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Expression of Immunoglobulin Genes Differs Between Chickens and Ducks Following H5N1 Avian Influenza Virus Infection

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Abstract: Different species have different susceptibilities to Avian Influenza Virus (AIV). In the present study, the expression profiles of Immunoglobulins (Igs) in different tissues of Specific Pathogen-Free (SPF) chickens and ducks infected with AIV were examined by quantitative PCR (qPCR) and compared with those of normal controls. The results showed that the copy numbers of IgA in AIV-infected chickens and ducks sharply increased in the spleen, thymus, bursa of Fabricius, lung and tracheal mucosa ($p<0.05$). Significant changes were also observed in the expression of IgM and IgY in the spleen, thymus and bursa of fabricius in AIV-infected chickens and ducks ($p<0.05$). Ducks had higher expression of all three Ig classes than chickens after AIV infection. Additionally, ducks expressed a unique form of IgY (ΔFc) that exhibited significant changes in the spleen, thymus, bursa of Fabricius and lungs following AIV infection ($p<0.05$). The unique form was expressed at higher levels than full-length IgY. The expression patterns of these Igs may explain why chickens and ducks have different disease signs following AIV infection. The expression of immunoglobulin could affect the susceptibility of chickens and ducks to the H5N1 influenza virus.

Key words: Avian influenza virus, chicken, duck, immunoglobulin, susceptibility, AIV infection

INTRODUCTION

Avian influenza is an infectious disease caused by type A influenza virus which infects species such as chickens, turkeys, ducks, geese, quails, wild birds and waterfowl (Webster *et al*., 1992). Waterfowl live in the most complex ecosystems of influenza viruses (Sturm-Ramirez *et al*., 2005). Since, the mid-1990s, waterfowl infected with Highly Pathogenic Avian Influenza (HPAI) H5N1 virus have become ill or died (Stallknecht *et al*., 1990). Waterfowl are not only highly susceptible to influenza and die from infection (Kim *et al*., 2009) but also can horizontally transmit the virus from terrestrial sources. Different influenza strains display different pathogenicities due to their high degree of variability. In addition, different hosts exhibit different susceptibilities toward the same influenza strains. For example, chickens and ducks infected with the same Avian Influenza Virus (AIV) react differently, resulting in asymptomatic wild ducks but seriously affected chickens.

The immune response against influenza viruses in birds involves CD8+cytotoxic T cells and antibodies against the surface antigens Hemagglutinin (HA) and Neuraminidase (NA) (Doherty *et al*., 2009). In chickens, protective immunity is mainly provided by antibodies against HA. However, the infection of ducks usually leads to a weak immune response with short-lived memory. The three isotypes in avian species are IgM, IgY and IgA. In mammals there are five Ig isotypes: IgM, IgD, IgG, IgA and IgE (Schaerlinger *et al*., 2008). IgM is the first antibody produced in an immune response but is quickly replaced by IgA and IgY. Duck IgM heavy chain cDNA has been cloned and sequenced and the membrane form and locus have also been determined (Magor *et al*., 1998; Lundqvist *et al*., 2001). The collection of sera from animals infected with duck/HongKong/75 showed that IgM contributed to the response in the first 3-5 days post-infection (Magor, 2011). Avian IgM exists as a pentameric structure and a membrane bound form serves as the B cell antigen receptor (Magor *et al*., 1998). IgY is the most abundant serum globulin and functions as the main antibody against bacteria, viruses and toxins. The two forms of IgY exist in some species, a full-length IgY and a truncated form, IgY(ΔFc) (Grey, 1963; Marchalonis and Edelman, 1966; Lykakis, 1968; Chartrand *et al*., 1971; Leslie and Clem, 1972). The

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truncated antibody IgY(ΔFc) of ducks lacks specificity for the hemagglutinin receptor and does not have sufficient steric hindrance to prevent erythrocyte agglutination (Magor, 2011). This observation implies that the ratio of IgY(ΔFc) in immune complexes may influence the resistance of AIV-infected ducks and cause different responses in chickens and ducks. Secretory IgA plays an important role in controlling local infections by preventing pathogenic microorganism adhesion to mucosal epithelial cells (Corthesy and Spertini, 1999). Pervious data have clearly proven that B cells can produce anti-influenza IgA responses independent of CD4 T cells (Sha and Compans, 2000; Lee *et al*., 2005). Therefore, host Igs play an important role against AIV infection. In this study, the expression levels of IgM, IgA, IgY and IgY(ΔFc) in different tissues of Specific Pathogen-Free (SPF) chickens and ducks infected with H5N1 avian influenza virus and healthy controls were analyzed by quantitative PCR to determine the changes in Igs between chickens and ducks.

MATERIALS AND METHODS

Animals and sample collection: Three chickens and three ducks that were 4 weeks old were infected with H5N1 avian influenza (A/Duck/Anhui/1/2006). An equal number of control chickens and ducks were also used in this study. There is a different MDT between the two species. The MDT for chickens is 3 days and the MDT for ducks is 7.3 days. Researchers collected the heart, liver, spleen, lung, kidney, mucosa of the upper part of the trachea, thymus and the bursa of Fabricius at the MDT. After the animals were bled and sacrificed they were quickly stored in liquid nitrogen until use.

The experimental infections of the animals were conducted in a bio-safety level 3 facility in Harbin Veterinary Research Institute, Chinese Academy of Agriculture.

Primer design: Primers were designed by primer premier 5.0 based on the sequence of the constant regions of the immunoglobulins. The accession numbers of the reference sequences are listed in Table 1. Primers were designed to cross intron-exon boundaries to avoid the genomic copies of the target genes. Primer IgY (1-2) was designed for exon 1 and exon 2 of the IgY constant region and primer IgY (3-4) targets exon 3 and exon 4.

RNA extraction and cDNA synthesis: Total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's protocol. To prepare cDNA, 1 μg of total RNA from each tissue was mixed with 1 μg of oligo dT primer, 1 μL of dNTP mixture (10 mM each) and RNase-free dH_2O up to 10 μL. The reaction was incubated at 65°C for 5 min and cooled rapidly on ice. Then, 4 μL of 5×prime script buffer, 0.5 μL of RNase inhibitor (40 U μL^{-1}) and 1 μL of prime script RTase (200 U μL^{-1}) were added to the mixture and RNase-free dH_2O was added to a final volume of 20 μL. The sample was incubated at 30°C for 10 min, 42°C for 1 h and 95°C for 5 min. The reaction was terminated by incubation in an ice bath for 5 min.

Preparation of the standards and real-time PCR: Plasmid DNA containing the target gene was serially diluted with dH_2O to create standards of 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 copies μL^{-1}. A standard curve was generated for each assay plate to assess the PCR efficiency. The copy number was calculated as follows: Copy number (copies/mL) = plasmid concentration (μg mL)×6.02×10^{23}/plasmid molecular weight where the plasmid molecular weight is the average molecular weight of a base pair (649)×the total length of the recombinant plasmid.

Absolute quantification was used to calculate the copy numbers of the Igs using the standard curve. A mix of 1 μL of forward primer, 1 μL of reverse primer, 9.5 μL of dH_2O and 12.5 μL of SYBR® Premix Ex Taq™ (Takara) was

Table 1: Primers for immunoglobulins of chicken and duck

Species	Gene name	Sequence (5'-3')	Length (bp)	Annealing temperature (°C)	GenBank accession No.
Chicken	*IgA-F*	GGAGAGCATCAGGAAGGAGAC	138	58	AH008322
	IgA-R	ATGGAAGAAGGGAGGAAGGAG			
	IgM-F	AGGATGGAGTGCGGATTAGAA	94	58	X01613
	IgM-R	CCGATTTGCTGATGAAGATGT			
	IgY-F	CAGCAAGAGCGTCTACAGGAA	148	60	X07174
	IgY-R	CCACCGGATCTCGATGTCA			
Duck	*IgA-F*	CGACATTTTGGTGACTTGGAC	111	58	U27222
	IgA-R	AACTTGCTGTAGACGCTGAAGA			
	IgM-F	GATGAGGAACAGCAGCAAGTC	176	60	U27213
	IgM-R	TGAGCCAGGAGATGACCAA			
	IgY(1-2)-F	TCACCGTCCCCGAGACCCAC	291	64	AJ534873
	IgY(1-2)-R	CGTCCACCAGCCACTCCACC			
	IgY(3-4)-F	CTCACCGAGCACTTCAACG	183	60	AJ534873
	IgY(3-4)-R	TGGGAAGGTGAAGATGTAGGG			

added to 1 μL of template cDNA in every well in a final volume of 25 μL. The PCR reaction conditions were as follows: 1 min at 95°C, 15 sec at 95°C, followed by 40 cycles of 64°C for 15 sec and 72°C for 45 sec.

Data analysis: SPSS 13.0 Software was used for the statistical analysis and significant differences were determined using the ONE-WAY ANOVA Variance Analysis and Duncan's LSD test. The results are expressed as the mean±standard error.

Fold changes were calculated by dividing the SPF-group copies by the AIV-group copies. The data show the degree of changes for the immunoglobulin copy numbers after AIV infection relative to the SPF controls. Researchers compared the differences in immunoglobulin expression between the two types of birds infected with the same type of avian influenza virus.

RESULTS

Comparison of IgA and IgM mRNA expression of SPF and AIV chickens and ducks: Under normal conditions, SPF chickens generated the highest IgA expression levels in the tracheal mucosa. The SPF chickens had moderate IgA expression in the spleen and bursa of fabricius but low levels in other tissues. AIV infection induced high-level IgA expression in all tissues. The expression of IgA increased significantly in the spleen, thymus, bursa of fabricius, lung and tracheal mucosa with $p<0.05$ for these tissues (Fig. 1a). IgA was expressed at the highest level in the tracheal mucosa of SPF ducks. After AIV infection, the expression of IgA in various tissues increased by varying degrees. The spleen and tracheal mucosa expressed the highest IgA level in the AIV duck group. Additionally, the lung, thymus and bursa of fabricius showed significant differences in expression (Fig. 1b). The IgA mRNA expression patterns in chickens and ducks were similar. However, the copy numbers in ducks were an order of magnitude higher than in chickens. The copy numbers of IgA in the AIV-infected groups increased sharply compared with the SPF groups. The IgA expression increases in the lung, immune tissues and tracheal mucosa in both chickens and ducks were significant (Fig. 1a, b). Researchers chose to use the tissues with significant expression changes to compare the IgA fold changes of the two species. The results

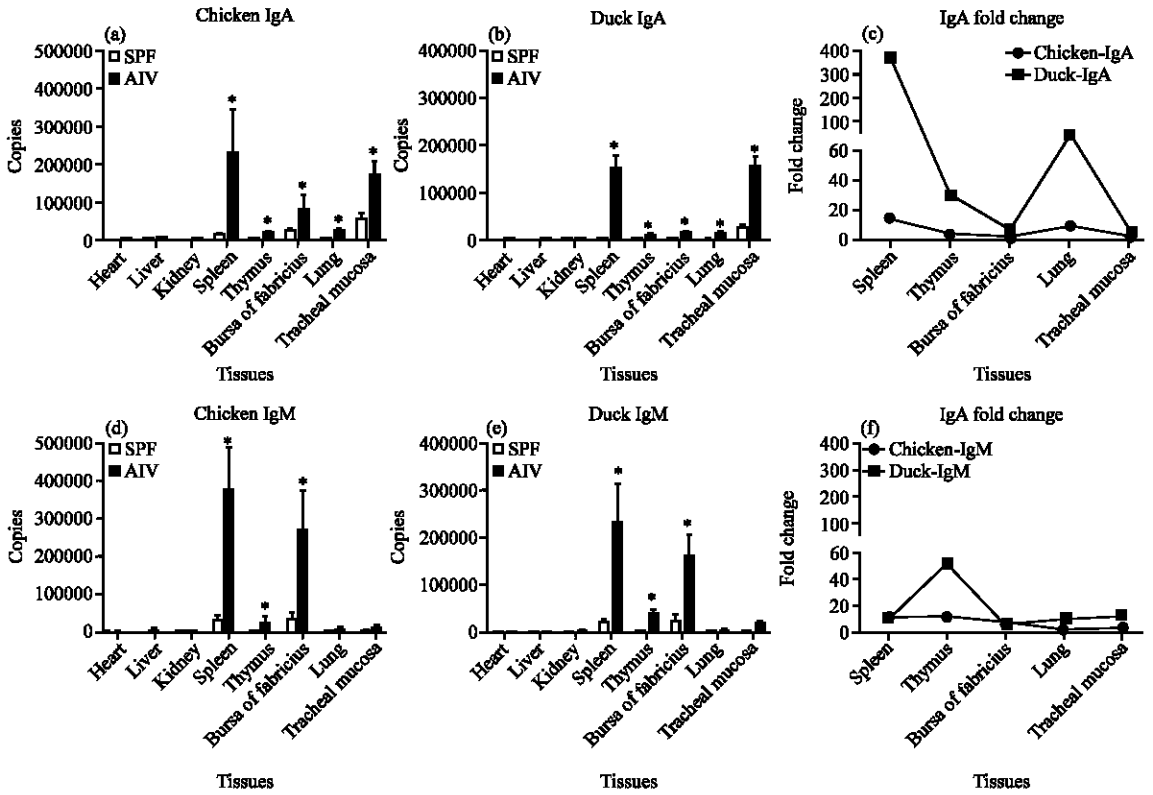

Fig. 1: The fold changes of IgA, IgM and mRNA expression of SPF and AIV-infected chickens and ducks in different tissues. qPCR was used to detect the mRNA expressions of a) IgA in chickens; b) IgA in ducks; c) IgM in chickens; d) and IgM in ducks; e) before and after AIV infection. The fold change calculations of; c) IgA and f) IgM were chosen for the significantly expressed tissues to compare between the two species. *$p<0.05$

indicate that the IgA expression changes for ducks in the three immune tissues, lung and tracheal mucosa were all higher than those for chickens. The greatest differences were found in the spleen, lung and thymus. There were no differences in IgA expression in the liver and kidney, although, chickens tended to have a higher expression (Fig. 1c).

After AIV infection, the expression of IgM also increased in all the organs of both chickens and ducks. However, significant increases ($p<0.05$) were observed only in the spleen, thymus and bursa of fabricius (Fig. 1d). The IgM tissue-specific patterns of expression in ducks were similar to those in chickens but ducks had a much higher level than chickens (Fig. 1e). A comparison of the IgM expression between chickens and ducks (Fig. 1d, e) showed that ducks had a nearly six-fold higher copy number than chickens. A comparison between these two species (Fig. 1a, b, d and e) showed that the expression levels of IgA or IgM were much higher in duck tissues than in chicken tissues. The fold change values of IgM are listed with significant changes in the tissues (Fig. 1f). The spleen and bursa of fabricius were not different between chickens and ducks. The IgM values of other duck tissues were higher than chicken tissues.

Comparison of IgY and IgY(ΔFc) mRNA expression in chickens and ducks in SPF and AIV groups: IgY was expressed in all tissues of chickens and ducks at low levels. Significant increases ($p<0.05$) were observed in the spleen, thymus and bursa of fabricius upon infection in chickens (Fig. 2a). The two isoforms of IgY exist in ducks, the full-length IgY and the truncated isoform IgY(ΔFc). The IgY mRNA levels of ducks were affected by AIV infection with significant increases found in the spleen, thymus and bursa of fabricius ($p<0.05$). The increased expression in the spleen of ducks was greater than in the thymus and bursa of fabricius (Fig. 2b). IgY(ΔFc), the peculiar isoform found in ducks, showed the maximum level in spleen and high levels in the bursa of fabricius after AIV infection (Fig. 2c). In addition, the expression levels of IgY(ΔFc) in the lung and thymus of the AIV group were greater than in the SPF group ($p<0.05$). In SPF chickens and ducks, both IgY and IgY(ΔFc) were expressed at low levels in all tissues. After AIV infection, the heart, liver, lung and kidney showed very small expression increases. The expression patterns of Ig Y in AIV-infected chickens and ducks were different as reflected by the increased copy numbers in the spleen, thymus and bursa of Fabricius (Fig. 2a, b). The IgY mRNA

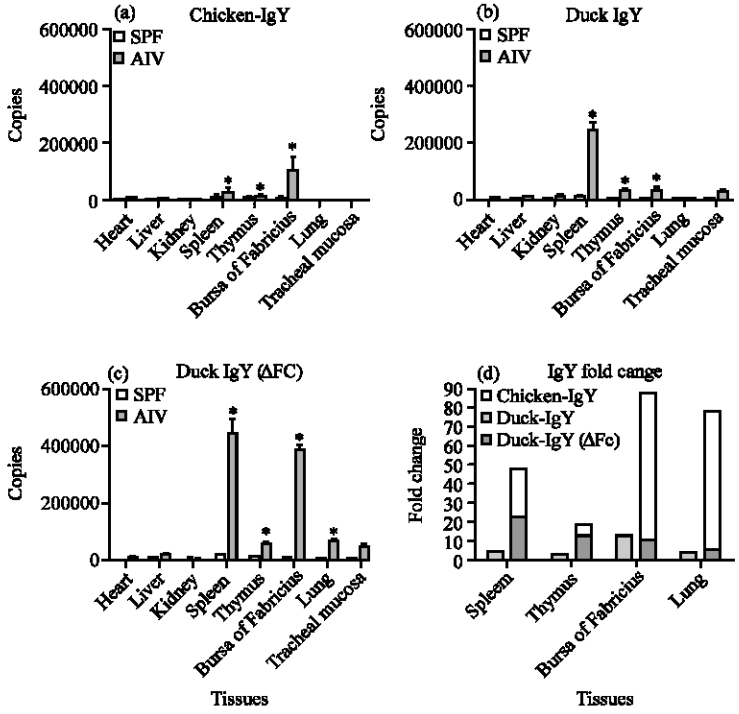

Fig. 2: IgY and IgY(ΔFc) mRNA expression in SPF and AIV-infected chickens and ducks in different tissues. The mRNA expresions of IgY of a) chickens, b) ducks, c) IgY(ΔFc) in only ducks using qPCR Method were compared and d) the fold changes of IgY were calculated. *$p<0.05$

levels of AIV-infected ducks were higher in the spleen than in the bursa of Fabricius. Conversely, the IgY mRNA levels in the AIV-infected chickens were higher in the bursa of fabricius than in the spleen. IgY and IgY(ΔFc) exhibited similar mRNA expression patterns in ducks and ducks produced more IgY(ΔFc) than IgY in the immune organs and lung (Fig. 2b, c). Researchers examined the expression of IgY in chickens, IgY in ducks and IgY(ΔFc) in ducks to compare the fold changes to determine the significant-change tissues (Fig. 2d). Researchers found that the values for IgY were higher in ducks than in chickens in nearly all tissues except the bursa of Fabricius. In ducks, IgY(ΔFc) was expressed more in the spleen, bursa of Fabricius and lung than IgY.

DISCUSSION

Ducks and chickens have the same hematopoietic tissues which consist of the bone marrow, gut-associated lymphoid tissue, spleen, thymus and bursa of fabricius. Birds have three classes of antibodies: IgM, IgY and IgA. Ducks also possess a truncated form of IgY called IgY(ΔFc). These antibodies are expressed in the serum and secretions in ducks and chickens with unique tissue distribution patterns. After influenza infection, viral clearance occurs through the interaction of Ig-mediated Immune Complexes (IC) with resident mononuclear phagocytic cells (Aguado and Mannik, 1987; Davies *et al*., 1995; Nash *et al*., 2001). IgA is considered to be representative of mucosal immunity. Secretory IgA exists mainly in milk, saliva, tears and the mucosal surfaces of the digestive tract, respiratory tract and reproductive tract. Mucosal IgA can inhibit the invasion of bacteria attached to the mucosa and inhibit toxins produced in the intestinal epithelium. IgA also directly kills bacteria by antibody-dependent cell-mediated cytotoxicity and participates in local mucosal immunity. IgA immunodeficiency leads to an increased susceptibility to influenza virus infection (Arulanandam *et al*., 2001). Recent studies have shown that the elimination of high toxin levels in AIV-infected birds occurs through the trachea (upper respiratory tract) rather than the cloaca. IgA primarily neutralizes the virus in the upper respiratory tract and IgY mainly work in the lower respiratory tract (Renegar *et al*., 2004). Moreover, IgA in the digestive tract plays an important role against avian influenza infection. The present data indicated that IgA is highly expressed in the spleen, thymus, bursa of fabricius, lung and tracheal mucosa after AIV infection in both chickens and ducks. However, the copy numbers found in AIV-infected ducks were much higher than those in AIV-infected chickens. This result suggests that the spleen and lungs of ducks infected with AIV secreted large amounts of IgA against the invasion of the virus and that there are some differences in local mucosal immunity between chickens and ducks. Mucosal immunity in ducks showed stronger activity than in chickens.

Chicken IgM is homologous to its mammalian counterpart based on structure and function (Magor, 2011). During embryonic development, IgM is the earliest expressed Ig in the bursa of Fabricius in 16 days duck embryos and 12 days chicken embryos (Bando and Higgins, 1996). IgM is often the first antibody produced during viral infection. Several studies have demonstrated that mice lacking IgM were more susceptible to infection by influenza virus (Kopf *et al*., 2002). In this study, the expression of IgM increased significantly in the spleen, thymus and bursa of fabricius in animals infected with AIV. The results suggest these three immune organs are the major source for antigen processing and B cell proliferation. The copy numbers of IgM after infection in ducks were six-fold higher than in chickens. IgM has a transient role during an immune response. It is produced first in an immune response but is soon replaced by IgY and IgA. The long course of disease for ducks after AIV infection suggests a different developmental process compared with the short-lived and acute infection of chickens. In this study, the IgM of AIV-infected ducks changed more than that of AIV-infected chickens, indicating that the infections were in the early stages. Furthermore, ducks possessed stronger resistance to AIV infection and produced large amounts of IgM in response to viral infection. IgM production could explain why soon after AIV infection chickens have more severe symptoms.

Poultry IgY is the major antibody component of the serum and extracellular fluid, representing 80% of the total serum immunoglobulins. Serum immunoglobulins can inhibit influenza from passing from mucosal surfaces and becoming a systemic infection. Conversely, IgY(ΔFc) is the major IgY form appearing later in an immune response, at least in hyperimmunized animals. It is produced predominantly when ducks are repeatedly exposed to an antigen with naive ducks mainly producing the full-length IgY (Grey, 1967a, b). IgY(ΔFc) lacks the Cυ3 and Cυ4 domains of the heavy chain and has lost the ability to bind to the complement. It is also incapable of mediating secondary effector functions (Grey, 1967a, b; Warr *et al*., 1995). The classical complement pathway is the main method of antibody-mediated humoral immune response. C1q binds the Fc portion of antibodies and is activated and the Fc segment links antibodies to complement activation. In pigs and mice, the spleen and liver are key organs for clearing immune complexes using mononuclear phagocytic cells (Aguado and Mannik,

1987; Davies *et al*., 1995; Nash *et al*., 2001). IgY (ΔFc) could shift inflammatory responses from acute to chronic responses by persisting at a high level and reducing the systemic acute phase response (Humphrey *et al*., 2004). Data from the present study showed that chickens express low levels of IgY. However, IgY(ΔFc) was expressed more than IgY. The expression of IgY(ΔFc) could weaken complement activation and reduce phagocytic function. In addition, IgY(ΔFc) does not bind to Fc receptors which makes antibody-dependent cell-mediated cytotoxicity impossible. In AIV-infected ducks, IgY(ΔFc) bound antigens only with Fab fragments and prevented the virus from being adsorbed by susceptible target cells to reduce viral infection. Thus, IgY(ΔFc) plays an important role in the different survival rates found for AIV-infected chicken and ducks.

CONCLUSION

The Ig expression patterns of chickens and ducks infected with AIV in eight tissues were compared with those of SPF animals. The levels of Igs increased significantly after infection, especially in the immune organs. The duck expression signature of Ig transcription differs from that of chickens because of the unique expression of IgY(ΔFc). In the experiments, all chickens died rapidly within 3 days after systemic infection and 70% of ducks died in 6-9 days. Researchers speculate the difference in the mortality rates between chickens and ducks may be related to the different types and different amounts of Igs expressed. This study indicates that the expression of Igs may one of the factors that affect the susceptibilities of chickens and ducks to the H5N1 influenza virus. Additional studies must be performed to confirm this correlation.

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