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**Transmission between chickens of an H7N1 Low Pathogenic  
Avian Influenza virus isolated during the epidemic of 1999  
in Italy**

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## 25 Abstract

26 The transmissibility of an H7N1 Low Pathogenic Avian Influenza (LPAI) virus  
 27 isolated from a turkey flock during the large epidemic in Italy in 1999, was  
 28 experimentally studied in chickens. Four group transmission experiments were  
 29 performed. Infection and transmission were monitored by means of virus isolation on  
 30 swab samples and antibody detection in serum samples. From the results of these  
 31 groups, we estimated the mean infectious period at 7.7 (6.7 – 8.7) days, the  
 32 transmission rate parameter at 0.49 (0.30 – 0.75) infections per infectious chicken per  
 33 day and the basic reproduction ratio at 3.8 (1.3 – 6.3). These estimates can be used for  
 34 the development of surveillance and control programmes of LPAI in poultry.

35  
 36 **Keywords:** Avian Influenza, LPAI, H7N1, Transmission, Transmission parameters,  
 37 Reproduction ratio

## 39 1. Introduction

40 Highly pathogenic Avian influenza (HPAI) is caused by AI viruses of H7 or  
 41 H5 subtypes, that have evolved from preceding Low Pathogenic Avian Influenza  
 42 viruses (LPAIv) (Banks et al., 2001; Capua and Maragon, 2000; DEFRA, 2008;  
 43 Garcia et al., 1996). The H7N1 LPAI epidemic in Italy in 1999, which preceded the  
 44 HPAI (1999 – 2000) epidemic, is one of the largest LPAI epidemics reported in a  
 45 western country. In the absence of control measures – no compulsory regulations for  
 46 control of LPAI outbreaks were in place – a total of 199 LPAI infected poultry farms  
 47 were detected before the HPAI virus (HPAIv) took over (Busani et al., 2009; Capua  
 48 and Maragon, 2000). Current knowledge does not allow to predict when an H5 or H7  
 49 LPAIv strain will mutate into a HPAIv strain. However, assuming that the molecular

changes necessary for the virulence transformation occur at random, the probability that a LPAIv strain will mutate into a HPAIv strain will depend on the number of virus replicates, which in turn is associated with the number of birds acquiring infection. Hence, besides knowledge of the molecular biology of these viruses, quantitative knowledge of the transmission of LPAIv is important for the development of surveillance programmes that target early detection (see for example Graat et al., (2001)), hence reducing the probability of both undesired mutations and/or large epidemics.

To our knowledge, until now only one study quantifying LPAI virus transmission between chickens has been published in the peer reviewed literature – a chicken-derived H5N2 LPAI precursor virus of the 1983 HPAI epidemic in Pennsylvania (Van der Goot et al., 2003) – and there is no information about the transmissibility between chickens of H7 LPAIv subtypes. The objective of this study was to characterize – by means of transmission experiments – the between-chicken transmission of an H7N1 LPAIv isolated from turkey during the 1999 epidemic in Italy.

## 2. Material and methods

### 2.1 Virus and inoculation

The LPAIv used for this study was the H7N1 LPAI A/turkey/Italy/1067/99 virus isolate. Its intravenous pathogenicity index and amino acid sequence of the hemagglutinin gene (GenBank accession number AF364134) have been published elsewhere (Banks et al., 2001; Capua and Maragon, 2000). Birds were inoculated both intranasally and intratracheally with 0.1 ml/route of inoculum containing  $10^6$  EID<sub>50</sub> (50% egg infectious dose)/ml.

75

76 *2.2 Birds and experimental procedure*

77 A total of two transmission experiments were performed and each experiment  
 78 consisted of two replicates (here referred to as trials). A trial comprised 10 six-week-  
 79 old specific pathogen free (SPF) white leghorn chickens (Charles Rivers, The  
 80 Netherlands). Five of the 10 birds were inoculated and the remaining five kept as  
 81 contacts. The experiments were carried out in the High Containment Unit at the  
 82 Central Veterinary Institute of Wageningen UR in Lelystad using biosafety level 3  
 83 procedures. Birds were housed in different pens in the same experimental room. Next  
 84 to each experimental pen, 2 pairs of birds, housed in separate smaller pens, were  
 85 placed as sentinels to detect whether between-pen (indirect) transmission should be  
 86 considered and monitor independence of the trials. Chickens were housed 1 week  
 87 before challenge to allow adaptation and during this period they were tested for  
 88 presence of antibodies against influenza A virus using an in-house competitive NP  
 89 ELISA (de Boer et al., 1990) and for virus by virus isolation. The day of inoculation  
 90 (day = 0), contacts were removed from their pen and placed back 24 hours later.  
 91 Trachea and cloaca swabs were collected daily from 1 to 10 days post inoculation  
 92 (dpi) and then on 14, 17 and 21 dpi. Serum samples were taken at 7, 14 and 21 dpi.  
 93 All surviving birds were euthanized at 21dpi. All experiments complied with the  
 94 Dutch Law on Animal Experiments and were reviewed by an ethical committee.

95 Trachea and cloaca swabs were used for virus isolation using SPF  
 96 embryonated chicken eggs that were incubated for 9 days. Serum samples were tested  
 97 for antibodies using the Hemagglutinin Inhibition test (HI) using 8 Hemagglutination  
 98 units (HAU) of H7N1 as antigen. Diagnostic procedures were similar as those  
 99 described by van der Goot et al. (2003). A bird was considered infected when it

100 scored positive in virus isolation or serology. A bird was considered infectious for the  
101 time it remained positive in virus isolation.

102

### 103 *2.3 Data analysis*

104 The basic reproduction ratio ( $R_0$ ), which is defined as the average number of  
105 new infections caused by one infectious individual in a susceptible population, was  
106 estimated by the final size (FS) and the generalized linear model (GLM) methods as  
107 described by van der Goot et al. (2003; 2005). The latent period was estimated as the  
108 time (in days) from inoculation to the first day the inoculated chicken was positive to  
109 virus isolation. The infectious period was estimated by fitting a parametric survival  
110 regression model with a Weibull distribution. In some cases, infected birds were  
111 detected positive by virus isolation, late, for example at 10 dpi and were tested again  
112 at 14 dpi when they became negative and assumed to have recovered (Table 1).  
113 These cases were treated as interval censoring in the survival analysis. Survival  
114 analysis and the GLM method were carried out with the statistical software R (R  
115 Development Core Team, 2005).

116

### 117 **3. Results**

118 In the first experiment, all inoculated birds in both trials became infected and  
119 transmitted virus to 4 and 5 contact birds in the first and second trial respectively  
120 (Tables 1a and 1b). In the second experiment (trials 3 and 4) all inoculated and  
121 contact birds became infected (Tables 1c and 1d). No apparent clinical signs were  
122 observed in infected birds. The sentinels remained negative during the course of the  
123 experiment, which confirmed the independence of the trials.

Inoculated birds were positive to virus isolation at 1 dpi. The estimated mean length of the infectious period (IP) was 7.7 (95% Confidence Interval (CI): 6.7 – 8.7) days and the mean estimate of the transmission parameter  $\beta$  was equal to 0.49 (95% CI: 0.30 – 0.75) infections/day per chicken.  $R_0$  was estimated to be 3.8 (95% CI: 1.3 – 6.3) by the GLM method and 4.0 (95% CI: 1.7 – 11.0) by the FS method (Table 2).

#### 4. Discussion

Herein the transmissibility in chickens of the H7N1 LPAIv, which caused a severe epidemic in poultry in Italy in 1999 and later evolved into a HPAI epidemic, was characterized. We have, in particular, estimated  $\beta$  and the IP. These parameters give an indication of how fast the virus would spread to another susceptible bird and how long an infected chicken would remain infectious. Due to, mainly, the subclinical presentation of LPAI infections, it is difficult to accurately follow the course of the infection in field conditions, and characterization of transmission in the field would be limited to estimates of the  $R_0$  (Comin et al., 2010). Our experimental approach allowed us to follow the course of the infection under controlled conditions and have objective estimates of the transmission parameters.

The mean estimates of the IP,  $\beta$  and  $R_0$  in chickens for this H7N1 LPAIv are higher than those observed for an H5N2 LPAIv (van der Goot et al., 2003). This is remarkable because the latter strain was isolated from a chicken whereas our strain originated from turkey. Upon introduction, the virus here studied would reach its peak (highest prevalence of infectious animals) faster and at a higher prevalence than the H5N2 virus. Thus, it could be hypothesized that the infectivity of the flock would be higher, resulting in a higher probability of transmission (assuming similar contact rate) to other flocks. In this scenario, surveillance programmes would need to sample



149 at a higher frequency if the objective is to detect in an early stage infection and to  
150 reduce the probability of between- flock transmissions.

151 Of the two methods used to estimate  $R_0$ , the GLM method yielded a more  
152 precise estimate (Table 2). This is because the GLM method uses all the detailed (day  
153 to day) information about the time course of the infection chain, while the FS method  
154 uses only the total number of animals that got infected throughout the experiment. We  
155 also used the FS method, because with that method there is no dependency on whether  
156 or not there is a latency period, as intrinsically assumed in the GLM method.

157 In conclusion, this study revealed valuable information on transmission  
158 parameters for LPAI virus in chickens. Such information can be used to improve or  
159 develop surveillance programmes and control measures.

160

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204

Table 1. Overview of the transmission process in the four group-transmission trials

Table 1a		Days post – inoculation											Serology
Trial 1	1	2	3	4	5	6	7	10	14	17	21		
Inoculated	+  <sup>a</sup>	+	+	++	++	+	-	-	nd	nd	nd	+ (7) <sup>c</sup>	
Inoculated	+	++	++	++	++	+	+	-	nd	nd	nd	+ (7)	
Inoculated	++	++	+	+	+	+	+	-	nd	nd	nd	+ (6)	
Inoculated	+	+	++	++	+	+	+	-	nd	nd	nd	+ (7)	
Inoculated	+	+	+	+	-	-	-	-	nd	nd	nd	+ (4)	
Contact	nd <sup>b</sup>	-	+	+	++	++	++	-	-	-	-	+ (7)	
Contact	nd	+	-	-	-	+	+	-	-	-	-	- (2)	
Contact	nd	-	-	-	-	-	-	+	-	-	-	+ (5)	
Contact	nd	-	-	-	-	-	-	-	+	-	-	+ (6)	
Contact	nd	-	-	-	-	-	-	-	-	-	-	-	

Table 1b		Days post – inoculation											Serology
Trial 2	1	2	3	4	5	6	7	10	14	17	21		
Inoculated	+	+	+	+	-	-	-	-	nd	nd	nd	+ (8)	
Inoculated	+	++	++	++	++	+	-	-	nd	nd	nd	+ (6)	
Inoculated	+	+	+	+	+	+	-	-	nd	nd	nd	+ (5)	
Inoculated	++	++	++	++	++	+	+	-	nd	nd	nd	+ (6)	
Inoculated	+	+	+	+	++	++	-	-	nd	nd	nd	+ (6)	
Contact	nd	-	-	-	-	+	++	-	-	-	-	-	
Contact	nd	-	-	+	++	++	++	++	-	+	-	+ (6)	
Contact	nd	-	-	-	-	+	+	+	-	-	-	+ (5)	
Contact	nd	-	-	-	-	-	++	+	+	-	-	+ (5)	
Contact	nd	-	-	-	-	++	++	+	-	-	-	+ (6)	

Table 1c		Days post – inoculation												Serology
Trial 3	1	2	3	4	5	6	7	8	9	10	14	17	21	
Inoculated	+	+	+	+	-	+	-	-	-	-	-	-	-	+ (6)
Inoculated	+	+	-	+	+	+	-	-	-	-	-	-	-	+ (3)
Inoculated	+	+	+	+	-	++	-	-	-	-	-	-	-	+ (5)
Inoculated	+	++	++	++	+	+	+	+	-	-	-	-	-	+ (7)
Inoculated	+	+	+	+	+	++	+	+	+	+	-	-	-	+ (5)
Contact	nd	-	-	+	+	-	-	+	-	-	-	-	-	+ (5)
Contact	nd	-	+	+	+	++	+	-	+	-	-	-	-	+ (4)
Contact	nd	-	+	++	+	+	++	+	+	+	-	-	-	+ (6)
Contact	nd	-	-	+	+	+	+	+	+	+	+	-	-	+ (7)
Contact	nd	-	-	+	-	+	-	+	-	-	-	-	-	- (2)

Table 1d		Days post – inoculation												Serology
Trial 4	1	2	3	4	5	6	7	8	9	10	14	17	21	
Inoculated	+	+	+	++	++	+	-	-	-	-	-	-	-	+ (7)
Inoculated	+	++	++	++	++	++	+	-	-	-	-	-	-	+ (7)
Inoculated	+	+	+	+	+	-	-	-	-	-	-	-	-	+ (6)
Inoculated	+	++	++	++	++	+	+	-	-	-	-	-	-	+ (7)
Inoculated	+	+	+	+	+	+	+	-	+	-	-	-	-	+ (6)
Contact	nd	-	-	-	+	++	++	+	+	+	+	+	-	+ (8)
Contact	nd	-	+	+	+	-	++	+	+	+	-	+	-	+ (6)
Contact	nd	-	-	-	-	+	+	+	+	+	-	-	-	+ (6)
Contact	nd	-	+	+	-	-	+	++	+	+	-	-	-	+ (6)
Contact	nd	-	-	++	+	+	+	+	+	+	+	-	-	+ (6)

<sup>a</sup> ++, +|, -|, - : virus isolation results of “trachea | cloaca” swab samples. The single “-” means that both type of samples were negative.

<sup>b</sup> nd: test not done

<sup>c</sup> HI results at day 21 post-inoculation. Number in parenthesis are log<sub>2</sub> HI titers. Titers higher than 2 were considered positive.

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Table 2 Estimates (95% confidence interval) of transmission parameters of H7N1 LPAI

Parameter	Estimates
Number of contacts infected per trial	4, 5, 5, 5
Latent period <sup>b</sup>	≤1
Mean length Infectious period	7.7 (6.7 – 8.7)
Transmission rate $\beta$ (day <sup>-1</sup> )	0.49 (0.30 – 0.75)
Reproduction ratio $R_0$ (GLM) <sup>a</sup>	3.8 (1.3 – 6.3)
Reproduction ratio $R_0$ (FS) <sup>a</sup>	4.0 (1.7 – 11.0)

<sup>a</sup>  $R_0$  estimates using the generalized lineal model (GLM) or the final size (FS) methods.

<sup>b</sup> Inoculated birds were swab-sampled 24 hours post inoculation.