LIIsimposiocientíficode avicultura
MÁLAGA del 28 al 30 de octubre de 2015

ASOCIACIÓN ESPAÑOLA DE CIENCIA AVÍCOLA
Sección Española de WPSA
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COM-02

Characterization of local immune response in the upper respiratory tract of different avian species experimentally infected with influenza A virus H7N9

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At the moment, H7N9 is considered the most prevalent avian influenza virus strain affecting human in China. Although it is well known that poultry is the main source of human infection, the host-pathogen interaction of H7N9 virus with its avian natural hosts remains unclear. The aim of this study was to determine the innate immune response in the nasal cavity of SPF chicken, European quail and Muscovy duck after an experimental inoculation of the novel avian-origin H7N9 virus of a human isolate. Despite the three avian species remained asymptomatic along the 8 days of infection, mild rhinitis was observed in the nasal cavity of quail and chicken, accordingly with higher viral load detected in this tissue. In contrast, no lesion and poor replication were seen in duck. Quail and chicken showed high expression of the proinflammatory IL-6 gene, coinciding with a higher presence of proinflammatory cells and tissue damage. The lower replication detected in chicken and duck compared to quail correlated with the early expression of TLR7 and IFNα genes that act as viral recognition and induction of an antiviral state, respectively. The results suggest that the viral load and lesion detected in the nasal cavity of the three avian species may be partially explained by host factors such as the local immune response pattern and that quail, together with chicken, could act as major sources of H7N9 virus to the human population.

En la actualidad, el virus H7N9 es considerada la cepa de influenza aviar que afecta humanos más prevalente en China. Aunque los estudios han demostrado que la principal fuente de infección para los humanos son las aves de corral, la interacción huésped-patógeno del virus H7N9 con dichas aves se conoce poco. El objetivo del presente estudio es determinar la respuesta inmune innata en la cavidad nasal de pollos SPF, codornices europeas y patos Muscovy tras la infección experimental con el virus H7N9 de origen humano. Aunque las tres especies aviares permanecieron asintomáticas durante los 8 días de infección, se detectó una leve rinitis en la cavidad nasal de codornices y pollos, en correlación con una mayor carga viral detectada en estas especies. Las codornices y los pollos mostraron alta expresión del gen proinflamatorio IL-6, coincidiendo con mayor presencia de células proinflamatorias y daño tisular. La baja replicación detectada en pollos y patos comparado con las codornices se relaciona con la temprana expresión de los genes TLR7 y IFNα que actúan, respectivamente, como detectores virales e induciendo un estado antiviral en el tejido. Los resultados sugieren que la carga viral y la lesión detectada en la cavidad nasal de las tres especies aviares pueden estar parcialmente

explicadas por factores del huésped tales como el patrón de respuesta inmune local y que la codorniz, junto con el pollo, podrían actuar como fuentes principales del virus H7N9 para la población humana.

Keywords: Avian Influenza; H7N9; Immune response

Introduction

On March 31, 2013, the National Health and Family Planning Commission of China notified World Health Organization (WHO) of three cases of human infection with the novel AIV type A H7N9 virus. Two cases were reported from Shangai and one case from the Anhui province. By October 2013, a second wave of human infection reemerged (Husain, 2014). At the moment, H7N9 is considered the most prevalent avian influenza virus strain affecting human in China (Chen *et al.*, 2015). The last available WHO risk assessment report indicates that H7N9 virus have caused 571 laboratory-confirmed infections, including 212 human deaths and severe economic losses (WHO, 2015), suggesting that H7N9 virus continues being a global threat. 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).

Most of the human infections are believed to result from exposure to infected poultry in live bird markets or contaminated environment (WHO, 2015). Unfortunately, H7N9 infection in poultry is asymptomatic, being more difficult to eradicate than other influenza viruses (Poovorawan *et al*., 2013). In a recent study, an active surveillance in fifteen cities across five Chinese provinces identified 493 H7N9 viruses from oropharyngeal swabs of market chickens, with an average isolation rate of 3.0%. For that, it is considered now as enzootic in Eastern Chinese poultry (Lam *et al.*, 2015).

Cytokines are necessary for recruiting inflammatory cells to the sites of infection, defending against virus infections and consequently help in disease recovery. However, deregulated balance between pro- and anti-inflammatory mediators produces massively and persistently activated and recruited inflammatory cells, causing a cytokine storm that may be fatal for the animal or human host. In H7N9 severe human infection cases, bilateral pneumonia that progresses to acute respiratory distress syndrome (ARDS) associated with a cytokine storm is reported (Sun *et al*. 2014). This clinical presentation is unusual for H7 viruses, which are low-pathogenic avian influenza (LPAI) in poultry that normally cause mild respiratory illness or conjunctivitis in humans (Belser *et al*. 2009). In poultry infection, little information about immune response is available. Due to that, H7N9 virus has onset worldwide public concern and efforts are needed to understand the host-pathogen interaction of H7N9 virus with the avian species that act as natural hosts.

Materials and methods

The virus used in this study was A/Anhui/1/2013 (H7N9), which was obtained from a patient during the 2013 H7N9 outbreak. The virus was passaged at least 3 times in eggs from the original patient sample and virus stocks were produced in embryonated SPF chicken eggs. The mean embryo infectious dose (EID_{50}) was determined by the Reed and Muench method (Reed and Muench, 1938).

SPF Chicken (*Gallus gallus domesticus*), European quail (*Coturnix coturnix*) and Muscovy duck (*Cairina moschata*) were used in this study. European quail and Muscovy duck had approximately 1 month and 5 days of age respectively and were kept one week for acclimation. SPF chicken were hatched and placed under BSL-3 containment conditions. Feed and water were provided *ad libitum* throughout the experiment. All the experiments were performed in *Centre de Recerca en Sanitat Animal* (CReSA) under Biosafety level 3 containment.

Before the infection serum samples of all quail and duck 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 duck were ensured to be negative for AIV by real time RT-PCR (RT-qPCR) prior to infection.

Twenty-five animals of each species were randomly separated into two groups with twenty challenged birds/group and one control group with 5 birds. For each challenged group birds were subdivided into two experimental groups A and B of 10 animals each. A-groups were used to evaluate morbidity, transmissibility, and viral shedding pattern and B-groups were used for pathological, viral load quantification and immunological studies. All animals in the challenged group were inoculated intranasally with 10^5 EID_{50} of H7N9 in a volume of 0.5 mL, except four birds of each A group which were used as contact animals.

All birds were monitored daily for clinical signs. At 1, 3, 5 and 8 day post-inoculation (dpi) 2 animals from B groups and 1 animal from the control groups were euthanized using intramuscular sodium pentobarbital (100mg/kg, Dolethal®, Vetóquinol, Cedex, France). All birds from the B groups were necropsied to evaluate gross lesions and histopathological examination. The tissues were fixed for 48 h in neutral-buffered 10% formalin, then embedded in paraffin wax, sectioned at 3 µm, and stained with haematoxylin and eosin (HE) for examination under light microscopy. Nasal turbinate were also stored at -70ºC on *RNA-later* until used for RNA extraction.

For the detection of influenza A virus AIV antigen by IHC, nasal turbinate were stained with a primary antibody against the influenza A nucleoprotein following described methods (Chaves *et al*., 2011). Briefly, an antigen retrieval step was performed using protease XIV (Sigma-Aldrich, UDA) for 10 minutes at 37ºC and blocked for 1 hour with 2% bovine serum albumin (85040C, Sigma-Aldrich Química, S.A., Spain) at room temperature (RT). Samples were then incubated with a commercially available mouse-derived monoclonal anti-IAV antibody (ATCC, HB-65, H16L-10-4R5) concentration (343mg/ml) in a 1:100 dilution at 4ºC overnight followed by 1 hour incubation with biotinylated goat anti-mouse immunoglobulin (Ig) G secondary antibody (Dako, immunoglobulins As, Denmark). Finally, a polymer-based non avidin-biotin peroxidase system, (Dako EnVision® + System, Peroxidase-HRP, Dako, Denmark) was used and applied directly to the slides, and incubated for 30 min at RT. The antigen–antibody reaction was visualized with 3,3'-diaminobenzidine tetrahydrichloride (DAB) as chromogen. Sections were counterstained with Mayer's Haematoxylin. The positive control consisted of a formalin-fixed paraffin-embedded heart from a chicken experimentally infected with influenza. The same section in which the specific primary antibodies were substituted with PBS were used as negative controls.

Total RNA was extracted from the nasal turbinate of infected and control animals using a purification kit (RNeasy mini RNA purification kit, Qiagen). This process was done following manufacturer's instructions. Total RNA of the samples were quantified by spectrophotometry (Biophotometer Eppendorf 230 V 50/60 Hz) using a concentration of 1 µL of sample and 99 µL of RNA*ase*-free water. Samples with an important outrange of quality values were also measured with laser cyte (Biodrop µLite) using 1 µL of sample without dilution.

cDNA transcription was developed following ImProm-II™ Reverse Transcription System, GeneAmp PCR System 9700 protocol. Briefly, 1 µL of random primer and RNAase-free water was added to the sample to form a primer mix of 10 µL at 70ºC during 5'. It was then incorporated to 10

µL of reaction mix to a final volume of 20 µL of cDNA following three steps: Annealing with reverse transcriptase mix (25ºC 5'), First-strand synthesis reaction (42ºC 60') and Inactivation of reverse transcriptase (70ºC 15'). The formed cDNA was stored at -30ºC until used for quantitative PCR.

RT-qPCR was used for detection of H7N9 virus in nasal turbinate. Published primers were used (Table 1). RT-qPCR was developed with a standard protocol of 4 µL of sample mix (0,25 µL of sample and 3,75 µL of H20) and 6 µL of primer mix (1 µL of primer and 5 µL of SYBR) to form a final volume of 10 µL of each sample (7500 Fast Real-Time PCR System). Temperatures and times used for quantification of H7N9 virus were the following: Holding Stage (50ºC 2'- 95ºC 10', 1 cycle), Cycling Stage (95ºC 15''-60 ºC 1', 40 cycles), Melt curve stage (95ºC 15''- 60ºC 1'- 60ºC 1'- 60ºC 15'', 1 cycle).

RT-qPCR was used for detection of different genes related with innate immune response in nasal turbinate. For the RT-qPCR, published primers for β-actin (ACTβ), interferon alfa (IFNα), interferon gamma (IFNγ), interleukin 6 (IL-6), Toll-like receptor 7 (TLR-7) and retinoic acid inducible (RIG-1) were used (Table 1). Primers were diluted at 2,5 mM following manufacturer instructions and primer efficiency was determined by dilution of samples. IFNγ primers showed lack of efficiency and were eliminated (data not shown).The RT-qPCR process was developed as previously described.

Average cycle threshold (Ct) values for each target gene were normalized by the Ct value of an endogenous control gene, β-actin, which is not affected by inflammation and cytokine response. It was then normalized with the median of negative control animals to obtain the expression fold change. It was considered up-regulated when the expression change was upon 1 and down-regulated when below 1. Cytokine quantification data of one chicken (1 dpi) and two duck (1 dpi and 3 dpi) is not shown due to have a deviation upon 3α and were consequently considered outliers. Data visualization was performed with GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

Table 1. Primers used for RT-qPCR assays in the three avian species.

Gen	Primer sequence Forward (5'-3')	Primer sequence Reverse (5'-3')	Reference
Chicken			(Cornelissen *et al*., 2012)
ACTβ	CAACACAGTGCTGTCTGGTGGTA	ATCGTACTCCTGCTTGCTGATCC	
IFNα	ATCCTGCTGCTCACGCTCCTTCT	GGTGTTGCTGGTGTCCAGGATG	
IFNγ	ACACTGACAAGTCAAAGCCGCACA	AGTCGTTCATCGGGAGCTTGGC	
IL-6	AGGACGAGATGTGCAAGAAG	TGCTGTAGCACAGAGACTCG	
TLR7	ATGCTGTTATCAGGACGTTGGTT	CCTTGAGGCGACGGTCACT	
Duck			(Cornelissen *et al*., 2012)
ACTβ	CCCCCGTGCTGTGTTCCCATCTATCG	GGGTGCTCCTCAGGGGCTACTCTCAC	
IFNα	GGGCCCCGCAACCT	CTGTAGGTGTGGTTCTGGAGGAA	
IFNγ	AAATACCCTTTCCAATGACTACAAGAA	AGCCTTGCGTTGGATTTTCA	
IL-6	TTCGACGAGGAGAAATGCTT	CCTTATCGTCGTTGCCAGAT	
TLR7	CCTTTCCCAGAGAGCATTCA	TCAAGAAATATCAAGATAATCACATCA	
RIG-1	GTGTATGGAGGAAAACCCTATTCTT	GGAGGGTCATACCTGTTGTTTGAT	
Quail			(Uno *et al*., 2012)
ACTβ	CTGGCACCTAGCACAATGAA	CTGCTTGCTGATCCACATCT	
IFNα	CCTTFCTCCTTCAACGACA	CGCTGAGGATACTGAAGAGGT	
IFNγ	CAACCTTAATGATGGCACGA	CTTTGCGGTGGATTCTCA	
IL-6	CAACCTCAACCTGCCCAA	GGAGAGCTTCCTCAGGCATT	
TLR7	AGATGTTTTCTGGGCAGACG	AATGACTTCAACGGTTACTGG	
H7N9	AGATGAGTCTTCTAACCGAGGTCG	TGCAAARACAYCTTCCAGTCTCTG	(Spackman *et al*., 2002)

Results and discussion

No quail, duck or chicken presented clinical signs throughout the experimental infection. No macroscopic lesions were observed in the organs of any bird species at necropsy, except one infected quail (1 dpi) which presented fibrinous secretion in the nasal turbinate.

Mild lymphoplasmacytic rhinitis was observed in the nasal turbinate of quail and chicken at different dpi. IAV detection by IHC was only observed in quail and chicken, accordingly with histopathological lesions. All infected quail presented AIV+ cells in nasal turbinate at 3 dpi. The histological lesion and H7N9 detection by IHC were higher in quail than chicken (Figure 1).

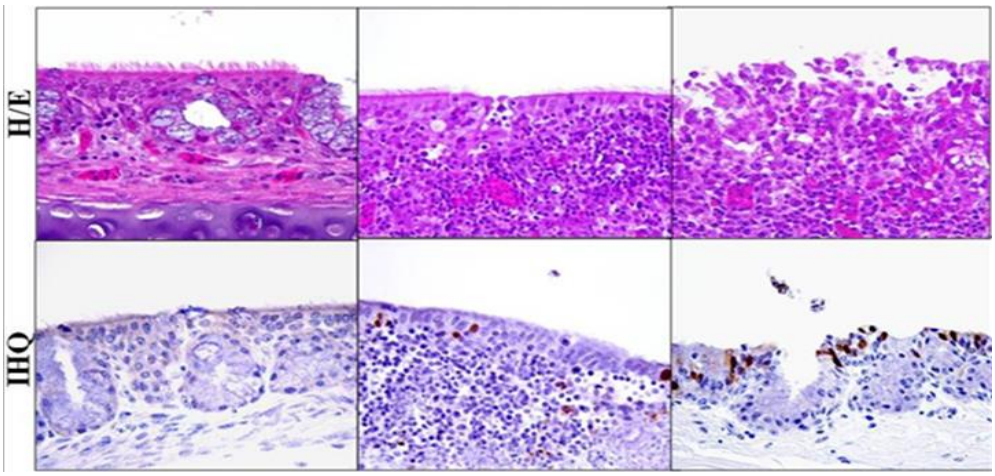

Figure 1. Histopathology and IHC of nasal cavity of ducks, chickens SPF and quails infected experimentally with AIV Influenza A (H7N9). Duck (left), chicken (central), quail (right). The images correspond with 5 dpi.

Differences in the viral RNA levels in nasal turbinate between the three avian species were observed (Figure 2). Higher levels of H7N9 RNA were found in quail, especially at third dpi. In chicken, viral RNA was only observed at days 1 and 5 dpi. In ducks, H7N9 RNA levels were lower than quail and chicken and were only present at days 5 and 8 dpi.

Compared to the control group, all the cytokines measured in the H7N9 infected animals were up-regulated at some point post-inoculation. Differences in the genes expression pattern were found (Figure 2).

The IFNα and TLR7 expression pattern were similar in chicken and duck. High early levels of IFNα and TLR7 were observed at 1 dpi. In chicken, but not in duck, the levels remained high until 8 dpi. The expression pattern in quail was found to be different. IFNα levels in quail were not up-regulated at 1 dpi and TLR7 increased later after infection.

IL-6 expression pattern was similar in chicken and quail. A strong up-regulation of IL-6 is observed at 1 dpi and 3 dpi in both species. The major expression was found at 3 dpi, but remained at high levels until 8 dpi especially in quail. In duck, later and lower up-regulation of IL-6 was observed.

RIG-1 was found to be slightly up-regulated at 1 dpi but not the remaining days.

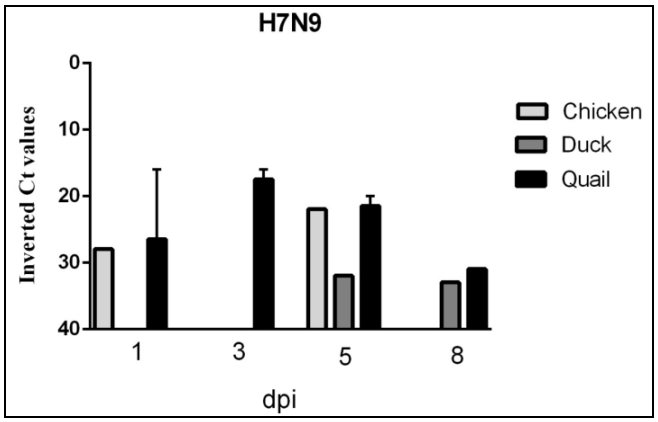

Figure 2. Viral RNA detected in nasal cavity of chicken, duck and quail (n=2) infected with AIV H7N9 at different times post inoculation. Data are presented as mean +- SEM. Dpi (day post-infection)

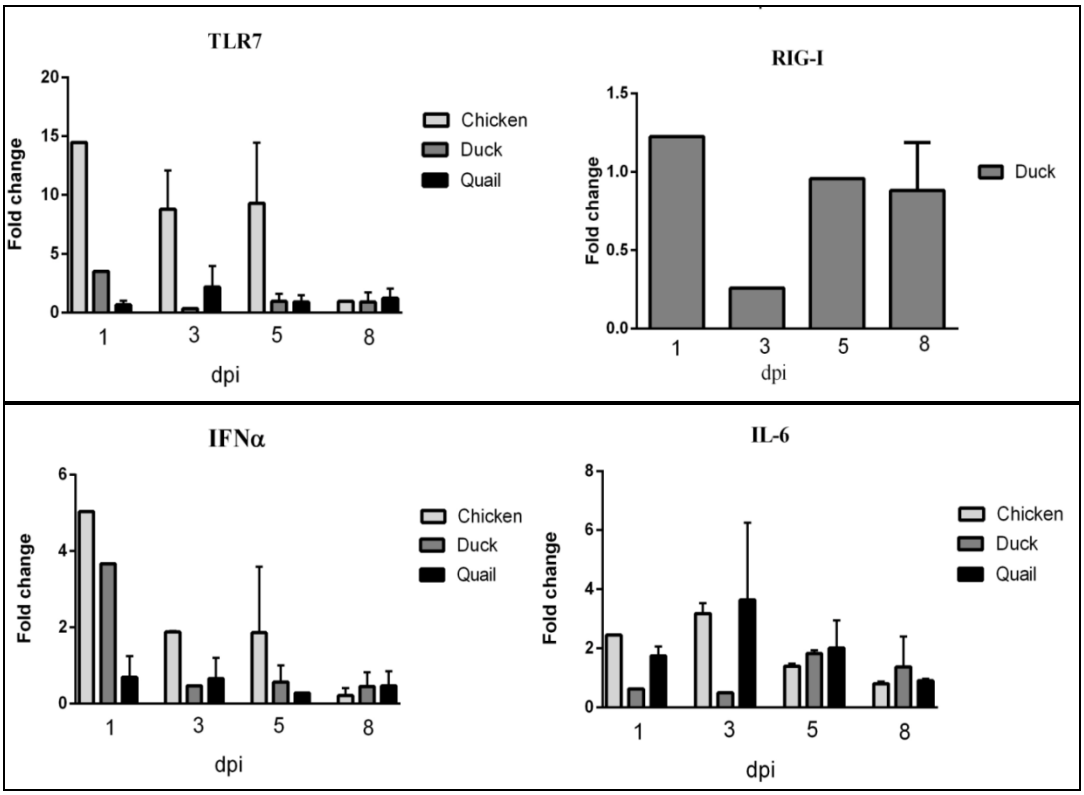

Figure 3. TLR7, RIG-1, pro-inflammatory cytokine IL-6 and IFNα mRNA expression in nasal cavity of chicken, duck and quail (n=2) infected with AIV H7N9 at different time post inoculation. mRNA expression is shown as the fold change normalized with ACTB (endogenous control) and compared with uninfected birds +- SEM. Dpi (day post-infection).

The understanding of the H7N9 epidemiology, including the main reservoirs of the virus, remains limited. However, it is likely that most human cases were exposed to the H7N9 virus through contact with infected poultry or contaminated environments, including live poultry markets (WHO, 2015).

Because of that, the H7N9 virus interaction with the avian species that can be a source of human infection must be clarified.

In our study, the three avian species challenged by intranasal route with H7N9 virus remained asymptomatic during the 8 days of infection, although active viral replication was detected along the experimental period in European quail, SPF chickens and Muskovy duck. These results are in agreement with previous experimental studies that demonstrate that quail and chicken are susceptible to H7N9 infection and shed large amounts of virus (Pantin-Jackwood *et al*., 2014). Altogether, these data suggest that quail and chicken, and to a minor extent duck, spread H7N9 virus and are likely sources of human infection.

By both RT-qPCR and IHC, our results indicate that quail is the species with higher viral replication in the nasal cavity, followed by chicken. In contrast, poor replication was seen in duck. It has been confirmed that H7N9 virus replication in the human nasal cavity, correlates with high expression of α2,6 sialic acid receptors (mammalian-like influenza virus receptors), suggesting that H7N9 virus has an increased tropism for mammalian hosts (Guan *et al*., 2014). This increased avidity of H7N9 for human receptors α2,6 instead of avian-like receptors α2,3 is partially due to Q226L and G186V mutations in HA surface glycoprotein (Josset *et al*., 2014; Xiong *et al*., 2013). Previous studies have also confirmed that European quail harbour a large quantity of sialic acid receptors in an α2,6 configuration in the nasal cavity, followed by chicken, and duck in a minor extent (Costa *et al*., 2012). Therefore, the sialic acid receptor pattern present in the epithelial cells of the nasal cavity might be determinant to explain the different viral loads detected in the upper respiratory tract of each avian species and their potential role as viral reservoirs.

The nose mucosal tissue is the first to come into contact with aerosol-associated H7N9 virus. 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 (B and T cell mediated) is activated to clear the infection (Davison *et al.*, 2008). In our study, the results show that chicken and duck but not quail have an early up-regulation of TLR7 and IFNα genes, which are higher in chicken. This cytokine pattern results are in agreement with previous AIV experimental infection in chicken and duck (Cornelissen *et a*l., 2012). TLR7 is a Pathogen Recognition Receptor (PRRS) present in the cells that senses single-stranded viral genomic RNA (ssRNA) and it is assumed that produces high levels of IFNα when activated, a type I interferon cytokine that has antiviral effect by inducing the synthesis of proteins that interfere with viral replication (Keestra *et al*., 2013). In our results, IFNα early up-regulation in chicken and duck nasal cavity correlates with lesser viral replication than quail in this tissue, suggesting that early type I interferon expression might also have an effect slowing down viral dissemination and consequently the viral replication in each avian species.

It has been proposed that RIG-1, a PRRS cytosolic receptor that is only present in ducks and that also leads the production of type I IFN in infected epithelial cells when recognizes the viral ssRNA, plays an important function in clearing influenza virus. Furthermore, NS1 protein of some AIV has evolved to block RIG-1 signalling, indicating that RIG-1 mediated recognition is a key antiviral determinant in infected hosts (Barber *et al*., 2010). Surprisingly, our results do not show an important up-regulation of this gene in ducks. Although the number of animals tested is low, it is likely that the natural resistance of duck to H7N9 infection is not only related to the early innate immune response but also to other viral or host factors.

In our results, epithelial cell necrosis associated with infiltration by inflammatory cells (rhinitis) was seen in quail and chicken nasal cavity, but was absent in duck. The histopathological lesions observed in the present study are in concordance with a previous H7N9 experimental study (Pantin-Jackwood *et al*. 2014). In addition, our results showed that quail and chicken but not duck have an

early and high IL-6 up-regulation in the nasal cavity, a pro-inflammatory cytokine produced by macrophages and heterophils that recruits inflammatory cells and induces an acute phase protein response (Kaiser & Staheli, 2008). In this study, the higher IL-6 levels present in quail and chicken at 3 and 5 dpi, correlated with abundant inflammatory cell infiltration, tissue damage and higher viral load in nasal cavity. Therefore, high IL-6 expression might explain the inflammatory infiltration detected in the nasal cavity of quail and chicken but not duck and could be correlated with tissue damage, which is in concordance with previous studies (Paquette *et al*., 2012).

In summary, the data show that the differences seen between European quail, SPF chicken and Muscovy duck after intranasal inoculation with H7N9 virus can be explained by host factors such as sialic acid receptors distribution present in upper respiratory cells surface and early host local innate immune response pattern, and suggest that quail, together with chicken, could be a major source of H7N9 virus for the human population. Further studies focused on the prevalence of H7N9 infection in different minor gallinaceous species in the endemic areas would be desirable to better understand and control the avian-human transmission.

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