Studies on the pathogenicity and host immune response in Japanese quail infected with H5N1 subtype highly pathogenic avian influenza virus

（ニホンウズラにおける H5N1 亜型高病原性鳥インフルエンザウイルス感染時の病態及び免疫応答に関する研究）

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CONTENTS

ABBREVIATION

BSL-3	biosafety level - 3
cDNA	complementary DNA
ConA	Concanavalin A
d.p.i.	days post-inoculation
EID_{50}	50% egg infective dose
ELISA	enzyme-linked immunosorbent assay
HA	hemagglutinin
HPAIV	highly pathogenic avian influenza virus
h.p.i.	hours post-inoculation
IFN	interferon
IL	interleukin
LPAIV	low pathogenic avian influenza virus
M1/M2	matrix protein 1/ matrix protein 2
MDT	mean death time
mRNA	messenger RNA
NA	neuraminidase
NK cell	natural killer cell
NP	nucleoprotein
NS	nonstructural protein
PA	polymerase acidic protein
PB1/PB2	polymerase basic protein 1/ polymerase basic protein 2

PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RIG-I	retinoic acid-inducible gene - I
RNA	ribonucleic acid
RT	reverse transcription
SE	standard error
SEM	standard error of the mean
SPF	specific pathogen-free
TLR	Toll-like receptor
Tm	melting temperature

GENERAL INTRODUCTION

Influenza viruses are members of the *Orthomyxoviridae* family and are the cause of Influenza. This family contains five genera, including *Influenzavirus* A, B and C, *Isavirus* and *Thogotovirus* [27]. Influenza viruses are classified as A, B and C based on antigenic differences in their nucleoprotein (NP) and matrix (M1) protein [31]. Only influenza A viruses are known to infect bird species [2]. Influenza A virus genomes are comprised of eight segments of single-stranded negative sense RNA [31]. These RNA segments basically encode 10 viral proteins, NP, RNA polymerase complex (PA, PB1 and PB2), M1, membrane proteins (M2), nonstructural proteins (NS1 and NS2) and external proteins, hemagglutinin (HA) and neuraminidase (NA). Influenza A viruses can be classified into subtypes based on the antigenic relationships of their surface glycoproteins, HA and NA. To date, sixteen subtypes of HA (H1 to H16) and nine of NA (N1 to N9) have been identified [11].

Influenza A viruses have been isolated from a variety of animals, including humans, pigs, horses, mink, sea mammals and a wide range of domestic and wild birds [67]. They were first isolated at the beginning of the 20th century from chickens (A/Brescia/1902(H7N7)), and since then were isolated from pigs in the late 1920s, humans in the early 1930s, horses and domestic ducks in the 1950s, terns (*Sterna hirundo*) in 1961, and many waterfowl and shorebirds since 1974 [19, 26]. All subtypes have been detected in isolates from avian species [2] and almost all isolates come from

the order *Anseriformes* and to a lesser extent *Charadriiformes* in the family *Laridae* [55]. Therefore, waterfowl (predominantly wild ducks) are considered a natural reservoir of influenza A viruses [65].

Influenza A viruses isolated from waterfowl replicated poorly in mammals and land-based birds [5, 40]. Therefore, when influenza A viruses in a natural reservoir are transmitted to other animals, the viruses need intermediate hosts where they can undergo gene mutation, including genetic reassortment to accomplish cross-species transmission and host adaptation. More recently, Japanese quail (*Coturnix japonica*) were reported to have the potential to support replication of at least 14 HA subtypes of influenza A viruses isolated from wild aquatic birds [35]. In addition, it was reported that once influenza A viruses adapted in quail, several of these viruses led to transmission and replication in chickens [45, 54]. Therefore, quail are considered to have the potential of playing a role as an intermediate host that permits the adaptation of avian influenza viruses from wild birds to chickens.

Avian influenza viruses can be divided based on their pathogenicity to naïve chickens. Low pathogenic avian influenza viruses (LPAIVs) mainly cause respiratory illness in poultry and generally low mortality. Highly pathogenic avian influenza viruses (HPAIVs) cause systemic disease, often resulting in high mortality in turkeys and chickens [59, 60]. The HPAIVs are restricted to H5 and H7 subtypes, and viruses of these two subtypes can be either low or highly pathogenic [25, 30, 56], whereas all other known HA subtypes have only low pathogenic forms.

Since 1997, H5N1 subtype HPAIVs have posed a serious threat to poultry, wild birds and mammals, including humans all over the world. To date, H5N1 HPAIVs have been isolated from several poultry species, including chickens, quail, turkeys and domestic ducks. More recently, some reports have documented that genetic analysis of H5N1 viruses isolated from humans indicated the possibility of a genetic reassortment event from their precursor viruses in quail [7, 14, 17, 32]. Additionally, it was reported that H5N1 HPAIVs derived from quail increased pathogenicity in mice after multiple experimental passages in quail [57]. These observations indicate that quail have the potential to serve as an intermediate host for generation of reassortant viruses with pandemic potential for humans. In Japan, several outbreaks have occurred in poultry and wild birds by H5N1 HPAIVs since 2004 [36, 51]. To date, H5N1 HPAIVs have never been isolated from quail in Japan. However, we should pay attention to quail as an important poultry species.

Quail have high sensitivity to H5N1 HPAIVs and show approximately 100% mortality from infection. However, it has been reported that quail infected with H5N1 HPAIV take longer than chickens to show signs of disease and die [15, 21, 22, 29, 68]. For example, H5N1 Hong Kong isolates that emerged in 2001 were more pathogenic to chickens and the mean death times (MDTs) of chickens were around 2 days without any prior clinical signs, whereas the MDTs of quail were 1-5 days longer than those of chickens [15]. Furthermore, the MDTs in chicken and quail infected with a more recent HPAIV isolate A/chicken/Korea/ES/03 (H5N1) were reported to be 2.0 and 3.8 days,

respectively [29]. 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 infected with the same virus could be explained in part by the innate immune responses induced against the virus. 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" [71]. In humans, clinical features associated with H5N1 virus infections have been linked to cytokine dysregulation [6, 62]. 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 clinical outcomes and cytokine responses from H5N1 virus infection [18, 24, 38, 44, 58]. However, in quail, little is known about the immune responses to H5N1 virus. Thus, the purpose of this thesis is to clarify the immune response of quail infected with H5N1 HPAIV.

To accomplish this study, in chapter 1, this author determined the nucleotide sequences of quail immune-related genes including *interferon* (*IFN*)-α (a type I interferon), *IFN-γ*, *interleukin* (*IL*)-*12a*, *IL-12b*, and *IL-18* (Th1 cytokines), *IL-4* and *IL-13* (Th2 cytokines), *IL-10* (a Treg cytokine), *IL-2* (a T cell proliferative cytokine), *IL-1β* and *IL-6* (pro-inflammatory cytokines), and *IL-8* (a chemokine) and established

quantitative real-time PCR assays for these immune-related genes. In chapter 2, this author examined the expression levels of innate immune-related genes in peripheral blood mononuclear cells (PBMC) associated with H5N1 virus infection in quail. These studies will provide useful information about avian immunology in the H5N1 HPAIV infection.

CHAPTER 1

Quantification of interferon, interleukin, and Toll-like receptor 7 mRNA in quail splenocytes using real-time PCR

INTRODUCTION

Japanese quail (*Coturnix japonica*) are farmed as poultry all over the world. Inbred strains of quail have been established and have served as animal models in avian toxicology, sexual conditioning, photoperiodicity, and microbiological studies [3, 41, 52]. Quail are sensitive to various avian infectious diseases such as avian influenza, Marek's disease, fowl pox, and Newcastle disease [35, 47]. Innate immune responses have been examined in virally infected chickens and ducks [1, 28, 48], whereas little is available on the immune response of quail.

Cytokines including interferon (IFN) and interleukin (IL) participate in the host immune system by modulating lymphocyte activation, proliferation, differentiation, survival, and apoptosis. The cytokine expression profile can clarify the interaction between host immunity and pathological pathways involved in many inflammatory responses associated with infectious disease and autoimmune reactions. In mammals, serum interferon and interleukin levels are usually measured with ELISA. This method uses a molecule-specific antibody to directly measure cytokine expression in specimens at the protein level. However, the method is not suitable for birds because of a lack of specific antibodies against avian cytokines. Alternatively, cytokines can be measured at the mRNA level by real-time PCR [42].

Pattern-recognition receptors, such as Toll-like receptors (TLR), also affect host immunity by triggering innate immune responses. Mammalian TLR-7 has been

implicated in recognizing single-stranded viral RNA such as the genome of influenza virus. The genomic organization and function of avian *TLR-7* have been characterized for chicken and duck [34, 46]. To date, nucleotide sequences of many cytokines of poultry chicken and domestic duck are available in the databases. However, most quail cytokines and *TLR-7* have not been documented and immunological features of quail have not been well characterized.

In the present study, this author determined nucleotide sequences of the quail *IL-1β*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12a*, *IL-12b*, *IL-13*, *IL-18*, and *TLR-7* genes. For immunological analysis of quail, this author also established quantitative real-time PCR assays for immune-related genes including *IFN-α* (a type I interferon), *IFN-γ*, *IL-12a*, *IL-12b*, and *IL-18* (Th1 cytokines), *IL-4* and *IL-13* (Th2 cytokines), *IL-10* (a Treg cytokine), *IL-2* (a T cell proliferative cytokine), *IL-1β* and *IL-6* (proinflammatory cytokines), and *IL-8* (a chemokine).

MATERIALS AND METHODS

Sequencing of Quail Immune-Related Genes

To amplify quail gene transcripts, which were synthesized in quail splenocytes, for *IL-1β*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12a*, *IL-12b*, *IL-13*, *IL-18*, and *TLR-7*, primers for each gene were designed based on published chicken mRNA sequences. The PCR was performed using a TaKaRa PCR Thermal Cycler Dice Version 3 (TaKaRa, Shiga, Japan). A typical PCR protocol consists of 4 min of polymerase activation at 95°C, followed by 40 cycles at 95°C for 30 sec, 52°C for 45 sec, and 72°C for 30 sec, followed by a 5 min final extension at 72°C. Each amplified PCR product was visualized by electrophoresis on 1.5% agarose gel with GelRed staining (Biotium, Hayward, CA), purified using MinElute Gel Extraction Kit (Qiagen, Mainz, Germany) according to the manufacturer's instructions and sequenced to determine quail immune-related gene mRNA sequences. Sequencing reactions were performed using Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems LLC, Foster City, CA). The determined sequences of *IL-1β*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12a*, *IL-12b*, *IL-13*, *IL-18*, and *TLR-7* mRNA were deposited in GenBank under the accession numbers shown in Table 1.

Splenocytes Culture, Total RNA Isolation, and cDNA Preparation

Quail splenocytes were isolated from spleen by a density gradient centrifugation on 60% Percoll (GE Healthcare Bioscience, Uppsala, Sweden) and suspended in RPMI-1640 medium (Wako, Osaka, Japan). Total RNA was extracted from splenocytes by using TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA was resuspended in 40 μl of RNase-free water and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and cDNA was synthesized with QuantiTect Reverse Transcription Kit (Qiagen) as directed by the manufacturer. In short, approximately 0.5 μg of total RNA was reverse transcribed in a final volume of 20 μl of reaction containing the following components: 7×gDNA Wipeout buffer, 5×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 PCR and real-time PCR.

Quantitative Real-Time PCR Assay

For quantitative real-time PCR assays, specific primers for quail *IL-1β*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12a*, *IL-12b*, *IL-13*, *IL-18*, and *TLR-7* genes were designed. Primers for

previously reported *IFN-α*, *IFN-γ*, and *IL-2* mRNA were designed based on published quail mRNA sequences. Primers of *IL-8* and *IL-13* antisense sequence were used as reported for chicken genes [20, 50]. β-Actin was used as a reference housekeeping gene.

Real-time PCR was performed with a MiniOpticon Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Details of the amplification conditions and products are shown in Table 1. The real-time PCR reaction mixture contained 1.0 *μl* of sample cDNA, 1.0 *μl* of forward and reverse primers (10 *μ*M each), and 10 *μl* of iQ SYBR Green Supermix (Bio-Rad) and 7.0 *μl* 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. Following amplification, melt curve analysis was performed and the melting temperature (Tm) of the sample was determined. After the completion of amplification step, the samples 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. A standard curve for quantification was established using serial dilutions ranging from 10^1 to 10^7 copies of an external standard plasmid. Each standard plasmid was prepared from pGEM-T Easy vector (Promega, Madison, WI) in which the interest target genes were cloned and each standard plasmid digested at a specific site in the vector by restriction enzymes *Pst* I or *Spe* I. Specificity of amplification was checked using a melting curve analysis of real-time PCR following the manufacturer's instructions. Each amplified PCR product was visualized by electrophoresis on 1.5% agarose gel with GelRed staining. 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.

ConA-Induced Immune-Related Gene Expression in Quail Splenocytes

Three specific pathogen-free Japanese quail (5-wk-old, female) were purchased from Nisseiken (Oume, Japan). Splenocytes prepared from the 3 quail were stimulated with RPMI-1640 medium containing concanavalin A (ConA, 5 μg/m*l*, Sigma, St. Louis, MO) for 0, 3, 6, 12, and 24 hr and collected at each time point. Total RNA was extracted from the stimulated splenocytes and the cDNA was synthesized as described above. The cDNA were used to quantify the expressions of *IFN-α*, *IFN-γ*, *IL-1β*, *IL-2*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12a*, *IL-12b*, *IL-13*, *IL-18*, and *TLR-7*. The mean value of the relative expression of the target gene at each time point was calculated. Student's t-test was used to determine the statistical differences between the group of 0 hr time point and the group of other time points.

RESULTS

Nucleotide Sequences of Quail Immune-Related Gene Transcripts

The lengths of the nucleotide sequences of quail *IL-1β*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12a*, *IL-12b*, *IL-13*, *IL-18*, and *TLR-7* mRNA determined in the present study ranged from 266 to 3,328 bp (Table 1). Among them, the sequences of *IL-4* and *TLR-7* mRNA contained the predicted start and stop codon. The coding sequence of quail *IL-4* (426 bp) included the insertion of 5 residues, at the C-terminal part, compared with the chicken sequence (411 bp). The nucleotide and amino acid sequence identities between quail *IL-4* and chicken *IL-4* are 90.5 and 93.0%, respectively. The coding sequence of quail *TLR-7* (3,144 bp) shares 94.6% nucleotide and 93.4% amino acid identity with chicken *TLR-7* (3,180 bp). The nucleotide and amino acid sequence identities between quail and human *IL-4* (74.9 and 75.8%, respectively) and between quail and human *TLR-7* (74.0 and 65.4%, respectively) are low. The DNA Data Bank of Japan (DDBJ) accession numbers of these sequences are shown in Table 1.

Specificity and Efficiency of Real-Time PCR Assay

For quantitative real-time PCR assays, specific primers for quail *IFN-α*, *IFN-γ*, *IL-1β*, *IL-2*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12a*, *IL-12b*, *IL-13*, *IL-18*, and *TLR-7* mRNA

were designed based on each sequence (Table 1). The annealing temperatures that this author found to work best for each primer pair are shown in Table 1. A single product with a distinct Tm was amplified for each gene. After gel electrophoresis, a single band of the expected size was obtained for each gene. The products were sequenced and their lengths are shown in Table 1. The r^2 values of each standard curve ranged from 0.985 to 0.998. The range in the number of mRNA over which the assay was valid was 10 to 10^7 for most assays (Table 1).

ConA-Induced Immune-Related Gene Expression

The assay was tested using mononuclear cells isolated from quail spleen stimulated with ConA (Fig. 1). The mRNA expression of *IL-18* increased approximately 4-fold at 3 to 6 hr and returned to the normal level by 24 hr. For *IL-18*, the expression at 6 and 12 hr was significantly greater than that at 0 hr ($P < 0.05$). The ConA did not affect *IL-12a* expression but increased *IL-12b* expression about 10-fold at 6 hr. The ConA increased the expressions of *IL-4* and *IL-2* approximately 40- and 12-fold, respectively, by 12 hr. In contrast, mRNA expression of *IL-13* did not increase at 3 hr and decreased after 6 hr. The expressions of *IL-10* and *IFN-α* peaked at 3 hr approximately 3- and 4-fold at 3 hr, respectively. The mRNA expression of *IL-6* gradually increased approximately 2-fold by 24 hr. The ConA stimulation decreased the expression of *IFN-γ*, *IL-1β*, *IL-8*, and *TLR-7*.

DISCUSSION

In this work, this author aimed to develop a real-time PCR method for quantifying immunological gene expression of Japanese quail. Quantitative real-time PCR has been the method of choice for the rapid analysis and quantification of cytokine gene expression. One of the advantages of quantitative real-time PCR is that it requires only partial sequence information for the target genes. However, nucleotide sequences of most quail cytokines were not available in the database. Therefore, this author first determined the nucleotide sequences of mRNA for quail immune-related genes. Cytokines and TLR are both important effector molecules of the host innate immune response, which establishes a state of inflammation to eradicate pathogens from host cells [61, 72]. In the present study, this author designed primers for 12 quail cytokines and *TLR-7* to develop a SYBR Green I-based quantitative real-time PCR method.

The specificity of real-time PCR was validated for each gene by the generation of a single PCR product from quail splenocytes, which was confirmed by running the product in an agarose gel and analyzing the melt curve. After gel electrophoresis, single fragments were visualized with the expected size and further confirmed by sequence analysis (data not shown). The melting curves showed only one peak for each gene. These results indicate that nonspecific amplification and primer-dimer formation had not occurred during PCR and guarantee the specificity of the assay. A standard curve can be used for absolute quantification. In this study, this author used plasmids

containing the PCR products of target genes as external standard samples. Consequently, a good regression coefficient ($r^2 \geq 0.985$) was achieved for each target gene. These results validate the real-time PCR assay to quantify the expressions of immune-related genes in quail. As a test of the method, this author measured gene expression in quail splenocytes in response to ConA treatment. Concanavalin A is described as a classical T-cell mitogen [12], which has been shown to induce *IL-2* mRNA expression in chicken lymphocytes [53, 69]. In chicken splenocytes, *IFN-γ* was slightly induced by ConA stimulation [43]. The expression of *IFN-γ* has been shown to be induced by *IL-12* and *IL-18* expression [8, 13]. In this study, however, ConA stimulation significantly reduced the expression of quail *IFN-γ*, significantly induced the expression of *IL-18*, and moderately induced the expressions of *IL-2* and *IL-12b*. The *IFN-γ* expression was not induced by ConA, possibly because *IL-10* inhibited *IFN-γ* expression. Concanavalin A induced the expression of regulatory cytokine *IL-10* in quail splenocytes. However, *IL-10* was also shown to inhibit the ConA-induced expression of *IFN-γ* in chicken splenocytes [49].

Further, *IL-18* was not essential for *IFN-γ* induction in chicken, although it is essential in mammals [24]. In addition, ConA remarkably induced the expressions of quail *IL-4*, *IL-6*, and *IFN-α* but decreased the expressions of quail *IL-1β*, *IL-8*, *IL-13*, and *TLR-7*. This variation in expression might be due to the presence of different cell types in the splenocytes.

The immune response against viral infection may affect the host defense against the virus. The immune system is affected by not only infectious disease but also the sexual cycle, stress, and growth of animals. Therefore, a better understanding of the quail's immune system may also make quail a more useful experimental animal and improve their breeding in farms. In conclusion, this author established real-time PCR assays for quantifying the gene expressions of quail cytokines and *TLR-7*. These assays should help to elucidate how the quail immune system responds to various pathogens.

Table 1. Primer sequences for real-time PCR amplification of quail target genes

Target gene	Primer sequence (5'-3')	Real-time PCR				Accession number [b]	Length of determined sequence (bp)
		Annealing temperature [a] (°C)	Length of product [a] (bp)	Melting temperature [a] (°C)	Quantification range [a] (copies)		
IFN-α	F: CCTTGCTCCTTCAACGACA R: CGCTGAGGATACTGAAGAGGT	58	100	82.5	10^1-10^7	AB154298	–
IFN-γ	F: CAACCTTAATGATGGCACGA R: CTTTGCGGTGGATTCTCA	58	86	79.5	10^1-10^7	AJ001678	–
IL-1β	F: CTTCCTCCAGCCAGAAAGT R: CAGCTTGTAGCCCTTGAT	60	116	88.0	10^1-10^7	AB559570 [c]	446
IL-2	F: GTGCAAAGTACTGATCTTCGCC R: CTTGGTGTGTAGAGCTCGAGATG	61	168	76.5	10^1-10^7	AY386204	–
IL-4	F: GAGAGCATCCGGATAGTGAAG R: TTCGCATAAGAGCTGGGTTC	60	114	76.5	10^1-10^7	AB559571 [c]	433
IL-6	F: CAACCTCAACCTGCCCAA R: GGAGAGCTTCCTCAGGCATT	53	85	83.0	10^1-10^7	AB559572 [c]	438
IL-8	F: CTGAGGTGCCAGTGCATTAG R: AGCACACCTCTCTTCCATCC	58	139	80.0	10^2-10^7	AB559573 [c]	266
IL-10	F: CACAACTTCTTCACCTGCGAG R: CATGGCTTTGTAGATCCCGTTC	55	96	78.0	10^2-10^7	AB559574 [c]	478
IL-12a	F: AAGACCTGAAAACCTACAAGGC R: GGCTTGCATCATGTCATCAA	60	83	81.0	10^1-10^7	AB727361 [c]	344
IL-12b	F: CACAGCAGCTTTTCATCAGAGA R: ATAGGACTTTGGTGTGCTCCAG	58	118	78.0	10^1-10^7	AB559575 [c]	276
IL-13	F: CTGCAAGAAGGACTATGAGCCC R: CAGTGCCGGCAAGAAGTT	55	115	82.0	10^2-10^7	AB559576 [c]	380
IL-18	F: GCAGCGGAATGTACTTCAAC R: CTCTTATCTTCTACCTGGACGCTG	53	94	79.0	10^1-10^7	AB559577 [c]	555
TLR-7	F: AGATGTTTTCTGGGCAGACG R: AATGACTTCAACCGGTTACTGG	60	177	78.0	10^1-10^7	AB553582 [c]	3,328
β-actin	F: CTGGCACCTAGCACAATGAA R: CTGCTTGCTGATCCACATCT	55	123	84.0	10^1-10^7	AF199488	–

a) The annealing temperature of primers, the length of real-time PCR products, the melting temperature of the specific amplification and the quantification range are indicated.
b) Accession numbers are given for the gene sequences from which primers are derived.
c) In the present study.

Fig. 1.

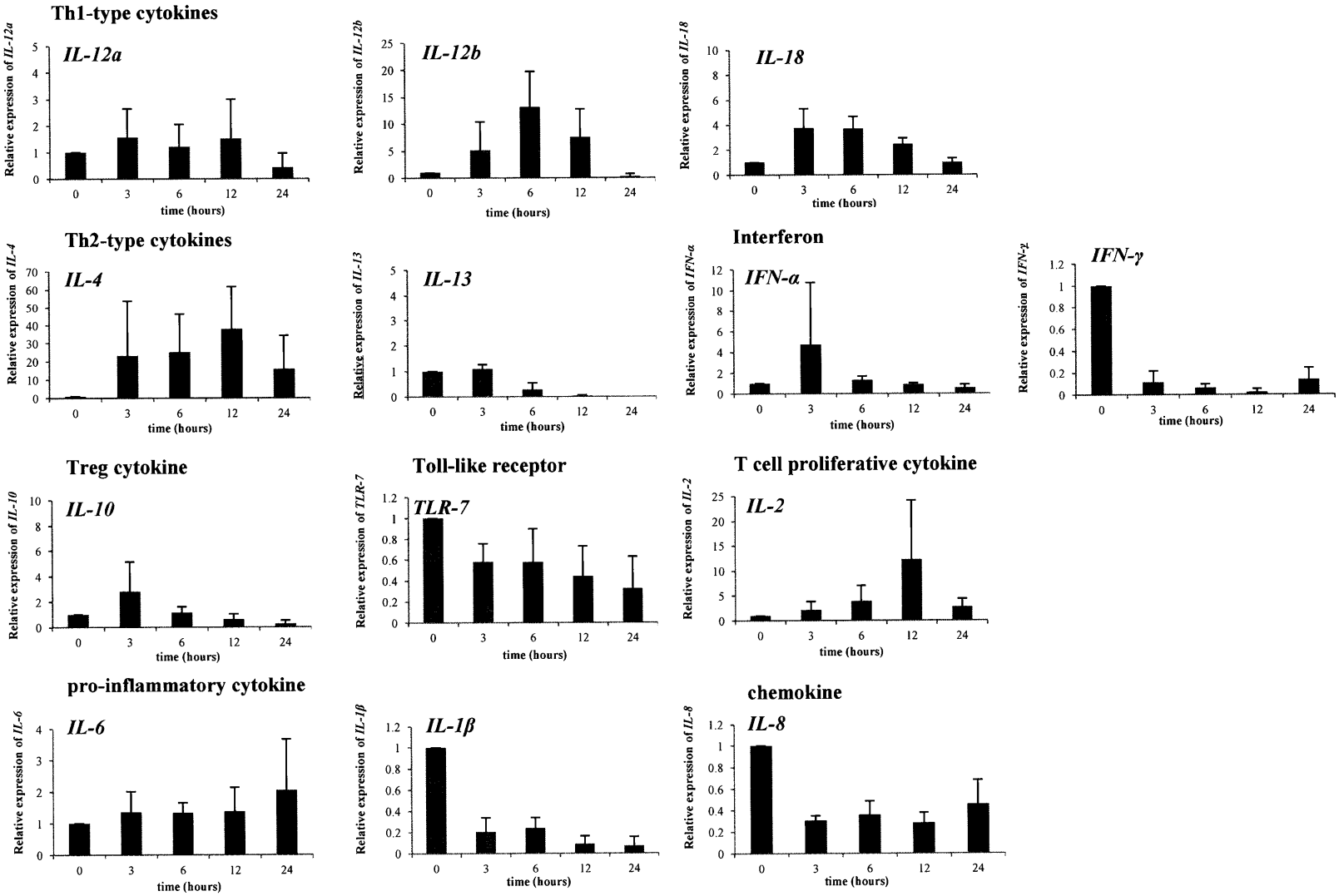
Th1-type cytokines
IL-12a
IL-12b
IL-18
Th2-type cytokines
IL-4
IL-13
Interferon
IFN-α
IFN-γ
Treg cytokine
IL-10
Toll-like receptor
TLR-7
T cell proliferative cytokine
IL-2
pro-inflammatory cytokine
IL-6
IL-1β
chemokine
IL-8
Relative expression of IL-12a
Relative expression of IL-12b
Relative expression of IL-18
Relative expression of IL-4
Relative expression of IL-13
Relative expression of IFN-α
Relative expression of IFN-γ
Relative expression of IL-10
Relative expression of TLR-7
Relative expression of IL-2
Relative expression of IL-6
Relative expression of IL-1β
Relative expression of IL-8
time (hours)
0
3
6
12
24

Fig. 1. Kinetics analysis of quail immune-related gene expression in Concanavalin A (ConA)-stimulated splenocytes. Splenocytes from three quail were stimulated with 5 μg/m*l* of ConA for the indicated times. Expression levels of each time point were measured relative to each splenocytes at 0 hr time point. Each bar represents the mean ± SD of three samples. Statistical significance was assessed using Student's t-test (*P < 0.05).

CHAPTER 2

The pathogenicity and host immune response associated with H5N1 highly pathogenic avian influenza virus in quail

INTRODUCTION

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 [68]. 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 [15, 21, 22, 29, 68]. 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" [71]. In humans, clinical features associated with H5N1 virus infections have been linked to cytokine dysregulation [6, 62]. 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 [18, 24, 38, 44, 58]. Type I interferon (IFN) was induced by H5N1 HPAIV in lung, spleen and plasma

of 6-week-old chickens [38]. Th1 and pro-inflammatory responses were observed in the lung and spleen of 4-week-old chickens infected with a human-derived H5N1 HPAIV [24]. 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 [58]. 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 [44]. 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 [18]. 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.

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, this author 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, this author 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) [64] 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 [39]. 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 EID_{50}/0.1 m*l* of the H5N1 virus and quail of control group were inoculated with 0.1 m*l* 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 m*l* 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 8 chickens (challenged group) and 4 chickens (control group). Chickens of challenged group were inoculated intranasally with 10^6 EID_{50}/0.1 m*l* of the H5N1 virus and chickens of control group were inoculated with 0.1 m*l* 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 m*l* 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 μg/m*l* Concanavalin A (ConA, Sigma, Saint Louis, MO, USA), 10% fetal bovine serum, 0.2 mM 2-mercaptoethanol (Sigma, Saint Louis, MO, USA), 100 unit/m*l* penicillin (Meiji, Tokyo, Japan) and 100 μg/m*l* streptomycin (Meiji, Tokyo, Japan) 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, USA) according to the manufacturer's instructions. The RNA was resuspended in 40 μl of RN*ase*-free water and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and cDNA was synthesized with QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) as directed by the manufacturer. In short, approximately 0.5 μg of total RNA was reverse transcribed in a final volume of 20 μl of reaction containing the following components; 7×gDNA Wipeout buffer, 5×Quantiscript RT Buffer, Quantiscript *Reverse Transcriptase* and RN*ase*-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-12a, IL-18* and *TLR-7* were quantified by real-time PCR using gene specific primers (Table 2) as described previously [63]. 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') [66]. 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, USA). The real-time PCR reaction mixture contained 2.0 *μl* of sample cDNA, 1.0 *μl* of forward and reverse primers (10 *μ*M each), 10 *μl* of iQ SYBR Green Supermix (BIO-RAD, Hercules, CA, USA) and 6.0 *μl* 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 m*l* of nutrient broth media containing 10 mg streptomycin and 10,000 units of penicillin and serially diluted with PBS by 10-fold. Twenty% (w/v) tissue homogenates were prepared with PBS and the supernatant was collected after centrifugation of 1,000×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 [39].

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 3). Six of the 11 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 hr and 66.0 hr, respectively) were significantly different (Table 3).

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. 2). 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

This author 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 4). All living quail shed virus in the laryngopharyngeal swabs on 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 4). 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. 3).

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. 4). *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-12a, 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. This author 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 [15, 21, 22, 29, 68], resulted in a late onset time and a prolonged MDT in quail. Interestingly, the expression of IFN-γ, an antiviral cytokine, increased in quail while it decreased in chicken after H5N1 virus inoculation (Fig. 4). Furthermore, the viral load in quail PBMC was significantly lower than that in chickens (Fig. 3), 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 this study (Fig. 4). IFN-γ has antiviral activity through activation of innate immune cells such as macrophages and NK cells [16]. Because these cells are reported to play essential roles in the control of influenza virus [37], IFN-γ is also thought to be associated with the pathogenesis of influenza [9, 23]. For example, H5N1

HPAIV was found to induce IFN-γ in mammals [10], and early administration of exogenous IFN-γ during influenza virus infection stimulates NK cell proliferation and function in infected lungs [70]. An HPAIV A/Vietnam/1203/2003 (H5N1) was also found to induce IFN-γ in the lung and spleen of chickens [24]. However, in the present study, infection caused a decrease in the expression of IFN-γ in chicken PBMC. Similarly, Suzuki *et al.* [58] 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 this 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 [58], 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, these 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 4). 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. [22], 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 4). 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 3). 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. 2). 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 1 IFN in innate immunity [33], and thus, it is considered that influenza viruses can easily replicate in chicken cells [4]. 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, these 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, this author 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.

Table 2. Sequences for primers used in the quantitative real-time PCR assays

Target gene			Quail	Chicken
Type 1 interferon	*IFN-α*	F [a)]	5'-CCTTGCTCCTTCAACGACA-3'	5'-CCAGCACCTCGAGCAAT-3'
		R	5'-CGCTGAGGATACTGAAGAGGT-3'	5'-GGCGCTGTAATCGTTGTCT-3'
Type 2 interferon	*IFN-γ*	F	5'-CAACCTTAATGATGGCACGA-3'	5'-CTCCCGATGAACGACTTGAG-3'
		R	5'-CTTTGCGGTGGATTCTCA-3'	5'-CTGAGACTGGCTCCTTTTCC-3'
Th1-type cytokine	*IL-2*	F	5'-GTGCAAAGTACTGATCTTCGCC-3'	5'-TTGGAAAATATCAAGAACAAGATTCATC-3'
		R	5'-CTTGGTGTGTAGAGCTCGAGATG-3'	5'-TCCCAGGTAACACTGCAGAGTTT-3'
	IL-12a	F	5'-AAGACCTGAAAACCTACAAGGC-3'	5'-AAGACCTGAAAACCTACAAGGC-3'
		R	5'-GGCTTGCATCATGTCATCAA-3'	5'-GGCTTGCATCATGTCATCAA-3'
	IL-18	F	5'-GCAGCGGAATGTACTTCAAC-3'	5'-GAAACGTCAATAGCCAGTTGC-3'
		R	5'-CTCTTATCTTCTACCTGGACGCTG-3'	5'-TCCCATGCTCTTTCTCACAACA-3'
Th2-type cytokine	*IL-10*	F	5'-CACAACTTCTTCACCTGCGAG-3'	5'-CATGCTGCTGGGCCTGAA-3'
		R	5'-CATGGCTTTGTAGATCCCGTTC-3'	5'-CGTCTCCTTGATCTGCTTGATG-3'
Pro-inflammatory cytokine	*IL-6*	F	5'-CAACCTCAACCTGCCCAA-3'	5'-CTGTTCGCCTTTCAGACCTACC-3'
		R	5'-GGAGAGCTTCCTCAGGCATT-3'	5'-CATGGTGATTTTCTCTATCCAGTCC-3'
Chemokine	*IL-8*	F	5'-CTGAGGTGCCAGTGCATTAG-3'	5'-CTGCGGTGCCAGTGCATTAG-3'
		R	5'-AGCACACCTCTCTTCCATCC-3'	5'-AGCACACCTCTCTTCCATCC-3'
Receptor	*TLR-7*	F	5'-AGATGTTTTCTGGGCAGACG-3'	5'-TTCTGGCCACAGATGTGACC-3'
		R	5'-AATGACTTCAACCGGTTACTGG-3'	5'-CCTTCAACTTGGCAGTGCAG-3'
β-actin		F	5'-CTGGCACCTAGCACAATGAA-3'	5'-CTGGCACCTAGCACAATGAA-3'
		R	5'-CTGCTTGCTGATCCACATCT-3'	5'-CTGCTTGCTGATCCACATCT-3'

a) F: forward primer, R: reverse primer

Table 3. 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	8/8
Cyanosis (comb or feet)	0/11	8/8
Neurological signs	6/11	0/8
Dead	11/11	8/8
MDT (hr)	91.2 ± 4.4 [a)]	66.0 ± 4.0

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

Table 4. 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 m*l*)			
		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

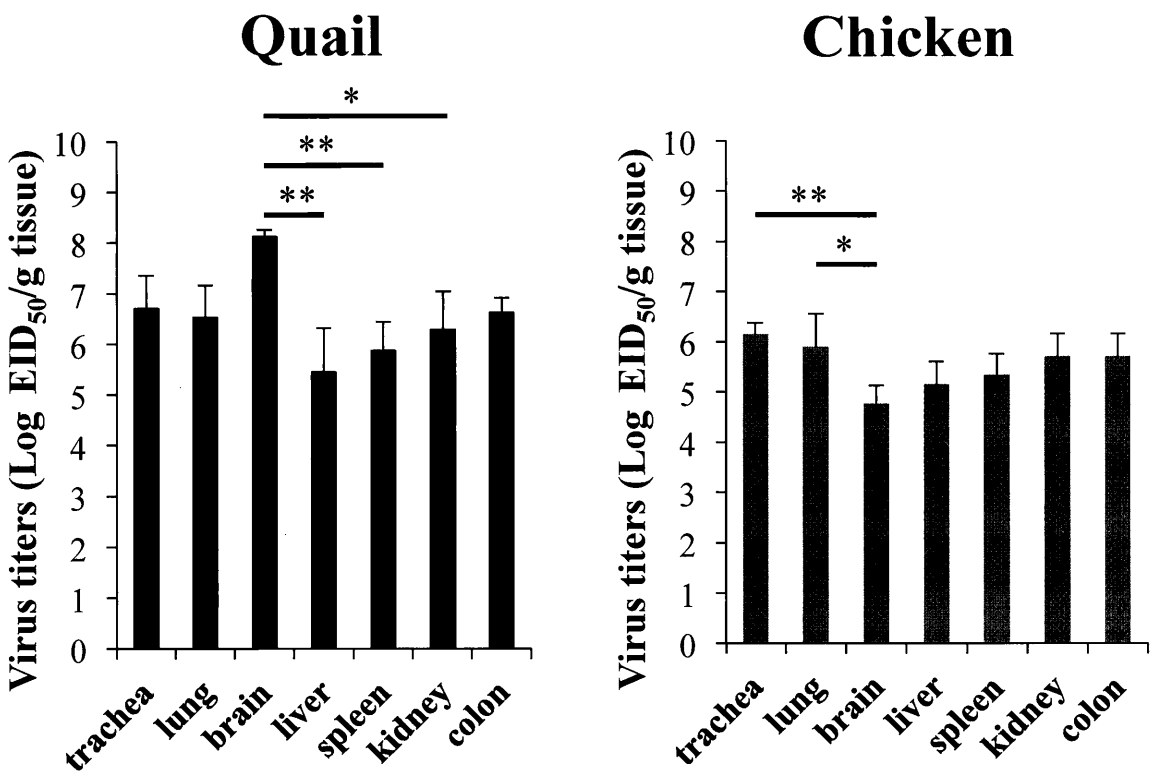

Fig. 2. Virus titers in tissues from quail and chickens intranasally inoculated with the A/whooper swan/Aomori/1/2008 (H5N1). The tissues were collected at 72 h.p.i. from quail and at 56 h.p.i. from chickens. The virus titer of brain in quail was significantly higher than the titers of liver, spleen 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).

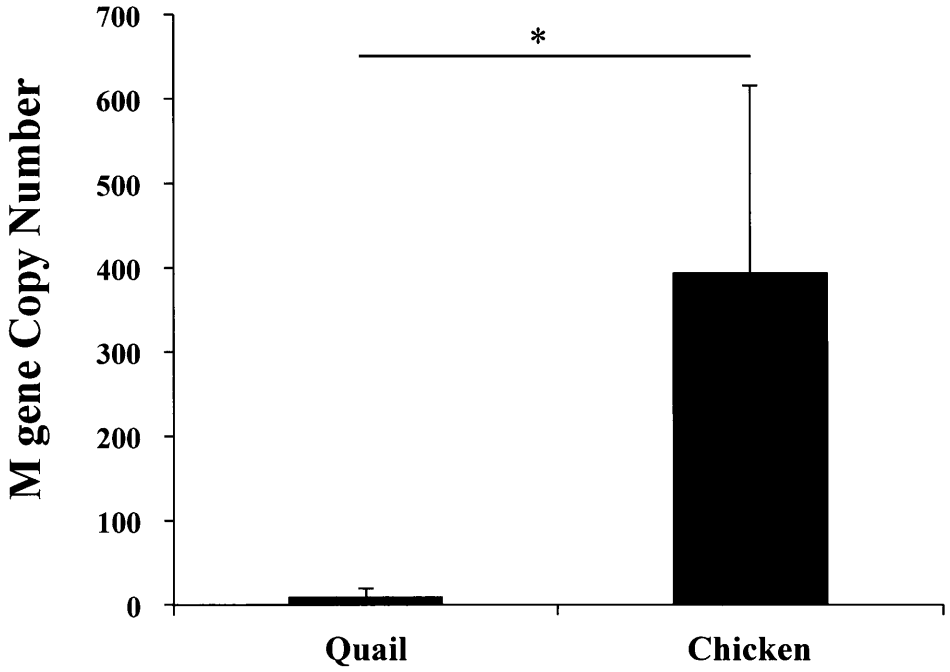

Fig. 3. Influenza A virus matrix 1 (M1) gene expression in quail and chicken PBMC at 24 h.p.i. The copy number of virus-derived RNA in PBMC collected at 24 h.p.i. was measured by quantitative real-time PCR. Error bars show standard error (SE). Values designated by an asterisk are significantly different between quail and chickens (*$P <$ 0.05, Student's t-test).

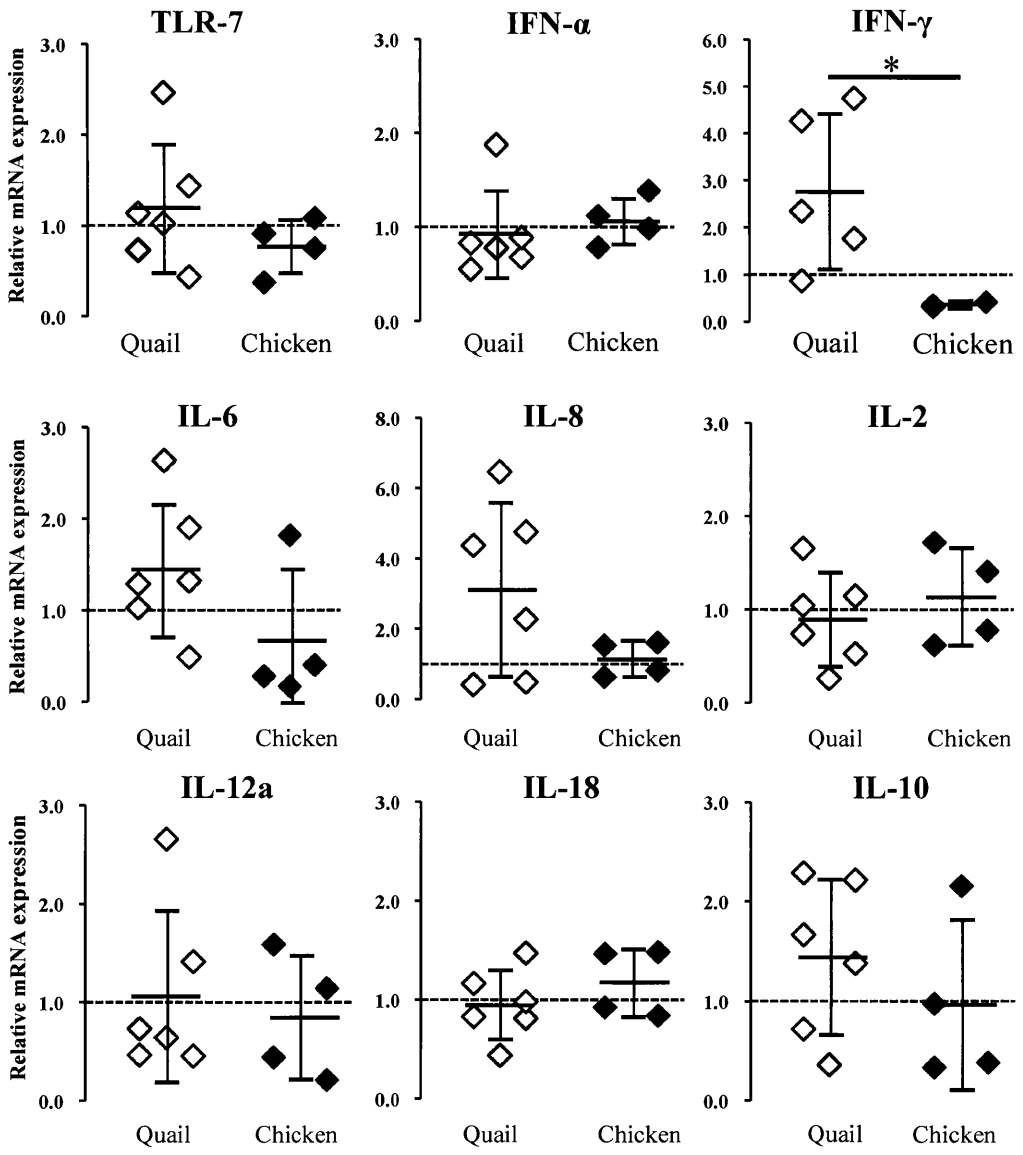

Fig. 4. Comparison of cytokines and TLR-7 mRNA responses of the PBMC between quail and chickens infected with A/whooper swan/Aomori/1/2008 (H5N1) at 24 h.p.i. The value of mRNA expression of each gene was quantified by real-time PCR. The mRNA expressions at 24 h.p.i relative to the mRNA expressions before infection were compared with control birds. Bars indicate the average ± 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.

GENERAL CONCLUSION

Quail is a major poultry species that is farmed all over the world. Whereas quail have high sensitivity to H5N1 HPAIVs and show approximately 100% mortality from infection, it has been reported that quail infected with H5N1 HPAIV take longer than chickens to show signs of disease and die. The reason for the different pathologies of quail and chicken by H5N1 HPAIV infection might be related to host immune response. However, the immunological information for quail has been scant. Hence, in this thesis, the nucleotide sequences of the quail immune-related genes including *IFN-α* (a type I interferon), *IFN-γ*, *IL-12a*, *IL-12b*, and *IL-18* (Th1 cytokines), *IL-4* and *IL-13* (Th2 cytokines), *IL-10* (a Treg cytokine), *IL-2* (a T cell proliferative cytokine), *IL-1β* and *IL-6* (pro-inflammatory cytokines), and *IL-8* (a chemokine) were determined and the quantitative real-time PCR assays for these immune-related genes and quail *IFN-α*, *IFN-γ* and *IL-2* genes was constructed. Subsequently, the induction of antiviral cytokine *IFN-γ* in quail PBMC at the early stage of H5N1 HPAI virus infection, which might inhibit viral replication, was shown by using real-time PCR. This information and the quantitative method for quail immune-related genes are very useful in the field of avian immunology. These studies will contribute to deeper understanding of avian immunology and clinical manifestation in the H5N1 HPAIV infection. In addition, this study has provided the first insight into host immune responses of quail against H5N1 HPAIV infection.

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