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

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This work was carried out at the "Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA".

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Abstract

H7N9 virus infection is a global concern, given that it can cause severe infection and mortality in humans. However, the understanding of H7N9 epidemiology, animal reservoir species and zoonotic risk remains limited. This work evaluates the pathogenicity, transmissibility and local innate immune response of three avian species harbouring different respiratory distribution of α2,6 and α2,3 SA receptors. Muscovy ducks, European quails and SPF chickens were intranasally inoculated with 10^5 embryo infectious dose $(EID)_{50}$ of the human H7N9 (A/Anhui/1/2013) influenza isolate. None of the avian species showed clinical signs or macroscopic lesions and only mild microscopic lesions were observed in the upper respiratory tract of quail and chickens. Quail presented more severe histopathologic lesions and avian influenza virus (AIV) positivity by immunohistochemistry (IHC), which correlated with higher IL-6 responses. In contrast, Muscovy ducks were resistant to disease and presented higher IFNα and TLR7 response. In all species viral shedding was higher in the respiratory than in the digestive tract. Higher viral shedding was observed in quail, followed by chicken and ducks, which presented similar viral titers. Efficient transmission was observed in all contact quail and half of the Muscovy ducks, while no transmission was observed between chicken. All avian species showed viral shedding in drinking water throughout infection.

Impacts

- All studied species exhibited viral shedding, pointing out their role as H7N9 virus reservoirs, despite not presenting clinical signs. European quail and Muscovy ducks were able to transmit infection to naïve counterparts, with a 100% and 50% transmission rate observed respectively. However, chickens were not able to transmit disease, in despite shedding more virus than Muscovy ducks.

- Drinking water was shown for the first time to be involved in viral transmission. Therefore, water and fomite transmission routes to humans and other animals should be considered in the control of the disease.

- European quails were shown to be more susceptible to disease. Susceptibility, as in humans, correlated with the higher presence of α2,6 SA receptors in the upper respiratory (indicating viral preference for α2,6 SA receptors *in vivo)* and with a local pro-inflammatory immune response. In contrast, ducks showed resistance to disease associated with the up-regulation of anti-viral genes.

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

Introduction

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

Analysis from several H7N9 human isolates indicates that H7N9 is a reassorted virus incorporating envelope genes from an H7N3 avian strain and a neuraminidase (NA) gene from an avian-adapted H7N9 Eurasian linage strain with the internal genes from at least two H9N2 avian-adapted influenza strains commonly found in chickens (Liu *et al.*, 2013). However, the H7N9 virus is more virulent in humans than H9N2, which suggests that the internal genes of H7N9 have mutated (Bi *et al.*, 2015). The sialic acid (SA) binding site in the hemaglutinin (HA) protein is a major determinant of the virus "host jump". Human influenza viruses predominantly bind to α2,6 SA host receptors expressed in the human respiratory tract while AIV predominantly bind to α2,3 SA receptors (de Wit & Fouchier, 2008). Epidemiological data has linked H7N9 transmission to humans who have been exposed to birds in live bird markets (LBM) (Lam *et al.*, 2013) and studies focused on the H7N9 viral receptor binding shift elucidated that human H7N9 recognizes both avian and human receptor analogues (Shi *et al.*, 2013, Liu *et al*., 2014).

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

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

in different commercial avian species, which present varying distribution of α2,6 and α2,3 SA receptors throughout the respiratory tract and also different susceptibility to avian influenza.

Material and Methods

Virus and Facilities

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

Animals

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

Experimental design

Twenty-five animals of each species were randomly separated into two groups with twenty challenged birds/group and one control group with 5 birds (Table 1). For each challenged group, birds were further subdivided into two experimental groups, A and B (n=10 animals/group). Groups A were used to evaluate morbidity, transmissibility, and viral shedding pattern. Groups B were used for the pathological studies. All animals in the challenged groups were inoculated intranasally with 10^5 EID_{50} of H7N9 diluted in PBS in a final volume of 0.05 ml (0.0025ml each nostril), except four birds of each group A which were used as contact animals. Thus, they were placed into the isolators 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 ºC 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^2 approx.)
199 were collected and immediately stored at 70ºC 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

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

Viral RNA detection by qRT-PCR

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

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

Results

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

Clinical signs and gross lesions

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

Pathologic examination and immunohistochemical testing

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

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

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

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

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

Immune gene expression profiles

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

Viral RNA detection by qRT-PCR

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

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

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

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

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

Serology

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

Discussion

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

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

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

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

The host restriction of AIV is determined by the distribution patterns of SA receptors in the upper respiratory tract. Ducks have mainly α2,3 receptors, quail possess mainly α2,6 receptors and chickens possess both α2,3 and α2,6 SA receptors in the upper respiratory tract, while humans possess α2,6 (de Graaf & Fouchier, 2014, Costa *et al.*, 2012). In general AIVs attach better to α2,3 SA receptors (de Wit & Fouchier, 2008). However, sequence analysis of the H7N9 influenza viral proteins revealed that the virus has acquired several amino acid changes associated with adaptation to human receptor binding α2,6 SA in the HA gene (Q226L and G186V) (Xu *et al.*, 2013, Watanabe *et al.*, 2013), indicating that H7N9 recognizes both α2,6 and α2,3 receptors (Shi *et al*., 2013, Liu *et al*., 2014, Josset *et al.*, 2014, Xiang *et al.*, 2013). Accordingly, the results obtained in this study showed that susceptibility of the different avian species used to H7N9 infection correlates with the presence of both receptors in the upper respiratory tract.

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

In this study, effective viral transmission from inoculated animals to naïve contact quail and Muscovy ducks has been confirmed, even though the original host was human. These findings suggest either quick H7N9 virus adaptation, or that adaptation may not be needed for H7N9 to replicate and be transmitted between human and avian species, confirming the substantial role of commercial avian species in H7N9 epidemiology. The fact that quail are more susceptible to human origin H7N9 infection may elucidate the preference of H7N9 for α2,6 receptors *in vivo,* as quail present a higher proportion of

435 α2,6 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 α2,3 and α2,6 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.

452 ## Financial Support

453 This work was founded by the Instituto Nacional de Investigación y Tecnología agraria (INIA),
454 Spanish Government, project "Avian influenza: relationship between the host, the
455 pathogen and the ecosystem" RTA2011-00111-C03.

456 ## Competing interest

457 The authors declare that they have no competing interests.

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