Transmission of Avian Influenza (H7N7) in vaccinated and unvaccinated pheasants (*Chrysolophus pictus*) and ducks (*Callonetta leucophrys*)

Transmissie van hoogpathogeen aviair influenzavirus in gevaccineerde en ongevaccineerde goudfazanten en roodschoudertalingen

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1. Nederlandstalige samenvatting (Dutch summary)

Achtergrond

In 2003 heeft in Nederland een grote uitbraak plaatsgevonden van een hoogpathogeen aviair influenzavirus (subtype H7N7). De epidemie heeft aanzienlijke economische verliezen tot gevolg gehad, en de praktijk van grootschalige ruimingen heeft geleid tot fundamentele ethische vragen. Naast de economische verliezen en ethische vragen heeft de epidemie ook aanzienlijke negatieve sociale gevolgen gehad, niet alleen voor de commerciële pluimveehouders als ook voor meer dan 17000 houders van niet-commercieel gehouden vogels.

Het is de vraag of de praktijk van het ruimen van alle vogelsoorten die mogelijk gevoelig zijn voor infectie met hoogpathogeen aviair influenzavirus vanuit epidemiologisch oogpunt effectief is geweest. Immers, van de meeste vogelsoorten staat nog geenszins vast of ze ook werkelijk kunnen worden geïnfecteerd door de verschillende hoogpathogene influenza virussen. Bovendien is het ook niet duidelijk of -indien infectie mogelijk is- het virus effectief kan worden doorgegeven aan niet-geïnfecteerde dieren.

Naast het ruimen van dieren is vaccinatie in theorie een aantrekkelijk alternatief om de verspreiding van hoogpathogeen aviair influenzavirus tegen te gaan. Echter, er is ook zeer weinig bekend over de effectiviteit van vaccinatie in verschillende diersoorten. Het is bijvoorbeeld niet duidelijk of gevaccineerde vogels nog kunnen worden geïnfecteerd, en of ze vervolgens dragers van het virus kunnen worden. Deze onzekerheid vormt een belangrijk bezwaar waar rekening mee moet worden gehouden bij het inzetten van vaccinatie als controlestrategie. De Europese Unie noemt deze onzekerheid zelfs het grootste bezwaar van vaccinatie, en heeft strenge regels opgesteld met betrekking tot vaccinatie voor lidstaten waar een epidemie van hoogpathogene aviaire influenza heerst. Zo moet in een uitbraaksituatie met behulp van testen het verschil kunnen worden aangetoond tussen infectie en vaccinatie. Alleen als aan deze voorwaarde wordt voldaan en de test laat zien dat er werkelijk geen virus meer circuleert, is het mogelijk dat de grenzen binnen afzienbare tijd na het einde van een epidemie weer worden opengesteld voor de export. Hoewel deze problemen het meest naar voren komen bij commercieel gehouden pluimvee zullen vergelijkbare problemen een rol gaan spelen bij een vaccinatieprogramma van niet-commercieel gehouden vogels.

Dit onderzoek is er op gericht om de vraag te beantwoorden of vaccinatie van niet-gedomesticeerde, hobbymatig gehouden watervogels en fazanten vanuit epidemiologisch oogpunt een effectieve controlestrategie zou kunnen zijn bij het voorkómen van infectie met hoogpathogeen aviair influenzavirus, en bij het voorkómen van het spreiden van virus van dier tot dier.

Vraagstelling

De vragen die in deze studie zijn onderzocht zijn de volgende:

- Kunnen goudfazanten en roodschoudertalingen worden besmet met hoogpathogeen H7N7 virus?
- Zijn er klinische symptomen te zien bij besmette goudfazanten en roodschoudertalingen?
- Kunnen besmette goudfazanten en roodschoudertalingen hoogpathogeen H7N7 virus efficiënt doorgeven aan niet-besmette soortgenoten?
- Beschermt vaccinatie goudfazanten en roodschoudertalingen tegen ziekte en sterfte veroorzaakt door infectie met hoogpathogeen H7N7 virus?
- Beschermt vaccinatie goudfazanten en roodschoudertalingen tegen infectie en verspreiding van hoogpathogeen H7N7 virus?

Om deze vragen te beantwoorden zijn in dit onderzoek transmissie-experimenten uitgevoerd. In een transmissie-experiment wordt een aantal kunstmatig geïnfecteerde dieren in een ruimte geplaatst met een aantal niet-geïnfecteerde dieren (contactdieren). Van alle dieren wordt dagelijks een monster genomen uit de trachea en uit de cloaca. Vervolgens wordt door middel van virusisolatie technieken bepaald welke dieren zijn geïnfecteerd. Het feit dat met deze opzet de hele transmissieketen in detail kan worden geobserveerd betekent dat er met een relatief klein aantal proefdieren al relatief sterke uitspraken kunnen worden gedaan over de verspreiding van influenzavirus in ongevaccineerde en gevaccineerde populaties. In het bijzonder kunnen er sterke uitspraken worden gedaan over het effect van vaccinatie op het inperken van transmissie van het virus.

Goudfazant

Roodschoudertaling

Resultaten

De uitkomsten van de experimenten zijn weergegeven in de Figuren 1-4 van het rapport. Tabellen 2 en 3 geven een overzicht van de statistische analyses.

Ongevaccineerde eenden: Figuur 1 laat zien dat alle kunstmatig besmette eenden vanaf dag 1 na de experimentele besmetting (inoculatie) positief zijn in de cloaca. De meerderheid is bovendien ook positief in de trachea. Daarnaast zijn alle contactdieren vanaf dag 2 ook positief. Dit geeft een indicatie dat het virus zeer snel spreidt in ongevaccineerde eenden. Zowel de geïnoculeerde dieren als de contactdieren zijn bovendien meer dan vier dagen besmettelijk. De statistische analyse laat zien dat het reproductiegetal (dat aangeeft of het virus kan spreiden in een populatie van ongevaccineerde eenden) significant groter is dan 1. Dit betekent dat het virus zich makkelijk verspreidt en dat een grote uitbraak waarschijnlijk is na introductie van virus in een populatie van eenden. Ondanks dat het virus eenden kan infecteren en goed kan spreiden, zijn gedurende het experiment bij geen van de eenden klinische verschijnselen waargenomen.

Gevaccineerde eenden: Figuur 2 geeft de resultaten voor de transmissie-experimenten met gevaccineerde eenden. Hier is het beeld geheel anders dan bij de ongevaccineerde eenden: Slechts zeven van de tien geïnoculeerde dieren wordt ook werkelijk positief. Bovendien zijn deze dieren doorgaans slechts één dag positief in de trachea. Van de contactdieren is geen enkel dier positief geworden. Dit duidt er op dat het virus niet meer kan spreiden in een gevaccineerde populatie. De statistische analyse laat zien dat dit inderdaad het geval is: het reproductiegetal is significant kleiner dan 1. Bovendien blijkt uit de analyse dat er een duidelijk verschil is tussen verspreiding van het virus in gevaccineerde versus ongevaccineerde groepen eenden.

Ongevaccineerde fazanten: Wat betreft transmissie lijken de resultaten in ongevaccineerde fazanten (Figuur 3) enigszins op de resultaten met ongevaccineerde eenden: De infectie slaat aan in alle geïnoculeerde dieren en het virus wordt efficiënt doorgegeven aan de contactdieren. Alle dieren zijn meerdere dagen infectieus, zowel in de trachea als in de cloaca. De statistische analyses bevestigen dat het reproductiegetal in ongevaccineerde fazanten groter is dan 1 en dat een grote uitbraak mogelijk is na een introductie. Er is echter ook een belangrijk verschil met de ongevaccineerde eenden: Waar bij de ongevaccineerde eenden geen tekenen van klinische ziekte zijn waargenomen zijn alle ongevaccineerde fazanten ernstig ziek geworden. Uiteindelijk zijn twaalf van de 20 fazanten voor het eind van het experiment overleden.

Gevaccineerde fazanten: De resultaten van de experimenten met gevaccineerde fazanten (Figuur 4) zijn het meest opvallend. Alle geïnoculeerde dieren zijn besmet geraakt en het virus wordt efficiënt doorgegeven aan de contactdieren. Dit geeft aan dat het virus nog makkelijk kan spreiden in een groep gevaccineerde fazanten. Deze conclusie wordt onderbouwd door de statistische analyse. Het blijkt echter ook dat geen van de gevaccineerde fazanten ziek wordt. Dit betekent, met andere woorden, dat het vaccin wel de ziekte maar niet de spreiding van het virus voorkomt.

Conclusies

Samenvattend kunnen we stellen dat het in dit rapport beschreven onderzoek de volgende conclusies toelaat:

- In zowel ongevaccineerde fazanten als ongevaccineerde eenden kan hoogpathogeen H7N7 influenzavirus makkelijk spreiden. Terwijl niet-gevaccineerde fazanten ernstig ziek worden en er doorgaans binnen één a twee weken ook aanzienlijke sterfte optreedt, zijn er in geïnfecteerde eenden geen klinische ziekteverschijnselen te zien. Dit betekent dat een infectie met hoogpathogeen H7N7 influenzavirus in een populatie van fazanten waarschijnlijk binnen korte tijd zal worden opgemerkt zonder dat daar actieve surveillance voor nodig is. In een populatie van ongevaccineerde eenden daarentegen is het zeer wel mogelijk dat zonder een actief surveillanceprogramma het virus geruime tijd ongezien kan spreiden.
- Terwijl ongevaccineerde fazanten ernstig ziek worden van een infectie met hoogpathogeen H7N7 virus, blijken gevaccineerde fazanten niet ziek te worden maar wel degelijk het virus onderling te kunnen doorgeven. Dat zou betekenen dat het vanuit epidemiologisch oogpunt voorlopig niet is aan te raden om fazanten te vaccineren in een eventuele nieuwe crisissituatie. Als er al sprake is van vaccinatie van fazanten, dan zal er naast het vaccinatieprogramma zeker een actief surveillanceprogramma moeten worden opgezet om er zeker van te zijn dat er niet ongezien transmissie van virus plaatsvindt.
- Vaccinatie werkt wel heel goed in eenden; terwijl ongevaccineerde eenden het virus makkelijk onderling doorgeven, blijkt dat hoogpathogeen H7N7 virus nauwelijks nog kan spreiden in een populatie van gevaccineerde eenden. Vaccinatie zou dus een goede optie zou kunnen zijn in een controleprogramma dat er op is gericht om eenden te beschermen tegen infectie met en verspreiding van hoogpathogeen H7N7 influenzavirus. Daarnaast is vaccinatie van eenden aantrekkelijk omdat ongevaccineerde eenden na infectie geen ziekteverschijnselen tonen, en omdat eenden algemeen worden gezien als een belangrijke bron van voor kippen en mensen gevaarlijke influenzavirussen.
- Strikt genomen gelden de uitkomsten van deze studie slechts voor de specifieke gastheer-virus-vaccin combinatie die in dit onderzoek is gebruikt (virus: A/chicken/Netherlands/03 H7N7, gastheer: roodschoudertaling/goudfazant, vaccin: geïnactiveerd H7N1). Hoewel het zonder aanwijzingen van het tegendeel plausibel is om aan te nemen dat de resultaten ook gelden voor verwante diersoorten en vergelijkbare virussen/vaccins, moet toch enige voorzichtigheid worden betracht bij het extrapoleren van de in dit rapport beschreven resultaten naar andere gastheersoorten, andere influenzavirussen en/of andere vaccins.

2. Materials and methods

Broadly speaking, this chapter is based on the experimental set-up and methodology of van der Goot *et al.* (2003, 2005). For completeness, we give a concise overview of the experimental procedures, materials used, and methods of analysis.

2.1 Virus and inoculation

As in our previous study (van der Goot *et al.* 2005) the influenza virus used in this study was strain A/chicken/Netherlands/621557/03 H7N7. This virus was isolated on the index farm of the outbreak in the Netherlands in March 2003. On a scale of 0-3 the virus had an intravenous pathogenicity index (IVPI) in chickens of 2.93, as determined by the procedure described elsewhere (van der Goot *et al.* 2003).

Briefly, ten specific pathogen free chickens were injected intravenously with 0.1 ml of tenfold diluted allantoic fluid. Chickens were examined at 24-hour intervals for ten days. At each observation each animal was recorded normal (0), sick (1), severely sick (2) or dead (3). The index is calculated by adding up all scores and by dividing the total by 100. When the index is greater than 1.2 the avian influenza is considered highly pathogenic (HPAI).

Inoculation dose and route were as follows. The animals were inoculated both intranasaly and intratracheally with 0.1 ml diluted allantoic fluid containing 106 median egg infectious dose (EID50) per ml.

2.2 Animals

The transmission experiments were carried out with two semi-feral bird species that are commonly kept in the Netherlands: ringed teal (*Callonetta leucophrys*) and golden pheasant (*Chrysolophus pictus*) (De Boeck-Pauchet 2003). The animals were kindly provided by the association of breeders of ornamental fowl 'Aviornis International Nederland' (www.aviornis.nl).

In short, the animals were bred by animal keepers of Aviornis. All animals were born in the Spring of 2004. Six weeks before inception of the experiments 50 pheasants and 50 teals were transported to a central location where they were housed together for acclimatisation. Two weeks before the start of the experiments the animals were subsequently transported to the Central Institute for Animal Disease Control (CIDC) in Lelystad where they were kept together until the start of the experiments.

All animal experiments were undertaken in a high containment unit under BSL3+ conditions at the Central Institute for Animal Disease Control Lelystad. The experiments comply with the Dutch law on animal experiments and were reviewed by an ethical committee.

2.3 Vaccine

A readily available inactivated oil emulsion H7N1 vaccine was used in the experiments. The dosage was 0.5 ml, as recommended by the manufacturer. The animals were injected in the muscles of the leg. In the hemagglutination assay the antigen content of the H7N1 was 80 hemagglutinating units (HAU). The homology at the protein level of the immunogenic part of the hemagglutinin (HA1) between the H7N1 vaccine and the challenge strain H7N7 was 98% (van der Goot *et al.* 2005).

2.4 Transmission experiments

Transmission experiments were performed with unvaccinated teals and pheasants (Exp. 1 and Exp. 3), and with teals and pheasants challenged two weeks after vaccination (Exp. 2 and Exp. 4). All experiments were done in duplicate. The design of the experiments was as follows: five animals were placed in a cage. At day 0 these animals were inoculated with virus and 24 hours later (day 1) five contact animals were added. The animals were monitored by taking tracheal and cloacal swabs daily during the first ten days and twice a week for the next 11 days. A blood sample was taken once a week. The experiment was terminated three weeks after the challenge. Table 1 gives an overview of the four experiments.

number of the experiment	number of replicates	experimental set-up
1	2	unvaccinated teals
2	2	vaccinated teals
3	2	unvaccinated pheasants
4	2	vaccinated pheasants

Table 1. Overview of the experiments.

2.5 Virus isolation

Swabs were put in 2 ml 2.95% tryptose phosphate buffer with 5 x 103 IU of penicillin-sodium and 5 mg streptomycin per ml. The swabs were stored at -70°C until analyzed. Three embryonated animal eggs incubated for 9 days were inoculated with 0.2 ml per egg. After 72h the allantoic fluid was harvested. A Hemagglutination Assay (HA) was performed following standard procedures. When at least one of the eggs was positive in the HA assay the swab was considered to be positive.

2.6 Hemagglutination Inhibition (HI) assay

A Hemagglutinin Inhibition (HI) test on the sera of the blood samples was done by standard methods. Briefly, the test was performed in V-bottom 96-well microtiter plates with 8 hemagglutinating units of H7N7 challenge virus and 1% SPF chicken erythrocytes.

2.7 Statistical analyses

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

We use two different methods to estimate the reproduction number: *(i)* final size methods and *(ii)* a Generalized Linear Model. The appeal of final size methods is that they are flexible and robust. For instance, the final size does not depend on whether or not there is a period of latency, and different assumptions on the infectious period distribution are easily incorporated. On the other hand, final size methods do not make use of all the information, and do not allow separate estimation of the transmission rate parameter and infectious period. For this purpose the Generalized Linear Model is appropriate.

2.7.1 Final size analysis

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

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

where $L[z]$ is the Laplace transform of the infectious period probability distribution. We make the flexible assumption that the infectious period is gamma distributed. Hence, $L[z]$ is given by

$$L[z] = (1+bz)^{-c}. \quad (2)$$

In this equation, the parameter b is commonly called the scale parameter, while the parameter c is referred to as the shape parameter. The mean of the infectious period is given by the product $T=bc$. Although the above formulation is flexible, in our calculations we focus on two extreme scenarios for the infectious period distribution that are specified by a single parameter. In the first, we assume that the infectious period is exponentially distributed so that there is considerable variation in individual infectious periods. In this case we take $c=1$ in Eqn. (2), and L is given by

$$L[z] = \frac{1}{1+Tz}. \quad (3)$$

In the second, we assume that the infectious period is fixed so that there is no variation in individual infectious periods. This is another special case of the gamma distribution for which

$$L[z] = e^{-Tz}. \quad (4)$$

Further, by rescaling the time-axis we may measure time in units of the infectious period (Ball 1986). As a consequence, $E(T_i)=1$ and $R=\beta E(TI)=\beta$. In other words we may, without loss of generality, take bc=1 and equate the transmission rate parameter β with the reproduction number R in the final size equation (1).

Insertion of either eqn (3) or eqn (4) in eqn (1) and using the above argument allows one to write down explicit equations for the final size in terms of the reproduction number by recursively solving eqn (1), starting with k=0. For instance, if s0=5 and i0=5 and the infectious period is fixed, then the probability of an outbreak of size 0 is given by

$$p(0) = e^{-\frac{5R}{2}}. \quad (5)$$

Insertion of $p(0)$ in eqn (1) then allows one to determine $p(1)$, etcetera.

With formulas for the final size at hand it is possible to obtain estimates of the reproduction number by Maximum Likelihood (Bailey 1975, Becker 1989, Kroese and de Jong 2001). Estimates of the reproduction number are labelled by R_{exp} in case of an exponentially distributed infectious period, and by R_{fix} in case of an infectious period of fixed duration. Exact 95% confidence intervals are obtained by finding all possible r values such that the hypothesis H_0: $R=r$ is not rejected, i.e. by finding all values of r with a p-value larger than 0.05.

In the same manner, exact tests of R against the threshold value 1 are performed (Kroese and de Jong 2001). Furthermore, taking the difference in the number of contact infections between treatments as a natural test statistic, it is possible to make comparisons between treatments based on R, i.e. to test whether $R_{vaccine}=R_{control}$ (Kroese and de Jong 2001). All calculations are carried out using the software package Mathematica 5.0.

2.7.2 Generalized Linear Model

To take the time course of the experimental epidemics into account we estimate the transmission parameter ß of the SEIR model by means of a Generalized Linear Model (GLM) (McCullagh and Nelder 1989, Becker 1989, van der Goot *et al.* 2003, 2005). To this end the data in Figures 1-4 are rendered into the format *(S(t),I(t),C(t), Δt)*, where *S(t)* is the number of susceptible chickens at the beginning of a certain time period of length Δt, *I(t)* represents the average number of infectious chickens in this time period, and *C(t)* represents the number of new infections that have appeared. In contrast to our previous studies (van der Goot *et al.* 2003, 2005) we here assume a latent period of one day since some of the contact animals were already infected at day 2. Finally, the total number of chickens that is alive is also relevant, and is denoted by *N(t)*. By standard reasoning (Bailey 1975, Becker 1989) we accept that the number of cases is binomially distributed with parameter

$$p_{\text{inf}}(t,t+\Delta t)=1-\exp\left(-\beta \frac{I(t)}{N(t)}\Delta t\right) \qquad (5)$$

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

$$C(t,t+\Delta t)\sim Bin\left(S(t), p_{\text{inf}}(t,t+\Delta t)\right). \qquad (6)$$

The model specified by eqns. (5) and (6) can be formulated as a GLM with a complementary log-log link function, taking *log(I(t)/(N(t)))* as offset variable. The intercept of this generalized regression estimates *log(β)*. The analyses are carried out using the software package GenStat 6.0.

The infectious periods are directly observed from the infected contact animals. Hence, estimation of the infectious period and the construction of confidence interval is straightforward. An estimate of the reproduction number is given by the product of the estimates of the transmission parameter and infectious period (Stegeman *et al.* 2004). Construction of the confidence intervals of the reproduction numbers follows the lines of van der Goot *et al.* (2003). Confidence intervals are only calculated if both the number of contact infections as well as the number of records in the Generalized Linear Model equals or exceeds 5.

3. Results

In sections 3.2-3.5 we present the result for each of the Experiments 1-4 separately. An overview of the analyses is given in the next section.

3.1 Overview of the analyses

Table 2 below gives an overview of the final size analyses, and Table 3 gives the results of the analyses based on the Generalized Linear Model (section 2.7.2). Serological data were also collected on a weekly basis but formed no part of this study. These will be analysed at a later stage.

exp.	final size	R_{fix}	R_{exp}	H_0: $R_{fix} \geq 1$	H_0: $R_{exp} \geq 1$	H_0: $R_{vaccine} = R_{control}$
1	5,5	∞ (1.3-∞)	∞ (1.3-∞)	NS	NS	**<0.001**
2	0,1	0.19 (0.005-1.1)	0.20 (0.005-1.4)	**0.036**	0.068	
3	5,5	∞ (1.3-∞)	∞ (1.3-∞)	NS	NS	NS
4	5,5	∞ (1.3-∞)	∞ (1.3-∞)	NS	NS	

Table 2. Overview of the final size analyses. The first and second column give the number of the experiment (see Table 1) and the final size outcome of the experiments, respectively. The third and fourth column give Maximum Likelihood estimates of the reproduction number based on a fixed and exponentially distributed infectious period, respectively. 95% confidence intervals of the estimates are given between brackets. The last column gives two-sided p-values of the hypothesis in the heading under the assumption of an exponentially distributed infectious period. NS: p>0.1. See sections 3.2-3.5 below for details.

exp.	transmission parameter (β)(day^{-1})	infectious period ($1/\gamma$)(day)	reproduction number (R)
1	*∞ (n=2)*	*>6 (n=10)*	*na*
2	*0.037 (n=4)*	*na (n=0)*	*na*
3	*0.62 (n=3)*	*>8 (n=10)*	*>5*
4	*3.3 (1.2-9.5) (n=7)*	*12.2 (9.6-14.8)* (n=10)*	*40*

Table 3. Overview of the Generalized Linear Model analysis. 95% confidence intervals and number of observations are given between brackets. No confidence intervals are calculated if the number of record was smaller than 5 or if not all contact animals had stopped shedding virus at the end of the experimental period. *: the infectious period was calculated by taking the difference between the last day that the animal

was positive minus the first day that the animal was positive plus *1*. In case that the last positive day could not be determined (e.g., if the animal was positive at day 10 and negative at day 14), the last positive day was calculated as (first day negative - last day positive - *1*) divided by *2*. See sections 3.2-3.5 below for details.

3.2 Unvaccinated ducks

Figure 1 shows the results of the transmission experiments with unvaccinated ducks. The rows in the figure refer to different animals, while the columns refer to different days. Animals denoted with an *I* in the first column represent inoculated animals, while animals denoted with an *S* represent contact animals. The contact animals were put in the cage *24* hours after inoculation of the inoculated animals.

	pre	D 1	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10	D 14
I	-/-	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-	nd	nd	nd
I	-/-	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-	nd	nd	nd
I	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	nd	nd	nd
I	-/-	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-	nd	nd	nd
I	-/-	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-	nd	nd	nd
S	-/-	nd	+/-	+/+	+/+	+/+	+/+	+/-	+/-	-/-	-/-	+/+
S	-/-	nd	+/+	+/+	+/+	+/+	+/+	-/+	-/-	-/-	-/+	-/-
S	-/-	nd	+/+	+/+	+/+	+/+	+/+	+/-	-/-	-/-	-/+	-/-
S	-/-	nd	+/+	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/+	-/-
S	-/-	nd	+/+	+/+	+/+	+/+	+/+	+/-	+/-	-/-	-/-	-/-

	pre	D 1	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10	D 14
I	-/-	+/+	+/+	+/+	+/+	+/-	+/-	-/-	-/-	nd	nd	nd
I	-/-	+/+	+/+	+/+	+/+	+/-	+/-	-/-	-/-	nd	nd	nd
I	-/-	+/+	+/+	+/-	+/+	+/-	-/-	-/-	-/-	nd	nd	nd
I	-/-	+/+	+/+	+/+	+/+	+/-	+/-	-/-	-/-	nd	nd	nd
I	-/-	+/+	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/+	nd
S	-/-	nd	+/+	-/+	+/-	+/+	+/+	+/+	+/+	-/-	-/-	-/-
S	-/-	nd	+/+	+/+	+/+	+/+	+/+	x/+	+/+	-/-	-/-	-/-
S	-/-	nd	+/+	+/+	+/+	+/+	+/-	+/-	-/-	-/-	+/+	-/-
S	-/-	nd	+/+	+/+	+/+	+/+	+/-	+/-	+/+	+/-	-/+	+/-
S	-/-	nd	+/+	+/+	+/+	+/+	+/+	+/+	+/-	-/-	+/+	-/-

Figure 1. Overview of the transmission experiments with unvaccinated ringed teals (experiment 1). Animals were challenged 14 days after vaccination (D0). pre: infection status before vaccination. I: inoculated animal, S: contact animal. a/b: trachea/cloaca swab. nd: not determined. x: not available. Animals that were scored negative on a certain day (-/-) but that had been positive on one or more days before and after that day were considered to be infected but not infectious (light-grey shaded cells).

As Figure 1 shows, all inoculated animals became positive by virus isolation. The mean infectious period of the inoculated animals was *6.6* days. There was little variation in the individual infectious periods of the

inoculated animals (range: *5-11.5* days; *CV=27%*), indicating that the inoculation route and dose resulted in a highly predictable infection pattern in the inoculated animals. (Note, however, that in the calculation of the infectious period we assumed that the fifth inoculated animal in the second replicate was negative on day 14.) From the inoculated animals the virus spread rapidly to the contact animals. In fact, at day 2 all 10 contact animals were already positive by virus isolation.

The mean infectious period of the contact animals could not be determined exactly, as two of the ten contact animals were still positive at day 14, and as several animals appeared to be shedding the virus intermittently in the second week of infection. More detailed studies including analysis of available data on days 17, 21, and 24 in conjunction with virus titration analyses are needed to resolve this issue. However, given the excretion patterns in Figure 1 it seems safe to state that the infectious period of the contact infected animals is at least *6.6* days. Using the available data up to day 14 there was no evidence that the mean infectious period of the contact animals was different from the infectious period of the inoculated animals ($p<0.05$, Welsch 1938).

The final size analysis of the experiments based on the SEIR model reveals that the reproduction number is estimated to be very large ($R_{fix}=\infty$, *95%CI=(1.33-∞)*, Table 2). Not surprisingly, the null hypothesis that the reproduction number exceeds the critical value of *1* cannot be rejected (Table 2).

Although all contact animals were infected rapidly (within one day), the value of the GLM analysis may be limited. Indeed, the virus spread so rapidly from the inoculated to the contact animals that the results of the GLM are based on two records only (one for each replicate experiment). Not surprisingly, the transmission rate parameter was estimated to be infinitely large (Table 3).

The experiments with unvaccinated ducks showed that the scores in the trachea correlate very well with the scores of the cloaca. In fact, a positive score in the cloacal swabs appeared an almost perfect indicator for a positive score in the trachea, while a positive score in the trachea was a good indicator for a positive score in the cloaca. Since this information was not central to the focus of this study, no formal analyses were carried out to investigate this phenomenon further.

Finally, there were no signs of clinical disease (breathing difficulties, conjunctivitis, lethargy) that could be scored by visual inspection of the animals. This is not wholly unsurprising in view of the classical literature in which it is shown that infected ducks usually are silent carriers of the virus that may excrete large amounts of virus in their faeces. On the other hand, recent reports from the H5N1 viruses circulating in Asia seem to indicate that this may not be a universal phenomenon since over the years these H5N1 viruses have become pathogenic in ducks.

3.3 Vaccinated ducks

The outcome of the experiments with vaccinated ducks (Figure 2) differed considerably from the experiments with unvaccinated ducks (Figure 1). In fact, while all animals were infected in the experiment with unvaccinated ducks, only 7 out of 10 inoculated animals were scored positive in the experiment with vaccinated ducks,. In addition, none of the vaccinated contact animals was found positive. Moreover, while the infected ducks were positive for several days in the unvaccinated ducks (see Table 3), the ducks that were scored positive in the experiments with vaccination were positive for a few days only (Table 3). There is a significant difference between the infectious period in the two groups ($p<0.05$, Welsch 1938).

	pre	D 1	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10	D 14
I	-/-	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	-/-	+/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	-/-	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	-/-	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	-/-	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	-/-	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	-/-	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-

	pre	D 1	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10	D 14
I	-/-	+/-	-/-	-/-	-/-	x/-	-/-	-/-	-/-	-/-	-/-	-/-
I	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
I	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	-/-	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	-/-	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	-/-	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	-/-	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
S	-/-	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-

Figure 2. Overview of the transmission experiments with vaccinated ringed teals (experiment 2). Animals were challenged 14 days after vaccination (D0). pre: infection status before vaccination. I: inoculated animal, S: contact animal. a/b: trachea/cloaca swab. nd: not determined. x: not available.

For the experiments with vaccinated ducks the GLM analysis was based on four records (Table 3). The transmission rate parameter was estimated at *0.037* (*day*$^{-1}$) which indicates that it would take about *27* days for an infected animal to infect a single susceptible animal. However, as with the experiments with unvaccinated ducks no confidence interval was calculated because of the small number of records.

As with the experiments with unvaccinated ducks, no clinical signs of infection or adverse effects of vaccination were observed in the vaccinated ducks.

3.4 Unvaccinated pheasants

Figure 3 shows the results for the transmission-experiments with unvaccinated pheasants. As the figure shows, all the contact animals were scored positive after a single day. Moreover, the virus also spread efficiently to the contact animals. Statistical analyses corroborate these findings: The reproduction number is estimated at *R=∞ (95%CI=(1.33-∞))* (Table 2) by the final size method, while it is larger than *5* when using the GLM (Table 3). Specifically, the GLM estimates that one infected chicken will infect about *3* susceptible chickens in five days, while the infectious period is well over one week.

	pre	D 1	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10	D 14
I	-/-	+/+	+/+	+/+	+/+	+/+	+/+					
I	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/-	-/-			
I	-/-	+/+	+/+	+/+	+/+							
I	-/-	+/-	+/+	+/+	+/+	+/+	+/+					
I	-/-	+/+	+/+	+/+	+/+	+/+	+/+					
S	-/-	nd	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+		
S	-/-	nd	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/+
S	-/-	nd	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/+
S	-/-	nd	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/-	-/-	-/-
S	-/-	nd	+/-	+/+	+/+	+/+	+/+	+/+	+/+			

	pre	D 1	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10	D 14
I	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/+
I	-/-	+/+	+/+	+/+	+/+	+/+						
I	-/-	+/-	+/+	+/+	+/+	+/+	+/+					
I	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/-				
I	-/-	-/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	+/+	+/+	-/-
S	-/-	nd	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/+
S	-/-	nd	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/-	-/-
S	-/-	nd	-/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/+
S	-/-	nd	-/-	+/+	+/+	+/+	+/+	+/+	+/-	+/-		
S	-/-	nd	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/-		

Figure 3. Overview of the transmission experiments with unvaccinated golden pheasants (experiment 3). Animals were challenged 14 days after vaccination (D0). pre: infection status before vaccination. I: inoculated animal, S: contact animal. a/b: trachea/cloaca swab. nd: not determined. x: not available. Black cells denote animals that died from the infection. Animals that were scored negative on a certain day (-/-) but that had been positive on days before and after that day were considered to be infected but not infectious (light-grey shaded cells).

Interestingly, the vast majority of samples were positive in the tracheal swabs as well as in the cloacal swabs. This indicates that infection with highly pathogenic H7N7 is not only limited to the trachea, but is able to spread and multiply throughout the gastro-intestinal tract. This is often observed in chickens infected with highly pathogenic avian influenza A viruses (van der Goot *et al.* 2003, 2005), and may well be a general

phenomenon of highly pathogenic avian influenza viruses in birds that sets low and high pathogenicity viruses apart.

In contrast to the transmission experiments with ducks, all the inoculated as well as the contact pheasants showed clear clinical disease signs. Ultimately, eight of the ten inoculated pheasants died, while four of the ten contact animals died.

3.5 Vaccinated pheasants

Figure 4 below shows the results for the vaccinated pheasants. All pheasants, the inoculated as well as the contact animals, became positive during the experiment. Moreover, in both replicates the contact animals became positive already during the first few days of the experiment (day 2 - day 5). This indicates that - although all animals had been vaccinated- the virus is still highly transmissible. These observations are corroborated by formal analyses: As Table 2 shows the reproduction ratio based on the final size method was estimated to be infinitely large ($R_{fix}=\infty$, *95%CI=(1.33-∞)*). The Generalized Linear Model yielded similar results (*R=40*), and showed that the virus was both highly transmissible ($\beta=3.3$ *(day^{-1}), 95%CI=(1.2-9.5)*) and induced a long infectious period in infected animals (*T=12.2 (day), 95%CI=(9.6-14.8)*.

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

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

Figure 4. Overview of the transmission experiments with vaccinated golden pheasants (experiment 4). Animals were challenged 14 days after vaccination (D0). pre: infection status before vaccination. I: inoculated animal, S: contact animal. a/b: trachea/cloaca swab. nd: not determined. Animals that were

scored negative on a certain day (-/-) but that had been positive on days before and after that day were considered to be infected but not infectious (light-grey shaded cells).

An interesting feature of the experiments with vaccinated pheasants was that some of the infected animals were scored negative on certain days while they were scored positive on earlier and later days. This could indicate that although vaccinated pheasants can be infected and can readily spread the virus, it may well be that the amount of virus spread may be lower than in the unvaccinated pheasants. Given what is known of the dynamics of avian influenza in a suite of host species it seems highly unlikely that these animals these phenomena could be due to reinfection of animals that were infected earlier.

A formal comparison of the transmission dynamics between the unvaccinated versus vaccinated pheasant was unable to detect differences in the transmission dynamics ($p>0.1$; Table 2). As would be expected, there is a significant difference between the outcome of the experiments with vaccinated ducks and vaccinated pheasants (Table 2).

With regard to disease symptoms the results differ diametrically from the experiments with unvaccinated pheasants: while all unvaccinated pheasants became severely ill and most of them died before the end of the experiments, no disease signs were observed in the vaccinated pheasants. This has important implications for vaccination as a potential control strategy, as will be explained in the Discussion.

4. Discussion

Transmission of H7N7 HPAI in unvaccinated pheasants and ducks.

Our results and analyses show that the highly pathogenic H7N7 avian influenza virus that caused a large outbreak in poultry in the Netherlands does not only spread rapidly in chickens (van der Goot *et al.* 2005), but can also spread efficiently in pheasants (*Chrysolophus pictus*) and ducks (*Callonetta leucophrys*). This finding shows that the host range of the virus is not limited to chickens, and it lends some credibility to the hypothesis of a recent origin of the virus in the avian reservoir (Kemink *et al.* 2004).

A detailed analysis of the experiments with H7N7 highly pathogenic avian influenza virus in unvaccinated ducks and pheasants showed that the infectious period of contact infected pheasants and ducks were comparable, and that for both species the mean infectious period was more than 7 days. The transmission rate parameters in unvaccinated pheasants and ducks were also comparable, since all contact animals were infected either at day 2 (all ducks and most pheasants) or else at day 3 (some pheasants). The statistical analyses corroborate these findings, as no significant differences could be demonstrated between the infectious period as well as the transmission rate parameter in unvaccinated pheasants as compared to unvaccinated ducks.

Although the highly pathogenic H7N7 virus spreads easily in both pheasants and ducks, the clinical manifestations of infection were wholly different in pheasants as compared to ducks. In fact, clinical signs were completely absent in ducks while pheasants showed all the major clinical signs, including death, that are commonly observed in chickens (Elbers *et al.* 2005). For control strategies this finding has important implications as it makes it likely that an outbreak of highly pathogenic avian influenza in pheasants will be detected rapidly, while it is quite conceivable that an outbreak of the same virus in ducks may spread unnoticed for a considerable amount of time.

Does vaccination reduce transmission of H7N7 HPAI in vaccinated pheasants and ducks?

The experiments with vaccinated ducks indicate that the vaccine used and vaccination scheme employed were highly effective in reducing virus spread from animal to animal. In fact, while in the group of unvaccinated ducks all the contact animals were positive from day 3 onwards, none of the contact animals in the vaccinated group were found positive on any day. This suggests that an introduction of highly pathogenic avian influenza virus in a population of vaccinated ducks will not be able to cause a major outbreak. The statistical analyses corroborate this indication: Vaccination significantly reduces the reproduction number ($p=0.0007$; Table 2), and the reproduction number in a population of vaccinated ducks in a one-sided test is significantly below 1 ($p=0.036$; Table 2).

The results of the experiments with vaccinated pheasants differed diametrically from the experiments with vaccinated ducks: While in vaccinated ducks no virus transmission was observed, in the experiments with vaccinated pheasants all contact animals were quickly infected. As a result, with regard to transmission no

significant effect of vaccination could be demonstrated in pheasants (Table 2), and, in addition, the reproduction number in vaccinated pheasants remains significantly above the threshold value of 1 ($p=1$). Therefore, we conclude that the vaccine and vaccination scheme employed in the present study is not sufficient to protect pheasants against spread of H7N7 highly pathogenic avian influenza virus. Whether other vaccines or vaccination schedules (e.g., multiple vaccination bouts or a longer interval between vaccination and challenge) would result in a decrease of transmission remains an open question.

Our results indicate that it may not be an easy task to decide whether or not vaccination is a viable control measure. On the one hand, from an epidemiological perspective vaccination works perfectly well in ducks. On the other hand, vaccination does not seem to work at all in pheasants. Even worse is the fact that vaccinated pheasants do not show overt clinical symptoms of disease, so that there is the real possibility that vaccination may lead to prolonged and unnoticed spread of the virus. All in all we conclude that it may not be easy to make general statements on the effectiveness of vaccination, and that for every host-influenza combination a meticulous weighing of the pros and cons of vaccination based on experimental evidence is called for.

Relevance for other host species and influenza viruses

Motivated by the Dutch outbreak of a highly pathogenic H7N7 avian influenza virus we have in this study focused on the transmission dynamics of A/chicken/Netherlands/03 in pheasants and ducks. In a previous study we had already investigated the transmission dynamics of this virus in chickens (van der Goot *et al.* 2005). The question arises to what extent our findings can be generalized to other host species, and other influenza A viruses.

At present most interest is focused on vaccination of chickens and ducks in Southeast Asia against the circulating highly pathogenic H5N1 viruses. In China, Indonesia, Taiwan, and other Asian countries several H5 vaccines are now in use, mostly to protect commercial poultry. However, up to now the evidence that vaccination programs are helpful in reducing virus transmission is fragmentary at best. An important open question that needs to be addressed is whether these H5 vaccines protect birds against infection and transmission. This question is pressing in view of our experimental results with vaccinated pheasants that show that it is quite conceivable that vaccination reduces morbidity rates but may do little or nothing in terms of preventing viral spread.

To improve our understanding of the dynamics of avian influenza A viruses key questions that need to be addressed are related to the evolutionary paths that influenza viruses take in different host species. Recent studies have stressed the role of chickens as an intermediate species from which influenza viruses in the reservoir in migratory birds may ultimately be transmitted to the human population. In Southeast Asia recurrent epizootics of highly pathogenic H5N1 virus in chickens are at present considered to be the main threat for transmission to the human population. This is mainly so because of the huge numbers of chickens

infected (tens of millions) and the intimate contact between chickens and humans in this region, presenting ample opportunities for transmission from chicken to man. Our study has provided evidence that not only highly pathogenic H5N1 viruses can spread in a wide variety of bird species, but that this is probably also true for highly pathogenic H7N7 virus. Hence, if one is to understand the dynamics of highly pathogenic H7N7 virus the focus should not solely be on chickens and other species in which high mortality is observed (e.g., pheasant, turkey, ostrich), but one should also include other species in which the virus may spread efficiently without showing overt symptoms of disease.

Summarizing, we have shown that, for each specific host-pathogen combination it is perfectly feasible to determine whether vaccination would be an epidemiologically effective tool. However, influenza A viruses are highly variable, especially those that have a recent origin in the reservoir species. Moreover, each influenza virus may have different characteristics in different host species. As a result, it may not be straightforward to translate results obtained with one host species and one virus strain to other host species or other virus strains.

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6. References

Note: All papers referred to in the main text of the report are mentioned in the reference list below, but not all papers in the reference list are explicitly mentioned in the text of the report. Apart from the papers mentioned in the main text the reference list includes major papers that deal with either (1) the spread of influenza in bird species other than poultry, or (2) the determination of the effect of vaccination in bird species. The main purpose of the reference list is to provide an overview of the literature relevant to this study.

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