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

4
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24

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

50 changes necessary for the virulence transformation occur at random, the probability
51 that a LPAIv strain will mutate into a HPAIv strain will depend on the number of
52 virus replicates, which in turn is associated with the number of birds acquiring
53 infection. Hence, besides knowledge of the molecular biology of these viruses,
54 quantitative knowledge of the transmission of LPAIv is important for the development
55 of surveillance programmes that target early detection (see for example Graat et al.,
56 (2001)), hence reducing the probability of both undesired mutations and/or large
57 epidemics.

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

66

67 **2. Material and methods**

68 *2.1 Virus and inoculation*

69 The LPAIv used for this study was the H7N1 LPAI A/turkey/Italy/1067/99
70 virus isolate. Its intravenous pathogenicity index and amino acid sequence of the
71 hemagglutinin gene (GenBank accession number AF364134) have been published
72 elsewhere (Banks et al., 2001; Capua and Maragon, 2000). Birds were inoculated
73 both intranasally and intratracheally with 0.1 ml/route of inoculum containing 10^6
74 EID₅₀ (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.

124 Inoculated birds were positive to virus isolation at 1 dpi. The estimated mean
125 length of the infectious period (IP) was 7.7 (95% Confidence Interval (CI): 6.7 – 8.7)
126 days and the mean estimate of the transmission parameter β was equal to 0.49 (95%
127 CI: 0.30 – 0.75) infections/day per chicken. R_0 was estimated to be 3.8 (95% CI: 1.3 –
128 6.3) by the GLM method and 4.0 (95% CI: 1.7 – 11.0) by the FS method (Table 2).

129

130 **4. Discussion**

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

141 The mean estimates of the IP, β and R_0 in chickens for this H7N1 LPAIv are
142 higher than those observed for an H5N2 LPAIv (van der Goot et al., 2003). This is
143 remarkable because the latter strain was isolated from a chicken whereas our strain
144 originated from turkey. Upon introduction, the virus here studied would reach its peak
145 (highest prevalence of infectious animals) faster and at a higher prevalence than the
146 H5N2 virus. Thus, it could be hypothesized that the infectivity of the flock would be
147 higher, resulting in a higher probability of transmission (assuming similar contact
148 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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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	+ ^a	+ -	+ -	+ +	+ +	+ -	-	-	nd	nd	nd	+ (7) ^c	
Inoculated	+ -	+ +	+ +	+ +	+ +	- +	- +	-	nd	nd	nd	+ (7)	
Inoculated	+ +	+ +	- +	- +	- +	- +	- +	-	nd	nd	nd	+ (6)	
Inoculated	+ -	+ -	+ +	+ +	- +	- +	- +	-	nd	nd	nd	+ (7)	
Inoculated	+ -	+ -	+ -	+ -	-	-	-	-	nd	nd	nd	+ (4)	
Contact	nd ^b	-	+ -	+ -	+ +	+ +	+ +	-	-	-	-	+ (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)

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

^b nd: test not done

^c HI results at day 21 post-inoculation. Number in parenthesis are \log_2 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 ^b	≤1
Mean length Infectious period	7.7 (6.7 – 8.7)
Transmission rate β (day ⁻¹)	0.49 (0.30 – 0.75)
Reproduction ratio R_0 (GLM) ^a	3.8 (1.3 – 6.3)
Reproduction ratio R_0 (FS) ^a	4.0 (1.7 – 11.0)

^a R_0 estimates using the generalized lineal model (GLM) or the final size (FS) methods.

^b Inoculated birds were swab-sampled 24 hours post inoculation.

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