Indirect transmission of highly pathogenic avian influenza in chickens

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Indirect transmission of highly pathogenic avian influenza in chickens

Indirecte transmissie van
hoog pathogene aviaire influenza bij kippen
(met een samenvatting in het Nederlands)

Proefschrift

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Voor Mik

Contents

Chapter 1

General Introduction

1.1. General Introduction

1.1.1. Avian influenza virus

Highly pathogenic avian influenza (HPAI) was first described as a poultry disease by Perroncito in 1878 (Perroncito, 1878), but it was not until 1955 when the causative agent was characterized as influenza A virus (Schäfer, 1955). Influenza viruses, which are included in three genera (*Influenzavirus A, Influenzavirus B, and Influenzavirus C*), have segmented, negative sense, single strand RNA genomes and are placed in the family *Orthomyxoviridae*. Influenza A viruses are further divided into different subtypes according to the antigenic characteristics of the surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). To date, 17 haemagglutinin subtypes (H1 – H17), and 9 neuraminidase subtypes (N1 – N9) have been described. All types except H17, which has been detected in bats (Tong et al., 2012), have been isolated as low pathogenic avian influenza (LPAI) viruses from aquatic wild birds, which are considered being the main reservoir of the virus (Webster et al., 1992; Fouchier et al., 2005; Alexander, 2007).

Generally, LPAI virus replicates in wild birds without causing disease, and infection of poultry usually causes only mild clinical signs and a reduction in weight gain and egg production (Alexander, 2000). LPAI virus of the subtypes H5 and H7 can, however, mutate in poultry into a highly pathogenic avian influenza (HPAI) virus strain when the amino acid sequence at the cleavage site of the haemagglutinin changes. If HPAI virus becomes the dominant virus in a flock, the clinical outcome shifts from a disease with little or no clinical signs into a severe disease characterized by respiratory distress, sinusitis, ocular and nasal discharges, neurological signs and diarrhoea, and mortality rates up to 100% (Swayne and Suarez, 2000). Classification of the pathogenicity of avian influenza viruses (HPAI or LPAI viruses) of the subtypes H5 or H7 occurs according to the OIE standard (OIE, 2009).

During the past decades several epidemics of HPAI have been documented by the World Organisation for Animal Health (OIE, 2012). Although the H5N1 pandemic is without any doubt the most devastating one up to now, huge epidemics have also been observed in poultry dense areas of Italy in 1999 (Marangon et al., 2004) and The Netherlands in 2003 (Stegeman et al., 2004). A movement ban of live birds in infected regions and culling of infected flocks

was, however, insufficient to stop those epidemics, and they could only be ended after massive culling of contiguous flocks or depopulating entire areas resulting in high economic losses (Capua and Marangon, 2003).

1.1.2. Routes of transmission

Although movement of infected birds between flocks is the most efficient way of virus transmission (Swayne and Halvorson, 2008), even after a movement ban transmission between flocks in poultry dense regions was observed. During the H7N7 epidemic in the Netherlands in 2003, between-farm transmission of HPAI virus through humans was found feasible, and also transport of vehicles and materials was associated with the spread of HPAI virus (Te Beest et al., 2011; Ssematimba et al., 2012). However, the contacts registered for these routes of transmission only associated with a limited part of the between-farm transmission (Ssematimba et al, 2012). This may have been the result of underreporting of diverse contacts between poultry farmers, or, alternatively, some routes of transmission may have yet to be identified.

Airborne transmission has been suggested as a possible example of the latter (Davison et al., 2003). During the HPAI epidemic in British Colombia, Canada, in 2004, live virus was detected in air samples taken up to 250 metres from an infected farm (Schoffield et al., 2005). However, in air samples, collected during the H5N2 HPAI epidemic in the USA in 1983 – 1984, no influenza virus was detected from samples taken more than 45 metres downwind from an infected poultry house (Brugh and Johnson, 1987). Nevertheless, the contribution of airborne spread to between-farm transmission in both above mentioned epidemics remains unclear.

1.1.3. Airborne transmission

Airborne transmission is defined a route of transmission in which an infectious agent is spread to a susceptible host through the air by means of droplets, droplet nuclei (aerosols), or environmental sources such as dander and dust. In human influenza it has been distinguished into short- and long-distance transmission. For short distance transmission an infection results from droplets containing influenza expelled by infected humans through coughing, sneezing or exhalation (Brankston et al., 2007). These droplets are subsequently deposited onto conjunctivae or mucous membranes of a susceptible host. This

type of transmission can only occur at close proximity between an infected and susceptible host, as droplets settle quickly on the ground (Tellier, 2006; Fabian et al., 2008; Gralton et al., 2011).

When droplets are expelled by coughing and sneezing, they may also shrink in size by evaporation; such particles are referred to as droplet nuclei or aerosols. These droplet nuclei can remain suspended in air for extended periods enabling them to travel long distances up to many kilometers if wind and other weather conditions are favorable, and thereby expose susceptible individuals to infection at a greater distance from the source (Gralton et al., 2011). Droplet nuclei are respirable and can transport the virus directly into the alveolar region, resulting in infection of the respiratory tract.

For chickens the mechanism of airborne transmission is probably different from that described for humans. Viruses from the respiratory tract of chickens can become expelled as droplets but the anatomy of the respiratory tract of the chicken, and the incomplete diaphragm, most likely prevents viruses to become suspended as droplet nuclei to the same extent as in humans. Consequently, long-distance virus transmission caused by virus that was directly aerosolized from the respiratory tract is considered less likely than in case of humans. However, virus attached to environmental sources such as dust might be a possible route of long-distance virus transmission by air.

1.1.4. Role of airborne transmission between poultry flocks

Poultry houses are an important source of dust for the environment. It originates from feed, bedding material, skin cells, feather debris and faeces. Dust particles can carry microorganisms (Aarnink et al, 1999) and consequently, avian influenza (AI) virus excreted by infected birds via the respiratory tract, in faeces, feathers and skin (Perkins and Swayne, 2001; Swayne and Halvorson, 2008) might become attached to dust. Ventilation of poultry houses emits dust into the environment that could subsequently serve as a mechanical vector for transmission of AI virus between farms (Cambra-Lopez et al., 2010).

Chen et al. (2010) found RNA of AI virus in dust originating from Asian dust storms, demonstrating the attachment of the virus to dust. However, demonstrating viral RNA in dust collected in the environment is insufficient to state that an infection may occur by airborne transmission as the virus may not be viable anymore. Viability is determined by the inactivation rate outside the

host, which depends on several environmental parameters, such as relative humidity (RH) and temperature. AI virus can survive in aerosols for several hours, but only little is known about the mechanism of inactivation in the airborne environment, and up to now studies on inactivation processes of AI virus have shown contradictory results (Weber and Stilianakis, 2008).

Under field circumstances it is difficult to demonstrate whether airborne transmission is a relevant causal transmission route of avian influenza. Avian influenza infections of poultry with H5 and H7 subtypes are notifiable to the World Organisation for Animal Health and if infection occurs in the European Union it should be controlled as soon as possible. Therefore, information on between-flock transmission routes can only be collected in outbreak situations and available data is limited. Furthermore, even if field data would be available, reconstruction of the transmission chain is often clouded by competing transmission routes, uncertainties of the infection dynamics in a flock (e.g. when was virus introduced?) and lack of information (underreporting of contacts). Consequently, experimental studies into airborne transmission of HPAI virus would be useful to help elucidating its role in HPAI epidemics.

1.2. Aim and outline of this thesis

The main goal of this thesis was to study and quantify airborne transmission of highly pathogenic avian influenza H5N1 infection in naive and vaccinated chickens under experimental conditions.

First it was studied how virus transmission to chickens in direct contact depends on the inoculation dose (Chapter 2). In the study described in Chapter 3 airborne transmission between chickens in the same room was quantified. During this study virus was detected in dust collected from the air and ground. In the next series of experiments (Chapter 4) transmission of virus through dust was further studied between groups of chickens in different rooms connected by a tube. Chapter 5 describes a vaccination study in which the chickens were vaccinated with a low dose of vaccine to estimate virus excretion into the environment and determine transmission of virus to susceptible cage mates.

Mapping of the spatiotemporal transmission of HPAI virus in chickens in the field using molecular epidemiological techniques is hampered by applying these phylogenetic analyses on a very small sample size from a limited number of

farms. In the sixth chapter the genetic variation of HPAI virus in the experimental population described in chapter 4 is reported. Not only does this provide information on the changes of the virus during infection of a flock, but also on changes after airborne transmission to another flock.

In the last chapter (Chapter 7), results, implications and critical elements presented in previous chapters are integrated and discussed. The results from these transmission experiments may help to increase our understanding of between-flock transmission during outbreaks and thus help improve control measures.

Chapter 2

The effect of an inoculation dose of a highly pathogenic avian influenza virus strain H5N1 on the infectiousness of chickens

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Abstract

Highly pathogenic avian influenza is of major concern for the poultry industry, as the virus can spread rapidly in and between flocks, causing high mortality and severe economic losses. The aim of this study was to determine the probability of infection and to determine dose-dependent virus transmission (direct transmission) for various inoculation doses.

Two transmission experiments with pair-wise housed layer type chickens were performed, in which one bird per pair was inoculated with an HPAI H5N1 virus and the other contact-exposed. Various inoculation doses were used to determine the susceptibility (ID_{50}), and possible relation between ID_{50}, and infectiousness, expressed as the amount of virus shedding and the probability of contact birds becoming infected.

The infectious H5N1 dose (CID_{50}) in this study was an estimated $10^{2.5}$ egg infectious dose (EID_{50}). Increasing the dose increased the probability of infection but survival from infection was independent of dose. In addition, increasing the dose decreased the mean latent period in the inoculated chickens significantly. This could be important for determining the time of onset of infection in a flock and thus allowing more accurate identification of the source of infection. Moreover, the amount of virus shed in trachea and cloaca by the inoculated chickens in the time between inoculation and contact infection, also differed between the various dose groups. Despite differences in latent period and virus shedding, the transmission rate parameter β and reproduction ratio R_0 did not differ significantly between the various dose groups. This implies that in this experiment the amount of virus shedding is not a measure to predict transmission or the infectiousness of chickens.

2.1. Introduction

Highly pathogenic avian influenza (HPAI) is a viral disease caused by influenza type A virus strains carrying the haemagglutinin H5 or H7. Although the virus can infect many animal species (Webster et al., 1992; Perdue and Swayne, 2005; Alexander, 2007), it is of major concern for the poultry industry, as the virus can spread rapidly in and between flocks causing high mortality and severe economic losses (Alexander, 2007). HPAI strains have also caused

human infection and fatalities, and taking into account the recent outbreaks and fatal human cases in Asia and other countries in the world, a human influenza pandemic caused by these virus strains is considered a risk (Claas et al., 1998; Subbarao et al., 1998). As a consequence, outbreaks of HPAI in poultry need to be controlled as quickly as possible, both to prevent more human cases and to protect poultry.

Rapid detection and control of new outbreaks require appropriate surveillance programmes and intervention strategies, which, in turn, require knowledge of the transmission dynamics of the infection. A key parameter herein is the reproduction ratio R_0 (Anderson and May, 1979) defined as the average number of secondary cases caused by a typical infectious individual during its entire infectious period in a totally susceptible population (Diekmann et al., 1990). This parameter, representing the spread of the virus within a population, depends on the infectiousness of infected birds, the susceptibility of the contact-exposed birds and the contact structure between the two (Anderson and May, 1979; Diekmann and Heesterbeek, 2000) and is often used in epidemiological studies in which the effect of various surveillance programs (Graat et al., 2001; Klinkenberg et al., 2003) or the efficacy of control measures is determined (Stegeman et al., 1999; de Jong and Kimman, 1994).

Several studies have been carried out to quantify the reproduction ratio for HPAI (van der Goot et al., 2005; Bos et al., 2008; Bouma et al., 2009). In the experimental studies, high inoculation doses have been used to ensure the start of the infection chain. These doses often resulted in rapid infection and death of the chickens (Poetri et al., 2008; van der Goot et al., 2008). It seems, however, reasonable to assume that an infection under field conditions may occur after exposure to lower amounts of virus than used in (challenge) experiments. Differences in (exposure to) infectious doses may result in differences in infectiousness, susceptibility and contact rate, and consequently in differences in the transmission.

Dose-response studies have been carried out before. These studies determined virus shedding and number of successful infections. In such studies, the amount of virus excreted after infection is usually considered to be the appropriate measure for the infectiousness of birds (Perdue and Swayne, 2005; Kelly et al., 2008; Swayne and Slemons, 2008). The link between virus shedding and infectiousness has, however, not been demonstrated in the

aforementioned studies, because infectiousness is not only related to virus production and shedding, but also to the probability of contact birds becoming infected when exposed to a certain virus dose. The virus dose able to infect 50% of the group, a common measure of susceptibility for infection, is often expressed as the ID_{50}. ID_{50} studies have also been performed previously (Swayne and Slemons, 2008). Although these studies provide relevant information about, for example, differences in virulence, the link between challenge dose and ID_{50} might not be straightforward, as under field conditions the exact dose with which they become infected remain unknown, as does the link with transmission in relation to the dose dependent probability of infection. A link between ID_{50} and excretion on the one side and virus transmission on the other side can be determined in a pair-wise transmission experiment (Velthuis et al., 2006).

Thus, the aim of this study was to determine both the probability of HPAI virus infection and to quantify direct virus transmission for various inoculation doses. To these ends, two series of transmission experiments with pair-wise housed layer type chickens were performed, in which one bird per pair was inoculated with a HPAI H5N1 virus and the other contact-exposed. Various inoculation doses were used to determine the susceptibility (ID_{50}), and possible relation between ID_{50}, and transmission.

2.2. Materials and methods

2.2.1. Animals

Eggs from White Leghorn chickens were purchased from a commercial avian influenza (AI) free poultry farm and hatched at the Central Veterinary Institute in Lelystad, The Netherlands. The first 5 weeks after hatching, the chickens were housed as a group. At 5 weeks of age, the chickens were tested for the absence of antibodies against AI using a blocking ELISA and then randomly divided in 4 groups of 22 chickens each, each housed in a separate room. In each room, chickens were housed pair-wises in one of 11 cages. A cage was constructed of hardboard and the cover was made of gaze. The cages were divided over two rows. Feed and water were provided ad libitum.

2.2.2. Inoculum

The HPAI virus strain A/turkey/Turkey/1/2005 H5N1 (clade 2.2) was used as challenge strain for inoculation (Londt et al., 2008). The virus was grown in embryonated SPF eggs, and vials with a known EID_{50} titer were stored at -70°C until use. On the day of challenge, one vial was thawed and diluted in 10-fold dilution steps in tryptose phosphate buffer (TPB) to obtain the necessary inoculation doses.

Two experiments were carried out consecutively, each with 4 groups of 22 chickens. Each group was housed in a separate room. Per group one inoculation dose was used. In experiment 1, the inoculation doses were 10^2, 10^3, 10^4 and 10^5 50% egg infectious dose (EID_{50}). In experiment 2, the range of the treatment groups was narrowed, and the doses used were $10^{2.5}$, 10^3, $10^{3.5}$ and 10^4 EID_{50} per chicken. The term ‘‘treatment group’’ is used to refer to the various dose groups. In both experiments, the chickens were inoculated with 0.1ml inoculum applied intra-nasally and 0.1ml inoculum applied intra-tracheally using a blunt needle, according to standard protocol (van der Goot et al., 2005).

2.2.3. Experimental design

Pairs of chickens were used, in which one was inoculated with one particular dose and one was contact-exposed to the inoculated chicken. In experiment 1, five pairs per treatment group were used, in experiment 2, six pairs. Between the cages in which these chickens were kept, non-inoculated ‘sentinel’ chickens were put pair-wise, to measure the independence of the transmission in the pairs. These sentinels were thereby indirectly exposed to the inoculated chickens in the neighbouring cages. Because of the maximum of available cages per room, in the second experiment the number of pairs in which one was inoculated and one was contact-exposed was interchanged with the number of pairs with sentinel chickens. Both animal experiments were carried out in the high containment unit under BSL^{3+} conditions at the Central Veterinary Institute of Wageningen UR in Lelystad. The experiments complied with Dutch law on animal experiments and were reviewed by an ethical committee.

2.2.4. Sampling procedures

At day 1 post-inoculation (p.i.), swabs from trachea and cloaca were collected from the inoculated chickens and at days 2 – 10, 14, 17 and 21 p.i. from all

chickens. The swabs were put in 2ml of 2.95% TPB with 5 x 10^3 IU of penicillin–sodium and 5mg streptomycin per ml and stored at -70°C until analysed. Serum blood samples were taken from the ulnar vein 7 days before and at days 7, 14 and 21 after inoculation from all chickens. These samples were stored at -20°C until analysed. The experiments were terminated 21 days post inoculation by euthanizing surviving birds with an intracardiac injection of T-61.

2.2.5. RNA isolation and quantitative real-time reverse transcriptase PCR

RNA isolation was performed with MagNA Pure LC 2.0 instrument (Roche Applied Science, Mannheim, Germany) with the MagNA Pure LC total Nucleic Acid Isolation Kit (Roche Applied Science, Mannheim, Germany). RNA was isolated from 200ml of swab fluid according to the manufacturer's instructions. The nucleic acids were collected in elution buffer and stored at -70°C or directly processed for the quantitative real-time reverse transcriptase PCR (RT-qPCR). The RT-qPCR and data analysis were performed using the MX4000 Quantitative PCR system (Stratagene) with version 4.20 software. We used 5ml of the elution buffer with extracted RNA for RT-qPCR as described in van der Goot (2008) to detect the matrix gene of the influenza virus. The viral RNA concentration of each sample could be calculated using a calibration curve of serial dilutions of a standard batch of the virus with a known EID_{50} titer. Dilutions of the standard batch were run along with the unknown samples. Quantification of the viral concentration in each sample was based on the calibration curve generated by plotting the cycle threshold value against the known virus titers. Titers of the samples were expressed as EID_{50} equivalents.

2.2.6. Laboratory tests

For virus isolation per swab, three embryonated SPF chicken eggs incubated for 9 days were inoculated with 0.2ml swab fluid per egg. After 72h the allantoic fluid was harvested and a standard hemagglutination assay (HA) with chicken red blood cells was performed (OIE, 2008). When at least one egg of three eggs was positive in HA the swab was considered to be positive. Sera were incubated for 30min at 56°C. A modified indirect double antibody sandwich (IDAS) nucleoprotein (NP)-blocking ELISA that detects antibodies against the nucleoprotein of influenza A was performed (de Boer et al., 1990).

2.2.7. Data analysis

Direct transmission was based on the number of infected contact chickens. Indirect transmission was based on the number of infected sentinel chickens. The method used to estimate transmission parameters was based on a generalized linear model (GLM) assuming a stochastic SEIR (susceptible-exposed-infectious-removed) transmission process (de Jong and Kimman, 1994). A chicken was considered to have been infected if it met one or more of the following criteria: the occurrence of AI-like symptoms, two or more positive serological tests, or a positive RT-qPCR; the correlation between the amount of viral RNA determined by RT-qPCR and virus titer has been shown by Lee and Suarez (2004). Chickens that tested positive in RT-qPCR but did not die from infection were confirmed by virus isolation (Table 1).

The transmission rate parameter (β) and basic reproduction ratio (R_0) were calculated for each treatment group. The transmission rate parameter was defined as the average rate at which an infected animal infects a susceptible animal per unit of time (Moerman et al., 1993; Diekmann and Heesterbeek, 2000; Klinkenberg et al., 2002). The day of transmission was determined by the first day of excretion of the contact chickens minus a latent period of 1 day. The basic reproduction ratio (R_0) is the expected average number of secondary cases per typical primary case in a totally susceptible population. R_0 was calculated by multiplying β with the infectious period (T_i), the number of days an inoculated chicken shed virus. Details of the method have been described previously (Becker, 1989; van der Goot et al., 2005; Velthuis et al., 2006).

The 50% chicken infectious dose (CID_{50}) and 50% chicken lethal dose (CLD_{50}) were calculated using a binomial regression with probit link function. Survival functions for the different treatment groups were calculated using the Kaplan–Meier method. The mean survival times for the different treatment groups were compared using a log rank test. Viral shedding after inoculation, measured in RT-qPCR on tracheal and cloacal swabs, was quantified over time by calculating the area under the 'excretion against time' curve (AUC) of the viral titers ($\log_{10}$ EID_{50}) as described by Brown et al. (2009). These AUC values, which estimate the amount of virus shedding during the total infectious period, were compared for the different treatment groups using ANOVA. The amount of virus shed in trachea and cloaca by the inoculated chickens in the time between inoculation and contact infection of the pen mate for the different

treatment groups were compared using ANOVA. All tests were performed assuming a 2-sided alternative hypothesis; *p* values smaller than 0.05 were considered statistically significant. Analysis was performed using commercially available statistical software (SPSS 16.0; SPSS Inc., Chicago, Illinois).

2.3. Results

2.3.1. Infection of chickens

In total 176 chickens were used; 44 inoculated, 44 contact and 88 sentinel chickens. None of these had pre-existing antibodies against AI. The 58 chickens that died during the experiments all showed AI-like symptoms and of these, 55 had one or more positive RT-qPCR results for the tracheal and/or cloacal swabs. The three chickens that failed to do so were all in the 10^3 treatment group. One pair of sentinel chickens in the $10^{3.5}$ treatment group shed virus from day 4 until day 6, showed AI-like symptoms and died during the experiment. The most common AI-like symptoms were losing appetite, depression and conjunctivitis.

None of the surviving chickens showed any clinical signs. One inoculated chicken in the 10^4 treatment group and its contact tested RT-qPCR positive in the trachea on day 3 p.i. Three surviving contact chickens in the 10^2, 10^3 and 10^4 treatment groups tested positive in the RT-qPCR once on day 3, 2 and 5 p.i., respectively (Table 2.1). One surviving sentinel in the 10^5 treatment group tested positive in the RT-qPCR on day 4 p.i. The positive RT-qPCR results were confirmed by virus isolation. Finally, one surviving sentinel chicken in the 10^2 treatment group tested positive in IDAS NP blocking ELISA at days 7, 14 and 21 p.i.

2.3.2. Survival data

In treatment group 10^2, 2 of 5 birds died after inoculation; in group $10^{2.5}$, 1 of 6 died; in groups 10^3 and 10^4, 10 of 11 died; in group $10^{3.5}$, 5 of 6 died; and in group 10^5, all 5 inoculated birds died (Table 2.2). The CID_{50} was $10^{2.5}$ EID_{50} (95%CI: $10^{1.7}$–$10^{2.8}$ EID_{50}) and the CLD_{50} was $10^{2.5}$ EID_{50} (95%CI: $10^{1.9}$–$10^{2.8}$ EID_{50}). The mean survival time for the infected inoculated chickens did not differ significantly between treatment groups.

Table 2.1: Summary of the RT-qPCR results of trachea and cloacal swabs, mortality data and serological response of both transmission experiments for the inoculated and contact chickens that tested positive in the RT-qPCR or died during the experiment.
I = inoculated chicken; C = contact chicken; nd = not determined; +/+ = positive trachea and cloacal swab; -/- = negative trachea and cloacal swab; +/- = positive trachea and negative cloacal swab; -/+ = negative trachea and positive cloacal swab; grey box = chicken tested positive but did not die from infection; † = chicken died; EID = egg infectious dose.

		Virus excretion in trachea and cloacal swabs										Serology
Inoculation dose		Days post-inoculation										
[log_{10} EID_{50}]		1	2	3	4	5	6	7	8	9	10	21
2	I	-/-	**+/+**	†								nd
	C	nd	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	Neg
	I	-/-	**+/+**	**+/+**	†							nd
	C	nd	-/-	-/-	-/-	**+/+**	†					nd
2.5	I	**+/+**	†									nd
	C	nd	-/-	**+/+**	†							nd
3	I	**+/+**	†									nd
	C	nd	**-/-**	**+/-**	†							nd
	I	-/-	†									nd
	C	nd	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	Neg
	I	-/-	**+/+**	†								nd
	C	nd	**+/+**	**+/-**	**+/+**	†						nd
	I	-/-	**+/+**	†								nd
	C	nd	-/-	**+/-**	**+/-**	**+/+**	†					nd
	I	-/-	**+/+**	†								nd
	C	nd	-/-	-/-	-/-	**+/+**	†					nd
	I	-/-	**+/+**	†								nd
	C	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	Neg
	I	-/-	**+/-**	**+/+**	†							nd
	C	nd	-/-	**+/-**	**+/-**	**+/-**	**+/-**	†				nd
	I	-/-	**+/-**	**+/+**	†							nd
	C	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	Neg
	I	-/-	-/-	-/-	-/-	†						nd
	C	nd	-/-	-/-	-/-	**+/-**	**+/+**	**+/+**	†			nd
	I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	†		nd
	C	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	**+/-**	†	nd
3.5	I	**+/+**	†									nd

Table 2.1 continued

	C	nd	**+/-**	**+/-**	**+/-**	†						nd
	I	**+/-**	†									nd
	C	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	Neg
	I	**+/-**	**+/+**	†								nd
	C	nd	-/-	**+/-**	†							nd
	I	**+/-**	**+/+**	**+/+**	†							nd
	C	nd	-/-	-/-	-/-	**+/+**	†					nd
	I	-/-	-/-	**+/+**	**-/+**	**-/-**	**+/-**	**-/-**	**+/+**	**+/+**	†	nd
	C	nd	-/-	-/-	-/-	**+/+**	**+/+**	†				nd
10^4	I	**+/+**	†									nd
	C	nd	-/-	**+/+**	†							nd
	I	**+/-**	†									nd
	C	nd	-/-	-/-	-/-	-/-	**+/-**	**+/+**	†			nd
	I	**+/-**	†									nd
	C	nd	-/-	-/-	-/-	+/-	-/-	-/-	-/-	-/-	-/-	Neg
	I	**+/-**	†									nd
	C	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	Neg
	I	**+/-**	†									nd
	C	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	Neg
	I	**+/+**	**+/+**	†								nd
	C	nd	-/-	**+/+**	†							nd
	I	**+/-**	**+/+**	†								nd
	C	nd	-/-	**+/-**	†							nd
	I	**+/-**	**+/+**	†								nd
	C	nd	**+/-**	**+/+**	**+/+**	†						nd
	I	**+/-**	**+/+**	†								nd
	C	nd	-/-	-/-	-/-	**+/+**	†					nd
	I	**+/-**	**+/+**	†								nd
	C	nd	-/-	**+/-**	**-/+**	**+/+**	†					nd
	I	**-/-**	**-/-**	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	Neg
	C	nd	**-/-**	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	Neg
10^5	I	**+/+**	†									nd
	C	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	Neg
	I	**+/-**	**+/+**	†								nd
	C	nd	-/-	**+/+**	†							nd
	I	**+/+**	**+/+**	†								nd
	C	nd	-/-	-/-	**+/-**	†						nd

Table 2.1 continued

I	**+/-**	**+/+**	†								nd
C	nd	-/-	**+/-**	**+/+**	**+/+**	†					nd
I	**+/+**	**+/+**	†								nd
C	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	Neg

2.3.3. Virus excretion

The combined amount of virus detected in the trachea and cloaca of infected inoculated chickens before the pen mate was contact-infected increased significantly as the inoculation dose (p = 0.04) increased. However, the total amount of virus shed during the entire infectious period did not differ significantly between inoculated infected birds in the various treatment groups. Information about the area under the 'excretion against time' curve (AUC) of the inoculated and contact chickens is summarized per group in Table 2.3. The mean latent period of the inoculated chickens decreased with increasing inoculation dose, from 2 days for treatment group 10^2 EID_{50} to 1 day for treatment group 10^5 EID_{50} (p = 0.01). Nevertheless, the mean infectious period (duration of shedding) did not differ significantly between the treatment groups, neither for the inoculated chickens or contact-infected chickens (Table 2.2).

2.3.4. Quantification of transmission characteristics

In treatment group 10^2, 1 of the 2 infected inoculated birds transmitted the virus to its cage mate. The transmission ratio in other treatment groups was 1:1 ($10^{2.5}$), 7:10 (10^3), 4:5 ($10^{3.5}$), 7:10 (10^4), and 3:5 (10^5). The transmission rate parameter β for treatment group 10^2 was estimated to be 1.7/day, for group $10^{2.5}$ 2.2/day, for group 10^3 0.9/day, for group $10^{3.5}$ 2.0/day, for group 10^4 1.6/day, and for group 10^5 2.7/day. These parameter estimates did not differ significantly between treatment groups. The reproduction ratio (R_0) for treatment group 10^2 was estimated at 2.5; for group $10^{2.5}$, 2.2; for group 10^3, 1.2; for group $10^{3.5}$, 5.6; for group 10^4, 2.1; and for group 10^5, 4.8. Again, these parameter estimates did not differ significantly between treatment groups (Table 2.2). The time between day of inoculation and day of presumed infection of the pen bird also, did not differ significantly between the groups. As the various mean length of shedding did not differ significantly with dose, we combined the results of the groups. The mean length of period between inoculation and infection was 2.3 days (95%CI: 1.9–2.7 days p.i.).

Table 2.2: Overview of number of infected inoculated and contact chickens. Estimates of the transmission rate parameter and basic reproduction ratio per inoculation dose and an overview of the mean latent and infectious period per inoculation dose.
EID = egg infectious dose; CI = confidence interval

Inoculation dose [$\log_{10}$ EID_{50}]	Number of infected inoculated chickens [n/total]	Pair with successful transmission [n/total]	Transmission rate parameter (95% CI) [day^{-1}]	Reproduction ration (95% CI)	Mean latent period	Mean infectious period	
					Inoculated chickens (95% CI) [day^{-1}]	Inoculated chickens (95% CI) [day^{-1}]	Contact chickens (95% CI) [day^{-1}]
2.0	2/5	1/2	1.7 (0.1-19.3)	2.5 (0-6.8)	2	1.5 (0-7.8)	1
2.5	1/6	1/1	2.2 (0.1-44.4)	2.2 (0-5.2)	1	1	1
3.0	10/11	7/10	0.9 (0.1-10.0)	1.2 (0-4.4)	1.9 (1.5-2.2)	1.3 (0.8-1.7)	2.1 (1.1-3.2)
3.5	5/6	4/5	2.0 (0.3-14.0)	5.6 (0-16.8)	1.4 (0.3-2.6)	2.8 (0.6-5.0)	1.8 (0.2-3.3)
4	10/11	7/10	1.6 (0.2-11.0)	2.1 (0-5.2)	1.1 (0.9-1.3)	1.4 (1.0-1.7)	1.6 (0.9-2.2)
5	5/5	3/5	2.7 (1.2-5.9)	4.8 (0-7.5)	1	1.8 (1.2-2.4)	1.7 (0-4.5)

2.4. Discussion

The aim of this research was to determine the association between the inoculation dose of an H5N1 HPAI virus strain and transmission of the virus to contact-exposed chickens. The results showed that the probability of infection increased with increasing inoculation dose. In inoculated birds, the mean latent period decreased significantly with increasing dose, which means that the higher the dose, the quicker infected birds started shedding virus after inoculation. Moreover, the amount of virus shed in trachea and cloaca in the time between inoculation and contact infection of the pen mate also differed between the various treatment groups. However, despite differences in latent period and virus shedding until contact infection occurred, the estimates for the transmission rate parameter β (varying between 0.9 [day^{-1}] and 2.7 [day^{-1}]) and the reproduction ratio R_0 (varying between 1.3 and 4.8) did not differ significantly between the various treatment groups. Apparently, the amount of virus shed by inoculated infected birds in all treatment groups was sufficient to induce infection in contact-exposed birds.

The probability of infection increased with increasing inoculation dose, which is consistent with previous findings (Swayne and Slemons, 2008). As the amount of virus shed until successful contact infection also increased with dose, it was expected that transmission rate would also have differed. However, although the transmission rate tended to show an ‘optimum’ in the median treatment groups, the estimates did not differ significantly.

Table 2.3. Overview of the total amount of viral RNA excreted during the entire infectious period of the inoculated and contact chickens, the amount of viral RNA excreted during the infectious period until day of transmission to the pen-mate of the inoculated chickens and the amount of viral RNA excreted in trachea and cloaca during the entire infectious period of the inoculated and the contact chickens.
EID = egg infectious dose; CI = confidence interval; AUC = area under the 'excretion against time' curve. [a] p = 0.04; [b] p = 0.01

Inoculation dose [$\log_{10}$ EID_{50}]	Mean AUC until transmission	Mean total AUC					
	Trachea and cloaca	Trachea and cloaca		Trachea		Cloaca	
	Inoculated chickens (95% CI) [$\log_{10}$]	Inoculated chickens (95% CI) [$\log_{10}$]	Contact chickens (95% CI) [$\log_{10}$]	Inoculated chickens [$\log_{10}$]	Contact chickens [$\log_{10}$]	Inoculated chickens [$\log_{10}$]	Contact chickens [$\log_{10}$]
2.0	4.8 (2.6-7.1)	4.8 (2.6-7.1)	3.3 (0-31.8)	4.8	3.3	3.1	4.1
2.5	5.1[a]	5.1	3.4	1.5	3.3	5.1	2.6
3.0	2.7[a] (1.0-4.3)	4.6 (3.1-6.1)	4.4 (2.3-6.4)	4.6[b]	3.9	2.7[b]	3.5
3.5	4.2[a] (1.6-6.9)	4.5 (2.4-6.7)	5.4 (3.2-7.7)	4.3	5.4	4.3	5.2
4.0	4.7[a] (3.4-6.1)	3.8 (2.7-4.9)	4.0 (2.2-5.8)	3.7	4.3	4.2	4.8
5.0	5.5[a] (4.3-6.7)	5.4 (4.7-6.1)	3.6 (0-9.7)	4.2	3.5	4.8	3.8

Small differences in transmission characteristics are difficult to demonstrate in this type of experiments, as the power is rather low if R_0 in all groups is above one (Velthuis et al., 2007). Another explanation could be that the amount of virus shed by infected birds was sufficient in all treatment groups to induce infection to contact-exposed birds, especially since birds were sampled only once a day, and may have produced more virus during the day. Measuring virus shedding more often during the first days after inoculation might show that differences in transmission rate exist, although it is doubtful whether small differences in shedding shortly after infection are biologically relevant.

The majority of the chickens that died from AI were positive in the PCR and virus isolation. However, three chickens from the 10^3 treatment group that died during the experiments did not test positive in the RT-qPCR. An explanation could be that these birds became virus positive and died in between two samplings. Another possible explanation is that the amount of sample material collected from the trachea was too low to detect virus particles or that the birds shed low amounts of virus. The CID_{50} of the strain used in this study was comparable to CID_{50} estimated in other studies with other isolates (Brown et al., 2007; Swayne and Slemons, 2008). It was thus expected that infected chickens would die from the infection.

However, in contrast to their and other studies (Nakamura et al., 2008), in our experiments not all chickens that shed detectable levels of virus died, indicating that CID_{50} and CLD_{50} were not similar. It is likely that virus had replicated in these birds, as RT-qPCR positive samples were confirmed in the virus isolation assay. The finding suggests a rapid immune response, but none of these AI-surviving chickens seroconverted. Possibly the virus did not trigger the systemic immune system sufficiently or only an effective T cell response had been induced. Another explanation is that local immune responses were able to clear the virus, as for example has been shown for infectious bronchitis virus (Cavanagh, 2003). Finally, contamination of the swab samples during sampling can never be excluded if virus was present in the air. Such cross-contamination is considered highly unlikely, as sentinels from the same room remained AI-free during the experiment.

The latent period is considered to be important in models applied to analyse epidemics and transmission studies (Bos et al., 2007; van der Goot et al., 2005), as it affects the course of the infection within a flock. The latent period differed between the treatment groups, a finding that could be useful as input parameters for when analysis of future epidemics as done by for example by Stegeman et al. (2004) and Boender et al. (2007) or as modelling scenario studies as for example carried out by Bos et al. (2007).

The parameters provided in this study could be used as input parameters in mathematical models for the analysis of ‘‘within-flock’’ transmission as for example done by Bos et al. (2007) or Tiensin et al. (2007). Of course, for the control of an HPAI epidemic, the ‘‘between-flock’’ transmission is more relevant than the ‘‘within-flock’’ transmission, but the two are likely to be linked. This is hypothesized for the following reason: the rate of ‘‘within-flock’’ transmission determines the rate at which the number of infectious chickens increases over time. It seems reasonable to assume that the higher the number of infectious birds, the higher the viral load in the environment and the higher the exposure of other flocks. Of course variables other than virus load may affect the risk of infecting neighbouring flocks, but the experiments described in this study will contribute risk analyses studies seeking to determine the risk of infection after exposure to infected flocks in combination with information about the amount of virus measured in the surroundings of a flock.

Chapter 3

Airborne transmission of a highly pathogenic avian influenza virus strain H5N1 between groups of chickens quantified in an experimental setting

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Abstract
Highly Pathogenic Avian Influenza (HPAI) is a devastating viral disease of poultry and quick control of outbreaks is vital. Airborne transmission has often been suggested as a route of transmission between flocks, but knowledge of the rate of transmission via this route is sparse. In the current study, we quantified the rate of airborne transmission of an HPAI H5N1 virus strain between chickens under experimental conditions. In addition, we quantified viral load in air and dust samples. Sixteen trials were performed, comprising a total of 160 chickens housed in cages, with three treatment groups. The first group was inoculated with strain A/turkey/Turkey/1/2005 H5N1, the second and third group were not inoculated, but housed at 0.2 and 1.1m distance of the first group, respectively. Tracheal and cloacal swabs were collected daily of each chicken to monitor virus transmission. Air and dust samples were taken daily to quantify virus load in the immediate surroundings of the birds. Samples were tested by quantitative RT-qPCR and virus isolation.
In 4 out of 16 trials virus was transmitted from the experimentally inoculated chickens to the non-inoculated chickens. The transmission rate was 0.13 and 0.10 new infections per infectious bird at 0.2m and 1.1m, respectively. The difference between these estimates was, however, not significant. Two air samples tested positive in virus isolation, but none of these samples originated from the trials with successful transmission. Five dust samples were confirmed positive in virus isolation. The results of this study demonstrate that the rate of airborne transmission between chickens over short distances is low, suggesting that airborne transmission over a long distance is an unlikely route of spread. Whether or not this also applies to the field situation needs to be examined.

3.1 Introduction

Highly Pathogenic Avian Influenza (HPAI), caused by avian influenza viruses of subtype H5 or H7, is one of the most important poultry diseases worldwide (Alexander, 2007). The infection spreads rapidly among chickens and between flocks, causing high mortality rates and severe economic losses. Moreover, HPAI virus strains have caused infections in humans (Kallthof et al., 2010) and are considered a risk for a human influenza pandemic. As a consequence, outbreaks of HPAI virus in poultry flocks need to be controlled quickly.

Control measures aiming to eliminate HPAI virus often include stamping out infected flocks, pre-emptive culling of flocks at risk to become infected, movement restrictions and bio-safety measures. These control measures may, however, not be sufficient to control a major epidemic in poultry dense regions (Capua et al., 2003; Stegeman et al., 2004; Boender et al., 2007). Moreover, the costs associated with pre-emptive culling are high and the killing of large numbers of uninfected birds evokes ethical discussion in society. Consequently, improvement of the culling strategy, making it both more efficient and acceptable is needed.

To increase the effectiveness of control strategies, quantitative information of the possible routes of virus transmission between farms is essential. It has been demonstrated that the probability of between-flock virus transmission decreases with increasing distance between an infected and an uninfected flock (Boender et al., 2007), but the underlying mechanism of transmission still shows considerable gaps. Several routes are considered to be important during HPAI epidemics, such as movements of visitors, materials, and fomites, but, as shown for some other viral diseases (Gloster et al., 2010; Otake et al., 2010; Li et al., 2009) also airborne transmission has been hypothesised (Chen et al., 2010; Tsukamoto et al., 2007; Yee et al., 2009). Although some of the routes could be controlled by stringent hygienic measures, prevention of virus introduction via airborne route seems hardly feasible in commercial poultry industry. It is therefore important to establish the contribution of airborne infection in the between-farm spread.

During an HPAI epidemic it is difficult to quantify the rate of airborne virus transmission between farms. The rate at which such an epidemic evolves, the need for immediate implementation of control measures and the presence of other routes of transmission that can act as confounding factors hamper a thorough investigation during epidemic episodes. An alternative way to quantify airborne transmission is by carrying out animal experiments. In experiments the occurrence of airborne transmission can be established and the relation between distance to an infectious bird and probability of infection can be quantified in the absence of confounding factors.

Tsukamoto et al. (2007) demonstrated the possibility of airborne transmission of HPAI H5N1 virus in an isolator. Moreover, they showed that the likelihood of infection was dependent on the number of infectious birds. From their results,

however, we cannot quantify the transmission probability. Moreover, they did not examine the presence of virus in the air.

In this paper we describe two experiments, which enabled us to quantify the rate of airborne transmission of H5N1 virus strain between chickens at various distances. In addition, we quantified virus concentrations in air and dust samples in the immediate surroundings of the birds.

3.2. Materials en methods

3.2.1. Animals

Embryonated eggs from White Leghorn chickens were purchased from a commercial avian influenza (AI) free poultry breeder farm and hatched at the Central Veterinary Institute (CVI), Lelystad, The Netherlands. After hatching, the chickens were housed in one room. At 5 weeks of age, the chickens were tested for the presence of antibodies against avian influenza using a modified indirect double antibody sandwich (IDAS) nucleoprotein (NP)-blocking ELISA.

The chickens were subsequently randomly divided in 4 groups, each housed in a separate room. Two experiments were carried out consecutively. In the first experiment 4 groups of 8 chickens were formed. Based on the results of this experiment, we increased the number of chickens in the second experiment in which 4 groups of 32 chickens were used. Feed and water were provided *ad libitum*.

3.2.2. Experimental design

Two experiments were carried out, each in 4 isolation rooms under BSL^{3+} conditions at the CVI. The layout of the rooms of the first and second experiment is shown in Figure 3.1. The volume of the rooms was $22m^3$, which were ventilated 0.8/h. The temperature was kept at 20ºC and the relative humidity at 55%. The rooms contained two rows of 3 cages each. A cage was constructed of gaze and hardboard and had a size of $1x0.5m^2$. The distance between the first and second cage was 0.2m and between the second and third cage 0.4m.

In the first experiment, 2 chickens were placed in the first cage of every row and 1 chicken in the second and third cage each. In the second experiment, 7 chickens were placed in the first and second cage and 2 chickens were placed in

the third cage of every row. Chickens from the first cage of every row were inoculated at day 0. Chickens in the second and third cage were not inoculated. The air circulation in the rooms was determined with a smoke test. The rows were placed in favour of the transmission route. The experiments complied with the Dutch law on animal experiments and were reviewed by an ethical committee.

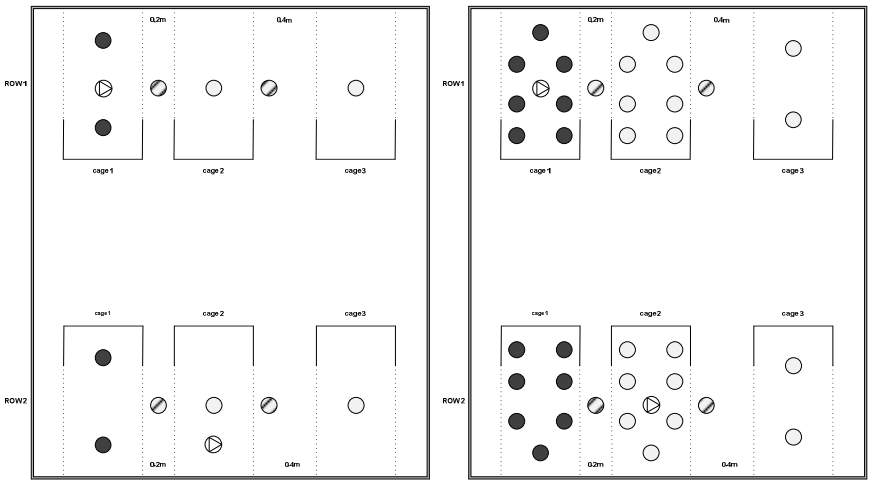

Figure 3.1. Layout of the isolation room of the first experiment (left) and second experiment (right).
● *represents the inoculated chickens;* ○ *represents the non-inoculated chickens;* ◁ *represents the location of air sampling;* ▨ *represents the location of dust sampling.*

3.2.3. Inoculum

The HPAI virus strain A/turkey/ Turkey/1/2005 H5N1 (clade 2.2) was used as challenge strain for inoculation (Londt et al., 2008; Spekreijse et al., 2011). The virus was grown in embryonated SPF eggs and vials with a known egg infectious dose (EID_{50}) titer were stored at -70ºC until use. On the day of challenge, one vial was thawed and diluted in 10-fold dilution steps in tryptose phosphate buffer (TPB) to obtain the necessary inoculation dose of 10^4 $EID_{50.}$ In both experiments, the chickens were inoculated with 0.1ml inoculum applied intra-nasally and 0.1ml inoculum applied intra-tracheally using a blunt needle, according to standard protocol (van der Goot et al., 2005; Spekreijse et al., 2011).

3.2.4. Sampling procedures

At day 1 post-inoculation (p.i.), swabs from trachea and cloaca were collected from inoculated chickens, and at days 2 to 10, 14, 17 and 21 p.i., from all chickens. The non-inoculated chickens were sampled first, and between the rooms clothes and gloves of the animal handlers were changed. The swabs were put in 2 ml of 2.95% TPB with 5 x 10^3 IU of penicillin-sodium and 5 mg streptomycin per ml and stored at -70^{o}C until analysed.

Serum blood samples were taken from the ulnar vein 7 days before and at days 7, 14 and 21 after inoculation from all chickens. The samples were stored at -20^{o}C until analysed.

In two of four rooms air samples were taken and in the remaining 2 rooms dust was sampled. Dust and air samples were collected from day 1 to day 10 p.i. Air samples were taken with an MD8 air-scan air sampling device (Sartorius, Nieuwegein, The Netherlands) using sterile gelatine filters of 80mm diameter and 3µm pore size. Samples were taken at an air speed of 8m^3/h for 10 minutes, according to the manual of the manufacturer[1]. In both rooms 2 samples were taken; one above the first cage of one row, another above the second cage of the second row. After sampling, the gelatine filters were dissolved in 10 ml of 2.95% TPB with 5 x 10^3 IU of penicillin-sodium and 5mg streptomycin per ml at a temperature of 37^{0}C. Dissolved filter solutions were stored at -70^{o}C until analysed.

Dust samples were taken using electrostatic dust cloths (Swiffer, Procter and Gamble, U.S.) that were placed in a Petri disk. Dust was sampled for 24 hours. In both rooms 4 samples were taken; two per row. The Petri disks were placed on both sides of the second cage. The dust cloths were put in 10ml of 2.95% TPB with 5 x 10^3 IU of penicillin-sodium and 5mg streptomycin per ml and stored at -70^{o}C until analysed.

The experiments were terminated 21 days p.i. by euthanizing surviving birds with an intracardiac injection of T-61.

1 Jaschhof, H. Sammlung von virusaerosolen – vergleichende untersuchungen zur effektivität von gelatine-membranfiltern, schlitzsammler und impinger. Biotec. October 1992

3.2.5. RNA isolation and quantitative real-time reverse transcriptase PCR (RT-qPCR)

RNA isolation was performed with the MagNA Pure LC 2.0 instrument (Roche Applied Science, Mannheim, Germany) with the MagNA Pure LC total Nucleic Acid Isolation Kit (Roche Applied Science, Mannheim, Germany). The viral RNA was isolated from 200µl of swab fluid or filter solution according to the manufacturer's instructions. The nucleic acids were collected in elution buffer and stored at -70°C or directly processed for the quantitative real-time reverse transcriptase PCR (RT-qPCR). The RT-qPCR and data analysis were performed using the MX4000 Quantitative PCR system (Stratagene) with version 4.20 software.

The matrix gene of the influenza was detected in 5µl of the elution buffer with extracted RNA by RT-qPCR as described in van der Goot (2008). A calibration curve of serial dilutions of a standard batch of the virus with a known EID_{50} titer was used to calculate the viral concentration of each sample. Dilutions of the standard batch were run along with the unknown samples. Viral concentration in each sample was quantified based on the calibration curve generated by plotting the cycle threshold value (C_t-value) against known virus titers. Titers of the samples were expressed as EID_{50} equivalents.

3.2.6. Laboratory tests

Sera were incubated for 30min at 56ºC. A modified indirect double antibody sandwich (IDAS) nucleoprotein (NP)-blocking ELISA that detects antibodies against the nucleoprotein of influenza A was performed as described by de Boer et al. (1990). For virus isolation, per swab three embryonated SPF chicken eggs incubated for 9 days were inoculated with 0.2ml swab fluid per egg. After 72h the allantoic fluid was harvested and a standard hemagglutination assay (HA) with chicken red blood cells was performed (OIE, 2008). When at least one egg was positive in HA the swab was considered to be positive.

3.2.7. The effect of the gelatine filter on the concentration of HPAI virus

The effect of the gelatine filters on the concentration of virus particles was determined. Gelatine filters were placed in Petri dishes and inoculated either with 1ml of 10^3, 10^4 or 10^5 EID_{50}/ml. Two gelatine filters per dose were used. The first filter per dose was dissolved in 9ml of PBS (at 37 degrees) after 10

minutes of incubation. The second filter per dose was dissolved in 9ml of PBS (at 37 degrees) after 30 minutes of incubation. This number of minutes was considered to be representative for the time between sampling and processing in the lab. As control, the virus stock was treated similarly and dissolved in the same volume of medium. Virus concentration was determined by RNA isolation and RT-qPCR.

3.2.8. Data analysis

Airborne transmission was based on the number of infected non-inoculated chickens. A chicken was considered infected if it met one or more of the following criteria: the occurrence of HPAI-like symptoms, a positive RT-qPCR, or both. Positive RT-qPCR results from chickens that did not die were confirmed by virus isolation.

The day of infection of the non-inoculated chickens was defined as the first day of virus excretion minus a one day latent period (Spekreijse et al., 2011). Non-inoculated chickens got infected either through air by the inoculated chickens, through air by infected non-inoculated chickens from another cage, or non-inoculated chickens were contact exposed by shedding cage mates.

A generalized linear model (GLM) assuming a stochastic SIR (susceptible-infectious-removed) transmission process was used to estimate a separate transmission rate parameter (the average number of infections caused by one infectious bird per day) for every distance (Velthuis et al., 2003).

The mean latent and infectious period and the total amount of virus detected in the samples of the inoculated chickens in the two experiments were compared using ANOVA. All statistical tests were performed assuming a 2-sided alternative hypothesis; p values smaller than 0.05 were considered significantly different. Analysis was performed using commercially available statistical software (SPSS 16.0; SPSS Inc., Chicago, Illinois).

3.3. Results

3.3.1. Infection of inoculated chickens

None of the inoculated chickens had pre-existing antibodies against avian influenza virus. In the first experiment 3 out of 16 inoculated chickens escaped infection and remained serologically negative; the other thirteen died. The mean

latent period of the inoculated chickens was 1.1 day (95% confidence interval (C.I.): 0.9 – 1.2 days). The mean infectious period of the inoculated chickens (days of virus shedding) was 1.6 day (95% C.I.: 1.4 – 1.7 days). In the first experiment, the mean amount of virus shedding on day 1 p.i. was $10^{3.5}$ EID_{50} (95% C.I.: $10^{2.5}$ – $10^{4.5}$ EID_{50}), and on day 2 p.i., $10^{5.3}$ EID_{50} (95% C.I.: $10^{5.2}$ – $10^{5.5}$ EID_{50}).

In the second experiment all 56 inoculated chickens died. The mean latent period of these chickens was 1.1 day (95% C.I.: 1.0 – 1.2 days). The mean infectious period was 1.7 day (95% C.I.: 1.6 – 1.8). In the second experiment, the mean shedding of virus on day 1 p.i. was $10^{4.3}$ (95% C.I.: $10^{3.8}$ – $10^{4.8}$ EID_{50}), and on day 2 p.i., $10^{6.3}$ EID_{50} (95% C.I.: $10^{5.3}$ – $10^{6.9}$ EID_{50}). The mean latent period, the mean infectious period and the total amount of virus shedding did not differ significantly between the two experiments.

3.3.2. Infection of non-inoculated chickens

None of the non-inoculated chickens had pre-existing antibodies against avian influenza (AI) virus. Chickens that died during the experiments showed AI-like symptoms, and had one or more positive RT-qPCR results for the tracheal and/or cloacal swabs. The most common AI-like symptoms were loss of appetite, depression and conjunctivitis.

In the first experiment none of the non-inoculated chickens became infected, showed clinical signs of infection or developed detectable amounts of antibodies. In the second experiment, 20 out of 72 non-inoculated chickens got infected. In total 17 of these 20 infected chickens died from infection. The infected chickens originated from 7 cages, 16 birds were located in cage 2, and 4 birds in cage 3. Based on the time of infection, we concluded that 6 of these 20 birds became infected by the inoculated chickens and 14 by either their cage mates or by 'air' with virus shed by infectious chickens from another cage.

Three non-inoculated infected chickens, one from a second cage and two from a third cage, tested positive once, both in the RT-qPCR and virus isolation, but did not die from infection nor seroconverted. From the moment of infection we derived that 2 birds became infected by the inoculated chickens, and 1 became infected from infected chickens from another cage.

In 5 rows, the virus did not transmit to the non-inoculated chickens. In 2 rows, the inoculated chickens succeeded in transmission to the non-inoculated

chickens in both cages. The distribution of the infected chickens over the different isolation rooms and rows are summarized in Figure 3.2.

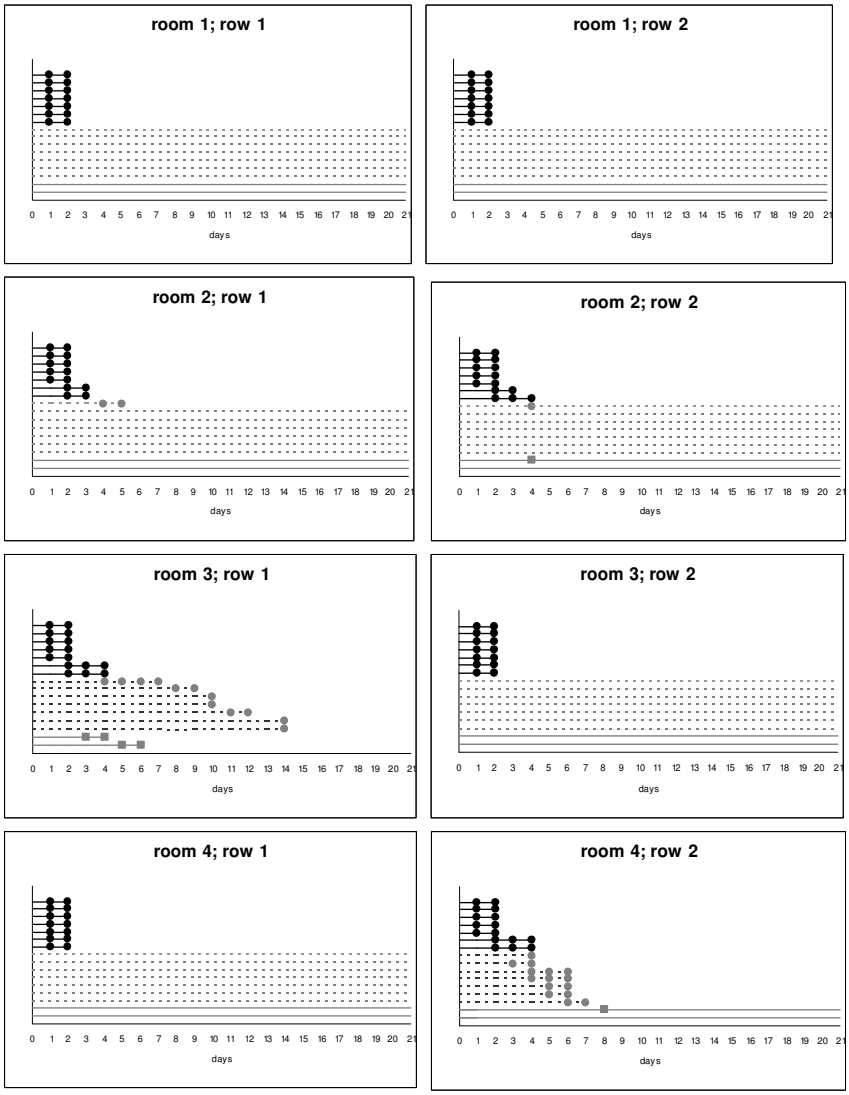

Figure 3.2. Summary of the RT-qPCR results of the swabs and mortality data of the second experiment. Each figure represents one row of a room. The black line represents an inoculated chicken, the grey dotted line represents a non-inoculated chicken at 0.2m, and the grey line represents a non-inoculated chickens at 1.1m. The dots and squares represent a positive RT-qPCR swab for trachea and/or cloaca. At the end of each timeline the chicken died either by infection or at day 21 chickens were euthanized.

Table 3.1. Effect of gelatine filters on the concentration of HPAI virus determined by RT-qPCR

Dose groups (EID_{50}/ml)	Time (min)	C_t value (cycles)		Titer (log_{10} EID_{50}/ml)	
		filter	virus stock	filter	virus stock
10^3	10	36.6	36.8	1.9	1.9
	30	36.6	37.6	1.9	1.7
10^4	10	29.8	30.0	3.8	3.7
	30	29.8	30.1	3.8	3.7
10^5	10	26.0	26.6	4.7	4.8
	30	26.2	26.6	4.7	4.8

3.3.3. Virus detection in air and dust samples

No effect of the gelatine filters was seen on the concentration of HPAI virus (Table 3.1) and processing filters after 30 minutes did not reduce the quantities measured in RT-qPCR.

In the first experiment, 2 air samples tested positive in RT-qPCR and virus isolation. On day 2 p.i., one air sample tested $10^{1.6}$ EID_{50}, and on day 3 p.i., one air sample tested $10^{1.3}$ EID_{50}. Three dust samples tested positive in RT-qPCR and virus isolation. On day 1 p.i., one dust sample tested $10^{1.6}$ EID_{50}, and on day 2 p.i., one dust sample tested $10^{1.7}$ EID_{50}, and on day 4 p.i., one dust sample tested $10^{2.4}$ EID_{50}. In the second experiment, on day 2 and day 3 dust samples tested positive in RT-qPCR and virus isolation ($10^{3.9}$ and $10^{2.2}$ EID_{50}, respectively), but none of the air samples tested positive.

3.3.4. Quantification of transmission parameters

In the first experiment, no transmission of virus from the inoculated chickens to non-inoculated chickens occurred. In the second experiment transmission did occur and combined with the first experiment, the transmission rate parameters for the various distances were calculated. The transmission parameter for the inoculated chickens to the non-inoculated chickens at a distance of 0.2m was estimated at 0.13 new infections per infectious chicken per day (95% C.I.: 0.01 – 2.73), for the distance of 0.4m between the inoculated and non-inoculated chickens at 0.21/day (95% C.I.: 0 – 9.31), and for the distance 1.1m at 0.10/day (95% C.I.: 0.02 – 0.40). The estimates did not differ significantly for the various distances. The transmission rate parameter for directly exposed chickens was estimated at 1.43/day (95% C.I.: 0.27 – 7.56). This parameter differed

significantly from the combined transmission rate parameter (0.12/day; 95% C.I.: 0.06 – 0.26) of the non-inoculated chickens ($p < 0.05$).

3.4. Discussion

The aim of this study was to quantify airborne transmission of an HPAI H5N1 virus strain between chickens housed at various distances. Virus was transmitted to chickens exposed to inoculated chickens over distances of 0.2, 0.4 and 1.1m, but most exposed chickens escaped infection. No statistical difference was found between the rates of transmission over the above mentioned distances. Nevertheless, the combined β of airborne infection was significant lower than the transmission rate between chickens in the same pen (β = 1.43/day). Our findings indicate that airborne transmission over a short distance can occur, but that the rate at which it takes place is low.

Throughout the experiments, strict hygienic measures were taken to exclude transmission via other routes than by air, and the flow in the rooms was in favour of airborne transmission. Inoculated chickens shed virus in large quantities, but the amount of virus detected in air samples was low and most times undetectable. Moreover, the test results of the air samples did not correlate with the occurrence of airborne transmission. An explanation for the low proportion of virus positive air samples could be the length of the sampling time. According to the manual of the manufacturer, samples were taken daily for 10', a protocol that had also been used by Weesendorp et al. (2008) to detect classical swine fever virus in ambient air. Moreover, chickens are exposed to virus containing particles much longer, which may have increased the probability of contracting the infection. Stochastic processes may also have occurred. Sampling for a longer time might have increased the number of positive air samples. Optimization of the sampling procedure may be an option for further research.

In the first experiment the amount of virus produced by the inoculated chickens was apparently not sufficient for transmission of virus over a distance of 0.2m. We therefore increased the number of inoculated chickens in the second experiment in order to increase the probability of airborne transmission (Tsukamoto et al., 2007). In the second experiment, virus shedding of the inoculated chickens resulted in airborne infection of 6 chickens in 6 cages. The

other 14 non-inoculated chickens that got infected were most likely infected either by cage mates (13 chickens) or by airborne-infected chickens from another cage (one chicken). In the second experiment, virus was isolated from three chickens exposed to airborne transmission that did not die from infection nor did they seroconvert. This is remarkable, but we made a similar observation in a previous experiment (Spekreijse et al, 2011). In that experiment a small number of chickens in direct contact with experimentally infected chickens tested positive in RT-qPCR and virus isolation, but did not die from infection, nor seroconverted. These findings suggest that airborne introduction of a HPAI virus may not always result in a major outbreak. In the field, however, the amount of dust produced by large poultry flocks may be high, and dust-borne infection may be facilitated via artificial ventilation. Moreover, in an infected flock the number of infectious birds may be much higher. Therefore, it is difficult to extrapolate the experimental results to a field situation.

In studies using influenza virus in other host species, airborne transmission was observed. Stark et al. (1999) for example showed that airborne transmission of influenza virus between pigs was feasible, and Tellier (2009) demonstrated airborne transmission of influenza virus among mice and guinea pigs. Our results showed that the probability of airborne transmission over a distance of 1m is low. Although in our study the relation between distance and probability of infection was not straightforward, as we did not find a significant difference between transmission over the various distances, our results can be used as input in transmission kernels to better understand the indirect transmission of HPAI virus (Keeling et al., 2005).

The possibility of long-range transport of other livestock viruses has been reported for other diseases such as foot-and-mouth disease (Amaral Doel et al., 2009; Gloster et al., 2010), porcine reproductive and respiratory syndrome virus (PRRSV) (Dee et al., 2009) and Newcastle disease (Li et al., 2009). This long-range transport was linked to aerosol transmission and meteorological data, but not with the dispersion of dust, suggesting at least air-born transmission. Our findings of transmission via air seem to be consistent with these field observations.

In our experiments, dust samples tested positive, although no clear link between transmission and positive dust samples was demonstrated. Infectious particles in dust were previously demonstrated by Sedlmaier et al. (2009) and,

moreover, Chen et al. (2010) suggested the possibility of long-range transport of influenza virus through air by dust storms as the attachment of viruses to dust particles could increase their chances of survival. Pitkin et al. (2009) confirmed in a production region model that the use of air filtration under controlled field conditions could significantly reduce airborne transmission of PRRSV between two pig populations. Their results could be useful for the implications of filter systems in the field. Unfortunately, the amount of dust produced by flocks of poultry exceeds the dust produced by pigs and, therefore, it is questionable if the filter system could be implicated easily in poultry houses. To substantiate the hypothesis of long-range dust-borne infection it would be necessary to quantify the amount of dust-borne virus produced by an infected flock and combine that with the dispersion pattern of dust emitted by that flock. This field research can, however, only be done with other viruses than AI virus, and whether or not this applies to AI virus should then be evaluated. Nevertheless, our experimental work showed that transmission via air is possible and our estimates may be of value for models used to simulate between flock transmission.

Chapter 4

Quantification of dust-borne transmission of highly pathogenic avian influenza virus between chickens

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Abstract

Understanding the transmission of Highly Pathogenic Avian Influenza (HPAI) virus between poultry flocks is essential to prevent and control epidemics. Dust, produced in infected chicken flocks, has been hypothesized to be an important mechanical vector for between-flock transmission of HPAI virus. The aim of our study was to quantify the amount of virus shed by infected birds and its relation to deposition of virus in the environment and the rate of dust-borne transmission between groups of chickens. Four replicate experiments were performed, each replicate with two groups of 14 chickens housed in two separate rooms. In one group, chickens were inoculated with HPAIv. Ventilation forced the air from that room to the second (recipient) group through a tube. Deceased birds in the inoculated group were replaced with new susceptible birds up to day 10 p.i. Dust samples were collected daily. Trachea and cloaca swabs were collected daily to determine virus shedding and virus spread to the recipient group. The amount of virus detected in dust samples in the recipient room was, on average, $10^{3.7}$ EID_{50} per m^3. Virus transmission from the inoculated to the recipient group occurred in two experiments. The transmission rate parameter for dust-borne transmission was estimated at 0.08 new infections per infectious chicken per day. The results of this study are a first step to elucidate the importance of dust-borne transmission of HPAI virus between flocks and help interpret environmental samples.

4.1 Introduction

Highly pathogenic avian influenza (HPAI) is a contagious and devastating viral disease of poultry caused by influenza type A strains carrying the haemagglutinin H5 or H7. Outbreaks of HPAI H5N1 virus have occurred in several countries in Europe, Asia and Africa and the virus is currently endemic in, amongst others, China, Egypt and Indonesia, causing economic losses and animal welfare problems (OIE, 2011). In addition, in 15 countries human cases were reported since the first outbreak in Hong Kong in 1997, and the disease is considered a threat for public health because of the risk of re-assortment with

other influenza A viruses which may result in a new pandemic (FAO, OIE and WHO, 2010).

Eradication of HPAI virus is difficult and is mainly achieved by culling of large numbers of (infected) poultry (Capua and Marangon, 2000; Stegeman et al., 2004; Webster et al., 2005; van der Goot et al., 2007; Fiebig et al., 2009). Because of this massive culling of flocks, improvement of control measures is necessary, which requires more knowledge on the epidemiology of the disease and the role of different routes of transmission between flocks.

One of the routes suggested is airborne transmission. During the 2004 HPAI outbreak in British Colombia live virus was found in dust samples collected around infected flocks (Schofield et al., 2005), and Chen et al. (2010) found RNA of influenza viruses attached to dust particles originating from Asian dust storms, and hypothesized the possibility of dust borne transmission of HPAI virus. Poultry flocks generate high concentrations of dust that consists of bedding, feathers and faeces, but can also contain high concentrations of micro-organisms (Takai et al., 1998; Aarnink et al., 1999; Olsen et al., 2009; Sedlmaier et al., 2009; Cambra-López et al., 2010). Previous studies detected *Campylobacter*, *Salmonella*, Newcastle disease and Marek's disease in dust samples collected from broiler flocks (Olsen et al., 2009; Anderson et al., 1966; Hietala et al., 2005; Renz et al., 2006; Iwabuchi et al., 2010). Due to artificial ventilation systems, but also during transport of birds or manure, dust is emitted into the environment and can become a mechanical vector for microorganisms. Dust particles may be subsequently transported by wind over large distances up to kilometres, and in that way may become a route for the spread of infectious particles between poultry flocks (Banhazi and Seedorf, 2008; Shao, 2008).

Up to now, however, evidence for transmission of HPAI virus by dust is sparse and more insight in this matter may be helpful for interpreting results from environmental samples and to develop prevention strategies, for example minimal acceptable distance between flocks. Moreover, it might improve control measures for personnel working on infected flocks, which may become infected after exposure to contaminated dust particles, or to provide insight in the risk of exposure for people living in the proximity to poultry flocks (van Riel et al., 2006; Tsukamoto et al., 2007; Kuchipudi et al., 2009; Liu et al., 2009; Yee et al., 2009; Chen et al., 2010; Spekreijse et al., 2011).

Studying dust-borne virus spread of HPAI virus during a disease-free period is difficult, and the only way of gaining insight in distance-related spread is by doing experiments. In a previous study, the rate of airborne transmission of HPAI virus over short distances (up to 1.2 metres) within an experimental unit was determined, and it was demonstrated that virus could be transmitted over a short distance, and that dust was the possible vector of transmission (Spekreijse et al., 2011). A study by Yee et al. (2009) demonstrated that airborne transmission of a low pathogenic avian influenza (LPAI) virus could be an important route of transmission of virus in life bird markets (LBM). However, in these studies transmission was only determined qualitatively, meaning that the relation between viral load and probability of infection remains unknown.

Therefore, quantitative data is necessary for the risk assessment on transmission of virus to human and animal. However, up to now no data is available on the amount of shedding by infected birds and its relation to environmental deposition of dust-borne virus and the rate of dust-borne transmission. In this study, we performed four animal experiments in which we quantified the amount of virus shed by infected birds and its relation to deposition of virus in the environment and the rate of dust-borne transmission between groups of chickens.

4.2. Materials and Methods

4.2.1. Animals

Embryonated eggs from White Leghorn chickens were purchased from a commercial avian influenza (AI) free poultry breeder farm and hatched at the Central Veterinary Institute (CVI) in Lelystad. After hatching, the chickens were housed in one room. At 5 weeks of age, the chickens were tested for the presence of antibodies against AI using a modified indirect double antibody sandwich (IDAS) nucleoprotein (NP)-blocking ELISA (de Boer et al., 1990). The chickens were housed on the floor with bedding material of wood shavings and sawdust. Irradiated rearing feed and tap water were provided *ad libitum*.

4.2.2. Inoculum

For inoculation, the HPAI virus strain A/turkey/ Turkey/1/2005 H5N1 (clade 2.2) was used as challenge strain (Londt et al., 2008; Spekreijse et al., 2011). The virus was grown in the embryonated SPF eggs and vials with a known egg

infectious dose (EID_{50}) titer were stored at -70ºC until use. On the day of challenge, one vial was thawed and diluted in 10-fold dilution steps in tryptose phosphate buffer (TPB) to obtain the necessary inoculation dose of 10^4 $EID_{50.}$ Chickens were inoculated with 0.1ml inoculum applied intra-nasally and 0.1ml inoculum applied intra-tracheally using a blunt needle, according to a standard protocol (Spekreijse et al., 2011; van der Goot et al., 2005).

4.2.3. Experimental design

Four replicate experiments, trials 1 to 4, were carried out consecutively (Table 4.1). The experiments complied with the Dutch law on animal experiments and were reviewed by an animal ethics committee. Each of the four replicate experiments consisted of two separately housed groups of 14 chickens. Each group was housed in an isolation unit (volume of 22 m^3) under BSL3+ conditions at the CVI in an open cage of 2 m^2. The two isolation units were connected with a tube (Figure 4.1). The inlet of the tube started above the open cage from the first isolation room and the outlet of the tube ended above the open cage from the second room. Ventilation forced all air from the first room to the second room through the tube. The units were ventilated at an average flow of 225 m^3/h (range from 217 – 233 m^3/h) through the tube. At this air flow dust deposition on the plastic smooth tube is unlikely. Room temperature was kept at 21^{o}C and a relative humidity of 55 %.

In the first room, 8 out of 14 chickens were inoculated with HPAI H5N1 virus. The remaining 6 birds were contact exposed p.i. Deceased birds in this inoculated group were replaced daily with new susceptible ones up to 10 days p.i. to generate a constant source of virus shedding that was verified by taking swabs. In the second room, birds were not treated. Deceased birds in this recipient group were removed but not replaced.

4.2.4. Sampling procedures

At day 1 p.i., swabs from trachea and cloaca were collected from inoculated chickens and at days 2 to 10 p.i., from all chickens. In experiment 3 and 4, swabs from trachea and cloaca were also collected on day 13 and 14 p.i. because of the results of experiment 1 and 2. The swabs were put in 2 ml of 2.95% TPB with 5 x 10^3 IU of penicillin-sodium and 5 mg streptomycin per ml and stored at -70^{o}C until analysed.

In experiments 3 and 4, from day 1 to 10, 13 and 14 p.i. additional dust samples were collected from the wall (Figure 4.1) of the inoculated and recipient units, using electrostatic dust cloths (Swiffer, Procter and Gamble, U.S.). Per room, a defined section of 0.1 m^2 of wall was marked and sampled daily to determine viral deposition in 24 hours. The dust cloths were put in 10 ml of 2.95% TPB with 5 x 10^3 IU of penicillin-sodium and 5 mg streptomycin per ml and stored at -70^{o}C until analysed.
Air samples were collected during days 1 - 10 p.i. from the air flowing to the recipient group (Figure 4.1) using either an MD8 air-scan air sampling device (Sartorius, Nieuwegein, The Netherlands) (experiments 1 and 3), or a two-stage viable Andersen Cascade Impactor (Thermo Scientific, Franklin, MA, USA) (experiments 2 and 4). In experiments 3 and 4, air samples were also collected on days 13 and 14.

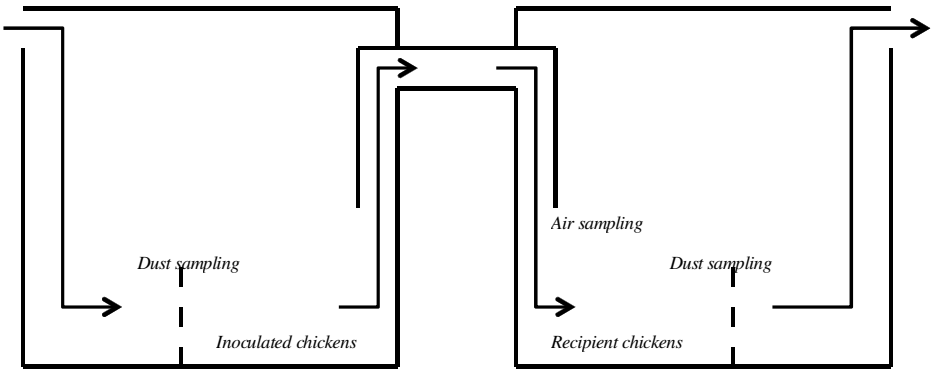

Figure 4.1. Overview of the connected rooms with locations of the groups of chickens, dust sampling and air sampling. The arrows represent the airflow from the inoculated to the recipient room.

MD8 air samples were taken at an air speed of 2 m^3/h using sterile gelatine filters of 80 mm diameter and 3 μm pore size, Andersen air samples were taken at an air speed of 1.7 m3/h (28.3 l/min) using sterile gelatine filters to collect the dust. In the latter, the particles were grouped by their aerodynamic diameters (Andersen, 1958). The fractions of dust collected with the Andersen were divided into a non-respirable fraction (PM > 8 μm) and a respirable fraction (PM ≤ 8μm). In experiments 1 and 2, air was sampled for 90min, in experiments 3 and 4 this time was increased to 270min.

After sampling, the gelatine filters were dissolved in 10 ml of 2.95% TPB with 5 x 10^3 IU of penicillin-sodium and 5mg streptomycin per ml at a temperature of 37°C. Dissolved filter solutions were stored at -70°C until analysed. Viral load was determined for each gelatine filter and positive RT-qPCR results were confirmed with virus isolation. The experiments were terminated 21 days p.i. by euthanizing surviving birds with an intracardiac injection of T-61.

4.2.5. RNA isolation and quantitative real-time reverse transcriptase PCR

RNA isolation was performed with the MagNA Pure LC 2.0 instrument (Roche Applied Science, Mannheim, Germany) using the MagNA Pure LC total Nucleic Acid Isolation Kit (Roche Applied Science, Mannheim, Germany). The viral RNA was isolated from 200µl of swab fluid or filter solution according to the manufacturer's instructions, collected in elution buffer and stored at -70°C or directly processed for the quantitative real-time reverse transcriptase PCR (RT-qPCR). The MX4000 Quantitative PCR system (Stratagene) with version 4.20 software was used to perform the RT-qPCR and data analysis. To detect the matrix gene of the influenza A virus, 5 µl of the elution buffer with extracted RNA was used for RT-qPCR as described in van der Goot (2008). The viral RNA concentration of each sample could be calculated using a calibration curve of serial dilutions of a standard batch of the virus with a known EID_{50} titer, that was run along with the unknown samples. Quantification of the viral concentration in each sample was based on the calibration curve generated by plotting the cycle threshold value against known virus titers. Results are given in ^{10}log virus titers, expressed as equivalent units of EID_{50}/ml.

4.2.5. Serology

Blood samples were taken from the ulnar vein 7 days before and at days 7, 14 and 21 p.i. from all chickens. The blood was coagulated at room temperature and serum collected and stored at -20°C until use. A modified indirect double antibody sandwich (IDAS) nucleoprotein (NP)-blocking ELISA to detect antibodies against the nucleoprotein of influenza A was used to test the sera after incubation for 30 min at 56 ºC (de Boer et al., 1990).

4.2.7. Virus isolation

For virus isolation, for each swab three embryonated SPF chicken eggs incubated for 9-11 days were inoculated in the allantoic cavity with 0.2ml swab fluid per egg and candled daily. Dead embryos were stored at 4^{o}C. After 72h the allantoic fluid was harvested from all eggs and a standard hemagglutination assay (HA) with chicken red blood cells was performed, and allantoic fluid was tested with RT-qPCR (OIE, 2008) When at least one egg of three eggs was positive in HA the swab was considered to be positive. Viral load in the different fractions of dust and on the gelatine filters used in the MD8 air scan was determined in RT-qPCR and confirmed by virus isolation.

4.2.8. Data analysis

Chickens from the recipient group were considered to have been infected with HPAI H5N1 virus if they tested positive in RT-qPCR and subsequently died from infection.

The day of infection of the chickens of the recipient group was defined as the first day of virus excretion minus a one day latent period (Spekreijse et al., 2011).

Transmission rate parameters (average number of new infections caused by an infected chicken per day) between chickens in the same group and between chickens in the inoculated group and chickens in the recipient group were estimated using a generalized linear model assuming a stochastic SIR (Susceptible – Infectious – Recovered) transmission process (Diekmann and Heesterbeek, 2000).

To determine whether the amount of virus excreted by the inoculated groups was comparable across the four experiments, the amounts of virus detected in the swabs of the chickens of the four inoculated groups were compared using ANOVA. To find the relation between time, amount of virus shed and virus present in the dust or air samples, log-transformed data of the amount of virus detected in swabs, on the Swiffer dust cloths or on the gelatine filters were analysed using a linear mixed effect model with experiment as random effect and days post inoculation as fixed effect. All statistical tests were performed assuming a 2-sided alternative hypothesis; p values smaller than 0.05 were considered significantly different. Analysis was performed using commercially available statistical software (SPSS 16.0; SPSS Inc., Chicago, Illinois).

Table 4.1. Summary of variables and virus detection with RT-qPCR (expressed as log_{10} EID_{50} per m^2 of wall or m^3 of air) in the different dust samples post inoculation of the four replicate experiments. Samples not available are 'na'.

Trial	Dust sampling						Virus titer (log_{10} EID_{50} equivalents per m^2 wall or per m^3 air)											
							days post inoculation											
	Room	Apparatus	time	m^2	number of infected birds	non-infected birds	1	2	3	4	5	6	7	8	9	10	13	14
1	Inoculated				48	0												
	Recipient	MD8	90		0	14	0	2.9	2.1	3.1	2.6	2.8	2.9	2.7	0	0	na	na
2	Inoculated				35	1												
	Recipient	Andersen – non-respirable	90		14	0	0	0	0	2.3	0	1.5	0	0	0	0	na	na
	Recipient	Andersen – respirable	90				0	0	0	0	2.0	0	0	0	2.2	2.8	na	na
3	Inoculated	Dust cloths		0.1	30	7	0	3.9	5.0	3.7	4.9	5.1	4.8	4.9	4.8	4.4	4.9	3.5
	Recipient	Dust cloths		0.1	0	14	0	2.9	4.0	3.9	3.9	4.5	4.0	4.4	4.5	3.9	3.8	2.7
	Recipient	Andersen – non-respirable	270				0	0	0	2.5	0	3.1	0	3.1	2.4	2.5	2.7	3.4
	Recipient	Andersen – respirable	270				0	2.4	0	2.9	2.7	0	0	3.3	2.5	3.2	2.5	2.8
4	Inoculated	Dust cloths		0.1	47	0	0	3.3	4.5	4.6	4.1	4.6	4.9	4.8	5.0	4.9	5.4	3.9
	Recipient	Dust cloths		0.1	14	0	0	0	3.5	3.2	3.9	4.1	4.7	4.8	3.9	4.2	4.7	3.5
	Recipient	MD8	270				0	2.5	2.9	3.5	3.2	4.1	3.9	3.5	4.0	3.7	3.5	3.7

4.3. Results

All inoculated and contact birds housed in the inoculated group became infected. The number of birds replaced in the inoculated groups is listed in Table 4.1. RT-qPCR detected on average $10^{5.9}$ EID_{50} per day (95% C.I.: $10^{5.7}$ – $10^{6.0}$ EID_{50}) in the swabs of the inoculated group up to 10 days p.i. No significant differences were detected between the four experiments. No correlation in time was found between the amount of virus shed and the amount of virus detected in the environment.

Transmission from the inoculated to the recipient group occurred in experiment 2 and 4. In experiment 2, one bird from the recipient group tested positive in RT-qPCR on day 3, and this bird died on the same day before infection was transmitted to cage mates. Next, a second infection in this group was observed on day 6 p.i. This infection initiated a chain of infections ending with infection and death of all birds in this group. In experiment 4, two birds of the recipient group were found positive in RT-qPCR on day 13 p.i. Finally, all other birds in the group became infected and all birds in the recipient group died before the end of the experiment. In experiments 1 and 3, no transmission from the inoculated to the recipient group was observed.

The transmission rate parameter for the inoculated chickens to the recipient chickens was estimated at 0.08 new infections per infectious chicken per day (95% C.I.: 0.04 – 0.14). The combined transmission rate parameter for direct transmission in the inoculated group was estimated at 1.71 per day (95% C.I.: 0.51 – 5.75) and in the recipient group at 1.45 per day (95% C.I.: 0.38 – 5.57). The estimated parameters for direct transmission differed significantly from the airborne transmission rate parameter ($p < 0.05$).

In Swiffer dust samples RT-qPCR detected virus from day 2 until day 10 p.i. at a mean level of $10^{4.8}$ EID_{50} per day per m^2 (95% C.I.: $10^{4.6}$ – $10^{4.9}$ EID_{50}) for the inoculated group, and $10^{4.2}$ EID_{50} per day per m^2 (95% C.I.: $10^{4.0}$ – $10^{4.4}$ EID_{50}) for the recipient group (Table 4.1). The amount of virus detected in Swiffer dust cloths in the inoculated room was significantly higher than in the recipient room. The amount of virus detected in the two rooms was correlated in time ($p < 0.05$). All positive RT-qPCR results from the dust cloths from the inoculated room tested positive in virus isolation, but no viable virus was found in any of the dust cloths from the recipient room.

MD8 samples contained on average $10^{3.7}$ EID_{50} per m^3 air (95% C.I. $10^{3.4}$ – $10^{3.8}$ EID_{50}) (Table 4.1). All positive RT-qPCR results of the MD8 samples tested positive in virus isolation. No change was detected in the amount of virus detected on MD8 samples during the course of the experiment ($p > 0.05$).

The non-respirable and respirable fraction, collected by the Andersen, tested in RT-qPCR on average $10^{2.8}$ EID_{50} per m^3 air (95% C.I.: $10^{0.5}$ – $10^{5.2}$) and $10^{2.9}$ EID_{50} per m^3 air (95% C.I.: $10^{0.9}$ – $10^{5.0}$), respectively (Table 4.1). No change was detected in the amount of virus detected in the different fractions during the course of the experiment. On day 2 no viable virus was detected in the RT-qPCR positive fractions. From day 3 to day 10 all fractions positive in RT-qPCR tested positive in virus isolation.

4.4. Discussion

In this study we determined the amount of virus shed by infected birds and its relation to environmental deposition of dust-borne virus and the rate of dust-borne transmission. Virus strain A/turkey/Turkey/1/2005 H5N1 was transmitted through air, but not all recipient groups became infected after exposure. The rate of indirect transmission associated with dust was 20-fold lower than the rate of direct transmission between chickens housed in the same cage, suggesting that indirect transmission can occur but is probably less efficient.

The amount of virus detected in air samples collected above the cages in the units with the recipient groups was on average $10^{3.7}$ EID_{50} per m^3. A chicken in this group might have been exposed to $10^{3.8}$ EID_{50} per day, given that a chicken inhales on average 1.2 m^3 per day (Sedlemaier et al., 2009). In a previous study the median infectious dose for the same virus strain (CID_{50}) was estimated to be $10^{2.5}$ EID_{50} /chicken after inoculation and falls within the range of $10^{1.2}$ – $10^{4.7}$ CID_{50} as median infectious doses for other HPAI virus strains, suggesting the model used to assess dust borne transmission has an application to other HPAI viruses (Swayne and Slemons, 2008; Spekreijse et al., 2011). If this CID_{50} also applied for infection after indirect exposure of chickens to dust-contaminated particles, we would expect a higher probability of infection. An explanation for this difference between inoculation dose and 'natural' infectious dose could be that the innate immune system may be better capable of clearing a small virus load inhaled over a prolonged period of time than when it is overwhelmed with

virus at one time (Pujol et al, 2009). Another explanation is that the amount of viable virus in the air samples was lower than measured in RT-qPCR, as the latter also detects inactivated virus particles. We detected viable virus in the air samples, but we did not determine the virus titre, and therefore cannot make a real association between RT-qPCR results and amount of infectious virus. Nevertheless, our trials can help to interpret results from environmental samples in the field like samples taken from live bird markets or during outbreaks, as it may help to get an indication of the probability of airborne transmission or exposure to infectious virus (Suarez et al., 2005; Nguyen et al., 2005; Chen et al., 2008; Indriani et al., 2010).

We collected dust samples using two methods. With the MD8 we collected air samples for 90 or 270min, in which live virus was detected. Samples from dust that was deposited on the wall of the rooms during 24 hours were collected using dust cloths. Dust samples collected from the wall of the recipient room tested only half-log lower than dust samples collected in the inoculated room ($10^{4.8}$ and $10^{4.2}$ EID_{50} per day per m^2, respectively). However, none of the positive RT-qPCR dust samples from the wall of the recipient room tested positive in virus isolation test in contrary to the dust samples of the inoculated room, despite infection of chickens in two of these recipient groups. Transport of dust particles into the environment depends on wind, and deposition of dust particles only occurs under certain conditions (Shao, 2008). Data from our study suggest that transmission of virus to other flocks during an outbreak may only occur during a short time after being produced. It also implies that during an outbreak the location and technique of environmental sampling in the surroundings of an infected flock is essential to determine the amount of infectious dust deposited in that environment and the implications of these findings for exposure. For example, during the HPAI H7N7 outbreak in British Columbia in 2004 dust samples were collected from the air around infected flocks and using high volume sampling virus was retrieved in 3 out of 16 samples at an average of $10^{2.5}$ viral doses per m^3. Nevertheless, this information was too sparse to explain the transmission of virus to other flocks as the sampling technique was not optimal.

Our results of dust-associated virus transmission may also have relevance for human health issues. Personnel working inside stables housing infected flocks may be exposed to large quantities of virus contaminated dust particles. Bos et

al. (2010) demonstrated that persons involved in inspection and culling of infected poultry during the Dutch H7N7 epidemic had a higher risk for infection. With the results of our study the level of exposure of humans can be better assessed which may contribute to the optimization of personal protective equipment for personnel working on infected flocks (Te Beest et al., 2010).

We also examined the amount of virus attached to particles of different size as smaller dust particles can travel larger distances, and as smaller particles can be deposited more easily in the lower respiratory tract of chickens affecting the probability of infection (Van Riel et al., 2006; Shao, 2008; Kuchipudi et al. 2009; Liu et al., 2009). Infectious virus was detected on both fractions after two days of shedding of virus by the infected birds, but no difference was found between the amount of virus detected in the respirable and non-respirable fraction. Understanding the biological relevance of these observations require more research on for example viral load, particle size and transmission. The results of this study are a first step to elucidate the importance of the transmission of dust-borne HPAI virus between poultry flocks. Mathematical modelling, using the results described here, can help extrapolating the results from these experimental flocks to the size of commercial flocks to examine whether they can, in part, explain the transmission during epidemics.

4.5. Acknowledgements

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Chapter 5

Vaccination with 1/3 dose of an H5N9 vaccine prevents cloacal shedding and transmission of Highly Pathogenic Avian Influenza H5N1 virus upon experimental infection of chickens

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Abstract

Vaccination can be used to control outbreaks of highly pathogenic avian influenza (HPAI) in chickens. There are, however, concerns of (sub-)clinical outbreaks in suboptimally vaccinated flocks. One explanation for outbreaks in vaccinated flocks is that birds were vaccinated with a dose lower than recommended. We carried out experiments in which we quantified transmission of an HPAI H5N1 virus strain after vaccination with an H5N9 vaccine, using either 1/3 or 2/3 of the recommended dose. Twelve groups of chickens were formed, with 10 chickens each. In nine groups, per group 5 chickens were vaccinated and inoculated with an HPAI H5N1 virus, and thereafter placed with 5 unvaccinated in-contact birds. In three control groups, the inoculated birds remained unvaccinated. Tracheal and cloacal swabs were collected daily of each chicken and tested in RT-qPCR to monitor virus transmission, and the amount of virus in dust samples from the environment was determined. None of the vaccinated birds showed clinical signs post inoculation, whereas all birds in the control groups did. In vaccinated birds, virus was only detected in tracheal swabs; cloacal swabs remained negative. The amount of virus in tracheal swabs after inoculation was $10^{3.1}$ EID_{50} in the 1/3 group, $10^{2.5}$ in the 2/3 group, and $10^{5.6}$ EID_{50} in the control group. None of the contact birds in the vaccinated groups became infected, whereas all contacts to the control groups did. Dust samples collected from the air and pen wall of the vaccinated groups remained negative in RT-qPCR, whereas these samples in the control group tested on average $10^{2.8}$ EID_{50} per m^2. This study showed that birds vaccinated with 1/3 dose of vaccine can become infected, but transmission to susceptible cage mates did not occur. Shedding of virus was reduced and limited to trachea. Possibly, the absence of cloacal shedding prevented transmission of virus to cage mates and deposition of virus in the environment.

5.1. Introduction

Highly pathogenic avian influenza (HPAI) is considered one of the most important infectious diseases of poultry, because of its rapid spread, high mortality rate and economic impact, and because the virus may cause human infections possibly resulting in a pandemic (Alexander, 2007). Outbreaks of

HPAI virus have been reported in 60 countries (OIE, 2012), of which most were controlled by culling of infected flocks and preemptive culling of contiguous flocks (Directive 92/40/EEC; Stegeman et al., 2004; Tiensin et al., 2005; Capua and Marangon, 2007; Pasick et al., 2009). The economic impact of both the disease and this intervention strategy is huge, as it usually results in death and culling of millions of chickens (Stegeman et al., 2004; Tiensin et al., 2005; Marangon and Capua, 2006).

In countries where HPAI virus infection is endemic, like Indonesia, Vietnam and China, vaccination is often used as an additional control measure to protect poultry from clinical signs, and to reduce production losses (Ellis et al., 2006; Domenech et al., 2009). In addition, in countries with incidental outbreaks vaccination may be considered as an emergency measure to reduce further spread of virus between flocks (Swayne, 2003; Suarez, 2005; Bruschke et al., 2007; Capua and Marangon, 2007).

Several vaccine studies have been carried out to investigate the efficacy of this strategy. Under experimental conditions, vaccination in chickens, using single or multiple doses of vaccine, has been found effective in inducing clinical protection, reduction of the amount of virus particles shed into the environment (Swayne et al., 1999; Suarez, 2005; Lee and Suarez, 2005; Tian et al., 2005; Lee et al., 2007; Abdelwab et al., 2011) and reduction of transmission (van der Goot et al., 2008; Bouma et al., 2009; Poetri et al., 2009). Vaccination in the field, however, appears less effective, as for example demonstrated in Mexico (Lee et al., 2004), Egypt (Abdelwab et al., 2011), and Indonesia (Bouma et al., 2008). Explanations given are, for example, failure of cold chain, the difference in homology between vaccine strain and circulating field virus, improper application of vaccines or application of a dose lower than the one recommended by the manufacturer.

To gain more insight in the cause of vaccine failure, the effect of these above mentioned factors on vaccine efficacy can be investigated. To determine whether chickens can become infected when vaccinated with a dose lower than recommended, and if so whether virus transmission may still be possible, two transmission experiments were performed in which we quantified virus excretion upon HPAI virus infection in chickens vaccinated with 1/3 or 2/3 of the recommended dose of an H5N9 vaccine, and studied transmission to non-vaccinated in-contact chickens and presence of the virus in the environment.

5.2. Materials en methods

5.2.1. Animals

Embryonated eggs from White Leghorn chickens from a commercial avian influenza (AI) free poultry breeder farm were hatched at the Central Veterinary Institute (CVI) in Lelystad. After hatching, the chickens were housed in one room. At 3 weeks of age, the chickens were tested for the presence of antibodies against AI using a modified indirect double antibody sandwich (IDAS) nucleoprotein (NP)-blocking ELISA. Chickens were kept on the floor with bedding, and feed and water were provided *ad libitum*. Room temperature was kept at 21°C and a relative humidity of 55 %.

5.2.2. Vaccine

A commercially available oil-emulsion adjuvanted vaccine formulated with the A/turkey/Wisconsin/1968 H5N9 strain was used for vaccinating the chickens at the age of 3 weeks. The recommended dose according to the manufacturer was 0.3 ml per bird administered intramuscular (IM). The efficacy of the vaccine was controlled by the manufacturer through serological conversion in 3-weeks-old SPF chickens, using 1 dose IM. After 4 weeks post vaccination, an average HI titer of 8 against a homologous H5N9 antigen was achieved. The vaccine dose used in the experiments was either 1/3 or 2/3 of the recommended dose, and was administered in the pectoral muscle.

5.2.3. Inoculum

The HPAI virus strain A/turkey/ Turkey/1/2005 H5N1 (clade 2.2.1) was used as challenge strain (Spekreijse et al., 2011). The virus was grown in embryonated SPF eggs and vials with a known egg infectious dose (EID_{50}) titer were stored at -70ºC until use. On the day of challenge, one vial was thawed and diluted in 10-fold dilution steps in tryptose phosphate buffer (TPB) to obtain an inoculation dose of 10^4 $EID_{50.}$ Chickens were inoculated at 5 weeks of age with 0.1ml inoculum applied intra-nasally and 0.1ml inoculum applied intra-tracheally using a blunt needle, according to standard protocol (van der Goot et al., 2005).

5.2.4. Experimental design

Two experiments were carried out consecutively, that complied with the Dutch

law on animal experiments and had been approved by an ethical committee. The experiments were carried out under $BSL3^+$ conditions at the CVI. Each one of the experiments consisted of 6 groups of 10 chickens, three groups per room. In the first experiment, per group, 5 chickens were vaccinated at the age of 3 weeks using 1/3 (room 1) or 2/3 (room 2) of the recommended dose of vaccine and inoculated at the age of 5 weeks with the challenge strain. After inoculation the 5 non-vaccinated in-contact chickens were placed in the cage with the inoculated vaccinated birds. In the second experiment, chickens were vaccinated with 1/3 dose of vaccine (room 2) or not vaccinated before challenge (room 1). Clinical inspection of the chickens was performed two times per day to determine the presence of clinical signs after inoculation. The experiments were terminated 21 days p.i. by euthanizing surviving birds with an intracardiac injection of T-61.

5.2.5. Sampling procedures

At day 1 post-inoculation (p.i.), swabs from trachea and cloaca were collected from the inoculated chickens and at day 2 to 10 p.i., from all chickens. Sampling was discontinued if the inoculated chicken remained negative in trachea or cloaca for two days in a row. The swabs were put in 2ml of 2.95% TPB with 5 x 10^3 IU of penicillin-sodium and 5mg streptomycin per ml and stored at -70°C until analyzed.

Air and dust samples were collected from day 1 to day 10 p.i. Air samples were taken with an MD8 air-scan air sampling device (Sartorius, Nieuwegein, The Netherlands) using sterile gelatin filters of 80mm diameter and 3µm pore size. The MD8 air-scan was placed in the middle of the rooms at a distance of approximately 50 cm from each cage and at a height of 80cm. Samples were taken at an air speed of 2 m^3/h for 270 minutes. After sampling, the gelatin filters were dissolved in 10ml of 2.95% TPB with 5 x 10^3 IU of penicillin-sodium and 5mg streptomycin per ml at a temperature of 37°C. Dissolved filter solutions were stored at -70°C until analyzed. Viral load was determined for each gelatin filter.

Dust samples were taken daily using electrostatic dust cloths (Swiffer, Procter and Gamble, U.S.). Per room, a defined section of 0.1m^2 of wall was marked and sampled daily to determine viral deposition in 24 hours. In the third and fourth experiment, additional dust samples were taken from inside the cages

from 0.5m^2. The dust cloths were put in 10ml of 2.95% TPB with 5 x 10^3 IU of penicillin-sodium and 5mg streptomycin per ml and stored at -70^{o}C until analyzed. Viral load was determined for each dust cloth.

5.2.6. RNA isolation, quantitative real-time reverse transcriptase PCR (RT-qPCR) and virus isolation

Trachea and cloaca swab fluid or filter solution (200µl) was used to extract the viral RNA with the MagNA Pure LC 2.0 instrument (Roche Applied Science, Mannheim, Germany) with the MagNA Pure LC total Nucleic Acid Isolation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The nucleic acids were collected in elution buffer and stored at -70°C or directly processed for the quantitative real-time reverse transcriptase PCR (RT-qPCR).

The matrix gene of influenza virus was detected in RT-qPCR in 5µl of the elution buffer with extracted RNA as described by van der Goot (2008). The RT-qPCR and data analysis were performed using the MX3005 Quantitative PCR system (Stratagene) with version 4.20 software. The viral RNA concentration of each sample could be calculated using a calibration curve of serial dilutions of a standard batch of the virus with a known EID_{50} titer that was run along with the unknown samples. The viral concentration in each sample was quantified based on the calibration curve generated by plotting the cycle threshold (Ct) value against known virus titers. The viral concentration was considered negative if no Ct value was measured or when the Ct was larger than 40. Titers of the samples were expressed as EID_{50}/ml equivalents.

Swabs from the vaccinated birds and dust samples that tested positive in RT-qPCR were submitted for virus isolation. Per swab or sample three embryonated SPF chicken eggs incubated for 9 days were inoculated with 0.2ml swab fluid per egg. After 72h the allantoic fluid was harvested and a standard hemagglutination assay (HA) with chicken red blood cells was performed (OIE, 2008). When at least one egg of three eggs was positive in HA the swab was considered to be positive.

5.2.7. Serology

Blood samples were taken from the ulnar vein at three weeks of age, and at days 7, 14 and 21 after inoculation from all chickens. In addition, the vaccinated

chickens were sampled 1 day prior to inoculation to determine the immune status. Sera were incubated for 30min at 56°C and stored at -20°C until analyzed.

A modified indirect double antibody sandwich (IDAS) nucleoprotein (NP)-antibody blocking ELISA that detects antibodies against the nucleoprotein of influenza A was performed as described by de Boer et al. (1990). In addition, sera from the vaccinated chickens were tested at 3, 5, 6, 7 and 8 weeks of age in a haemagglutination inhibition (HI) test against an A/turkey/Wisconsin/68 H5N9 according to Maas et al. (2009) to determine serological response against the vaccine strain. All sera were tested in N1 ELISA (ID Screen® Influenza N1 Antibody Competition ELISA, FLUAcN1 of ID-Vet, Montpellier, France) according to the protocol of the manufacturer to differentiate infected birds from vaccinated birds (DIVA). A cut-off value of 40% was chosen according to the manufacturer's procedures.

5.2.8. Data analysis

A bird was considered infected if it tested positive in RT-qPCR. In the inoculated birds, the association between vaccination status (1/3, 2/3 or control) and infection status upon inoculation (infected or not infected) was tested by Chi-square test. The amount of virus shed in trachea after inoculation of the positive birds, measured in RT-qPCR, was quantified over time by calculating the area under the excretion against time curve (AUC) of the viral titers. The AUC values, which estimate the amount of virus shedding during the total infectious period, were compared for the vaccinated and non-vaccinated groups using ANOVA.

All tests were performed assuming a 2 sided-alternative hypothesis; *p* values smaller than .05 were considered statistically significant. Analysis was performed using commercially available statistical software (SPSS 16.0; SPSS Inc., Chicago, Illinois).

5.3. Results

None of the birds had antibodies against avian influenza virus before vaccination. After vaccination all vaccinated birds developed antibody titers in

HI test using H5N9 and H5N1 antigen. Serological data on HI from the vaccinated birds are summarized in Table 5.1.

Table 5.1. Mean HI-antibody titers (log^2) of vaccinated chickens of the 1/3 and 2/3 dose groups against H5N1 and H5N9 before and after inoculation with H5N1.

Vaccine	Antigen	Mean HI titer (95% C.I.) weeks after vaccination /days after challenge				
		week 0/ day -14	week 2/ day -1	week 3/ day 7	week 4/ day 14	week 5/ day 21
1/3	H5N9	0	3.7 (3.2 – 4.1)	8.6 (8.2 – 9.0)	7.8 (7.4 – 8.2)	7.4 (7.1 – 7.8)
	H5N1	0	1.7 (1.4 – 2.0)	7.3 (6.7 – 8.0)	6.7 (6.2 – 7.3)	6.4 (6.0 – 6.8)
2/3	H5N9	0	4.9 (4.2 – 5.5)	7.9 (7.2 – 8.5)	8.1 (7.5 – 8.6)	7.4 (6.9 – 7.9)
	H5N1	0	1.9 (1.3 – 2.4)	7.8 (7.2 – 8.4)	6.7 (6.0 – 7.5)	6.8 (6.1 – 7.6)

No clinical signs were observed in any of the vaccinated birds of the 1/3 and 2/3 groups, and none of them died after inoculation. Virus was detected in tracheal swabs of 18 out of 30 birds vaccinated with 1/3 dose with a mean AUC of $10^{3.1}$ EID_{50} (95% C.I.: $10^{2.5}$ – $10^{3.6}$ EID_{50}). Ten of these 18 chickens, that tested positive in RT-qPCR, developed antibodies against N1; one bird at 1 week p.i., two birds at 2 weeks p.i., and 7 at birds 3 weeks p.i. Seven of these 18 chickens, that tested positive in RT-qPCR, tested positive in virus isolation.

Virus was detected in tracheal swabs of 11 out of 15 birds vaccinated with 2/3 dose with a mean AUC of $10^{2.5}$ EID_{50} (95% C.I.: $10^{1.8}$ – $10^{3.1}$ EID_{50}). Of these 11 birds 3 developed antibodies against N1, one bird at 2 weeks p.i., and two birds at 3 weeks p.i. Of these 11 birds, 3 tested positive in virus isolation. The results from N1-ELISA, on the amount of virus detected in trachea swabs from the inoculated birds and virus isolation are listed in Table 5.2.

An association between vaccination and infection status determined by RT-qPCR was found between the vaccinated and the non-vaccinated group, but this association did not differ between the 1/3 and 2/3 groups. All swabs taken from the cloaca of the vaccinated birds from the 1/3 and 2/3 groups tested negative in RT-qPCR.

None of the contact birds from the 1/3 or 2/3 groups showed clinical signs, tested positive in RT-qPCR, in (NP) - antibody blocking ELISA on day 7, 14 and 21 p.i., or died. In contrast, all inoculated non-vaccinated birds tested positive in trachea and cloaca in RT-qPCR and died within three days after inoculation from infection p.i., as did all cage mates from these birds within 5 days p.i.

Table 5.2. N1 ELISA antibody response and the amount of virus shed via trachea (log^{10} EID_{50} equivalents) of the chickens vaccinated with 1/3 or 2/3 of recommended dose of vaccine.[*] = positive virus isolation; [+] = positive N1 ELISA; [-] = negative N1 ELISA or RT-qPCR; [na] = data not available; [†] = death of chicken

Dose	N1 ELISA antibody response				RT-qPCR tracheal swabs ($log^{10}EID_{50}$ equivalents)						
	days p.i.				days p.i.						
	-1	7	14	21	1	2	3	4	5	6	7
1/3	-	-	+	+	1.9	1.5	-	-	-	-	-
1/3	-	-	-	-	-	-	-	-	-	-	-
1/3	-	-	-	-	2.6	-	-	-	-	-	-
1/3	-	-	-	-	-	-	-	-	-	-	-
1/3	-	-	-	-	-	-	-	-	-	-	-
1/3	-	-	-	-	0.8	-	-	-	-	-	-
1/3	-	-	-	-	-	-	-	-	-	-	-
1/3*	-	-	-	+	-	0.8	4.5	4.4	3.1	3.2	-
1/3*	-	-	+	+	2.3	-	4.9	3.5	2.1	-	-
1/3	-	-	-	-	-	-	-	-	-	-	-
1/3	-	-	-	-	-	-	-	-	-	-	-
1/3	-	-	-	-	-	-	-	-	-	-	-
1/3	-	-	-	-	-	-	-	-	-	-	-
1/3	-	-	-	-	-	-	-	-	-	-	-
1/3	-	-	-	-	-	-	-	-	-	-	-
1/3*	-	+	-	+	4.4	-	2.8	-	-	-	-
1/3	-	-	-	-	1.5	0.6	1	-	-	-	-
1/3*	-	-	-	+	-	4.5	3.3	3.3	3.4	-	-
1/3*	-	-	-	+	3.3	3.5	2.1	4.2	1.5	-	-
1/3	-	-	-	+	-	3.6	2.8	3.6	-	-	-
1/3	-	-	-	-	0.3	-	-	-	-	-	-
1/3	-	-	-	-	1.6	-	-	-	-	-	-
1/3	-	-	-	+	-	1.8	2.8	1.5	0.5	-	-
1/3	-	-	-	-	3.4	-	2.0	-	-	-	-
1/3*	-	-	-	+	1.4	3.6	2.6	3.6	-	-	-
1/3	-	-	-	-	-	-	-	-	-	-	-
1/3	-	-	-	-	-	-	-	-	-	-	-
1/3	-	-	-	-	0.1	1.6	2.1	-	-	-	-
1/3	-	-	-	-	1.6	-	-	-	-	-	-
1/3*	-	-	-	+	3.8	4.6	5.3	4.6	1.4	-	-
2/3*	-	-	-	+	-	3.4	2.7	-	-	-	-
2/3	-	-	-	-	-	-	-	-	-	-	-
2/3	-	-	-	+	-	1.0	1.2	2.1	1.1	-	-
2/3	-	-	-	-	-	1.1	1.2	1.2	-	-	-
2/3	-	-	-	-	1.8	2.3	-	-	-	-	-
2/3	-	-	-	-	0.8	-	-	-	-	-	-
2/3	-	-	-	-	1.9	1.6	-	-	-	-	-
2/3*	-	-	+	-	2.8	4.2	3.7	3.4	-	-	-
2/3	-	-	-	-	-	3.0	1.2	-	-	-	-
2/3*	-	-	-	-	0.6	3.4	0.6	2.2	0.2	-	-
2/3	-	-	-	-	-	-	-	-	-	-	-
2/3	-	-	-	-	0.7	1.5	0.5	2.2	0.1	-	1.3
2/3	-	-	-	-	0.3	-	-	-	-	-	-

Table 5.2 continued

2/3	-	-	-	-	-	-	-	-	-	-	-
2/3	-	-	-	-	-	-	-	-	-	-	-
0	na	na	na	na	2.8	6.4	†				
0	na	na	na	na	2.1	4.6	†				
0	na	na	na	na	2.7	5.7	†				
0	na	na	na	na	4.0	5.8	†				
0	na	na	na	na	3.5	†					
0	na	na	na	na	4.6	6.2	†				
0	na	na	na	na	2.3	5.3	†				
0	na	na	na	na	2.8	6.4	†				
0	na	na	na	na	2.9	6.4	†				
0	na	na	na	na	3.0	6.9	†				
0	na	na	na	na	3.2	6.5	†				
0	na	na	na	na	2.9	6.1	†				
0	na	na	na	na	3.2	6.1	†				
0	na	na	na	na	4.6	6.6	†				
0	na	na	na	na	2.4	4.9	†				

These unvaccinated chickens shed virus after inoculation with a mean AUC of $10^{5.6}$ EID_{50} (95% C.I.: $10^{5.0}$ – $10^{6.2}EID_{50}$). The mean AUC of the vaccinated and non-vaccinated birds differed significantly. The mean AUC of 1/3 and 2/3 dose did not differ significantly.

In the groups vaccinated with 1/3 or 2/3 dose, all samples taken from the walls of the pens and all samples collected by the MD8 air scan tested negative in RT-qPCR. In the non-vaccinated group, positive samples were found from day 2 until day 5 p.i. and virus was detected at on average of $10^{2.8}$ EID_{50} per m^2 (95% C.I.: $10^{2.1}$ – $10^{3.1}$ EID_{50} per m^2) on the wall of the room and on average of $10^{1.5}$ EID_{50} per m^3 (95% C.I.: $10^{0.3}$ – $10^{1.8}$ EID_{50} per m^3). All RT-qPCR positive dust samples tested positive in virus isolation

5.4. Discussion

The aim of this study was to quantify virus excretion upon HPAI virus infection in chickens vaccinated with 1/3 or 2/3 of a recommended dose of an A/turkey/Wisconsin/1968 H5N9 vaccine, and to determine transmission to unvaccinated in-contact chickens. All vaccinated chickens inoculated with A/turkey/ Turkey/1/2005 H5N1 virus strain were protected against clinical signs, but not against infection as virus was detected in tracheal swabs. However, none of the unvaccinated cage mates became infected, indicating that

the amount of virus shed by the inoculated birds was not sufficient for virus transmission to occur. This implies that, under experimental conditions, 1/3 dose of vaccine is sufficient to stop transmission of virus, even of a heterologous strain, suggesting that vaccine dose is not an explanation for observed outbreaks in vaccinated flocks in endemically infected regions. However, it should be mentioned, that it is possible that the applied doses in the field are even lower than the one used here. Moreover, differences in vaccine efficacy are observed when measured under experimental conditions, implying that 1/3 dose in the field might not be as effective as determined here. The explanation for observed outbreaks in vaccinated populations remains unclear.

We considered birds infected when one or more of the trachea or cloacal swabs of a bird were positive for virus, and regarded positive swabs as indication for shedding in respiratory and alimentary tract, respectively. Another indication for infection, which is often used, is a fourfold increase in HI antibody titer. The immune response of the vaccinated inoculated birds in our trials showed a fourfold increase of antibody titer in the HI test against H5N1. However, it could also be possible that this increase was due to vaccination instead of response on challenge, because the interval between vaccination and inoculation was only 2 weeks whereas the vaccination titer levels can increase up to week 4 after vaccination (van der Goot et al., 2005). We cannot discriminate between these two responses, as no vaccinated but unchallenged control group was included in our experiment. Another indication for infection is a positive N1 antibody response, as the strain used for inoculation was an H5N1, whereas the vaccine strain contained H5N9. However, only 56% of the birds with a positive RT-qPCR swab result in the 1/3 dose group, and only 18 % of the RT-qPCR positive birds in the 2/3 dose group developed antibodies against N1, suggesting N1 ELISA has a lower sensitivity when compared to RT-qPCR, or virus isolation for the detection of infected birds. However, neither RT-qPCR nor N1 ELISA or virus isolation results were suitable to predict infectious birds correctly, as in none of the cages with inoculated birds that tested positive in RT-qPCR, N1 ELISA or virus isolation, transmission of virus occurred.

Vaccines can reduce the amount of virus shed from respiratory and gastro-intestinal tract after challenge (Webster et al., 1996; Swayne et al., 1999; Swayne et al., 2000; Maas et al., 2009). It may be argued that birds in our trials

received a dose that was too low for successful infection. The vaccinated birds were challenged with 10^4 EID_{50}, which is considered to be sufficient for unvaccinated birds to become infected, and to shed sufficient virus to infect contact birds (Spekreijse et al., 2011). In a previous study, no association was found between the inoculation dose of HPAI H5N1 virus and transmission of the virus to contact exposed chickens, except for the probability of infection (Spekreijse et al., 2011). In our study, virus shedding in trachea in the vaccinated birds was reduced with 2 log_{10} compared to the non-vaccinated birds, but we expected this amount sufficient for infection of the unvaccinated contact birds and virus transmission in the group, as the mean infectious dose for this virus strain has been estimated to be $10^{2.5}$ EID_{50} (Spekreijse et al., 2011) and the amount shed by the vaccinated birds exceeded this estimation. Of course, it remains unknown what the 'real' amount of virus was to which contact birds were actually exposed. Furthermore, it could be argued that the virus detected by RT-qPCR was not viable. We did, however, show that the swabs contained live virus, as samples tested positive in virus isolation, but we did not quantify the amount of viable virus, so it could have been lower than the amount determined with RT-qPCR.

We assumed that positive swabs were an indication for virus shedding in the lumen of the trachea and cloaca from which the sample was taken. Vaccinated inoculated birds were, however, only positive in trachea swabs, and shedding via the cloaca did not occur. Dust samples collected in the surroundings of the infected vaccinated birds remained negative as well. An explanation for the absence of transmission, despite positive virus detection, is that virus shed in the trachea might not reach cage mates. Birds shedding virus in the tracheal lumen did not show any signs of avian influenza and may have swallowed the virus instead of excreting it into the environment. An explanation for the absence of cloacal shedding is that the vaccine-induced immune response protected birds against a systemic infection preventing virus shedding via the gastro-intestinal tract. Our findings might be an indication that only birds that shed virus via the alimentary tract are infectious to other birds, as for example seen in the unvaccinated group. This suggests that when measuring infectiousness, virus determination on cloacal swabs might be a more appropriate indication than determination of virus in tracheal swabs.

Our study showed that even a lower vaccine dose than recommended was sufficient to prevent transmission. Outbreaks of HPAI in the field may, however, still occur, as has also been observed for other livestock viruses, like foot-and-mouth disease (Yadin et al., 2007), Aujeszky's disease (Stegeman et al., 1994) and Newcastle disease (Kapczynski and King, 2005). Extrapolation of experimental results may therefore be difficult, and an explanation for vaccine failure in the field remains unclear.

Chapter 6

Genetic variation of a highly pathogenic avian influenza H5N1 virus strain in groups of chickens directly or indirectly exposed to the virus

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In preparation

Abstract
Highly pathogenic Avian Influenza (HPAI) is a devastating viral disease of poultry and outbreaks have a large socioeconomic impact and need to be controlled as quickly as possible. The improvement of control measures is only possible if mechanisms of the transmission of HPAI virus within and between poultry flocks are understood. However, the knowledge on the genetic variation of HPAI virus in groups of birds still shows considerable gaps. Therefore, in this study we carried out experimental studies in which we applied next generation sequencing to examine the genetic variation of virus after introduction in the host and during between-host transmission. In addition, isolates originating from chickens infected by the airborne route were examined. Described study showed that phylogenetic studies used to reconstruct indirect transmission pathways during an outbreak of HPAI are hampered by the genetic variation of the virus as different lineages can already evolve within a host, and multiple lineages can be transmitted between hosts.

6.1. Introduction

Highly pathogenic avian influenza (HPAI) is a devastating poultry disease (Alexander, 2007) that not only poses a risk for the commercial poultry industry but also for public health (FAO, WHO, OIE, 2010; OIE, 2011). The disease is caused by a virus belonging to the Influenza virus A genus of the Orthomyxoviridae family that has a negative single-stranded RNA genome consisting of 8 segments that encode for 11 proteins. (Bouvier and Palese, 2008; Höper et al., 2011). Segment 4 encodes the surface glycoprotein hemagglutinin (HA), which is responsible for the attachment of the virus to the host cell and fusion of the virus membrane with the host cell membrane. Segment 6 encodes another surface glycoprotein called neuraminidase (NA) that enhances the release of viral particles from an infected cell. Influenza A viruses are classified into subtypes based on the characteristics of these two surface glycoproteins (Webster et al., 1992). HPAI virus strains either have the H5 or H7 glycoprotein.

Like other RNA viruses, influenza A viruses, have a high error rate during transcription, a million times higher than vertebrates, because of the lack of proofreading ability in RNA polymerases (Parvin et al., 1986). Mutations can

involve single nucleotide changes, but deletions or insertions of several contiguous nucleotides also occur. Most of the mutations are deleterious or lead to viruses that are less fit and as a consequence will disappear quickly from the population, a process that is indicated as negative selection. However, it is observed that mutations that lead to changes in amino acid residues appear significantly more often in the evolution of some segments; for instance in the region of the HA gene that determines the antigenic domains. This observation indicates that antigenic drift of influenza A viruses is driven by the equilibrium between virus and host immunity. The production of many different viral genotypes in just a few days with each genotype a different level of fitness enables the virus to swiftly adapt to a new environment (Domingo et al., 1985; McHardy and Adams, 2009; Suarez, 2000).

As a result of the high mutation rate, evolutionary and ecological behaviour of the virus is closely linked. The increasing possibilities to obtain viral genome sequence data of high quality and resolution, and development of advanced statistical models make it feasible to link the evolutionary dynamics of HPAI virus (HPAIv) and epidemiological data. Reconstructing evolutionary changes on a timescale enables to link epidemiologically important events, provide insight in the spatial distribution of a disease and discern the routes of transmission (Pybus and Rambaut, 2009). For example, evolutionary analysis has helped to track the global spread of HPAI H5N1 (Catolli et al., 2009; Liang et al., 2010; Wallace et al., 2007; Vijaykrishna et al., 2008), and combining temporal data with genome sequences pointed out transport of infected livestock as the main cause of between-farm transmission during the UK outbreaks of foot-and-mouth-disease (Cottam et al., 2008). Unfortunately, often epidemiological data are insufficient, when for instance data of outbreaks are missing, investigations fail to utilize all epidemiological information, or sequence data are skewed toward the shorter genes and surface glycoproteins (Starick et al., 2007; Pybus and Rambaut, 2009). Even if a sample is available from the vast majority of infected farms, like in the 2003 Dutch H7N7 outbreak (Bataille et al., 2011; Jonges et al., 2011), evolutionary studies may be hampered because of the availability of only one virus sequence per farm, whereas each infected farm and each individual host within this farm most likely contains a wide variety of closely related virus variants. The clonal sequencing of samples showed that the dominant virus strain originally

sequenced per farm was not always the only variant present on the farm (Bataille et al., 2011).

To improve control measures understanding mechanisms of transmission of HPAI virus within and between poultry flocks is essential. Nevertheless, the full potential of molecular epidemiology to elucidate transmission mechanisms cannot be used yet, because the knowledge on the genetic variation of HPAI virus in groups of birds still shows considerable gaps. In particular the variation that arises shortly upon introduction of a virus into a flock is unknown, because it cannot be observed under field conditions. In this study we carried out experimental studies in which we applied next generation sequencing to examine the genetic variation of virus after introduction in the host and during between-host transmission. In addition, isolates originating from chickens infected by the airborne route were examined.

6.2. Materials and Methods

6.2.1. Direct and indirect transmission experiments

Two experiments were carried out in which per experiment two groups of 14 chickens were formed. In the first group of both experiments, 8 out of 14 chickens were inoculated with 10^4 EID_{50} of HPAIv strain A/turkey/ Turkey/1/2005 H5N1 (clade 2.2.1) according to a standard protocol (Spekreijse et al., 2011). The remaining 6 birds were contact exposed post inoculation (p.i.). Deceased birds were replaced daily with new susceptible ones up to 10 days p.i. to generate transmission of the virus through subsequent generations of infected chickens. Virus shedding was verified by taking daily swabs from trachea and cloaca. The second group in both experiments consisted of 14 susceptible chickens in a separate room that was interconnected by a tube to the room with the inoculated and direct contact chickens. Ventilation forced all air from the room with the inoculated birds to the room with the second indirectly exposed group through the tube and these birds were indirectly exposed. The chickens were housed on the floor with bedding material of wood shavings and sawdust. Rearing feed that was irradiated and tap water were provided *ad libitum*. Room temperature was kept at 21^{o}C and the relative humidity at 55 %. Both experiments complied with the Dutch law on animal experiments and were reviewed by an animal ethics committee. The experiments were terminated 21

days p.i. by euthanizing surviving birds with an intracardiac injection of T-61. Further details on the transmission experiments can been found in Spekreijse et al. (2012) and in Table 6.1 and 6.2.

Table 6.1. Results of RT-qPCR swabs collected after inoculation and day of introduction of directly exposed contact birds of experiment 1.
I = inoculated chicken; C = directly exposed chicken; R = indirectly exposed chicken; na = not available data; + = positive RT-qPCR result of trachea and/or cloaca swab; - = negative RT-qPCR result of trachea and cloaca swab. Samples analyzed in next generation sequencing are marked with a black box.

EXPERIMENT 1

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
I1	challenge	+	†																			
I2	challenge	+	†																			
I3	challenge	+	†																			
I4	challenge	+	†																			
I5	challenge	+	†																			
I6	challenge	-	+	†																		
I7	challenge	+	+	†																		
I8	challenge	-	+	†																		
C1			-	+	†																	
C2			-	-	+	+	+	+	†													
C3			+	+	+	†																
C4			-	+	†																	
C5			-	-	-	+	†															
C6			-	+	+	+	+	+	†													
			C7	-	+	†																
			C8	-	+	+	+	†														
			C9	-	+	+	+	†														
			C10	-	+	†																
			C11	-	+	+	+	+	+	†												
				C12	-	-	+	+	†													
				C13	+	+†																
				C14	-	-	+	+	+	†												
					C15	-	-	+	+	+	†											
					C16	-	-	+	†													
						C17	-	+	+	†												
						C18		+	+	†												
						C19	-	+	+	†												
						C20	+	+	+	†												
							C21	+	+	+												
								C22	+	+	†											
								C23	-	+	+	†										
									C24	+	+	†										
									C25	+	+	†										
									C26	+	+	na	na	†								
									C27	+	+	†										
									C28	-	+	†										
										C29	-	na	na	†								
										C30	-	na	na	+†								
										C31	-	na	na	†								
										C32	+	na	†									
										C33	+	na	†									
										C34	+	na	†									
											C35	na	na	+	+	†						
											C36	na	na	+	†							
											C37	†										
											C38	na	na	+	+	†						
											C39	na	na	+	+	†						
R1			-	-	-	-	-	-	-	-	-	na	na	-	-	na	na	na	†				
R2			-	-	-	-	-	-	-	-	-	na	na	-	+	†							
R3			-	-	-	-	-	-	-	-	-	na	na	+	†								
R4			-	-	-	-	-	-	-	-	-	na	na	-	-	na	na	na	na	na	na	†	
R5			-	-	-	-	-	-	-	-	-	na	na	-	+	na	na	†					
R6			-	-	-	-	-	-	-	-	-	na	na	-	+	na	†						
R7			-	-	-	-	-	-	-	-	-	na	na	-	+	na	na	†					
R8			-	-	-	-	-	-	-	-	-	na	na	-	-	na	na	na	†				
R9			-	-	-	-	-	-	-	-	-	na	na	-	-	na	na	na	na	†			
R10			-	-	-	-	-	-	-	-	-	na	na	+	+	na	†						
R11			-	-	-	-	-	-	-	-	-	na	na	+	+	†							
R12			-	-	-	-	-	-	-	-	-	na	na	-	-	na	na	na	†				
R13			-	-	-	-	-	-	-	-	-	na	na	+	-	na	†						
R14			-	-	-	-	-	-	-	-	-	na	na	-	-	na	na	na	na	na	†		

As the financial resources were limited, a selection of RNA samples from the chickens was made based on viral titer, route of transmission, and day of infection. From the first experiment, one sample from an inoculated bird (I7), 17 samples from 15 direct contact infected chickens (from C2, C6 and C20 two samples from 2 different sampling days), and 2 samples from 2 indirect contact infected birds (R3 and R11) were sequenced. From the second experiment, one sample from an inoculated bird (I6), 4 samples from direct contact infected birds, and 3 samples from indirect contact infected birds (R4, R10, R14) were sequenced.

6.2.2.RNA isolation and quantitative real-time reverse transcriptase PCR

Viral RNA extraction from trachea and cloaca swab fluid was performed with the MagNA Pure LC 2.0 instrument (Roche Applied Science, Mannheim, Germany) with the MagNA Pure LC total Nucleic Acid Isolation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The nucleic acids were collected in elution buffer and processed for the quantitative real-time reverse transcriptase PCR (RT-qPCR) according to van der Goot et al. (2008). The viral RNA titer of each sample could be calculated using a calibration curve of serial dilutions of a standard batch of the virus with known EID_{50} titer. Tracheal or cloacal swabs containing more than 10^4 EID_{50} per ml were suitable for next generation sequencing.

6.2.3. RNA isolation, reverse-transcription-PCR and next generation sequencing

For next generation sequencing new batches of viral RNA from trachea and cloacal swab fluid were isolated with a high pure viral DNA kit version 15.0 (Roche, Minneapolis, USA) according to the manufacturer's instructions. Sequencing libraries were prepared according to Höper et al. (2009). An additional RT-PCR was performed with the Expand High Fidelity PCR system (Roche, Minneapolis, USA) according to the manual of the manufacturer with primers according to Höper et al. (2009). The PCR products were purified with a High Pure PCR product purification kit according to the manufacturer's instructions (Roche, Minneapolis, USA). PCR products were pooled in equimolar amounts and prepared for Illumina Next Generation Sequencing

according to standard procedure for paired-end sequencing. The samples were sequenced on the illumina HiSeq 2000 sequencer.

Table 6.2. Results of RT-qPCR swabs collected after inoculation and day of introduction of directly exposed contact birds of experiment 2. See legend of Table 6.1.

EXPERIMENT 2

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
I1	challenge	-	+	†																		
I2	challenge	+	†																			
I3	challenge	+	†																			
I4	challenge	-	+	+	†																	
I5	challenge	+	+	+	†																	
I6	challenge	**+**	+	†																		
I7	challenge	+	+	†																		
I8	challenge	-	-	-	-	+	+	†														
C1		-	-	-	-	-	-	-	+	†												
C2		-	-	-	**+**	†																
C3		-	-	+	+	+	†															
C4		-	-	-	-	-	-	+	+	+	†											
C5		+	+	+	†																	
C6		-	-	-	-	-	-	-	-	†												
			C7	-	+	†																
			C8	-	-	-	+	+	+	†												
			C9	-	-	-	**+**	†														
			C10	-	+	+	†															
			C11	-	-	-	-	+	+	†												
					C12	+	+	†														
					C13	-	-	-	†													
					C14	-	+	+	†													
						C15	-	-	-	-	-	na	†									
						C16	-	-	-	-	-	na	na	na	na	na	†					
						C17	-	+	†													
							C18	+	**+**	†												
								C19	-	-	-	na	†									
								C20	-	-	-	na	na	na	na	na	na	na	†			
								C21	-	**+**	+	†										
									C22	-	+	†										
									C23	-	+	†										
										C24	-	na	na	na	na	na	na	na	na	na	na	na
										C25	-	na	na	†								
										C26	-	na	†									
										C27	-	na	na	na	†							
R1		-	-	+	†																	
R2		-	-	-	-	-	-	-	-	-	-	na	na	na	†							
R3		-	-	-	-	-	-	-	-	-	-	na	na	na	na	na	na	na				
R4		-	-	-	-	-	-	-	-	+	**+**	†										
R5		-	-	-	-	-	-	-	-	-	-	na	na	†								
R6		-	-	-	-	-	-	-	-	-	-	†										
R7		-	-	-	-	-	-	-	-	-	-	na	na	na	†							
R8		-	-	-	-	-	-	-	-	-	-	na	na	†								
R9		-	-	-	-	-	-	-	-	-	-	na	na	na	na	na	na	na				
R10		-	-	-	-	-	-	+	**+**	†												
R11		-	-	-	-	-	-	-	-	-	-	na	na	na	na	na	na	na				
R12		-	-	-	-	-	-	-	-	-	-	na	na	na	na	na	na	na				
R13		-	-	-	-	-	-	-	-	-	-	na	†									
R14		-	-	-	-	-	**+**	†														

A single-end data set of 12 million unfiltered sequence tags with a length of 50 bases per sample was created. Data was further processed using SNP/Indel analysis. Reads of each sample were aligned to the reference sequence based on Burrows-Wheeler Transform and SNP discovery was performed. Default parameters were used with maximal mismatch of 4% during alignment, and a minimal depth of 10 for variant analysis.

6.2.4. Phylogenetic and selection pressure analyses

A/turkey/ Turkey/1/2005 H5N1 (genbank: EF619973 - EF619980) was used as a reference sequence in the assembly process. The reference sequence was not complete and missing nucleotides were reconstructed by alignment of the reference sequence from the database with other H5N1 strains. Numbering of the nucleotides or amino acids throughout the paper is according to the reconstructed H5N1 genes whereby the start of the open reading frames is set at 1.

The alignment of the inoculum with the reference sequence was performed using MEGA5. All positions containing gaps and missing data were eliminated. Mutations that were positioned in the regions of the primers were excluded from the analysis. The alignment of the reference sequence and the inoculum revealed that ten nucleotides differed with a frequency of more than 50% and were integrated into the reference sequence for the analysis of the other samples of the two experiments. The average numbers of nucleotide differences within and between the phylogenic clusters identified were calculated using MEGA5. The analysis involved 20 RNA samples from the first experiment, and 8 RNA samples from the second experiment (Table 6.1 and Table 6.2).

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used (Tamura et al., 2011). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis of the first experiment involved 21 nucleotide sequences, and for the second 9 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 17 mutations in the final dataset for the first experiment and 13 mutations for the second experiment. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Selection pressure on the segments was investigated by estimating the ratio of non-synonymous to

synonymous nucleotide substitutions by calculating the dN/dS ratio conducted in MEGA5.

6.3. Results

The assembled sequences of all samples together differed at 142 positions from the inoculum with frequencies between 0.1 and 100. The sequence depth of one of the segments of the inoculum is visualized in Figure 6.1 as a representative example for the sequence depth of the other segments and samples. The depth is the number of times that every nucleotide in the final sequence was sequenced.

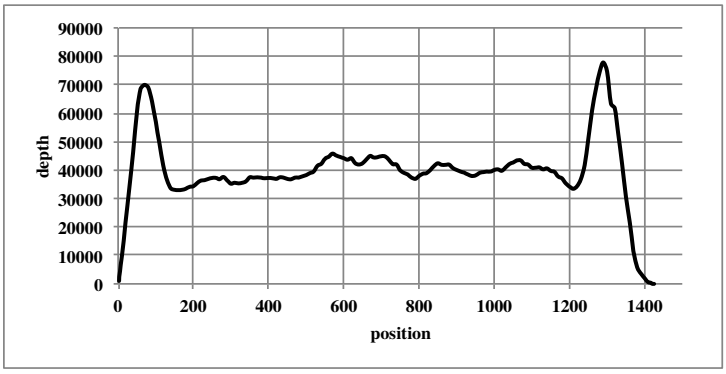

Figure 6.1. The sequence depth of segment NA from the inoculum. The sequence depth is plotted against the nucleotide position of the mRNA within the sequence of the segment.

Compared to the sequence of the inoculum, we identified in total 22 mutations which were present in more than 95% of the sequence fragments; 8 of these 22 mutations were found in both experiments, 17 in experiment 1, and 13 in experiment 2, and are listed for experiment 1 and experiment 2 in Table 6.3 and 6.4, respectively. None of these mutations were found in the inoculum.

The non-synonymous/synonymous ratio for these mutations is 1.4, indicating that positive selection is driving the amino acid mutations. The amino acid mutations are listed in Table 6.5. We detected in total 11 amino acid mutations; 3 in PB2, 1 in PA, 6 in NP and 1 in NS1. Five of these 11 amino acid mutations are found independently in both experiments, of which 4 are present in the NP and 1 in PB2.

The maximum likelihood phylogenetic trees for experiment 1 is visualised in Figure 6.2 and for experiment 2 in Figure 6.3, The sequences of both experiments were divided, compared to the sequence of the inoculum, into 3 lineages. For experiment 1, the sequence of the inoculated bird was placed in the same lineage as the inoculum, but for experiment 2, the sequence of the inoculated bird, 2 days post inoculation, was placed in another lineage than the inoculum. The airborne infected birds of both experiments were placed in the same lineage but in different sublineages.

In experiment 1, three contact birds (C2, C6 and C20) were tested at two different time points with an interval of 2 days. Remarkably, C2 had mutations A180T, A183G, G190A on segment NP at day 4, which were not present in the sample collected from the same bird two days later. Similar, C6 had mutations A106G, C180T and A183G at day 4 which were not present at day 6, and C20 had mutations C1398T, T1399G, C1415T on segment NP and T1269A on segment PA at day 6 which were not present at day 8. Thus no obvious relation was found between both genotypes in these three birds.

Table 6.4. Intra- and interhost genetic variability of HPAI H5N1 virus in chickens of experiment 2.

		Frequency of mutation in inoculated (I), contact (C) or R(indirect contact) chickens day after inoculation							
segment	mutation	I6-day1	C2-day4	C9-day6	C18-day8	C21-day9	R4-day10	R10-day8	R14-day6
PB2	A138G	0	0	0	0	10	98	47	100
PB2	G1901A	0	30	0	0	100	0	0	0
PB1	A1047C	100	0	0	0	0	0	0	0
PA	G635A	0	0	0	0	39	0	100	0
PA	T1269A	96	83	30	14	84	100	97	39
NP	C180T	23	21	0	98	60	100	10	98
NP	A183G	21	19	9	99	5	100	100	100
NP	G190A	0	0	0	100	13	100	100	100
NP	C1398T	71	48	65	100	0	100	100	100
NP	T1399G	71	48	66	100	0	100	100	100
NP	C1415T	11	0	16	99	0	100	100	98
NA	G375A	0	0	0	0	0	59	0	100
NA	T1278C	0	100	100	98	0	88	100	10

In the first experiment, samples of 2 birds (R3 and R11) were analyzed from the group that became infected through air. In this experiment, R3 had 1 mutation that was also present in C14, R11 had 2 mutation (in PB2) that were also present in C15 (Table 6.3).

Table 6.3. Intra- and interhost genetic variability of HPAI H5N1 virus in chickens of experiment 1.

		Frequency of mutation in inoculated (I), contact (C) or indirect contact (R) chickens day after inoculation																			
segment	mutation	I7-day2	C2-day4	C2-day7	C4-day3	C6-day4	C6-day7	C8-day6	C9-day6	C13-day5	C14-day8	C15-day10	C20-day6	C20-day8	C21-day9	C23-day10	C24-day10	C30-day13	C39-day14	R3-day13	R11-day14
PB2	G178A	1	14	0	<1	14	0	<1	0	<1	0	100	0	<1	<1	<1	0	99	<1	<1	98
PB2	A1299G	0	0	0	0	0	0	0	99	0	0	0	0	0	0	0	0	0	0	0	0
PB2	C1901A	<1	0	0	<1	0	78	0	0	<1	100	98	94	100	<1	100	99	99	100	100	100
PB1	G381A	<1	0	0	<1	0	0	<1	0	<1	0	0	0	<1	99	<1	0	<1	<1	0	<1
PB1	T873C	<1	0	0	<1	0	0	<1	0	<1	0	0	0	<1	<1	<1	0	98	<1	<1	<1
PA	T1269A	0	75	84	0	99	92	0	13	0	0	0	99	0	0	0	16	0	0	0	0
NP	A106G	0	0	0	0	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP	C180T	0	100	0	0	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP	A183G	0	100	0	0	94	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP	G190A	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NP	G1207A	0	0	0	0	0	0	0	98	0	0	0	23	0	0	0	0	0	0	0	0
NP	C1398T	0	0	91	0	0	98	0	32	0	75	0	100	0	0	0	0	0	0	0	0
NP	T1399G	0	0	91	0	0	98	0	30	0	75	0	100	0	0	0	0	0	0	0	0
NP	C1415T	0	0	46	0	0	95	0	0	0	0	0	95	0	0	0	0	0	0	0	0
NA	T228C	<1	0	0	<1	0	0	<1	0	<1	0	0	0	<1	<1	<1	0	<1	100	<1	<1
NA	T273C	<1	0	0	<1	<1	0	<1	0	<1	0	0	0	<1	<1	<1	0	<1	100	<1	<1
NS1/2	G349A	<1	24	0	<1	0	0	<1	0	<1	0	42	0	<1	<1	<1	0	98	<1	<1	<1

In the second experiment, samples of 3 birds (R4, R10 and R14) from the indirectly exposed group were sequenced. These indirectly exposed birds exhibit 7-8 mutations compared to the sequence of the inoculum. Six mutations in R4 were also present in C18 whereas the mutation T1269 was present in 14% of the C18 reads. The silent mutation NA T1278C is present in 98% of the reads of C18 and in 88% of the reads of R4. Furthermore, 6 mutations present in R4, R10 and R14 were already present between 11% and 96% of the sequence reads of an inoculated bird (T1269A on PA, and C180T, A183G, C1398T, T1399G, C1415T on NP) sampled at day 1 p.i. The PB2 silent mutation, A138G, was present in 10% of the reads of C21 at day 9.

Table 6.5. The amino acid mutations compared to the inoculum per segment for both experiments. Bold positions are mutations that occurred in both experiments.

segment	Experiment 1	Experiment 2
PB2	D60N; **S634Y**	N46D; **S634Y**
PA		R222H
NP	I36V; **I61M; E64K**; A403T; **S467A; T472M**	**I61M; E64K; S467A; T472M**
NS1/NS2	A117T	

6.4. Discussion

This study describes the genetic variation of HPAI virus shortly upon introduction into two populations of chickens (n = 30 - 40) and showed that a high number of mutations can appear and disappear in a short period of time. Furthermore, the with-in host variation may exceed the between-host variation suggesting that host immunity drives the genetic variation of HPAI virus. The detectability of genetic differences in just a few days after virus introduction into a flock could be able to help resolve the order and timing of transmission events during an outbreak, although exact implications are yet unclear.

Within-host evolution remains the source of viral genetic diversity, and models that link epidemiological events and evolutionary dynamics can only be optimized if the within-host evolution is sufficiently understood. The exceptional high mutation rate of RNA viruses makes it possible to study viral evolution for the duration of an infection. Analysis of different samples taken from the same animal at different times post-infection helped studying the evolutionary dynamics in the host over very short periods of time.

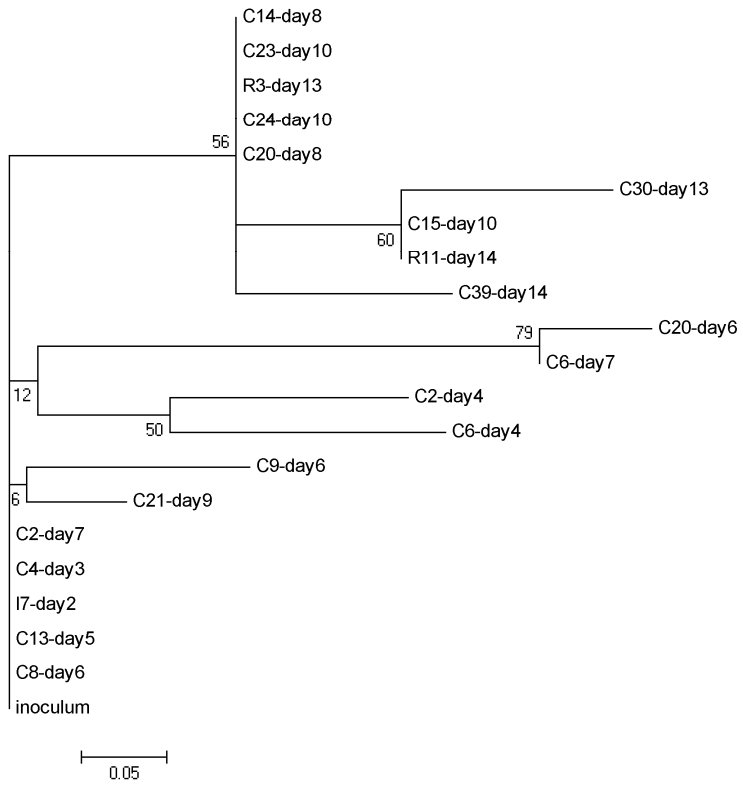

Figure 6.2. Molecular Phylogenetic analysis of experiment 1.

Figure 6.3. Molecular Phylogenetic analysis of experiment 2.

Remarkably, the phylogenetic tree placed different samples taken from the same animal at a two-day interval based on their mutations in different branches of

the phylogenetic tree, because mutations detected in the first sample could not be shown in the 2nd sample. This has also been described in a study on the genetic variation of Equine Influenza Virus within a population of horses, in which novel lineages bearing distinct antigenicity were observed to evolve within a single individual (Murcia et al., 2010). Besides by within-host evolution, our observation might be explained by super-infection of these birds by other circulating virus variants as the observed mutations also were shown in other birds from the population.

The number of different mutations found within-host was high compared to the observed between-host variation. This discrepancy between with-in and between-host rates has been shown for other viruses, as evolutionary rates are slower when measured at the epidemic level than when measured at the with-in host level (Lemey et al., 2006). Our data showed that chickens that shed virus with the highest number of emerged mutations compared to the inoculum (>15 based on the total number of mutations in the analysis) had a longer infectious period (4.2 days (95%: 3.5 – 4.9) compared to chickens that shed virus with less emerged mutations (<15) (2.6 days (95% C.I.: 2.1 – 3.0). Mutations in the NS1 segment may have an attenuating effect on the virus (Hale et al., 2008; Keiner et al., 2010), possibly leading to an increase in the mean time to death of the host. However, in our study only one amino acid mutation on this segment, and one that has not yet been described was observed.

The non-synonymous/synonymous (dN/dS) ratio for the mutations was estimated 1.4. The comparison of synonymous and non-synonymous substitution rates is the common approach to determine the existence of positive selection. A dN/dS ratio greater than one is a strong indication for selective pressure on protein level (Li et al., 2011). However, the absence of non-synonymous mutations in the HA nor NA segments suggests that the timescale was too short to introduce mutations in the surface glycoproteins. The NP segment has been shown to be involved in many aspects of viral replication (Portela and Digard, 2002), but the mechanism remains unclear. In experiment 1, 6 mutations in the NP segment emerged, and of these mutations 4 also emerged in experiment 2. However, none of these mutations have yet been described as being important for functions of NP nor to have an effect on viral fitness. Another mutation emerged in both experiments on the basic polymerase 2 (PB2) at amino acid position 634. The PB2 genes have been identified among

the most consistent host-range determinants in influenza viruses (Hatta et al., 2001; Shinya et al., 2004; Schat et al., 2012). Mutation at position 627, 7 amino acids upstream from 634 has been shown to determine polymerase activity. However the effect of mutation at position 634 has not yet been described. All samples from the airborne infected group in experiment 1 contained this mutation, but it was not detected in the airborne infected group from experiment 2, even though the mutation was found in one of the chickens from the source group. According to Russel et al. (2012) the majority of H5 viruses in clade 2.2 are only 3 nucleotide mutations away from becoming transmissible via respiratory droplets in mammals. None of these mutations were found in the sequences from the populations of chickens in our experiments although airborne transmission was observed. Possibly the mechanism of airborne transmission between chickens (and populations of chickens) is different from that of indirect transmission between mammals, and more dependable on the transmission of virus via dust (Spekreijse et al., 2012). Consequently, viruses that use dust as a mechanical vector might adapt differently from viruses that use droplets as a vector for transmission resulting in different mutations due to natural selection.

Studying the genetic variability of HPAI virus under controlled conditions is necessary to be able to improve the predictive value of molecular epidemiology in the field. By combining the results of the two transmission experiments we tried to identify by use of a maximum likelihood phylogenetic analysis the source of infection of both airborne infected groups (Figure 6.4).

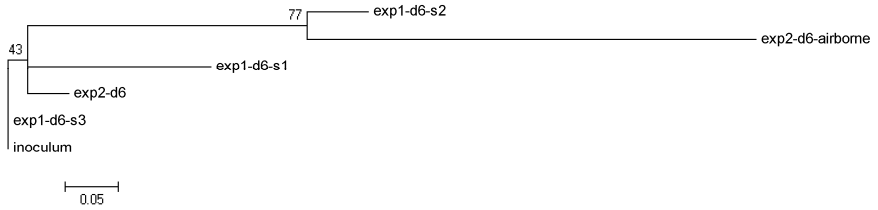

Figure 6.4. Molecular phylogenetic analyses by maximum likelihood method with bootstrap on a selection of samples from both experiments on day 6 after introduction of virus in both flocks.

However, the phylogenetic analyses failed to distinguish the inoculated groups of experiment 1 and experiment 2 as source group. An explanation could be that the reconstruction of the route of transmission was hampered because of missing data as not all samples were analysed.

Finally, understanding within-host and between host genetic variation will assist to develop molecular epidemiological models to reconstruct transmission routes after direct and indirect transmission of HPAI virus.

Chapter 7

General Discussion

7.1. Summary of the results

The research described in this thesis focused on airborne transmission of HPAI H5N1 in groups of chickens kept under experimental conditions. A dose-response study, carried out with pair-wise housed chickens, indicated how the probability of infection increased with increasing inoculation dose and showed that, once a bird was infected, the inoculation dose was neither associated with the amount of virus shed, nor with the rate of virus transmission to in-contact chickens. Airborne transmission was observed between chickens housed in the same room over distances up to 1.2 meters, the longest distance studied. The rate of airborne transmission was 20 times lower than the direct transmission rate measured in pair-wise housed chickens. Virus was detected in dust samples collected from the air and ground, and dust-borne transmission between groups of chickens housed in two adjacent different rooms connected by a tube was observed. Phylogenetic analysis of these experiments showed that within-host variation may exceed between-host variation. Within a single chicken different lineages can evolve and multiple lineages can be transmitted between hosts, hampering genotype based reconstruction of the infection pathways of HPAI virus. Furthermore, in vaccinated chickens that received only 1/3 of the recommended vaccine dose no virus could be detected upon challenge in environmental dust despite virus detection in tracheal swabs.

In this chapter results presented in previous chapters are integrated and implications and critical elements are discussed.

7.2. Dose response of highly pathogenic avian influenza virus

Knowledge of the relationship between the infectious virus dose and the probability of infection is relevant to determine the risk of infection of a poultry flock due to exposure to fomites or air originating from infected flocks. Figure 7.1 shows the dose response curve constructed from data of the trials described in Chapter 2. The median infective dose (ID_{50}), the dose that infects 50% of the birds after inoculation, for *A/turkey/ Turkey/1/2005 H5N1* was estimated $10^{2.5}$ EID_{50} (egg infectious dose) (95% C.I.: $10^{1.9}$ – $10^{2.8}$ EID_{50}). This value falls within the range of $10^{1.2}$ - $10^{4.7}$ as median infectious doses reported for other

HPAI H5N1 virus strains (Swayne and Slemons, 2008), suggesting that the findings of this thesis can be extrapolated to other strains.

The fitted dose response curve shows a minimum infective dose (MID) at $10^{1.4}$ EID_{50} (95% C.I.: $10^{0.5}$ – $10^{1.8}$ EID_{50}). The uncertainty around this value may be larger than indicated by the confidence interval, as the relatively low number of observations makes it difficult to estimate the shape of the dose–response curve at low doses.

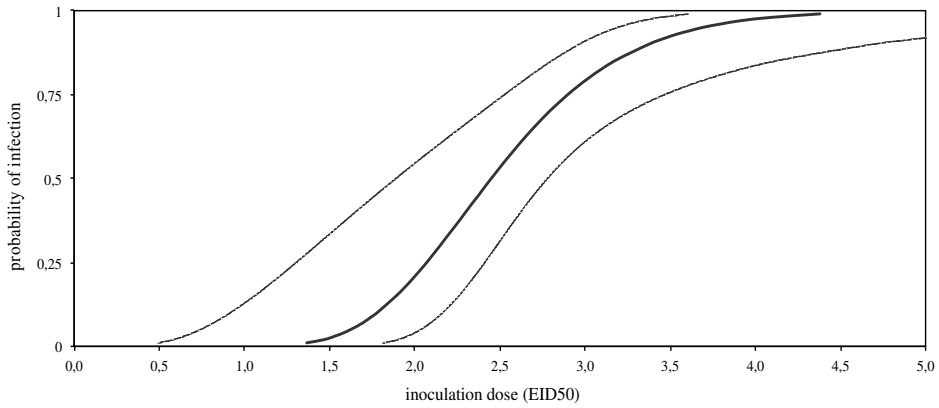

Figure 7.1. Dose response curve for A/turkey/ Turkey/1/2005 H5N1 based on Spekreijse et al.(2011) (grey lines represent 95% confidence interval)

It would be valuable to have a better estimate of the shape of the curve in that low dose region because low infection probabilities might be relevant in large poultry flocks. The reasoning is as follows. Suppose that each chicken in a flock of size n has a probability p to become infected by an amount of virus introduced into that flock. The probability that all chickens escape infection is $(1 - p)^n$ which implies that even when the probability of infection is very low, the probability of at least one chicken becoming infected can be high. For example, if the probability of infection were 0.0001 for each chicken, the probability of infection for a flock of size n = 20.000 would be close to 1. However, to estimate infection probabilities per chicken that low in experiments is unfeasible because of the high number of animals needed.

During outbreaks air samples have been collected and virus load has been quantified (Schoffield et al., 2005). It is however unclear how to interpret the virus load with respect to the probability of infection of a bird or flock. The

dose-response curve might facilitate interpretation of this quantitative measure. In the experiments, described in Chapter 4, airborne virus transmission occurred occasionally. The virus load in air samples was $10^{3.7}$ EID_{50}/m^3, which was considered to be the exposure load for chickens in the contact group. A chicken inhales on average 1.2 m^3 of air per day (Sedlemaier et al., 2009) implying that the expected cumulative exposure to virus of an individual chicken was $10^{3.8}$ EID_{50} per day. In the dose-response curve this reflects an infection probability of 0.96 per day. According to this reasoning one would have expected a higher transmission rate between the two rooms than was observed. An explanation could be that chickens were exposed to the above estimated dose of virus over a period of 24 hours, whereas in the challenge experiments chickens are inoculated (exposed) with the total amount of virus at one time. Exposure to small amounts of virus distributed over a longer time might influence the virus activation by the (innate) immune system resulting in an apparently higher resistance against infection than in case of exposure to the whole dose at once (Pujol et al., 2009; Marois et al., 2012).

7.3. Infectiousness of chickens

In poultry, replication of HPAI virus occurs in various tissues (MacLachlan and Dubovi, 2010). Tracheal and gut epithelium is considered to be of relevance for transmission from one host to another. Infections of avian influenza virus are therefore usually detected by examination of oropharyngeal, tracheal or cloacal swabs (OIE, 2008; Jindal et al., 2010; Van Dalen et al., 2010). The study described in Chapter 5 on direct transmission suggests, however, that virus presence does not necessarily reflect infectiousness of chickens accurately. In the study in Chapter 5, viable virus was detected in tracheal swabs, collected from vaccinated infected birds, but was not detected in cloacal swabs. Despite virus presence in the trachea, these chickens did not spread virus to cage mates. In clinically healthy birds respiratory fluid collected from the trachea possibly remains in the respiratory tract of the birds, or is swallowed, preventing infectious virus to be expelled into the environment. The occurrence of the latter was indicated by the absence of viral RNA in air and environmental dust samples. Consequently, in birds, virus load measured in swabs from the cloaca appears to reflect more accurately infectiousness than virus load measured in

samples from the trachea, as virus excreted from the gastro-intestinal tract is always deposited into the environment via faeces.

In Chapter 2, it was found that the amount of virus detected in infected chickens was not related to the initial inoculation dose, but only a reduction of latent period was seen upon inoculation with a high dose (>10^4 EID_{50}). The innate immune cells play an essential role in the control of infection through their ability to interfere with early viral replication (McGill et al., 2009). Most likely, when inoculated with a high dose, a large proportion of viral particles will escape the chicken's innate immunity, and subsequently, as virus replication and release of virus occurs within 6 hours after infection of the cell (Roizman and Palese, 1996) the number of copies released increases within 24 hours to 10^5 EID_{50}/ml (Spekreijse et al., 2011). For a low inoculation dose (<10^2 EID_{50}) only a small number of viral particles will escape this innate immunity response, fewer respiratory cells will be invaded and, consequently, more generations and thus more time is needed for the excretion to arrive at the above mentioned titer. Most likely the amount of virus produced after a low inoculation dose will be initially below the detection level of the test, resulting in a longer latent period. Although the latent period was extended for a low inoculation dose, no difference was found in the total amount of virus shed during the infectious period of an infected chicken. Furthermore, the initial inoculation dose had no influence on the probability of transmission of virus.

7.4. Extrapolating indirect transmission from experiment to field

In the study described in chapter 3 HPAI H5N1 virus transmission was observed between chickens housed in cages up to a distance of 1.2 m. Although this suggest the possibility of airborne transmission of HPAI virus infections, it is difficult to derive the rate of airborne transmission from these results to field conditions.

Boender et al. (2007) proposed the transmission kernel $h(r) = \frac{h_0}{1 + (r / r_0)^{\alpha}}$ to model spatial transmission of HPAI H7N7 virus between farms during the 2003 epidemic in The Netherlands. In this model *h(r)* is the probability of

infection at a distance *r* from an infected farm. Applying the data reported in chapter 3 to this model reveals the transmission kernel shown in Figure 7.2. It must be realized that in contrast to the transmission kernel presented by Boender et al. (2007) this kernel reflects the probability of infection from one infected chicken to another susceptible chicken at the specified distance instead of that from one infected flock to another susceptible flock.

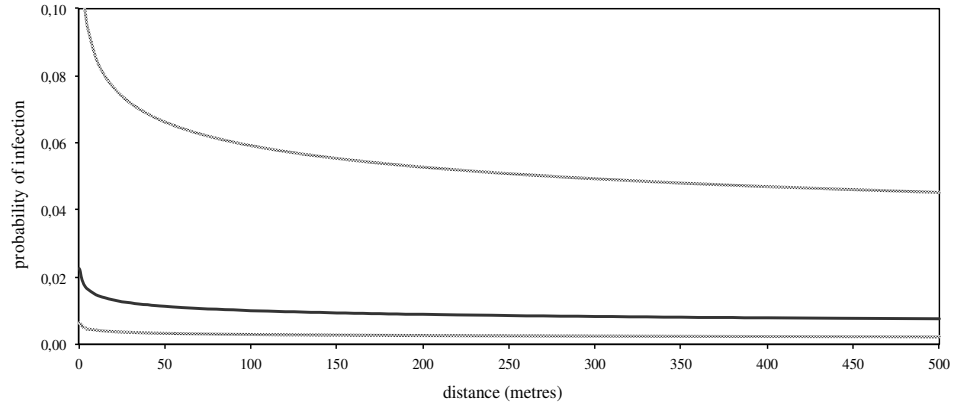

Figure 7.2. Transmission kernel based on data from Spekreijse et al. (2011). (grey lines represent 95% confidence intervals)

Bearing this in mind it is remarkable that the infection probabilities resulting from modeling results of the present study are strikingly higher than those reported by Boender et al. (2007). The probability of infection for a susceptible flock at a distance of 100 meters from an infected farm was according to Boender et al. 0.002/day (95% C.I.: 0.001 – 0.004), whereas the probability of infection of a susceptible chicken at a distance of 100 meters from an infected bird is 0.01/day (95% C.I.: 0.002 – 0.04). Several reasons for this difference can be hypothesized. First of all, the environmental barriers present between two farms may result in a completely different airflow between two neighboring farms than between two chickens in the same room. In addition, the estimates of the transmission kernel derived from the experiments were rather imprecise and, consequently, the probabilities might have been considerably lower. Moreover, the infection kernel associated with H5N1 might be different from the one associated with H7N7.

Knowledge of the contribution of individual transmission routes is important to improve measures to control HPAI epidemics. Moreover, transmission kernels can be used to derive critical farm densities, e.g. distinguish areas where HPAI outbreaks can be effectively controlled by traditional control methods and areas where such control may not be effective. Ssematimba et al. (2012) recently modeled data of HPAI H7N7 epidemic in The Netherlands in 2003, using a Gaussian plume model that incorporated windborne dispersal and deposition of viral dust, and Ypma et al. (in press) did a phylo-geographical analysis with information on wind directions. Both studies showed that the contribution of windborne spread to between-farm transmission would have been around 20%.

7.5. Infection chains and virus evolution

Because of the absence of a cellular proofreading mechanism, influenza viruses have a high error rate during replication of their viral RNA, and as viruses undergo an ongoing series of replication cycles when transmitted from host to host, genetic variants are continually generated and a cloud of genetically related but non-identical viruses will co-circulate in a flock (Suarez, 2000; Bataille et al., 2011). The genetic diversity enables the virus to adapt to the selection pressure of a new environment in order to optimize transmission (Suarez, 2000). In chapters 4 and 6, an experimental setting was used in which eight chickens were inoculated with HPAI H5N1 virus and placed within a group of six susceptible chickens. Next, up to day 10 post inoculation, deceased chickens were removed and replaced with new susceptible ones. In this way, an infection chain was created which enabled detailed observation of the virus evolution during the initial generations of infected birds in a flock.

In the field, virus evolution shortly upon introduction into a flock cannot be assessed, because at that time it is usually unknown that a flock is infected, and also virtually impossible to identify the infected birds. Consequently, only the dominant virus type that emerged during the outbreak will be identified, and not the way it has evolved in the flock during the first generations of infected birds. Knowledge of the latter is valuable to better understand changes necessary for cross species transmission or to enable tracing of transmission routes of viruses between flocks.

The results of whole genome sequencing reported in this thesis showed the emergence of HPAI H5N1 virus that contained a mutation on the basic polymerase 2 (PB2) at amino acid position 634, suggesting that this viral genotype had a selective advantage under the experimental conditions. The PB2 protein is a key component of the RNA transcription and replication process, and exerts an important role in determining virulence and host range (Machlachan and Dubovi, 2010). Table 7.1 shows a decrease in the length of the latent period of the contact-infected chickens in time that could have been associated with the emergence of the mutation at position 634, as it is positioned 7 amino acids downstream from the amino acid at position 627, that has been associated with enhanced replication of the virus after substitution (Schat et al., 2012). A shorter latent period will shorten the time between infected birds of sequential generations and, consequently, such a mutant will have a selective advantage in the population. However, the power of this study is too small to draw a statistically sound conclusion.

Table 7.1. Latent period based on the results of Spekreijse et al. (2012).

Chickens	days post inoculation	latent period (days)	95 % confidence interval
Inoculated chickens	0	1.3	0.9 – 1.6
contact chickens	0	2.3	1.5 – 3.2
contact chickens	2	2.0	2.0 – 2.0
contact chickens	5	1.8	1.3 – 2.2
contact chickens	9	1.2	0.8 – 1.6

The evolutionary study reported in this thesis has its limitations. First of all, restraints in the budget and the limited amount of virus present in a considerable number of the samples did not allow sequencing of all samples. Consequently, important information may have been lost. In addition, because of the study design, we do not know the infection chain beforehand. For that purpose a pair-wise design would have been more appropriate. However, in such a design timing of the replacement of deceased animals by new susceptible ones is much more critical than in the study reported in this thesis. The reason is that further transmission can only take place if the remaining bird is still excreting virus, but the length of the infectious period for HPAI is short and infected animals often

die within 2 days after becoming infected (e.g. Bouma et al., 2009). However, not all infected animals need to become infectious, moreover, even if a bird excretes virus, its susceptible cage mate could still escape from infection (van der Goot et al., 2003; Bouma et al., 2009), which asks for a large and costly experiment to end up with a reasonable number of infected birds in generations 4 and beyond.

7.6. Outbreaks in vaccinated populations

Vaccination can be successful in the (local) eradication of infectious animal diseases as has been demonstrated for several diseases, e.g. rinderpest (Roeder et al., 2004), foot-and-mouth disease (Parida, 2009), classical swine fever (Van de Putte and Chappuis, 1999), and Aujeszky's disease (Stegeman, 1995). In western countries usually outbreaks of HPAI are controlled by stamping out infected and contiguous flocks and vaccination is not applied. The economic and social impact of both the disease and this control strategy is huge, as usually far more flocks are culled than are actually infected (Stegeman et al., 2004; Tiensin et al., 2005; Marangon and Capua, 2006).
In Indonesia, Vietnam and China, stamping out has not been applied extensively because of lack of resources, the socio-economic impact and with respect to securing food supplies (Capua and Marangon, 2007). In these countries vaccination is used as a control measure (OIE, 2012), and the major goal of the vaccination campaigns is to prevent clinical HPAI, associated production losses and reduction of human exposure rather than eradication.

Despite vaccination outbreaks in vaccinated flocks in these countries still occur (Lee et al., 2004; Bouma et al., 2008; Abdelwab et al., 2011). Several reasons could account for the persistence of infection in the population. First, vaccination coverage may not be high enough; the proportion of vaccinated birds needs to be above a threshold to prevent major outbreaks. This threshold equals $1 - 1/R_0$, where R_0 is the basic reproduction ratio (average number of secondary infections caused by one infectious individual in a susceptible population) (Anderson & May, 1985). According to Tiensin et al. (2007), R_0 of H5N1 infections varied between 2.26 and 2.64 in Thai poultry types, leading to an estimate that at least 60% of the population should be vaccinated (and 80% if it were to be based on the upper confidence levels of the above mentioned

values of R_0). In the four countries that account for 99% of vaccination use (China, Egypt, Indonesia and Vietnam) the vaccination coverage rate varies between 14% for Indonesia (2004 to 2010), 55% for China (2004 to 2009), 58% for Vietnam (2005 to 2009), and 67% for Egypt (2006 to 2009), meaning that the coverage rate in most countries is below the minimal coverage percentage (Swayne et al., 2011).

The estimates given above assume perfect vaccines for which the R_0 in a fully vaccinated population is zero. The ability of vaccines to reduce transmission can be determined experimentally by comparing virus transmission between vaccinated and unvaccinated animals in a controlled environment. Several of such transmission experiments have been carried out for HPAI; examples are van der Goot et al. (2005), Bos et al. (2008), Poetri et al. (2009), and Swayne et al. (2000). These studies provide useful information on vaccine efficacy, but the extrapolation of the results to the field is not always straightforward. In experimental settings, although not in all species (van der Goot et al., 2007), most vaccines and vaccine strategies are highly effective. The HI titers induced are rather high, and the coverage is usually 100%. For poultry vaccinated in the field, however, this appears different (Lee et al., 2004; Bouma et al., 2008; Rudolf et al., 2009, 2010; Abdelwab et al., 2011). Vaccination efficacy is influenced by several factors including co-infections, antigenic relatedness between vaccine and field strains, host species, age and chicken breed. These factors may result in reduced vaccination efficacy and subsequent spread of HPAI virus in vaccinated populations (Lee et al., 2004; Bouma et al., 2008; Rudolf et al., 2010).

The studies described in Chapter 5 aimed at investigating the effect of insufficient vaccination on direct and indirect transmission of HPAI H5N1 virus. In an attempt to create insufficiently protected vaccinated birds, chickens were vaccinated with only 1/3 of the dose recommended by the manufacturer. Although the subsequently acquired level of immunity did not protect the chickens from infection upon inoculation, infectiousness was reduced to such a level that even unvaccinated contact birds did not become infected, and virus could neither be detected in the environment, nor in air samples. The average HI titers found in the vaccinated birds two weeks after vaccination (and 1 day before inoculation) were $3.7\log_2$ against the vaccine antigen (95% C.I.: 3.2 – 4.1) and was below a level of the so-called and as such considered solid state

immunity of $5\log_2$ (Kumar et al., 2007). Nevertheless, the level of immunity was sufficient to eliminate fecal excretion of the virus and reduce overall excretion to an extent that naïve in-contact chickens could not be infected. The average HI titers found in the vaccinated birds three weeks after vaccination (and one week post-inoculation) were $7.9\log_2$ (95% C.I.: 7.2 – 8.5) suggesting that the titers measured before inoculation were still increasing due to vaccination and sufficient to create a solid state immunity. Apparently, the lower vaccine dose was not the appropriate way to create chickens that can silently spread virus. Other ways to achieve this might be the application of a shorter period between vaccination and inoculation, comparable to a field situation with emergency vaccination, or birds should be vaccinated with a dose even lower than used in the experimental set-up.

7.7. Concluding remarks

The experiments described in this thesis demonstrate that the inoculation dose of HPAI virus determines the probability of infection but does not influence the amount of virus excreted after infection nor does it influence subsequent transmission. Moreover, it was shown that airborne transmission is a possible causal pathway in the transmission of HPAI virus between poultry and dust can act as a mechanical vector of the virus.

Although avian influenza virus replicates extensively within the respiratory tract, for vaccinated chickens this excretion appears not to result in transmission. Finally, phylogenetic studies used to reconstruct indirect transmission pathways during an outbreak may be hampered by the large genetic variation of the virus than can arise even within a single host.

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Nederlandse samenvatting

Hoogpathogene aviaire influenza (HPAI), ook wel vogelgriep genoemd, is een ernstige luchtwegaandoening van kippen en ander pluimvee, die gepaard gaat met zeer hoge mortaliteit. De ziekte wordt veroorzaakt door Influenza A virus van de subtypen H5 en H7. In de afgelopen decennia hebben zich wereldwijd verschillende HPAI epidemieën voorgedaan, met de nog steeds actieve H5N1 pandemie als belangrijkste exponent. De bestrijding van HPAI is vaak moeilijk en gaat gepaard met hoge kosten; in pluimvee-dichte gebieden kan het virus slechts worden gestopt door stringente zoösanitaire maatregelen, waaronder het ruimen van besmette pluimveekoppels en koppels blootgesteld aan het virus. Om die reden, en vanwege de risico's voor de volksgezondheid van een vogelgriep uitbraak, is het noodzakelijk tot betere bestrijdingsmaatregelen te komen. Beter begrip van de overdrachtsroutes van het virus is daarvoor de eerste stap.

Aanvoer van besmette kippen is de efficiëntste manier om HPAI virus een pluimveebedrijf binnen te brengen, maar het virus kan ook worden geïntroduceerd door personen en voertuigen afkomstig van een besmet bedrijf. Dergelijke persoons- en vervoerscontacten verklaarden echter maar een klein deel van alle infecties die plaatsvonden tijdens de HPAI H7N7 epidemie in 2003 in Nederland. Mogelijk is dit het gevolg van onderrapportage van contacten, maar wellicht was er ook overdracht via contacten die niet kunnen worden vastgelegd tijdens een bestrijdingscampagne, zoals contacten via de lucht. Bij humane influenza is transmissie door middel van aerosolen een bekende route. Door de afwijkende anatomie van de respiratietractus en het incomplete middenrif verloopt het proces van aerosolisering van influenzavirus bij vogels waarschijnlijk anders dan bij zoogdieren. Echter, in pluimveestallen wordt veel stof geproduceerd en gekoppeld aan stof zou het virus door mechanische ventilatie in de omgeving kunnen worden geblazen zodat andere koppels pluimvee aan virus worden blootgesteld.

Het experimentele onderzoek beschreven in dit proefschrift is uitgevoerd om meer inzicht te krijgen in de aerogene verspreiding van HPAI virus. Onder praktijkomstandigheden is het erg moeilijk om de causaliteit van aerogene transmissie van HPAI virus aan te tonen. Tijdens een uitbraak ligt de nadruk op het zo snel mogelijk bestrijden van de ziekte en niet op onderzoek. Daarnaast wordt onderzoek tijdens een uitbraak gehinderd door bias (bijvoorbeeld de

eerder genoemde onderrapportage), onzekerheid (bijvoorbeeld het moment van virus insleep) en confounding.

Eerst werd bestudeerd hoe de virustransmissie tussen kippen in direct contact afhing van de hoeveelheid virus waarmee de infectieketen werd opgewekt. Hiervoor werden twee transmissie-experimenten uitgevoerd waarbij kippen in paartjes werden gehuisvest. In elk paartje werd een kip besmet met HPAI H5N1 virus H5N1 (A/turkey/Turkey/1/2005), waarbij verschillende doses werden gebruikt. De infectieuze dosis waarmee 50% van de kippen kan worden geïnfecteerd (CID_{50}) bleek $10^{2.5}$ ei infectieuze doses (EID_{50}). Toename van de infectiedosis vergrootte de kans op infectie en verkorte de latente periode (de periode tussen infectie en virusuitscheiding), maar was niet van invloed op de kans om infectie te overleven. Ook bleek de totale hoeveelheid virus die werd gemeten in trachea en cloaca afhankelijk van de inoculatiedosis, maar was de kans op virusoverdracht niet significant verschillend tussen de diverse dosisgroepen. De hoeveelheid virusuitscheiding bleek in dit experiment dus geen goede manier om de transmissie van virus te voorspellen.

Vervolgens werd de aerogene overdracht van een HPAI H5N1-virusstam tussen kippen gekwantificeerd en werd de hoeveelheid virus in lucht- en stofmonsters vastgesteld. Zestien experimenten, met in totaal 160 kippen gehuisvest in kooien, werden uitgevoerd. Binnen een experiment werden de kippen verdeeld over drie behandelingsgroepen. De eerste groep werd besmet met stam H5N1 A/turkey/Turkey/1/2005, de tweede en derde groep werden niet geïnoculeerd, maar gehuisvest op een afstand van respectievelijk 0.2 en 1.1 meter van de eerste groep. Dagelijks werden de trachea en cloaca van de kippen met een swab bemonsterd en werden stofmonsters uit de lucht en van het hok oppervlak in de directe omgeving van de kippen verzameld. De monsters werden onderzocht op de aanwezigheid van virus met behulp van RT-qPCR (viraal RNA) en virusisolatie (levensvatbaar virus). In 4 van de 16 experimenten werd virusoverdracht van de experimenteel besmette kippen naar de kippen op 0.2 of 1.1 meter vastgesteld. De kans op overdracht werd berekend op 0.13 en 0.10 infecties per infectieuze kip op 0.2 en 1.1 meter, respectievelijk. Het verschil tussen beide schattingen was echter niet significant. Twee stofmonsters uit de lucht bleken positief in virusisolatie, maar geen van deze monsters was afkomstig uit de experimenten waar de transmissie succesvol was. Tevens bleken 5 oppervlaktestofmonsters positief in virusisolatie. De resultaten van

deze studie tonen aan dat aerogene transmissie tussen kippen over korte afstanden mogelijk is, maar de kans op transmissie betrekkelijk laag.

In een vervolgstudie werd de aerogene transmissie van virus tussen koppels kippen gehuisvest in verschillende ruimtes onderzocht. Vier experimenten werden uitgevoerd, elk met twee groepen van 14 kippen ondergebracht in twee aparte ruimtes. Binnen een experiment werden de kippen in een van de twee groepen besmet met H5N1 A/turkey/Turkey/1/2005. Alle lucht uit de ruimte van deze kippen werd via een buis naar de ruimte met de tweede groep kippen geventileerd. Tot dag 10 na de start van het experiment werden gestorven kippen in de groep geïnoculeerde kippen vervangen door nieuwe vatbare kippen. Dagelijks werden de trachea en cloaca van de kippen geswabt voor onderzoek op virus om transmissie van virus vast te kunnen stellen. Virusoverdracht van de groep met geïnoculeerde kippen naar de groep met niet geïnoculeerde kippen deed zich voor in twee experimenten. De kans op overdracht voor de transmissie via de lucht werd geschat op 0.08 infecties per besmettelijke kip per dag. De hoeveelheid virus gedetecteerd in stofmonsters in de ruimte van de groepen niet geïnoculeerde kippen was gemiddeld $10^{3.7}$ EID_{50} per m^3.

Met een deel van de monsters van deze experimenten werd een phylogenetische studie met next generation sequencing uitgevoerd om vast te stellen of de transmissie van virus tussen de kippen en tussen de groepen gevolgd kon worden. Het bleek echter niet mogelijk het infectie-pad te reconstrueren. De belangrijkste reden hiervoor was de grote genetische variatie die al binnen een enkele gastheer ontstaat; verschillende virus lineages werden waargenomen binnen een enkele kip en meerdere virus lineages bleken te kunnen worden verspreid tussen kippen.

Vaccinatie is een van de maatregelen die kunnen worden gebruikt om uitbraken van HPAI bij kippen te bestrijden. Er bestaat echter twijfel of altijd wel goed genoeg wordt gevaccineerd, bijvoorbeeld als ze een lagere dosis vaccin dan aanbevolen krijgen. Een dergelijke suboptimale vaccinatie zou tot suboptimale immuniteit en mogelijk tot onzichtbare virusverspreiding kunnen leiden. Om dit te onderzoeken werden 12 groepen van elk 10 kippen gevormd. In negen groepen werden 5 van de 10 kippen gevaccineerd, de overige 3 groepen dienden als controle, er werd geen enkele kip gevaccineerd. Twee weken na vaccinatie werden de vijf gevaccineerde dieren geïnoculeerd met

HPAI H5N1-virus en geplaatst bij de 5 ongevaccineerde kippen. In de drie controlegroepen werden 5 ongevaccineerde kippen geïnoculeerd en geplaatst bij de andere 5 dieren. Dagelijks werden trachea- en cloacaswabs verzameld van elke kip, evenals stofmonsters uit de omgeving (lucht en oppervlak). In tegenstelling tot de ongevaccineerde controle kippen, vertoonde geen van de gevaccineerde kippen enig klinisch symptoom na inoculatie. Wel werd in de tracheaswabs van de gevaccineerde dieren virus aangetoond, in RT-qPCR en virusisolatie; alle cloacaswabs van deze dieren bleven echter negatief. De hoeveelheid virus in de tracheaswabs was $10^{3,1}$ EID_{50} in de groep gevaccineerd met 1/3 dosis, $10^{2,5}$ in de groep gevaccineerd met 2/3 dosis en $10^{5,6}$ EID_{50} in de controlegroep. Ondanks de virusuitscheiding via de trachea raakten geen van de ongevaccineerde contact kippen in de gevaccineerde groep besmet, terwijl alle contacten in de controle groepen besmet raakten. In tegenstelling tot de controle groep kon met RT-qPCR geen virus worden aangetoond in stofmonsters afkomstig uit de lucht en van de wand van het hok waarin de gevaccineerde groepen verbleven, Deze studie suggereert dat uitscheiding van virus via de cloaca belangrijk is voor de virusoverdracht en de afzetting van virus in het milieu.

De studies beschreven in dit proefschrift laten zien dat de inoculatiedosis van HPAI virus de kans op infectie bepaalt, maar de hoeveelheid virus uitgescheiden na infectie, noch de transmissie na infectie beïnvloed. Bovendien werd aangetoond dat aerogene transmissie een causaal overdrachtsmechanisme van HPAI virus tussen pluimvee is en dat stof kan optreden als mechanische vector van het virus. Hoewel vogelgriepvirus goed kan repliceren in de luchtwegen van suboptimaal gevaccineerde kippen hoeft uitscheiding niet te leiden tot transmissie. Ten slotte worden phylogenetische studies om indirecte transmissieroutes te reconstrueren tijdens een uitbraak bemoeilijkt door de grote genetische variatie van het virus die voorkomt in een koppel kippen en zelfs al in een individuele kip.

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Curriculum vitae

Dieuwertje Spekreijse werd op 28 September 1974 geboren in Amsterdam. In 1994 behaalde zij het VWO diploma aan het Commenius College in Hilversum. Ondanks toelating tot de officiersopleiding aan de Koninklijke Militaire Academie, en een jaar Psychologie aan de Universiteit van Amsterdam, trad zij uiteindelijk in 1997 in dienst bij de Vrije Universiteit medisch centrum als laborant Klinische Neurofysiologie dat zij in 2001 beëindigde om te starten met de opleiding Diergeneeskunde aan de Universiteit Utrecht. Na het behalen van het doctoraal Diergeneeskunde in 2006 trad zij in dienst bij het RIVM waar zij als beleidsmedewerkster werkte aan de microbiologische veiligheid van het Nederlandse drinkwater. In 2008 begon ze aan haar promotieonderzoek aan de faculteit Diergeneeskunde van de Universiteit Utrecht waarbij het experimentele werk werd uitgevoerd op het Centraal Veterinair Instituut. Dit proefschrift is daar het resultaat van. Naast haar promotieonderzoek heeft zij in 2010 eveneens haar dierenartsexamen, met differentiatie veterinair wetenschappelijk onderzoek, behaald. Vanaf augustus 2012 werkt zij als onderzoeker veterinaire infectieuze ziekten op het Centraal Veterinair Instituut, onderdeel van Wageningen UR.

List of Publications

Publications and manuscripts printed in this thesis

Spekreijse, D., Bouma, A., Stegeman, J.A., Koch, G., de Jong, M.C.M., 2011. The effect of inoculation dose of a highly pathogenic avian influenza virus strain H5N1 on the infectiousness of chickens. Veterinary Microbiology 147. 59 – 66.

Spekreijse, D., Bouma, A., Koch, G., Stegeman, J.A., 2011. Airborne transmission of a highly pathogenic avian influenza virus strain H5N1 between groups of chickens quantified in an experimental setting. Veterinary Microbiology 152. 88 – 95.

Spekreijse, D., Bouma, A., Koch, G., and Stegeman, J.A., 2012. Quantification of dust-borne transmission of highly pathogenic avian influenza between chickens. Influenza and other respiratory viruses. doi: 10.1111/j.1750-2659.2012.00362.x

Spekreijse, D., Bouma, A., Koch, G., Stegeman, J.A.. Vaccination with 1/3 dose of a H5N9 vaccine prevents cloacal shedding and transmission of Highly Pathogenic Avian Influenza H5N1 virus upon experimental infection of chickens. Submitted.

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Conference contributions

Proceedings of 7th International Symposium on Avian Influenza: Avian Influenza in Poultry and Wild Birds. The University of Georgia, Athens, Georgia, USA. Association between inoculation dose, infection and excretion of Highly Pathogenic Avian Influenza Virus Strain H5N1 in chickens. Spekreijse, D., Stegeman, A., de Jong, M.C.M., Bouma, A. and Koch, G. [Poster presentation]

Airborne transmission of a HPAI H5N1 between groups of chickens quantified in an experimental setting. Spekreijse, D., Bouma, A., Koch, G., Stegeman, J.A.. 5th Annual meeting EPIZONE 2011 (Arnhem, The Netherlands). [Poster presentation]

Dust related transmission of a highly pathogenic avian influenza virus strain H5N1 between groups of chickens. Spekreijse, D., Bouma., A., Koch, G., Stegeman, J.A. 4th Oxford Influenza 2011 (Oxford, UK). [Oral presentation]

8th International Symposium on Avian Influenza, University of Londen, UK. HPAI H5N1 virus from suboptimally vaccinated chickens was not transmitted to unvaccinated pen mates and not detected in environmental dust. Spekreijse, D., Bouma, A., Koch, G., Stegeman, J.A. [Oral presentation]

HPAI H5N1 virus from suboptimally vaccinated chickens was not transmitted to unvaccinated pen mates and not detected in environmental dust. Spekreijse, D., Bouma, A., Koch, G., Stegeman, J.A. 12th ISVEE 2012 (Maastricht, The Netherlands). [Oral presentation]