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

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

INTRODUCTION
The primary reservoir of influenza A viruses is generally considered to be in wild aquatic birds of the orders Anseriformes and Charadriiformes [1]. In these species all major subtypes of the virus can be found, and the virus causes no or only limited mortality [2–3]. A subset of the subtypes is found also in a variety of other avian and mammalian species. In these hosts some strains induce considerable mortality. The close relationships between strains found in different species suggest that interspecies transmission events are fairly common (e.g., [4–8]).

In poultry certain strains of influenza A are especially noted for their ability to cause high mortality. Indeed, the term ‘fowl plague’ is colloquially used for disease caused by these highly pathogenic avian influenza A strains (HPAI). In Hong Kong a large outbreak of such an HPAI strain occurred in 1997. Besides leading to substantial economic losses, the virus also spread to at least 18 humans. This led to the suggestion that outbreaks of HPAI virus in poultry could constitute a ‘pandemic threat’ to the human population [9].

Up to now, HPAI virus in poultry has been limited to strains of the H5 and H7 subtype. Eighteen outbreaks of HPAI virus have been recorded in chickens and turkeys since 1959 [10–11]. Virulence is mainly determined by the hemagglutinin protein (which defines the H type of the strain), although there are other determinants of virulence [12–15]. In order to attain efficient replication the hemagglutinin needs post translational cleavage by proteases of the host. HPAI strains have multiple basic amino acids at the cleavage site of the hemagglutinin [16–18], making
cleavage by ubiquitous proteases possible and allowing the virus to spread systemically and cause high mortality.

It is known that outbreaks of HPAI virus can arise out of outbreaks of the corresponding LPAI virus (e.g., [11, 19]). An example is the outbreak of LPAI in chickens in Pennsylvania in 1983. The outbreak started in April 1983 with a low pathogenicity virus that caused only limited mortality. In October of the same year the virus population had transformed into a state of high pathogenicity, causing over 80% mortality [19–20]. The most recent example of such a transformation event is the Italian outbreak of HPAI H7N1 in December 1999 that had started as an outbreak of LPAI H7N1 in April of the same year [11].

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

We tackle the aforementioned questions by means of transmission experiments. Transmission experiments form an invaluable tool to study the effect of a single factor (strain type, vaccination, host genotype) as they offer a controlled setting in which confounding variation due to other factors is kept to a minimum [21]. In the field of avian influenza transmission experiments have been carried out before [22–27]. However, these previous studies were qualitative and the conclusions were not based on a sound statistical analysis. Here we base the analysis on a well-founded epidemic model (the susceptible–infected–removed or SIR model). As a consequence, all parameters have a clear-cut biological interpretation (susceptibility, infectiousness, infectious period), making it possible to ascribe differences between strains or treatments to specific, biologically interpretable parameters.

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

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

METHODS

Materials and experimental setup

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

Viruses

Two avian H5N2 strains were used, a low pathogenicity strain isolated from the index case in April 1983 (A/Chicken/Pennsylvania/21525/83), and a high pathogenicity strain isolated in October 1983 (A/Chicken/Pennsylvania/1370/83). Both strains were sent from the USDA (Ames, Iowa) to our laboratory in 1984. The strains were grown in allantoic fluid and stored at −70 °C. We performed a standard intravenous pathogenicity test with both strains. The intravenous pathogenicity index (IVPI) of the LPAI strain was 0-0 implying low pathogenicity, while the IVPI of the HPAI strain was 2-0 implying high pathogenicity. We checked for mutations in the hemagglutinin gene by sequencing the cleavage and glycosylation sites. No differences were found with published sequences [28–29].
**Animals**

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

**Experimental procedures**

**Inoculation route and dose**

Animals were inoculated both intranasally and intra-tracheally with 0·1 ml diluted allantoic fluid containing 10⁶ EID50 per ml.

**Virus isolation and serology**

Swabs were put in 2 ml 2·95% tryptose phosphate buffer with 5 × 10³ IU of penicillin–sodium and 5 mg streptomycin per ml. The swabs were stored at −70 °C until analysed. Three embryonated chicken eggs incubated for 9 days were injected with 0·2 ml of the solution per egg. The eggs were then incubated for another 72 h. A standard hemagglutination assay was carried out on the allantoic fluid of the eggs.

The Hemagglutinin Inhibition (HI) test on the sera was done by standard methods.

**RNA isolation and PCR**

RNA isolation and reverse transcriptase PCR were done as described in [30].

**Statistical analyses**

**Final size analysis**

In first instance, the analysis of the experiments is based on the final size of the experiments, i.e. the number of contact animals that have been infected when the infection chain has ended. The final sizes are used to obtain estimates of the (basic) reproduction ratio, i.e. the number of infections that would be caused by a single infected individual in a large population of susceptibles. A forte of final size methods is that they are robust (e.g., inclusion of a latent period does not alter the results) and that different assumptions on the distribution of the infectious period are easily incorporated.

The methods are based on maximum likelihood estimation (MLE). That it is possible to use MLE hinges on the fact that final size distributions can be determined under a wide range of assumptions [31]. We focus on two extreme scenarios, one in which the infectious period is exponentially distributed, and one in which the infectious period is of fixed duration. The estimator of the reproduction ratio is labelled by \( R_{MLE1} \) in case of the first scenario, and by \( R_{MLE2} \) in case of the second scenario.

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

**Generalized Linear Modelling**

Although final size methods are flexible, they do not make use of all the available information. To take the time course of the experimental epidemics into account, we estimate the transmission parameter \( \beta \) of the stochastic SIR model by means of a Generalized Linear Model [32–33].

To this end the data in Figures 1–3 are first rendered into the format \((S, i, C)\). Here \( S \) is the number of susceptible animals in a certain time period, \( i \) is the prevalence of infection (i.e. the average number of infectious animals divided by the total number of animals), and \( C \) represents the number of new infections that have appeared at the end of the time period. By standard reasoning we assume that the number of cases \( C \) arising in a day is binomially distributed with parameter \( p_{inf} = 1 - e^{-\beta} \) (the probability of infection) and binomial totals \( S \):}

\[
C \sim Bin(S, 1 - e^{-\beta}).
\] (1)

Notice that the above model entails the following assumptions: (i) all susceptible birds are equally susceptible; (ii) all infected birds are equally infectious; and (iii) each infected bird poses an independent risk of infection to each susceptible bird. These assumptions can be relaxed, but this seems wise only if the fit of the model is unsatisfactory or if a large amount of data is available.

In the model (1), \( \log(\beta) \) is estimated using a complementary log–log link function while taking \( \log(i) \) as offset variable. The fit of the model is checked by inspection of the (residual) deviance that,
### Fig. 1. Summary of the LPAI transmission experiments (A/chicken/Pennsylvania/83 H5N2). Four replicate experiments are carried out. In each experiment five chickens are inoculated at day 0 (I). From day 1 onwards five contact animals (S) are placed in the cage with the inoculated chickens, and the infection chain is monitored. The data X/Y/Z represent the following: X/Y/Z = egg culture trachea/PCR trachea/egg culture cloaca. An animal is marked positive in the serology if

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under standard assumptions ([32], p. 118), is approximately \( \chi^2 \)-distributed. The analyses are carried out in GenStat 5.4.

The infectious periods are directly observed. Hence, estimation of the infectious periods and corresponding confidence intervals is straightforward.

The estimate of the reproduction ratio is given by the product of the estimates of the transmission parameter and infectious period: \( \hat{R} = \hat{\beta} \hat{\gamma} \). Construction of the corresponding confidence intervals is based on the identity \( \text{Var}(\hat{\beta} \hat{\gamma}) = (E\gamma)^2 \text{Var}(\hat{\beta}) + E\beta^2 \text{Var}(\gamma) \) [34]. Insertion of the estimated means and variances of \( \beta \) and \( \gamma \) into this formula yields an estimate for the variance of \( R \), thereby allowing the construction of confidence intervals. Notice that as the model yields estimates of \( \log(\beta) \) we need to take into account that (asymptotically) the estimator of \( \beta \) is lognormally distributed.

**RESULTS**

**The transmission characteristics of LPAI virus**

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

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

The mean of the infectious period of the contact infected animals is comparable to the infectious period of the inoculated animals (4.3 days vs. 4.8 days). However, variation in the infectious period is considerably higher for the contact infected animals than for the inoculated animals (CV = 55% for contact infections vs. CV = 9% for inoculated animals; \( F_{(9,18)} = 27.9, P < 0.001 \)). This indicates that contact infections are more variable than artificially induced infections.

The infectiousness of infected animals is determined by the transmission parameter \( \beta \). Loosely

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

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Let us now compare the LPAI and HPAI experiments on a more quantitative basis. A comparison of the infectious period of the LPAI contact infected animals with the HPAI contact infected animals shows that there is evidence that the infectious period in HPAI contact infected animals is significantly longer than the infectious period of LPAI contact infected animals ($t=2.28$, df = 18, $P<0.05$). Hence, the fact that HPAI efficiently exploits the host and that 9 out of 10 HPAI contact infected animals die within two weeks after infection in effect does not reduce the infectious period.

In addition, there is evidence that transmission parameter of HPAI virus is higher than that of LPAI virus. This is true if we assume that the period of latency is at most one day and compare the transmission parameters of LPAI virus and HPAI virus ($t=3.48$, df $=6$, $P<0.05$; [35]), or if we assume that the latent period is between one and two days ($t=3.07$, df $=4$, $P<0.05$).

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

Prior infection with LPAI virus provides effective protection against HPAI virus

Inspection of Figures 2 and 3 indicates that there are considerable differences between Experiments 5 and 6 where fully susceptible contact animals were used, and Experiments 7 and 8 where the contact animals had been infected before. Two features are noteworthy. First, only 4 of the contact animals are infected in Experiments 7 and 8, while all 10 infected contact animals died in Experiments 5 and 6. Second, none of the four infected contact animals die in Experiments 7 and 8, while 9 out of 10 infected contact animals died in Experiments 5 and 6.
animals die in Experiments 5 and 6. Formal tests corroborate these findings: the infectious period, transmission rate, and pathogen induced host mortality are higher in Experiments 7 and 8 than in Experiments 5 and 6. We conclude that an infection with LPAI provides effective protection against infection and mortality caused by HPAI virus.

DISCUSSION

The transmission characteristics of LPAI and HPAI virus

We have shown, on the basis of an epidemic model, that fully susceptible birds infected with HPAI virus are more infectious than birds infected with LPAI virus, and that the infectious period induced by the HPAI virus is longer than the infectious period induced by LPAI virus. The latter finding is somewhat surprising as one could have argued that the fact that HPAI efficiently exploits the host and causes rapid death (within 4–10 days) would in effect result in a reduction of the infectious period.

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

Third, birds infected by contact with HPAI virus shed virus in the trachea as well as in the cloaca, while birds infected by contact with LPAI virus shed virus in the trachea only (see also [19]). It is tempting to speculate that the observed differences in the transmission characteristics of LPAI virus and HPAI virus are a consequence of differences in excretion patterns of these particular LPAI and HPAI strains. To what extent these results hold for other combinations of low and high pathogenicity viruses (e.g., Mexico 1995...
(H5N2) or Italy 1999 (H7N1)) is still an open question.

The value and limitations of experimental transmission studies

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

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

Implications for the transmission dynamics of influenza A

The main value of the reproduction ratio is that it combines different aspects of the host–pathogen interaction into a single summary parameter that gives insight in the transmission dynamics of the pathogen. Consider, for instance, the following question. How many animals should have had an infection with LPAI virus to prevent an outbreak of HPAI virus? In the SIR model the critical fraction of the population that needs to be immune to obtain herd immunity, $p_c$, is related to the reproduction ratio $R_{HPAI}$ through $p_c = 1 - 1/R_{HPAI}$ [36]. Assuming that our estimates of the reproduction ratio of HPAI virus (e.g., $R_{HPAI} = 31.7$; Table 2) are indicative of the reproduction ratio in the field, this implies that the critical fraction of animals that need to be infected previously with LPAI virus to prevent an outbreak of HPAI virus is $1 - 1/31.7 = 0.97$.

In view of the large differences in the reproduction ratios of LPAI and HPAI virus it is unlikely that the critical infection fraction will be reached in the field. In fact, the fraction of susceptibles $S^*$ that is left at the end of a major outbreak of LPAI virus is related to the reproduction ratio $R_{LPAI}$ through a ‘final size equation’ (e.g., $\ln S^* = -R_{LPAI} (1 - S^*)$ in case of an exponentially distributed infectious period [31]). Our estimates of the reproduction ratio of LPAI do not exceed 2.39 (Table 1), implying that at most 88% of the individuals will have protective immunity after an outbreak. This is considerably lower than the required 97% to prevent an outbreak of HPAI. The practical implication is that a major outbreak of LPAI virus is unlikely to provide herd immunity against a major outbreak of HPAI virus.

Evolution of avian influenza A viruses

Why do low pathogenicity influenza A strains in poultry that are quite capable to transform into highly pathogenic counterparts prevail in the field, and how can this fact be reconciled with the apparent selective advantage of highly pathogenic strains over strains of low pathogenicity? An intuitive explanation states that ‘the main goal of parasitic microbes is not to multiply in their host as much as possible, but to ensure that they can still do so after years, decades, or even centuries’ ([37], p. 218). Other authors have expressed similar ideas (e.g., [1, 7]). The argument, however, does not stand scrutiny as it invokes a group selectionist argument that is known to be flawed [38]. In fact, the argument suggests that outbreaks of HPAI are artifacts that are a consequence of the virus not yet being well adapted to its host. It may even give a false sense of security as it suggests that, given enough time, the pathogen population will eventually evolve so as to minimize the damaged inflicted upon the host.

In theory, one may still rescue the argument that the pathogen population will evolve to be able to persist on the longer time scale [39]. The conditions under which this may happen are, however, restrictive. First, it requires that the number of lineages that have become extinct because of overexploitation of the host is large, and second, the time scale on which
the phenomenon could be effective is very long. It is therefore unlikely that selection has moulded influenza strains of poultry to a state where they exploit the host prudently. In fact, it is questionable whether such a state will ever be reached as highly pathogenic H5 and H7 viruses do not form unique lineages but share a recent common ancestor with nonpathogenic H5 and H7 viruses [3, 40], and as interspecies transmission events are common [7, 41]. In view of the above reasoning it remains enigmatic why we do not observe outbreaks of highly pathogenic avian influenza A more often.

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