Airborne transmission of a highly pathogenic avian influenza virus strain H5N1 between groups of chickens quantified in an experimental setting

D. Spekreijse, A. Bouma, G. Koch, J.A. Stegeman

HAL Id: hal-00719080
https://hal.science/hal-00719080

Submitted on 19 Jul 2012

Accepted Manuscript

Title: Airborne transmission of a highly pathogenic avian influenza virus strain H5N1 between groups of chickens quantified in an experimental setting

Authors: D. Spekreijse, A. Bouma, G. Koch, J.A. Stegeman

Please cite this article as: Spekreijse, D., Bouma, A., Koch, G., Stegeman, J.A., Airborne transmission of a highly pathogenic avian influenza virus strain H5N1 between groups of chickens quantified in an experimental setting, *Veterinary Microbiology* (2010), doi:10.1016/j.vetmic.2011.04.024

1   **Airborne transmission of a highly pathogenic avian influenza virus strain**

2   **H5N1 between groups of chickens quantified in an experimental setting**

3

4   Spekreijse D.[1*], Bouma A.[1], Koch G.[2], Stegeman J.A.[1]

5

6

7   *[1]Department of Farm Animal Health, Faculty of Veterinary Medicine, University of Utrecht,*

8   *P.O. Box 80151, 3508 TD Utrecht, The Netherlands.*

9   *[2]Central Veterinary Institute (CVI), Lelystad, Wageningen University and Research Centre,*

10   *The Netherlands.*

11

12

13   *Corresponding author at: Department of Farm Animal Health, Faculty of Veterinary

14   Medicine, P.O. Box 80151, 3508 TD, Utrecht, The Netherlands. Tel.: +31 30 2531014; fax:

15   +31 30 2539185.

16   E-mail address: d.spekreijse@uu.nl

17

18

19   **Abstract**

20   Highly Pathogenic Avian Influenza (HPAI) is a devastating viral disease of poultry and quick

21   control of outbreaks is vital. Airborne transmission has often been suggested as a route of

22   transmission between flocks, but knowledge of the rate of transmission via this route is

23   sparse. In the current study, we quantified the rate of airborne transmission of an HPAI H5N1

24   virus strain between chickens under experimental conditions. In addition, we quantified viral

25   load in air and dust samples. Sixteen trials were done, comprising a total of 160 chickens

26   housed in cages, with three treatment groups. The first group was inoculated with strain

27   A/turkey/Turkey/1/2005 H5N1, the second and third group were not inoculated, but housed at

28   0.2 and 1.1m distance of the first group, respectively. Tracheal and cloacal swabs were

29   collected daily of each chicken to monitor virus transmission. Air and dust samples were

30   taken daily to quantify virus load in the immediate surroundings of the birds. Samples were

31   tested by quantitative RRT-PCR and virus isolation. In 4 out of 16 trials virus was transmitted

32   from the experimentally inoculated chickens to the non-inoculated chickens. The transmission

33   rate was 0.13 and 0.10 new infections per infectious bird at 0.2m and 1.1 m, respectively. The

34   difference between these estimates was, however, not significant. Two air samples tested

35   positive in virus isolation, but none of these samples originated from the trials with successful

36   transmission. Five dust samples were confirmed positive in virus isolation. The results of this

37   study demonstrate that the rate of airborne transmission between chickens over short distances

38   is low, suggesting that airborne transmission over a long distance is an unlikely route of

39   spread. Whether or not this also applies to the field situation needs to be examined.

40

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

42

43   **1. Introduction**

44

45   Highly Pathogenic Avian Influenza (HPAI), caused by avian influenza viruses of

46   subtype H5 or H7, is one of the most important poultry diseases worldwide (Alexander,

47   2007). The infection spreads rapidly among chickens and between flocks, causing high

48   mortality rates and severe economic losses. Moreover, HPAI virus strains have caused

49   infections in humans (Kallthof et al., 2010) and are considered a risk for a human influenza

50   pandemic. As a consequence, outbreaks of HPAI virus in poultry flocks need to be controlled

51   quickly.

52   Control measures aiming to eliminate HPAI virus often include stamping out infected

53   flocks, pre-emptive culling of flocks at risk to become infected, movement restrictions and

54   bio-safety measures. These control measures may, however, not be sufficient to control a

55   major epidemic in poultry dense regions (Capua et al., 2003; Stegeman et al., 2004; Boender

56   et al., 2007). Moreover, the costs associated with pre-emptive culling are high and the killing

57   of large numbers of uninfected birds evokes ethical discussion in society. Consequently,

58   improvement of the culling strategy, making it both more efficient and acceptable is needed.

59   To increase the effectiveness of control strategies, quantitative information of the

60   possible routes of virus transmission between farms is essential. It has been demonstrated that

61   the probability of between-flock virus transmission decreases with increasing distance

62   between an infected and an uninfected flock (Boender et al., 2007), but the underlying

63   mechanism of transmission still shows considerable gaps. Several routes are considered to be

64   important during AI epidemics, such as movements of visitors, materials, and fomites, but, as

65   shown for some other viral diseases (Gloster et al., 2010; Otake et al., 2010; Li et al., 2009)

66   also airborne transmission has been hypothesised (Chen et al., 2010; Tsukamoto et al., 2007;

67   Yee et al., 2009). Although some of the routes could be controlled by stringent hygienic

68   measures, prevention of virus introduction via airborne route seems hardly feasible in

69   commercial poultry industry. It is therefore important to establish the contribution of airborne

70   infection in the between-farm spread.

71   During an AI epidemic it is difficult to quantify the rate of airborne virus transmission

72   between farms. The rate at which such an epidemic evolves, the need for immediate

73   implementation of control measures and the presence of other routes of transmission that can

74   act as confounding factors hamper a thorough investigation during epidemic episodes. An

75   alternative way to quantify airborne transmission is by carrying out animal experiments. In

76   experiments the occurrence of airborne transmission can be established and the relation

77   between distance to an infectious bird and probability of infection can be quantified in the

78   absence of confounding factors.

79   Tsukamoto et al. (2007) demonstrated the possibility of airborne transmission of HPAI

80   H5N1 virus in an isolator. Moreover, they showed that the likelihood of infection was

81   dependent on the number of infectious birds. From their results, however, we cannot quantify

82   the transmission probability. Moreover, they did not examine the presence of virus in the air.

83   In this paper we describe two experiments which enabled us to quantify the rate of

84   airborne transmission of H5N1 virus strain between chickens at various distances. In addition,

85   we quantified virus concentrations in air and dust samples in the immediate surroundings of

86   the birds.

87

88

89 **2. Materials en methods**

90

91 *2.1. Animals*

92

93   Embryonated eggs from White Leghorn chickens were purchased from a commercial

94 AI-free poultry breeder farm and hatched at the Central Veterinary Institute (CVI), Lelystad,

95 The Netherlands. After hatching, the chickens were housed in one room. At 5 weeks of age,

96 the chickens were tested for the presence of antibodies against AI using a modified indirect

97 double antibody sandwich (IDAS) nucleoprotein (NP)-blocking ELISA. The chickens were

98 subsequently randomly divided in 4 groups, each housed in a separate room. Two

99 experiments were carried out consecutively. In the first experiment 4 groups of 8 chickens

100 were formed. Based on the results of this experiment, we increased the number of chickens in

101 the second experiment in which 4 groups of 32 chickens were used. Feed and water were

102 provided *ad libitum*.

103

104 *2.2. Experimental design*

105

106   Two experiments were carried out, each in 4 isolation rooms under BSL3+ conditions

107 at the CVI . The lay-out of the rooms of the first experiment is shown in Figure 1, of the

108 second experiment in Figure 2. The volume of the rooms was $22m^3$, which were ventilated

109 0.8/h. The temperature was kept at 20ºC and the relative humidity at 55%. The rooms

110 contained two rows of 3 cages each. A cage was constructed of gaze and hardboard and had a

111 size of $1x0.5m^2$. In the first experiment, 2 chickens were placed in the first cage of every row

112 and 1 chicken in the second and third cage each. In the second experiment, 7 chickens were

113 placed in the first and second cage and 2 chickens were placed in the third cage of every row.

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

115   cage 0.4m. Chickens from the first cage of every row were inoculated at day 0. Chickens in

116   the second and third cage were not inoculated. The air circulation in the rooms was

117   determined with a smoke test. The rows were placed in favour of the transmission route. The

118   experiments complied with the Dutch law on animal experiments and were reviewed by an

119   ethical committee.

120

121   *2.3. Inoculum*

122

123   The HPAI virus strain A/turkey/ Turkey/1/2005 H5N1 (clade 2.2) was used as

124   challenge strain for inoculation (Londt et al., 2008; Spekreijse et al., 2011). The virus was

125   grown in embryonated SPF eggs and vials with a known egg infectious dose (EID_{50}) titer

126   were stored at -70ºC until use. On the day of challenge, one vial was thawed and diluted in

127   10-fold dilution steps in tryptose phosphate buffer (TPB) to obtain the necessary inoculation

128   dose of 10^4 $EID_{50.}$ In both experiments, the chickens were inoculated with 0.1 ml inoculum

129   applied intra-nasally and 0.1 ml inoculum applied intra-tracheally using a blunt needle,

130   according to standard protocol (van der Goot et al., 2005; Spekreijse et al., 2011).

131

132   *2.4. Sampling procedures*

133

134   At day 1 post-inoculation (p.i.), swabs from trachea and cloaca were collected from

135   inoculated chickens, and at days 2 to 10, 14, 17 and 21 p.i., from all chickens. The non-

136   inoculated chickens were sampled first, and between the rooms clothes and gloves of the

137   animal handlers were changed. The swabs were put in 2 ml of 2.95% TPB with 5 x 10^3 IU of

138   penicillin-sodium and 5 mg streptomycin per ml and stored at -70ºC until analysed.

139   Serum blood samples were taken from the ulnar vein 7 days before and at days 7, 14

140   and 21 after inoculation from all chickens. The samples were stored at -20°C until analysed.

141   In two of four rooms air samples were taken and in the remaining 2 rooms dust was

142   sampled. Dust and air samples were collected from day 1 to day 10 p.i.

143   Air samples were taken with an MD8 air-scan air sampling device (Sartorius,

144   Nieuwegein, The Netherlands) using sterile gelatine filters of 80mm diameter and 3μm pore

145   size. Samples were taken at an air speed of 8m^{3}/h for 10 minutes, according to the manual of

146   the manufacturer[1]. In both rooms 2 samples were taken; one above the first cage of one row,

147   another above the second cage of the second row. After sampling, the gelatine filters were

148   dissolved in 10 ml of 2.95% TPB with 5 x 10^{3} IU of penicillin-sodium and 5 mg streptomycin

149   per ml at a temperature of 37^{0}C. Dissolved filter solutions were stored at -70°C until analysed.

150   Dust samples were taken using electrostatic dust cloths (Swiffer, Procter and Gamble,

151   U.S.) that were placed in a Petri disk. Dust was sampled for 24 hours. In both rooms 4

152   samples were taken; two per row. The Petri disks were placed on both sides of the second

153   cage. The dust cloths were put in 10 ml of 2.95% TPB with 5 x 10^{3} IU of penicillin-sodium

154   and 5 mg streptomycin per ml and stored at -70°C until analysed.

155   The experiments were terminated 21 days p.i. by euthanizing surviving birds with an

156   intracardiac injection of T-61.

157

158   *2.5. RNA isolation and quantitative real-time reverse transcriptase PCR (RT-qPCR)*

159

160   RNA isolation was performed with the MagNA Pure LC 2.0 instrument (Roche

161   Applied Science, Mannheim, Germany) with the MagNA Pure LC total Nucleic Acid

162   Isolation Kit (Roche Applied Science, Mannheim, Germany). The viral RNA was isolated

---

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

163   from 200µl of swab fluid or filter solution according to the manufacturer's instructions. The

164   nucleic acids were collected in elution buffer and stored at -70°C or directly processed for the

165   quantitative real-time reverse transcriptase PCR (RT-qPCR). The RT-qPCR and data analysis

166   were performed using the MX4000 Quantitative PCR system (Stratagene) with version 4.20

167   software. We used 5µl of the elution buffer with extracted RNA for RT-qPCR as described in

168   van der Goot (2008) to detect the matrix gene of the influenza virus. The viral RNA

169   concentration of each sample could be calculated using a calibration curve of serial dilutions

170   of a standard batch of the virus with a known EID_{50} titer. Dilutions of the standard batch were

171   run along with the unknown samples. Quantification of the viral concentration in each sample

172   was based on the calibration curve generated by plotting the cycle threshold value (C_t-value)

173   against known virus titers. Titers of the samples were expressed as EID_{50} equivalents.

174

175   *2.6. Laboratory tests*

176

177   Sera were incubated for 30min at 56 ºC. A modified indirect double antibody

178   sandwich (IDAS) nucleoprotein (NP)-blocking ELISA that detects antibodies against the

179   nucleoprotein of influenza A was performed as described by de Boer et al. (1990).

180   For virus isolation, per swab three embryonated SPF chicken eggs incubated for 9

181   days were inoculated with 0.2 ml swab fluid per egg. After 72 h the allantoic fluid was

182   harvested and a standard hemagglutination assay (HA) with chicken red blood cells was

183   performed (OIE, 2008). When at least one egg was positive in HA the swab was considered to

184   be positive.

185

186   *2.7. The effect of the gelatine filter on the concentration of HPAI virus*

187

188   The effect of the gelatine filters on the concentration of virus particles was determined.

189   Gelatine filters were placed in Petri dishes and inoculated either with 1 ml of 10^3, 10^4 or 10^5

190   EID_{50}/ml. Two gelatine filters per dose were used. The first filter per dose was dissolved in 9

191   ml of PBS (at 37 degrees) after 10 minutes of incubation. The second filter per dose was

192   dissolved in 9 ml of PBS (at 37 degrees) after 30 minutes of incubation. This number of

193   minutes was considered to be representative for the time between sampling and processing in

194   the lab. As control, the virus stock was treated similarly and dissolved in the same volume of

195   medium. Virus concentration was determined by RNA isolation and RT-qPCR.

196

197   *2.8. Data analysis*

198

199   Airborne transmission was based on the number of infected non-inoculated chickens.

200   A chicken was considered infected if it met one or more of the following criteria: the

201   occurrence of HPAI-like symptoms, a positive RT-qPCR, or both. Positive RT-qPCR results

202   from chickens that did not die were confirmed by virus isolation.

203   The day of infection of the non-inoculated chickens was defined as the first day of

204   virus excretion minus a one day latent period (Spekreijse et al., 2011). Non-inoculated

205   chickens got infected either through air by the inoculated chickens, through air by infected

206   non-inoculated chickens from another cage, or non-inoculated chickens were contact exposed

207   by shedding cage mates.

208   A generalized linear model (GLM) assuming a stochastic SIR (susceptible-infectious-

209   removed) transmission process was used to estimate a separate transmission rate parameter

210   (the average number of infections caused by one infectious bird per day) for every distance

211   (Velthuis et al, 2003).

212   The mean latent and infectious period and the total amount of virus detected in the

213   samples of the inoculated chickens in the two experiments were compared using ANOVA. All

214   statistical tests were performed assuming a 2-sided alternative hypothesis; p values smaller

215   than 0.05 were considered significantly different. Analysis was performed using

216   commercially available statistical software (SPSS 16.0; SPSS Inc., Chicago, Illinois).

217

218   **3. Results**

219

220   *3.1. Infection of inoculated chickens*

221

222   None of the inoculated chickens had pre-existing antibodies against avian influenza

223   virus. In the first experiment 3 out of 16 inoculated chickens escaped infection and remained

224   serologically negative; the other thirteen died. The mean latent period of the inoculated

225   chickens was 1.1 day (95% confidence interval (C.I.): 0.9 – 1.2 days). The mean infectious

226   period of the inoculated chickens (days of virus shedding) was 1.6 day (95% C.I.: 1.4 – 1.7

227   days). In the first experiment, the mean amount of virus shedding on day 1 p.i. was $10^{3.5}$

228   EID_{50} (95% C.I.: $10^{2.5}$ – $10^{4.5}$ EID_{50}), and on day 2 p.i., $10^{5.3}$ EID_{50} (95% C.I.: $10^{5.2}$ – $10^{5.5}$

229   EID_{50}).

230   In the second experiment all 56 inoculated chickens died. The mean latent period of

231   these chickens was 1.1 day (95% C.I.: 1.0 – 1.2 days). The mean infectious period was 1.7

232   day (95% C.I.: 1.6 – 1.8). In the second experiment, the mean shedding of virus on day 1 p.i.

233   was $10^{4.3}$ (95% C.I.: $10^{3.8}$ – $10^{4.8}$ EID_{50}), and on day 2 p.i., $10^{6.3}$ EID_{50} (95% C.I.: $10^{5.3}$ – $10^{6.9}$

234   EID_{50}). The mean latent period, the mean infectious period and the total amount of virus

235   shedding did not differ significantly between the two experiments.

236

237   *3.2. Infection of non-inoculated chickens*

238

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

240   that died during the experiments showed AI-like symptoms, and had one or more positive RT-

241   qPCR results for the tracheal and/or cloacal swabs. The most common AI-like symptoms

242   were loss of appetite, depression and conjunctivitis.

243   In the first experiment none of the non-inoculated chickens became infected, showed

244   clinical signs of infection or developed detectable amounts of antibodies. In the second

245   experiment, 20 out of 72 non-inoculated chickens got infected. In total 17 of these 20 infected

246   chickens died from infection. The infected chickens originated from 7 cages, 16 birds were

247   located in cage 2, and 4 birds in cage 3. Based on the time of infection, we concluded that 6 of

248   these 20 birds became infected by the inoculated chickens and 14 by either their cage mates or

249   by 'air' with virus shed by infectious chickens from another cage.

250   Three non-inoculated infected chickens, one from a second cage and two from a third

251   cage, tested positive once, both in the RT-qPCR and virus isolation, but did not die from

252   infection nor seroconverted. From the moment of infection we derived that 2 birds became

253   infected by the inoculated chickens and 1 became infected from infected chickens from

254   another cage.

255   In 5 rows, the virus did not transmit to the non-inoculated chickens. In 2 rows, the

256   inoculated chickens succeeded in transmission to the non-inoculated chickens in both cages.

257   The distribution of the infected chickens over the different isolation rooms and rows are

258   summarized in Figure 3.

259

260   *3.3. Virus detection in air and dust samples*

261

262   No effect of the gelatine filters was seen on the concentration of HPAI virus

263   (Table 1) and processing filters after 30 minutes did not reduce the quantities measured in

264   RT-qPCR.

265   In the first experiment, 2 air samples tested positive in RT-qPCR and virus isolation.

266   On day 2 p.i., one air sample tested $10^{1.6}$ EID$_{50}$, and on day 3 p.i., one air sample tested $10^{1.3}$

267   EID$_{50}$. Three dust samples tested positive in RT-qPCR and virus isolation. On day 1 p.i., one

268   dust sample tested $10^{1.6}$ EID$_{50}$, and on day 2 p.i., one dust sample tested $10^{1.7}$ EID$_{50}$, and on

269   day 4 p.i., one dust sample tested $10^{2.4}$ EID$_{50}$.

270   In the second experiment, on day 2 and day 3 dust samples tested positive in RT-qPCR

271   and virus isolation ($10^{3.9}$ and $10^{2.2}$ EID$_{50}$, respectively), but none of the air samples tested

272   positive.

273

274   *3.4. Quantification of transmission parameters*

275

276   In the first experiment, no transmission of virus from the inoculated chickens to non-

277   inoculated chickens occurred. In the second experiment transmission did occur and combined

278   with the first experiment, the transmission rate parameters for the various distances were

279   calculated. The transmission parameter for the inoculated chickens to the non-inoculated

280   chickens at a distance of 0.2m was estimated at 0.13 new infections per infectious chicken per

281   day (95% C.I.: 0.01 – 2.73), for the distance of 0.4m between the inoculated and non-

282   inoculated chickens at 0.21/day (95% C.I.: 0 – 9.31), and for the distance 1.1m at 0.10/day

283   (95% C.I.: 0.02 – 0.40). The estimates did not differ significantly for the various distances.

284   The transmission rate parameter for directly exposed chickens was estimated at 1.43/day

285   (95% C.I.: 0.27 – 7.56). This parameter differed significantly from the combined transmission

286   rate parameter (0.12/day; 95% C.I.: 0.06 – 0.26) of the non-inoculated chickens ($p < 0.05$).

287

Discussion

290   The aim of this study was to quantify airborne transmission of an HPAI H5N1 virus

291   strain between chickens housed at various distances. Virus was transmitted to chickens

292   exposed to inoculated chickens over distances of 0.2, 0.4 and 1.1m, but most exposed

293   chickens escaped infection. No statistical difference was found between the rates of

294   transmission over the above mentioned distances. Nevertheless, the combined β of airborne

295   infection was significant lower than the transmission rate between chickens in the same pen (β

296   = 1.43/day). Our findings indicate that airborne transmission over a short distance can occur,

297   but that the rate at which it takes place is low.

298   Throughout the experiments, strict hygienic measures were taken to exclude

299   transmission via other routes than by air, and the flow in the rooms was in favour of airborne

300   transmission. Inoculated chickens shed virus in large quantities, but the amount of virus

301   detected in air samples was low and most times undetectable. Moreover, the test results of the

302   air samples did not correlate with the occurrence of airborne transmission. An explanation for

303   the low proportion of virus positive air samples could be the length of the sampling time.

304   According to the manual of the manufacturer, samples were taken daily for 10', a protocol that

305   had also been used by Weesendorp et al. (2008) to detect classical swine fever virus in

306   ambient air. Moreover, chickens are exposed to virus containing particles much longer, which

307   may have increased the probability of contracting the infection. Stochastic processes may also

308   have occurred. Sampling for a longer time might have increased the number of positive air

309   samples. Optimization of the sampling procedure may be an option for further research.

310   In the first experiment the amount of virus produced by the inoculated chickens was

311   apparently not sufficient for transmission of virus over a distance of 0.2m. We therefore

312   increased the number of inoculated chickens in the second experiment in order to increase the

313 probability of airborne transmission (Tsukamoto et al., 2007). In the second experiment, virus

314 shedding of the inoculated chickens resulted in airborne infection of 6 chickens in 6 cages.

315 The other 14 non-inoculated chickens that got infected were most likely infected either by

316 cage mates (13 chickens) or by airborne-infected chickens from another cage (one chicken).

317 In the second experiment, virus was isolated from three chickens exposed to airborne

318 transmission that did not die from infection nor did they seroconvert. This is remarkable, but

319 we made a similar observation in a previous experiment (Spekreijse et al, 2011). In that

320 experiment a small number of chickens in direct contact with experimentally infected

321 chickens tested positive in RT-qPCR and virus isolation, but did not die from infection, nor

322 seroconverted. These findings suggest that airborne introduction of a HPAI virus may not

323 always result in a major outbreak. In the field, however, the amount of dust produced by large

324 poultry flocks may be high, and dust-borne infection may be facilitated via artificial

325 ventilation. Moreover, in an infected flock the number of infectious birds may be much

326 higher. Therefore, it is difficult to extrapolate the experimental results to a field situation.

327   In studies using influenza virus in other host species, airborne transmission was

328 observed. Stark et al. (1999) for example showed that airborne transmission of influenza virus

329 between pigs was feasible, and Tellier (2009) demonstrated airborne transmission of influenza

330 virus among mice and guinea pigs. Our results showed that the probability of airborne

331 transmission over a distance of 1m is low. Although in our study the relation between distance

332 and probability of infection was not straightforward, as we did not find a significant

333 difference between transmission over the various distances, our results can be used as input in

334 transmission kernels to better understand the indirect transmission of HPAI virus (Keeling et

335 al., 2005).

336   The possibility of long-range transport of other livestock viruses has been reported for

337 other diseases such as foot-and-mouth disease (Amaral Doel et al., 2009; Gloster et al., 2010),

338   porcine reproductive and respiratory syndrome virus (PRRSV) (Dee et al., 2009) and

339   Newcastle disease (Li et al., 2009). This long-range transport was linked to aerosol

340   transmission and meteorological data, but not with the dispersion of dust, suggesting at least

341   air-born transmission. Our findings of transmission via air seems to be consistent with these

342   field observations.

343   In our experiments, dust samples tested positive, although no clear link between

344   transmission and positive dust samples was demonstrated. Infectious particles in dust were

345   previously demonstrated by Sedlmaier et al. (2009) and, moreover, Chen et al. (2010)

346   suggested the possibility of long-range transport of influenza virus through air by dust storms

347   as the attachment of viruses to dust particles could increase their chances of survival. Pitkin et

348   al. (2009) confirmed in a production region model that the use of air filtration under

349   controlled field conditions could significantly reduce airborne transmission of PRRSV

350   between two pig populations. Their results could be useful for the implications of filter

351   systems in the field. Unfortunately, the amount of dust produced by flocks of poultry exceeds

352   the dust produced by pigs and, therefore, it is questionable if the filter system could be

353   implicated easily in poultry houses. To substantiate the hypothesis of long-range dust-borne

354   infection it would be necessary to quantify the amount of dust-borne virus produced by an

355   infected flock and combine that with the dispersion pattern of dust emitted by that flock. This

356   field research can, however, only be done with other viruses than AI, and whether or not this

357   applies to AI should then be evaluated. Nevertheless, our experimental work showed that

358   transmission via air is possible and our estimates may be of value for models used to simulate

359   between flock transmission.

360

361

362

367 **References**

368

369 Alexander D.J., 2007. An overview of the epidemiology of avian influenza. Vaccine 25.

370 5637-44.

371

372 Amaral Doel, C.M., Gloster, J., Valarcher, J.F., 2009. Airborne transmission of foot-and-

373 mouth disease in pigs: evaluation and optimisation of instrumentation and techniques. Vet. J.

374 179 (2): 219-24.

375

376 Boender, G.J., Hagenaars, T.J., Bouma, A., Nodelijk, G., Elbers, A.R.W., de Jong, M.C.M.,

377 van Boven, M., 2007. Risk maps for the spread of highly pathogenic avian influenza in

378 poultry. PLoS Comput. Biol. 3 (4): e71, doi:10.1371/journal.pcbi.0030071.

379

380 Capua, I., Marangon, S., 2003. The use of vaccination as an option for the control of avian

381 influenza. Avian Pathol. 32 (4), 335-343.

382

383 Chen, P.-S., Tsai, F.T., Lin, C.K., Yang, C.-Y., Chan, C.-C., Young, C.-Y., Lee, C.-H., 2010.

384 Ambient influenza and avian influenza virus during dust storm days and background days.

385 Environ. Health Perspect. 118, 1211-1216. doi:10.1289/ehp.0901782.

386

387   de Boer, G.F., Back, W., Osterhaus, A.D.M.E., 1990. An ELISA for detection of antibodies

388   against influenza A nucleoprotein in humans and various animal species. Arch. Vir. 115, 47-

389   61.

390

391   Dee, S., Otake, S., Oliveira, S., Deen, J., 2009. Evidence of long distance airborne transport of

392   porcine reproductive and respiratory syndrome virus and Mycoplasma hyopneumoniae. Vet.

393   Res. 40: 39.

394

395   Gloster, J., Jones, A., Redington, A., Burgin, L., Sorensen, J.H., Turner, R., Dillon, M.,

396   Hullinger, P., Simpson, M., Astrup, P., Garner, G., Stewart, P., D'Amours, R., Sellers, R.,

397   Paton, D., 2010. Airborne spread of foot-and-mouth disease-model intercomparison. Vet. J.

398   183 (3): 278-86.

399

400   Kalthoff, D., Globig, A., Beer, M., 2010. (Highly pathogenic) avian influenza as a zoonotic

401   agent. Vet. Microbiol. 140, 237-245.

402

403   Keeling, M.J., 2005. Models of foot-and-mouth disease. Proc. Biol. Sci. 272 (1569): 1195-

404   202.

405

406   Li, X., Chai, T., Wang, Z., Song, C., Cao, H., Liu, J., Zhang, X., Wang, W., Yao, M., Miao,

407   Z., 2009. Occurrence and transmission of Newcastle disease virus aerosol originating from

408   infected chickens under experimental conditions. Vet. Microbiol. 136 (3-4), 226-32.

409

410   Londt, B.Z., Nunez, A., Banks, J., Nili, H., Johnson, L.K., Alexander, D.J., 2008.

411   Pathogenenis of highly pathogenic avian influenza A/turkey/Turkey/1/2005 H5N1 in Pekin

412   Ducks (*Anas platyrhynchos*) infected experimentally. Avian Path. 37(6), 619-627.

413

414   OIE, 2008. Avian influenza. In: Manual of diagnostic tests and vaccines for terrestrial animals

415   2008. Office International des Epizooties (OIE).

416   http://www.oie.int/eng/normes/mmanual/A_summry.thm.pp.470-471.

417

418   Otake, S., Dee, S, Corzo, C., Oliveira, S., Deen, J., 2010. Long-distance airborne transport of

419   infectious PRRSV and *Mycoplasma hyopneumoniae* from a swine population infected with

420   multiple viral variants. Vet. Microbiol. 145, 198-208.

421

422   Pitkin, A., Deen, J., Dee, S., 2009. Use of a production region model to assess the airborne

423   spread of porcine reproductive and respiratory syndrome virus. Vet. Microbiol. 136, 1-7.

424

425   Sedlmaier, N., Hoppenheidt, K., Krist, H., Lehmann, S., Lang, H., Büttner, M., 2009.

426   Generation of avian influenza virus (AIV) contaminated fecal fine particulate matter (PM

427   2.5): genome and infectivity detection and calculation of immission. Vet. Microbiol. 139,

428   156-64.

429

430   Spekreijse, D., Bouma, A., Stegeman, J.A., Koch, G., de Jong, M.C.M., 2011. The effect of

431   inoculation dose of a highly pathogenic avian influenza virus strains H5N1 on the

432   infectiousness of chickens. Vet. Microbiol. 147 (1-2), 59-66.

433

434   Stark, K.D.C., 1999. The role of infectious aerosols in disease transmission in pigs. The Vet.

435   Journal, 158, 164-181.

436

437   Stegeman, A., Bouma, A., Elbers, A.R., de Jong, M.C., Nodelijk G., de Klerk, F., Koch, G.,

438   van Boven, M., 2004. Avian influenza A virus (H7N7) epidemic in The Netherlands in 2003:

439   course of the epidemic and effectiveness of control measures. J. Infect. Dis. 190 (12), 2088-

440   95.

441

442   Tellier, R., 2009. Aerosol transmission of influenza A virus: a review of new studies. J. R.

443   Soc. Interface 6, S783-S790.

444

445   Tsukamoto, K., Imada, T., Tanimura, N., Okamatsu, M., Mase, M., Mizuhara, T., Swayne, D.,

446   Yamguchi, S., 2007. Impact of different husbandry conditions on contact and airborne

447   transmission of H5N1 highly pathogenic avian influenza virus to chickens. Avian Dis. 51,

448   129-132.

449

450   van der Goot, J.A., Koch, G., de Jong, M.C.M., van Boven, M., 2005. Quantification of the

451   effect of vaccination on transmission of avian influenza (H7N7) in chickens. PNAS 102 (50),

452   18141-18146.

453

454   van der Goot, J.A., van Boven, M., Stegeman, J.A., van de Water, S.G.P., de Jong, M.C.M.,

455   Koch, G., 2008. Transmission of highly pathogenic avian influenza H5N1 virus in Pekin

456   ducks is significantly reduced by a genetically distant H5N2 vaccine. Virology 382, 91-97.

457

458   Velthuis, A.G.J., De Jong, M.C.M., Kamp, E.M., Stockhofe, N., Verheijden, J.H.M., 2003.

459   Design and analysis of an *Actinobacillus pleuropneumoniae* transmission experiment. Prev.

460   Vet. Med. 60, 53 - 68.

461

462   Weesendorp, E., Landman, W.J.M., Stegeman, A., Loeffen, W.L.A., 2008. Detection and

463   quantification of classical swine fever virus in air samples originating from infected pigs and

464   experimentally produced aerosols. Vet. Microbiol. 127, 50-62.

465

466   Yee, K.S., Carpenter, T.E., Farver, T.B., Cardona, C.J., 2009. An evaluation of transmission

467   routes for low pathogenic avian influenza virus among chickens sold in live bird markets.

468   Virology 394, 19-27.

Table 1 revised version   ACCEPTED MANUSCRIPT

1   *Table 1: Effect of gelatine filters on the concentration of HPAI virus determined by RT-qPCR*

Dose groups (EID_{50}/ml)	Time (min)	C_t value (cycles)		Titer (log_{10} EID_{50}/ml)	
		filter	virus stock	filter	virus stock
10^3	10	36.6	36.8	1.9	1.9
	30	36.6	37.6	1.9	1.7
10^4	10	29.8	30.0	3.8	3.7
	30	29.8	30.1	3.8	3.7
10^5	10	26.0	26.6	4.7	4.8
	30	26.2	26.6	4.7	4.8

2

3

Figure 1 revised version

Figure 1. Lay-out of the isolation room of the first experiment. ● *represents the inoculated chickens;* ○ *represents the non-inoculated chickens;* ◁ *represents the location of air sampling;* ◎ *represents the location of dust sampling.*
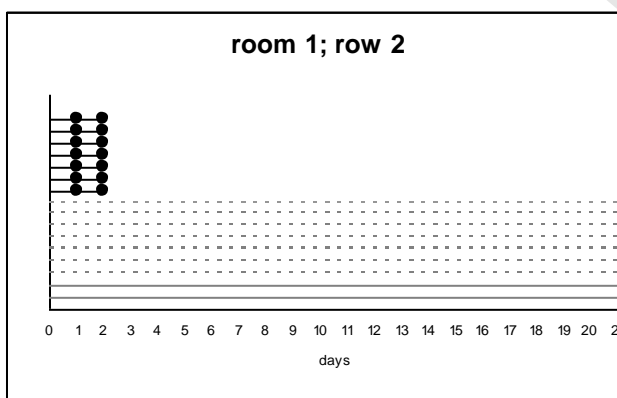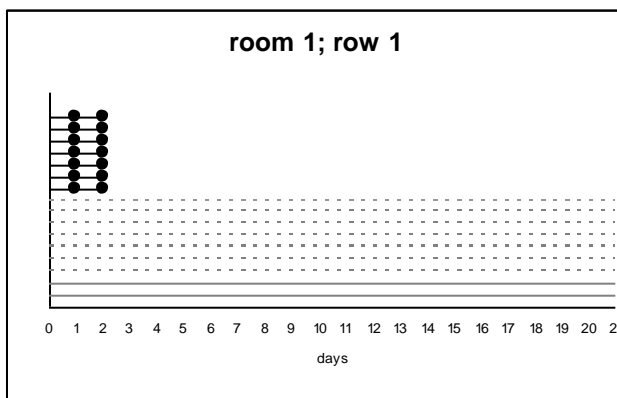
Figure 2 revised version ACCEPTED MANUSCRIPT
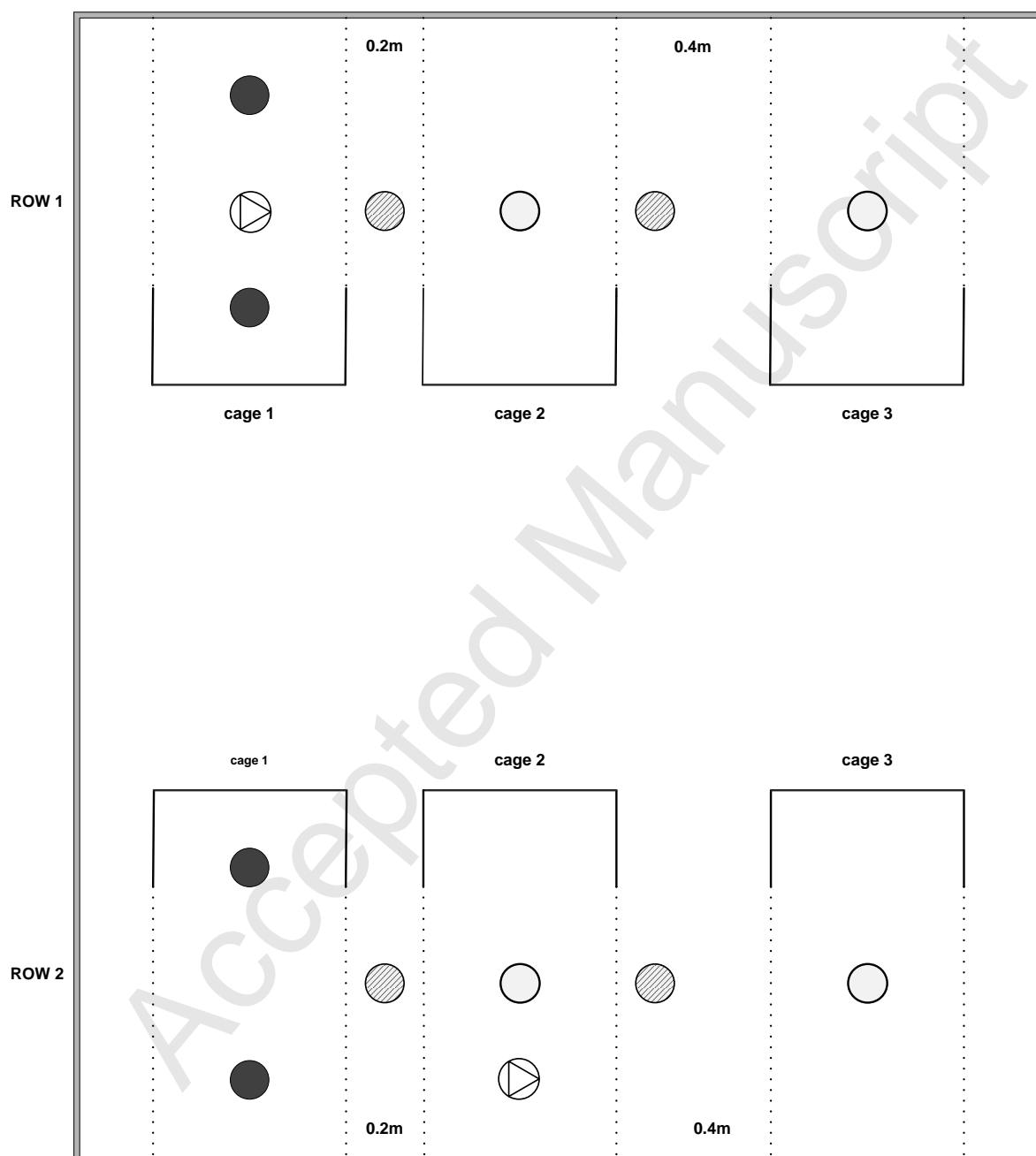
Figure 2. Lay-out of the isolation room of the second experiment. ● *represents the inoculated chickens;* ○ *represents the non-inoculated chickens;* ▷ *represents the location of air sampling;* ▧ *represents the location of dust sampling.*
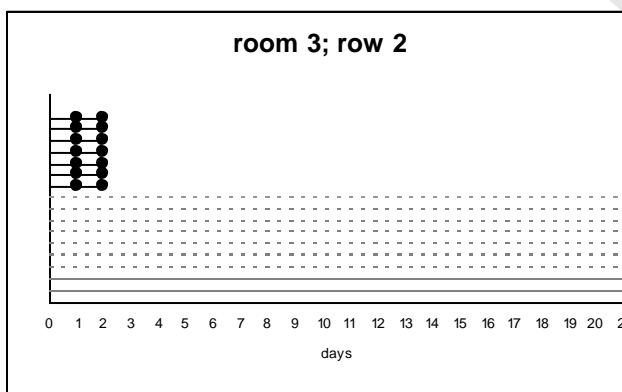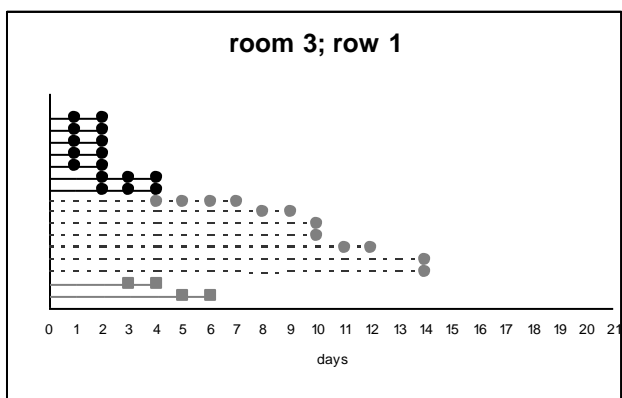
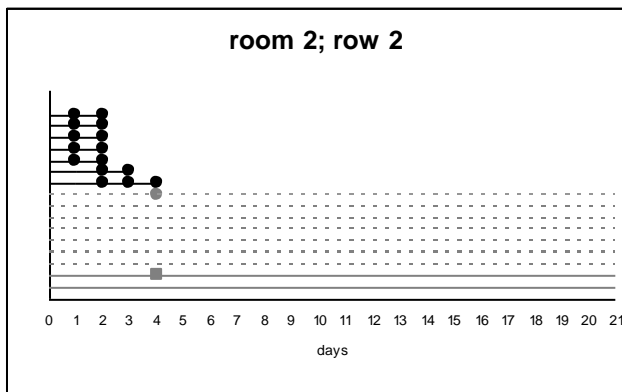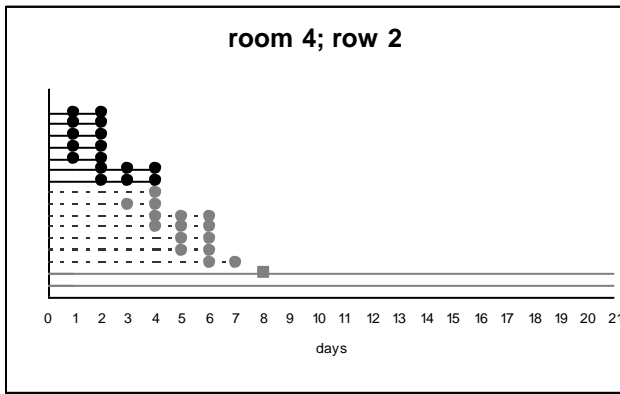**Figure 3 revised version**   ACCEPTED MANUSCRIPT
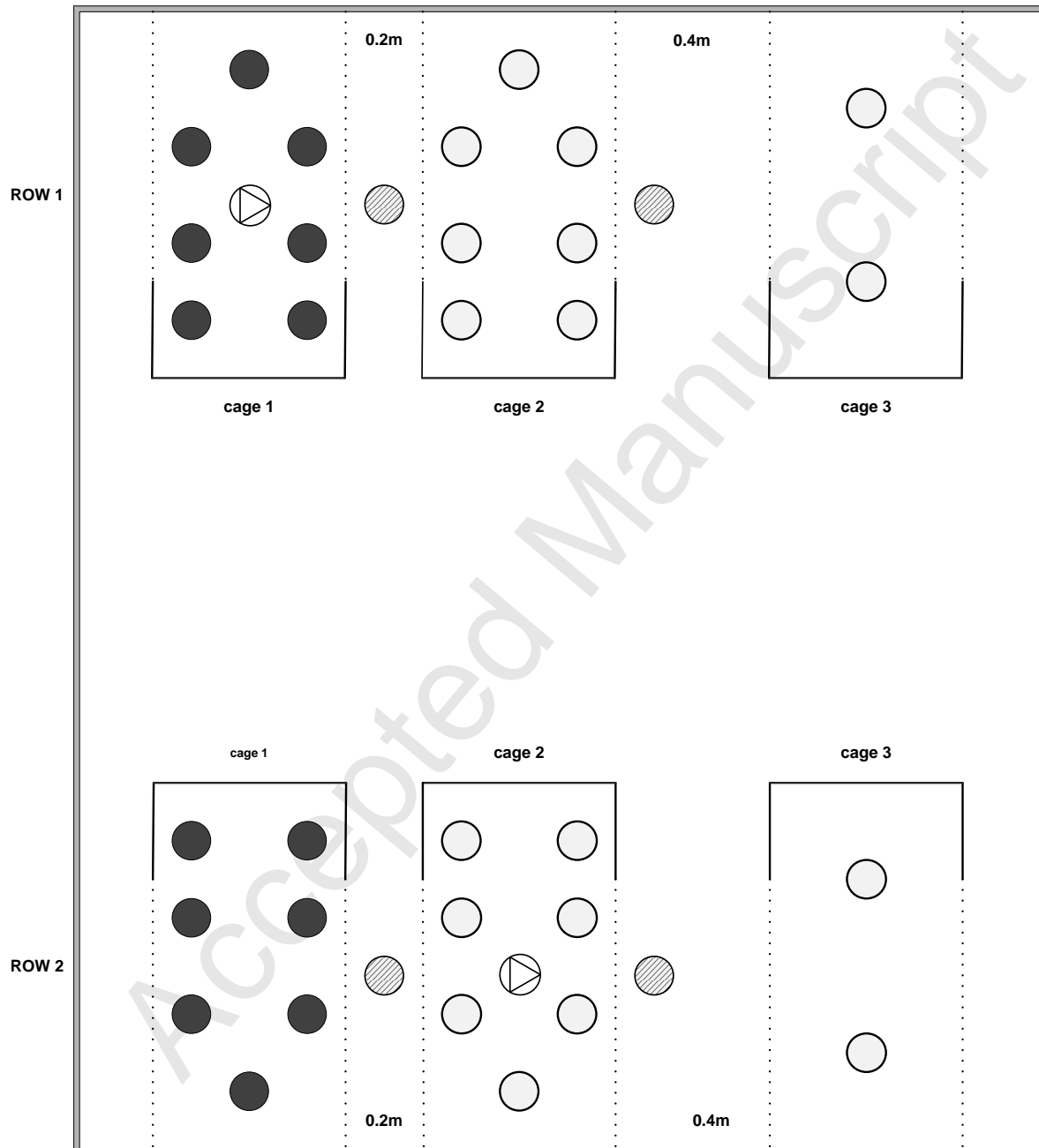
Figure 3. Summary of the RT-qPCR results of the swabs and mortality data of the second experiment. Each figure represents one row of a room. The black line represents an inoculated chicken, the grey dotted line represents a non-inoculated chicken at 0.2m, and the grey line represents a non-inoculated chickens at 1.1m. The dots and squares represent a positive RT-qPCR swab for trachea and/or cloaca. At the end of each timeline the chicken died either by infection or at day 21 chickens were euthanized.

room 2; row 2

room 3; row 1

room 3; row 2

room 4; row 1

room 4; row 2