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RESEARCH ARTICLE

Changes in Serum Acute Phase Reactants, Inflammatory Mediators and Gangliosides in Japanese quail (*Coturnix japonica*) with Retained Yolk Sac

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ABSTRACT

Twenty-two 2-week-old Japanese quails (*Coturnix japonica*) with retained yolk sac have been assessed using validated standard procedures to quantify the serum concentration of the acute phase reactants (haptoglobin and serum amyloid A), inflammatory mediators (tumor necrosis factor-alpha, gamma interferon) and gangliosides (total sialic acid, lipid bound sialic acid and protein bound sialic acid). The present findings show that the concentrations of all measured parameters in diseased birds were significantly higher than the control group. Inflammation following yolk sac infection/retention leads to stimulation of the synthesis of inflammatory mediators, acute phase reactants and gangliosides. Among the study variables, lipid bound sialic acid had the most obvious change, so it is the most sensitive parameter.

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INTRODUCTION

Bacterial infection of the navel and yolk sac of newly hatched chicks by varying agents as a result of contamination before healing of the navel leads to yolk sac infection/omphalitis which assumed as economically important disease. The members of family Enterobacteriaceae such as *Escherichia coli* (the main one), *Salmonella*, *Pseudomonas*, other bacteria such as *Staphylococcus* species and/or *Aspergillus fumigatus* cause natural cases of yolk sac infection (Khan *et al.,* 2004). Poor breeder farm nest hygiene, use of floor eggs, inadequate hatchery hygiene or poor incubation conditions, for example poor hygiene of setters, hatchers or chick boxes are some of the predisposing factors. Transmission of bacteria through unhealed naval, infection through blood stream and contamination of yolk before it is internalized into the chick have been reported as routes of infection (Anjum, 1997).

Yolk retention is not the cause of death only in chicken but also in other species of poultry including guinea fowl, duck, turkey, quail and goose (Khan *et al.,* 2004). In the other hand, the game bird industry is going to continue to grow. The number of quails which are annually being produced for commercial purposes have increased and raising quails become important to the economy. Unabsorbed yolk is observed as principal lesion in quails died up to one week of age.

Acute phase proteins (APPs) are blood proteins primarily synthesized by hepatocytes as part of the acute phase response (APR). The APR is part of the early-defense or innate immune system, which is triggered by different stimuli and after cytokine stimulation (Cray *et al*., 2009). The acute phase response with its changes in blood plasma composition is thought to be beneficial to the organism by preventing microbial growth and helping to restore homeostasis (Gruys *et al*., 2005). Releasing immune modulators and pro-inflammatory cytokines also induce the release of acute-phase glycoproteins with sialic acid (SA) from the liver into the general circulation, leading to an increase in SA concentrations (Crook *et al.,* 2001). SA can be used as a marker for the determination acute phase protein concentrations because it is localized at the end chain of many acute phase proteins (Enjuanes *et al.,* 2000; Ekin *et al.,* 2003).

Similar to mammals, chickens produce APPs in the liver, secrete it to the blood and up regulate the production during acute stages of infections (Nielsen *et al.,* 1999; Juul-Madsen *et al.,* 2003). It has been suggested that determining the concentrations of acute phase proteins could help to monitor poultry health. In large animals, APPs have been proposed as markers of 'herd health' (Murata *et al*., 2004; Ganheim *et al*., 2007). In companion animals, APPs have especially been identified for prognostic applications (Ceron *et al*., 2005). Although

several research studies have been published about APPs changes in association with some common poultry diseases (Barnes *et al.,* 2001; Koutsos and Klasing, 2001; Rath, 2005; Kokosharov, 2006; Rath *et al.,* 2007; Nazifi *et al.,* 2010; 2011) however, APPs changes in avian infection and disease are not known completely. To the best of our knowledge, no study has examined the changes in the concentrations of acute phase reactants (haptoglobin (Hp) and serum amyloid A (SAA)), inflammatory mediators (tumor necrosis factor-alpha (TNF-α) and gamma interferon (IFN-γ)) and gangliosides (total sialic acid (TSA), lipid bound sialic acid (LBSA), and protein bound sialic acid (PBSA)) for quails with yolk sac retention. Therefore, this study evaluates the alteration in the acute phase reactants (Hp and SAA), inflammatory mediators (TNF-α and IFN-γ) and gangliosides (TSA, LBSA, PBSA) in quails with yolk sac retention in comparison with healthy quails.

MATERIALS AND METHODS

The diseased chicks (n=22), involved in this study, were from a 2-week-old Japanese quail (*Coturnix japonica*) flock which had a history of anorexia, weakness, dehydration and weight loss. The total mortality rate within two weeks was 6.8%. Necropsy was performed to determine the cause of the disease**.**

Blood sampling and processing: Prior to necropsy, blood samples were collected from the jugular vein of the diseased as well as clinically healthy birds as control group (n=13). Sera were harvested by centrifugation at 750×*g* for 15 min and stored at – 20ºC until use. In the serum of different groups of birds, acute phase reactants, gangliosides and inflammatory mediators were assayed by using validated standard procedures as follows:

Acute Phase reactants (Hp and SAA) determination: Hp was measured according to prevention of the peroxidase activity of hemoglobin, 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).

Gangliosides (TSA, LBSA, PBSA) determination: Serum TSA concentration was determined by the thiobarbituric acid method. The amount of TSA was determined by use of a standard curve developed from a standard sample 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.

Inflammatory mediators (IFN-γ **and TNF-α) determination:** 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).

Bacterial isolation and identification: After necropsy, samples from retained yolk sacs were taken by sterile loop and aseptically cultured on MacConkey agar and Blood agar. To enrich *Salmonella spp*., another swab was inoculated into Selenite-F broth. All cultured media were incubated at 37°C. Cultures in Selenite-F were subcultured on the MacConkey and Brilliant-green agar. After the 24-48 h incubation of the solid media, the plates were observed for colony formation. The identification of the isolated colonies was performed using standard bacteriological and biochemical procedures (Quinn *et al.,* 2002).

Statistical analysis: Descriptive statistics including mean, standard deviation, standard error, minimum and maximum were calculated for all variables. Data were evaluated for normality by Kolmogrov-Smirnov test. However, due to the concurrent occurrence of unequal sample size and non-homogenous variances, for some other variables, non-parametric Kruskal-Wallis test was used for comparison of the two groups. For comparison of different parameters the ANOVA test and Tukey were used. Association between the studied variables was investigated using Pearson's correlation coefficients, and only statistically significant correlations were reported. To evaluate which factor is more sensitive to change in diseased quails compared with healthy control quails, receiver operating characteristic (ROC) analysis was done and area under the curve (AUC) were compared. Data were analyzed by SPSS software, version 11.5. A P-value less than 0.05 was considered as statistically significant.

RESULTS

The postmortem examination revealed unabsorbed yolk sac in diseased chicks. The unabsorbed yolk sacs were distended with yellow/yellow brown, concentrated and fetid content. This lesion was also associated in some cases with perihepatitis and pericarditis indicating systemic spread of organism from the yolk sac.

Summary statistics and results for the comparison of the study variables in healthy and diseased quails are presented in Table 1. All measured parameters showed significant differences between the diseased and control group. Results showed that the concentrations of all of these variables were significantly higher in diseased quails compared with the control group ($P<0.05$). According to the area under the curve (AUC), LBSA was the most sensitive factor to change in the diseased quails.

Bacterial cultures of the sampled yolk sacs showed 100% bacterial contamination. *Escherichia coli* was the most common bacterium recovered from all samples. However, *Salmonella spp*. and other bacteria were not isolated from any of the eggs.

DISCUSSION

Several acute phase proteins have been analyzed in chicken in association with common poultry diseases (Chamanza *et al.,* 1999; Holt and Gast, 2002; Xie *et al.,* 2002; Nazifi *et al.,* 2010, 2011). Most of these APPs do not change to the same level as mammalian APPs and could not be analyzed in natural infections.

Table1: Summary statistics and comparison between parameters in diseased and control group

Parameters	Mean	SD	SE	Min	Max
Control group (n=13)					
Hp (g/l)	0.066	0.017	0.0047	0.04	0.09
SAA (µg/ml)	1.543	0.127	0.0354	1.35	1.75
TSA (mmol/L)	0.00038	0.00008	0.00002	0.00030	0.00050
PBSA (mmol/L)	0.00016	0.00005	0.00001	0.00010	0.00020
LBSA (mmol/L)	0.00022	0.00008	0.00002	0.00010	0.00030
TNF-α (pg/dl)	14.26	2.72	0.75	10.29	18.62
IFN-γ (pg/dl)	8.42	2.06	0.57	5.64	11.49
Treated group (n=25)					
Hp (g/l)	0.0960	0.01225	0.00245	0.07	0.12
SAA (µg/ml)	2.3360	0.30301	0.06060	1.93	2.93
TSA (mmol/L)	0.00162	0.00029	0.00005	0.00110	0.00210
PBSA (mmol/L)	0.00056	0.00010	0.00002	0.00040	0.00080
LBSA (mmol/L)	0.00106	0.00034	0.00006	0.00040	0.00170
TNF-α (pg/dl)	21.4696	1.59578	0.31916	18.26	24.35
IFN-γ (pg/dl)	15.6876	1.41037	0.28207	13.06	17.87

Based on the results of this research, significant changes in Hp, SAA, TSA, LBSA, PBSA, TNF-α, and IFN-γ concentrations were indicated in quails with retained yolk sac.

During bacterial infections, larger amounts of peptidoglycan (PG) or lipopolysaccharide (LPS), which are the major components of bacterial cell wall, may be released into the bloodstream. The host immune system is activated by LPS and PG in an indirect manner. They are capable of stimulating mononuclear phagocytes and endothelial cells to release immune modulators such as TNF-α, members of the interleukin family and interferon-α. On the other hand, the massive release of LPS and PG during a severe bacterial infection into the bloodstream results in pathophysiological reactions due to an over stimulation of immune system (Hamann *et al.,* 1998). During yolk sac infection larger amounts of PG or LPS may be released into the bloodstream, leading to increasing TNF-α and IFN-γ. Spread of *E. coli* by extension into the body cavity or systemically to produce colisepticemia may also lead to increase these factors. The concentration of TNF-α and IFN-γ increased 1.5- and 1.86-fold respectively, compared with control group. Koutsos and Klasing (2001) determined the effect of LPS or Muramyl peptides (MDP), the minimum immuno-adjuvant structure from the cell wall PG of gram-positive bacteria, and administration on the systemic acute phase response in Japanese quail. They indicate that Japanese quail are less sensitive to MDP than LPS, and quail demonstrate tolerance to LPS following repeated injections. In addition, these pro-inflammatory cytokines induce the synthesis of APPs by the liver (Zetterstrom *et al.,* 1998). Therefore, the elevation in SAA and Hp could be explained. Inflammatory reaction in yolk sac causes release and elevation of SAA concentrations, as a positive APP. Number of antimicrobial properties such as opsonization of bacteria, activation of complement, enhancement of phagocytosis, and scavenging of minerals from the bloodstream that are limiting for bacterial growth and replication (Baumann and Gauldie, 1994) were explained for APPs. Significant increase in SAA and α1-acid glycoprotein levels in gumboro virus-infected chicks has been reported (Nazifi *et al.,* 2010). In relation to infectious bronchitis disease, chickens infected with infectious bronchitis virus show a significant increase in SAA, Hp, LBSA, PBSA and TSA (Nazifi *et al.,* 2011). The elevation in SAA and transferrin concentrations following administration of terpentin to the pullet and *Staphylococcus aureus* infection in chicks have been reported (Chamanza *et al.,* 1999). Kovacs *et al.* (2007) showed a mildly increase of SAA levels in goose by administration of a fowl cholera vaccine containing inactivated *Pasteurella multocida*.

Significant changes in the levels of serum LBSA, PBSA and TSA also found in the current study. The increase in serum SA concentrations was in good agreement with other inflammatory parameters including SAA, Hp, TNF-α and IFN-γ. Serum gangliosides (TSA, PBSA and LBSA) concentrations in quails with retained yolk sac were 4.26-, 3.5- and 4.8-fold higher than those in healthy quails, respectively, which revealed apparent inflammatory disorders. The results are in agreement with the previous studies (Farsang *et al.,* 2002; Nazifi *et al.,* 2011). These significant changes in the levels of serum gangliosides indicate that they may be a valuable indicator of the inflammatory process associated with yolk sac retention in quails. According to the area under the curve (AUC), LBSA was the most sensitive factor to change in the diseased quails. Therefore, increase in serum LBSA concentration may be a good indicator of the inflammatory process in quails with retained yolk sac.

It is well documented that different types of bacterial agents are attributed for causation of yolk sac infection. *Escherichia coli* were frequently the main one involved (Khan *et al.,* 2004). In the current study, *E. coli* was the most common bacteria recovered from all samples.

In conclusion, inflammation following yolk sac infection leads to stimulation of the synthesis of acute phase reactants, gangliosides and inflammatory mediators. Significant increases in levels of serum Hp, SAA, TSA, LBSA, PBSA, TNF-α, and IFN-γ were observed in quails with retained yolk sac as expected. Among study variables, LBSA had the most obvious change, so it is the most sensitive parameter. Although the reaction of APPs in birds is different from the reaction in mammals, they are useful in monitoring the health status of birds. More research is needed to refine the best reacting proteins to be used for health monitoring in different bird species.

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