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EFFECT OF H9N2 VIRUS INFECTION ON THE ACUTE PHASE RESPONSE IN CHUKAR PARTRIDGES (*ALECTORIS CHUKAR*)

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Summary

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To determine the effect of H9N2 avian influenza virus (AIV) infection on the acute phase response, the serum concentrations of the inflammatory mediators, acute phase proteins and gangliosides in the acute stage of experimentally-induced AIV infection in adult partridges (*Alectoris chukar*) was measured. The birds were challenged intranasally with 10^7 EID_{50} dose of the A/Chicken/Iran/772/1998(H9N2) virus. Serum samples were obtained prior to challenge, at days 1, 3, and 6 post challenge (*pc*) and assayed for inflammatory mediators (TNF-α and IFN-γ), acute phase protens: haptoglobin (Hp), serum amyloid A (SAA) and gangliosides: total sialic acid (TSA), lipid-bound sialic acid and protein-bound sialic acid ((LBSA; PBSA) using validated standard procedures. All measured parameters showed significant differences from the baseline level in different days post challenge. Elevation in serum concentration of all measured parameters was observed after the 24th hour *pc*. In infected birds, the levels of Hp peaked at 24 h *pc*, while the concentrations of SAA, TNF-α and IFN-γ – 3 days *pc*. The level of TSA, PBSA and LBSA remained relatively constant during the experiment. The findings suggest that IFN-γ and TNF-α may have a role in immunopathogenesis of the H9N2 influenza virus and that Hp and SAA are induced by cytokines. Cytokines and acute phase proteins (APPs) could be therefore, useful indicators of H9N2 infection.

Key words: acute phase response, avian influenza, H9N2, partridge (*Alectoris chukar*)

INTRODUCTION

The H9N2 influenza viruses, which are low pathogenic avian influenza (LPAI) viruses, are present worldwide in poultry populations. They have become established and maintain long-term endemicity in terrestrial poultry in Asian countries (Alexander, 2007). Occasionally, these viruses are transmitted to other mammals, including humans (Butt *et al.*, 2005).

The farmed partridge population size has dramatically increased during the last decades in Asia, although it is still considered minor poultry species in comparison with chicken. Recent studies have suggested that partridges are susceptible to both avian and swine origin influenza viruses (Humberd *et al.,* 2006) and could have an important role in the interspecies transmission and adaptation of different influenza viruses of avian origin to the mammal host, because both α-2,3 and α-2,6 receptors are present in the respiratory

and intestinal tracts of the partridge (Costa *et al.*, 2012).

Despite the widespread occurrence of the disease, the virus potential to mutate to a highly pathogenic form and its transmission to humans (Lin *et al.,* 2000), our understanding of the mechanisms of the disease development, the pathogenesis of the virus and host immunity is limited. The first reaction of the body to immunological stress is the innate, non-specific response preceding specific immune reactions (Gruys *et al.,* 2005). Several investigators have tried to establish a correlation between cytokines and disease during influenza or to confirm the role of acute phase proteins (APPs) and inflammatory mediators in influenza infections (Van Reeth, 1998). The acute phase response (APR) is a prominent systemic reaction of the organism to local or systemic disturbances in its homeostasis (Gruys *et al.,* 2005). These immune responses may be important in protecting chickens from severe clinical signs or the lethal effects of influenza viruses.

Influenza virus has been shown to induce the production of cytokines in cell culture (Bussfeld *et al.,* 1998). Researchers have also used various animal models and experimental approaches to evaluate APR against influenza viruses. All studies in mice, humans and pigs assert a role of multiple cytokines in influenza symptoms and pathology (Van Reeth, 1998). Inflammatory cytokines are suggested to be involved in pathogenesis in humans, pigs and horses (Barbe *et al.,* 2011). In horses, elevation of haptoglobin (Hp) concentrations (Kent & Goodall, 1991) or serum amyloid A (SAA) (Hulten *et al.,* 1999), during the acute stage of equine influenza infection were reported. Barbe *et al.* (2011) investigated the cytokines and acute phase proteins associated with the acute stages of experimentally-induced swine influenza virus infection in piglets.

Hp, SAA, sialic acids, IFN-γ and TNF-α are the most important APPs in many animal species and their changes due to various inflammatory and non-inflammatory conditions have been intensively studied (Kaneko *et al.,* 2008). Some previous studies have clearly demonstrated changes in selected APPs in some common poultry diseases including ovotransferrin (Rath *et al.*, 2007), serum total proteins (Kokosharov, 2006), SAA (Nazifi *et al.*, 2010) and LBSA (Nazifi *et al.*, 2011; Mosleh *et al.,* 2012). However, there are few studies published about APPs or inflammatory mediators changes (TNF-α, transforming growth factor-beta; interferon alpha, beta, and gamma) in association with avian influenza infection in birds (Nang *et al.,* 2011; Sylte & Suarez, 2012).

The present study was set out to evaluate the inflammatory mediators, acute phase reactants and gangliosides in the acute stage of experimentally induced H9N2 avian influenza virus (AIV) infection in adult partridges (*Alectoris chukar*) to further understand its early pathogenesis and host immune responses.

MATERIALS AND METHODS

Virus

The virus isolate used in this study was A/Chicken/Iran/772/1998(H9N2). It was propagated two times in 9- to 11-day-old embryonated chicken eggs. The embryo infective dose (EID_{50}) was calculated according to the Reed & Muench (1938) formula.

Birds and experimental design

Thirty two clinically healthy, adult chukar partridges (*Alectoris chukar*) were used in

this study. They were reared in the Animal Research Unit of the Veterinary School of Shiraz University and received feed and water *ad libitum* during the experiment.

Serum samples from each bird were harvested and tested by haemagglutination inhibition (HI) assay (Burleson *et al.,* 1992) before inoculation to ensure that the birds were serologically negative for avian influenza virus. Inflammatory mediators (IFN-γ and TNF-α), acute phase proteins (Hp and SAA) and gangliosides (TSA, LBSA, PBSA) were also measured prior to challenge as a baseline using validated standard procedures.

All birds were challenged intranasally with 0.4 mL allantoic fluid containing 10^7 EID_{50} of the H9N2 virus. Following challenge, the birds were monitored daily for overt clinical signs of disease and mortality. Serum samples were obtained by wing bleed at days 1, 3 and 6 post-challenge (*pc*) to measure inflammatory mediators, acute phase reactants and gangliosides.

Serum samples were also tested for the presence of antibodies to the challenge virus antigen using the HI test on days 7 and 14 *pc* (Burleson *et al.,* 1992).

Blood parameters and analyses

Inflammatory mediators (IFN-γ and TNF-α) were measured by a solid phase sandwich ELISA (AbC 606 and AbC 607, respectively; Votre fournisseur AbCys S. A. Paris, France).

Hp was measured according to prevention of the peroxidase activity of haemoglobin, which is directly proportional to the amount of Hp. The analytical sensitivity of this test in serum has been determined as 0.0156 mg/mL for Hp by the manufacturer (Tridelta Development Plc, Wicklow, Ireland).

SAA was measured by a solid phase sandwich ELISA. The analytical sensitivity of this test in serum has been determined as 0.3 μg/mL for SAA by the manufacturer (Tridelta Development Plc, Wicklow, Ireland).

Serum TSA concentration was determined by the thiobarbituric acid method previously described by Warren (1959). The amount of TSA was determined by use of a standard curve developed from standard concentrations of N-acetyl neuraminic acid. LBSA concentration was determined by the method described by Katopodis *et al.* (1982). The amount of LBSA was determined by use of a standard curve developed from a standard sample of N-acetyl neuraminic acid. PBSA concentration was measured by subtracting serum TSA from LBSA.

Statistical analysis

All data are presented as means ± SD. Data analysis was carried out by using paired sample Student's *t* test (SPSS 11.5 for Windows software). Differences were considered significant at $P<0.05$.

RESULTS

Clinical signs

Only 4 birds displayed sneezing from day 5 to 9 *pc* and no other clinical signs or mortality were observed during the 14-day observation period.

Serological findings

To determine the immune status of the chukar partridges, the blood of all experimental birds was tested for seroconversion to H9N2 influenza A virus before challenge, and 7 and 14 days *pc*.

All pre challenged sera tested negative against the homologous virus by HI. Antibodies to AIV were detected in the challenged birds. The mean antibody titre was

increased at day 7 *pc* ($2^{-4.2}$) and reached 2^{-6} at day 14 *pc* The results for the HI antibody titre are detailed in Table 1.

Acute phase responses

The concentrations of the inflammatory mediators, acute phase proteins and gangliosides, prior to and after challenge are presented in Table 2. Results showed that the concentrations of all variables were significantly higher in challenged partridges compared with baseline levels (P<0.05). In general, the concentrations of the study variables in challenged birds were approximately 1.3 –1.4 fold higher than baseline levels. The findings of the present study showed elevation in the serum concentration of the inflammatory mediators, acute phase reactants and gangliosides from 24 h *pc* on. In infected birds, the levels of Hp peaked at 24 h *pc*, while the concentrations of SAA, TNF-α and IFN-γ peaked at 3 days *pc*. The level of TSA, PBSA and LBSA remained relatively constant during the experiment.

DISCUSSION

Innate immune responses are essentially involved in orchestrating and modulating the immune response towards a pathogen. For chickens, proper activation of innate

Table 1. Serum HI titres prior to and after challenge with H9N2 AI virus

HI antibody titre (–log 2)					
Prior to challenge		Day 7 post challenge		Day 14 post challenge	
Mean	Range	(Mean)	Range	(Mean)	Range
1.4	1–2	4.2	4–5	5.9	5–6

Table 2. Serum levels of acute phase proteins, inflammatory mediators and gangliosides (mean±SD; n=32) of partridges prior to challenge (baseline) and on days 1, 3 and 7 post challenge with H9N2 avian influenza virus

Parameters	Prior to challenge	Post challenge		
		Day 1	Day3	Day 6
Hp (g/L)	0.07±0.0042^a	0.088±0.005^b	0.086±0.006^b	0.085±0.0066^b
SAA (μg/mL)	1.59±0.056^a	2.08±0.1^b	2.11±0.083^b	2.06±0.12^b
TSA (μmol/L)	0.38±0.03^a	0.52±0.03^b	0.52±0.04^b	0.52±0.05^b
PBSA (μmol/L)	0.16±0.02^a	0.23±0.02^b	0.23±0.03^b	0.23±0.04^b
LBSA (μmol/L)	0.22±0.02^a	0.29±0.02^b	0.29±0.07^b	0.28±0.06^b
TNF-α (pg/dL)	14.38±1.74^a	19.88±1.68^b	20.12±1.37^b	19.81±1.7^b
IFN-γ (pg/dL)	8.28±1.47^a	11.77±0.89^b	11.91±1.42^b	11.69±0.9^b

Hp – haptoglobin; SAA – serum amyloid A; TSA – total sialic acid; PBSA – protein bound sialic acid; LBSA – lipid bound sialic acid; TNF-α – tumor necrosis factor-alpha; IFN-γ – gamma interferon. Different superscript letters denote significant differences (P<0.05) with baseline level (prior to challenge).

immune reactions is essential for subsequent triggering of adaptive immunity (Juul-Madsen *et al.,* 2008). Several components such as mucus, macrophages, interferon and other cytokines, natural killer cells and complement are involved in the innate immune system (Tamura & Kurata, 2004). Infection elicits a cascade of host immune defenses leading to mucosal inflammation and the influx of polymorphonuclear cells, lymphocytes, and macrophages into the mucosa. Subsequently, the production of proinflammatory mediators leads to local elevation of these mediators as well as serum levels (Hayden *et al.,* 1998).

Our approach was to infect clinically healthy partridges with a LPAI virus and then to quantify serum levels of inflammatory mediators, acute phase proteins and gangliosides.

In the current study, detectable haemagglutination inhibition antibodies were found on days 7 and 14 *pc*. Humberd *et al.* (2006) reported low levels of serum haemagglutination inhibition antibody titres in chukar partridges. Virus neutralising antibodies are believed to be largely responsible for protection of immunised hosts, but not essential (Graham & Braciale, 1997). Although antibody production is clearly an important mechanism for limiting viral infection, it is often insufficient to completely protect against viral replication. Xing *et al.* (2008) suggested that neutralising antibodies and humoral immunity may not be developed efficiently in H9N2-infected chickens. Seo *et al.* (2002) showed that the currently circulating H9N2 influenza viruses provide chickens with cross-reactive protective immunity against the currently circulating H5N1 influenza viruses and that this protective immunity is closely related to the percentage of pulmonary $CD8^{++}$ T cells expressing gamma interferon. On the basis of our findings, it seems that increasing levels of IFN-γ in the serum after H9N2 inoculation may make it a good candidate to account for the early diagnosis before the induction of virus-specific neutralising antibodies and cellular immunity. However, it is important to note that the quantity of a cytokine in the blood circulation does not necessarily reflect the amount produced in the mucosa, since some cytokines may be bound and cleared more rapidly and completely at mucosal sites than others (Hayden *et al.*, 1998).

Our results indicate that both IFN-γ and TNF-α are detectable in the serum 24 h after H9N2 infection of partridges. The concentrations of TNF-α and IFN-γ were significantly higher than basal levels in all days, but showed no significant changes in the different days of sampling in challenged birds. The early production of IFN-γ as measured in serum might result from innate immune responses (Unanue, 1997). The chicken IFN-γ role in the immune response has been well-documented (Kaiser & Staheli, 2008). IFN-α, TNF-α, IL-1 and IL-6 are known to participate in non-specific and specific antiviral immune responses (Le & Vilcek, 1987). There is growing evidence that the so-called early cytokines produced at the site of infection mediate many of the clinical and pathological manifestations (Van Reeth, 1998). H9N2 virus has also been shown that to induce high levels of TNF-α and IFN-γ in trachea, lung and intestine of infected chickens which fulminate by day 4 *pc* when viral titre peaked (Nang *et al.,* 2011). In contrast, Sylte & Suarez (2012) demonstrated that vaccination and homologous or heterologous challenge with LPAI significantly reduce serum concentration of acute phase mediators such as alpha-1 acid glucoprotein

(AGP), PGE2 and ovotransferrin in chickens. The production of various cytokines has also been reported in response to influenza infection (Gambotto *et al.,* 2008; Barbe *et al.,* 2011).

TNF-α, another inflammatory mediator which is well known for its profound stimulating effects on neutrophil and macrophage functions (Van Reeth, 1998), showed significant changes compared to baseline. Previous studies revealed a synergism between influenza virus and lipopolysaccharide from *Escherichia coli* or *Haemophilus influenzae* for TNF-α production. This finding supports the idea that the complications of combined influenza virus and bacterial infections may be partially due to excessive TNF-α production (Nain *et al.,* 1990).

The cytokines IL-1, IL-6 and TNF-α are the main inducers of APPs (Petersen *et al.,* 2004). In the current study, elevation of SAA in the challenged birds was in accordance with the TNF-α and IFN-γ responses, which is in agreement with previous studies (Hulten *et al.,* 1999). Certain viral infections including influenza have been shown to cause substantial increases in both CRP and SAA concentrations in men (Sarov *et al.,* 1982) and animals like horses (Hulten *et al.,* 1999).

In the present study, elevation in the serum Hp was observed from 24th hour *pc*. Although Hp levels in the challenged group were significantly higher by all days compared to baseline, the Hp concentration had remained relatively constant and did not return to baseline values by day 9 *pc* while Hp concentrations peaked 7–10 days after the infection in horses following experimental infection with influenza virus (Kent & Goodall, 1991).

We also found elevated plasma TSA, LBSA and PBSA. Sialic acid (SA) concentration increases rapidly following the inflammatory and injury process (Ekin *et al.,* 2003) and high serum SA level is an important factor for certain diseases. In the present study, serum TSA, PBSA and LBSA concentrations in partridges infected with H9N2 avian influenza virus were 1.36, 1.43 and 1.32 times higher than baseline levels, respectively, which revealed apparent tissue damage and inflammatory disorders. These significant changes in the levels of serum LBSA, PBSA and TSA indicate that they may be a valuable indicator of the inflammatory process associated with influenza infection in partridges. In this study, the increase in serum sialic acid concentrations was in good agreement with other inflammatory parameters including SAA, Hp, TNF-α and IFN-γ. Although speculative, the constancy in SA level observed from day 1 to 6 *pc*, may be due to low pathogenicity of the virus and its inability to induce stronger responses. Inflammatory mediators, acute phase reactants and gangliosides are not specific parameters and increased in animals subjected to external or internal challenges such as infection, inflammation, surgical trauma or stress (Kaneko *et al.,* 2008). Cytokines are probably useful indicators of influenza infection in partridges as well as inflammatory mediators, acute phase proteins and gangliosides production. Examination of respiratory secretions and evaluation of correlation with virus replication and disease symptoms is recommended. More research is needed to refine the best inflammatory mediators or reacting proteins to be used in influenza infection in different bird species.

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