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**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 done, 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 RRT-PCR 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 1.1 m, 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.

Keywords: Avian Influenza; H5N1; HPAI; transmission; chicken; airborne.

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 AI 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 AI 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.

2. Materials en methods

2.1. Animals

Embryonated eggs from White Leghorn chickens were purchased from a commercial 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 AI 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*.

2.2. Experimental design

Two experiments were carried out, each in 4 isolation rooms under BSL3+ conditions at the CVI. The lay-out of the rooms of the first experiment is shown in Figure 1, of the second experiment in Figure 2. The volume of the rooms was 22m³, which were ventilated 0.8/h. The temperature was kept at 20°C 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 1x0.5m². 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.

The distance between the first and second cage was 0.2m and between the second and third cage 0.4m. 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.

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; Spekrijse et al., 2011). The virus was grown in embryonated SPF eggs and vials with a known egg infectious dose (EID₅₀) 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 dose of 10⁴ EID₅₀. In both experiments, the chickens were inoculated with 0.1 ml inoculum applied intra-nasally and 0.1 ml inoculum applied intra-tracheally using a blunt needle, according to standard protocol (van der Goot et al., 2005; Spekrijse et al., 2011).

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 10³ IU of penicillin-sodium and 5 mg 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. The samples were stored at -20°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 8m³/h for 10 minutes, according to the manual of the manufacturer¹. 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 10³ IU of penicillin-sodium and 5 mg streptomycin per ml at a temperature of 37°C. Dissolved filter solutions were stored at -70°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 10 ml of 2.95% TPB with 5 x 10³ IU of penicillin-sodium and 5 mg streptomycin per ml and stored at -70°C until analysed.

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

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

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

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. We used 5µl 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 EID₅₀ 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 (C_t-value) against known virus titers. Titers of the samples were expressed as EID₅₀ equivalents.

2.6. Laboratory tests

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 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 0.2 ml swab fluid per egg. After 72 h 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.

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 1 ml of 10^3 , 10^4 or 10^5 EID₅₀/ml. Two gelatine filters per dose were used. The first filter per dose was dissolved in 9 ml of PBS (at 37 degrees) after 10 minutes of incubation. The second filter per dose was dissolved in 9 ml 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.

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. Results

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 $10^{3.5}$ EID₅₀ (95% C.I.: $10^{2.5} - 10^{4.5}$ EID₅₀), and on day 2 p.i., $10^{5.3}$ EID₅₀ (95% C.I.: $10^{5.2} - 10^{5.5}$ EID₅₀).

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.: $10^{3.8} - 10^{4.8}$ EID₅₀), and on day 2 p.i., $10^{6.3}$ EID₅₀ (95% C.I.: $10^{5.3} - 10^{6.9}$ EID₅₀). The mean latent period, the mean infectious period and the total amount of virus shedding did not differ significantly between the two experiments.

3.2. Infection of non-inoculated chickens

None of the non-inoculated chickens had pre-existing antibodies against AI. 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.

3.3. Virus detection in air and dust samples

No effect of the gelatine filters was seen on the concentration of HPAI virus (Table 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 $10^{1.6}$ EID₅₀, and on day 3 p.i., one air sample tested $10^{1.3}$ EID₅₀. Three dust samples tested positive in RT-qPCR and virus isolation. On day 1 p.i., one dust sample tested $10^{1.6}$ EID₅₀, and on day 2 p.i., one dust sample tested $10^{1.7}$ EID₅₀, and on day 4 p.i., one dust sample tested $10^{2.4}$ EID₅₀.

In the second experiment, on day 2 and day 3 dust samples tested positive in RT-qPCR and virus isolation ($10^{3.9}$ and $10^{2.2}$ EID₅₀, respectively), but none of the air samples tested positive.

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 ($p < 0.05$).

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 ($\beta = 1.43/\text{day}$). 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 seems 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, and whether or not this applies to AI 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.

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1 *Table 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

2

3

Figure 1. Lay-out of the isolation room of the first experiment. ● represents the inoculated chickens; ○ represents the non-inoculated chickens; ⊕ represents the location of air sampling; ⊙ represents the location of dust sampling.

Figure 2. Lay-out of the isolation room of the second experiment. ● represents the inoculated chickens; ○ represents the non-inoculated chickens; ⊕ represents the location of air sampling; ⊙ represents the location of dust sampling.

Figure 3. 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.

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