Assessment of the pathogenicity and the host innate immune response in the respiratory tract of european quails experimentally infected with different strains of H5 and H7 avian influenza A virus

R. SÁNCHEZ[1*], N. ERAM[2], M. NOFRARÍAS[1], A. RAMIS[1,3], M. PÉREZ[1] and N. MAJÓ[1,3]

[1]Centre de Recerca en Sanitat Animal (CReSA) - Institut de Recerca i Tecnología Agroalimentàries (IRTA), Campus de la UniversitatAutònoma de Barcelona (UAB), Catalonia, Spain
[2]Department of Avian Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
[3]Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona (UAB), Catalonia, Spain
***Corresponding author: raul.sanchezgo@e-campus.uab.cat**

Recently, European quail (*Coturnix c.coturnix*) has been suggested to share with Japanese quail (*Coturnix c.japonica*) its potential as an intermediate host in the epidemiology of avian influenza viruses (AIV). On the other hand, a deregulated innate immune response in AIV infections related to fatal outcomes has been detected in several species. In the present study, we determined the microscopic lesions, the viral load and the innate immune response in samples from nasal cavity and lung of E.quails that had been experimentally infected with low pathogenic (LP) (H5N2, H7N9) or high pathogenic (HP) (H5N1, H7N1) AIV. The quails inoculated with H7N9/LP, which was isolated from a human patient, presented more severe histopathological findings, antigen positive cells and higher viral load in nasal cavity, suggesting an increased avidity of H7N9/LP to sialic acid receptors in an α2,6 configuration. Viral RNA in the lung was only detected in HPAIV-inoculated quails, indicating a wider distribution capacity of these viruses. Regarding the innate response, the quails inoculated with LPAIV strains showed a strong expression of IFNα in nasal cavity. Moreover, H7N9/LP-inoculated quails presented an early up-regulation of TLR7 in the nasal cavity. Lastly, the expression levels of IFNα, IL-6 and TLR7 in lung were highly and long-term up-regulated in the quails inoculated with the HPAIV strains. These results highlight that European quail supports the replication of AIV isolated from human and diverse avian species. Differential innate immune-related genes expression between HPAIV and LPAIV may influence viral replication and clinical outcome, supporting the current idea that the pathological outcomes are related to both viral and host factors.

Keywords: Avian influenza; Quail; Respiratory tract; Innate immune response.

Recientemente, se ha sugerido que la codorniz europea (*Coturnix c.coturnix*) comparte con la codorniz japonesa (*Coturnix c.japonica*) el potencial como huésped intermediario en la epidemiología del virus de la influenza aviar (IA). Asimismo, una respuesta inmune innata desregulada en infecciones fatales de IA ha sido reportada en diversas especies. En el presente estudio, se determinaron las lesiones microscópicas, la carga viral y la respuesta inmune innata en muestras de cavidad nasal y pulmón de codornices europeas infectadas previamente con virus de IA de alta patogenicidad (AP) (H5N1,H7N1) y baja patogenicidad (BP) (H5N2,H7N9). Las codornices inoculadas con H7N9/BP, aislado de un paciente humano, presentaron lesiones

histopatológicas más severas, mayor presencia de células positivas a antígeno viral y mayor detección de RNA vírico en cavidad nasal, sugiriendo un incremento de la afinidad por los receptores de ácido siálico en una configuración α2,6. Se detectó RNA vírico en pulmón de las codornices infectadas con las cepas de AP, indicando una mayor capacidad de diseminación de estos virus. Respecto la respuesta innata, las codornices inoculadas con las cepas de BP mostraron una alta expresión de IFNα en la cavidad nasal. Las codornices infectadas con H7N9/BP mostraron una elevada y temprana expresión de TLR7 en la cavidad nasal. Finalmente, la expresión de IFNα, IL-6 y TLR7 fue altamente incrementada y prolongada en el tiempo en el pulmón de las codornices infectadas con las cepas de AP. Los resultados demuestran que la codorniz europea permite la replicación de cepas de IA aisladas de humanos y diversas especies aviares. La expresión diferencial de genes relacionados con la respuesta inmune innata entre las codornices infectadas con cepas de AP y BP podría influenciar en la sintomatología clínica y replicación viral, confirmando la hipótesis de que la patogenicidad producida por el virus de IA está asociada tanto a factores del virus como del huésped.

Palabras clave: Influenza aviar; codorniz; tracto respiratorio; respuesta inmune innata.

Introduction

Avian influenza (AI) causes severe economic losses in domestic poultry farms worldwide, being a concern to food-producing avian bird's industry, but also to wildlife conservation and to global human health (Capua & Alexander, 2006). AI viruses (AIV) are able to overcome the species-specific barrier and be transmitted from waterbirds to domestic poultry population, especially when they are raised in outdoor production systems which provide these animals the opportunity to contact with wild migratory birds. Although AIV can be divided into low-pathogenic (LP) or high-pathogenic (HP) depending on the mortalities that cause in poultry, HPAIV infections have been limited to H5 and H7 subtypes (Reperant, Kuiken & Osterhaus, 2012).

Recently, Japanese quail (J.quail, *Coturnix c. japonica*) has been considered the most important intermediate host in the epidemiology of AIV (Nguyen *et al.*, 2016). The first case of AIV infection in J.quail was reported so far in Italy and since then several subtypes have been isolated in Asia, Europe and America in the routine surveillance programs (Suarez *et al*., 1999). It has been reported that J.quail supports the replication of a broad range of AIV, along with several mammal influenza viruses (Makarova *et al.*, 2012), suggesting that quail may act as "mixing vessel". The recent study performed by Thontiravong *et al.* (2012) confirmed the capacity of J.quails to produce reassortant viruses after the inoculation with pandemic H1N1, duck H3N2 and swine H1N1 viruses. Moreover, serial passages of AIV of duck-origin in quail enabled its capacity to replicate in chicken and human bronchial epithelial cells (Yamada *et al.*, 2012).

European quail (E.quail, *Coturnix c. coturnix)* is bred in all European Union member states for meat and egg production and hunting purposing. In Spain, E.quail comprised the 5,0% of the total meat poultry farms on December 2014, with an increasing tendency through the years (SGPG, 2015). Bertran *et al.* (2013) reported that E.quails inoculated with H5N1, H7N1 and H7N2 showed long-term viral shedding and transmission to naïve quails, suggesting that E.quail could share with J.quail its potential as a reservoir or intermediate host in the epidemiology of AIV.

The pathogenic outcome after AIV infection is a result of complex processes involving both viral and host factors that include components of the innate immune system. Recently, a detrimental immune response has been considered a factor that contributes to fatal AIV outcomes in several mammal and avian species (Jong *et al.*, 2006; Kuribayashi *et al.*, 2013; Vidaña *et al.*, 2014), indicating that a correct balance between inflammatory and anti-inflammatory cytokines is important for the induction of an equilibrated innate immune response. Regarding the innate response in minor gallinaceous species, a

small number of studies are available. In order to elucidate the host-pathogen interaction of E.quail with H5 and H7 AIV, we measured the histological findings, the viral replication, the viral load and the innate immune response pattern in nasal cavity and lung of E.quails that had been previously experimentally challenged with HP (H5N1, H7N1) or LP (H5N2, H7N9) AIV.

Materials and methods

Samples. Formalin-fixed, paraffin-embedded (FFPE) samples from nasal cavity and lung obtained from E.quails infected with different strains of AIV were used. Samples from animals experimentally inoculated with H5N1/HP and H7N1/HP (Bertran *et al*., 2013), H5N2/LP (García *et al*., 2014) and H7N9/LP (Vidaña *et al*., 2014) were evaluated in this study. H7N1/HP (A/Chicken/Italy/5093/1999) was obtained during the epidemics that affected Italy in 1999-2000. H5N1/HP (A/Great crested grebe/ Basque Country/06.03249/2006) was isolated from the only case of H5N1/HP infection reported in wild birds in Spain. H7N9/LP (A/Anhui/1/2013) was obtained from a human patient during the 2013 H7N9 outbreak. H5N2/LP (A/Anas platyrhynchos/2420/2010) was isolated during the course of the avian influenza surveillance program in wild birds in Catalonia.

Pathologic examination and immunohistochemical testing. Cut sections of 3 µm (Leica RM2255) from FFPE nasal cavities and lungs were processed, stained with haematoxylin and eosin and then examined under light microscopy. An immunohistochemical technique was performed in nasal cavity and lung as previously described (Bertran *et al.*, 2013). Briefly, a mouse-derived monoclonal commercial antibody against nucleoprotein (NP) of influenza A virus (ATCC, HB-65, H16L-10-4R5) was used as a primary antibody. The samples were then incubated with biotinylated goat anti-mouse immunoglobulin (Ig) G secondary antibody (Dako, immunoglobulins As, Denmark). The antigen–antibody reaction was visualized using the chromogen 3,3'-diaminobenzidine tetrahydrichloride (DAB). Tissues that were previously ensured to be positive to influenza A virus and samples that were incubated with PBS instead of the primary antibody were used as positive and negative controls, respectively. A semi-quantitative score was used to evaluate the grade of positive cells: no positive cells (-), single positive cells (+), scattered groups of positive cells (++), widespread positivity (+++).

Viral RNA detection and innate immune response genes quantification by qRT-PCR. Total RNA from FFPE nasal cavity and lung was extracted using a FFPE purification kit and DNase reagents (RNeasy FFPE Kit, Qiagen). This process was done following manufacturer's instructions. The eluted RNA was quantified by spectrophotometry (BioDrop µLite). For the detection of viral RNA and the relative quantification of the innate immune response-related genes, two-steps real time reverse transcription polymerase chain reaction (qRT-PCR) were performed following procedures reported previously (Vidaña *et al.*, 2014). RNA was first transcribed to cDNA using a standard reverse transcriptase protocol (ImProm-II^{TM} Reverse Transcription System, GeneAmp PCR System 9700). The qPCR reactions were performed using a PowerSYBR green kit (Applied Biosystems) in 10 µL final reaction volumes and analyzed in Fast7500 equipment (Applied Biosystems, Foster City, CA, USA) through the following steps: Holding Stage (50ºC 2'- 95ºC 10', 1 cycle), Cycling Stage (95ºC 15''-60 ºC 1', 45 cycles), Melt curve stage (95ºC 15''- 60ºC 1'- 60ºC 1'- 60ºC 15'', 1 cycle. A FFPE-positive sample and Nuclease-free H_2O were used as positive and negative controls, respectively. The primers used for the detection of a highly conserved region of the matrix (M) gene of influenza A virus, β-actin (ACTβ), alpha interferon (IFNα), interleukin 6 (IL-6) and toll-like receptor 7 (TLR7) are listed in Table 1. The average cycle thresholds (Ct) of the innate immune-related genes were normalized to a housekeeping gene (ACTβ) taking into account primer efficiencies and compared to control animals by means of relative quantification (Štefan, Bukovská & Koppel, 2007). Data was presented as fold change expression and visualized as the means of individuals + SEM using GraphPad Prism 6 software (GraphPadSoftware, La Jolla, CA, USA).

Table 1. 5'-3' primers sequences used in two-steps qRT-PCR for the detection of viral RNA and innate immune-related genes in nasal cavity and lung. IFNα (alpha interferon), TLR7 (toll-like receptor 7), IL6 (interleukin 6), ACTβ (β-actin).

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	Publication source
Influenza A M	AGATGAGTCTTCTAACCGAGGTCG	TGCAAAAACATCTTCAAGTCTCTG	(Spackman *et al.*, 2002)
IFNα	CCTTGCTCCTTCAACGACA	CGCTGAGGATACTGAAGAGGT	(Uno *et al.*, 2012)
IL-6	CAACCTCAACCTGCCCAA	GGAGAGCTTCCTCAGGCATT	
TLR7	AGATGTTTTCTGGGCAGACG	AATGACTTCAACGGTTACTGG	
ACTβ	CTGGCACCTAGCACAATGAA	CTGCTTGCTGATCCACATCT	

Results and discussion

Quails inoculated with H7N9/LP presented mild to severe rhinitis from 1 to 8 days post-inoculation (dpi). More severe lesions were detected at 5 dpi. In contrast, quails challenged with H7N1/HP, H5N1/HP and H5N2/LP showed minor histological findings in nasal cavity that were restricted to the earliest stages of infection. In agreement with the histological findings detected in nasal cavity, scattered to widespread cell positivity was observed from 1 to 5 dpi in the respiratory epithelium in the H7N9/LP-group, whereas single positive cells were detected in H7N1/HP, H5N1/HP and H5N2/LP-inoculated quails. Moreover, higher levels of Influenza A RNA were found from 1 to 5 dpi in the nasal cavity of the H7N9-inoculated group (Figure 1). These results suggest a positive correlation between lesional findings and viral load, as stated previously (Vidaña *et al.*, 2014).

Costa *et al*. (2012) detected a strong expression of α-2,6 (mammal-type) sialic acid receptors in the epithelial cells of the nasal cavity in E.quail, while lower levels of sialic acid in a α-2,3 configuration (avian-type) were found. Considering that the H7N9/LP strain was isolated from a human patient, the high viral replication observed in the present study may indicate a human-adaptation of the virus that results in an increased avidity for α-2,6 sialic acid receptors. H7N1/HP, H5N1/HP and H5N2/LP strains were isolated from wild and domestic avian species and thus they might retain an α-2,3 specificity. Overall, our results confirm that E.quail, similarly to J.quail (Makarova *et al.*, 2003), support the replication of H5 and H7 AIV strains isolated from wide-ranging bird species and human.

No microscopic lesions or NP+ cells were found in the lung in any of the assessed animals. The results showed the long-term detection of viral RNA in the lung of quails challenged with the HPAIV strains (Figure 1). In contrast, no viral RNA was found in the lung of LPAIV-inoculated quails. Therefore, our results indicate that HPAIV show a wider tissue distribution capacity.

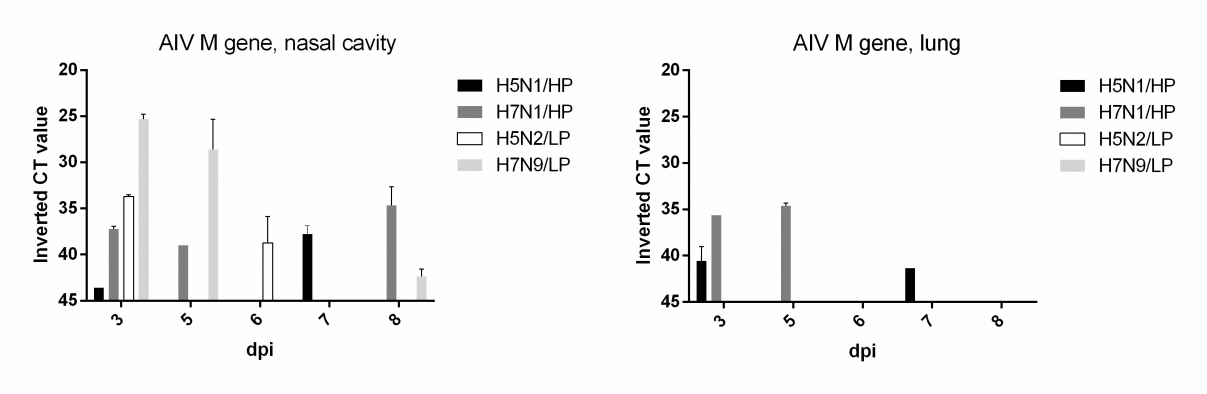

Figure 1. AIV M gene detection by two-steps qRT-PCR at different dpi in nasal cavity and lung. Data is expressed as inverted Ct values and shown as the means of individual quails + SEM. Ct, cycle threshold, dpi, day post-inoculation.

The innate immune response plays a pivotal role in both viral clearance and tissue damage through the release of antiviral proteins, pro-inflammatory cytokines and chemokines that act as natural mediators of the inflammatory process. Lately, a deregulated and prolonged activation of the innate immune response has been considered a host factor that contributes to the lethal outcomes detected in severe AIV infections (Kuribayashi *et al.*, 2013).

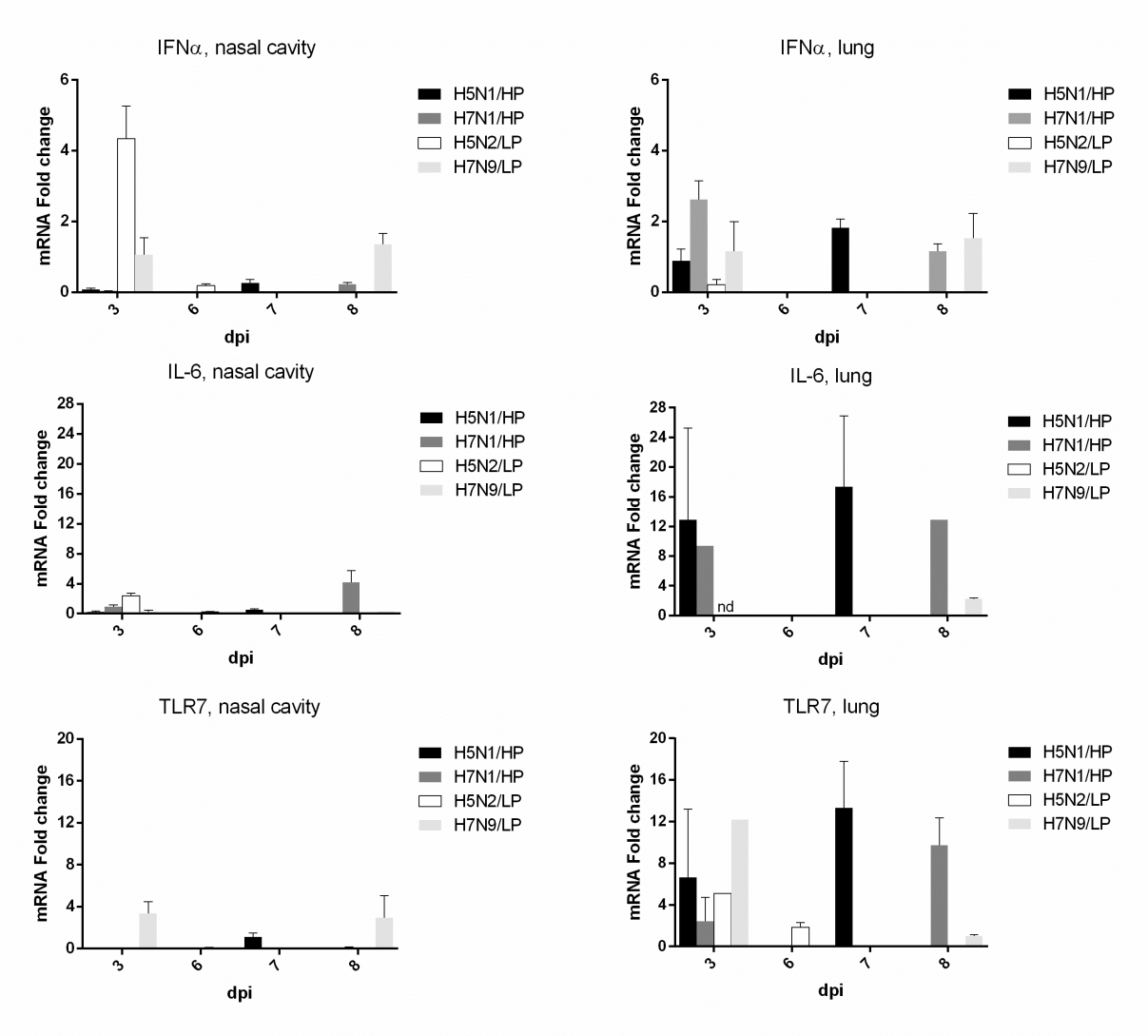

Figure 2. Innate immune-related genes expression at different dpi in nasal cavity and lung of quails experimentally infected with different strains of AIV. Data is presented as the fold change expression compared to control animals and shown as the means of individual animals + SEM. IFNα, alpha interferon, TLR7, toll-like receptor 7, IL-6, interleukin 6, dpi, day post-inoculation, nd, not determined.

IFNα has been reported to reduce AIV replication and mortality rates when delivered as a prophylactic therapy (Beilharz, Cummins & Bennett, 2007; Szretter *et al.*, 2009; Jiang, Yang & Kapczynski, 2011). In our study, the quails inoculated with the LPAIV showed a marked up-regulation of IFNα in nasal cavity (Figure 2), which may explain the decreasing levels of viral RNA during the course of infection in these animals and suggesting that IFNα plays a pivotal role in viral clearance. In contrast, relatively stable amounts of viral RNA in nasal cavity were detected along the experiment in H5N1/HP and H7N1/HP-inoculated quails (Figure 1). Furthermore, the absence of viral RNA in the lung of quails inoculated with the LPAIV strains suggests that IFNα up-regulation may slow down viral dissemination, as is reported in a mice model (Szretter *et al.*, 2009).

The viral signaling was assessed by measuring TLR7, a Pattern Recognition Receptor (PRRS) that senses single-stranded viral genomic RNA (Keestra *et al*., 2013). The quails inoculated with H7N9/LP showed a strong expression of TLR7 in the nasal cavity (Figure 2), which correlates with the widespread immunohistochemical positive staining and suggesting that the viral replication cycle up-regulates TLR7-signalling pathway.

In the lung, a strong and prolonged expression of all the immune-related genes was observed in HPAIV-inoculated quails (Figure 2), which correlates with the long-term presence of viral RNA. In contrast, despite variable early up-regulation of IFNα and TLR7 has been observed in the lung of quails inoculated with the LPAIV strains, only IFNα in the H7N9-group persisted at high levels later after infection.

The proinflammatory cytokine IL-6, the main mediator of the acute phase response and fever, has been broadly detected at high levels in severe AIV cases, thus suggesting that may be considered as a disease severity biomarker (Paquette *et al.*, 2012). Hence, the severe clinical signs and fatal outcomes observed by Bertran *et al*. (2013) in the E.quails inoculated with HPAIV may be partially explained by the overexpression of IL-6 in the lung, which may be tightly linked to a systemic expression.

In conclusion, the data shows that E.quail supports the replication of AIV isolated from human and avian species, which could be explained by the presence of sialic acid receptors in both avian and mammal configuration. The differential innate immune-related genes expression between HPAIV and LPAIV may influence the differences in clinical outcome, viral replication and dissemination between the four AIV strains, supporting the current idea that the pathological outcomes are related to both viral and host factors.

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