

The Pathogenicity and Host Immune Response Associated with H5N1 Highly Pathogenic Avian Influenza Virus in Quail

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ABSTRACT. Quail, like chickens, are susceptible to H5N1 subtype highly pathogenic avian influenza virus (HPAIV). Both birds experience high mortality, but quail usually survive a few more days than chicken. To understand why, we monitored quail and chickens after inoculation with 10⁶ fifty-percent egg infectious doses of HPAIV A/whooper swan/Aomori/1/2008 (H5N1). The clinical course initiated as depression at 48 hr post inoculation (h.p.i.) in quail and at 36 h.p.i. in chicken, and all infected birds died. Mean death time of quail (91 hr) was significantly longer than that of chicken (66 hr). The virus titers of most tissue samples collected before death were not significantly different. At 24 h.p.i., interferon gamma (IFN- γ) mRNA expression in peripheral blood mononuclear cells (PBMC) was up-regulated in the quail but down-regulated in the chicken, although TLR-7 and seven other cytokines showed no significant differences between quail and chicken. The viral load in quail PBMC was significantly lower than that in chickens. These results suggest that the induction of IFN- γ after HPAIV infection in quail is related to lower titer of HPAIV. In conclusion, the different clinical courses observed between quail and chicken infected with H5N1 HPAIV might be caused by different IFN- γ responses against the HPAIV infection.

KEY WORDS: cytokine, H5N1, influenza virus, pathogenicity, quail.

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Since 1997, H5N1 subtype highly pathogenic avian influenza viruses (HPAIVs) have caused outbreaks in poultry in Asia, Europe and Africa, causing great losses. Japanese quail (*Coturnix japonica*) is an important poultry species worldwide and, like chicken, is highly susceptible to H5N1 HPAIVs [23]. Both quail and chicken infected with H5N1 HPAIVs show the approximately 100% mortality. However, quail take longer than chickens to show signs of disease and to die [5, 8, 9, 12, 23]. The longer survival time of quail may result in a longer period of viral excretion and a higher probability of transmission. However, the reason for the different pathologies of quail and chicken is not well understood.

The different clinical manifestations observed among individual animals could be explained in part by the innate immune responses induced against viral infection. In mammals, host immunity appears to play an important role in the pathogenesis of H5N1 HPAIVs. For example, high virulence of HPAIV in mammals has been associated with induction of high levels of pro-inflammatory cytokines in blood or tissues, commonly referred to as “cytokine storms” [25].

In humans, clinical features associated with H5N1 virus infections have been linked to cytokine dysregulation [2, 19]. On the other hand, the role of cytokines in the pathology caused by H5N1 HPAIVs in avian species is less well understood. To date, several studies have shown that different avian species exhibit different pathologies and cytokine responses from H5N1 virus infection [7, 11, 15, 17, 18]. Type I interferon (IFN) was induced by H5N1 HPAIV in lung, spleen and plasma of 6-week-old chickens [15]. Th1 and pro-inflammatory responses were observed in the lung and spleen of 4-week-old chickens infected with a human-derived H5N1 HPAIV [11]. Furthermore, a recently emerged Asian H5N1 HPAIV was shown to quickly induce antiviral and pro-inflammatory cytokines in the lung of 4-week-old chickens as early as 24 hr after inoculation, but 8 hr later, just before death, their mRNA levels decreased dramatically [18]. In Pekin duck infected with three H5N1 strains, younger ducks expressed cytokine mRNA more weakly than older ducks, which were more resistant to the pathological conditions caused by HPAIV infection [17]. In pigeons, which are thought to be less susceptible to H5N1 HPAIV, H5N1 HPAIV infection did not strongly induce the expression of innate immune and inflammatory-related genes in the lung [7]. Although these studies have shown multiple immune responses against H5N1 virus infection among avian host species, the expressions of cytokine genes in quail infected with H5N1 HPAIVs have not yet been examined.

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In the present study, the clinical course, viral growth and cytokine response in quail and chicken infected with an H5N1 HPAIV were compared to elucidate the reason for the different clinical courses of H5N1 virus infection in quail and chicken. To this end, we examined the expressions of innate immune-related genes in peripheral blood mononuclear cells (PBMC) associated with H5N1 virus infection in quail and chicken by quantitative real-time PCR. Additionally, we measured the virus titer in major tissues of quail and chickens to understand the difference of pathology between quail and chickens.

MATERIALS AND METHODS

Influenza virus: The HPAIV A/whooper swan/Aomori/1/2008 (H5N1) [21] used in this study was isolated in Tottori University, propagated in 10-day-old embryonated chicken eggs and stored at -80°C . Values of 50% egg infectious doses (EID_{50}) were calculated by the Reed-Muench method [16]. All experiments using HPAIV were carried out in BSL-3 facilities at Tottori University.

Quail experiment: Eighteen 6-week-old specific-pathogen-free (SPF) Japanese quail were purchased from Nisseiken Co., Ltd., Tokyo, Japan, and divided into two groups of 14 quail (challenged group) and 4 quail (control group). Quail of challenged group were inoculated intranasally with $10^6 \text{ EID}_{50}/0.1 \text{ ml}$ of the H5N1 virus, and quail of control group were inoculated with 0.1 ml of phosphate buffered saline (PBS). At 72 hr post-inoculation (h.p.i.), 3 quail of challenged group were sacrificed and trachea, lung, brain, liver, spleen, kidney and colon were collected for virus titration. Other quail were observed for clinical symptoms of disease every day for 10 days. Laryngopharyngeal and cloacal swabs were collected at 1, 2, 3, 4, 5, 7 and 10 days post-inoculation (d.p.i.) until die or period of observation. At 24 h.p.i., approximately 1 ml of heparinized blood was collected from 6 quail of challenged group and 4 quail of control group and used to isolate PBMC. The other 5 quail in challenged group were monitored every 8 hr to determine the mean death time (MDT). All of tissues and swabs collected for virus titration were treated as described below.

Chicken experiment: Eleven 6-week-old white leghorn chickens were purchased from N. G. C. Inc., Hyogo, Japan, and divided into two groups of 7 chickens (challenged group) and 4 chickens (control group). Chickens of challenged group were inoculated intranasally with $10^6 \text{ EID}_{50}/0.1 \text{ ml}$ of the H5N1 virus, and chickens of control group were inoculated with 0.1 ml of PBS. At 56 h.p.i., 3 chickens of challenged group were sacrificed, and the seven organs described in quail experiment section were collected for virus titration. Other chickens were observed for clinical symptoms for 10 days. Laryngopharyngeal and cloacal swabs were collected at 1, 2, 3, 4, 5, 7 and 10 d.p.i. until die or period of observation. At 24 h.p.i., approximately 1 ml of heparinized blood was collected from 4 chickens of both groups by wing venipuncture and used to isolate PBMC. Concomitantly, these 4 chickens of challenged group were monitored for determination of MDT.

PBMC cell culture: PBMC were purified by a density gradient centrifugation on 60% Percoll (GE healthcare bioscience, Uppsala, Sweden) and suspended in RPMI-1640 medium (Wako, Osaka, Japan). Approximately 10^6 cells of PBMC were stimulated with RPMI-1640 medium containing $5 \mu\text{g/ml}$ Concanavalin A (ConA, Sigma, St. Louis, MO, U.S.A.), 10% fetal bovine serum, 0.2 mM 2-mercaptoethanol (Sigma), 100 unit/ml penicillin (Meiji, Tokyo, Japan) and $100 \mu\text{g/ml}$ streptomycin (Meiji) for 6 hr in 24-well tissue culture test plates (TPP, Trasadingen, Switzerland) and collected for RNA extraction.

Total RNA isolation and cDNA preparation: Total RNA was extracted from PBMC by using TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. The RNA was resuspended in $40 \mu\text{l}$ of RNase-free water and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A.), and cDNA was synthesized with QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) as directed by the manufacturer. In short, approximately $0.5 \mu\text{g}$ of total RNA was reverse transcribed in a final volume of $20 \mu\text{l}$ of reaction containing the following components; $7\times$ gDNA Wipeout buffer, $5\times$ Quantiscript RT Buffer, Quantiscript Reverse Transcriptase and RNase-free water. To control for genomic DNA contamination, every reaction set contained an RNA sample without Quantiscript Reverse Transcriptase. The resulting cDNA was stored at -30°C until use for real-time PCR.

Quantification of cytokines, TLR-7 and influenza A virus M1 gene: The mRNA expressions of *IFN- α* , *IFN- γ* , *IL-2*, *IL-6*, *IL-8*, *IL-10*, *IL-12 α* , *IL-18* and *TLR-7* were quantified by real-time PCR using gene specific primers (Table 1) as described previously [20]. To determine viral load in PBMC, viral RNA and mRNA of influenza A virus was quantified by using a matrix 1 (M1) gene-specific primer pair (forward: 5'-AAG ACC AAT CCT GTC ACC TCT GA-3' and reverse: 5'-CAA AGC GTC TAC GCT GCA GTC C-3') [22]. Expression of β -actin mRNA was also quantified as a reference housekeeping gene. Real-time PCR was performed on a MiniOpticon Real-Time PCR Detection System (BIO-RAD, Hercules, CA, U.S.A.). The real-time PCR reaction mixture contained $2.0 \mu\text{l}$ of sample cDNA, $1.0 \mu\text{l}$ of forward and reverse primers ($10 \mu\text{M}$ each), $10 \mu\text{l}$ of iQ SYBR Green Supermix (BIO-RAD) and $6.0 \mu\text{l}$ of nuclease free water. A typical thermal profile consists of one cycle and 10 min of polymerase activation at 95°C , followed by 40 cycles of PCR at 95°C for 15 sec and specific annealing temperature for 60 sec. After the completion of amplification step, the real-time PCR products were heated to 95°C for 15 sec and then cooled to 60°C for 5 sec before ramping back to 95°C in 0.5°C increments. The relative expression of each target gene was normalized by dividing the copy number of the target gene by that of quail β -actin gene in the same cDNA sample.

Virus isolation and titration: Laryngopharyngeal and cloacal swab samples were taken in 1 ml of nutrient broth media containing 10 mg streptomycin and $10,000$ units of penicillin and serially diluted with PBS by 10-fold. Twen-

Table 1. Sequences for primers used in the quantitative real-time PCR assays

Target gene			Quail	Chicken
Type 1 interferon	<i>IFN-α</i>	F ^{a)}	5'-CCTTGCTCCTTCAACGACA-3'	5'-CCAGCACCTCGAGCAAT-3'
		R	5'-CGCTGAGGATACTGAAGAGGT-3'	5'-GGCGCTGTAATCGTTGTCT-3'
Type 2 interferon	<i>IFN-γ</i>	F	5'-CAACCTTAATGATGGCAGCA-3'	5'-CTCCCGATGAACGACTTGAG-3'
		R	5'-CTTTGCGGTGGATTCTCA-3'	5'-CTGAGACTGGCTCCTTTTCC-3'
Th1-type cytokine	<i>IL-2</i>	F	5'-GTGCAAAGTACTGATCTTCGCC-3'	5'-TTGGAATAATCAAGAACAAGATTCATC-3'
		R	5'-CTTGGTGTGTAGAGCTCGAGATG-3'	5'-TCCCAGGTAACACTGCAGAGTTT-3'
	<i>IL-12α</i>	F	5'-AAGACCTGAAAACCTACAAGGC-3'	5'-AAGACCTGAAAACCTACAAGGC-3'
		R	5'-GGCTTGCATCATGTCATCAA-3'	5'-GGCTTGCATCATGTCATCAA-3'
	<i>IL-18</i>	F	5'-GCAGCGGAATGTACTTCAAC-3'	5'-GAAACGTCAATAGCCAGTTGC-3'
		R	5'-CTCTTATCTTCTACCTGGACGCTG-3'	5'-TCCCATGCTCTTCTCACAACA-3'
Th2-type cytokine	<i>IL-10</i>	F	5'-CACAACCTTCTTACCTGCGAG-3'	5'-CATGCTGCTGGGCCTGAA-3'
		R	5'-CATGGCTTGTAGATCCCGTTC-3'	5'-CGTCTCCTTGATCTGCTTGATG-3'
Pro-inflammatory cytokine	<i>IL-6</i>	F	5'-CAACCTCAACCTGCCCAA-3'	5'-CTGTTTCGCTTTTTCAGACCTACC-3'
		R	5'-GGAGAGCTTCTCAGGCATT-3'	5'-CATGGTGATTTTCTCTATCCAGTCC-3'
Chemokine	<i>IL-8</i>	F	5'-CTGAGGTGCCAGTGCATTAG-3'	5'-CTGCGGTGCCAGTGCATTAG-3'
		R	5'-AGCACACCTCTCTCCATCC-3'	5'-AGCACACCTCTCTCCATCC-3'
Receptor	<i>TLR-7</i>	F	5'-AGATGTTTTCTGGGCAGACG-3'	5'-TTCTGGCCACAGATGTGACC-3'
		R	5'-AATGACTTCAACCGGTTACTGG-3'	5'-CCTTCAACTTGGCAGTGCAG-3'
<i>β-actin</i>		F	5'-CTGGCACCTAGCACAATGAA-3'	5'-CTGGCACCTAGCACAATGAA-3'
		R	5'-CTGCTTGCTGATCCACATCT-3'	5'-CTGCTTGCTGATCCACATCT-3'

a) F: forward primer, R: reverse primer.

ty% (w/v) tissue homogenates were prepared with PBS, and the supernatant was collected after centrifugation of 1,000 \times g for 10 min. These samples were inoculated into allantoic cavity of 10-day-old embryonated chicken eggs. After 48 hr incubation, allantoic fluid was collected and tested for hemagglutination (HA) activity. The virus titer of each specimen was calculated by the Reed-Muench method [16].

Statistical analysis: The significance of differences in virus titers was determined with the Tukey-Kramer multiple comparisons test. Other differences were analyzed with Student's *t*-tests. Statistical significance was set at $P < 0.01$ or $P < 0.05$.

RESULTS

Clinical symptoms and MDT in quail and chicken: All of the quail and chickens that were inoculated intranasally with A/whooper swan/Aomori/1/2008 (H5N1) died at 88 to 96 h.p.i. and at 64 to 72 h.p.i., respectively. Infected quail presented loss of appetite, depression and weakness after 56 h.p.i. (Table 2). Six of the eleven quail in the challenged group displayed neurological signs characterized by tremors, lack of coordination, seizures and head tilt after 80 h.p.i. On the other hand, the chickens presented loss of appetite, depression and weakness at 48 h.p.i., but no neurological signs. The MDTs of the quail and chickens (91.2 and 66.0 hr, respectively) were significantly different (Table 2).

Virus titers just before death: Virus was recovered from each of the tissues examined (trachea, lung, brain, liver, spleen, kidney and colon). The virus titers in the brain of infected quail were significantly higher than those in the liver ($P < 0.01$), spleen ($P < 0.01$) and kidney ($P < 0.05$) (Fig.

Table 2. Fractions of quail and chickens showing clinical symptoms following infection with A/whooper swan/Aomori/1/2008 (H5N1), and mean death time (MDT)

Clinical symptom	Number of positive/ total number	
	Quail	Chicken
Loss of appetite, Depression and Weakness	11/11	4/4
Cyanosis (comb or feet)	0/11	4/4
Neurological signs	6/11	0/4
Dead	11/11	4/4
MDT (hr)	91.2 \pm 4.4 ^{a)}	66.0 \pm 4.0

a) The MDT was significantly longer in quail than in chicken ($P < 0.01$).

1). On the other hand, the virus titers in the brain of chicken were significantly lower than those in the trachea ($P < 0.01$) and lung ($P < 0.05$).

Virus shedding in quail and chicken: We measured virus titers in the laryngopharyngeal and cloacal swabs collected from living quail at 1, 2, 3 and 4 d.p.i. and from living chickens at 1 and 2 d.p.i. (Table 3). All living quail shed virus in the laryngopharyngeal swabs at 1, 2, 3 and 4 d.p.i. In the cloacal swabs, H5N1 virus was detected in 2 out of 14 quail at 1 d.p.i. and from all quail at 2, 3 and 4 d.p.i. (Table 3). All living chickens shed virus in both laryngopharyngeal and cloacal swabs at 1 and 2 d.p.i. The mean virus titer of cloacal swabs in chickens at 2 d.p.i. was significantly higher than that in quail ($P < 0.01$).

Expression of influenza virus M1 gene in PBMC: At 24 h.p.i., the copy number of M1 gene in chicken was significantly greater than that in quail (Fig. 2).

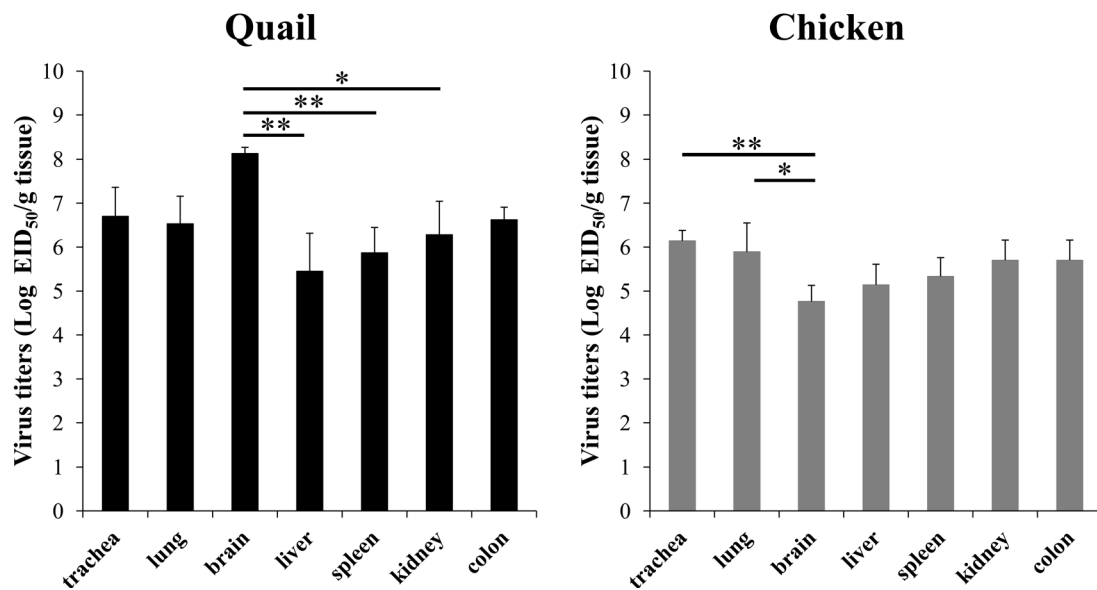


Fig. 1. Virus titers in tissues from quail and chickens intranasally inoculated with the A/whooper swan/Aomori/1/2008 (H5N1). The tissues were collected at 72 h.p.i. from quail and at 56 h.p.i. from chickens. The virus titer of brain in quail was significantly higher than the titers of liver, spleen and kidney. The virus titer of brain in chickens was significantly lower than the titers of lung and trachea (* $P < 0.05$, ** $P < 0.01$, Tukey-Kramer test).

Table 3. Virus titers in laryngopharyngeal and cloacal swabs from quail and chickens infected with A/whooper swan/Aomori/1/2008 (H5N1)

Animal	Swab	Number of positive/total number (Log EID ₅₀ /0.1 ml)			
		1 d.p.i.	2 d.p.i.	3 d.p.i.	4 d.p.i.
Quail	laryngopharyngeal	14/14 (3.52 ± 0.59)	14/14 (3.93 ± 0.58)	14/14 (4.79 ± 0.80)	11/11 (5.57 ± 0.34)
	cloacal	2/14 (1.25 and 0.50)	14/14 (1.20 ± 0.70) ^a	14/14 (2.88 ± 1.10)	11/11 (3.32 ± 0.96)
Chicken	laryngopharyngeal	4/4 (2.81 ± 0.52)	4/4 (2.50 ± 0.20)	ND ^b	ND
	cloacal	4/4 (1.00 ± 0.46)	4/4 (2.50 ± 0.74)	ND	ND

a) The mean virus titer of the cloacal swabs in quail was significantly lower than that in chickens at 2 d.p.i. ($P < 0.01$). b) ND, not determined.

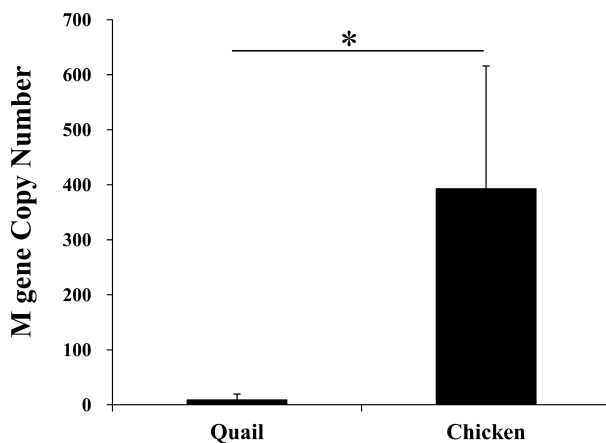


Fig. 2. Influenza A virus matrix 1 (M1) gene expression in quail and chicken PBMC at 24 h.p.i. The copy number of virus-derived RNA in PBMC collected at 24 h.p.i. was measured by quantitative real-time PCR. Error bars show standard error (SE). Values designated by an asterisk are significantly different between quail and chickens (* $P < 0.05$, Student's *t*-test).

Cytokines and TLR-7 responses to H5N1 HPAIV infection: At 24 h.p.i., the mRNA expressions of *IFN-γ* and *IL-8* were up-regulated in quail, while the mRNA expression of *IFN-γ* was down-regulated in chickens (Fig. 3). *IFN-γ* mRNA expression was significantly higher in quail than in chickens. The mRNA levels of TLR-7 and the other cytokines quantified (*IFN-α*, *IL-2*, *IL-6*, *IL-8*, *IL-10*, *IL-12α* and *IL-18*) were not appreciably changed by the inoculation.

DISCUSSION

In the present study, the clinical course, viral growth and cytokine response in quail infected with A/whooper swan/Aomori/1/2008 (H5N1) were compared with chicken to clarify why quail shows a different pathology from chicken. We focused on the cytokine response, because it is considered to have an important role in the pathology caused by H5N1 HPAIV in mammals and several avian species. The H5N1 virus used in this study, like other H5N1 HPAIV strains [5, 8, 9, 12, 23], resulted in a late onset time and a prolonged MDT in quail. Interestingly, the expression of

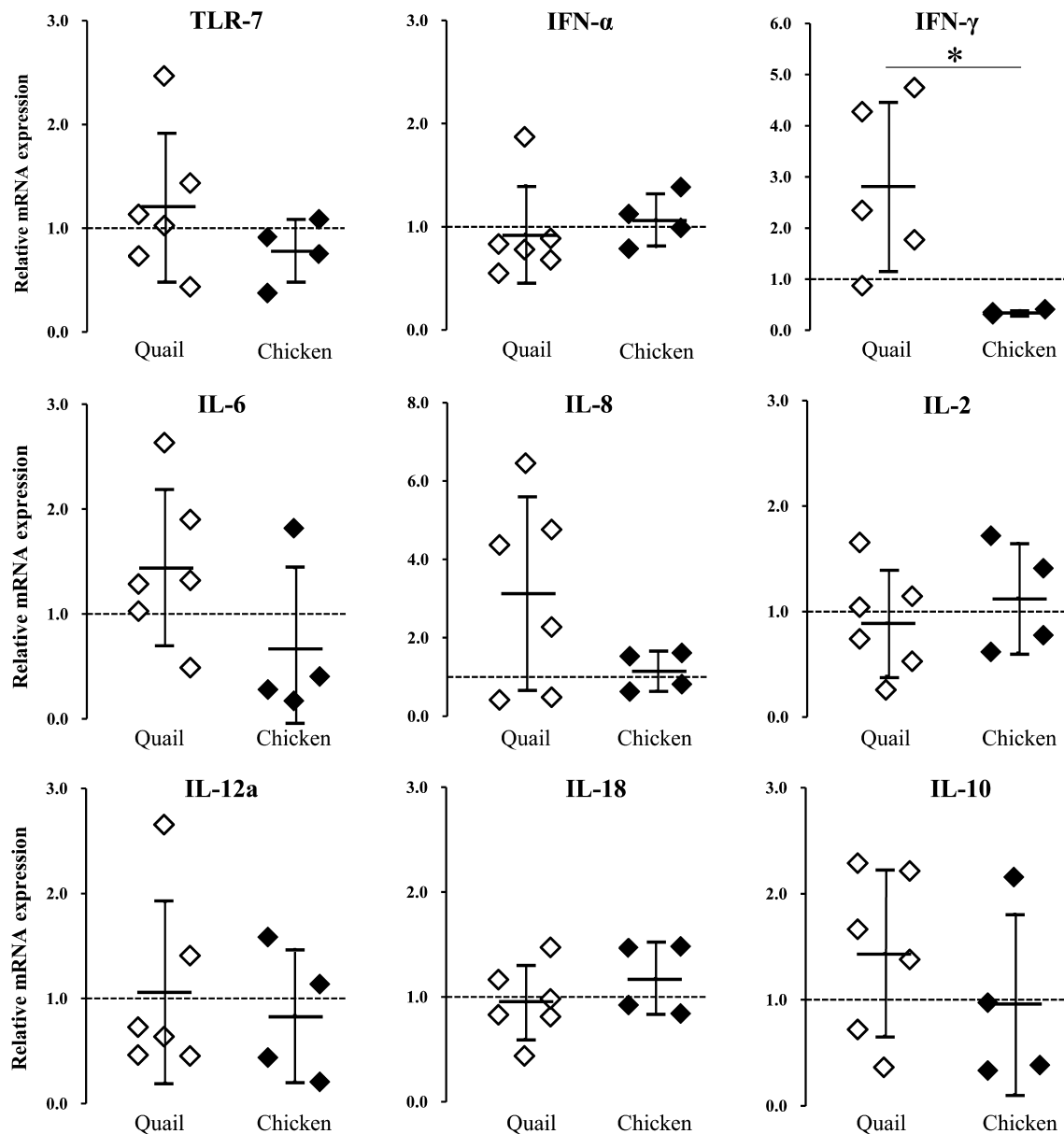


Fig. 3. Comparison of cytokines and TLR-7 mRNA responses of the PBMC between quail and chickens infected with A/whooper swan/Aomori/1/2008 (H5N1) at 24 h.p.i. The value of mRNA expression of each gene was quantified by real-time PCR. The mRNA expressions at 24 h.p.i. relative to the mRNA expressions before infection were compared with control birds. Bars indicate the average \pm standard error of the mean (SEM) for each group. The asterisk indicates a significant difference between quail and chickens (* P <0.05). All points above and below the dashed lines indicate increases and decreases in expression, respectively, at 24 h.p.i.

IFN- γ , an antiviral cytokine, increased in quail, while it decreased in chicken after H5N1 virus inoculation (Fig. 3). Furthermore, the viral load in quail PBMC was significantly lower than that in chickens (Fig. 2), indicating that the virus had hardly propagated in quail PBMC at the early stage of infection. These results indicate that the induction of IFN- γ expression in quail PBMC is related to inhibition of viral replication, which may be related to the late onset time and

the prolonged MDT in quail. Therefore, the different clinical courses in quail and chicken infected with H5N1 HPAIV might be caused by a difference in IFN- γ responses.

The expression of IFN- γ was increased in quail at the early stage after H5N1 virus inoculation in our study (Fig. 3). IFN- γ has antiviral activity through activation of innate immune cells such as macrophages and NK cells [6]. Because these cells are reported to play essential roles in

the control of influenza virus [14], IFN- γ is also thought to be associated with the pathogenesis of influenza [3, 10]. For example, H5N1 HPAIV was found to induce IFN- γ in mammals [4], and early administration of exogenous IFN- γ during influenza virus infection stimulates NK cell proliferation and function in infected lungs [24]. An HPAIV A/Vietnam/1203/2003 (H5N1) was also found to induce IFN- γ in the lung and spleen of chickens [11]. However, in the present study, infection caused a decrease in the expression of IFN- γ in chicken PBMC. Similarly, Suzuki *et al.* [18] found that IFN- γ decreased in the lung of chicken infected with HPAIV A/chicken/Yamaguchi/7/2004 (H5N1). Both this virus and the virus used in our study were isolated in Asia in recent years. These recent Asian H5N1 viruses are thought to cause early destruction of the innate immune response in chicken [18], and have been increasing in pathogenicity in diverse avian species. On the other hand, in quail PBMC, the increase of IFN- γ expression appears to have activated the antiviral function in the innate immune response. Because all the quail in this study eventually died, the immune response against H5N1 HPAIV induced in quail might be overwhelmed by virus replication at the late phase of infection. However, our results indicate that IFN- γ could be recognized as one of host antiviral factors to be induced against influenza virus infection in quail.

In the present study, laryngopharyngeal swabs indicated that both quail and chicken were shedding virus at 1 d.p.i. (Table 3). Similarly, in quail and chickens experimentally infected with H5N1 virus, the virus was detected in both laryngopharyngeal and cloacal swabs at 1 d.p.i. [9], and the virus titers were rather higher in quail. In the present study, almost all quail first shed virus from the cloaca at 2 d.p.i., one day later than in chicken, and the virus titers were lower than those in chickens (Table 3). Because the amount of virus shedding from the cloaca is considered to reflect the level of virus replication in the colon, the delay of virus shedding from the cloaca in quail might be related to the antiviral function of IFN- γ induced in quail PBMC at the early stage of infection. These differences between quail and chickens probably account for the longer survival time of quail. However, the longer survival time would increase the period of viral excretion and the probability of transmission.

The quail in this study frequently showed neurological signs, whereas the chickens did not (Table 2). Additionally, the virus titer in the brain of quail before death was significantly higher than the titers in the other tissues examined, whereas the titer in the brain of chickens was the same as or less than the titers in the other tissues (Fig. 1). Histopathological examination revealed the degeneration of many of the cerebellar Purkinje cells in quail showing neurological sign after infection with A/whooper swan/Aomori/1/2008 (H5N1) (data not shown). Together, these results suggest that the neurological signs in the quail were caused by damage to brain tissues by the virus.

Recently, chickens were reported to lack retinoic acid-inducible gene I (RIG-I), which is triggered by influenza virus and which leads to production of type I IFN in innate immunity [13], and thus, it is considered that influenza vi-

ruses can easily replicate in chicken cells [1]. It is not known whether quail have RIG-I. It would be helpful to answer this question, because RIG-I has the potential to affect viral replication in quail cells.

In conclusion, our results suggest that the induction of antiviral cytokine IFN- γ at the early stage of H5N1 virus infection inhibits viral replication in quail PBMC. In this study, we focused the cytokine responses only at 24 h.p.i. for limit of sequential blood sampling. Further studies are needed to investigate the cytokine responses after 24 h.p.i. to better understand the correlation between the induction of cytokines and pathogenicity of HPAIV. A better understanding of how IFN- γ expression is related to the different H5N1 pathologies in quail and chicken might also help to predict the pathogenesis of various infectious diseases in quail.

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REFERENCES

1. Barber, M. R., Aldridge, J. R. Jr., Webster, R. G. and Magor, K. E. 2010. Association of RIG-I with innate immunity of ducks to influenza. *Proc. Natl. Acad. Sci. U.S.A.* **107**: 5913–5918. [[Medline](#)] [[CrossRef](#)]
2. Chan, M. C., Cheung, C. Y., Chui, W. H., Tsao, S. W., Nicholls, J. M., Chan, Y. O., Chan, R. W., Long, H. T., Poon, L. L., Guan, Y. and Peiris, J. S. 2005. Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. *Respir. Res.* **6**: 135. [[Medline](#)] [[CrossRef](#)]
3. Du, N., Zhou, J., Lin, X., Zhang, Y., Yang, X., Wang, Y. and Shu, Y. 2010. Differential activation of NK cells by influenza A pseudotype H5N1 and 1918 and 2009 pandemic H1N1 viruses. *J. Virol.* **84**: 7822–7831. [[Medline](#)] [[CrossRef](#)]
4. Evseenko, V. A., Sharshov, K. A., Bukin, E. K., Zaykovskaya, A. V., Ternovoy, V. A., Ignatyev, G. M., Shestopalov, A. M., Netesov, S. V., Shkurupiy, V. A. and Drozdov, I. G. 2008. Pathogenesis of infectious disease of mice caused by H5N1 avian influenza virus. *Bull. Exp. Biol. Med.* **146**: 766–769. [[Medline](#)] [[CrossRef](#)]
5. Guan, Y., Peiris, J. S., Lipatov, A. S., Ellis, T. M., Dyrting, K. C., Krauss, S., Zhang, L. J., Webster, R. G. and Shortridge, K. F. 2002. Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. *Proc. Natl. Acad. Sci. U.S.A.* **99**: 8950–8955. [[Medline](#)] [[CrossRef](#)]
6. Guidotti, L. G. and Chisari, F. V. 2001. Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu. Rev. Immunol.* **19**: 65–91. [[Medline](#)] [[CrossRef](#)]
7. Hayashi, T., Hiromoto, Y., Chaichoune, K., Patchimasiri, T., Chakritbudsabong, W., Prayoonwong, N., Chaisilp, N., Wiriyarat, W., Parchariyanon, S., Ratanakorn, P., Uchida, Y. and Saito, T. 2011. Host cytokine responses of pigeons infected with highly pathogenic Thai avian influenza viruses of subtype H5N1 isolated from wild birds. *PLoS ONE* **6**: e23103. [[Medline](#)] [[CrossRef](#)]
8. Isoda, N., Sakoda, Y., Kishida, N., Bai, G. R., Matsuda, K.,

- Umemura, T. and Kida, H. 2006. Pathogenicity of a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals. *Arch. Virol.* **151**: 1267–1279. [[Medline](#)] [[CrossRef](#)]
9. Jeong, O. M., Kim, M. C., Kim, M. J., Kang, H. M., Kim, H. R., Kim, Y. J., Joh, S. J., Kwon, J. H. and Lee, Y. J. 2009. Experimental infection of chickens, ducks and quails with the highly pathogenic H5N1 avian influenza virus. *J. Vet. Sci.* **10**: 53–60. [[Medline](#)] [[CrossRef](#)]
10. Julkunen, I., Sareneva, T., Pirhonen, J., Ronni, T., Melen, K. and Matikainen, S. 2001. Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. *Cytokine Growth Factor Rev.* **12**: 171–180. [[Medline](#)] [[CrossRef](#)]
11. Karpala, A. J., Bingham, J., Schat, K. A., Chen, L. M., Donis, R. O., Lowenthal, J. W. and Bean, A. G. 2011. Highly pathogenic (H5N1) avian influenza induces an inflammatory T helper type 1 cytokine response in the chicken. *J. Interferon Cytokine Res.* **31**: 393–400. [[Medline](#)] [[CrossRef](#)]
12. Lee, C. W., Suarez, D. L., Tumpey, T. M., Sung, H. W., Kwon, Y. K., Lee, Y. J., Choi, J. G., Joh, S. J., Kim, M. C., Lee, E. K., Park, J. M., Lu, X., Katz, J. M., Spackman, E., Swayne, D. E. and Kim, J. H. 2005. Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. *J. Virol.* **79**: 3692–3702. [[Medline](#)] [[CrossRef](#)]
13. Loo, Y. M., Fornek, J., Crochet, N., Bajwa, G., Perwitasari, O., Martinez-Sobrido, L., Akira, S., Gill, M. A., Garcia-Sastre, A., Katze, M. G. and Gale, M. Jr. 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *J. Virol.* **82**: 335–345. [[Medline](#)] [[CrossRef](#)]
14. McGill, J., Heusel, J. W. and Legge, K. L. 2009. Innate immune control and regulation of influenza virus infections. *J. Leukoc. Biol.* **86**: 803–812. [[Medline](#)] [[CrossRef](#)]
15. Moulin, H. R., Liniger, M., Python, S., Guzylack-Piriou, L., Ocana-Macchi, M., Ruggli, N. and Summerfield, A. 2011. High interferon type I responses in the lung, plasma and spleen during highly pathogenic H5N1 infection of chicken. *Vet. Res.* **42**: 6. [[Medline](#)] [[CrossRef](#)]
16. Reed, L. J. and Muench, H. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27**: 493–497.
17. Pantin-Jackwood, M. J., Smith, D. M., Wasilenko, J. L., Cagle, C., Shepherd, E., Sarmiento, L., Kapczynski, D. R. and Afonso, C. L. 2012. Effect of age on the pathogenesis and innate immune responses in Pekin ducks infected with different H5N1 highly pathogenic avian influenza viruses. *Virus Res.* **167**: 196–206. [[Medline](#)] [[CrossRef](#)]
18. Suzuki, K., Okada, H., Itoh, T., Tada, T., Mase, M., Nakamura, K., Kubo, M. and Tsukamoto, K. 2009. Association of increased pathogenicity of Asian H5N1 highly pathogenic avian influenza viruses in chickens with highly efficient viral replication accompanied by early destruction of innate immune responses. *J. Virol.* **83**: 7475–7486. [[Medline](#)] [[CrossRef](#)]
19. To, K. F., Chan, P. K., Chan, K. F., Lee, W. K., Lam, W. Y., Wong, K. F., Tang, N. L., Tsang, D. N., Sung, R. Y., Buckley, T. A., Tam, J. S. and Cheng, A. F. 2001. Pathology of fatal human infection associated with avian influenza A H5N1 virus. *J. Med. Virol.* **63**: 242–246. [[Medline](#)] [[CrossRef](#)]
20. Uno, Y., Usui, T., Fujimoto, Y., Ito, T. and Yamaguchi, T. 2012. Quantification of interferon, interleukin, and Toll-like receptor 7 mRNA in quail splenocytes using real-time PCR. *Poult. Sci.* **91**: 2496–2501. [[Medline](#)] [[CrossRef](#)]
21. Usui, T., Yamaguchi, T., Ito, H., Ozaki, H., Murase, T. and Ito, T. 2009. Evolutionary genetics of highly pathogenic H5N1 avian influenza viruses isolated from whooper swans in northern Japan in 2008. *Virus Genes* **39**: 319–323. [[Medline](#)] [[CrossRef](#)]
22. Ward, C. L., Dempsey, M. H., Ring, C. J., Kempson, R. E., Zhang, L., Gor, D., Snowden, B. W. and Tisdale, M. 2004. Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. *J. Clin. Virol.* **29**: 179–188. [[Medline](#)] [[CrossRef](#)]
23. Webster, R. G., Guan, Y., Peiris, M., Walker, D., Krauss, S., Zhou, N. N., Govorkova, E. A., Ellis, T. M., Dyrting, K. C., Sit, T., Perez, D. R. and Shortridge, K. F. 2002. Characterization of H5N1 influenza viruses that continue to circulate in geese in southeastern China. *J. Virol.* **76**: 118–126. [[Medline](#)] [[CrossRef](#)]
24. Weiss, I. D., Wald, O., Wald, H., Beider, K., Abraham, M., Galun, E., Nagler, A. and Peled, A. 2010. IFN-gamma treatment at early stages of influenza virus infection protects mice from death in a NK cell-dependent manner. *J. Interferon Cytokine Res.* **30**: 439–449. [[Medline](#)] [[CrossRef](#)]
25. Yuen, K. Y. and Wong, S. S. 2005. Human infection by avian influenza A H5N1. *Hong Kong Med. J.* **11**: 189–199. [[Medline](#)]