Assessment of 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

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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 ARN vírico en cavidad nasal, sugiriendo un incremento de la afinidad por los receptores de ácido siálico en una configuración α2,6. Se detectó ARN 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 H5N2/BP mostraron una mayor expresión de IFNα en la cavidad nasal. Las codornices infectadas con H7N9/BP mostraron una elevada expresión de TLR7 en la cavidad nasal. Finalmente, la expresión de 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 de aves. 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.

Keywords: Avian influenza; European quail; Respiratory tract; Innate immune response.

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). Although AIV can be divided as 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). 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.quail to produce reassortant 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). Bertran *et al.* (2013) described that E.quails (E.quail, *Coturnix c. coturnix)* inoculated with H5N1/HP, H7N1/HP and H7N2/LP presented 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. 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). In order to elucidate the host-pathogen interaction of E.quail with H5 and H7 AIV, we measured the histological findings, the viral replication and the innate immune response 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 (A/Great crested grebe/Basque Country/06.03249/2006) and H7N1/HP (A/Chicken/Italy/5093/1999) (Bertran *et al*., 2013), H5N2/LP (A/Anas platyrhynchos/2420/2010) (García *et al*., 2014) and H7N9/LP (A/Anhui/1/2013)(Vidaña *et al*., 2014) were evaluated.

Pathologic examination and immunohistochemical testing. Cut sections of 3 µm (Leica RM2255, Nussloch, Germany) 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. 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 IgG secondary antibody (Dako, immunoglobulins As, Denmark).The antigen–antibody reaction was visualized using the chromogen 3,3'-diaminobenzidine tetrahydrichloride.

Viral RNA detection and innate immune response genes quantification. Total RNA was extracted from nasal cavities and lungs using a FFPE purification kit and DNase reagents (RNeasy FFPE Kit, Qiagen, CA, USA). This process was done following manufacturer's instructions. The eluted RNA was quantified by spectrophotometry (BioDrop µLite, Cambridge, UK). 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. RNA was first transcribed to cDNA using a standard reverse transcriptase protocol (ImProm-II™ Reverse Transcription System, Promega, WI, USA). The qPCR reactions were performed using a PowerSYBR green kit (Applied Biosystems, CA, USA) in 10 µL final reaction volumes and analyzed in Fast7500 equipment (Applied Biosystems, 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).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 the housekeeping gene (ACTβ) taking into account primer efficiencies and compared to control animals by means of relative quantification. Data was presented as fold change expression and visualized as the means of individuals + SEM using GraphPad Prism 6 software (GraphPadSoftware, CA, USA).

Table 1. 5'-3' primers sequences used in 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), IL-6 (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 H5N1/HP, H7N1/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-inoculated quails, 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 quails (Figure 1). These results suggest a positive correlation between lesional findings and viral load.

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 used 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.

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), indicating that HPAIV present a wider tissue distribution capacity.

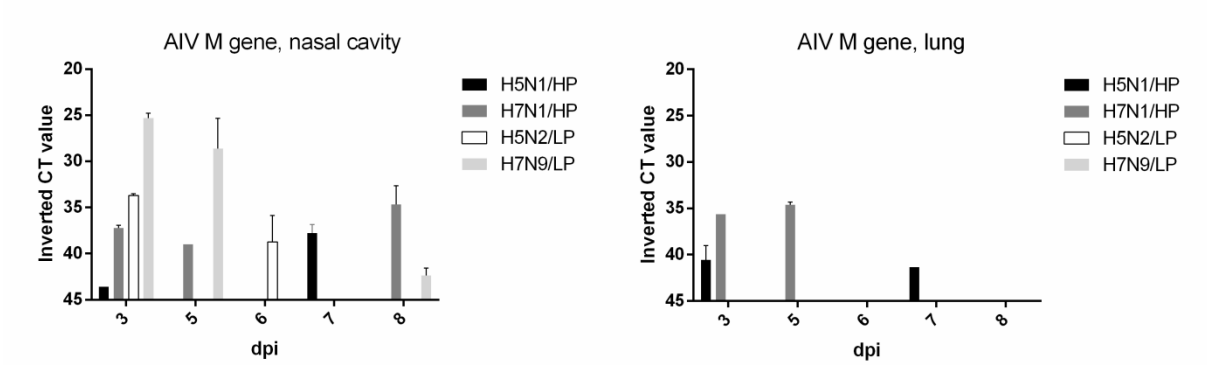

Figure 1. AIV M gene detection at different dpi by two-steps qRT-PCR. Data is expressed as inverted Ct values and shown as the means of individual quails + SEM. Ct, cycle threshold, dpi, day post-inoculation.

IFNα has been reported to reduce AIV replication and mortality rates when delivered as a prophylactic therapy (Szretter *et al.*, 2009). In our study, a strong up-regulation of IFNα was observed in the nasal cavity of the H5N2/LP-inoculated quails and, in a minor extent, in H7N9/LP-inoculated quails (Figure 2). The high expression of IFNα may explain the decreasing levels of viral RNA detected in nasal cavity during the course of infection in these animals. In comparison, relatively stable amounts of viral RNA were observed through the experiment in HPAIV-inoculated quails. 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 mouse model (Szretter *et al.*, 2009).

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.

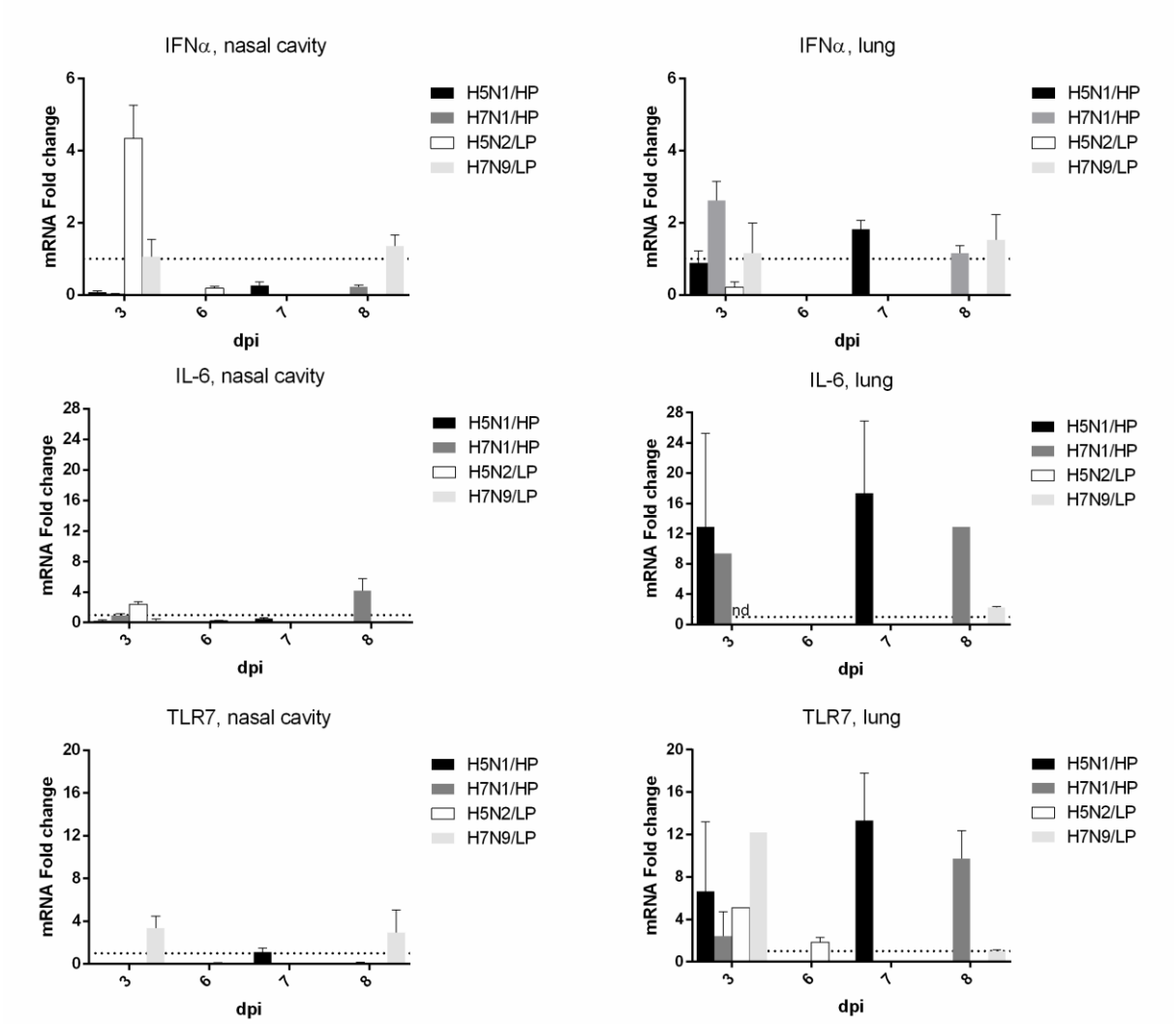

Figure 2. Innate immune-related genes expression at different dpi. Data is presented as the fold change expression compared to control animals and shown as the means of individual animals + SEM. IFNα, alpha interferon, TLR7, toll-like receptor 7, IL-6, interleukin-6, dpi, day post-inoculation, nd, not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

In the lung, a prolonged up-regulation of IL-6 and TLR7 genes was observed in HPAIV-inoculated quails (Figure 2), which correlates with the long-term detection of viral RNA. 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 overexpression of IL-6 in the lung of the HPAIV-inoculated quails detected in the present study, which may be tightly linked to a systemic expression, could partially explain the severe clinical signs and fatal outcomes observed by Bertran *et al.* (2013) in the E.quails inoculated with H5N1/HP and H7N1/HP AIV.

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 response-related genes expression between HPAIV and LPAIV-inoculated quails may influence the clinical outcome and viral replication, supporting the current idea that the pathological outcomes are related to both viral and host factors.

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