Special Issue:
Emerging and Re-emerging Animal Health Challenges in Low and Middle-Income Countries

Comparison of the Immune Response to Two Different Vaccination Programs in Broiler Chickens

Mohammed Salih Obaid Al-Mansouri, Hayder Abd Al-Emier Almremdhy*

Department of Pathology and Poultry Diseases, Collage of Veterinary Medicine, Al-Qasim Green University, Babylon 51013, Iraq.

Abstract | One of the approved methods for controlling viral endemic diseases, which cause huge economic losses, is the use of vaccines to protect birds against these diseases. This study aimed to evaluate the immune response to two vaccination programs commonly used in Iraq. To achieve this goal, one-day-old Rose 308 broiler chicks were brought in and divided into three groups. The first group was vaccinated in the hatchery at one day old with an intensive vaccination program against avian influenza, Newcastle disease, infectious bronchitis, and infectious bursitis. The chicks in the second group were vaccinated with the same vaccination program as the first group, but on the fifth day of the chicks' life, with the addition of two doses of Newcastle disease vaccine at the age of 15 and 25. As for the chicks in the third group, they were left without vaccination as a control group. The humoral immunity of the chicks was measured at the age of 10, 20, and 30 days by ELISA and the cellular immunity was measured on the 35th day of the chicks' age by measuring the concentration of CD8 in the chicks' blood by ELISA. The ratio of heterozygous cells to lymphocytes was also calculated as an indicator of stress and the weight index of the lymphoid organs was also measured. The results showed no significant differences between the two vaccinated groups in terms of humoral immunity, cellular immunity, H/L ratio, and average weights of lymphoid organs, and there was a clear significant difference with the control group. we conclude that both vaccination programs induce a good cellular and humoral immune response. Furthermore, neither vaccination program induces stress in vaccinated chicks.

Keywords | Broiler, Vaccination program, CD8, Humoral immunity, H/L

Received | November 18, 2025; **Accepted** | October 24, 2025; **Published** | November 02, 2025
***Correspondence** | Mohammed Salih Obaid Al-Mansouri, Department of Pathology and Poultry Diseases, Collage of Veterinary Medicine, Al-Qasim Green University, Babylon 51013, Iraq; **Email:** mohamedsalih@vet.uoqasim.edu.iq
Citation | Al-Mansouri MSO, Almremdhy HAA-E (2025). Comparison of the immune response to two different vaccination programs in broiler chickens. J. Anim. Health Prod. 13(s1): 740-749.
DOI | https://dx.doi.org/10.17582/journal.jahp/2025/13.s1.740.749
ISSN (Online) | 2308-2801

INTRODUCTION

Poultry species, especially domestic chickens are one of the most widely reared animals for either their meat or eggs (Abdallah *et al*., 2023). The poultry industry is crucial to the human food supply due to its quick production and cost protein sources. It is also a key economic sector, particularly in developing nations (Nkukwana, 2018). To meet the growing demand for poultry protein, genetic selection for higher egg production and larger breast meat has led to problems with metabolic and structural diseases, compromising overall health and immune function in poultry, making them susceptible to disease (Korver, 2023). Environmental changes caused by climate change have exacerbated the emergence and spread of poultry diseases, rendering the immune system of poultry species unable to protect birds from infection and sudden death (Collett *et al*., 2020). On the other hand, the growth of this

industry faces a significant threat due to various diseases such as bacterial, fungal, viral and parasitic and that affect poultry health and productivity (Aruwa and Sabiu, 2024). To address these challenges, two primary strategies are being adopted: Biosecurity measures and vaccination practices. Vaccination plays a crucial role in combating viral diseases that affect birds. Vaccination involves administering antigenic molecules, which may be whole pathogens or parts thereof, to stimulate the host's immune response (Abdul-Cader *et al*., 2018). To manage or prevent the spread or outbreak of diseases, the poultry sector routinely vaccinates against some known and persistent poultry pathogens of specific economic importance that are common within its geographical boundaries which include viral diseases such as MD, ND, IB, IBD, ILT and FP, bacterial diseases such as fowl cholera and salmonella, and parasitic diseases such as coccidiosis (Isebe *et al*., 2014; Abdelaziz *et al*., 2024). Furthermore, many factors must take into consideration before choosing the right vaccination programs which includes the type of poultry production (commercial or rural), the organization of the industry (vertical integration),the bird species and age, the prevailing diseases situation, status of maternal immunity, status of immune system at the time of vaccination, vaccine availability, their types, storage, preparation and routes of vaccination, intervals and interference between vaccines, antigenic difference between field virus and the vaccine, immunogenicity of vaccine strain (Abdallah *et al*., 2023; Ali and Rabee, 2024; Muhammed and Rabee, 2024). On other hand, Almremdhy *et al*., (2024) referred to that immunosuppression agents specially the stress factors and mycotoxins must be considered when vaccination programs design. While as Aljuhaishi and Albawi (2024) pointed to that found of antibiotics in water or feed pre or post vaccination effect in poultry immune response. The main aim of using vaccines is to enhance immunity leading to resistance, prevention or avoidance of the spread of infectious diseases leading to healthier flocks and improved productivity also enhances consumer confidence in poultry products by ensuring food safety (Collett *et al*., 2020). Vaccination can be performed at the hatchery or at the production units and the type of vaccine to be used as well as the mode of application has to be critically examined to ensure no errors or harmful effects on both the animals as well as the farmer (Abdallah *et al*., 2023). All the different methods of vaccine administrations have their advantages and disadvantages. Vaccination does not guarantee immunity as there are several other factors such as stress, maternal immunity, handling and storage that may inhibit or cause immunosuppression (Abdallah *et al*., 2023).

This research seeks to compare the immune responses of broiler chicks vaccinated at one day old with those experiencing a vaccination programme spread throughout their rearing period.

MATERIALS AND METHODS

This experiment was conducted at Al-Qasim Green University, College of Veterinary Medicine, Department of pathology and poultry diseases. A total of two hundred one-day-old broiler chicks of Ross 308 hybrid were brought from a local hatchery in Babylon province for conducting this experiment. The chicks were reared on sawdust litter in separate pens after being equipped with all broiler breeding equipment for a period of 35 days in good hygienic conditions. The chicks were fed ad-libitum on stander balanced nutrient ration commensurate with the age of the chicks, in addition to providing clean drinking water throughout the experiment. The birds were offered a starter diet from 1 to 20 days and finisher diet from day 21 to day 35 as out lined by national research council requirement (Council, 1994). On the first day of the experiment, twenty chicks were randomly selected to be sacrificed to collect blood samples for the purpose of serum separation to estimate the maternal antibody titer (MAT) against Newcastle Disease (NDV), Avian Influenza (AI), Infectious Bronchitis (IB) and infectious bursal disease (IBD) using the indirect enzyme-linked immunosorbent assay (ELISA) BIOTEK® through used specific kits for each them as explained in (Table 3) according to the manufacturer's instructions. The remaining 180 chicks were randomly divided into three groups with 60 chicks in each group (1, 2, 3), with three replicates (n = 20) for each group. The chicks in G1 were vaccinated viral disease (ND, AI, IB, IBD) at one day of age with an intensive vaccination program as shown in (Figure 1), while the chicks in G2 vaccinated against same disease in G1 but at different time as shown in (Figure 2). The chicks in G3 left without vaccination as control groups.

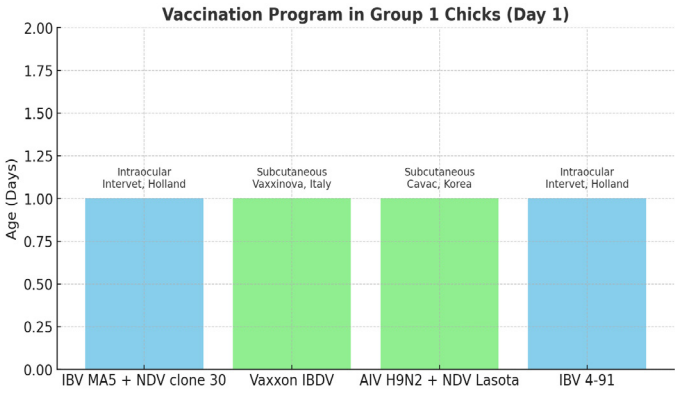

Figure 1: The vaccination program applied in group 1 chicks on day 1 of age, including four essential vaccines against respiratory and immunosuppressive diseases (IBV, NDV, IBDV, and AIV H9N2) administered via subcutaneous injection and intraocular routes to ensure early and broad immunity.

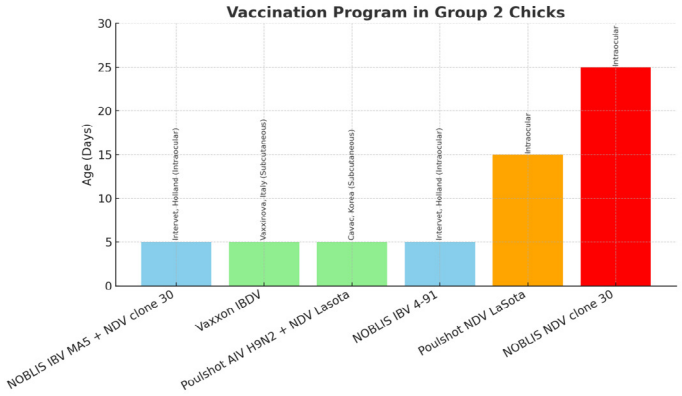

Figure 2: Vaccination program applied in chicks of Group 2, showing different vaccines administered at specific ages (Day 5, Day 15, and Day 25) through various routes (subcutaneous and intraocular) from different manufacturers.

Ten blood samples were collected from the jugular vein of chickens in each group after being randomly selected on the 10th, 20th and 30th day of chicks' life for serum separation to estimate the humoral immune response against ND, AI, IB and IB diseases using indirect ELISA test by using special kits for each one according to the manufacturer's instructions. at 35 days, blood samples (3ml) were collected from jugular vein then divided into two parts, once put in gel tube (without anticoagulant) to measured concentrations of CD8 molecules in serum, by competitive ELISA kit (chicken cluster of differentiation ELISA kits for measure cellular immune response according to manufacture instruction. While the other part of blood sample was put in K3EDTA tubes to estimate heterophils / lymphocytes (H/L) ratio by automated veterinary hematology analyzer BC-5000 vet five-part blood test medical CBC machine according to (Post *et al*., 2003). The lymphoid organs index (spleen, bursa of Fabicia and thymus) was also examined at the end of the experiment at 35 days of age. The living broiler chicks from all groups were weighted at the end of the experiment then the weights of bursa of fabricious, spleen, and thymus were detected after slaughter of the broilers and the lymphoid organs to body weight ratios were calculated by the equation: Organ weight in grams x 1000 / final body weight in grams (Sharma *et al*., 1989).

Statistical analyses

The collected numerical data were analyzed by using the SAS statistical program (SAS, 2020). Significant differences between the coefficients of the studied traits were found using Duncan's multiple range test (Duncan, 1955). A comprehensive evaluation of treatment effects on various immunological parameters in broiler chickens, as measured through ELISA antibody titers, hematological indices, and immune organ metrics. Statistical significance of differences was determined at two levels: *p < 0.05 and **p< 0.01, with post-hoc comparisons indicated by superscript letters (A, B, C).

RESULT AND DISCUSSION

Viral epidemics pose a significant threat to the poultry industry worldwide, negatively impacting poultry production performance, such as feed consumption, feed conversion ratio, body weight gain, and egg and meat quality. poultry in Iraq, are attacked by many of viral diseases most of them became endemic diseases like Newcastle disease, Avian Influenza, infectious bronchitis and Infectious Bursal disease (Almremdhy, 2014; Al-Shareef and Abawi, 2024; Faraj *et al*., 2024) these diseases cause great losses in most commercial flocks. In an effort to prevent infection with the above-mentioned viral pathogens, preventive measures for disease spread include mass vaccination, surveillance, and physical separation or preventive culling of infected birds (Chung *et al*., 2021; Colvero *et al*., 2018; Olukotun *et al*., 2018). Vaccination plays a crucial role in protecting poultry from viral diseases endemic in a particular geographical area. Based on this, vaccination programs are designed to significantly reduce the spread of the pathogens against which they have been vaccinated, as well as improve flock health and reduce economic losses resulting from these diseases (Saif *et al*., 2020). For that, this study aimed to evaluated immune response gained through applied two different vaccination programs, once include all vaccines administration for chicks at day one of their life in hatchery (intensive vaccination program) as explained in Table 1 while as, the other program include vaccines administration for chicks at different time of rearing period (Field vaccination programs) which prolonged 35 days. It is worth noting that both vaccination programs are commonly used in vaccinating chickens in Iraq to protect them against several viral diseases such as Newcastle disease, infectious bronchitis, avian influenza, and infectious bursal diseases, which cause huge economic losses. ELISA have been employed for the detection of antibodies against AI, ND, IB and IBD viruses due to ELISA technique is more accurate, sensitive and rapid as confirmed by (Alexander *et al*., 2004; Tabidi *et al*., 2004).

Humoral immune response against AI, ND, IB and IBD

The titers of maternal antibody titer (MAT) against AI, ND, IB and IBD viruses at day one old of broiler chickens which measure by indirect ELISA were 8440.6, 5579.2, 5471 and 6728.2, respectively as shown in Table 1.

Table 1: Explain ELISA maternal antibody titers of AI, ND, IB and IBD virus at day 1.

Virus	AI	ND	IB	IBD
Sample number	10	10	10	10
Titer	8440.6	5579.2	5471	6728.2

Table 2: ELISA antibodies titer against AIV, NDV, IBV and IBD at days (10,20 and 30) among experiment groups.

Traits	G1	G2	G3
No. of samples	**10**	**10**	**10**
ELIZA AI AB-10-DAY**	2078.4 ± 62.64 A	2089.5 ± 127.60 A	1456.0 ± 141.22 B
ELIZA ND AB-10-DAY**	3023.70 ± 339.47 A	2579.70 ± 357.60 AB	1862.70± 108.46 B
ELIZA IBV AB-10 DAY*	2659.40 ± 373.94B	4496.10 ± 644.503 A	1305.33 ± 191.844 C
ELIZA IBD AB-10-DAY*	3186.40 ± 248.31 AB	3357.20 ± 208.28 A	2533.80 ± 251.33 B
ELIZA AI AB-20DAY**	2981.2 ± 57.51 A	2836.9 ± 100.14 A	216.6 ± 19.41 B
ELIZA ND AB-20 DAY**	3518.70 ± 208.36 B	4063.30 ± 310.89 A	772.70 ± 59.41 C
ELIZA IBV AB-20 DAY*	3550.60 ± 234.93 B	5039.50 ± 315.218 A	468.800 ± 36.953 C
ELIZA IBD AB-20 DAY*	4233.60 ± 381.32 A	4403.80 ± 265.98 A	1543.70 ± 185.09 B
ELIZA AI AB-30 DAY**	4232.5 ± 227.57 A	4472.7 ± 579.26 A	49.0 ± 7.82 B
ELIZA ND AB-30 DAY*	4376.70 ± 296.93 B	5200.30 ± 241.12 A	231.50 ± 29.44 C
ELIZA IBV AB-30 DAY	3829.20 ± 387.37 B	5147.00 ± 443.542 A	211.500 ± 18.386 C
ELIZA IBD AB-30 DAY*	5412.10 ± 292.97 A	6047.60 ± 384.26 A	898.80 ± 60.01 B

NS: Non significant *: Significant differences at 5%. **: Significant differences at 1%.

When the parent flocks are pre-vaccinated several times with live and killed vaccines against the pathogens included in the vaccination program during the rearing period, this will result in a high concentration of IgY in her blood, which will be transferred to the blood of her chicks after hatching via the yolk sac (Gharaibeh and Mahmoud, 2013). The titer of IgY in chicks blood is proportional to the IgY titer in blood of their mothers (Hamal *et al*., 2006). Maternal antibodies play an important role in protecting chicks, especially during the first few weeks of life when their immune system is still not fully functional (Hamal *et al*., 2006). The level of maternal antibodies in the serum of unvaccinated chickens gradually decreases until it reaches an insignificant level on the twenty-first day of the chicks' life (Al-Shahery *et al*., 2008; Banu *et al*., 2009; Gharaibeh and Mahmoud, 2013; Magda *et al*., 2013; Deka *et al*., 2020). The result of these studies corresponded with present study result which found that antibodies titer in serum of chickens in G3 which unvaccinated against AI, ND, IB and IBD viruses declined gradually from one day old to reach undetectable titer in day 30 of age. Gharaibeh and Mahmoud (2013) confirmed that there are significant differences among half-lives of maternal antibody titers against certain pathogens. Where revealed the half-life estimates of maternal antibody titers were 4.2, 5.1, 3.9, 6.3d for AIV, IBDV, IBV and NDV, respectively.

The results of humoral immune response against AI, ND, IB, IBD viruses was estimated by indirect ELISA at days 10, 20, and 30. these results were summarized in Table 2.

The result of current study shows there are significant difference in level ($p \leq 0.01$) in ELISA antibodies titer against AIV, NDV, IBV and IBDV at days 10, 20, and 30 between vaccinated groups (G1 and G2) and unvaccinated group (G3) except against NDV at day 10 there isn't significant difference ELIZA antibodies titer between chicks in G2 and G3. At the same period, also, there isn't significant difference ELIZA antibodies titer against IBD between chicks in G1 and G3 as shown in Table 5. While as, there is non- significant difference in ELISA antibodies titer against AIV and IBDV at days 10, 20, and 30 between chickens in vaccinated groups (G1 and G2) indicating that the applied vaccination in G1 and G2 both enhanced the humoral immune response against AI and IBD as shown in Table 5. While as, there is a significant difference in ELISA antibodies titer against IB between chickens in G2 and G1 at days 10, 20, 30 as shown in Table 5. Also, there is a significant difference in ELISA antibodies titer against ND between chickens in G2 and G1 at days 20, 30 as shown in Table 5. The results of this study are consistent with those obtained by other studies (Magda *et al*., 2013; Anebo *et al*., 2014; Zhao *et al*., 2017; Al-Zuhariy, 2017; Cahyani *et al*., 2020; Mahamud *et al*., 2023); who indicated that vaccination of chickens with live vaccine, inactivated vaccine, or inactivated bivalent vaccine (NDV + AI) induces a safe and effective immune response against Newcastle disease and H9N2 avian influenza. Also, the results of present study agree with result obtain by (Chung *et al*., 2021b) who found that vaccination against ND+IB and IBD induce good immune response as well as preventing disease outbreaks and promoting health and productivity in broiler. The results of the current study differ from those of previous researchers (El-Khantour *et al*., 2021), who concluded that vaccination administered on the first day of the hatchery with influenza, Newcastle disease, bronchitis, and bursitis vaccines did not provide acceptable protection compared to the unvaccinated control group. This may explain the observed vaccination failure in the field. On other hand, the results of our study agree with the results obtained by (Amer *et al*., 2012; Talat *et al*., 2020), who found that vaccination at seven days of

age stimulates a better immune response in chicks than if they were vaccinated at one day of age, where, our results indicated there is a significant difference in level ($p \leq 0.01$) in ELISA antibodies titer against ND between chickens in G2 (vaccinated at older ages) and G1(vaccinated at one day of age) at days 20, 30. The significant difference in the humoral immune response between the chicks in the G2 at the expense of the G1 may be attributed to the decrease in maternal antibodies and their lack of interference with the immune response resulting from the vaccine. It may also be attributed to the booster doses of the live vaccine at 15 days of age and the other at 25 days of age against the Newcastle disease virus. Regarding humoral immunity against NDV (Mahamud *et al*., 2023) concluded that the inactivated Lasota vaccine against Newcastle disease virus (NDV) was able to generate a significant antibody response in chicks, 28 days after vaccination, provided the vaccine dose was 0.5 ml per bird. While as Khodayari and Feizi (2017) and Deka *et al*. (2020) found that vaccination of chicks with live or inactivated vaccines at 7 days of age induces significant humoral immune responses at 35 days of age.

These results is similar to the results obtained in the current study, where a high immune response against Newcastle disease virus was obtained in chicks at 30 days of age after they were vaccinated with inactivated vaccines at early ages. While, (Talib and Thwiny, 2023) found that the antibody titers against NDV were higher in the chickens vaccinated twice with a live vaccine administered by eye drop at 7th day of age and by drinking water at 21st day of age (live vaccine). induced the highest antibody levels in broiler. Regarding humoral immunity against avian influenza virus, (Raheel *et al*., 2024) concluded that the inactivated oil-emulsion avian influenza H9N2 vaccine rapidly and strongly stimulates innate and humoral immunity, and that this vaccine can contribute to protecting broiler chickens from early H9N2 infection. While as, Mirzaie *et al*. (2020) referred to antibody titers in the vaccinated farms did not reach the protective level until the end of the rearing period. For that, the results of current study may be concur with results obtained by Mirzaie *et al*. (2020) because in our study observed increased antibody titer against avian influenza H9N2 gradually until reach at higher titer at day 30 as shown in Table 5. However, the results of Allaoui *et al*. (2022) indicated that the H9N2 vaccine should be reserved for immunizing flocks of parent chickens because the level of antibodies in the chicks' serum remains insufficient and will not reach protective levels until the 50th day of the chicks' life, which is the slaughter date. through the Table 5, there is a significant difference in ELISA antibodies titer against IB between chickens in vaccinated groups (G2 and G1) and control group (G3) at days 10, 20, 30. Smialek *et al*. (2016), confirmed that vaccinating broiler chicks against infectious bronchitis using vaccines containing Ma5 and 4/91 strains simultaneously is an effective strategy to stimulate a good immune response. This result is completely similar to the results of the current study, where we note that there is a significant difference in the level of antibodies against infectious bronchitis virus serotype MA5+4/91 between the two vaccinated groups and the unvaccinated control group.

On other hand, the results of our study differed from those of (Saiada *et al*., 2019), who indicated that vaccination of chicks against infectious bronchitis virus on the first day of life elicits significantly lower systemic and membrane antibody responses compared to vaccination at later time points. Also, there is a significant difference in ELISA antibodies titer against IB between chickens in G2 and G1 at days 10, 20, 30. This can be attributed to the effect of maternal immunity, which neutralizes the vaccine virus, thus reducing the antibodies produced by active immunity. There is a significant difference ($p \leq 0.01$) in ELISA antibodies titer against IBD between chickens in G2 and control G3 at days 10, 20, 30. While as, there is not a significant difference ($p \leq 0.01$) between chickens in G1 and control G3 at day10 only, other period there are significant difference. This result similar with results obtain by (Muniz *et al*., 2018; Abou El-Fetouh *et al*., 2020; Isihak *et al*., 2021; Avdosieva *et al*., 2023; Wang and Bo, 2024) whom pointed to that vaccination broiler chickens at day one against IBD by the immune complex vaccine which works in the absence of or with different levels of passive antibodies can induce active immune response with a high level of protection against the disease. Sun *et al*. (2024) referred to the ability of vaccines to stimulate immune system of chickens to produce both humoral and cellular immune responses more quickly and effectively. On other hand, (Dalgaard et al., 2010) confirmed to that cell-mediated immunity plays a key role in protective immunity against viral infection. For that, in this study, CD8 has been measured by competitive ELISA by specific kits according to manufacture instruction also, this method was previously used by (Sheehan *et al*., 2025). These results were explained in Table 5. The results appeared significant different at level ($p \leq 0.05$) between CD8 concentration in G2 and CD8 concentration in G3 (Control Group). Whereas, there is non-significant different between CD8 concentration in G2 and G3, also, there is non-significant different between CD8 concentration in G1 and G3.

Table 3: Explain the concentrations of CD8 µg/ml in serum of chickens among experiment groups measured by competitive ELISA at day 35.

Traits	G1	G2	G3
No. of samples	10	10	10
Cons*	2290.82 ± 204.72 AB	3965.42 ± 917.54 A	1551.57 ± 113.23 B

NS: Non significant *: Significant differences at 5% . **: Significant differences at 1%.

This result may be attributed to the vaccination program for chicks in G2, which included the same vaccination program for chicks in G1, plus two doses of live attenuated Newcastle disease vaccine (LaSota after 15 days and Clone 30 after 25 days), which led to the stimulation of a strong cellular immune response that led to an increase in CD8+ cells. This staggered immunization schedule likely provided a more sustained antigen exposure and immune stimulation, promoting a stronger CD8+ T cell response. In contrast, G1 received all vaccines at 1 day, which might have led to less optimal immune priming. On the other hand, chicks in G2 were vaccinated in a staggered manner, which provided more sustained exposure to the antigen, leading to a stronger CD8+ T cell immune response. Meanwhile, chicks in G1 received all vaccines on the same day, which may have led to less cellular immunity. Saiada *et al.* (2019) showed that vaccinating chicks at 7 days of age stimulates the immune system of birds to produce more serum antibodies, helper T cells (CD3+CD4+) and cytotoxic T cells (CD3+CD8+) compared to the immune response of chicks when vaccinated at one day of age. He also indicated that the recruitment or differentiation of the CD4+, CD8+ and CD4+/CD8+ T cell populations increased in different sites of effective immunity with age.

Table 4: Explain heterophil / lymphocyte (H/L) ratio in blood of chickens among experimental groups measure by automated veterinary hematology analyzer BC-5000 at day 35.

Groups	G1	G2	G3
No. of samples	10	10	10
Heterophils	69.344±40.67A	41.79±17.01A	28.14±5.29A
Lymphocytes	106.756±27.61A	70.748±20.76A	51.21±2.25A
H/L	0.65±0.041A	0.59±0.07A	0.54±0.086A

NS: Nonsignificant *: Significant differences at 5% . **: Significant differences at 1%.

In this study the heterophil to lymphocyte (H/L) ratio was estimated as indicator of stress, the H/L measured by automated hematology analyzers. There is a significant difference among groups in the H/L ratio ($p < 0.01$). G2 had the lowest ratio, while as G1 recorded higher ratio followed by G3 as shown in Table 4. These results suggested reduced physiological stress and improved immune status in chickens in G2, a lower H/L ratio is generally associated with better immune competence. Leukocytes are immune-related cells that are involved in defense of the body against foreign materials and infections, killing virus-infected cells, and enhancing the antibody production (Olugbemi *et al.*, 2010; Salim et al., 2013). Heterophils are phagocytic lymphocytes and play a role in mediating acute inflammation response in poultry (Scanes, 2016). Lymphocytes are further divided into B lymphocytes and T lymphocytes. B lymphocytes are important in humoral immunity, whereas T lymphocytes trigger cell-mediated immunity (Chung *et al.*, 2019). The circulating levels of lymphocytes are affected by stress. In the case of vaccination, stress can be in the form of improper handling techniques or administration of vaccines, besides immune stress. Such stress can be worse in heat stress environments, which can cause a drastic decline in circulating lymphocytes, leading to immunosuppression (Krams *et al.*, 2012). The H/L ratio is used to measure stress, and could be a viable parameter to measure the health of vaccinated broilers (Chung *et al.*, 2020). For that H/L ratio used in this study as stress indicator due to vaccination. The H/L measured by automated hematology analyzers Post *et al.* (2003) asserted that automated blood analyzers can be used to measure absolute heterophil cells counts and are a valuable tool in stress-related research. In current study, there is no significant difference in H/L ratio among experiment groups but there is non-statistic difference recorded in G1 followed by G2 then G3 which had the lowest ratio, (0.65 ± 0.041, 0.59 ± 0.07 and 0.54 ± 0.086), respectively that may be attributed to effect of vaccine, this explanation corresponded with (Riad *et al.*, 2010) who observed there is a significant increase in H/L ratio in biological additives groups than control ones. This can also be attributed to the fact that the type of vaccine, time of vaccination, vaccine administration methods, and age of the chickens are important factors that may have an impact on the H/L ratio. This explanation corresponded with (Krams *et al.*, 2012; Gottstein *et al.*, 2015) whom referred to that improper handling of vaccination techniques or vaccine administration methods, as well as immune stress, are potential causes of stress to chickens which lead to increase of H/L ratio. On the other hand (Ojiezeh *et al.*, 2014; Chung *et al.*, 2021; Rabee and Abdulameer, 2018), pointed to that, there was a reduction of the H/L ratio with age of vaccinated chickens.

Table 5: Explain lymphoid organs indices among experiment groups at day 35.

Traits	G1	G2	G3
No. of samples	5	5	5
Bursa index*	0.2540± 0.008A	0.2500± 0.006AB	0.2240± 0.02B
Thymus index*	0.3160± 0.016AB	0.2900± 0.007B	0.2920± 0.006
Spleen index**	0.1680± 0.012BC	0.1960± 0.010AB	0.1360± 0.008C
Body weight (g)	2340	2344	2220

NS: Non significant *: Significant differences at 5% . **: Significant differences at 1%.

The results of relative weight of lymphoid organs index

(bursa, thymus, and spleen) were summarized in Table 5. In this table show there was no a significant difference at ($p \leq 0.05$) in relative weight bursa index and thymus index among G2, G1 and G3 while as, there is a significant difference at ($p \leq 0.05$) in relative weight of spleen index between G2 and G3. Lymphoid tissue in poultry is essential for the immune response and consists of central and peripheral components (Ratcliffe, 2006). The central components include the thymus which is the site of T-cell maturation and differentiation, contributing to cell-mediated immunity, and while as the other part of the central components is bursa of Fabricius, which plays significant roles in immunoglobulin synthesis and antibody production. The size and mass of the thymus, and bursa of Fabricius provide important insights into the maturation and structure of the immune system (Cheng *et al*., 2023; Park and Kim, 2014). They are most active during the first few weeks, peaking at 4 to 6 weeks before gradually regressing (Cooper et al., 1966). The spleen, a secondary immune organ, is essential for filtering blood and mounting immune responses. It serves as a blood filter, removes aged red blood cells and pathogens (Lillehoj, 2018). Abdel-Fattah *et al*. (2008) indicated that the relative weight of lymphoid organs is usually used to predict the immune status of the bird, while (Fasina *et al*., 2006), indicated that changes in the weight of lymphoid organs may indicate changes in the function of lymphoid organs, as an increase in the weight of lymphoid organs indicates an increased immune response due to natural infection or vaccination, While a decrease in the weight of lymphoid organs indicates a decreased immune response or immunosuppression. For that, the vaccine is able to enhance lymphocyte proliferation, and this can reflect in the weight of lymphoid organs, impacting on immune function and disease resistance ability. On the other hand, (Kabir *et al*., 2004; Makram *et al*., 2010) pointed out that it is important to keep in mind that low weight may not necessarily be associated with low lymphocyte production; therefore, it is necessary to relate this variable to other measures of immune status. The lack of significant differences between the vaccinated groups and the control group in this study can be attributed to the difference in chick weight. The results of this study differed from the results reached by (Talib and Thwiny, 2023) who found the higher values of lymphoid organs (Bursa of Fabricius, thymus and spleen) indices were in vaccinated groups compared to non-vaccinated groups, but there was no significant difference between vaccinated groups ($P < 0.05$) at day 35 of chickens age.

CONCLUSIONS

From the results of this study, we conclude that both vaccination programs induce a good cellular and humoral immune response. Furthermore, neither vaccination program induces stress in vaccinated chicks. Although the lymphoid organ weight index is a contributing indicator in chickens, it cannot be fully relied upon without reference to other evidence.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to the staff of the Department of Pathology and Poultry Diseases, College of Veterinary Medicine, Al-Qasim Green University, for their technical support and assistance during the course of this study.

NOVELTY STATEMENT

This study provides the first comparative evaluation of two commonly used vaccination programs in Iraq, demonstrating that both protocols stimulate similar levels of humoral and cellular immune responses in broiler chickens without inducing stress. These findings may guide poultry producers in selecting practical vaccination strategies suitable for local field conditions.

AUTHORS' CONTRIBUTIONS

Both authors contributed to the preparation of this manuscript, its final review, and its approval for publication.

Ethical statement

All study animal samples were treated and handled following the required biosafety and security protocols. Prior to commencing this study, the Ethics and Scientific Committee in the Department of Pathology and poultry diseases in the College of Veterinary Medicine at Al-Qasim Green University, Ministry of Higher Education and Scientific Research, Iraq, approved the research protocol (No.2650 on 21/10/2024). These guidelines for the Care and Use of Laboratory Animals and the specific guide for broiler chickens were fully adhered to throughout the research.

Generative AI and AI-assisted technology statement

We did not use AI technology for the current research, except Grammarly for Grammar and spell check.

Conflict of interest

The authors have declared no conflict of interest.

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