

## Evaluation of the effect of simultaneous infection with *E. coli* O<sub>2</sub> and H<sub>9</sub>N<sub>2</sub> influenza virus on inflammatory factors in broiler chickens

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### Abstract

This study was conducted to evaluate the effect of experimental infection with *Escherichia coli* O<sub>2</sub> and H<sub>9</sub>N<sub>2</sub> influenza virus on inflammatory factors in broiler chickens. A total of 120 one-day-old Cobb broiler chicks were divided randomly to 6 groups. Inoculation program with 10<sup>9</sup> EID<sub>50</sub>/bird of the A/Chicken/Iran/772/1998 (H<sub>9</sub>N<sub>2</sub>) virus and 10<sup>9</sup> CFU/mL/bird of *E. coli* O<sub>2</sub> was carried out as follows: the chicks in group 1 were inoculated with virus and bacteria simultaneously on day 26, group 2 received virus on day 26 and then bacteria 3 days later, group 3 were inoculated with bacteria on day 23 and then virus on day 26, group 4 received only bacteria on day 26, group 5 were inoculated with only virus on day 26 and group 6 served as control. Serum samples were collected from wing vein at days 20, 30, and 40. Sera were examined for inflammatory mediators (TNF- $\alpha$  and INF- $\gamma$ ), acute phase reactants (haptoglobin and serum amyloid A) and gangliosides (total, lipid-bound and protein-bound sialic acids) using validated standard procedures. Among the measured parameters, serum gangliosides showed significant differences between the challenged and control groups in different days post inoculation ( $P < 0.05$ ). Significant increase in serum concentrations of serum sialic acids was observed on the 30<sup>th</sup> day in challenged groups. Elevations were found in the concentrations of serum gangliosides on day 40 compared to their first concentrations. The most obvious increase in serum sialic acids was observed in group 1 challenged with avian influenza virus and *E. coli* O<sub>2</sub> simultaneously. Bacterial infected group showed more significant changes in comparison with viral infected one. These findings suggest that serum sialic acids may be a useful indicator of H<sub>9</sub>N<sub>2</sub> avian influenza virus and avian pathogenic *E. coli* O<sub>2</sub> co-infection.

### Introduction

Avian influenza is an important poultry disease with the potential to cause major epi-

demics resulting in significant economic losses. Avian influenza viruses belong to type A of *Orthomyxoviridae* family, and 18 hemagglutinins (H1 to H18) and 11 neuraminidase subtypes (N1 to N11) have been reported.<sup>1</sup>

Avian H<sub>9</sub>N<sub>2</sub> influenza viruses have circulated widely in domestic poultry around the world.<sup>2</sup> Although avian H<sub>9</sub>N<sub>2</sub> influenza virus is pathotyped as low pathogenic, experiences over the last decade show serious disease and high mortality in broilers associated with this subtype in many regions of Iran. One of the possible explanations for such high mortality and great economic losses could be mixed infection with other respiratory pathogens.<sup>3</sup>

A member of *Enterobacteriaceae* family, pathogenic *E. coli* O<sub>2</sub> is one of the factors that may affect the pathogenicity of avian H<sub>9</sub>N<sub>2</sub> influenza virus. This bacterium is categorized based on its somatic (O) and flagellar (H) antigens. Any local or generalized infection caused by avian pathogenic *E. coli* O<sub>2</sub> is called colibacillosis, the most common bacterial infection in poultry.<sup>4</sup> Colibacillosis is usually seen with other infections resulting in disease deterioration. Simultaneous infection of colibacillosis with paratyphoid and histomoniasis results in high mortality.<sup>5</sup> Colibacillosis with Gaboro cause increase in mortality.<sup>6</sup> Infectious bronchitis virus increases the mortality due to avian H<sub>9</sub>N<sub>2</sub> influenza viruses.<sup>3</sup>

Acute phase proteins are a group of blood proteins whose concentrations are affected by infections and inflammatory conditions.<sup>7-9</sup> These proteins are mainly synthesized in liver. Acute phase protein mediators are pro-inflammatory cytokines.<sup>7,8,10</sup> In addition to mammals, acute phase proteins are also synthesized in birds and their concentrations change during different stages of infection.<sup>11</sup> Therefore, the concentration of acute phase proteins can be used to evaluate poultry health.

Investigators have reported that sialic acids, localized at the end chain of many acute phase proteins (APPs), can be used as a marker for the determination of acute phase protein concentrations.<sup>12-14</sup> The majority of sialic acids are found in either protein-bound (PBSA) or lipid-bound (LBSA) forms, while a small amount is in the free form. In addition, sialic acids are localized at the end chain of many acute-phase proteins (APPs) and are present in normal serum of human and animals;<sup>12,13</sup> their content in serum is changed in various diseases.<sup>14,15</sup> Therefore, the detection of sialic acid may be a valuable indicator for the diagnosis and prognosis of inflammatory diseases.<sup>14,15</sup>

This research was conducted to assess the inflammatory mediators in simultaneous infection with avian H<sub>9</sub>N<sub>2</sub> influenza virus and *E. coli* O<sub>2</sub> in broilers.

Although avian H<sub>9</sub>N<sub>2</sub> influenza virus in Iran's farms is pathotyped as a low pathogenic

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virus, the reports indicate that this subtype causes severe disease and results in 20-65% mortality rate.<sup>16</sup> The possibility of disease deterioration due to simultaneous infection of avian H<sub>9</sub>N<sub>2</sub> influenza virus with other bacterial or viral diseases is supposed. Therefore, diagnosis and control of simultaneous infections would be of great help in decreasing economic losses due to avian H<sub>9</sub>N<sub>2</sub> influenza virus.

Bano et al.<sup>17</sup> showed the role of *E. coli*, *Ornithobacterium rhinotrachealea* and bronchitis virus in increasing avian H<sub>9</sub>N<sub>2</sub> influenza virus virulence. The synergistic role of staphylococcus proteases on hemagglutinin cleavage was evaluated by Tashiro et al.<sup>18</sup> Kishida et al.<sup>19</sup> reported that simultaneous infection of avian H<sub>9</sub>N<sub>2</sub> influenza virus with *Staphylococcus aureus* or *Haemophilus paragallinarum* results in more severe clinical signs in broilers and affects the virus proliferation. In the research of Barbour et al.,<sup>20</sup> broilers were inoculated with different doses of *E. coli* and the same doses of avian H<sub>9</sub>N<sub>2</sub> influenza virus. The highest mortality was observed in the group that received the highest dose of *E. coli*. Moreover, clinical findings decreased with the decrease of *E. coli* doses. Nazifi et al.<sup>21</sup> showed that the concentrations of acute

phase proteins and sialic acids in broilers infected with bronchitis virus were significantly higher compared to controls.

The aims of the present study were to determine: i) whether simultaneous infection of *E. coli* O<sub>2</sub> and avian H<sub>9</sub>N<sub>2</sub> influenza virus affects the inflammatory factors in broilers or not; ii) which inflammatory factor is affected more by simultaneous infection of *E. coli* O<sub>2</sub> and avian H<sub>9</sub>N<sub>2</sub> influenza virus, and whether this factor has a diagnostic value or not.

## Materials and Methods

### Experimental design

A total of 120 one-day-old Cobb broiler chicks were purchased from a local hatchery. They were randomly divided into 6 groups and each group was kept in a separate room. They were reared up to 6 weeks in cages and fed on pelleted antibiotic-free broiler diet. The birds did not receive any vaccine. Feed and water were provided *ad-libitum*. A continuous twenty four hour light was supplied.

### Bacteria

*E. coli* strain O<sub>2</sub> was provided by Razi Serum and Vaccine Research Institute, Karaj, Iran. The culture was prepared as described by Matthijs *et al.*<sup>22</sup> and was used at a concentration of 1×10<sup>9</sup> CFU/mL.

### Avian influenza virus

Avian influenza virus subtype H<sub>9</sub>N<sub>2</sub> (A/chicken/Iran/772/1998) was proliferated in 9-day-old chicken embryo. For this purpose, 0.2 mL virus was inoculated to allantoic cavity and the eggs were incubated at 37°C for 48 hours. The allantoic fluid was harvested and EID<sub>50</sub> was calculated according to Reed and Muench method. The viral source was kept in -70°C until used.

### Inoculation program

Table 1 presents the inoculation program with avian influenza virus subtype H<sub>9</sub>N<sub>2</sub> and *E. coli*. Day 20 is a baseline data (prior infection). At 26 days of age, groups 1, 2, 3 and 5 were challenged intranasally with 10 µL of allantoic fluid containing 1×10<sup>9</sup> EID<sub>50</sub>/mL of the virus. *E. coli* O<sub>2</sub> was sprayed on each group separately. Each bird received 1 mL of 1×10<sup>9</sup> CFU/mL of *E. coli*.

### Blood samples

Blood samples were taken from wing vein into tubes without anticoagulant 3 times; on days 20 (before viral inoculation), 30 and 40 of the chicken age. The sera were separated following centrifugation at 750 g for 15 min and stored at -20°C until used.

## Biochemical assays

### Acute phase proteins determination

Haptoglobin (Hp) was measured by prevention of peroxidase activity of hemoglobin which is directly proportional to the amount of Hp. The analytical sensitivity in serum was 0.0156 mg/mL for Hp as stated by the manufacturer (Tridelata Development Plc, Wicklow, Ireland).

Serum amyloid A (SAA) was measured by a solid phase sandwich-ELISA with a sensitivity in sera of 0.3 µg/mL (Tridelata Development Plc, Wicklow, Ireland).

### Inflammatory mediators 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)).

Table 1. Inoculation program with avian influenza virus subtype H<sub>9</sub>N<sub>2</sub> and *E. coli*.

Group	H <sub>9</sub> N <sub>2</sub> inoculation	<i>E. coli</i> inoculation	Time of <i>E. coli</i> O <sub>2</sub> inoculation
1	+	+	Simultaneous with viral inoculation
2	+	+	3 days after viral inoculation
3	+	+	3 days before viral inoculation
4	-	+	Simultaneous with group 1
5	+	-	-
6	-	-	-

Viral infection starts in day 26 in Group 5.

Table 2. Mean ± standard errors of Hp, SAA, IFN-γ, TNF-α and TBSA concentrations in different groups experimentally infected with/without avian influenza virus subtype H<sub>9</sub>N<sub>2</sub> and *E. coli*. Day 20 is a baseline data (prior infection).

Group	Day 20	Day 30	Day 40
<b>Hp (g/L)</b>			
1	0.21±0.11 <sup>a</sup>	0.22±0.02 <sup>a</sup>	0.30±0.18 <sup>a</sup>
2	0.14±0.010 <sup>a</sup>	0.13±0.06 <sup>a</sup>	0.22±0.21 <sup>a</sup>
3	0.16±0.011 <sup>a</sup>	0.13±0.07 <sup>a</sup>	0.17±0.20 <sup>a</sup>
4	0.13±0.030 <sup>a</sup>	0.09±0.04 <sup>a</sup>	0.27±0.21 <sup>a</sup>
5	0.15±0.04 <sup>a</sup>	0.16±0.07 <sup>a</sup>	0.23±0.15 <sup>a</sup>
6	0.11±0.06 <sup>a</sup>	0.18±0.11 <sup>a</sup>	0.18±0.14 <sup>a</sup>
<b>SAA (µg/mL)</b>			
1	1.38±0.14 <sup>a</sup>	1.50±0.19 <sup>a</sup>	1.45±0.42 <sup>a</sup>
2	1.41±0.23 <sup>a</sup>	1.18±0.97 <sup>a</sup>	1.68±0.31 <sup>a</sup>
3	1.48±0.30 <sup>a</sup>	1.61±0.11 <sup>a</sup>	1.75±0.39 <sup>a</sup>
4	1.54±0.30 <sup>a</sup>	1.24±0.17 <sup>a</sup>	1.35±0.11 <sup>a</sup>
5	1.54±0.17 <sup>a</sup>	1.31±0.22 <sup>a</sup>	1.31±0.25 <sup>a</sup>
6	1.34±0.11 <sup>a</sup>	1.68±0.13 <sup>a</sup>	1.49±0.25 <sup>a</sup>
<b>IFN-γ (pg/dL)</b>			
1	15.88±2.71 <sup>a</sup>	18.18±2.97 <sup>a</sup>	19.10±3.46 <sup>a</sup>
2	12.47±2.09 <sup>a</sup>	14.82±2.25 <sup>a</sup>	14.68±2.25 <sup>a</sup>
3	13.60±2.33 <sup>a</sup>	14.62±2.59 <sup>a</sup>	22.03±3.43 <sup>a</sup>
4	36.93±4.06 <sup>a</sup>	36.15±4.30 <sup>a</sup>	20.65±3.34 <sup>a</sup>
5	12.55±1.43 <sup>a</sup>	18.98±1.98 <sup>a</sup>	24.18±3.07 <sup>a</sup>
6	15.53±2.31 <sup>a</sup>	20.40±2.04 <sup>a</sup>	21.46±2.43 <sup>a</sup>
<b>TNF-α (pg/dL)</b>			
1	32.64±2.65 <sup>a</sup>	30.93±2.26 <sup>a</sup>	25.90±0.14 <sup>a</sup>
2	40.98±2.56	39.20±2.42 <sup>a</sup>	43.22±2.84 <sup>a</sup>
3	38.46±2.14	39.24±2.40 <sup>a</sup>	38.53±2.27 <sup>a</sup>
4	29.57±9.35	29.91±1.12 <sup>a</sup>	27.92±9.54 <sup>a</sup>
5	28.00±9.49	25.85±5.65 <sup>a</sup>	28.72±9.75 <sup>a</sup>
6	27.65±1.02	29.10±1.06 <sup>a</sup>	24.49±1.00 <sup>a</sup>
<b>TBSA (µmol/L)</b>			
1	1.00±0.08 <sup>aC</sup>	1.7±0.06 <sup>aB</sup>	0.4±0.07 <sup>aA</sup>
2	0.9±0.06 <sup>bC</sup>	1.2±0.05 <sup>bB</sup>	0.4±0.13 <sup>bA</sup>
3	0.9±0.10 <sup>bC</sup>	1.2±0.05 <sup>bB</sup>	0.4±0.05 <sup>bA</sup>
4	0.6±0.10 <sup>cC</sup>	0.9±0.10 <sup>cB</sup>	0.4±0.11 <sup>cA</sup>
5	0.5±0.04 <sup>dC</sup>	0.7±0.04 <sup>dB</sup>	0.5±0.09 <sup>dA</sup>
6	0.4±0.06 <sup>cC</sup>	0.4±0.08 <sup>cB</sup>	0.4±0.05 <sup>cA</sup>

Different small letters in each column indicates significant difference. Different capital letters in each column indicates significant difference.

### TSA, LBSA, PBSA determination

Serum TSA concentration was determined by the thiobarbituric acid method. The amount of TSA was determined using a standard curve developed from a standard sample of N-acetyl neuraminic acid.<sup>23</sup> LBSA was determined as described by Katopodis *et al.*<sup>24</sup> using 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

Data were analyzed by SAS software. For comparison between different parameters, Tukey test was used. P value less than 0.05 was considered as statistically significant.

## Results

Mean $\pm$ SE of blood factor measurements are presented in Table 2 and Figure 1. Total, lipid bound and protein bound sialic acids were significantly different between groups ( $P < 0.05$ ). However, no significant difference was observed in Hp, SAA, TNF- $\alpha$  and INF- $\gamma$  between groups.

The concentrations of total, lipid-bound and protein-bound sialic acids in challenged groups revealed a significant increase on day 30, and then decreased (Figure 1). However, elevations were found in the concentrations of these factors on day 40 compared to their first concentrations. The highest increases were observed in group 1 (simultaneous inoculation of virus and bacteria).

## Discussion and Conclusions

No significant changes were observed in TNF- $\alpha$  and INF- $\gamma$  concentrations in challenged groups in comparison with control one. However, the concentrations of these 2 factors in group 1 showed an increase in the 2<sup>nd</sup> sampling day (day 30, 5 days after simultaneous inoculation of virus and bacteria) compared with the previous and next samplings.

Innate immunity plays an important role in coordination of immune response against pathogens. Many components including mucosal immunity, macrophages, interferon, cytokines, natural killer cells and complement systems have roles in native immune system. Infection induces the immune cascade system including mucosal inflammatory response and aggregation of polymorph nuclear cells, lymphocytes and macrophages in mucosal tissue resulting in producing and increasing the pre inflammatory mediators in blood.<sup>25</sup>

INF- $\gamma$ , TNF- $\alpha$ , interleukine-1 and interleukine-6 are involved in specific and non specific immune response against viruses.<sup>26</sup> Elevation in human cytokines was observed after infection with H<sub>1</sub>N<sub>1</sub> influenza virus.<sup>27</sup> The first stage of infection with swine influenza does not lead to interferon production. Increase in  $\gamma$ -interferon is observed 14 days post inoculation.<sup>28</sup>

Although humoral immunity, antibody production is one of the major mechanisms in limiting the infection, it has no sufficient efficiency against virus proliferation.<sup>29</sup> Cellular immunity due to T cells plays a role in many immune responses against viruses with different mechanisms such as cytokine production.<sup>30</sup> Therefore, cytokines are considered as important indicators in the diagnosis of influenza infection.<sup>25</sup>

INF- $\gamma$ , produced by macrophages and NK cells, is a cytokine produced during the initial stages of infection and induces Th1 stimulation.<sup>28</sup>

TNF- $\alpha$  is another inflammatory mediator that plays an important role in neutrophil and macrophage stimulation.<sup>31</sup> The synergistic effect of influenza virus and *E. coli* in stimulation of TNF- $\alpha$  production has been shown.<sup>32</sup> In the research of Fritz *et al.*,<sup>33</sup> an increase was observed in INF-c in human nasal lavage 2-5 days after nasal inoculation of H<sub>1</sub>N<sub>1</sub>. Monterio *et al.*<sup>34</sup> reported the elevation in IL-12 and INF-c in alveolar lavage in mice 3-7 days after H<sub>1</sub>N<sub>1</sub> inoculation.

During bacterial infection, a large amount of peptidoglycan or lipopolysaccharide is released in blood resulting in indirect stimulation of immune system. Peptidoglycan and lipopolysaccharide have the ability to stimulate mononuclear phagocytic and epithelial cells resulting in secretion of immune mediators such as TNF- $\alpha$ ,  $\gamma$ -INF and interleukins. Moreover, in severe bacterial infections, heavy secretion of proteoglycan and lipopolysaccharide in blood initiates pathologic processes and finally results in systematic immune stimula-

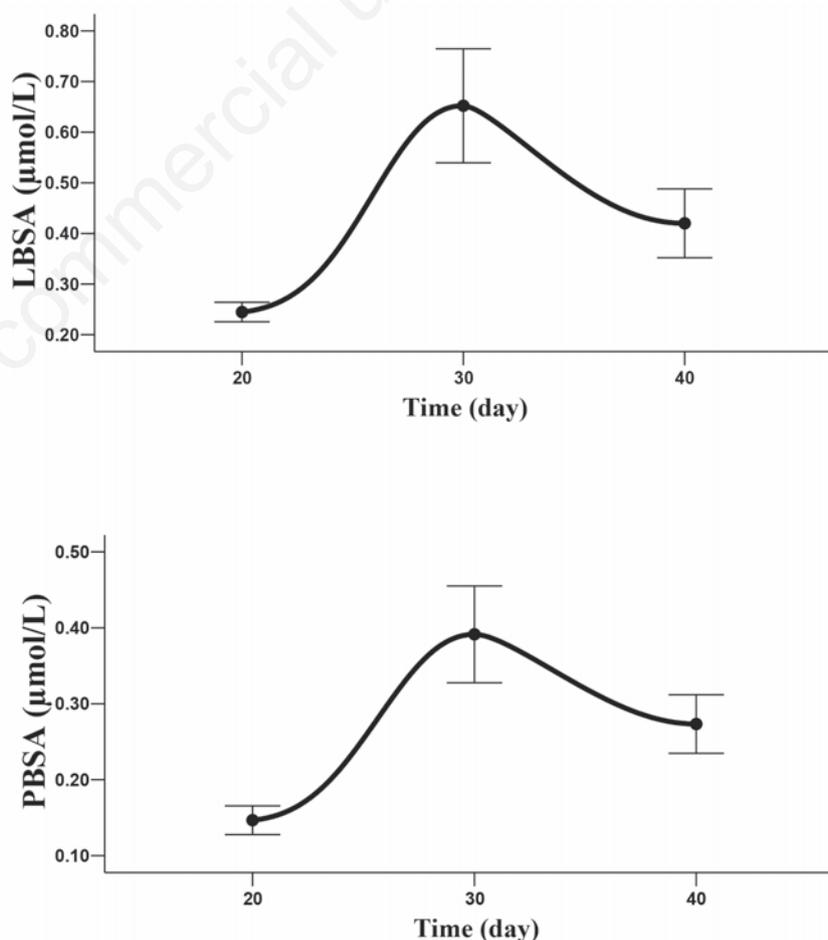


Figure 1. The concentrations of LBSA and PBSA ( $\mu\text{mol/L}$ ) in different times after experimental infection with avian influenza virus subtype H<sub>9</sub>N<sub>2</sub> and *E. coli*.

tion.<sup>25</sup> Koutsos and Klasing evaluated the effect of LPS and MDP on acute phase response in quail.<sup>35</sup> They reported that quail revealed less sensitivity to MDP in comparison with LPS and showed resistance to the repeat of LPS inoculation.

In the present study, no significant changes occurred in acute phase protein (Hp and SAA) concentrations. However, the highest concentration of these factors was observed in the last blood sampling (15 days after simultaneous inoculation of virus and bacteria).

Changes in acute phase proteins are considered as a major part of acute phase response. Acute phase proteins can be used to determine the prognosis of diseases. In birds, these proteins can also be evaluated as indicators to determine birds' health. Acute phase response is caused due to pro-inflammatory cytokines. The most important cytokines include interleukin-1, interleukin-6 and TNF- $\alpha$  resulting in stimulation of acute phase protein synthesis in liver.<sup>36</sup> Changes in most acute phase proteins in birds are not similar to those of mammals and cannot be evaluated in natural infections. In birds, changes in a few acute phase proteins have been described including alpha-1-acid glycoprotein, SAA, transferrin and ovotransferrin.<sup>21,37-40</sup>

Whicher *et al.*<sup>41</sup> reported an elevation in SAA in all people infected with influenza virus type A and the highest concentrations of ceruloplasmin and SAA were observed on the 3<sup>rd</sup> day. It was concluded that SAA measurement could be a valuable indicator in viral infection. In the study of Barbe *et al.*<sup>42</sup> on pigs infected with swine influenza, the concentration of acute phase proteins reached a peak 30-48 hours post inoculation and then decreased. However, the concentration of ceruloplasmin and Hp reached a peak after 48 hours. Hulthen *et al.*<sup>43</sup> reported that the highest increase in SAA in horses infected with equine influenza was observed in the first 48 hours of clinical symptoms appearance.

After increase in acute phase proteins, antimicrobial processes occur including opsonization, activation of complement system, increase in phagocytosis and removal of minerals from blood resulting in bacterial proliferation being limited.

Nazifi *et al.*<sup>21</sup> reported significant increase in Hp and SAA concentrations in broilers infected with infectious bronchitis. In another study by Nazifi *et al.*<sup>40</sup> infection with gamboro virus resulted in elevation of SAA and alpha-1-acid glycoprotein concentrations. Chamanza *et al.*<sup>38</sup> showed increase in SAA and transferrin concentrations in pullets after inoculation of triparentin and infection with *S. aureus*.

In the present study, significant changes in concentrations of total, lipid-bound and protein-bound sialic acids were observed between challenged groups and control one. These find-

ings are in agreement with recent researches.<sup>21</sup> The concentrations of serum sialic acids showed significant increase in all challenged groups and the highest concentrations were found in the second sampling on day 4 post infection. Main changes were seen in group 1, the group that was inoculated simultaneously with virus and bacteria.

The concentration of serum sialic acid showed more increase in bacterial infected group compared with viral infected one. Generally, inflammatory response in viral infections is milder than bacterial ones.<sup>44,45</sup> Also, increase in serum sialic acid concentration was more considerable in the group that received first virus and then bacteria.

Sialic acids increase rapidly after inflammatory interactions.<sup>14,46</sup> Significant increase in total, lipid-bound and protein-bound sialic acids were observed in broilers infected with infectious bronchitis.<sup>21</sup> In the study of Mosleh *et al.*<sup>47</sup> increase in total, lipid-bound and protein-bound sialic acids concentrations were reported after omphalitis in Japanese quail. The most significant changes were observed in lipid-bound sialic acids levels. They concluded that changes in serum gangliosides, especially lipid-bound sialic acids can be considered a valuable indicator in inflammatory response in quail omphalitis.

Simultaneous infection of *E. coli* O<sub>2</sub> and avian H<sub>9</sub>N<sub>2</sub> influenza virus had possible additive effects on the modulation of inflammatory responses.

In the present study, significant changes in sialic acid concentrations reveal that measurement of serum gangliosides can be considered a useful factor in evaluating the inflammatory response in broilers with avian influenza virus and *E. coli* co-infection. Furthermore, inflammatory factors were affected more by simultaneous infection of *E. coli* O<sub>2</sub> and avian H<sub>9</sub>N<sub>2</sub> influenza virus.

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