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1 **Transmission and immunopathology of the avian influenza**  
2 **virus A/Anhui/1/2013 (H7N9) human isolate in three**  
3 **commonly commercialized avian species.**

4  
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31 **Abstract**

32 H7N9 virus infection is a global concern, given that it can cause severe infection and  
33 mortality in humans. However, the understanding of H7N9 epidemiology, animal  
34 reservoir species and zoonotic risk remains limited. This work evaluates the  
35 pathogenicity, transmissibility and local innate immune response of three avian species  
36 harbouring different respiratory distribution of  $\alpha 2,6$  and  $\alpha 2,3$  SA receptors. Muscovy  
37 ducks, European quails and SPF chickens were intranasally inoculated with  $10^5$  embryo  
38 infectious dose (EID)<sub>50</sub> of the human H7N9 (A/Anhui/1/2013) influenza isolate. None  
39 of the avian species showed clinical signs or macroscopic lesions and only mild  
40 microscopic lesions were observed in the upper respiratory tract of quail and chickens.  
41 Quail presented more severe histopathologic lesions and avian influenza virus (AIV)  
42 positivity by immunohistochemistry (IHC), which correlated with higher IL-6  
43 responses. In contrast, Muscovy ducks were resistant to disease and presented higher  
44 IFN $\alpha$  and TLR7 response. In all species viral shedding was higher in the respiratory  
45 than in the digestive tract. Higher viral shedding was observed in quail, followed by  
46 chicken and ducks, which presented similar viral titers. Efficient transmission was  
47 observed in all contact quail and half of the Muscovy ducks, while no transmission was  
48 observed between chicken. All avian species showed viral shedding in drinking water  
49 throughout infection.

50

51 **Impacts**

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- 53 • All studied species exhibited viral shedding, pointing out their role as H7N9 virus  
54 reservoirs, despite not presenting clinical signs. European quail and Muscovy ducks  
55 were able to transmit infection to naïve counterparts, with a 100% and 50%  
56 transmission rate observed respectively. However, chickens were not able to  
57 transmit disease, in despite shedding more virus than Muscovy ducks.
- 58
- 59 • Drinking water was shown for the first time to be involved in viral transmission.  
60 Therefore, water and fomite transmission routes to humans and other animals should  
61 be considered in the control of the disease.
- 62
- 63 • European quails were shown to be more susceptible to disease. Susceptibility, as in  
64 humans, correlated with the higher presence of  $\alpha 2,6$  SA receptors in the upper  
65 respiratory (indicating viral preference for  $\alpha 2,6$  SA receptors *in vivo*) and with a  
66 local pro-inflammatory immune response. In contrast, ducks showed resistance to  
67 disease associated with the up-regulation of anti-viral genes.

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78 **Keywords:** Avian influenza, H7N9, water, quail, duck, chicken.

79 **Introduction**

80 The emergence of novel influenza virus strains from the avian reservoir remains a  
81 constant threat to human and animal health. In March 2013, several humans in China  
82 were reported to be infected with an avian A (H7N9) virus (Gao *et al.*, 2013) and  
83 transmission from poultry to humans was confirmed by phylogenetic analyses (Liu *et*  
84 *al.*, 2014). Since the emergence of the epidemic, and up to August 2016, 798 human  
85 cases have been reported, including at least 319 deaths; yet no strong evidence of  
86 human –to –human transmission has been detected (ECDC, 2016). At present, H7N9  
87 virus has become the most prevalent avian influenza virus (AIV) strain affecting human  
88 in Eastern China (Chen & Wen, 2015). Although the majority of human infections have  
89 been reported in Eastern China, there have been few confirmed cases in Taiwan, Hong  
90 Kong, Malaysia and Canada. All cases were either imported from or had a travel history  
91 to Eastern Chinese provinces (Jeyanthi *et al.*, 2014). Regarding poultry, a recent study  
92 monitoring fifteen cities across five Chinese provinces identified 493 H7N9 viruses  
93 from oropharyngeal swabs of market chickens, with an average isolation rate of 3.0%,  
94 suggesting that the H7N9 virus are also becoming enzootic in Chinese poultry (Lam *et*  
95 *al.*, 2015) .

96  
97 Analysis from several H7N9 human isolates indicates that H7N9 is a reassorted virus  
98 incorporating envelope genes from an H7N3 avian strain and a neuraminidase (NA)  
99 gene from an avian-adapted H7N9 Eurasian lineage strain with the internal genes from at  
100 least two H9N2 avian-adapted influenza strains commonly found in chickens (Liu *et al.*,  
101 2013). However, the H7N9 virus is more virulent in humans than H9N2, which suggests  
102 that the internal genes of H7N9 have mutated (Bi *et al.*, 2015). The sialic acid (SA)  
103 binding site in the hemagglutinin (HA) protein is a major determinant of the virus “host  
104 jump”. Human influenza viruses predominantly bind to  $\alpha$ 2,6 SA host receptors  
105 expressed in the human respiratory tract while AIV predominantly bind to  $\alpha$ 2,3 SA  
106 receptors (de Wit & Fouchier, 2008). Epidemiological data has linked H7N9  
107 transmission to humans who have been exposed to birds in live bird markets (LBM)  
108 (Lam *et al.*, 2013) and studies focused on the H7N9 viral receptor binding shift  
109 elucidated that human H7N9 recognizes both avian and human receptor analogues (Shi  
110 *et al.*, 2013, Liu *et al.*, 2014).

111 Along with the different distribution and differing affinities of influenza viruses for host  
112 SA receptors, the host immune response has been considered as an important  
113 contributor to the final clinico-pathological outcome of avian influenza virus infection  
114 in avian species (Vandervén *et al.*, 2012, Smith *et al.*, 2015). In that sense, dramatic  
115 differences in host responses to avian influenza infection have been found along  
116 different avian species, where duck species are considerably more resistant to avian  
117 influenza viruses (Smith *et al.*, 2015). In the case of H7N9 infection, only one study has  
118 examined which species of poultry are most likely to be infected, or shed sufficient  
119 levels of virus to infect humans (Pantin-Jackwood *et al.*, 2014) but no data about water  
120 transmission and the immune response of the different avian species towards infection  
121 has been presented.

122 The reason why different avian species show very different tolerance to avian influenza  
123 infection has important implications for animal and human health. Here, we evaluated  
124 the pathogenicity, host immune response and transmissibility of an H7N9 human isolate

125 in different commercial avian species, which present varying distribution of  $\alpha$ 2,6 and  
126  $\alpha$ 2,3 SA receptors throughout the respiratory tract and also different susceptibility to  
127 avian influenza.

## 128 **Material and Methods**

### 129 **Virus and Facilities**

130 The A/Anhui/1/2013 (H7N9) was isolated from a Chinese patient during the 2013  
131 H7N9 outbreak in China and was kindly provided by the Instituto de Salud Carlos III  
132 (Madrid). The virus was passaged at least 3 times in specific pathogen free (SPF)  
133 embryonating chicken eggs (ECEs) from the original patient sample and virus stocks  
134 were produced in SPF ECEs. Viral titration was performed following standard  
135 procedures. Briefly, inoculated SPF ECEs allantoic fluids were harvested at 96 hours  
136 post-inoculation (hpi) and tenfold diluted in phosphate buffer saline (PBS) for titration  
137 in 9-day-old SPF ECEs. The mean embryo infectious dose (EID<sub>50</sub>) was determined by  
138 the Reed and Muench method (Reed, 1938). Viral titer was 10<sup>8,79</sup> EID<sub>50</sub>. All  
139 experiments were performed under Biosafety level 3 (BSL3) containment facilities at  
140 the *Centre de Recerca en Sanitat Animal (CRESA)*, Barcelona, Spain.

### 141 **Animals**

142 European quail (*Coturnix coturnix*) (Urgasa S.A., Lleida, Spain) and Muscovy ducks  
143 (*Cairina moschata*) (Miquel Avícola, Girona, Spain) of approximately 1 month and 11  
144 days of age respectively were used in this study together with SPF chickens (*Gallus*  
145 *gallus domesticus*) of 14 days of age (Charles River, SPAFAS, MA, USA) that were  
146 hatched and subsequently placed in negative pressure isolators under BSL-3  
147 containment conditions at CRESA. Quail and ducks were kept one week for  
148 acclimatization. Feed and water were provided *ad libitum* throughout the experiment.  
149 All procedures were performed according to the requirements of the Ethical  
150 Commission of Animal Experimentation of the Autonomous Government of Catalonia.  
151 At 14 days of age, chickens were randomly divided into groups. After acclimatization,  
152 animals were divided into experimental groups and each group was housed in a  
153 different negative pressured isolator with HEPA-filtered air in the animal BSL-3  
154 facilities of CRESA. Before the infection, serum samples of all quail and ducks were  
155 confirmed to be seronegative for AIV by a competition ELISA test (c-ELISA) (IDVET,  
156 Montpellier, France). Furthermore, oropharyngeal (OS) and cloacal (CS) swabs of 5  
157 quail and 5 ducks were ensured to be negative for AIV by real time RT-PCR (qRT-  
158 PCR) prior to infection.

### 159 **Experimental design**

160 Twenty-five animals of each species were randomly separated into two groups with  
161 twenty challenged birds/group and one control group with 5 birds (Table 1). For each  
162 challenged group, birds were further subdivided into two experimental groups, A and B  
163 (n=10 animals/group). Groups A were used to evaluate morbidity, transmissibility, and  
164 viral shedding pattern. Groups B were used for the pathological studies. All animals in  
165 the challenged groups were inoculated intranasally with 10<sup>5</sup> EID<sub>50</sub> of H7N9 diluted in  
166 PBS in a final volume of 0.05 ml (0.0025ml each nostril), except four birds of each  
167 group A which were used as contact animals. Thus, they were placed into the isolators  
168 24 hours after inoculating the other birds and after changing drinking water. Control

169 birds were inoculated intranasally with 0.05ml of PBS solution. Amounts of virus were  
170 verified by performing a qRT-PCR of both the original non-diluted viruses and the  
171 inocula.

## 172 **Sampling**

173 All birds were daily monitored for clinical signs. OS, CS and feather pulp (FP) samples  
174 were obtained daily until 8 dpi and at 10, 12 and 14 dpi in all animals belonging to the  
175 groups A to measure viral shedding by qRT-PCR. Drinking water was changed on a  
176 daily basis after sampling 0.5ml of water collected with a 1 ml syringe at the same time  
177 points. The same samples were collected from control groups. Swabs and FP samples  
178 were placed in 0.5 mL of Dulbecco's Modified Eagle's Medium (DMEM)  
179 (BioWhittaker®, Lonza, Verviers, Belgium) with 600µg/ml penicillin and streptomycin.  
180 All samples and drinking water were stored at -75 °C until further use.

181 At 14 dpi, blood samples were collected before euthanasia in animals belonging to  
182 groups A to detect AIV antibodies by c-ELISA testing. As it was terminal, bleeding was  
183 done from the heart after previous anaesthesia with intramuscular injection of  
184 ketamine/xylazine (10g/kg body weight, Imalgene® 1000 and 1g/kg body weight,  
185 Xilagesic® 2%).

186 At 1, 3, 5, 8 and 14 dpi, 2 animals from groups B and 1 animal from the control group  
187 were euthanized using intramuscular sodium pentobarbital (100mg/kg, Dolethal®,  
188 Vetóquinol, Cedex, France). All birds from B and control groups were necropsied to  
189 evaluate gross lesions and samples were taken for histopathological and  
190 immunohistochemical examination as well as to assess immune response.

## 191 **Pathologic examination and immunohistochemical testing**

192 Necropsies and tissue sampling were performed according to standard protocols. Brain,  
193 trachea, nasal turbinate, lung, heart, skin, thymus, bursa of Fabricius, liver, kidney,  
194 adrenal gland, gonad, duodenum-pancreas, jejunum-ileum, cecum/cecal tonsil, colon,  
195 and rectum were taken for histological examination according to standard protocols.  
196 The tissues were fixed (for 48 h) in neutral-buffered 10% formalin, then embedded in  
197 paraffin wax, sectioned at 3 µm, and stained with haematoxylin and eosin (HE) for  
198 examination under light microscopy. Alongside, nasal turbinate samples (5mm<sup>2</sup> approx.)  
199 were collected and immediately stored at 70°C on *RNA-later* (RNAlater®, Invitrogen,  
200 Thermo-Fisher Scientific, MA, US) until used for RNA extraction. For the detection of  
201 IAV antigen by immunohistochemistry (IHC), the trachea, nasal turbinate, lung,  
202 duodenum-pancreas, jejunum-ileum, cecum/cecal tonsil, colon and rectum were stained  
203 with a primary antibody against the influenza A nucleoprotein (NP) as previously  
204 described (Haines & Chelack, 1991, Bertran *et al.*, 2013). The positive control consisted  
205 of a formalin-fixed paraffin-embedded heart from a chicken experimentally infected  
206 with influenza virus. The same section in which the specific primary antibodies were  
207 substituted with PBS was used as negative controls.

## 208 **Immune gene expression profiles**

209 Gene expressions of interleukin 6 (IL-6), toll like receptor (TLR) 7 and interferon (IFN)  
210 α in each avian species and retinoic acid inducible (RIG-I) in Muscovy ducks were  
211 assessed by qRT-PCR. Primer sequences are described in (Cornelissen *et al.*, 2012, Uno

212 *et al.*, 2013). Primers were diluted at 2,5 mM following manufacturer instructions.  
213 Briefly, RNA extraction was performed on the nasal turbinate tissue samples of control  
214 and infected animals on B groups. RNA extraction was performed with an RNeasy mini  
215 RNA purification (Qiagen, Valencia, CA) using RNA stabilization and on-column  
216 DNase digestion protocols (Qiagen, Valencia, CA). Reverse transcription was  
217 performed using an ImProm-II reverse transcription system (Promega, Madison, WI) at  
218 0.5 µg RNA. PCR was performed using a Power SYBR green kit (Applied Biosystems,  
219 Foster City, CA) and Fast 7500 equipment (Applied Biosystems, Foster City, CA,  
220 USA). The expression levels were normalized using the house-keeping gene β-actin  
221 (ACTB), and the results were expressed as arbitrary units. Gene expression profiles  
222 from infected animals were then normalized with the median gene expression of control  
223 animals. It was considered up-regulated when the expression change was upon 1 and  
224 down-regulated when below 1. Data visualization was performed with GraphPad Prism  
225 6 (GraphPad Software, La Jolla, CA, USA).

## 226 **Viral RNA detection by qRT-PCR**

227 Viral RNA from OS, CS, FP, and drinking water samples was extracted with  
228 NucleoSpin® RNA Virus Kit (Macherey-Nagel, Düren, Germany) following the  
229 manufacturer's instructions. The resulting viral RNA extracts were tested by a TaqMan  
230 one-step qRT-PCR. Briefly, a qRT-PCR assay was used to detect the viral (*M*) gene  
231 fragment in Fast7500 equipment (Applied Biosystems, Foster City, CA, USA) using the  
232 primers and probe previously described (Spackman *et al.*, 2002) and the amplification  
233 conditions described by (Busquets *et al.*, 2010). The limit of detection of the technique  
234 was 1.89 log<sub>10</sub> viral RNA copies/sample.

235 **Serology**  
236 At 14 dpi, serum was collected from animals belonging to the A and control groups and  
237 tested by c-ELISA to detect antibodies against the NP of AIV using the commercially  
238 available kit ID Screen® Influenza A antibody competition (IDVET, Montpellier,  
France), according to manufacturer's instructions.

## 239 **Results**

240 Main results including mortality, transmission rate, viral shedding peak day and serology  
241 are summarized in Table 1.

## 242 **Clinical signs and gross lesions**

243 Any quail, duck or chicken presented clinical signs throughout the experimental  
244 infection. In general, no macroscopic lesions were observed in the organs of any bird  
245 species at necropsy. Except for one infected quail, on group B, which presented  
246 fibrinosuppurative nasal secretion at 1 dpi.

## 247 **Pathologic examination and immunohistochemical testing**

248 Histopathological examination of animals within groups B revealed that microscopic  
249 lesions were restricted to the upper respiratory tract of quail and chicken.

250 In general, quail presented more severe histopathological lesions than chickens. Lesions  
251 were characterized by a mild to moderate catarrhal and/or lymphoplasmacytic rhinitis  
252 and sinusitis (Figure 1) that in quail lasted from 1 to 8 dpi and in chicken from 1 to 5

253 dpi. In addition, inoculated quails also presented lymphocytic tracheitis at 5 dpi. The  
254 remaining organs lacked significant histopathologic lesions.

255 Viral detection by IHC was performed in respiratory and digestive organs of infected  
256 and control birds. AIV antigen detection was only observed in the upper-respiratory  
257 tract of infected quail and chicken. Quails presented the higher detection of AIV  
258 positive cells by IHC. In quail, positivity was observed in the nucleus of glandular and  
259 respiratory/olfactory epithelial cells of the nasal turbinates from 1 to 5 dpi (Figure 1),  
260 and in the epithelial cells of the trachea at 5 dpi. Of infected chickens, AIV positivity by  
261 IHC was only observed on scarce respiratory and olfactory epithelial cells of the nasal  
262 turbinates at 3 and 5 dpi (Figure 1).

263 Muscovy ducks did not present any histopathological lesion or detection of AIV antigen  
264 by IHC in any examined organ (Figure 1).

### 265 **Immune gene expression profiles**

266 Immune gene expression levels observed in the nasal turbinates of chicken, ducks and  
267 quail are represented in (Figure 2). Two animals per group and day were tested. Higher  
268 expression levels of all cytokines and PRRs were observed in infected animals in  
269 comparison to control animals. Chickens followed by ducks presented the higher  
270 expression of IFN $\alpha$  and TLR7, particularly at 1dpi. In general, quail showed low  
271 expression of IFN $\alpha$  and TLR7 throughout infection. In contrast, quail presented the  
272 highest expression of IL-6 at 3 and 5 dpi. RIG-I was found to be slightly up-regulated at  
273 1 dpi but not the remaining days.

### 274 **Viral RNA detection by qRT-PCR**

275 Results for viral shedding in OS, CS and FP of animals in A groups are represented in  
276 (Figure 3). Quail presented the higher levels of viral shedding followed by chicken and  
277 Muscovy duck, which presented similar viral shedding levels.

278 All inoculated and contact quail presented high levels of viral shedding on OS from 1 to  
279 10 dpi. All contact quail also presented higher levels of viral excretion on OS, from 3 to  
280 11 days post contact (dpc). OS viral shedding levels on contact quail were higher than  
281 in inoculated animals. Most inoculated and contact quail presented viral shedding on  
282 CS. Challenged quail presented viral shedding on CS throughout the experimental  
283 infection, whilst contact quail only showed viral secretion on CS from 5 dpc onwards.  
284 Two out of six challenged and all contact quail showed viral shedding on FP. Quail  
285 presented the higher levels of viral genome on drinking water during all the  
286 experimental infection.

287 Most of inoculated chickens showed viral shedding on OS which was sustained until 10  
288 dpi. Viral shedding on CS of inoculated chickens was only observed in 1 animal at 5  
289 dpi. No viral shedding was observed in the FP of inoculated chicken or in the CS, OS  
290 and FP of contact chicken at any time point. In contrast, viral genome was detected in  
291 the drinking water of chicken throughout all the experimental infection.

292 Half of the inoculated Muscovy ducks showed high levels of viral excretion in OS, from  
293 1 to 8 dpi and half of the contact ducks presented viral shedding in OS from 3 dpc  
294 onwards. Only one duck presented viral shedding in CS, at 1 and 8 dpi, and, in FP at 10



295 and 14 dpi. Interestingly, viral RNA was detected on the drinking water of Muscovy  
296 ducks throughout infection.

## 297 **Serology**

298 All inoculated and contact quails presented detectable titers of antibodies against AIV at  
299 14 dpi and 13 dpc. One inoculated and one contact Muscovy duck seroconverted at 14  
300 dpi and 13 dpc, respectively. All contact chicken had undetectable antibody titers at 13  
301 dpc. Serology results from inoculated chickens are not available.

## 302 **Discussion**

303 The constant AIV outbreaks detected around the world in poultry and humans pose a  
304 significant economic threat to poultry industry, and to public health (Liu *et al.*, 2014).  
305 The H7N9 virus subtype is a major global concern, given that it has led to severe  
306 infection and mortality in humans, but causes no clinical disease in avian species  
307 (Morens *et al.*, 2009). The understanding of the H7N9 epidemiology, including the  
308 main reservoirs of the virus, remains limited. For this reason, the H7N9 virus interaction  
309 with the avian species that can be a source of human infection should be clarified. Here,  
310 we evaluated the pathobiology, host immune response and transmission capacity of a  
311 human H7N9 viral isolate in 3 different avian species (chickens, quail and Muscovy  
312 ducks) commonly commercialized in LBM.

313 In this study, none of the tested avian species presented any clinical sign or macroscopic  
314 lesion throughout the experiment. Quail followed by chicken were shown to be more  
315 susceptible to disease presenting microscopic lesions and detection of AIV antigen in  
316 the respiratory tract, while Muscovy ducks were shown to be resistant to the infection.  
317 These results are in agreement with a previous study on H7N9 susceptibility in different  
318 avian species (Pantin-Jackwood *et al.*, 2014). Microscopic lesions were restricted to the  
319 upper-respiratory tract of quail and chicken, and were consistent with low-pathogenic  
320 AIV infection in these species (Bertran *et al.*, 2013).

321 The nasal mucosal tissue is the first to come into contact with aerosol-associated  
322 viruses. If H7N9 virus is successful in invading the respiratory epithelial cells, it can  
323 spread to both non-immune and immune cells. Due to that, the correct function of the  
324 innate immune system is required in the earliest phases of microbial infection for  
325 limiting the spread of the pathogen until adaptive responses are activated to clear the  
326 infection. Albeit, this study evaluated the immune response of a limited number of  
327 animals, our results showed an association between the immune profiles in the different  
328 avian species and the corresponding histopathological lesions and viral replication  
329 pattern. Quail, followed by chicken, presented the higher up-regulation of the pro-  
330 inflammatory cytokine IL-6, which is released after influenza infection and has been  
331 associated with the recruitment of inflammatory cells, and severe pathology in chickens  
332 (Kuribayashi *et al.*, 2013, Kuchipudi *et al.*, 2014, Fukuyama & Kawaoka, 2011).  
333 Besides, chickens followed by Muscovy ducks, presented an early up-regulation of  
334 TLR7 and IFN $\alpha$  genes in the nasal cavity. This is in agreement with results obtained in  
335 previous LPAIV infections in chickens and Pekin ducks (Cornelissen *et al.*, 2012) and,  
336 in our study, correlated with the lower viral replication observed in these species in  
337 comparison to quail. TLR7 is a pathogen recognition receptor (PRR), activated by the  
338 recognition of single-stranded RNA. TLR7 activation has been associated with the up-  
339 regulation of IFN $\alpha$  after AIV infection, that promotes an antiviral effect by inducing the

340 synthesis of proteins that interfere with viral replication (Keestra *et al.*, 2013). However,  
341 in the present study, IFN $\alpha$  and especially TLR7 up-regulation in ducks was not as  
342 relevant as in chickens, despite ducks being less susceptible to infection.  
343

344 Recently it has been suggested that the limited pathogenicity of AIV in ducks reflects a  
345 successful antiviral innate immune response (Smith *et al.*, 2015). This differential  
346 immune response towards infection has been related to different *in vivo* host immune  
347 responses matched by differences in selection pressures and evolutionary history of  
348 interferon-stimulated genes (Barber *et al.*, 2010, Vanderven *et al.*, 2012, Smith *et al.*,  
349 2015). In that sense, the up-regulation of RIG-I, an intracellular receptor for viral  
350 ssRNA that is present in ducks and leads to IFN $\alpha$  release (Magor *et al.*, 2013, Barber *et al.*,  
351 2010), has been related to the ability of ducks to resist or delay infection with avian  
352 influenza viruses (Smith *et al.*, 2015). Furthermore, the NS1 protein of some AIV has  
353 evolved to block RIG-1 signalling, indicating that RIG-I mediated recognition is a key  
354 antiviral determinant in infected hosts (Barber *et al.*, 2010, Mibayashi *et al.*, 2007).  
355 However, RIG-I was only slightly up-regulated in ducks' nasal turbinate at 1 dpi, in this  
356 study. These results may indicate that the natural resistance of duck to H7N9 infection  
357 is not only related to the early innate immune response but also to other viral or host  
358 factors, such as the presence and affinity of influenza virus receptors. Further studies  
359 increasing the number of animals analysed should be carried out in order to clarify this  
360 hypothesis.

361 In correspondence with the above results, quail showed the highest levels of viral  
362 shedding throughout infection, followed by chickens and Muscovy ducks. In general,  
363 viral shedding was much higher in the upper respiratory tract than in the digestive  
364 system in all avian species studied. This was expected taking into account that poultry-  
365 adapted AIVs are normally highly shed in the respiratory tract of gallinaceous poultry  
366 (Johnson & Mueller, 2002, Morens *et al.*, 2008) and as previous studies have shown for  
367 H7N9 infection (Pantin-Jackwood *et al.*, 2014). High viral shedding levels were  
368 observed in OS of all inoculated quail throughout infection, and effective viral  
369 transmission was observed to all contact animals, which presented even higher viral  
370 shedding levels in OS. In addition, most challenged and all contact quail showed low  
371 viral load at FP and higher viral titers in drinking water during infection. This data  
372 provides more evidence of the high susceptibility of quail to AIVs (Bertran *et al.*, 2013,  
373 Bonfante *et al.*, 2013) and their important role in H7N9 epidemiology.

374 After quail, chickens presented the higher levels of viral shedding in OS. However, viral  
375 shedding was only observed on 4 of 6 inoculated chickens and only one animal  
376 presented viral shedding on CS. More importantly, no transmission was observed  
377 between inoculated and contact chickens throughout infection, even though viral titers  
378 were detected in the drinking water during infection. These results are striking since  
379 chickens are considered as the primary source of H7N9 infection in humans (Husain,  
380 2014). In addition, a previous report showed that H7N9 virus was successfully  
381 transmitted from infected to naïve contact chickens through direct contact (Kalthoff *et al.*,  
382 2014). However, recent studies have suggested that the H7N9 virus is poorly  
383 adapted to chickens and could not be transmitted efficiently to naïve chickens or ferrets  
384 (Ku *et al.*, 2014, Spackman *et al.*, 2015). In that sense, the use of different virus  
385 inoculation routes has been suggested as an explanation for the different results (Husain,  
386 2014, Spackman *et al.*, 2015).

387 Of particular interest are the results observed in Muscovy ducks. Chicken-adapted AIVs  
388 replicate better in chickens than in ducks (Spackman *et al.*, 2010, Jackwood *et al.*, 2010,  
389 Pillai *et al.*, 2010). However, our results showed similar viral shedding levels in chicken  
390 and Muscovy duck, which also presented higher transmission capacities, despite the fact  
391 that chickens were more susceptible to disease. In this study, half of challenged and  
392 contact Muscovy ducks showed relatively high levels of viral shedding in OS  
393 throughout infection and 1 inoculated and contact animal also presented viral shedding  
394 on CS. In addition, Muscovy ducks showed to shed the virus in the water throughout  
395 infection. This confirms efficient viral transmission between Muscovy ducks despite not  
396 presenting any clinical signs, or histopathological lesions. Muscovy ducks are domestic  
397 waterfowl frequently present in LBM in China and are known to be more susceptible to  
398 infection with highly pathogenic H5N1 than other domestic ducks (Cagle *et al.*, 2012).  
399 In correlation with our results, it has been shown that Muscovy ducks presented more  
400 H7N9 viral shedding than other duck waterfowl, namely Pekin and Mallard ducks  
401 (Pantin-Jackwood *et al.*, 2014).

402 The host restriction of AIV is determined by the distribution patterns of SA receptors in  
403 the upper respiratory tract. Ducks have mainly  $\alpha 2,3$  receptors, quail possess mainly  $\alpha 2,6$   
404 receptors and chickens possess both  $\alpha 2,3$  and  $\alpha 2,6$  SA receptors in the upper respiratory  
405 tract, while humans possess  $\alpha 2,6$  (de Graaf & Fouchier, 2014, Costa *et al.*, 2012). In  
406 general AIVs attach better to  $\alpha 2,3$  SA receptors (de Wit & Fouchier, 2008). However,  
407 sequence analysis of the H7N9 influenza viral proteins revealed that the virus has  
408 acquired several amino acid changes associated with adaptation to human receptor  
409 binding  $\alpha 2,6$  SA in the HA gene (Q226L and G186V) (Xu *et al.*, 2013, Watanabe *et al.*,  
410 2013), indicating that H7N9 recognizes both  $\alpha 2,6$  and  $\alpha 2,3$  receptors (Shi *et al.*, 2013,  
411 Liu *et al.*, 2014, Josset *et al.*, 2014, Xiang *et al.*, 2013). Accordingly, the results  
412 obtained in this study showed that susceptibility of the different avian species used to  
413 H7N9 infection correlates with the presence of both receptors in the upper respiratory  
414 tract.

415 Human H7N9 transmission has been related to incidental poultry exposure, particularly  
416 in LBM (Lam *et al.*, 2013). However, the exact role of poultry and the environment in  
417 the transmission of H7N9 to human is not well understood. This is due to the fact, that  
418 high H7N9 incidence in humans is observed, despite H7N9 prevalence in birds has been  
419 reported to be low (Ministry of Agriculture, 2014). Recent reports have suggested that  
420 indirect contact may play a role in the transmission of H7N9 from birds to humans,  
421 pointing to contaminate water sources as possible source of transmission as observed  
422 between wild birds (Alexander, 2007, Jones *et al.*, 2015). In that sense, viral persistence  
423 of H7N9 in water has been reported in our study, during the entire experimental  
424 infection, suggesting that this may play an important role of in viral transmission,  
425 particularly in Muscovy ducks. In addition, recent studies have confirmed that H7N9  
426 was found to be highly tolerant to moderately water acidic and alkali conditions (Zou *et al.*,  
427 2013).

428 In this study, effective viral transmission from inoculated animals to naïve contact quail  
429 and Muscovy ducks has been confirmed, even though the original host was human.  
430 These findings suggest either quick H7N9 virus adaptation, or that adaptation may not  
431 be needed for H7N9 to replicate and be transmitted between human and avian species,  
432 confirming the substantial role of commercial avian species in H7N9 epidemiology. The  
433 fact that quail are more susceptible to human origin H7N9 infection may elucidate the  
434 preference of H7N9 for  $\alpha 2,6$  receptors *in vivo*, as quail present a higher proportion of

435  $\alpha$ 2,6 receptors on the upper respiratory tract therefore being more susceptible through  
436 the oronasal route infection. On the contrary, chickens did not show transmission, in  
437 spite the fact that infected chickens presented high viral oral shedding, viral shedding in  
438 water, were susceptible to H7N9 disease, and express both  $\alpha$ 2,3 and  $\alpha$ 2,6 receptors in  
439 their respiratory tract. Finally, Muscovy ducks appeared to play an important role as  
440 possible biological vector of H7N9, showing efficient viral shedding and transmission.  
441 These differences in response, shedding and transmission of AIV in different avian  
442 species should be taken into account when determining which species are involved in  
443 the transmission of emerging viruses.

444 Control of H7N9 is complicated by the lack of disease signs in poultry. In the case of  
445 H7N9 surveillance, OS swab and water testing could be used as a tool for successful  
446 virus detection in active H7N9 surveillance in quail, Muscovy ducks and chickens, as it  
447 has been assessed for other avian species in which pathogenesis is still poorly  
448 understood (Bertran *et al.*, 2013, Kilbourne, 1975, Scholtissek *et al.*, 1978). Further  
449 studies focused on the prevalence of H7N9 infection in different minor gallinaceous  
450 species in the endemic areas would be desirable to better understand and control the  
451 avian-human transmission.

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## 456 **Competing interest**

457 The authors declare that they have no competing interests.

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