# Inactivated oil emulsion H9N2 vaccine in broiler chickens: Pathogenesis and Clinicopathological studies

**Hesham A. Sultan[1]; Allaa G. Abd El-Razik[1]; Tamer S. Allam[2*]; Hanan A. El-Deeb [3]**

[1]Department of birds and rabbits medicine, Faculty of Vet. Medicine, University of Sadat city, Egypt
[2]Department of Clinical pathology, Faculty of Vet. Medicine, University of Sadat city, Egypt
[3]Department of Virology, Faculty of Veterinary medicine, Cairo University, Egypt
***Corresponding** author: Tamer s. Allam, Email: Tamer_sma@yahoo.com

**Abstract**

Avian influenza (H9N2 subtype) considered one of the most economic problems threats poultry industry in the world. This study was conducted to evaluate the pathogenesis, serology, hematolology and blood biochemical changes of an inactivated oil emulsion avian influenza H9N2 vaccine prepared from a circulating H9N2 virus isolated from the field against challenge with avian influenza H9N2. Chickens were divided into three groups; two groups were given the prepared vaccine at 10 days of age with 0.5 or 0.7 ml /bird and a control group while at the age of 30-days of age each group was divided into two groups one were kept non challenged with H9N2 isolate and the other group were subjected to challenge. Serological response were monitored in vaccinated chicken at 2, 3 and 4 weeks post vaccination and we noticed that vaccination with 0.7ml /bird give higher HI titer than group vaccinated with 0.5ml /bird. Shedding of the virus was checked out in the trachea and the cloacal swabs using reverse transcriptase/ polymerase chain reaction RT/PCR at 2, 4 and 7 days post challenge, all tracheal and cloacal swabs collected 2, 4 and 7 days post challenge from vaccinated challenge group with 0.5 or 0.7 ml/bird shown negative reaction while in non vaccinated group showed positive reaction only in 33.3% in tracheal swab samples versus negative reactions with the cloacal swabs, at 7 days post challenge all tracheal samples of vaccinated challenge groups and non vaccinated group were positive. Hematological and blood biochemical parameters were noticed at 21 day and 35 day old age before and after challenge, respectively. H9N2 caused anemia, leucopenia, and lymphocytopenia. H9N2 also caused elevation of ALT, AST, A/G ratio and uric acid, with the decrease of total protein and total globulin. The present study concluded that H9N2 caused alteration in hematological and blood biochemical parameters besides immunosuppression.

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**Introduction**

Avian influenza (AI) is a contagious viral disease. Influenza viruses are members of family Orthomyxoviridae. These viruses are further categorized into three distinct genus "type" A, B and C based on serologic reactions to the internal proteins, principally NP and M1 proteins (Calnek et al., 1997). All avian influenza viruses are type A. Type A influenza viruses are divided into subtypes based on the antigenic relationship in the surface glycoprotein haemagglutinin (H) and neuraminidase (N). The virus is additionally subjected into two distinct pathogenic groups; highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) (Swayne and Halvorson, 2008). AIV are known to produce two different types of diseases in poultry birds on the bases of their virulence and pathogenicity. Due to this unique characteristic, the viruses are further classified into two types known as a highly pathogenic avian influenza virus (HPAIV) and a low pathogenic avian influenza virus (LPAIV) (Capua and Alexander, 2004). HPAIV are responsible for rapid and fatal systemic infection inducing mortality up to 100% in broilers, layers and breeders while LPAIV produce asymptomatic infection. Several experimental studies have demonstrated that inactivated AI vaccines are capable of inducing antibody response, which aids in the protection of the infected birds from mortality, and egg production decline (Capua and Alexander, 2008). H9N2 avian influenza virus strains have caused outbreaks in poultry since 1990, resulting in serious economic losses in Asia and the Middle East (Das and Suares, 2007). The causal strains, however, non specific-pathogen-free chickens experimentally infected with H9N2 isolates from diseased chickens showed any clinical symptoms (Mo et al., 1998). Co-infection of H9N2 viruses with bacteria such as Staphylococcus aureus and Haemophilus paragallinarum or with attenuated coronavirus vaccine exacerbated the disease (Haghighat-Jahromi et al., 2008). The mentoring of H9N2 virus shedding in infected bird is a

valuable tool for evaluation of AI vaccine effectiveness, because an effective vaccine not only prevents clinical disease, but also reduces the viral shedding, thereby decreasing the likelihood of transmission of the virus to new susceptible hosts (Subtain et al., 2011).

The present study was conducted to study the pathogenesis, serology, hematolology, blood biochemical changes and the effectiveness of inactivated oil emulsion vaccine prepared from field isolate on viral shedding in broiler chickens challenged with an H9N2 low pathogenic avian influenza virus isolate using RT/ PCR.

## Material and methods

### H9N2 Virus and vaccine

H9N2 avian influenza virus was isolated and identified by using classical laboratory methods. Briefly, swab samples were collected from apparently healthy chickens by taking smears from the trachea and cloaca. The swab were placed in a transport medium. The samples were clarified by centrifugation and the supernatants were inoculated in 10-day-old specific-pathogen-free chicken embryonated eggs via the allantoic sac route. The eggs were further incubated for 4 days. The allantoic fluids of the embryos were collected and subjected to hemagglutination and hemagglutination inhibition tests with H9N2 antisera obtained from from Intervet international (Inter. B. V. Boxmeer, Holland). Viruses were passaged twice and then titrated in 10-day-old SPF embryonating eggs to determine the 50% embryo infectious dose titer (EID50) calculated by the Reed and Muench .Virus was diluted with sterile phosphate buffered saline (PBS, pH 7.4) to adjust the amount of inoculum to 1 x106 EID50 per 0.1 ml per bird. 0.1 ml of undiluted virus per bird was given. An inactivated oil emulsion commercial H9N2 vaccine used in this experiment was supplied from local agencies.

### Chickens

One hundred and fifty one-day-old commercial broiler chicks were randomly divided into six groups including five experimental and one control group (25 chicks in each group) Feed and water were available adlibitum.

**Table (1):Experimental design used for Evaluation of H9N2 inactivated oil emulsion vaccine**

| Group No. | No. of birds | Vaccine regime | | | Challeng at 30-day of age | Assessment of protection |
|---|---|---|---|---|---|---|
| | | Type | Age/ days | Dose/bird | | |
| 1 | 25 | H9N2 | 10 | 0.5 S/C | ++ | 1-Seroconversion HI test.<br>2-Monitoring virus shedding 2,4,6 days post challenge using RT/PCR<br>3- Hematological parameters<br>4- Blood biochemical parameters. |
| 2 | 25 | | | | --- | |
| 3 | 25 | H9N2 | 10 | 0.7 S/C | ++ | |
| 4 | 25 | | | | --- | |
| 5 | 25 | --- | ---- | ---- | ++ | |
| 6 | 25 | ---- | ---- | ---- | --- | |

**RNA extraction and RT/PCR**

Total RNA from cloacal and tracheal swabs collected from vaccinated challenged chickens from groups 1, 3 and from non vaccinated non challenged group using RNA extraction kit (Viral gene spin TM viral RNA extraction kit), according to the manufacturer's instructions. The cDNA was synthesized followed by amplification of a 200 a specific fragment using the forward primer 5`-CTYCACACAGARCACAATGG-3` and the reverse primer 5`-GTCACACTTGTTGTRTC -3`. After amplification, PCR product was subjected to electrophoresis on 2% agarose gels and stained with ethidium bromide.

**Haemagglutination inhibition test**

Sera were examined for HA-specific antibodies by HI test according to OIE manual (OIE, 2005) using either the commercial H9N2 (A/chicken/Mexico/232/94/CPA) for avian influenza A subtype H9 (Inter. B. V. Boxmeer, Holland). Serial two-fold serum dilutions in PBS were subsequently mixed with equal volumes (25 µl) containing 4 haemagglutinating units (HAU) of the virus. 25 µl of washed chicken red blood cells was added and incubated for 40 min at room temperature. The HI titres were determined as reciprocals of highest serum dilutions in which inhibition of haemagglutination was observed. Know positive AIV antisera were obtained from Intervet international (Inter.B. V. Boxmeer, Holland).

**Blood samples**

Blood samples were obtained from shank vein at 21 and 35 day old age. Each Blood sample was divided into two portions. The first one was placed into tubes containing EDTA for evaluation of hematological parameters. The second portion was obtained in

plain centrifuge tubes allowed to clot, centrifuged at 3000 rpm for 15 minutes for separation of serum for determination of the blood biochemical parameters.

**Hematological studies**

The evaluated hematological parameters in the study included estimation of the packed cell volume (PCV) according to Campbell and Coles (1986), Hemoglobin concentration (Hb) according to Campbell (1988), Erythrocyte and Leukocyte count (RBCs & WBCs) according to Natt and Herrick (1952), Thrombocyte count according to Campbell (1995) and Differential Leukocytic count (DLC) according to Mulley (1979).

**Blood biochemical parameters**

The evaluated biochemical parameters in the study included estimation of serum alanine aminotransferase activity (ALT) and aspartat aminotransferase activity (AST) according to Reitman and Frankel (1957), Serum uric acid according to Caraway (1963), Serum total proteins (TP) according to Henry et al. (1978), serum albumin according to Doumas (1971), and serum globulins and albumin : globulin ratio according to Benjamin (1978).

**Statistical analysis**

All data were presented as mean ± standard error (SE) and were subjected to analysis of variance in one and two way (ANOVA) test according to Snedecore and Cochran (1969). Treatments means were compared by the least significant difference test (LSD) at 0.05 level of probability.

**Results**

The geometrical mean of HI titers log.2 in chicken vaccinated with inactivated oil emulsion H9N2 at 0.5ml/bird were 2.8, 3.2 and 3.8 at 2, 3 and 4 weeks post challenge, respectively. The HI titers were 3, 4.2 and 4 at 2, 3 and 4 weeks post challenge, respectively in chicken vaccinated with 0.7 ml/bird, versus o.4 in non vaccinated non challenged chicken at 4 weeks after challenge (table 2).

**Table (2): Serological response of broiler chickens following vaccination with H9N2 oil inactivated vaccine at 10 days of age with 0.5 or 0.7ml/bird subcutaneously**

| Group treatment | Vaccinal regime | | | G.M. of HI titers log.2 at weeks post challenge | | |
|---|---|---|---|---|---|---|
| | Type | Age/days | Dose | 2 w. | 3 w. | 4 w. |
| Vaccinated challenged | H9N2 | 10 | 0.5 | 2.8 | 3.2 | 3.8 |
| Vaccinated challenged | H9N2 | 10 | 0.7 | 3 | 4.2 | 4 |
| Non Vaccinated non challenged | ---- | --- | --- | --- | ---- | 0.4 |

The geometrical mean of HI titers log.2 in chickens vaccinated with inactivated oil emulsion H9N2 at 0.5 or 0.7 ml/bird were (3.8 and 4), respectively at 37 days of age, while were 4.5 and 4.8 in H9N2 inactivated oil emulsion vaccinated with 0.5 or 0.7 ml/bird at 7 days post challenge with H9N2 field isolate, versus o.4 in non vaccinated non challenged chicken at 37 days of age and 3 in non vaccinated challenged group (table 3).

**Table (3): Serological response following vaccination with 0.5 or 0.7 of H9N2 oil inactivated vaccine at 10 days of age and challenged at 30 days of age with H9N2 local field isolate**

| Group treatment | Vaccinal regime | | | G.M. of HI titers log.2at 7D. post challenge |
|---|---|---|---|---|
| | Type | Age/days | Dose | |
| Vaccinated non challenged | H9N2 | 10 | 0.5 | 3.8 |
| Vaccinated challenged | H9N2 | 10 | 0.5 | 4.5 |
| Vaccinated non challenged | H9N2 | 10 | 0.7 | 4 |
| Vaccinated challenged | H9N2 | 10 | 0.7 | 4.8 |
| Non Vacc.challenged | ---- | --- | --- | 3 |
| Non treated | --- | --- | ---- | 0.4 |

**Results of shedding of H9N2 virus 2, 4 and 7 days post challenge in chickens vaccinated with 0.5 or 0.7 ml/bird of H9N2 inactivated oil emulsion vaccine**

The shedding of the H9N2 were detected using RT/PCR collected cloacal and tracheal swabs from vaccinated challenged and non vaccinated challenged groups at 2, 4 and 7 days post challenge (table 4) . All cloacal swabs collected at 2, 4 and 7 days post challenge from vaccinated chickens with 0.5 or 0.7 ml/bird show negative for virus detection. While tracheal swabs of the same groups showed negative reaction at 2 and 4 days post challenge, but it gave positive reactions at 7 days of challenge. In group non vaccinated challenged shown positive reaction on 1/3 of collected tracheal swabs at 2 and 4 days with negative reaction in cloacal swabs, while at 7 days post challenge all collected tracheal swabs showed positive reaction and only 1/3 of the cloacal swabs showed positive reaction.

**Table(4):Determination of the degree of protection of broiler chicken following vaccination with 0.5 or 0.7ml/bird of oil inactivated H9N2 vaccine and challenge at 30-days of age with H9N2 virus by monitoring the H9N2 replication and shedding in vaccinated challenged groups**

| Group treatment | Vaccinal regime | | | Assessment of protection | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | type | age | dose | 2d.post chall. | | | | 4 d.post chall | | | | 7-d.post chall | | | |
| | | | | Tracheal swabs | | Cloacal swabs | | Tracheal swabs | | Cloacal swabs | | Tracheal swabs | | Cloacal swabs | |
| | | | | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| Vaccinated chall. | H9N2 | 10 | 0.5 | 0/3 | 0% | 0/3 | 0% | 0/3 | 0% | 0/3 | 0% | 3/3 | 100% | 0/3 | 0% |
| Vaccinated Chall | H9N2 | 10 | 0.7 | 0/3 | 0% | 0/3 | 0% | 0/3 | 0% | 0/3 | 0% | 3/3 | 100% | 0/3 | 0% |
| Non Vacc. chall. | --- | --- | -- | 1/3 | 33.3% | 0/3 | 0/3 | 1/3 | 33.3% | 0/3 | 0% | 3/3 | 100% | 1/3 | 33.3 |
| Non treated | --- | ---- | -- | 0/3 | 0% | 0/3 | 0% | 0/3 | 0% | 0/3 | 0% | 0/3 | 0% | 0/3 | 0% |

**The effect of avian influenza virus H9N2 on hematological parameters**

According to table 5**,** there was a significant decrease of packed cell volume (PCV), hemoglobin (Hb) and red blood cells (RBCs) in non vaccinated challenged group at 35 day old age when compared with control group and other groups. There was no significant change of PCV, Hb & RBCs in vaccinated challenged group (H9N2 0.5 ml) and vaccinated challenged group (H9N2 0.7 ml) when compared with control group. There was no significant change of thrombocytes count in all groups even at 21 day or 35 day old age when compared with each others or with control group.

**Table (5): The effect of avian influenza virus H9N2 on hematological parameters**

| Group treatment | PCV | | Hb | | RBCs | | Thrombocytes | |
|---|---|---|---|---|---|---|---|---|
| | Day-old age | | Day-old age | | Day-old age | | Day-old age | |
| | 21 | 35 | 21 | 35 | 21 | 35 | 21 | 35 |
| Vaccinated H9N2 (0.5) challenged | 26.00±0.57 aA | 25.33 ±0.66 aA | 11.03± 0.64 aA | 10.54± 0.32 aA | 2.04±0.10 aA | 2.04±0.13 aA | 22.16± 0.61 aA | 22.16± 0.614 aA |
| Vaccinated H9N2 (0.5) non challenged | 25.33±0.88 aA | 25.33 ±0.88 aA | 10.41± 0.44 aA | 9.68±0.32 aA | 2.14±0.15 aA | 1.97±0.03 aA | 22.40± 0.40 aA | 21.93± 0.61 aA |
| Vaccinated H9N2 (0.7) challenged | 25.00±0.00 aA | 25.33 ±0.33 aA | 10.54± 1.06 aA | 11.03± 0.76 aA | 2.08±0.08 aA | 2.07±0.13 aA | 20.76± 0.23 aA | 21.46± 0.46 aA |
| Vaccinated H9N2 (0.7) non challenged | 25.00±0.00 aA | 25.33 ±0.33 aA | 10.41± 0.64 aA | 10.66± 0.76 aA | 2.06±0.10 a | 2.02±0.12 aA | 22.16± 0.23 aA | 22.40± 0.40 aA |
| Non vaccinated challenged | 26.00±0.57 aA | 21.66 ±0.88 bB | 11.52± 0.32 aA | 7.80±0.19 bB | 2.11±0.09 aA | 1.63±0.00 bB | 21.46± 0.84 aA | 21.93± 0.61 aA |
| Non vaccinated non challenged (control) | 26.00±0.57 aA | ±0.88 25.33 aA | 11.52± 0.88 aA | 11.27± 0.44 aA | 2.17±0.12 aA | 2.17±0.09 aA | 22.16± 1.01 aA | 21.93± 0.46 aA |

PCV: Packed cell volume.
Hb: Hemoglobin. RBCs: Red blood cells.
Values are means ± standard errors.
Means in a column without a common small letter differ significantly (P<0.05).
Means in a row without a common capital letter differ significantly (P<0.05).

According to table 6, there was a significant decrease of total leukocytes and lymphocytes in vaccinated challenged group (H9N2 0.5 ml), vaccinated challenged group (H9N2 0.7 ml) and non vaccinated challenged group at 35 day old age when compared with control group and other groups. There was no significant change of heterophils, monocytes, esinophils and basophiles in all groups when compared with each others or with control group.

**Table (6): The effect of avian influenza virus H9N2 on hematological parameters**

| Group treatment | WBCs | | Hterophils | | Lymphocytes | | Monocytes | | Esinophils | | Basophiles | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Day-old age | | Day-old age | | Day-old age | | Day-old age | | Day-old age | | Day-old age | |
| | 21 | 35 | 21 | 35 | 21 | 35 | 21 | 35 | 21 | 35 | 21 | 35 |
| Vaccinated H9N2 (0.5) challenged | 17.33±0.66 aA | 13.66±0.33 bB | 5.95±0.82 aA | 4.57±0.14 aA | 10.49±0.12 aA | 8.27±0.31 bB | 0.85±0.07 aA | 0.82±0.02 aA | 0.03±0.03 aA | 0.00±.0000 aA | 0.00±0.00 aA | 0.00±0.00 aA |
| Vaccinated H9N2 (0.5) non challenged | 17.00±1.00 aA | 16.66±0.88 aA | 5.42±0.99 aA | 5.48±1.12 aA | 10.71±0.04 aA | 10.28±0.25 aA | 0.86±0.04 aA | 0.84±0.03 aA | 0.00±0.00 aA | 0.05±0.05 aA | 0.00±0.00 aA | 0.00±0.00 aA |
| Vaccinated H9N2 (0.7) challenged | 17.00±0.57 aA | 14.00±0.57 bB | 5.69±0.63 aA | 4.94±0.70 aA | 10.46±0.19 aA | 8.22±0.21 bB | 0.84±0.06 aA | 0.83±0.03 aA | 0.00±0.00 aA | 0.00±0.00 aA | 0.00±0.00 aA | 0.00±0.00 aA |
| Vaccinated H9N2 (0.7) non challenged | 17.33±0.66 aA | 17.00±0.57 aA | 5.71±0.73 aA | 5.70±0.59 aA | 10.74±0.02 aA | 10.41±0.06 aA | 0.87±0.05 aA | 0.83±0.04 aA | 0.00±0.00 aA | 0.04±0.04 aA | 0.00±0.00 aA | 0.00±0.00 aA |
| Non vaccinated challenged | 16.66±0.66 aA | 13.66±0.33 bB | 5.12±0.70 aA | 4.35±0.13 aA | 10.69±0.16 aA | 8.49±0.30 bB | 0.84±0.03 aA | 0.82±0.03 aA | 0.00±0.00 aA | 0.00±0.00 aA | 0.00±0.00 aA | 0.00±0.00 aA |
| Non vaccinated non challenged (control) | 17.66±0.33 aA | 17.00±0.57 aA | 6.00±0.13 aA | 5.58±0.65 aA | 10.83±0.14 aA | 10.50±0.13 aA | 0.83±0.05 aA | 0.85±0.03 aA | 0.00±0.00 aA | 0.05±0.05 aA | 0.00±0.00 aA | 0.00±0.00 aA |

WBCs: White blood cells.
Values are means ± standard errors.
Means in a column without a common small letter differ significantly (P<0.05).
Means in a row without a common capital letter differ significantly (P<0.05).

**The effect of avian influenza virus H9N2 on blood biochemical parameters**

According to table 7, there was a significant elevation of serum alanin aminotransferase (ALT) and serum aspartat aminotransferase (AST) enzymes in non vaccinated challenged group at 35 day old age when compared with control group or with other groups. Also in non

vaccinated challenged group, there was a significant decrease of total protein and total globulin at 35 day old age when compared with control group and other groups. On the other hand, there was no significant change of albumin in all groups when compared with each other or with control group even at 21 day or 35 day old age. The study also revealed that, a significant increase of albumin/globulin ratio was noticed in non vaccinated challenged group at 35 day old age when compared with control group and other groups. Moreover, there was a significant elevation of serum uric acid in vaccinated challenged group (H9N2 0.5 ml), vaccinated challenged group (H9N2 0.7 ml) and non vaccinated challenged group at 35 day old age when compared with control group and other groups.

**Table (7): The effect of avian influenza virus H9N2 on blood biochemical parameters**

| Group treatment | ALT | | AST | | Total protein | | Albumin | | Globulin | | A/G ratio | | Uric acid | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Day-old age | | Day-old age | | Day-old age | | Day-old age | | Day-old age | | Day-old age | | Day-old age | |
| | 21 | 35 | 21 | 35 | 21 | 35 | 21 | 35 | 21 | 35 | 21 | 35 | 21 | 35 |
| Vaccinated H9N2 (0.5) challenged | 6.66± 0.05 bB | 7.20± 0.27 bB | 10.94± 0.49 bB | 10.93± 0.38 bB | 3.46± 0.21 aA | 3.65± 0.28 aA | 1.58± 0.36 aA | 1.54± 0.03 aA | 1.88± 0.19 aA | 2.11± 0.32 aA | 0.89± 0.26 bB | 0.77± 0.15 bB | 8.49± 0.73 bB | 16.37± 0.58 aA |
| Vaccinated H9N2 (0.5) non challenged | 6.40± 0.09 bB | 6.82± 0.28 bB | 10.32± 0.18 bB | 11.05± 0.43 bB | 3.56± 0.17 aA | 3.83± 0.17 aA | 1.34± 0.21 aA | 1.52± 0.06 aA | 2.22± 0.14 aA | 2.31± 0.12 aA | 0.61± 0.12 bB | 0.66± 0.02 bB | 10.23± 1.15 bB | 9.70± 0.38 bB |
| Vaccinated H9N2 (0.7) challenged | 6.98± 0.50 bB | 7.04± 0.09 bB | 10.85± 0.46 bB | 10.85± 0.10 bB | 3.41± 0.08 aA | 3.58± 0.23 aA | 1.50± 0.12 aA | 1.55± 0.01 aA | 1.91± 0.17 aA | 2.03± 0.23 aA | 0.80± 0.13 bB | 0.78± 0.08 bB | 8.05± 0.44 bB | 15.87± 1.67 aA |
| Vaccinated H9N2 (0.7) non challenged | 6.88± 0.27 bB | 7.20± 0.40 bB | 10.73± 0.30 bB | 10.95± 0.56 bB | 3.46± 0.13 aA | 3.71± 0.30 aA | 1.55± 0.15 aA | 1.54± 0.06 aA | 1.91± 0.25 aA | 2.17± 0.30 aA | 0.86± 0.21 bB | 0.74± 0.12 bB | 8.25± 2.18 bB | 9.08± 0.77 bB |
| Non vaccinated challenged | 6.40± 0.18 bB | 8.78± 0.33 aA | 10.73± 0.28 bB | 12.95± 0.34 aA | 3.70± 0.19 aA | 2.58± 0.18 bB | 1.71± 0.12 aA | 1.54± 0.07 aA | 1.99± 0.12 aA | 1.03± 0.11 bB | 086± 0.06 bB | 1.51± 0.09 aA | 8.36± 1.69 bB | 17.48± 1.40 aA |
| Non vaccinated non challenged (control) | 6.68± 0.15 bB | 6.89± 0.15 bB | 10.77± 0.29 bB | 10.66± 0.38 bB | 3.78± 0.15 aA | 3.67± 0.45 aA | 1.64± 0.18 aA | 1.55± 0.04 aA | 2.13± 0.28 aA | 2.11± 0.41 aA | 0.81± 0.17 bB | 0.78± 0.11 bB | 9.83± 2.57 bB | 11.32± 0.52 bB |

ALT: Alanin aminotransferase AST: aspartate aminotransferase.
A/G: Albumin/globulin ratio. Values are means ± standard errors.
Means in a column without a common small letter differ significantly (P<0.05).
Means in a row without a common capital letter differ significantly (P<0.05).

**Discussion**

The results of the present study indicated that there is very good antibodies response against H9N2 after single vaccination with oil emulsion inactivated vaccine which agreed with Lee et al., (2011) who mentioned that although a single administration of oil-based inactivated H9N2 LPAI vaccine is very immunogenic and highly protective in laboratory trials using SPF chickens, use of that vaccine in broiler breeder farms induced poor antibody response. The results of the H9N2 AIV shedding pattern revealed that there is demarked reduction in virus shedding especially in early tracheal shedding and late cloacal shedding.

Regarding the hematological and blood biochemical changes, it included evaluation of packed cell volume (PCV); hemoglobin (Hb); red blood cells count (RBCs); thrombocytes; white blood cells count (WBCs) and differential leukocytic count (DLC), also through investigation of blood biochemical parameters that included evaluation of liver enzymes, serum alanin aminotransferase and aspatat aminotransferase; total protein; albumin; globulin; albumin to globulin ratio and uric acid.

The present study according to table 1 revealed that non vaccinated chickens challenged with H9N2 virus showed anemia at 35 day old age when compared with control group and other groups indicated by presence of a significant decrease of packed cell volume (PCV), hemoglobin (Hb) and red blood cells (RBCs) and that supported by Barbour et al. (2006) who Found that 13 out of 14 randomly selected commercial layers in flock infected H9N2 antigen had hematocrite values $\leq$ 27%, an indicator of anemia, also suggested that anew regimen should be introduced to increase iron levels in the feed, or soluble formulas in drinking water to help in homeostasis of red blood cells in infected flocks manifesting hemorrhagic lesions. Anemia could be attributed to decrease in feed consumption due to H9N2 virus infection, could be the effect of viral infection on pancreatic tissue which results in decrease production of pancreatic enzymes essential for efficient digestion (Shinya et al., 1995; Silvano et al., 1997 and Vasfi Marandi et al., 2002). Also Morales Jr et al. (2009) reported that chickens and commercial turkeys infected with H4, H6, and H9 type low pathogenic avian influenza viruses (LPAI) produced clinical signs ranged from no clinical signs to moderate depression, decreased activity, and decreased food and water consumption. Anemia may be attributed to hemorrhage as Pazani et al. (2008) reported petechial to ecchymotic hemorrhages in spleen, bursa of fabricious and thymus.

The present study according to table 2 investigated that non vaccinated and vaccinated chickens challenged with H9N2 virus showed a significant decrease of total leukocytes and

lymphocytes at 35 day old age when compared with control group and other groups. The decrease in leukocytes could be attributed to H9N2 infection or secondary infections and supported by Naeem et al. (1999) who indicated that avian influenza virus (AIV) serotype H9N2 although it does not fall under the definition of highly pathogenic avian Influenza (HPAI) viruses, it has caused severe infection in broilers, layers and broiler breeders in various countries, also Bano et al. (2003) indicated that H9N2 subtype of AIV as a nonpathogenic virus can cause a severe infection in field condition in presence of opportunist secondary pathogens and Subtain et al. (2011) also observed that birds inoculated at 31$^{st}$ day of age with H9 virus showed histopathological lesions such as deciliation, congestion and infiltration of leukocytes in trachea, lung, kidney and liver. The reduction of white blood cell count also may be due to reduced numbers of lymphocytes (McNulty, 1991). Lymphopenia supported with studies of Morales Jr et al. (2009) and Pazani et al. (2008) who observed that LPAI cause lymphocyte depletion and necrosis or apoptosis of lymphocytes in the cloacal bursa, thymus and other areas with lymphocyte accumulations. There are two mechanisms by which nucleated eukaryotic cells die: necrosis and apoptosis (Wyllie et al., 1980, and Duvall and Wyllie, 1986), necrosis is considered as a pathological reaction that occurs in response to major disturbances in the cellular environment, such as a lytic viral infection, while apoptosis, on the other hand, is considered as a physiological process that is part of homeostatic regulation during normal tissue turnover **(**Wyllie et al., 1980). Also Swayne and Halverson (2003) indicated that in experimental studies in mallard Ducks, LPAI virus infections suppressed T cell function. Immunosuppression of chickens due to H9N2 infection as well as bacterial co infection such as M. gallisepticum and E. coli might increase the pathogenicity of H9N2 infection (Callan et al., 1997). Park et al. (2011) stated that challenge studies revealed that most of the novel H9N2 genotypes were able to replicate in H9N2-vaccinated birds, suggesting that commercial vaccine (Ck/Korea/01310/01) does not provide full or cross-protection against infection from these newly emergent H9N2 viruses under experimental conditions.

According to table 3 the study revealed that non vaccinated chickens challenged with H9N2 virus showed a significant elevation of serum alanin aminotransferase (ALT) and serum aspartat aminotransferase (AST) enzymes at 35 day old age, also a significant decrease of total protein and total globulin at 35 day old age when compared with control group and other groups. The elevation of liver enzymes besides hypoproteinemia and hypoglobuliemia indicate liver affection supported by Pazani et al. (2008) who observed at necropsy of H9N2 infected chickens, carcasses were congested and severe congestion was noticed in

visceral organs especially the liver. Also Subtain et al. (2011) observed that birds inoculated at 31st day of age with H9 virus showed histopathological lesions such as deciliation, congestion and infiltration of leukocytes in liver. Hypoproteinemia and hypoglobuliemia may be attributed to liver damage because liver is the major organ in production of proteins such as albumin and globulins (Sastry, 1983). However, hypoproteinemia may be associated with chronic renal disease or hepatic disease, mal nutrition, mal absorption or chronic blood loss (Campbell and Coles, 1986). A significant increase of albumin/globulin ratio that noticed in non vaccinated challenged group at 35 day old age when compared with control group and other groups may be attributed to hypoglobulinemia, that may be attributed to liver damage because liver is the major organ in production of proteins such as albumin and globulin (Sastry, 1983).

The present investigation also revealed a significant elevation of serum uric acid in non vaccinated and vaccinated challenged groups at 35 day old age when compared with control group could be supported by Swanye & Slemons (1998); Subtain et al. (2011) and Hablolvarid et al. 2003 who indicated that the main target organ of H9 virus is kidney where lesions are more pronounced. Also Pazani et al. (2008) observed that kidneys of chickens infected with H9N2 virus were severely swollen with urate deposition. Uric acid is the end product of nitrogen metabolism and is the major nitrogenous component excreted by fowl and is the primary catabolic product of protein, nonprotein, and purines in birds (Campbell and Coles, 1986). This elevation of uric acid (Hyperuricemia) may be attributed to dehydration, starvation, gout, massive tissue destruction and renal disease as a result of reduced tubular excretion (Rivetz et al., 1977).

Although the present study stated that H9N2 caused alteration in hematological and blood biochemical parameters beside immunosuppression, the vaccination with 0.7ml /bird is better than vaccination with 0.5ml/bird due to the vaccination with 0.7ml/ bird give higher HI titer than the vaccination with 0.5ml /bird.

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