	Infectious period (day) (95% CI)	Transmission rate parameter (day^{-1}) (95% CI)	*R* (GLM)
Unvaccinated pheasants	5,5	>1.5[b]	ns		12.2 (7.7-16.7)	2.8 (1.4-5.5)	34
Unvaccinated teals	5,5	>1.5[b]	ns		10.4 (7.6-13.2)	>2.7[b]	>28
Vaccinated pheasants	5,5	>1.5[b]	ns	ns	13.3 (9.6-16.9)	1.0 (0.4-2.5)	13
Vaccinated teals	0,0	< 0.7[c]	0.017	<0.001	na	<0.5[c]	na

R, reproduction number; CI, confidence interval; GLM, generalized linear model; ns, not significant; na, not applicable. [a]R_v, reproduction ratio among vaccinated birds; R_c, reproduction ratio among unvaccinated birds. [b]The lower bound of the (one-sided) 95% confidence interval is given. [c]The upper bound of the (one-sided) 95% confidence interval is given.

at day 14 the mean HI titer of the teals is 5.7 (95% CI: 5.0-6.3) (Figure 1).

A virus neutralisation (VN) test was performed with the sera of day 14 (moment of challenge). For the teals the mean VN titer was 4.5 (95% CI: 3.6-5.3), and for the pheasants the mean VN titer was 1.9 (95% CI: 1.2-2.7). The Spearman's rank correlations between the HI titers and the VN titers were 0.78 for the teals and 0.85 for the pheasants. Both correlations differed significantly ($p < 0.05$) from 0, showing that there is an (approximately monotone) association between the two tests.

4.4 Discussion

In this paper we have studied the relation between infection, disease and transmission in vaccinated and unvaccinated golden pheasants and ringed teals.

Our results show that both pheasants and teals are permissive to infection by highly pathogenic A/Chicken/Netherlands/03 H7N7 virus, and excrete virus by the trachea and cloaca for a long period (pheasants: 4-23 days, teals: 5-17 days). Although both species are susceptible to HPAI infections, the difference in disease symptoms is considerable: most pheasants show severe morbidity and mortality, while in the teals only conjunctivitis is seen in a minority of birds. This variation in disease symptoms has been reported before (Wood et al., 1985; Alexander et al., 1986; Perkins and Swayne, 2001, 2002, 2003; Editorial, 2005). Despite the difference in disease symptoms the spread of virus is extensive in both species: the reproduction number is estimated to be at least 1.5, which implies that the virus is able to spread epidemically after a primary introduction. The finding that epidemic spread is possible corresponds well with our earlier study with the same HPAI H7N7 virus in unvaccinated chickens (Van der Goot et al., 2005). The fact

Table 4. Transmission of H7N7 in unvaccinated teals

Teal	D 1[a]	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10	D 14	D 17	D 21
I	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-	-/+	-/-	-/-	-/-	-/-
I	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-	+/+	-/-	-/-	-/-
I	+/+	+/+	+/+	+/+	+/+	+/-	-/-	-/-	-/-	+/+	†[b]		
I	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/+	-/-	-/-	-/-
S	nd	+/-	+/+	+/+	+/+	+/+	+/-	+/-	-/-	-/-	+/+	-/-	-/-
S	nd	+/+	+/+	+/-	+/+	+/+	-/+	-/-	-/-	-/+	-/-	-/-	-/-
S	nd	+/+	+/+	+/+	+/+	+/+	+/-	-/-	-/-	-/+	-/-	-/-	-/-
S	nd	+/-	+/+	+/-	+/+	+/+	-/-	-/-	-/-	-/+	-/-	-/-	-/-
S	nd	+/+	+/+	+/+	+/+	+/+	+/-	+/-	-/-	-/-	-/-	-/-	-/-

Teal	D 1[a]	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10	D 14	D 17	D 21
I	+/+	+/+	+/+	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	+/+	+/+	+/+	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	+/+	+/+	+/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	+/+	+/+	+/+	+/+	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	+/+	+/-	+/+	+/+	+/+	+/+	+/-	+/+	+/+	-/+	-/-	-/+	-/-
S	nd	+/+	+/+	+/-	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-
S	nd	+/+	+/+	-/+	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-
S	nd	+/+	+/+	+/+	+/-	+/-	+/-	-/-	-/-	+/+	-/-	-/+	-/-
S	nd	+/+	+/+	+/+	+/+	+/-	+/-	+/+	+/-	-/+	+/-	-/-	-/-
S	nd	+/+	+/+	+/+	+/+	+/+	+/+	+/-	-/-	+/+	-/-	-/-	-/-

Teals were inoculated with A/Chicken/Netherlands/621557/03 H7N7 virus. I, inoculated bird; S, contact bird; x/y, tracheal swab/cloacal swab; nd, not determined. [a]day after challenge. [b]Teal was suffering from severe foot problems from day 5, and died on day 12.

that teals can spread and transmit HPAI virus without overt disease symptoms has implications for the detection and control of avian influenza infections. One of the implications is that a passive surveillance system in an infected area may not always be sufficient to detect HPAI infections.

Our results furthermore show that a single vaccination with an inactivated H7N1 vaccine does not protect pheasants and teals from infection with HPAI H7N7 virus. All inoculated pheasants and 90% of the inoculated teals still became infected and excreted virus. This is in contrast with our earlier study in chickens, in which it was found that two weeks after a single vaccination all inoculated chickens were completely protected against infection (Van der Goot et al., 2005). Vaccination does, however, reduce the disease symptoms in both pheasants and teals. This reduction is most obvious in the pheasants in which disease symptoms are almost absent

Table 5. Transmission of H7N7 in vaccinated pheasants

Pheasant	D 1[a]	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10	D 14	D 17	D21	D 24
I	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/-	-/-	-/-	-/-	-/-
I	+/-	+/-	+/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/-
I	+/-	+/-	+/+	+/-	+/+	+/-	-/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-
I	+/-	+/-	+/-	-/+	-/+	-/+	-/+	+/-	+/+	+/+	-/+	-/+	-/-	-/-
I	+/-	-/+	+/+	-/-	-/+	-/-	-/-	+/+	+/-	+/-	-/-	-/-	-/-	-/+
S	nd	-/-	-/-	+/+	+/+	+/+	+/+	-/-	+/-	+/+	+/-	-/-	-/-	-/-
S	nd	-/-	-/+	+/+	+/+	+/+	+/+	+/-	+/+	+/-	-/-	-/-	-/-	-/-
S	nd	-/-	+/+	+/+	+/-	+/+	+/+	-/-	+/+	+/+	-/+	-/+	-/-	-/-
S	nd	-/-	-/-	+/+	+/-	+/-	+/-	+/-	+/+	+/+	-/+	-/+	-/-	-/-
S	nd	-/-	-/-	-/+	-/-	-/+	-/+	-/-	-/+	-/+	-/-	-/-	-/-	-/-

Pheasant	D 1[a]	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10	D 14	D 17	D21	D 24
I	+/-	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/-
I	+/-	+/-	+/+	+/+	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	+/-	+/-	+/+	+/+	-/+	-/+	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/-
I	+/-	+/-	-/+	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/-	-/-
S	nd	-/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/-	-/-	-/-	-/-
S	nd	+/-	-/-	+/+	+/+	+/-	+/+	+/+	+/+	-/+	-/-	-/-	-/-	-/-
S	nd	-/-	-/-	-/-	+/-	+/-	+/+	+/+	+/+	-/+	-/-	-/+	-/-	-/-
S	nd	-/-	+/-	+/+	+/+	+/+	+/+	+/+	-/+	-/-	-/-	-/-	-/-	-/+
S	nd	+/-	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/+	-/-	-/+	-/-	-/-

Pheasants were challenged with A/Chicken/Netherlands/621557/03 H7N7 virus, 14 days after a single vaccination with an inactivated H7N1 vaccine (A/Chicken/Italy/99).
I, inoculated bird; S, contact bird; x/y, tracheal swab/cloacal swab; nd, not determined. [a]day after challenge.

after vaccination. With respect to the effect of vaccination on transmission there is a clear difference between teals and pheasants. In the teals the reproduction number is significantly reduced by vaccination (p<0.001), while in the pheasants the reproduction number is not reduced. The experiment with vaccinated pheasants illustrates the risk of suboptimal vaccination which reduces disease symptoms while doing very little in terms of a reduction of virus transmission.

Immunity to influenza is mediated by both humoral and cell-mediated responses, but protection against avian influenza in chickens is mainly determined by antibodies against the hemagglutinin (Webster et al., 1991). Both pheasants and teals develop an HI titer after vaccination (Figure 1), but there is a difference in the onset of the antibody response. Seven days after vaccination there are already detectable HI titers in 55% of the teals but no HI titers in the

Table 6. Transmission of H7N7 in vaccinated teals

Teal	D 1[a]	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10
I	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-

Teal	D 1[a]	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10
I	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-

Teals were challenged with A/Chicken/621557/03 H7N7 virus, 14 days after a single vaccination with an inactivated H7N1 vaccine (A/Chicken/Italy/99). I, inoculated bird; S, contact bird; x/y, tracheal swab/cloacal swab; nd, not determined. [a]day after challenge.

pheasants. This difference still persists at the moment of challenge. It is tempting to conclude that the difference in HI titers is one of the reasons why the transmission of virus is significantly reduced in teals and not in pheasants. However, an earlier study showed that in chickens a mean HI titer of 0.25 (95% CI: 0.00-0.52) at the moment of challenge already reduced transmission significantly (Van der Goot et al., 2005). In pheasants the HI titer at the moment of challenge was 3.8 (95% CI: 2.9-4.8), and transmission was not reduced. Based on these findings we conclude that there is a difference in the relation between HI titers, and reduction of transmission in chickens and pheasants. Another way to look at vaccine-induced immunity is to measure VN titers, in vaccinated zoo birds it was found that there is a good degree of agreement between post vaccination HI titers and VN titers (Philippa et al., 2005). We performed a VN test with the sera of day 14 (the moment of challenge), and we also found a good correlation between the HI titers

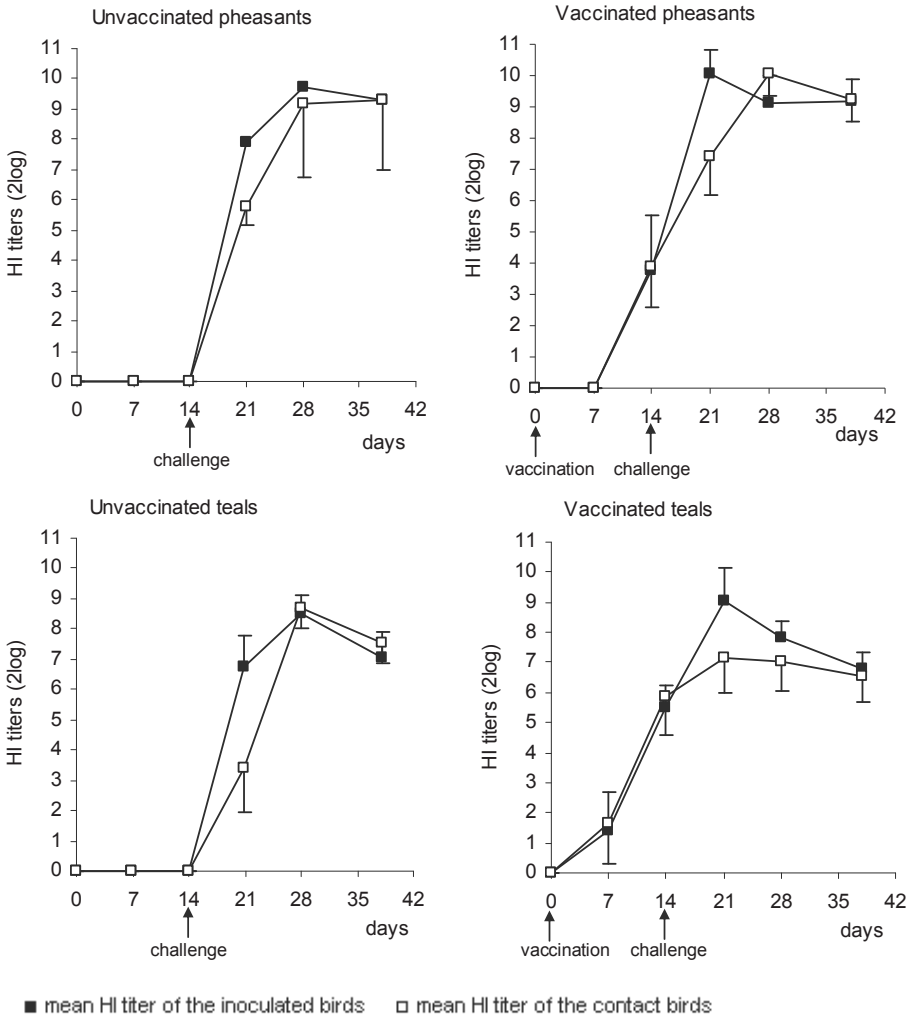

Figure 1. Mean HI titers of the golden pheasants and ringed teals. At day 0 birds in the vaccinated groups were vaccinated with A/Chicken/Italy/99 H7N1 (0.5 ml i.m.). At day 14 birds in all groups were challenged with 0.2 ml of diluted allantoic fluid containing 10^6 EID_{50} per ml (0.1 ml intranasaly and 0.1 ml intratracheally) of A/Chicken/Netherlands/621557/03 H7N7 virus. Vertical bars represent the 95% confidence interval, in the inoculated unvaccinated pheasant group no 95% confidence intervals are given because only 2 birds survived.

and the VN titers in pheasants (Spearman's correlation is 0.85) and teals (Spearman's correlation is 0.78). Despite the good correlation the VN titers are lower than the HI titers especially in the pheasants, where the VN titers at the moment of challenge have a mean of 1.9 and the HI titers have a mean of 3.8. In the teals the difference between VN titers (mean 4.5) and HI titers (mean 5.7) is less obvious. This might be an indication that in pheasants there is a different relation between HI titers, VN titers and protection against infection, disease and transmission than in teals or chickens, but whether this is significant and relevant needs further investigation.

The reproduction number of H7N7 virus in ringed teals and golden pheasants was estimated in an experimental setting. Extrapolation to the field, however, should always be done carefully. There are many factors that may influence the reproduction number: concurrent infections, density of the birds, heterogeneity in species and age, climate etc. The main advantage of an experimental setting is that it is possible to change just one variable, e.g. vaccination, and that the differences found between groups can be assigned directly to the treatment.

In all, our experiments have shown that the relation between disease and transmission may not be straightforward, and that infected birds without overt disease symptoms may still shed virus in such quantities that transmission is possible. With regard to vaccination it should be noted that the absence of symptoms does not necessarily imply that the virus spread is reduced or blocked.

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Chapter 5

Transmission of highly pathogenic avian influenza H5N1 virus in Pekin ducks is significantly reduced by a genetically distant H5N2 vaccine

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Abstract

Domestic ducks play an important role in the epidemiology of H5N1 avian influenza. Although it is known that vaccines that have a high homology with the challenge virus are able to prevent infection in ducks, little is yet known about the ability of genetically more distant vaccines in preventing infection, disease, and transmission. Here we study the effect of a widely used H5N2 vaccine (A/Chicken/Mexico/232/94/CPA) on the transmission of H5N1 virus (A/Chicken/China/1204/04) in ducks. The quantitative analyses show that despite the low level of homology between the virus and vaccine strain transmission was significantly reduced two weeks after a single or double vaccination. Mortality and disease rates were reduced markedly already one week after a single vaccination.

5.1 Introduction

Wild aquatic birds are the natural host species for avian influenza (Webster et al., 1992; Alexander, 2000). Most highly pathogenic avian influenza (HPAI) viruses replicate but do not cause disease symptoms in ducks (Alexander et al., 1978; Westbury et al., 1979; Wood et al., 1985; Alexander et al., 1986; Wood et al., 1995). In accordance with this finding, early Asian HPAI H5N1 viruses were non-pathogenic to ducks. However, since 2002 several H5N1 strains emerged that induce severe disease and mortality, while other H5N1 strains still cause asymptomatic infections (Sturm-Ramirez et al., 2004; Hulse-Post et al., 2005; Kishida et al., 2005; Sturm-Ramirez et al., 2005; Tian et al., 2005; Webster et al., 2006b; Beato et al., 2007; Middleton et al., 2007). Most HPAI viruses can be transmitted from infected to uninfected ducks, as has been shown in experimental studies (Alexander et al., 1978; Westbury et al., 1979; Alexander et al., 1986; Chen et al., 2004; Sturm-Ramirez et al., 2004; Sturm-Ramirez et al., 2005; Beato et al., 2007; Pantin-Jackwood et al., 2007). In the group of Asian H5N1 viruses there does not seem to be a clear correlation between the pathogenicity and the ability to spread from duck to duck. In fact, transmission is observed in strains that cause subclinical infections as well as in strains that cause severe disease and mortality (Sturm-Ramirez et al., 2004; Hulse-Post et al., 2005; Sturm-Ramirez et al., 2005; Beato et al., 2007; Pantin-Jackwood et al., 2007).

Domestic ducks play an important role in the epidemiology of HPAI H5N1 viruses. It has been shown that free-ranging ducks act as a reservoir of H5N1 avian influenza viruses (Songserm et al., 2006), and are a risk factor for the presence of H5N1 virus infections of domestic poultry (Gilbert et al., 2006; Gilbert et al., 2008). Since not all H5N1 infections are symptomatic, the

chance that infections in adult ducks are not detected is substantial. This was illustrated by Kwon et al. (Kwon et al., 2005), who showed that an H5N1 infection in adult breeder ducks was only detected after the 9 day old offspring showed signs of infection.

Vaccination is a potentially attractive tool for the prevention and control of avian influenza outbreaks. At present, vaccination against HPAI H5N1 is practiced in several countries e.g. Indonesia, People's Republic of China and Vietnam (OIE, 2008b). Vaccines based on different seed viruses are used, with different antigenic homology with the circulating field strains. In chickens it has been shown that the homology between the hemagglutinin of the vaccine strain and the challenge strain is an important factor in the reduction of virus shedding (Swayne et al., 1999; Swayne et al., 2000a).

Experimental studies investigating the effect of vaccination in ducks show that virus excretion is significantly reduced after vaccination (Tian et al., 2005; Webster et al., 2006a; Beato et al., 2007; Middleton et al., 2007). In fact, in most studies no virus could be isolated from the swabs at all. But it should be noticed that in most of these studies the homology between vaccine and virus strains is high, and that not all currently used vaccines have this high homology with the recent Asian H5N1 viruses. For this reason we are interested in the effectiveness of vaccines that are currently used and that have a larger genetic and antigenic distance from the Asian H5N1 viruses.

In this paper we report results of transmission experiments that were carried out to quantify the effectiveness of vaccination with a widely used H5N2 vaccine (A/Chicken/Mexico/232/94/CPA) in reducing disease and transmission of H5N1 virus (A/Chicken/China/1204/04) in Pekin ducks. In a transmission experiment a number of infected ducks is housed together with a number of uninfected ducks, and the infection chain is monitored on a regular basis. In our experiments disease symptoms and excretion of virus were monitored daily, while the antibody response was determined weekly. The aim of transmission experiments is to obtain estimates of the basic reproduction ratio (R), which is defined as the number of secondary infections that would be caused by one primary infected individual in a large population of susceptible individuals. If R exceeds 1, an infected animal infects on average more than one susceptible animal, and a chain reaction of infections may occur. If R is smaller than 1, a prolonged chain reaction of infections is not possible, and an epidemic cannot occur.

We show that a vaccine that is genetically and antigenically distant from the HPAI H5N1 virus protects the ducks against severe illness and mortality and significantly reduces the transmission of HPAI H5N1 in Pekin ducks.

5.2 Materials and methods

5.2.1 Ducks

Pekin ducks (*Anas platyrhynchos*) obtained from a duck farm were used in all experiments. The ducks were tested in the ELISA to detect antibodies against influenza prior to the experiments, and all were negative. An overview of the ages of the ducks is given in Table 6. The experiments were undertaken in a high containment unit under BSL3+ conditions at the Central Veterinary Institute of Wageningen UR. The experiments comply with the Dutch law on animal experiments and were reviewed by an ethical committee.

5.2.2 Virus and vaccine

The virus used in this study was A/Chicken/China/1204/04 H5N1. The virus was obtained from the Veterinary Laboratories Agency in Weybridge, is deposited in GenBank (accession number CAJ75442), and belongs to clade 2.4. The virus is also known under the name: A/Chicken/GxLA/1204/04, but since this is not a standard designation we did not use this name. For the transmission experiments ducks were inoculated both intranasaly and intratracheally with 0.1 ml diluted allantoic fluid containing $10^{6.0}$ (experiments with a single vaccination) or $10^{6.5}$ (experiments with a double vaccination) median egg-infectious dose (EID_{50}) virus per ml. The inoculation dose per duck was $10^{5.3}$ and $10^{5.8}$ EID_{50} respectively.

An inactivated oil emulsion vaccine was used based on the strain A/Chicken/Mexico/232/94/CPA H5N2 (Intervet Schering-Plough Animal Health, The Netherlands). The vaccine is commercially available, and widely used. A dosage of 0.5 ml vaccine was injected subcutaneously in the lower part of the neck, as recommended by the manufacturer. The protein homology of the genetically most variable region of the hemagglutinin (HA1) between the vaccine strain and the challenge strain is 84%.

5.2.3 Virus isolation

Swabs were put in 2 ml 2.95% tryptose phosphate buffer with 5 x 10^3 IU of penicillin-sodium and 5 mg streptomycin per ml. The swabs were stored at -70 °C until analyzed. Three embryonated chicken eggs (ECEs) incubated for 9 days were injected with 0.2 ml of the swab fluid per egg. After three days the allantoic fluid was harvested and a standard hemagglutination assay (HA) with chicken red blood cells was performed (OIE, 2008a). When at least one of the eggs was positive in the HA the swab was considered to be positive.

Table 1. Overview of the experiments

Treatment groups[a]	Age at vaccination	Age at challenge
Unvaccinated	na	8 weeks
Single vaccination, one week[b]	7 weeks	8 weeks
Single vaccination, two weeks[c]	6 weeks	8 weeks
Double vaccination, two weeks[d]	6 and 9 weeks	11 weeks

[a]Every treatment group consisted of two groups of 10 ducks; [b]Challenge one week after vaccination; [c]Challenge two weeks after vaccination; [d]Challenge two weeks after the second vaccination; na, vaccination was not applied.

5.2.4 RNA extraction and real time RT-PCR

Viral RNA was extracted using the MagNA Pure LC Total Nucleic Acid Isolation Kit and the MagNA Pure LC Instrument (Roche®). Fluid from the swabs (200 µl) was mixed with 300 µl lysis buffer. The rest of the procedure was followed as recommended by the manufacturer. Five µl of the extracted RNA was used in the RRT-PCR.

A one-tube RRT-PCR was performed to detect the matrix gene of the influenza virus. The Qiagen one-step RT-PCR kit was used with a 25 µl reaction mixture containing 1 µl of kit-supplied enzyme mixture, 1 µl dNTP mix, 4 units of RNase inhibitor (Promega, Madison, WI), 0.5 µM of each primer M-Fw (5`-CTTCTAACCGAGGTCGAAACGTA-3`), M-Rev (5`-CACTGGGCACGGTGAGC-3`), and 0.3 µM of probe M (5`-FAM-CTCAAAGCCGAGATCGCGCAGA-3`-TAMRA). The RRT-PCR was performed with the MX4000 (Stratagene®, Texas). The RT-PCR program consisted of 30 min at 50°C and 15 min at 95°C. A three-step cycling protocol was used as follows: 95°C for 5s, 58°C for 15s, and of 72°C for 20s for 45 cycles.

5.2.5 Logistic regression

The relation between virus isolation (y) and the RRT-PCR (x) was modeled by logistic regression:

$$\log\left(\frac{P(y=+)}{1-P(y=+)}\right) = a + b * \frac{x-1}{44}$$

Here, x = 1 was the lowest and x = 45 was the highest C_t value (for technical reasons a negative RRT-PCR result was given a C_t value of 45). The intercept *a* is associated with the highest probability for positive virus isolation (for C_t value x = 1), and (*a*+*b*) is associated with the lowest

probability for positive virus isolation (for C_t value x = 45). The logistic regression model was fitted with the statistical programming language GenStat (2007).

5.2.6 Antibody assays

The sera were incubated for 30 minutes at 56 °C, and subsequently incubated with 20% chicken erythrocytes at 4 °C overnight. The hemagglutination inhibition assay was performed by standard methods (OIE, 2008a). Briefly, the test was performed in V-bottom 96 well microtiter plates with 8 hemagglutinating units of the H5N1 challenge virus or the H5N2 vaccine virus and 1% v/v specific-pathogen-free chicken erythrocytes. A competitive ELISA that detects antibodies against the nucleoprotein of influenza A was performed, based on the ELISA that has been described before (De Boer et al., 1990).

5.2.7 Transmission experiments and quantification of transmission

An overview of the experiments is given in Table 6. Within every experimental group all ducks were given the same treatment, and all experiments were performed in duplicate. The design of the experiments is as follows: five ducks are inoculated with A/Chicken/China/1204/04 H5N1 HPAI virus, and 24 hours later these five ducks are housed together with five uninfected contact birds. To monitor the infection chain tracheal and cloacal swabs are taken daily during the first ten days and twice a week for the next 11 days. The experiments were terminated 21 days after challenge.

The statistical analyses are based on a stochastic SEIR epidemic model in which individuals are susceptible (S), latently infected (i.e. infected but not yet infectious) (E), infected and infectious (I), and recovered and immune or dead (R) (Anderson and May, 1991). The aim is to obtain estimates of the basic reproduction ratio (R), as well as the infectious period and the transmission rate parameter (β). Here we rely on a final size method and a Generalized Linear Model (GLM). Technical details can be found in previous papers (Becker, 1989; De Jong and Kimman, 1994; Van der Goot et al., 2003; Van der Goot et al., 2005; Van der Goot et al., 2007; Velthuis et al., 2007). The analyses are based on the results of the virus isolation in ECEs. For the final size method we took an exponentially distributed infectious period. For the GLM we have assumed a latent period of 1 day, and the infectious periods are directly observed from the infected contact birds. The analyses were carried out in Mathematica 6.0 (final size analysis) and SPSS 15.0 (GLM).

5.3 Results

5.3.1 Clinical symptoms

In the unvaccinated groups several birds showed clinical symptoms (Table 1). Two inoculated ducks died, and eight other birds showed depression and/or conjunctivitis. In all vaccinated groups there was a marked reduction in the number of birds that showed symptoms. In the groups that were challenged one week after a single vaccination only two ducks showed conjunctivitis (Table 2). In the groups that were challenged two weeks after a single vaccination three ducks showed symptoms: one duck showed conjunctivitis, one duck had a swollen oropharynx (possibly due to the swabbing), and one duck showed conjunctivitis and a swollen oropharynx (Table 3). In the groups that received a double vaccination two ducks showed conjunctivitis (Table 4).

5.3.2 Effect of vaccination on virus excretion and transmission

In the unvaccinated groups virus was isolated from all inoculated and all contact ducks (Table 1). The reproduction ratio based on the final size method is $R > 1.5$ with 95% confidence. The transmission rate parameter calculated using the Generalized Linear Model (GLM) is 4.7 (day^{-1}) (95% CI: 2.3-9.4), and the infectious period is 4.3 days (95% CI: 3.8-4.8) (Table 5).

In the groups that were challenged one week after a single vaccination virus was also isolated from all inoculated and all contact ducks (Table 2). Again, the estimate of the reproduction ratio based on the final size method is $R > 1.5$ with 95% confidence. The transmission rate parameter calculated using the GLM is 2.7 (day^{-1}) (95% CI: 0.87-8.6), and the infectious period is 3.4 days (95% CI: 2.9-3.9) (Table 5).

In the groups that were challenged two weeks after a single vaccination virus was isolated from nine of the ten inoculated ducks and from three contact ducks (Table 3). The estimate of the reproduction ratio R based on the final size method is 0.6 (95% CI: 0.1-2.2). The transmission rate parameter calculated using the GLM is 0.23 (day^{-1}) (95% CI: 0.09-0.55), and the infectious period is 3.3 days (95% CI: 1.9-4.8) (Table 5). There is a significant difference ($p = 0.01$) between the reproduction ratios R of the unvaccinated groups and the groups that were challenged two weeks after a single vaccination.

In the groups that were challenged two weeks after a double vaccination virus was isolated from four of the nine inoculated ducks and from one contact duck (Table 4). In one group one duck died before the start of the experiment, therefore four instead of five ducks were inoculated. The estimate of the reproduction ratio based on the final size method is 0.2 (95% CI: 0.005-1.5) (Table 5).

Table 2. Transmission of H5N1 in unvaccinated ducks

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10	Symptoms
I	+/-	+/+	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	a
I	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-
I	+/+	+/+	+/+	+/+	†						b
I	+/-	+/-	+/+	+/+	+/-	-/+	-/-	-/-	-/-	-/-	a,c
S	nd	+/-	+/-	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	a
S	nd	+/-	+/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	+/+	+/+	+/-	+/-	+/-	-/-	-/-	-/-	a
S	nd	+/-	+/-	+/+	+/-	+/-	-/-	-/-	-/-	-/-	-

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10	Symptoms
I	+/-	+/-	+/+	+/+	+/+	+/-	-/-	-/-	-/-	-/-	a
I	+/+	+/+	+/+	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-
I	+/+	+/+	+/+	+/-	+/+	†					b
I	+/-	+/+	+/+	+/+	+/-	+/-	-/-	-/-	-/-	-/-	a
I	+/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	a,c
S	nd	+/-	+/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-
S	nd	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	a
S	nd	+/-	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-

Pekin ducks were inoculated with A/Chicken/China/1204/04 H5N1 virus. Two duplicate experiments were performed. [a]Day after challenge; I, inoculated bird; S, contact bird; nd, not determined; †, duck died; x/y, results of the virus isolation in ECE's of the tracheal swab (x) and the cloacal swab (y); grey box, trachea and/or cloacal swab was positive in the virus isolation; a, conjunctivitis; b, bird died; c, depressed; -, no symptoms were observed.

The GLM could not be applied because one contact duck became positive before inoculated ducks became positive in the virus isolation. Here we also see that there is a significant difference (p = 0.002) between the reproduction ratios R of the groups that received a double vaccination and the unvaccinated groups (Table 5).

The results of the RRT-PCR are shown in Figure 1, and in Tables 7-10 of the supplementary data. The mean cycle threshold (C_t) values of the tracheal swabs of the inoculated ducks of each group are given, and it shows that vaccination reduces virus excretion of the ducks already at one week after a single vaccination (Figure 1). Logistic regression was applied to the results of the virus isolation (positive or negative) and the RRT- PCR (C_t value) of the tracheal swabs of the experiments with the unvaccinated ducks and the ducks that received a single vaccination

Table 3. Transmission of H5N1 in ducks one week after a single vaccination

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10	Symptoms
I	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	-/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	-/-	-/-	+/-	+/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10	Symptoms
I	+/+	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	a
I	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	-/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/+	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	a

Pekin ducks were inoculated with A/Chicken/China/1204/04 H5N1 virus, one week after a single vaccination with an inactivated H5N2 vaccine (A/Chicken/Mexico/232/94/CPA). Two duplicate experiments were performed. [a]Day after challenge; I, inoculated bird; S, contact bird; nd, not determined; x/y, results of the virus isolation in ECE's of the tracheal swab (x) and the cloacal swab (y); grey box, trachea and/or cloacal swab was positive in the virus isolation; a, conjunctivitis; -, no symptoms were observed.

(n=617). The estimates for the intercept (*a*) and slope (*b*) parameters are: $a = 19.34$ (SE: 2.17), $b = -27.87$ (SE: 3.14). The analyses show that when the C_t value of the swab is ≥ 36.2 the probability of a positive result in the virus isolation is smaller than 5%, and this can be used as a cut-of value for the RRT-PCR. When the result of the RRT-PCR is negative, the probability of a positive result in the virus isolation is 0.0002. This implies that the RRT-PCR can be used as a pre-screening, and we decided that only swabs with a positive RRT-PCR result from the experiment with a double vaccination were inoculated in ECEs.

Table 4. Transmission of H5N1 in ducks two weeks after a single vaccination

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10	Symptoms
I	-/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	d
I	-/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	+/-	+/+	+/+	+/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10	Symptoms
I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	a
I	+/-	+/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	a,d
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	+/-	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-

Pekin ducks were inoculated with A/Chicken/China/1204/04 H5N1 virus, two weeks after a single vaccination with an inactivated H5N2 vaccine (A/Chicken/Mexico/232/94/CPA). Two duplicate experiments were performed. [a]Day after challenge; I, inoculated bird; S, contact bird; nd, not determined; x/y, results of the virus isolation in ECE's of the tracheal swab (x) and the cloacal swab (y); grey box, trachea and/or cloacal swab was positive in the virus isolation; a, conjunctivitis; d, swollen oropharynx; -, no symptoms were observed.

5.3.3 Serology of the ducks after vaccination

The hemagglutination inhibition (HI) assay was performed with the challenge virus (H5N1) or the vaccine virus (H5N2) as antigen. In the groups that were challenged one week after a single vaccination only 2/20 ducks had a measurable HI titer with the H5N1 antigen ($\log_2$ titers: 1.0 and 1.7) at the moment of challenge (Figure 2). When the HI test was performed with the H5N2 antigen 11/20 ducks had a measurable titer with a $\log_2$ geometric mean titer (GMT) of 1.5 (95% CI: 0.8-2.1) (Figure 2). In the groups that were challenged two weeks after a single vaccination

Table 5. Transmission of H5N1 in ducks two weeks after a double vaccination

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10	Symptoms
I	-	-	-	-	-	-	-	-	-	-	-'
I	-	-	-	+/-	-	-	-	-	-	-	-'
I	-	-	-	-	+/-	-	-	-	-	-	-'
I	-	-	-	-	-	-	-	-	-	-	-'
S	nd	+/-	+/-	+/+	+/-	+/-	-	-	-	-	a
S	nd	-	-	-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	a

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10	Symptoms
I	-	-	-	-	-	-	-	-	-	-	-'
I	+/-	+/-	+/-	+/-	+/-	-	-	-	-	-	-'
I	-	-	-	-	-	-	-	-	-	-	-'
I	-	-	-	-	-	-	-	-	-	-	-'
I	+/-	+/-	+/-	+/-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	-'

Pekin ducks were inoculated with A/Chicken/China/1204/04 H5N1 virus, two weeks after a double vaccination with an inactivated H5N2 vaccine (A/Chicken/Mexico/232/94/CPA). Two duplicate experiments were performed. [a]Day after challenge; I, inoculated bird; S, contact bird; nd, not determined; -, swab was negative in the RRT-PCR; x/y, results of the virus isolation in ECE's of the tracheal swab (x) and the cloacal swab (y); grey box, trachea and/or cloacal swab was positive in the virus isolation; a, conjunctivitis; -', no symptoms were observed.

3/20 ducks had a $\log_2$ titer of 1.0 or 1.7 with the H5N1 antigen at the moment of challenge. When the HI test was performed with the H5N2 antigen 12/20 ducks had a measurable HI titer, with a $\log_2$ GMT of 2.5 (95% CI:1.4-3.6) (Figure 2). In the groups that received a double vaccination 17/19 ducks had a measurable HI titer with the H5N1 antigen with a $\log_2$ GMT of 3.1 (95% CI: 2.1-4.1) and 18/19 ducks had a measurable HI titer with the H5N2 antigen with a $\log_2$ GMT of 6.6 (95% CI: 5.5-7.7) at the moment of challenge (Figure 2).

Table 6. Overview of the statistical analysis of the transmission experiments

Treatment group	Final size	*R* (final size) (95% CI)	H_0:$R \geq 1$	H_0:R_v=R_c	Infectious period (day) (95% CI)	Transmission rate parameter (day^{-1}) (95% CI)	*R* (GLM)
Unvaccinated	5,5	>1.5	ns		4.3 (3.8-4.8)	4.7 (2.3-9.4)	20
Single vaccination (challenge: 1 week)	5,5	>1.5	ns	ns	3.4 (2.9-3.9)	2.7 (0.87-8.6)	9.2
Single vaccination (challenge: 2 weeks)	1,2	0.6 (0.1-2.2)	ns	0.01	3.3 (1.9-4.8)	0.23 (0.09-0.55)	0.76
Double vaccination (challenge: 2 weeks)	1,0	0.2 (0.005-1.5)	0.08	0.002	5 (n=1)	na	na

CI, confidence interval; R_v, reproduction ratio amongst vaccinated ducks; R_c, reproduction ratio amongst unvaccinated ducks; GLM, generalized linear model; ns, not significant; na, not applicable.

5.4 Discussion

The effect of the protein homology of the HA1 of the vaccine and challenge strain on virus shedding after challenge has been studied systematically in chickens (Swayne et al., 1999; Swayne et al., 2000a; Swayne et al., 2000b; Lee et al., 2004). When the protein homology of the HA1 varied from 96.8 to 100% no positive correlation was found between the sequence identity of the HA1 from the vaccine strain and challenge virus, and the ability to reduce the quantity of challenge virus shed from the cloaca or oropharynx (Swayne et al., 1999). When a fowl pox vectored vaccine containing the hemagglutinin (HA) of H5 and a variety of challenge strains was used with a HA1 protein homology varying from 87.3 to 100% there was a significant positive correlation in hemagglutinin sequence similarity between challenge viruses and vaccine, and the ability to reduce titers of challenge virus isolated from the oropharynx (Swayne et al., 2000a). In a study where the percentage of amino acid similarities of the HA1 ranged from 84.6 to 99.7% it was also found that the level of virus shedding in the trachea was correlated with the antigenic differences of vaccine and challenge strains (Lee et al., 2004). Transmission in chickens was completely stopped after a single vaccination with vaccine strains that had a protein homology of the HA1 of 92 and 98% with the challenge strain (Van der Goot et al., 2005).

Vaccination against HPAI virus in ducks has been studied less systematically. Previous studies in ducks generally used vaccines that had a high homology with the challenge virus. In most experiments the hemagglutinin of the vaccine and challenge strains both belonged to the

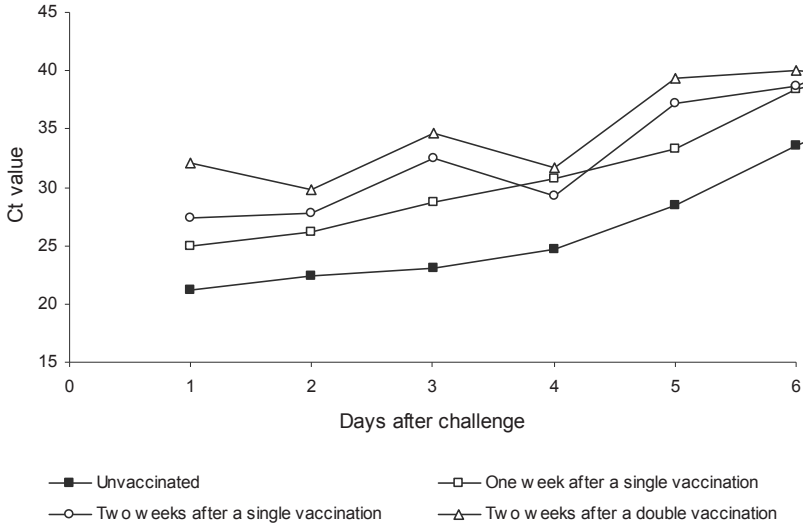

Figure. 1. C_t values of the RRT-PCR of the tracheal swabs of the inoculated ducks. The mean C_t values of the groups are given.

Eurasian H5N1 subtype, and these vaccines were able to completely prevent virus excretion (Tian et al., 2005; Webster et al., 2006a; Middleton et al., 2007). Two studies used viruses and vaccines that were genetically more distant (Beato et al., 2007; Middleton et al., 2007). Middleton et al. used a vaccine with an H5 virus (A/Chicken/Italy/22A/98 H5N9) with a protein homology of the HA1 of ~89% with the H5N1 challenge virus, and did find some virus shedding. Beato et al. used a vaccine based on the A/Duck/Potsdam/1402/86 H5N2 virus with a protein homology of the HA1 of 89% with the H5N1 challenge strain and did not isolate any virus. When the effect of vaccination on transmission was studied, no transmission from inoculated to contact ducks was detected (Webster et al., 2006b; Beato et al., 2007).

In these previous studies vaccines that had a high degree of homology (89-100%) with the HA1 of the challenge strain were able to completely protect ducks against morbidity and mortality, virus excretion and transmission. However, not all currently used vaccines have such a high degree of homology with the circulating H5N1 field viruses. Reasons to choose a genetically more distant vaccine may be the availability of vaccines or the choice for a vaccine

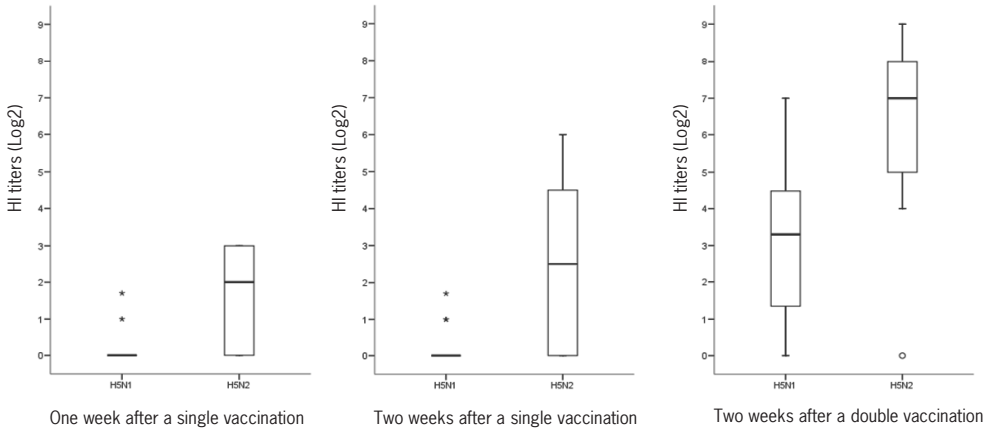

Figure. 2. Hemagglutination inhibition titers of the ducks prior to challenge. The HI test is performed with the challenge strain (H5N1) or the vaccine strain (H5N2). Each box shows the median value of the HI titers (black line), the interquartile range (upper and lower boundaries of the box), and the range of the HI titers (whiskers). ○, outlier, defined as cases with values between 1.5 and 3 box lengths from the upper or lower edge of the box; ⁎, extreme value, defined as cases with values more than 3 box lengths from the upper or lower edge of the box.

with a different neuraminidase to be able to adopt a DIVA (Differentiating Infected from Vaccinated Animals) strategy (Capua et al., 2002). In ducks little is known about the effectiveness of genetically more distant vaccines in preventing infection, disease, and transmission. In this paper we show that a widely used H5N2 vaccine strain that has a HA1 protein homology of 84% with the H5N1 challenge virus not only prevented severe morbidity and mortality but also significantly reduced virus excretion and transmission of H5N1 in ducks two weeks after vaccination.

Viruses and vaccines can be compared based on protein sequences of the HA1. It should be kept in mind, however, that although there is a correlation between genetic and antigenic distance, it is also possible that specific genetic differences of only one amino acid lead to a substantial difference in antigenic distance (Smith et al., 2004). For influenza it has been observed that antigenic distance is linearly related to the logarithm of the HI measurement. This principle is the basis for the construction of antigenic maps in which antigenic distances are visualized (Smith et al., 2004). In our study the low level of genetic homology between virus and vaccine is confirmed antigenically in the HI assay. Figure 2 shows that with the use of the H5N2 antigen in the HI test titers are substantially higher than if it is carried out with the H5N1 challenge antigen.

Although there is a clear relation between HI titers and antigenic distance it is difficult to relate antigenic distance to the level of protection and reduction of transmission. We find a substantial difference (3.5 twofold dilutions) in the HI assay between the H5N1 and the H5N2 viruses, but the vaccine is still able to reduce transmission and virus excretion and to protect the birds against mortality and severe morbidity. Human influenza vaccines are updated when there is an antigenic difference of at least 2 twofold dilutions in the HI assay (Smith et al., 2004). It would be a challenge to develop corresponding criteria for avian vaccines. However it should be borne in mind that there are various differences between avian and human vaccination such as host differences, vaccination schedules, and the use of different adjuvants.

The experimental set up could potentially influence the outcome of transmission experiments. One of the factors that are of importance is the moment of adding the contact birds to the inoculated birds. In our experiments contact ducks were added at 1 day after inoculation while in previous studies, where no transmission was found, the contact animals were added at day 3 after inoculation (Webster et al., 2006a; Beato et al., 2007). Since the dynamics of avian influenza virus transmission in birds occur on a fast time scale of a few days (Van der Goot et al., 2003; Van der Goot et al., 2005; Van der Goot et al., 2007), it is important that the contact birds are added quickly after inoculation. Tables 1-4 indicate that the infectious periods of the inoculated birds are short (1-5 days). Hence, if the contact birds are added late, this may result in an underestimation of the overall transmission level.

Two ducks in our experiments remained negative in both HI tests and in the Np-antibody Elisa after vaccination, which makes the fraction of non responders in our experiments 0.034 (95% CI: 0-0.08). Both ducks were infected, shed virus and seroconverted in the experiments. One of these birds suffered from conjunctivitis while the other showed no symptoms. These facts may seem of little importance but if the reproduction ratio in unvaccinated birds is high enough, even a low fraction of non responders may turn attempts to prevent outbreaks by vaccination futile. For instance, epidemiological theory states that the critical fraction of the population that

needs to be immune to obtain herd immunity, p_c, is related to the reproduction ratio R of an unvaccinated population through: $p_c=1-1/R$. Our point estimate of the reproduction ratio of the unvaccinated group is 20. This implies that at least 95% of the ducks should be fully protected by vaccination to obtain herd immunity. This may be difficult to achieve in an experimental setting, let alone under field conditions.

Overall, our results demonstrate that a widely used H5N2 vaccine strain with a low level of genetic and antigenic homology with the H5N1 challenge virus is able to reduce transmission in ducks significantly. Whether this is true in general remains to be investigated, and therefore it is important to explore the effectiveness of avian vaccines related to their genetic and antigenic distance to circulating field viruses.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2008.08.037.

Table S7. C_t values of the unvaccinated ducks

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10
I	22.2	23.3	27.4	31.2	28.1	34.1	38.0	-	-	-
I	19.8	20.1	20.5	25.1	27.6	28.1	40.3	37.0	39.3	-
I	20.6	23.0	22.7	26.6	30.9	32.1	38.8	40.8	-	-
I	20.2	21.0	21.7	21.9	†					
I	21.1	22.1	26.2	25.1	31.2	36.4	37.7	36.4	-	-
S	nd	26.2	23.7	22.4	29.0	35.2	37.1	-	-	-
S	nd	17.2	23.8	25.3	29.9	32.3	36.0	40.7	39.9	42.1
S	nd	32.0	25.7	23.4	32.7	33.2	40.3	-	38.8	-
S	nd	39.1	30.4	27.6	22.5	27.4	29.8	36.1	39.1	42.0
S	nd	25.0	25.1	25.5	28.3	28.1	35.9	36.2	40.5	-

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10
I	21.0	22.1	20.9	22.2	24.9	35.3	37.3	-	40.6	40.2
I	19.4	21.0	21.5	25.4	29.2	33.4	39.3	41.1	-	-
I	22.3	25.5	23.2	22.2	24.8	†				
I	22.1	21.4	22.2	23.1	31.0	36.3	38.3	-	38.3	-
I	22.7	24.9	24.1	23.8	28.9	33.2	33.4	36.7	-	-
S	nd	21.3	24.8	32.9	30.7	34.7	35.4	39.8	36.5	-
S	nd	32.3	26.9	28.6	30.0	31.5	34.9	39.7	40.4	-
S	nd	27.5	23.1	22.5	26.5	33.3	36.2	40.8	40.8	-
S	nd	20.4	22.7	22.4	21.5	35.2	37.6	36.4	39.0	35.1
S	nd	27.6	24.4	22.0	24.2	29.0	33.6	37.7	-	39.2

Pekin ducks were inoculated with A/Chicken/China/1204/04 H5N1 virus. Two duplicate experiments were performed. [a]Day after challenge; I, inoculated bird; S, contact bird; nd, not determined; †, duck died; dark grey box: tracheal and cloacal swab positive in ECEs; light grey box: tracheal swab positive in ECEs, cloacal swab negative in ECEs; dotted box: tracheal swab negative in ECEs, cloacal swab positive in ECEs; no colour: tracheal and cloacal swab negative in ECEs; number in the box, cycle threshold value (RRT-PCR) of the tracheal swab; -, tracheal swab negative in the RRT-PCR.

Table S8. C_t values of the ducks that were challenged one week after a single vaccination

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10
I	26.9	33.4	36.4	37.1	-	-	-	-	-	-
I	24.4	24.3	27.3	24.3	31.8	-	-	-	-	-
I	23.9	25.1	31.4	29.6	-	-	-	-	-	-
I	38.9	24.6	23.7	26.3	31.7	36.3	40.8	-	-	-
I	24.9	30.6	-	35.8	-	-	-	-	-	-
S	nd	39.8	-	39.8	26.3	25.7	34.6	-	40.1	-
S	nd	24.2	28.8	31.6	36.9	40.2	-	-	-	-
S	nd	26.3	26.7	29.5	30.4	-	43.6	-	-	-
S	nd	24.1	26.6	26.5	31.1	34.9	40.0	-	-	-
S	nd	23.9	26.0	21.4	23.4	38.1	41.9	34.6	-	-

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10
I	21.5	21.3	22.4	25.2	30.1	38.3	-	-	-	39.9
I	22.1	25.1	25.8	33.6	36.7	40.7	-	-	-	-
I	21.6	24.2	26.4	25.3	36.2	-	-	41.0	-	-
I	23.6	27.6	29.4	30.3	-	-	-	-	-	-
I	21.5	25.2	36.3	40.2	-	-	-	-	-	-
S	nd	31.0	29.9	31.4	36.5	-	-	-	-	-
S	nd	22.1	25.5	19.5	26.3	40.3	40.6	40.9	-	-
S	nd	27.4	28.7	31.7	32.4	-	-	-	-	-
S	nd	18.6	25.2	24.5	25.8	-	-	-	-	-
S	nd	25.3	26.2	26.4	33.3	-	-	-	-	-

Pekin ducks were inoculated with A/Chicken/China/1204/04 H5N1 virus, one week after a single vaccination with an inactivated H5N2 vaccine (A/Chicken/Mexico/232/94/CPA). Two duplicate experiments were performed. [a]Day after challenge; I, inoculated bird; S, contact bird; nd, not determined; dark grey box: tracheal and cloacal swab positive in ECEs; light grey box: tracheal swab positive in ECEs, cloacal swab negative in ECEs; no colour: tracheal and cloacal swab negative in ECEs; number in the box, cycle threshold value (RRT-PCR) of the tracheal swab; -, tracheal swab negative in the RRT-PCR.

Table S9. C_t values of the ducks that were challenged two weeks after a single vaccination

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10
I	-	22.6	28.4	26.0	32.6	38.7	45.0	-	40.0	-
I	24.4	29.8	35.2	38.8	-	-	-	-	-	-
I	24.3	27.5	39.1	30.6	-	40.2	-	-	-	-
I	27.3	32.1	37.5	-	-	-	-	41.1	-	-
I	39.5	21.1	27.2	28.8	37.5	-	-	-	-	-
S	nd	-	26.4	28.7	23.6	29.7	37.6	39.8	37.4	-
S	nd	-	-	-	-	-	-	41.0	-	-
S	nd	-	34.5	34.5	35.6	-	-	-	-	-
S	nd	-	-	-	-	-	-	-	-	-
S	nd	-	-	-	-	-	-	-	-	-

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10
I	-	-	-	-	-	-	40.2	-	-	-
I	24.5	28.0	29.2	29.3	40.0	-	-	-	-	-
I	27.5	38.8	40.5	-	-	-	-	-	-	-
I	24.5	24.7	24.3	24.1	38.7	40.5	-	-	-	-
I	27.2	25.3	30.6	27.1	-	-	43.7	-	-	-
S	nd	-	-	-	-	-	-	-	-	-
S	nd	-	-	-	-	-	-	-	-	-
S	nd	-	-	-	-	-	-	-	-	-
S	nd	29.8	27.4	28.6	34.2	37.1	-	-	-	-
S	nd	-	29.5	25.1	24.5	28.9	39.5	39.3	40.1	-

Pekin ducks were inoculated with A/Chicken/China/1204/04 H5N1 virus, two weeks after a single vaccination with an inactivated H5N2 vaccine (A/Chicken/Mexico/232/94/CPA). Two duplicate experiments were performed. [a]Day after challenge; I, inoculated bird; S, contact bird; nd, not determined; dark grey box: tracheal and cloacal swab positive in ECEs; light grey box: tracheal swab positive in ECEs, cloacal swab negative in ECEs; no colour: tracheal and cloacal swab negative in ECEs; number in the box, cycle threshold value (RRT-PCR) of the tracheal swab; -, tracheal swab negative in the RRT-PCR.

Table S10. C_t values of the ducks that were challenged two weeks after a double vaccination

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10
I	37.8	-	-	32.6	-	-	-	-	-	-
I	-	41.0	40.0	33.0	-	-	-	-	-	-
I	39.7	-	34.6	34.7	38.5	-	-	-	-	-
I	-	-	40.1	36.7	-	-	-	-	-	-
S	nd	25.6	19.2	19.3	21.6	27.9	37.0	38.1	39.8	39.9
S	nd	-	36.4	-	-	-	-	-	-	-
S	nd	-	-	-	-	-	-	-	-	-
S	nd	-	-	-	-	-	-	-	-	-
S	nd	-	34.3	-	-	-	-	-	-	-

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10
I	28.9	-	-	-	-	-	-	-	-	-
I	31.7	21.0	27.3	27.9	38.7	40.1	39.1	-	-	-
I	-	-	43.2	-	-	-	-	-	-	-
I	-	-	-	-	-	-	-	-	-	-
I	22.6	27.4	22.4	25.2	40.8	-	-	-	-	-
S	nd	-	-	-	-	-	-	-	-	-
S	nd	-	-	-	-	-	-	-	-	39.3
S	nd	-	-	-	-	-	-	-	-	-
S	nd	-	40.1	-	-	-	-	-	-	-
S	nd	-	40.5	-	-	-	-	-	-	-

Pekin ducks were inoculated with A/Chicken/China/1204/04 H5N1 virus, two weeks after a double vaccination with an inactivated H5N2 vaccine (A/Chicken/Mexico/232/94/CPA). Two duplicate experiments were performed. [a]Day after challenge; I, inoculated bird; S, contact bird; nd, not determined; -, swab was negative in the RRT-PCR; dark grey box: tracheal and cloacal swab positive in ECEs; light grey box: tracheal swab positive in ECEs, cloacal swab negative in ECEs; number in the box, cycle threshold value (RRT-PCR) of the tracheal swab; -, tracheal swab negative in the RRT-PCR.

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Chapter 6

Validation of diagnostic tests for detection of avian influenza in vaccinated chickens using Bayesian analysis

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Abstract

Vaccination is an attractive tool for the prevention of outbreaks of highly pathogenic avian influenza in domestic birds. It is known, however, that vaccination does not always provide perfect protection against infection, and that the detection of infection in vaccinated birds can be problematic. Here, we investigate the characteristics of three serological tests (immunofluorescent antibody test (iIFAT), neuraminidase inhibition (NI) assay, and NS1 ELISA) that are able to differentiate infected from vaccinated animals. To this end, data of H7N7 infection experiments are analyzed using Bayesian methods of inference. These Bayesian methods enable validation of the tests in the absence of a gold standard, and allow one to take into account that infected birds do not always develop antibodies after infection. The results show that the N7 iIFAT and the NI assay have sensitivities for detecting antibodies of 0.95 (95% CI: 0.89-0.98) and 0.93 (95% CI: 0.78-0.99), but substantially lower sensitivities for detecting infection: 0.64 (95% CI: 0.52-0.75) and 0.63 (95% CI: 0.49-0.75). The NS1 ELISA has a low sensitivity for both detecting antibodies (0.55 (95% CI: 0.34-0.74)) and infection (0.42 (95% CI: 0.28-0.56)). The estimated specificities of the N7 iIFAT and the NI assay are 0.92 (95% CI: 0.87-0.95) and 0.91 (95% CI: 0.85-0.95), and 0.82 (95% CI: 0.74-0.87) for the NS1 ELISA. Additionally, our analyses suggest a strong association between the duration of virus excretion of infected birds and the probability to develop antibodies.

6.1 Introduction

Vaccination against highly pathogenic avian influenza (HPAI) is one of the tools that can be used to prevent and control epidemics of HPAI. However, it is known that vaccinated birds may still be infected and shed virus without showing clinical symptoms (Swayne et al., 2000; Van der Goot et al., 2005; Van der Goot et al., 2007). This implies that there is a risk of silent spread of virus in vaccinated populations (Savill et al., 2006). Therefore, it is increasingly believed that vaccination programs should always be accompanied by active surveillance.

Surveillance programs can be based on the use of unvaccinated sentinels that act as indicators of virus circulation, on virus detection in the vaccinated birds, or on serological DIVA tests (differentiating infected from vaccinated animals) (CEC, 2006). Although the use of sentinels is relatively simple, it is often inconvenient, and the method is not validated. Virus detection in vaccinated birds has the disadvantage that it is only successful in the acute phase of

the infection. Therefore, serological DIVA tests are promising because birds can be randomly selected from a vaccinated flock and antibodies might persist for a long time.

DIVA tests for avian influenza can be based on the detection of antibodies against a heterologous neuraminidase or antibodies against NS1 (CEC, 2006; Suarez, 2005). The heterologous neuraminidase approach aims at detection of antibodies against the neuraminidase of the field strain that differ from those elicited by the neuraminidase of the vaccine strain. Such an approach was developed and applied as an indirect immunofluorescent antibody test (iIFAT) in LPAI outbreaks in Italy for the N1 serotype (Capua et al., 2003) and the N3 serotype (Cattoli et al., 2006). The neuraminidase inhibition (NI) assay, can also be used as a DIVA test in case of a heterologous neuraminidase (Van Deusen et al., 1983; World Health Organization, 2002). The NS1 DIVA strategy is based on the fact that the NS1 protein is produced during virus replication, and is not incorporated into virus particles. Consequently, after vaccination with inactivated vaccines no NS1 is produced, and no antibody response to the NS1 protein will be evoked. Thus NS1 antibodies in vaccinated animals indicate a preceding infection with field virus (Birch-Machin et al., 1997; Ozaki et al., 2001; Tumpey et al., 2005). A test based on antibodies against NS1 has the major advantage that it can be applied to every combination of a field strain and an inactivated vaccine strain. However, several disadvantages were reported: vaccinated chickens had low but detectable antibody titers to the NS1 protein, caused by the presence of small amounts of NS1 protein in the vaccine (Tumpey et al., 2005). Watson et al. (2008) showed that negative commercial flocks showed a high level of background in the NS1 fluorescence microsphere immunoassay (FMI), and cross reactivity with IBDV and avian pneumovirus was seen. Furthermore, a large variation in antibody levels between individual birds was observed, and the persistence of NS1 antibodies was poor (Watson et al., 2008).

Validation of serological DIVA tests is difficult to perform, because there is no gold standard test, and the true disease status of the animals is unknown. As a consequence, published information about the sensitivity and specificity of different DIVA tests for avian influenza is limited. Moreover, previous studies focused mainly on estimation of the specificity of DIVA tests, by testing sera of vaccinated uninfected birds, and did not or only sparingly consider test sensitivity. For instance, the specificity of the iIFAT was estimated at 95.0% (95% CI: 92.8-97.3) in vaccinated turkeys (Capua et al., 2003), and at 99.5% (95% CI: 98.5-100) in vaccinated chickens (Cattoli et al., 2006). The specificity of a NS1 ELISA was estimated at 94.4% (95%CI: 89.2-99.7) in vaccinated chickens (Zhao et al., 2005). In addition, Cattoli et al. (2006) reported an estimated sensitivity of 97.7% (95% CI: 96.3-99.0) for the N3 iIFAT in vaccinated turkeys. Their data set for chickens, however, was too small to enable estimation of the sensitivity with reasonable precision, even more because the true infection status of the individual animals was not known.

During the last decade Bayesian statistics and latent class models have been used increasingly to quantify the sensitivity and specificity of diagnostic tests in the absence of a gold standard test (Engel et al., 2008; Enoe, Georgiadis, and Johnson, 2000; Swildens et al., 2005). Using a Bayesian method, and sera of inoculated and contact-exposed vaccinated chickens we validated the above mentioned DIVA tests for the detection of avian influenza virus (H7N7) infections. Moreover, Suarez (2005) suggested that vaccinated and infected birds may not have enough virus replication to stimulate an antibody response to the novel viral proteins, and therefore may not seroconvert or seroconvert only at low levels. To take this into account our model allows for the possibilities whether or not antibodies are produced in infected birds.

Our results show that the N7 iFAT and the NI assay have sensitivities for detecting antibodies that exceed 0.90, with the accompanying specificity also exceeding 0.90. However, the sensitivities for detecting infection are substantially lower. The NS1 ELISA performed poor in this test validation. Additionally, our analyses suggest a strong association between the duration of virus excretion of infected birds and the probability to develop antibodies.

6.2 Materials and methods

6.2.1 Sera and swabs

Sera and swabs were obtained from transmission experiments with A/Chicken/Netherlands/621557/03 H7N7 HPAI (Van der Goot et al., 2005). In these experiments, groups of five inoculated chickens were housed together with five contact chickens, and the infection chain was monitored by virus isolation and serology during 21 days. All chickens were vaccinated with a commercial inactivated oil emulsion H7N1 (A/Chicken/Italy/99) or H7N3 (A/Chicken/Pakistan/95) vaccine one or two weeks prior to the inoculation. Tracheal and cloacal swabs were taken daily during the first ten days, and on days 14, 17 and 21 after inoculation. Swabs were cultured in embryonated chicken eggs. Sera were collected on the day of inoculation (n = 76), and 21 days after inoculation (n = 79). Details of the experimental procedures are given in (Van der Goot et al., 2005).

6.2.2 N7 iIFAT

The N7 iIFAT is based on the protocol of Capua et al. (2003), with minor modifications. The gene coding for the N7 neuraminidase of the A/Chicken/Netherland/621557/03 H7N7 HPAI virus was amplified by RT-PCR using the forward primer 5'-ACTAGT CCCGGG GGG TGA TTG AGA ATG AAT CCT AAT C-3', and the reverse primer 5'-GGTACC CCCGGG C GTT TTT KCA TTT TAC GAA AAG TAT TKG-3'. The forward primer contained *SpeI* and *XmaI*

restriction sites (underlined), and the reverse primer contained *KpnI* and *XmaI* restriction sites (underlined). For the transfection we used SF21 insect cells and FuGENE 6 transfection reagent (Roche). Plates were prepared and sera were tested as described by Capua et al. (2003).

6.2.3 NS1 ELISA

Cloning and expression of the NS1

A NS1 ELISA was developed using a recombinant NS1 antigen expressed with the PinPoint™ Xa Protein Purification System (Promega). The NS1 coding sequence of the A/Chicken/Netherlands/621557/03 H7N7 HPAI virus (from amino acid 8 to 230) was amplified by PCR using the forward primer 5'-TGTGTCAAGCTTTCAGGTAGACTGC-3' and the reverse primer 5'-CAGCCATCAGATCTCTTCAAACTTC-3'. The restriction sites *Hin*dIII (forward primer) and *Bgl*II (reverse primer) are underlined. The PCR fragment was ligated with the pGEM®-T Vector System I (Promega) in a pGEM vector. Plasmids were transformed in *E. Coli* DH5α cells, and plated on LB plates containing ampicillin, Isopropyl-β-D-thiogalactopyranoside (IPTG) and X-Gal. A miniprep was performed, and clones were digested with *Hin*dIII and *Bgl*II. The digested fragment was ligated in the Pinpoint vector Xa2 (PinPoint™ Xa Protein Purification System, Promega) with a Rapid DNA ligation kit (Roche). The Xa2 vector with the NS1 as well as an empty Xa2 vector were transformed into competent *E. Coli* JM109 cells (Promega). For protein expression the *E. Coli* JM109 cells were grown on Luria Broth (LB) plates containing 100 ug of ampicillin per ml. After one night of incubation the colonies from the plates were grown in 250 ml LB medium with biotin (2 uM final concentration) and ampicillin (100 ug/ml) at 32°C for 5 hours. IPTG (0.6M) was added, and the cultures were incubated at 20°C for 3 hours. The bacterial cultures were centifugated, and the pellets were stored at -20°C. After thawing the pellets were lysated in lysis buffer (50 mM Tris buffer pH 7.5, 50 mM NaCl, 5% glycerol) with protease inhibitor (Complete Mini tablet, 1 tablet in 10 ml lysis buffer, Roche), as a 10% v/v solution. The lysated pellets were sonificied, centrifuged and the supernatants were stored at -70°C.

NS1 ELISA

The working dilution of the NS1 protein is based on the OD_{450} of the positive control serum used in the test, of about 0.5. Streptavidin coated plates (Streptawell, Roche) were washed three times with PBST (PBS with 0.05% Tween 80). Hundred µl diluted NS protein and in the corresponding control wells 100 ul of diluted supernatant from the negative control (empty Xa2 vector) was added to the plates, and plates were incubated at 37°C for 1 h. Plates were washed six times with PBST. Sera were diluted 250 times in ELISA buffer (0.87 M NaCl with 5% horse serum), and 100 µl was added to the wells and control wells. Plates were incubated at 37°C for 1 h. Plates were washed six times with PBST. Goat anti-chicken IgG (H+L) conjugated to horseradish

peroxidase (SouthernBiotech) was diluted 5000 times in ELISA buffer. Plates were incubated at 37°C for 1 h. Plates were washed six times with PBST. Hundred µl of 3,3,5,5-tetramethyl benzidine (TMB, ID VET) was added as substrate, and the plates were incubated for at room temperature in the dark for 15 minutes. The reaction was stopped by adding 100 µl 0.025 M hydrofluoric acid solution (Stopsolution, ID VET). The OD_{450} of the control well with the empty Xa2 vector (background) was subtracted from the OD_{450} of the well with the Xa2 vector with the NS1 insert. Subsequently, the OD_{450} of each sample was expressed as a percentage of a positive control serum (S/P%).

6.2.4 Neuraminidae inhibition assay

The NI assay is based on the NI assay of the WHO (World Health Organization, 2002), with some modifications.

Day 1: As antigen A/Mallard/Denmark/75-64650/03 H5N7 was used. The amount of antigen was standardized based on the OD_{540} of a dilution series in phosphate buffer, and the dilution with an OD_{540} of 0.7 was chosen. Sera were diluted 5 times in phosphate buffer. Twenty five µl diluted serum and 25 µl diluted antigen were added in a micronic tube and mixed using a pipette. The mixture was incubated at 37 °C for 1 h, and 50 µl fetuin was added and mixed using a pipette. The mixture was then incubated at 37 °C o/n (18-19 hours).

Day 2: The micronics were cooled to 20 °C, and 25 ul periodate reagent was added and mixed by using a pipette. Then the mixtures were incubated at room temperature for exactly 20 minutes. Fifty µl of arsenite reagent was added and mixed by using a pipette until the brown color disappeared. Thiobarbituric acid reagent was added (250 µl) and mixed by using a pipette. The mixtures were then incubated in a boiling water bath for exactly 8 minutes. After 8 minutes the micronics were cooled in a -70 freezer for 5-10 minutes. Warenoff reagent was added (350 µl), the micronics were shaken vigorously, and centrifuged at 800 rpm for 20 minutes. Two hundred µl of the supernatant was added to a 96 wells microtiter plate. Extinctions were measured at 540 nm. In every plate 4 wells without antigen and serum were tested, the mean OD_{540} value of these wells was subtracted from the OD_{540} values of the samples. Then the percentage inhibition was calculated by the formula: $100- (OD_{sample} / OD_{neg}*100)$, where OD_{neg} is the mean OD_{540} of wells with negative chicken serum (Gibco).

6.2.5 The statistical model

An overview of the statistical model is shown in Table 1; technical details are given in the Appendix. Two variables D and d are introduced that relate to the state of the animals. Suppose that $D = 1$ when an animal is truly infected, and $D = 0$ otherwise, and that $d = 1$ when an animal is seropositive (antibodies present), and $d = 0$ otherwise. Because $D = 0$ implies $d = 0$, an animal

is in one of three possible true states: (D, d) = (0, 0), (1, 0) or (1, 1). D and d are unknown, except before challenge where animals are uninfected: $D = d = 0$. Note that e.g. $d = 0$ refers to the *true* antibody state of the animal and not to any (fallible) negative test result for that animal. So, although animals are not infected before challenge, some test results may be positive, when test specificity is below 1. The analysis contains results from two sera per chicken, collected just before inoculation, and 21 days later. The different parts of the model will be discussed below.

Prevalence of true infection

The prevalence (q) of true infection (D) within an experimental group of animals depends on the success of vaccination (π). In a group with a successful vaccination the prevalence is low, and in a group with an unsuccessful vaccination the prevalence is high.

Virus isolation (VI) and seroconversion

The number of days of positive virus isolation (VI) are entered in the analysis as data (Table 1). We distinguish between $VI = 0$, $1 \le VI \le 2$ and $VI \ge 3$, for a truly infected animal. P is the probability that a truly infected animal is positive in the virus isolation ($VI > 0$). Q is the probability that when a truly infected animal is positive in the virus isolation, it will be so for more than two days ($VI \ge 3$). P and Q relate to the sensitivity of virus isolation. With respect to specificity of virus isolation (β), probabilities β and (1-β) are assumed for a truly non-infected animal to show no virus isolation or one day of virus isolation. For a truly infected animal, the probabilities of seroconversion ($d = 1$) are p_0, p_1 and p_2, depending on whether $VI = 0, 1 \le VI \le 2$ or $VI \ge 3$ is observed. The Bayesian analysis will use days VI as (imperfect) information about the unknown true states D and d.

Sensitivity and specificity of the serological tests

The other source of information about antibody status (d), and indirectly about infection status (D) as well, are the test results. The model describes the (probabilistic) behavior of the tests in relation to antibody status d. Through the model we are able to estimate test specificity and sensitivity with respect to seroconversion (d), but also with respect to infection (D). Through the relationship in the model between infection and possible production of antibodies, the sensitivity of a test with respect to infection status D can be derived as well. The specificity of a test with respect to d and D is the same, because $D = 0$ implies $d = 0$. Details are given in the Appendix.

6.2.6 Bayesian inference

Bayesian inference involves combination of information in the data (days VI and + / - test results) with prior information about each of the parameters. Prior information was mainly derived from authors Jeanet van der Goot and Guus Koch, and based on results of related experiments. The combination of data and priors offers an up-to-date summary of all available information in the

Table 1. The general structure of the model

Data		Model structure	Parameters
		Vaccination for the i-th group is successful or not.	π
		↓	
		Prevalence q for the i-th group is generated depending on success of vaccination.	μ_1, μ_2, σ^2
		↓	
		Status *D* for infection is established for each animal. Each animal has a probability *q* to be infected and there are *qn* infected animals in a group.	q_i
		↓	
days *VI*	→	*VI* is generated, depending on *D*.	*P, Q,* β
		↓	
		Status *d* for seroconversion is established for each animal, depending on *D* and *VI*.	p_0, p_1, p_2
		↓	
test data *y*	→	Binary test results are generated, depending on status *d* [(a)]	$\delta_{-1}, \delta_{-2}, \delta_{-3}, \delta_{+1}, \delta_{+2}, \delta_{+3},$ $\varepsilon_{-12}, \varepsilon_{-13}, \varepsilon_{-23}, \varepsilon_{+12}, \varepsilon_{+13}, \varepsilon_{+23}$

[(a)] Note that the model describes the stochastic process that generates the data. This model is used in the MCMC process for inference about the unknown parameters (on the right). The actual data that feed the process are the observed days *VI* and test data *y* (on the left). Sensitivity and specificity follow as derived parameters (details in the appendix).

form of a posterior distribution for each parameter. Because this posterior is analytically intractable, a large sample from this distribution was generated by a Markov chain Monte Carlo (MCMC) algorithm, as implemented in WinBUGS (Spiegelhalter et al., 2003). The median of this sample was presented as a parameter estimate and the 2.5 and 97.5 percentile points as Bayesian confidence bounds (a 95% credible interval).

6.2.7 Prior distributions

Technical specifications of the priors are given in Table A1 of the Appendix.

Priors for the prevalence of infection (q) in the experimental groups depend on the success of vaccination and range from 0.1-0.4 in groups with a successful vaccination, and from 0.6-0.9 in groups with an unsuccessful vaccination. A diffuse prior was chosen for the probability of a successful vaccination (π), i.e. this prior nearly ranges from 0 to 1 and is not informative. The prior for the sensitivity of virus isolation (P) ranges from 0.95-1.00. It should be noted that this is the cumulative sensitivity for the whole excretion period, based on daily swabbing. The prior for the specificity of the virus isolation (β) ranges from 0.99-1.00, allowing for false positive results due to e.g. a very low aspecific hemagglutination of the allantoic fluid. The prior for Q, the probability that a truly infected animal that is positive in the VI will be so for three days or longer, ranges from 0.01 to 0.25. A possible relation was assumed between the duration of the infection, and the probability of the presence of antibodies. Sera from truly infected animals that were negative in the virus isolation had a prior for the probability of antibodies (p_0) from 0.00 to 0.30. When the duration of virus excretion was one or two days the prior for the probability of antibodies (p_1) had a range of 0.10-0.70. When the duration of virus excretion was three days or more the prior for the probability of antibodies (p_2) had a range of 0.95-1. The priors for the sensitivity and the specificity of the iIFAT were based on previously reported test results of Capua et al. (2003) and Cattoli et al. (2006). However, there are some differences between our study and the aforementioned studies. Our iIFAT test was based on the N7 neuraminidase, while the other studies were based on N1 and N3 neuraminidases, we used chicken sera while part of the other studies used turkey sera, and we used experimental sera while in the other studies field sera were used. These were all reasons to choose the priors for the sensitivity and specificity of the iIFAT somewhat wider (more diffuse), both priors having a median of 0.95 and a range of 0.92-0.98.

6.3 Results

6.3.1 Sensitivity and specificity of the serological DIVA tests in detecting antibodies

Results of the virus isolation and the serological DIVA tests are given in Table 2.

Estimates for the sensitivity in detecting antibodies are 0.95 (95% CI: 0.89-0.98) for the N7 iIFAT, 0.93 (95% CI: 0.78-0.99) for the NI assay, and 0.55 (95% CI: 0.34-0.74) for the NS1 ELISA (Figure 1 and Table 3). Estimates for the specificity are 0.92 (95% CI: 0.87-0.95) for the iIFAT, 0.91 (95% CI: 0.85-0.95) for the NI assay and 0.82 (95% CI: 0.74-0.87) for the NS1 ELISA (Figure 1 and Table 3). The sensitivity and specificity of the tests with respect to antibodies (*d*) prove to be stable under the different priors (Table 3). Differences between estimated values using different priors are small, compared with the width of the associated intervals (95% CI).

6.3.2 Sensitivity of the serological DIVA tests in detecting infection

Estimates of the sensitivity for detecting infection are 0.64 (95% CI: 0.52-0.75) for the iIFAT, 0.63 (95% CI: 0.49-0.75) for the NI assay, and 0.42 (95% CI: 0.28-0.56) for the NS1 ELISA (Figure 1 and Table 3). The estimates for sensitivity in detecting infection are sensitive to the choice of priors for *P* and *Q* (both related to sensitivity of *VI*) (Table 3). This will be discussed later.

6.3.3 Infection, virus isolation, and the production of antibodies

When a bird is considered infected, the probability that the bird is positive in the virus isolation during one or more days is estimated to be 0.78 (95% CI: 0.66-0.87). The estimated probability (*Q*) that an infected bird that is positive in the VI is so during 3 days or more, is 0.75 (95% CI: 0.55-0.88).

When a chicken was truly infected and negative in the VI, the estimated probability to produce antibodies was 0.03 (95% CI: 0.00-0.14) (Figure 1 and Table 3). When virus was detected for one or two days, the estimated probability to produce antibodies was 0.38 (95% CI: 0.11-0.74). When virus was detected for three days or more the estimated probability to produce antibodies was 0.98 (95% CI: 0.95-0.99). The aforementioned results do not critically depend on the choice of priors: similar results were found with less informative priors (Table 3).

Table 2. Frequency of combinations of test results for the iIFAT, NI assay, and NS1 ELISA

Days of positive VI	N7 iIFAT	NI assay	NS1 ELISA	Number of sera
0	-	-	-	29
	-	-	+	12
	-	+	-	4
	+	-	-	1
	+	-	+	7
	+	+	+	1
1-2	-	-	-	2
	-	+	-	1
	+	+	-	3
≥ 3	+	+	-	8
	+	+	+	11

6.4 Discussion

In this study we estimated the sensitivity of the N7 iIFAT in detecting antibodies at 0.95 (95% CI: 0.89-0.98), and in detecting infection at 0.64 (95% CI: 0.52-0.75). Previously published data by Cattoli et al. (2006) report a sensitivity for the N3 iIFAT in turkeys of 0.98 (95% CI: 0.96-0.99), based on sera taken from flocks with sentinels with a positive HI titer and a positive virus isolation. In chickens, from an infected flock of 24 birds, 11 tested positive in the N3 iIFAT (Cattoli et al., 2006). The specificity of the N7 iIFAT was estimated to be 0.92 (95% CI: 0.87-0.95), which is slightly lower than previously published data: 0.95 (95% CI: 0.93-0.97) (Capua et al., 2003), 1 and 0.995 (95% CI: 0.985-1) (Cattoli et al., 2006). Although the test is not particularly suitable for high-throughput, this could be improved by converting it into an ELISA (Ozaki et al., 2001) or a fluorescence microsphere immunoassay (FMI) (Watson et al., 2008).

The NI assay has an estimated sensitivity in detecting antibodies of 0.93 (95% CI: 0.78-0.99), and an estimated sensitivity in detecting infection of 0.63 (95% CI: 0.49-0.75), which makes it comparable to the iIFAT. The estimated specificity is 0.91 (95% CI: 0.86-0.95), possible reasons for false positive observations are cross reactivity between different N types, and antibodies against HA that non-specifically block neuraminidase activity (World Health

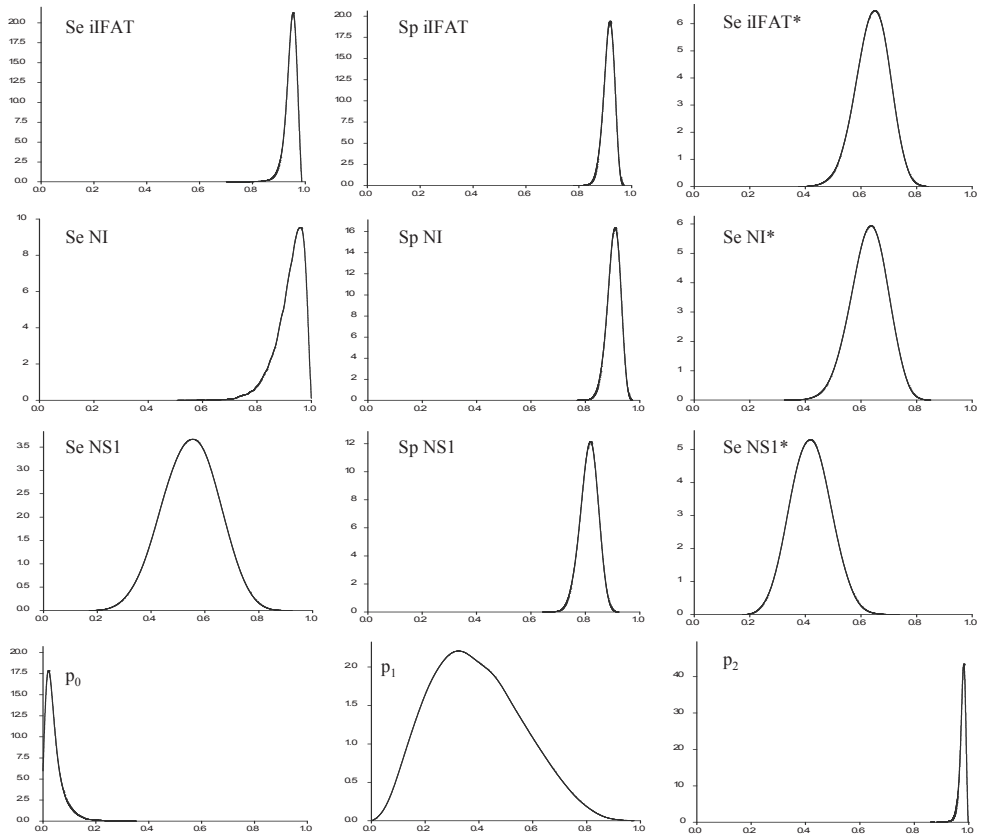

Figure 1. Probability density of the posterior distributions. Se, sensitivity with respect to seroconversion; Sp, specificity of the test; Se*, sensitivity with respect to infection; p_0, probability of an infected chicken to produce antibodies when VI is negative; p_1, probability to produce antibodies when VI is positive during 1 or 2 days; p_2, probability to produce antibodies when VI is positive during 3 or more days.

Organization, 2002).

The NS1 test has not been standardized yet, different antigens are used ranging from expression of the whole NS1 in baculovirus (Watson et al., 2008) or in *E. Coli* (Tumpey et al., 2005; Zhao et al., 2005) to peptides (Dundon et al., 2006; Tumpey et al., 2005). The NS1 ELISA we used has a sensitivity in detecting antibodies of 0.55 (95% CI: 0.34-0.74), and a sensitivity in detecting infection of 0.42 (95% CI: 0.28-0.56). To our knowledge the information published about the sensitivity of the NS1 ELISA is only limited, in studies using peptides small numbers

Table 3. Results for the original and alternative priors

Parameters	Priors (range)	Posterior estimates (95% CI)			
		Original priors	Diffuse prior for *P*	Diffuse prior for *Q*	Diffuse priors for p_0, p_1 and p_2
P	0.95-1.00	0.78 (0.66-0.87)	0.51 (0.36-0.66)	0.77 (0.66-0.86)	0.79 (0.68-0.88)
Q	0.01-0.25	0.75 (0.55-0.88)	0.78 (0.59-0.90)	0.90 (0.75-0.98)	0.75 (0.55-0.88)
β	0.99-1.00	0.99 (0.97-0.99)	0.98 (0.97-0.99)	0.98 (0.97-0.994)	0.99 (0.97-0.996)
p_0	0.00-0.30	0.03 (0.00-0.14)	0.03 (0.01-0.14)	0.03 (0.01-0.14)	0.08 (0.01-0.24)
p_1	0.10-0.70	0.38 (0.11-0.74)	0.28 (0.07-0.68)	0.28 (0.07-0.67)	0.50 (0.10-0.89)
p_2	0.95-1.00	0.98 (0.95-0.99)	0.98 (0.95-0.99)	0.98 (0.95-0.99)	0.94 (0.80-0.99)
Se iIFAT (d)[a]	0.92-0.98	0.95 (0.89-0.98)	0.95 (0.89-0.98)	0.95 (0.89-0.98)	0.95 (0.89-0.98)
Se NIA (d)[a]	Diffuse	0.93 (0.78-0.99)	0.93 (0.77-0.99)	0.93 (0.77-0.99)	0.93 (0.77-0.99)
Se NS1 (d)[a]	Diffuse	0.55 (0.34-0.74)	0.57 (0.36-0.77)	0.57 (0.36-0.77)	0.55 (0.34-0.75)
Sp iIFAT	0.92-0.98	0.92 (0.87-0.95)	0.91 (0.86-0.95)	0.91 (0.86-0.95)	0.92 (0.87-0.95)
Sp NIA	Diffuse	0.91 (0.85-0.95)	0.90 (0.84-0.94)	0.90 (0.84-0.94)	0.91 (0.85-0.95)
Sp NS1	Diffuse	0.82 (0.74-0.87)	0.82 (0.75-0.87)	0.82 (0.75-0.87)	0.82 (0.75-0.87)
Se iIFAT (D)[b]		0.64 (0.52-0.75)	0.46 (0.34-0.59)	0.70 (0.58-0.79)	0.66 (0.51-0.77)
Se NIA (D)[b]		0.63 (0.49-0.75)	0.46 (0.34-0.59)	0.68 (0.55-0.79)	0.64 (0.49-0.77)
Se NS1 (D)[b]		0.42 (0.28-0.56)	0.35 (0.25-0.47)	0.46 (0.31-0.61)	0.42 (0.28-0.58)

[a]sensitivity with respect to seroconversion; [b]sensitivity with respect to infection.

of birds (n = 20) were used for validation (Dundon et al., 2007; Tumpey et al., 2005). The estimate of the specificity of our test of 0.82 (95% CI: 0.74-0.87) is lower than the previously reported specificity of 0.94 (95% CI: 0.89-0.997) (Zhao et al., 2005).

In Bayesian statistics, sensitivity of the results to the choice of prior distributions warrants some attention. Therefore, additional analyses using less informative (more diffuse) priors have been performed (Table 3). The posterior estimates for the sensitivity and specificity of the tests with respect to antibodies (*d*) did not critically depend on the choice of prior. The same applies to the probability of producing antibodies in relation to days *VI*: differences between the parameter estimates are relatively small, compared to the width of the associated intervals. However, the sensitivity of the tests in detecting infection (*D*) is rather sensitive to the choice of priors for the sensitivity of VI (*P*), and to a lesser extent, to the proportion of birds with a longer excretion period (*Q*). There is a marked difference between the priors and posteriors of these parameters, and the analyses with wider, diffuse priors show that the data point towards lower *P* and higher *Q* values than the original priors. When the prior for *P* was made fairly non-informative (*logit* (*Q*) ~ N(0,0.5)), implying that practically all the information came from the data, the estimate for *P* decreased from 0.78 (95% CI: 0.66-0.87) to 0.51 (95% CI: 0.36-0.66). Likewise, the estimates for the sensitivity of the DIVA tests in detecting infection (*D*) decreased as well (Table 3). In table 2

it can be seen that this is in agreement with the data, because based on the results of the DIVA tests, a number of sera in rows 2-6 shall be considered positive. When the prior for *Q* was made fairly non-informative, the estimate for *Q* increased from 0.75 (95% CI: 0.55-0.88) to 0.90 (95% CI: 0.75-0.98) (Table 3). The results for *Q* indicate that the data (*VI* and test results) point to a markedly higher proportion of birds with a longer excretion period. In table 2 it can be seen that this is in agreement with the VI data, and this can be explained by the fact that a lot of sera were taken from birds that were infected shortly (one week) after vaccination. In these birds immunity against infection is still developing, and is not able to prevent longer excretion periods.

Analyses without a gold standard (see e.g. Engel et al., 2006, for references) tend to assume two true states, while our model assumes three true states, as represented by infection (*D*) and antibody status (*d*). This allows us to derive a test sensitivity with respect to both *d* and *D*. Where in the two-state models, posteriors will necessarily offer a summary based on a mix of different potential mixtures (infected or not and seroconverted or not), the present model has the possibility to distinguish between these mixtures. Although test sensitivity and specificity are of primary importance, the analysis offers additional information, e.g. about the relationship between the infected and antibody states *D* and *d*. This is of importance for the use of DIVA tests, because these tests rely on the fact that infected vaccinated birds produce antibodies. Suarez (2005) suggested that vaccinated and infected birds may not have enough virus replication to stimulate an antibody response to the novel viral proteins, and therefore may not seroconvert or seroconvert only at low levels. With our model we demonstrated that the probability of antibody production is associated with the number of days of positive VI. Birds that are positive in the VI for three days or more, being relevant for virus spread in the flock, have a high probability to produce antibodies, whereas infected birds that are negative in the VI have a low probability of producing antibodies. Most likely, it only has minor consequences that infected but VI negative birds are not detected by the DIVA test, because the contribution of these birds to the spread of virus is probably very limited.

When tests are validated the reference population should reflect the target population (Greiner and Gardner, 2000). Our sera were obtained from birds that were inoculated or contact exposed shortly after vaccination, which lead to a high proportion of infected birds with a longer excretion period. To what extent this sample of infected birds is representative for the target population depends mainly on the use of the DIVA test. If, for example, the test is used for a final screening to show freedom of disease after vaccination, the proportion of birds with a longer shedding period will probably be lower. On the other hand, in case of silent transmission due to a low vaccination efficiency the number of excreting birds will be higher. Moreover, our dataset is modestly sized, but on the basis of simulation results in Engel, Backer, and Buist (2008), it is reasonable to assume that estimated sensitivities and specificities and associated 95% credible

intervals have acceptable properties. In addition, a major advantage of our data is that they offer detailed information about the results of the VI of each individual bird, which allowed us to study the association between days of virus shedding and the probability of antibody production with, as far as we know, unique results.

According to our analysis, not all infected animals in the population produce antibodies, which implies that improving the tests in detecting antibodies will only have a limited effect on the sensitivity in detecting infection. However, because the likelihood of producing antibodies is associated with the duration of virus excretion, the birds missed by the test are probably not the birds contributing most to the spread. Moreover, a low sensitivity can be compensated by increasing the number of samples from the flock, although at the cost of a lower flock level specificity. The latter, however, could be overcome by using two or more independent tests in a serial way (Dohoo, Martin, and Stryhn, 2003). It is concluded that the N7 iIFAT and the N7 NI assay are useful tests in detecting silent transmission, because the birds that are most significant in spreading virus will be detected. On the other hand, due to the lower specificity, the N7 iIFAT and the N7 NI are less suitable for declaring freedom of disease after vaccination.

Appendix

A.1 Prevalence within a group

The logit transformed true prevalence of a group is sampled from a mixture of two Normal distributions: $logit(q) = \log(q/(1-q)) \sim \pi\ N(\mu_1, \tau) + (1-\pi)\ N(\mu_2, \tau)$, where $\tau = 1/\sigma^2$ is the precision and π is the probability for successful vaccination.

If seeded with one or only a few animals, infectious disease data are expected to follow a bimodal distribution (Diekmann and Heesterbeek, 2000). Here, however, the experiments were seeded with five experimentally infected animals and five contact animals, and the assumption of a unimodal distribution of the prevalence seems reasonable (De Jong and Kimman, 1994). Because of confounding with *VI* there are no additional parameters in the model to distinguish between contact and inoculated animals.

A.2 Test results

There are separate sets of probabilities for the joint test results (from + + + to - - -) of the three tests, for animals that are truly seropositive (d = 1) or truly seronegative (d = 0). Note that the model allows for dependence between tests, conditional upon true state d for seroconversion, cf. Engel et al. (2006).

A.3 Sensitivity and specificity of the serological tests

When P_{+ijk} is the probability for test results i, j, k (0 / 1 for a - / + test result) for a true seropositive animal ($d = 1$), the sensitivity of, say, test 3 is $a_3 = \sum_{i=0}^{1}\sum_{j=0}^{1} P_{+ij1}$. Likewise, the specificity follows from probabilities P_{-ijk} for animals that are not seropositive ($d = 0$), e.g. the specificity for test 3 is $b_3 = \sum_{i=0}^{1}\sum_{j=0}^{1} P_{-ij0}$. P_{+ijk} and P_{-ijk} are specified cf. Engel et al. (2006) in terms of 'main effects' $\delta_{+1}, \delta_{+2}, \delta_{+3}$ and $\delta_{-1}, \delta_{-2}, \delta_{-3}$ (one for each test, associated with sensitivity and specificity) and 'interactions' $\varepsilon_{+12}, \varepsilon_{+13}, \varepsilon_{+23}$ and $\varepsilon_{-12}, \varepsilon_{-13}, \varepsilon_{-23}$ (one for each pair of tests, associated with dependence between tests, for $d = 1$ or 0). The sensitivity of, say, test 3 with respect to D is

$$A_3 = P(y_3 = 1 \mid D = 1) = \frac{P(y_3 = 1, D = d = 1) + P(y_3 = 1,\ D = 1,\ d = 0)}{P(D = d = 1) + P(D = 1,\ d = 0)},$$

where

$$P(y_3 = 1, D = d = 1) = a_3\ P(D = d = 1),$$
$$P(y_3 = 1,\ D = 1,\ d = 0) = (1 - b_3)\ P(D = 1,\ d = 0),$$
$$P(D = d = 1) = P(D = d = 1, VI = 0) + P(D = d = 1, 1 \le VI \le 2) + P(D = d = 1, VI \ge 3) =$$
$$\big(p_0 P(VI = 0 \mid D = 1) + p_1 P(1 \le VI \le 2) \mid D = 1) + p_2 P(VI \ge 3 \mid D = 1)\big) P(D = 1) =$$
$$\big(p_0(1 - P) + p_1 P(1 - Q) + p_2 PQ\ \big)\, q,$$

and

$$P(D = 1,\ d = 0) = \big((1 - p_0)(1 - P) + (1 - p_1)P(1 - Q) + (1 - p_2)PQ\big)\, q,$$

where q in the numerator and denominator of A_3 cancels out.

A.4 Construction of prior distributions

Priors for parameters in the probabilities for joint test results were chosen similar to Engel et al. (2006). Normal priors were used for logit transformed parameters π, P, Q, p_1, p_2, p_3, e.g. $logit(P) = log(P/(1 - P)) \sim N(3.77, 3.97)$. Mean 3.77 and precision (inverse of the variance) 3.97 are such that the logit transformed lower and upper bounds of the specified range for P

Table A1. Technical specifications of prior distributions

Description	Prior
Parameters for prevalence q	$logit(\pi) \sim N(0, 0.5)$ [(a)] $\mu_1 \sim N(-1.3, 9)$ $\mu_2 \sim N(1.3, 9)$ $\tau \sim gamma(0.85, 0.25)$ [(b)]
Parameters for *VI*, conditional upon *D*	$logit(P_{12}) \sim N(3.77, 3.97)$ $logit(Q) \sim N(-2.85, 0.89)$ $\beta \sim beta(364.9,1.36)$ [(c)]
Parameters for *d*, conditional upon *VI* and *D*	$logit(p_0) \sim N(-2.72, 0.77)$ $logit(p_1) \sim N(-0.68, 1.17)$ $logit(p_2) \sim N(3.77, 3.97)$
Parameters for 'main effects' δ (associated with sensitivity and specificity) and 'interactions' ε (associated with dependence between tests) for probabilities for joint test results, conditional upon *d*.	$\delta_+ \sim N(2.944, 8)$, for the iIFAT, $\delta_+ \sim N(0, 0.5)$, for the other tests $\delta_- \sim N(2.944, 8)$, for the iIFAT $\delta_- \sim N(0, 0.5)$, for the other tests $\varepsilon_-, \varepsilon_+ \sim gamma(0.48, 1)$

[(a)] Normal distribution, mean 0 and precision 0.5, i.e. variance 2. [(b)] Gamma distribution, mean μ =0.85/0.25 and variance μ /0.25. [(c)] Beta distribution, mean μ = 364.9/(364.9+1.36), variance μ(1-μ)/(364.9+1.36+1)

correspond to the 5 and 95% points of the Normal distribution. For β a beta distribution was used as a prior. This prior was chosen very close to value 1; with 95th percentile and mode equal to 0.99 and 0.999 respectively. For π a reasonably diffuse prior was chosen. Priors for μ_1, μ_2 and σ^2 are such that prevalence q broadly covers the ranges specified for vaccinations that were successful or not. All priors are listed in A.1.

A.5 Priors and choice of cut-off values

For two of the tests (NI assay and NS1 ELISA), cut-off points were chosen equal to the 95 percentile points of the test results for the true negative sera collected on the day of challenge.The original, say *N*, test results for true negatives are put in increasing order. The cut-off g is chosen as the r-th observation, such that $r / N \approx b_0$, where b_0 (0.95 in the analysis) is the target value for the specificity. Without loss of generality, we assume that after transformation test data follow a uniform (0, 1) distribution. The r-th order statistic follows a beta distribution (Kendall and Stuart, 1969) with parameters *r* and (*N* +1 - *r*), which motivates the following prior for the specificity:

$$b \sim beta(r,\, N+1-r)\,. \qquad \text{(A5.1)}$$

As if *g* was established externally, we combine the diffuse prior $b \sim$ beta(1, 1) with the number of negative (according to cut-off *g*) test results $x|\, b \sim \text{binomial}\,(N,\, b)$, yielding the posterior

$$b \mid x \sim beta(x+1,\, N+1-x)\,. \qquad \text{(A5.2)}$$

For *N* not too small, $x = r \approx Nb_0$, and based on the similarity between (A5.1) and (A5.2), we conjecture that a diffuse prior, in combination with the binary test results from the true negatives, is a reasonable choice in the analysis.

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Chapter 7

General discussion

7.1 Introduction

For decades HPAI was considered an exotic disease of poultry with a low incidence of epidemics. However, during the last 35 years the frequency and magnitude of HPAI epidemics have seemed to increase, and several very large epidemics of HPAI have occurred. Moreover, the H5N1 HPAI virus, that had its origin in South East Asia in 1996, now has become endemic in large parts the world. Next to the worldwide spread in poultry the H5N1 virus appeared to be capable of infecting and killing humans. The increasing magnitude of HPAI epidemics and the endemicy of H5N1 ask for alternative strategies for prevention and control of HPAI next to measures like culling of infected poultry flocks, pre-emptive culling, and the imposement of movement restrictions. Such an alternative is offered by vaccination, which has been applied during some HPAI epidemics (Capua, 2007). Over the last years, experimental studies have yielded important information about the effect of vaccination on disease symptoms and excretion (Swayne et al., 1999; Swayne et al., 2000b; Webster et al., 2006). However, little is known about the effect of vaccination on transmission, which is crucial when an epidemic has to be controlled by vaccination.

The main goal of this thesis was to gain insight in the transmission parameters of HPAI viruses in birds, and in the effect of vaccination on transmission. To this end, transmission experiments with different HPAI strains in unvaccinated and vaccinated birds of different species were performed.

To better understand the origin of an HPAI outbreak from an LPAI outbreak, the transmission characteristics of genetically closely related HPAI and LPAI strains (A/Chicken/Pennsylvania/83) from the subtype H5N2 were compared (Chapter 2). The LPAI and HPAI strains appear to differ quite substantially in their key epidemiological parameters, with the HPAI strain spreading more efficiently and having a longer infectious period. In Chapters 3 and 4 the effect of vaccination on transmission of an H7N7 virus that caused a major epidemic in the Netherlands in 2003 was studied. It appears that the effect of vaccination on transmission depends on the bird species, the vaccine, and timing of vaccination relative to the moment of challenge. A single vaccination was able to sufficiently reduce transmission in chickens (Chapter 3), and in teals (Chapter 4), while in pheasants no significant reduction in transmission was observed (Chapter 4). In chickens a difference in the effect of vaccination on transmission was observed between two vaccines that differed in homology and HA content, and between the time points after vaccination (Chapter 3). Chapter 5 elaborates on this theme by focusing on the transmission dynamics of HPAI H5N1 virus in Pekin ducks. The results show that a genetically distant vaccine is able to significantly reduce transmission, although the analyses did not allow us to conclude that transmission is reduced to the extent that no major outbreaks can occur. The

results of Chapters 3-5, furthermore, have shown that silent transmission can occur in vaccinated birds. This implies that vaccination programs should be accompanied by monitoring based on a DIVA (Differentiating Infected from Vaccinated Animals) strategy. Therefore, in Chapter 6 three different DIVA tests were validated using sera that were obtained from the experiments in Chapter 3. The analyses show that two serological tests based on a heterologous neuraminidase have sensitivities and specificities of more than 0.90 in detecting antibodies, while the sensitivity in detecting infection is substantially lower. The discrepancy between the sensitivity in detecting antibodies and in detecting infection can be explained by the finding that chickens with no virus excretion or a short period of virus excretion after infection only have a low probability to develop antibodies.

7.2 Transmission experiments as a tool to study avian influenza in birds

Transmission experiments offer a way to study the transmission dynamics of an infectious agent in a given host in a controlled experimental setting, and are ideally suited to show the effect of an intervention (e.g. vaccination) on transmission (De Jong and Kimman, 1994). For instance, transmission experiments have yielded important insights in the dynamics and control options of animal infectious diseases, such as pseudorabies virus in pigs (De Jong and Kimman, 1994), foot and mouth disease virus in cattle and sheep e.g. (Hughes, Kitching, and Woolhouse, 2002; Orsel et al., 2005; Streftaris and Gibson, 2004), and classical swine fever virus in pigs (Hohle, Jorgensen, and O'Neill, 2005). In the following section I will discuss the potential and limitations of transmission experiments when used for studying avian influenza in birds.

To be able to perform a transmission experiment with HPAI one needs to be able to reliably detect infected birds, and to have a reliable infection model for the creation of infected birds. For the detection of infected birds primarily virus isolation (VI) on tracheal and cloacal swabs was used. The use of VI is convenient because it is still considered a gold standard for the detection of the presence of avian influenza virus. However, the analyses in Chapter 6 indicate that the sensitivity of VI in detecting infected birds is relatively low 0.78 (95% CI: 0.66-0.87). Whether this has implications for the analysis of the transmission experiments depends on the infectivity of the undetected infected birds: when these are not infectious the analysis will not change much. Furthermore, virus isolation in the way we used it, is not a quantitative test but yields binary outcome data, making it impossible to study gradual differences in virus excretion. Therefore, in Chapter 5 a quantitative PCR was used as a second virus detection test. Next to

generating quantitative data, the PCR seemed to have a higher sensitivity than VI, since swabs that were positive in the PCR with a high cycle threshold (ct) value were negative in the VI (Chapter 5, Tables S7-S10). However, it is difficult to directly compare VI and the PCR because the PCR detects infectious virus as well as non-infectious particles, while VI detects infectious virus only. Both the results from the Bayesian analyses and the PCR point to a low sensitivity of VI, and this might have implications for the conclusions of our experiments. In our transmission experiments most swabs are missed by VI, when compared to the PCR, at the end of the excretion period of the birds, when the ct values of the swabs are higher (Chapter 5, Tables S7-S10). This might lead to an underestimation of the duration of the infectious period. On the other hand, it is questionable whether these birds with high ct values still can be considered infectious. In table 10 of Chapter 5 it appears that some birds are positive in the PCR during one or more days, while they remain undetected by VI. When this happens equally often in both the inoculated as well as in the contact infected birds, the estimate for the reproduction ratio and the comparisons of treatments may not be affected very much by using VI to detect infected birds. Overall I conclude, that the use of VI to detect infected birds may lead to an underestimation of the duration of the infectious period. However, it should be kept in mind that any detection test is not really measuring the infectivity of the birds. Whether or not a bird is infectious can only be determined in conjunction with positive test results for contact birds and estimation of transmission from these data.

In all transmission experiments the inoculation dose was set at $10^{5.3}$ EID_{50}. In unvaccinated birds this inoculation dose always led to infection of the inoculated birds, but inoculation of vaccinated birds led to more variable results. The number of infected inoculated birds ranged from none out of ten in the experiments with vaccinated chickens that were challenged two weeks after vaccination (Chapter 3) to ten out of ten in the experiments with vaccinated pheasants (Chapter 4). When these inoculated birds remained negative in the VI, they were not classified as susceptible (S), but as exposed (E) or recovered (R). These birds were considered exposed (E) when they became positive in the VI later in the experiment, and recovered (R) when they remained negative in the VI. An example of this is given in the experiments with Pekin ducks where two inoculated ducks became positive in the VI on day 4 and 5 respectively, but both ducks were positive in the PCR from day 2 and 1 respectively (Chapter 5, Table S10), making it likely that they were already infected by the inoculation.

The statistical analyses of the transmission experiments of Chapters 3-5 are based on the classical stochastic SEIR model. This model assumes, among other things, that all infected birds are equally infectious during their infectious period, and that all susceptible birds are equally susceptible. In most transmission experiments the infectious period is determined using a positive VI as a proxy for a bird being infectious. In addition to VI, virus excretion was quantified by a

semi-quantitative PCR in the experiments with ducks (Chapter 5). Figure 1 shows that there are only minor differences in the ct values of inoculated and contact infected ducks of the unvaccinated groups and groups that received a single vaccination. However, in the groups that received a double vaccination, one contact infected duck shed virus for a prolonged period with high levels of excretion (indicated by low ct levels). This suggests that the assumption of equal infectiousness may not always hold. In the particular case of the transmission experiments with doubly vaccinated ducks no other contact infections were observed, and the overall conclusion that vaccination significantly reduced transmission is not affected. Nevertheless, the results of the PCR in Chapter 5 demonstrate that quantification of excretion could be valuable to further analyze the validity of the assumption of equal infectiousness.

The second assumption that needs scrutiny is that all uninfected birds are assumed equally susceptible. Since protection against avian influenza is mainly determined by antibodies against the hemagglutinin (Webster et al., 1991), one would expect there to be a relation between susceptibility and the hemagglutination inhibition (HI) titers. In turkeys, it was demonstrated that birds with a positive HI titer after vaccination were less susceptible to infection with H7N3 virus than naïve birds without an HI titer (Capua et al., 2004). For Newcastle disease a similar relation between HI titers after vaccination and transmission was shown (van Boven et al., 2008). In the experiments HI titers after vaccination varied between individual birds, and this variation seemed species dependent. In vaccinated pheasants the HI titers 14 days after vaccination ranged between 0 and 7, in teals titers ranged between 3 and 8, while in chickens there was less variation in titers with a range from 5 to 7 (using H7N1) and from 2 to 5 (using H7N3). However, the number of birds was fairly small, making it difficult to draw definitive conclusions.

In the statistical analyses assumptions also have to be made about the duration of the latent period. Specifically, since samples were taken once every day, and since we analyzed the data using a Generalized Linear Model the duration of the latent period was restricted to multiples of one day (the sampling interval). Models with a latent period of 0-1 day or 1-2 days were compared, and the model with the best fit (the lowest deviance) was presented in the results. A latent period of at most 1 day was assumed in the experiments with H7N7 in golden pheasants and teals, and with H5N1 in Pekin ducks (Chapter 4 and 5). A latent period of between 1 and 2 days was assumed in the experiments with H5N2 (HPAI) and H7N7 in chickens (Chapter 2 and 3). The same H7N7 virus in turkeys had a latent period of at most 1 day (Bos et al., 2008). Analyses based on Bayesian methods of inference do not require the latent period to be multiples of the sampling interval (Hohle, Jorgensen, and O'Neill, 2005), and an application of these methods show that for H5N1 virus in chickens the estimated latent period was 0.24 days (95% CI: 0.099-0.48) (Bouma et al., 2009). The results of the experiments with the H7N7 virus suggest that the length of the latent period is species dependent, but again, the number of experiments is

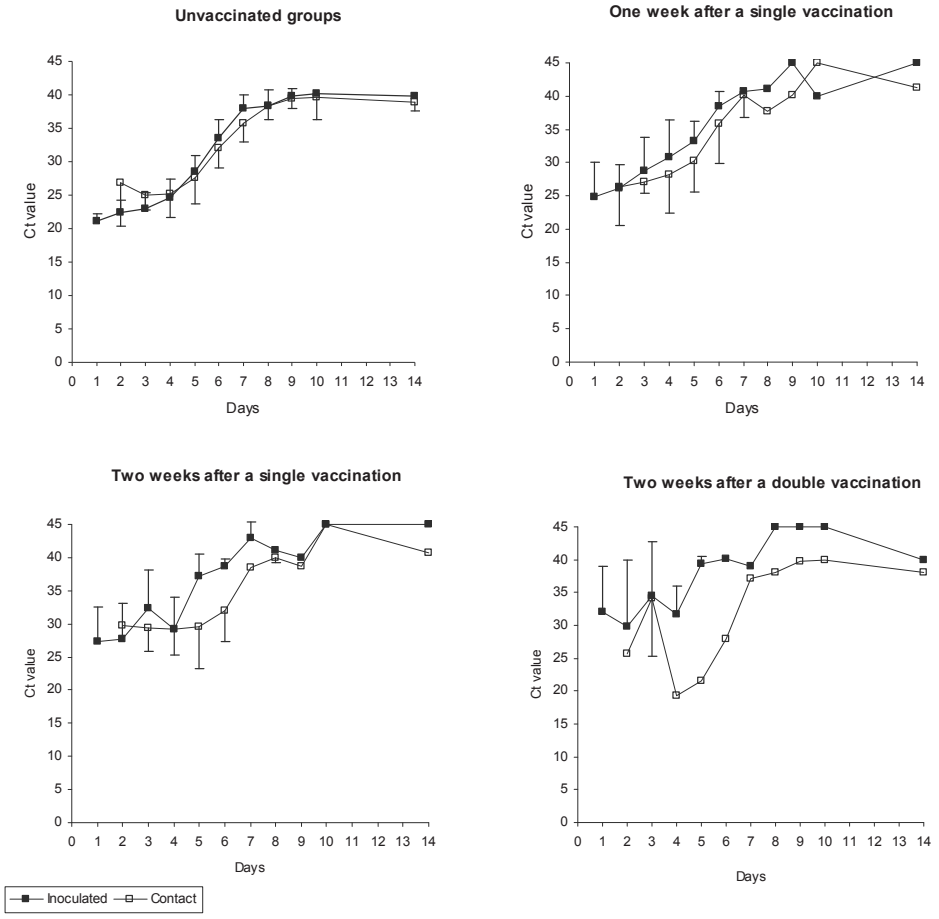

Figure 1. Ct values of the RRT-PCR of the tracheal swabs of the transmission experiments with Pekin ducks (Chapter 5). Days, days after inoculation; Standard deviations are given by vertical bars.

too small to allow definitive conclusions. When there are indications that the latent period is shorter than 1 day, like in the H5N1 experiments with chickens, the sampling interval should be shortened to, for example, 0.5 day. In this way the latent period and the transmission rate can be estimated more precisely.

Our results were obtained in an experimental setting. Compared to a field study this has both advantages and disadvantages. An advantage of an experimental setting over a field study is that it is possible to work under controlled circumstances. This makes it plausible that observed

differences between groups can be attributed to treatment differences, because variation caused by other influences is minimized. Factors such as vaccination, vaccination schedules, virus strains, vaccine doses or animal species, can be compared and their effects on transmission dynamics can be tested. When a causal relation between a treatment and an effect on transmission has been demonstrated the next step would then be to measure the magnitude of the effect of the treatment in the target population with a clinical trial. This was, for example, done in case of pseudorabies virus (Stegeman et al., 1995) and BHV1 (Bosch et al., 1998). However, for notifiable diseases like HPAI a field trial is a problem since field experiments are not allowed in most countries, or are difficult to perform (Bouma et al., 2008).

7.3 Transmission of LPAI and HPAI viruses

In order to successfully prevent and control epidemics of HPAI it is important to understand the origin of HPAI outbreaks. HPAI viruses do not constitute separate phylogenetic lineages but appear to arise from strains of low pathogenicity (Banks et al., 2000; Banks et al., 2001; Rohm et al., 1995). Because HPAI viruses arise from an environment of LPAI viruses, they must have a selective advantage to be able to replace the LPAI virus. Our results provide evidence that the H5N2 HPAI virus is more easily transmitted and induces a longer infectious period than the corresponding H5N2 LPAI virus (Chapter 2). Using estimates of the reproduction ratios of both the LPAI and the HPAI strains it was concluded that a major outbreak of H5N2 LPAI virus is unlikely to provide herd immunity against a major outbreak of H5N2 HPAI virus.

The understanding that HPAI viruses arise from LPAI viruses has lead to legislation (OIE, 2005), which describes that LPAI epidemics should be controlled in an appropriate manner. In this thesis I give another ground for eradication and control of LPAI outbreaks. The LPAI virus transmits less efficiently than the HPAI virus, which allows for more time for eradication of the LPAI outbreak compared to a HPAI outbreak. Furthermore, compared to HPAI, a smaller proportion of infected birds in the flock will become infected due to a lower reproduction ratio. This leads to a lower total amount of virus in the flock and consequently to a lower infection pressure to other farms. These differences in the transmission between the LPAI and HPAI viruses show that the control of an HPAI epidemic offers more difficulties than the control of a LPAI epidemic, and is another reason to eradicate H5 and H7 LPAI outbreaks at all time. However, detection of LPAI outbreaks based on clinical symptoms may offer difficulties, because disease symptoms are not always obvious.

Although it was shown that differences in transmission dynamics contribute to the selective advantage of H5N2 HPAI over H5N2 LPAI, it remains to be investigated whether this

is true in general. Given the economic, animal health, and potentially also public health implications of outbreaks of HPAI, it is surprising that up to now not more attention has been paid to this question.

7.4 The relation between disease symptoms and transmission

During epidemics of HPAI worldwide several domestic bird species may be involved, depending on cultural and geographical differences. Next to birds that are kept for food production, ornamental birds may also be involved in HPAI epidemics (Editorial, 2005; Ellis et al., 2004; Van Borm et al., 2005). In experiments it was shown that several bird species can be infected by avian influenza, and that disease symptoms may vary from subclinical to severe illness and mortality (Alexander, 2000; Alexander, Parsons, and Manvell, 1986; Editorial, 2005; Ellis et al., 2004; Perkins and Swayne, 2001; Perkins and Swayne, 2002; Perkins and Swayne, 2003; Wood, Webster, and Nettles, 1985). However, much less is known about the ability of HPAI viruses to spread among different bird species, and the relation between the severity of disease and transmission.

In this thesis transmission experiments were performed with chickens (Chapter 3), ringed teals, and golden pheasants (Chapter 4), using the same HPAI H7N7 virus strain and inoculation dose in all experiments, making it possible to directly compare the results of the experiments. In agreement with previous findings, there were major differences in morbidity and mortality between species. In chickens and pheasants the infection led to severe morbidity and high mortality, while in teals only conjunctivitis was seen in a minority of the birds. This is in agreement with earlier findings that most HPAI viruses replicate in ducks but do not cause severe disease symptoms (Alexander et al., 1978; Alexander, Parsons, and Manvell, 1986; Hulse-Post et al., 2005; Westbury, Turner, and Kovesdy, 1979; Wood, Parsons, and Alexander, 1995; Wood, Webster, and Nettles, 1985). However, when the transmission dynamics of HPAI in the different species were compared, it appeared that all contact birds from the chicken, pheasant and teal experiments became positive within 1-2 days. Statistical analyses based on the final size show that the virus is highly transmissible in all bird species. Hence, it appears that there need not necessarily be a correlation between the severity of disease symptoms and transmission. This implies that the detection of HPAI infections by passive surveillance based on clinical symptoms or mortality will not be sufficient in all bird species, and that surveillance programs have to be based on virus detection or on serology.

7.5 The effect of vaccination on transmission

7.5.1 Reduction of clinical symptoms and reduction of transmission

Our experiments demonstrate that in chickens the reduction of clinical symptoms by vaccination precedes the reduction of transmission. One week after a single vaccination with an H7N3 vaccine all chickens were protected against disease caused by H7N7 HPAI virus, but transmission was not significantly reduced yet. One week later (two weeks after vaccination), however, no transmission was observed and it could be concluded that transmission was significantly reduced (Chapter 3). The same was observed in experiments with Pekin ducks: one week after a single vaccination disease symptoms were reduced and mortality was prevented, but transmission was not significantly reduced yet. One week later (two weeks after vaccination) there was a significant reduction of transmission by the vaccination (Chapter 5). Apparently, vaccination reduces disease symptoms and mortality before it reduces transmission, which implies that the absence of clinical symptoms can not be used as a marker for the effect of vaccination on transmission.

7.5.2 Vaccine

For avian influenza, it has been shown that there is a relation between the antigen content of a vaccine and the level of protection (Swayne et al., 1999), and between genetic virus-vaccine homology and virus excretion (Lee, Senne, and Suarez, 2004; Swayne et al., 2000a). In Chapter 3 two vaccines with different antigen contents (45 μg/ml and 13 μg/ml) and different protein homology between the HA1 of the vaccine and challenge virus (98% and 93%) were compared in chickens. One week after vaccination the vaccine with the highest antigen content and homology gave a higher reduction of transmission than the vaccine with the lower antigen content and homology. However, two weeks after vaccination both vaccines gave sufficient reduction of transmission. Apparently, an antigen content of 13 μg/ml and a protein homology of the HA1 of 93% are enough to sufficiently prevent transmission two weeks after a single vaccination. The separate effects of antigen content and genetic homology have been studied systematically in chickens (Lee, Senne, and Suarez, 2004; Swayne et al., 1999; Swayne et al., 2000a), but it will be difficult to predict the effect of both variables together. An alternative would be the use of HI titers induced by vaccination as a proxy for protection. For Newcastle disease it has been shown that there is a relation between HI titers and transmission (van Boven et al., 2008). It would be interesting to similarly explore the relation between HI titers and the reduction of transmission by vaccination for avian influenza. This is discussed in more detail below.

7.5.3 Emergency vaccination

When emergency vaccination is considered, it should be realized that it takes time to implement the vaccination campaign, to vaccinate all susceptible poultry, and for the vaccine to provide effective protection against transmission. In addition, other factors are also of importance, such as the availability of staff, vaccine and equipment, and a registration system of all poultry in the intended vaccination area. To gain insight in the time needed for the vaccine to be effective in reducing transmission, chickens and Pekin ducks were challenged one or two weeks after vaccination. In chickens the effect of vaccination was already significant with the reproduction ratio below the threshold value 1 at one week after vaccination with one of the two vaccines used (Chapter 3). Two weeks after a single vaccination transmission among chickens was sufficiently prevented with both vaccines (Chapter 3). Hence, in chickens it is possible to prevent a major outbreak already at one week after vaccination, which might be sufficient for an emergency vaccination program. However, whether the vaccine prevents a major outbreak already at one week after vaccination depends on the vaccine and the match between vaccine and virus strain as discussed in the previous section.

In Pekin ducks there was not a significant reduction of transmission at one week after a single vaccination, but it was significant one week later (Chapter 5). Because a genetically distant vaccine was used no conclusions can be drawn whether a genetically more related vaccine will prevent major outbreaks at one week after vaccination. To study this transmission experiments with a genetically related vaccine should be performed.

7.5.4 Bird species

When vaccination against HPAI is applied different bird species may be vaccinated depending on the purpose of the vaccination and local circumstances. In The Netherlands free range chickens were vaccinated (Capua et al., 2009), in several European countries zoo birds were vaccinated (Philippa et al., 2007; Philippa et al., 2005), and in France (Anonymous, 2008) and Vietnam ducks were vaccinated (Pfeiffer et al., 2007). Our experiments show that the effect of vaccination on transmission varies depending on the bird species. Three different bird species (chickens, golden pheasants, and ringed teals) were compared under identical experimental conditions (Chapter 3 and 4). In experiments with chickens and teals transmission was significantly reduced two weeks after a single vaccination, while in pheasants there was no significant reduction in transmission. In Pekin ducks transmission was also significantly reduced two weeks after a single or a double vaccination, but it should be noted that in the experiments with Pekin ducks another vaccine and challenge virus were used (Chapter 5).

The results of this thesis show that the effect of vaccination varies substantially from species to species. A possible explanation for this is the genetic diversity among different bird

species, which may very well be reflected in differences in the immune system. Obviously it is impossible and undesirable to study every birds species and its reaction to vaccination. Therefore, like in poultry, a monitoring program to detect virus circulation should be implemented, which is in line with the importance of the species in the epidemic. However, I conclude that for the main groups of domestic birds species, like chickens (Chapter 3), turkeys (Bos et al., 2008) and Pekin ducks (Chapter 5) it was demonstrated that vaccination is effective in reducing transmission.

7.5.5 Serology as a correlate of protection

As was previously discussed, several factors influence the effect of vaccination on transmission: the timing of vaccination, the bird species considered, the match between vaccine and field virus, the vaccine used, and the number of vaccinations. All these factors make it difficult to predict the effect of vaccination on transmission, because the overall effect may be determined in a complex manner by the above factors. Clearly, it is neither practical nor desirable to perform transmission experiments for every possible combination of vaccine, field virus, and bird species. Therefore, it would be interesting to explore the value of HI titers after vaccination as a proxy determining the reduction of transmission. The choice for HI titers is based on the fact that antibodies against the hemagglutinin evoke the major protection against infection with HPAI (Webster et al., 1991), although they are not the only protective immune response. Antibodies against the neuraminidase were also found to be partly protective against HPAI in chickens (Pavlova et al., 2008; Qiao et al., 2003). Next to the humoral response other mechanisms like cellular immunity may also be involved in protection as was suggested in Chapter 3, and in previous studies (Webster et al., 2006). In Table 1 an overview of the HI titers at the moment of challenge and the reproduction ratio is given for all experiments. HI titers are given in an ascending order, and although the number of experiments is small it appears that there is a relation between the HI titers after vaccination and the final size of the experiments. However, there is variation between different species. In chickens a GMT of 0.25 (95%CI: 0-0.52) at seven days after vaccination was enough to significantly reduce transmission and reduce the reproduction ratio below 1, while in Pekin ducks, with a comparable low GMT of 0.19 (95% CI: 0-0.40) the reproduction ratio was not significantly below 1. In chickens a GMT of 3.6 (95% CI: 3.2-4.1) reduced transmission completely while in pheasants a GMT of 3.8 (95% CI: 2.9-4.8) did not lead to a significant reduction of transmission.

From these data I conclude that there are indications for a relation between HI titers after vaccination and reduction of transmission. The relation between transmission and HI titers should be explored systematically for the most important domestic bird species. In this way a standard can be developed for the HI titers that should be achieved by vaccination, in order to prevent outbreaks of HPAI in vaccinated birds. This will cost a lot of effort, but given that already a lot of

Table 1. HI titers of the birds at the moment of challenge and the final size of the experiments

HI titer* (95% CI)	Final Size	Species	Vaccination†	R_v‡	pH_0: $R_c = R_v$§	pH_0: $R_v \geq 1$
0	5, 1	Chickens	H7N3, 1 week	1.4 (0.4-2.9)	ns (0.10)	ns
0.14 (0-0.34)	5, 5	Pekin ducks	H5N2, 1 week	> 1.5	ns	ns
0.19 (0-0.40)	1, 2	Pekin ducks	H5N2, 2 weeks	0.6 (0.1-2.2)	0.01	ns
0.25 (0-0.52)	1, 0	Chickens	H7N1, 1 week	0.2 (0.005-1.1)	< 0.001	0.04
3.1 (2.1-4.1)	1, 0	Pekin ducks	H5N2, 2 weeks	0.2 (0.005-1.5)	0.002	ns (0.08)
3.6 (3.2-4.1)	0, 0	Chickens	H7N3, 2 weeks	< 0.7	< 0.001	< 0.017
3.8 (2.9-4.8)	5, 5	Pheasants	H7N1, 2 weeks	> 1.5	ns	ns
5.7 (5.0-6.3)	0, 0	Teals	H7N1, 2 weeks	< 0.7	< 0.001	0.017
5.8 (5.5-6.1)	0, 0	Chickens	H7N1, 2 weeks	< 0.7	< 0.001	< 0.017

* The geometric mean titer of the birds is given; † The vaccine used, time between vaccination and challenge; ‡ R is estimated based on the final size, for the chickens a fixed infectious period was assumed, for the Pekin ducks, teals and pheasants an exponentially distributed infectious period was assumed; § R_c , reproduction ration among unvaccinated birds; R_v, reproduction ration among vaccinated birds.

experimental work is undertaken worldwide to establish the efficacy and effectiveness of vaccines against HPAI it may well be worth the while (Spickler, Trampel, and Roth, 2008).

7.6 The use of DIVA tests

The results of Chapters 3-5 show that different mechanisms may lead to silent transmission in vaccinated birds. Silent transmission is undesirable because the epidemic is not controlled, and

because it may lead to antigenic variants (Lee, Senne, and Suarez, 2004). A way to detect silent transmission is by using a DIVA strategy, and in this thesis three serological DIVA tests were validated with sera from the experiments in Chapter 3 using Bayesian analyses. The estimated sensitivities of the N7 iFAT and the N7 NI assay are above 0.90 in detecting antibodies, but the sensitivity in detecting infection is substantially lower. The reason for this reduced sensitivity is that infected chickens with none or only a short period of detectable virus excretion (1-2 days) have a small change of developing antibodies (Chapter 6). This implies that a high proportion of these infected birds will not develop antibodies and, consequently, cannot be detected by a serological DIVA test. Whether these birds play a major role in spreading the disease remains to be investigated. On the other hand, infected chickens that do shed virus for three days or longer will most likely (0.95-0.99) develop antibodies and will be detected by the serological DIVA tests with a probability of over 0.90 (Chapter 6). Based on these findings, I conclude that the N7 iFAT and the N7 NI assay are useful tests in detecting silent transmission, because the birds that are most significant in spreading virus will be detected. On the other hand, due to the lower specificity the N7 iFAT and the N7 NI are less suitable for declaring freedom of disease after vaccination.

7.7 Conclusions

The experiments in this thesis demonstrate that vaccination of birds can be useful in reducing transmission of HPAI, already at one week after vaccination. The effect of vaccination on transmission depends on the vaccine used and the time between vaccination and challenge. Moreover, considerable differences were observed between bird species, but for the main groups of domestic bird species it was demonstrated that vaccination is effective in sufficiently reducing transmission.

The understanding that HPAI viruses arise from LPAI viruses has lead to legislation which describes that LPAI epidemics should be controlled. Based on the differences in the transmission between the H5N2 LPAI and HPAI viruses it was concluded that the control of an HPAI epidemic may offer more difficulties than the control of a LPAI epidemic, giving another reason to eradicate H5 and H7 LPAI outbreaks at all time.

It is concluded that the N7 iIFAT and the N7 NI assay are useful tests in detecting silent transmission. On the other hand, due to the lower specificity, the N7 iIFAT and the N7 NI are less suitable for declaring freedom of disease after vaccination.

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Samenvatting

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Samenvatting

Hoog pathogene aviaire influenza werd voor het eerst beschreven in 1878 in Italië onder de naam vogelpest, als een ernstige ziekte bij kippen. Hierna werden uitbraken beschreven in 1894 en 1901 in Italië, die later spreidden naar Oostenrijk, Duitsland, België en Frankrijk. In de jaren 20 kwam de ziekte ook voor in de Verenigde Staten, Afrika en het verre oosten. Vanaf de jaren 30 bleef het lange tijd stil totdat er in 1959 weer een uitbraak werd gerapporteerd.

Tegenwoordig is bekend dat hoog pathogene aviaire influenza wordt veroorzaakt door aviaire influenza virussen. Deze virussen kunnen worden ingedeeld op basis van twee oppervlakte eiwitten, het hemagglutinine (H) en het neuraminidase (N). Elk virus heeft één soort hemagglutinine en één soort neuraminidase. Op dit moment zijn er 16 verschillenden H types en 9 verschillenden N types bekend, die in principe in elke combinatie op een virus kunnen voorkomen (bijvoorbeeld H5N1, H7N7). De meeste aviaire influenza virussen veroorzaken een milde ziekte bij vogels, laag pathogene aviaire influenza (LPAI), maar sommige varianten van H5 en H7 virussen kunnen hoog pathogene aviaire influenza (HPAI) veroorzaken.

Hoog pathogene aviaire influenza veroorzaakt ernstige ziekte en sterfte bij gevoelige vogelsoorten zoals kippen en kalkoenen. Vaak is het eerste symptoom van de ziekte een plotselinge hoge sterfte van de dieren, tot wel 100%. Andere symptomen zijn eilegdaling, luchtwegproblemen, tranende ogen, bijholteontsteking, opgezwollen kop en lellen, onderhuidse bloedingen, diarree en hersenverschijnselen. De ernst van de symptomen hangt onder andere af van de vogelsoort, de leeftijd van de dieren, en de virusstam.

Wilde vogels worden beschouwd als het reservoir van laag pathogene aviaire influenza virussen. De primaire introductie van een LPAI virus in pluimvee vindt plaats vanuit wilde vogels, waarna het virus kan gaan spreiden binnen de pluimvee populatie. Phylogenetische studies hebben aangetoond dat HPAI virussen na mutatie ontstaan uit LPAI virussen tijdens uitbraken van LPAI in pluimvee. Er is een aantal uitbraken beschreven waarbij in eerste instantie een laag pathogeen virus werd geïsoleerd, maar waarbij later in de uitbraak een door mutatie ontstaan hoog pathogeen virus werd gevonden. Deze overgang van LPAI virus naar HPAI virus kan maanden na de introductie in pluimvee plaatsvinden, maar gebeurt soms ook al op het eerste bedrijf.

HPAI is lang beschouwd als een exotische ziekte met een lage uitbraakfrequentie, maar dit beeld is sterk veranderd in de afgelopen 10 jaar. De grootte en frequentie van uitbraken van HPAI lijken toe te nemen, en bovendien is een HPAI virus (H5N1) endemisch geworden in grote delen van de wereld. Deze ontwikkelingen vragen om alternatieve strategieën ter bestrijding en preventie van uitbraken naast maatregelen zoals ruimen, het instellen van beperkingsgebieden, vervoersverboden, en hygiënische maatregelen. Een aanvullende strategie is het gebruik van

vaccinatie.

Er is al veel onderzoek gedaan naar het effect van vaccinatie op het voorkomen van ziekte en sterfte en op het verminderen van virusuitscheiding door besmette dieren. Echter, er is nog niet zoveel bekend over het effect van vaccinatie op spreiding (transmissie) van virus tussen dieren, terwijl juist de spreiding verminderd zou moeten worden om een uitbraak te kunnen stoppen. In dit proefschrift is gekeken naar het effect van vaccinatie op transmissie van HPAI, hiertoe zijn transmissie experimenten uitgevoerd met verschillende HPAI virussen in verschillende vogelsoorten.

Om beter te begrijpen hoe een LPAI uitbraak kan overgaan in een HPAI uitbraak zijn in hoofdstuk 2 twee H5N2 stammen uit één uitbraak vergeleken. Deze stammen zijn afkomstig uit een uitbraak in Pennsylvania in 1983, die begon als een LPAI uitbraak en na zes maanden overging in een HPAI uitbraak. De transmissie van deze LPAI en HPAI virussen zijn met elkaar vergeleken in groepen kippen en grote verschillen tussen de laag en de hoog pathogene stam werden gevonden. De hoog pathogene stam spreidt gemakkelijker dan de laag pathogene stam en de geïnfecteerde dieren blijven langer besmettelijk. Deze verschillen kunnen verklaren waarom een hoog pathogeen virus, zodra het ontstaan is, een laag pathogeen virus kan verdringen. Ook blijkt dat als er al een uitbraak met laag pathogeen virus in een stal heeft plaatsgevonden, dat er dan nog voldoende niet geïnfecteerde dieren overblijven voor een hoog pathogeen virus om in diezelfde stal te kunnen spreiden. Vanwege de betere spreiding van het hoog pathogene virus is het aannemelijk dat dit virus moeilijker te bestrijden is dan de laag pathogene variant. Op basis hiervan concludeer ik dat dit een extra reden is om een uitbraak met een laag pathogeen H5 of H7 virus snel en afdoende te bestrijden.

In hoofdstukken 3 en 4 is gekeken naar het effect van vaccinatie op de spreiding van het H7N7 virus dat in Nederland in 2003 een grote uitbraak heeft veroorzaakt. Uit de experimenten blijkt dat het effect van vaccinatie afhangt van de vogelsoort, het gebruikte vaccin en de tijd tussen vaccinatie en besmetting. Een enkelvoudige vaccinatie gaf al een significante afname van de spreiding in kippen en talingen, maar in fazanten werd geen afname van de spreiding waargenomen. In kippen zijn twee verschillende vaccins vergeleken en beide vaccins voorkwamen transmissie op twee weken na vaccinatie. Een van de vaccins bleek de transmissie zelfs al te verminderen op een week na vaccinatie terwijl dat van het andere vaccin niet kon worden aangetoond.

Gedomesticeerde eenden worden gebruikt om te grazen op rijstvelden na de oogst in Zuidoost Azië, en zij blijken een grote rol te spelen in het optreden van uitbraken van H5N1 in pluimvee. Bovendien kan in eenden een infectie met een hoog pathogeen H5N1 virus symptoomloos verlopen, afhankelijk van de H5N1 stam. Dit waren redenen om te kijken naar het effect van vaccinatie op transmissie van H5N1 bij pekingeenden. Er werd een vaccin gebruikt dat

toegepast wordt in Azië, maar wat op basis van een lage genetische verwantschap met het H5N1 virus niet als ideaal kan worden beschouwd. Uit de experimenten blijkt echter dat het vaccin wel degelijk in staat is om de spreiding te verminderen, er kon niet worden aangetoond dat de spreiding zodanig verminderd werd dat een uitbraak voorkomen kan worden.

Uit de proeven met de kippen, fazanten en pekingeenden blijkt ook dat vaccinatie al heel snel beschermt tegen ziekte, maar dat dat nog niets zegt over vermindering van uitscheiding en spreiding van het virus. In al deze diersoorten werd spreiding gezien terwijl er geen (of minimale) ziektesymptomen werden gezien. Dit houdt in dat er tijdens vaccinatie altijd monitoring moet plaatsvinden naar veldinfecties. Dit kan gedaan worden door toepassing van een DIVA (Differentiating Infected from Vaccinated Animals) principe. Er zijn verschillende manieren om een DIVA principe toe te passen, bijvoorbeeld door middel van een serologische DIVA test. Bij een serologische DIVA test wordt gekeken naar antistoffen die wel ontstaan na infectie door het veldvirus, maar niet door het vaccin. Dit kan gebaseerd zijn op een verschil in neuraminidase: bij gebruik van een H7N1 vaccin worden er antistoffen gevormd tegen H7 en N1. Als deze gevaccineerde dieren vervolgens besmet worden door een H7N7 virus zullen er ook antistoffen gevormd worden tegen N7. Door deze N7 antistoffen aan te tonen met behulp van een serologische DIVA test kan een veldinfectie worden aangetoond. Een andere serologische DIVA test is gebaseerd op het aantonen van NS1 antistoffen die wel ontstaan na infectie met een veldvirus maar niet na vaccinatie met een geïnactiveerd vaccin. In hoofdstuk 6 van dit proefschrift zijn de prestaties van drie verschillende serologische DIVA testen onderzocht. Er is een statistische methode gebruikt waarmee niet alleen iets gezegd kon worden over de sensitiviteit en de specificiteit van de testen in het aantonen van antilichamen, maar waarmee ook een uitspraak kon worden gedaan over de waarschijnlijkheid dat een dier antilichamen maakt na infectie. Het blijkt dat geïnfecteerde gevaccineerde dieren die maar kort virus uitscheiden een kleine kans hebben om antilichamen te vormen. De sensitiviteit van twee van de drie onderzochte DIVA testen in het opsporen van antilichamen blijkt goed te zijn, de sensitiviteit in het opsporen van infectie ligt een stuk lager omdat een aantal geïnfecteerde dieren geen antilichamen maakt. De specificiteit van de testen is aan de lage kant, wat kan leiden tot een groot aantal vals positieve testuitslagen.

Geconcludeerd kan worden dat vaccinatie van vogels gebruikt kan worden om de spreiding van hoog pathogene aviaire influenza te verminderen, zelfs al op een week na vaccinatie. Het effect van vaccinatie op transmissie hangt af van het gebruikte vaccin, de tijd tussen vaccinatie en besmetting, maar vooral van de vogelsoort. Grote verschillen werden gevonden tussen verschillende vogelsoorten, echter voor de belangrijkste groepen blijkt dat vaccinatie een effectief middel is om spreiding te verminderen. In hoofdstuk 2 werd aangetoond dat het hoog pathogene H5N2 virus gemakkelijker spreidt dan de laag pathogene variant. Dit

impliceert dat de bestrijding van het hoog pathogene virus meer problemen kan geven en is een goede reden om laag pathogene H5 en H7 virussen afdoende te bestrijden. Twee van de drie onderzochte serologische DIVA testen blijken gevoelig genoeg om transmissie in gevaccineerde dieren op te sporen. Echter, beide testen geven behoorlijk wat vals positieve resultaten waardoor ze minder geschikt zijn om bijvoorbeeld een gebied vrij te verklaren.

Dankwoord

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Curriculum Vitae

Janke Antje (Jeanet) van der Goot werd op 24 november 1963 geboren in Balk. In 1982 deed zij eindexamen Atheneum en begon in 1984 aan de studie Diergeneeskunde aan de Rijksuniversiteit Utrecht. In 1996 ronde zij haar studie af, met als differentiatie gezelschapsdieren. Tijdens deze studie kreeg ze de gelegenheid om acht maanden stage te lopen bij de vakgroep Infectieziekten en Immunologie van de faculteit Diergeneeskunde op de afdeling Bacteriologie bij Wim Gaastra. Vervolgens werkte ze eerst nog gedurende 3 ½ jaar als dierenarts in diverse praktijken, om in 1999 te gaan werken bij het toenmalige ID-Lelystad. Van 1999 tot 2008 is zij werkzaam geweest op het aviaire virologie laboratorium bij Guus Koch, waar zij zich voornamelijk heeft beziggehouden met de diagnostiek van en onderzoek aan aviaire influenza. Sinds september 2008 is zij werkzaam bij het cluster Kwantitatieve veterinaire epidemiologie en risico analyse (QVERA) van het Centraal Veterinair Instituut van Wageningen UR.

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