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Measurement of the ELISA antibody titers and histopathological changes in the lymphoid tissues of broiler chickens vaccinated with different vaccination programs

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Abstract
One of the approved methods for controlling viral diseases endemic in Iraq, 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 in 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 and the histopathological changes of lymphoid organs was also measured. The results showed no significant differences between the two vaccinated groups in terms of humoral immunity and histopathological changes of lymphoid organs, and there was a clear significant difference with the control group. we conclude that both vaccination programs induce a good humoral immune response. the vaccination programs (time of vaccination, type of vaccine, routs of administration) applied in poultry houses lead to histopathogical changes in lymphoid organ may be used in estimated immune response in chickens. Further future studies need on vaccination programs designs until reach to suitable program can protecting chickens against infectious diseases.

Keywords: Broiler, vaccination program, CD8, humoral immunity, avian influenza, Newcastle disease, IBD, IB

Introduction
The poultry industry is a vital sector that is important for providing human food due to its rapid production and the high nutritional value of its products, which has made it a major economic sector, especially in developing countries (Abdallah *et al*., 2023; Nkukwana, 2018) [1, 50]. On the other hand, the rapid growth of this industry is facing a major threat due to various diseases such as bacteria, fungi, viruses and parasites that affect the health and productivity of poultry (Aruwa & Sabiu, 2024) [14]. To address these challenges, two main strategies are being adopted: biosecurity measures and vaccination practices. Vaccination plays a crucial role in combating viral diseases affecting birds (Abdul-Cader *et al*., 2018) [3]. To prevent the spread of diseases among chicken flocks, chickens are routinely vaccinated against known pathogens endemic within the geographical boundaries of an area, which include viral diseases such as MD, ND, IB, IBD, ILT, and FP, bacterial diseases such as avian cholera and salmonella, and parasitic diseases such as coccidiosis (Abdelaziz *et al*., 2024; Isegbe *et al*., 2014) [2, 37].
Furthermore, many factors must take into consideration before choosing the right vaccination programs which includes the type of poultry production, 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 & Rabee, 2024; Muhammed & Rabee, 2024) [1, 5, 47].

On other hand, Almremdhy *et al*., (2024) [8] referred to that immunosuppression agents specially the stress factors and mycotoxins must be consider when vaccination programs designs. The main goal of using vaccines is to enhance immunity, which leads to increased resistance of chickens to diseases and prevents their spread, resulting in healthier flocks with high egg and white meat production that meet human needs (Collett *et al*., 2020) [23]. Vaccination can be done at different times, at day one in the hatchery facilities or at vary ages at the production units (Abdallah *et al*., 2023) [1].

This study aims to compare the immune responses of broiler chicks vaccinated at one day of age with those subjected to an extended vaccination program throughout their rearing period by measuring humoral and cellular immunity and studying the histopathological changes that appear in the lymphoid organs.

Materials and Methods

This experiment was conducted at Al-Qasim Green University, College of Veterinary Medicine, Department of Poultry Diseases. Two hundred one-day-old broiler chicks of the Ross 308 hybrid were brought from a local hatchery in Babylon Governorate for the 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 *et al*., 1994) [25].

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 (table 1), while the chicks in G2 vaccinated against same disease in G1 but at different time as shown in (table 2). The chicks in G3 left without vaccination as control groups.

Table 1: Explain vaccination program used in vaccinated chicks in group 1

Vaccine	Origin	Route of administration	Age of chicks
NOBLIS IBV MA5 Massachusetts (3.0 $\log_{10}$ EID50) + NDV clone 30 (6.0 $\log_{10}$ EID50)	Intervet-Holland	Intraocular	Day 1
Vaxxon IBDV ((1×$10^{2.5}$ EID50)	vaxxinova-Italy	Subcutaneously injection	Day 1
Poulshot AIV H9N2+ NDV Lasota (1×$10^{9.5}$ EID50)	Cavac-korea	Subcutaneously injection	Day 1
NOBLIS IBV 4-91 (3.6 $\log_{10}$ EID50)	Intervet-Holland	Intraocular	Day 1

Table 2: Explain vaccination program used in vaccinated chicks in group 2

Vaccine	Origin	Route of administration	Age of chicks
NOBLIS IBV MA5 Massachusetts (3.0 $\log_{10}$ EID50) + NDV clone 30 (6.0 $\log_{10}$ EID50)	Intervet-Holland	Intraocular	Day 5
Vaxxon IBDV ((1×$10^{2.5}$ EID50)	vaxxinova-Italy	Subcutaneously injection	Day 5
Poulshot AIV H9N2+ NDV Lasota (1×$10^{9.5}$ EID50)	Cavac-korea	Subcutaneously injection	Day 5
NOBLIS IBV 4-91 (3.6 $\log_{10}$ EID50)	Intervet-Holland	Intraocular	Day 5
Poulshot NDV LaSota (1×$10^{6.0}$ EID50)	Cavac-korea	Intraocular	Day 15
NOBLIS NDV clone 30 (1×10^{6} EID50)	Intervet-Holland	Intraocular	Day 25

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 as shown in (Table 3) according to the manufacturer's instructions.

Table 3: Explain ELISA kits of IB, ND, IBD, AI used estimated humoral immune response

(ID Screen® infectious bronchitis disease Indirect ELISA Kit 2.0-IBVARSV2 ver 0223 EN
(ID Screen® Newcastle disease Indirect ELISA Kit-NDVS-CV 0416 EN
(ID Screen® Infectious bursal disease Indirect ELISA Kit-IBDS ver 0416 EN
(ID Screen® Avian influenza A Nucleoprotein Indirect ELISA Kit-FLUNPS ver 0620 EN

At 35 day, blood samples (2ml) were collected from jugular vein then 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.

At the end of the experiment, at day 35 of age, the histopathological changes in the lymphoid organs (spleen, bursa of Fabicia, and thymus) were also investigated. Tissue samples from the bursa of Fabicia and spleen and thymus were preserved in 10% neutral buffered formalin as soon as they were collected and they were subsequently wrapped in paraffin wax. Hematoxylin and eosin (H&E) stain was applied to thin slices of 4-5 µm thickness in accordance with the method described by (Guo *et al*., 2018) [33] in order to microscopically examine the changes.

Statistical Analyses

We evaluated the collected numerical data using the SAS statistical software (SAS, 2020) [55]. We identified significant disparities among the coefficients of the examined qualities using Duncan's multiple range test (Duncan, 1955) [27].

ELISA antibody titers were used to quantify the comprehensive evaluation of treatment effects on immunological parameters in broiler chickens. The statistical significance of differences was assessed at two levels: $p<0.05$ and $*p<0.01$, with post-hoc differences denoted by superscript letters (A, B, C).

Results 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) [7, 10, 29] 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 and strict biosecurity (Colvero *et al*., 2018; Olukotun *et al*., 2018; Chung *et al*., 2021) [24, 51, 22].

in this study, the immune response measured by ELISA and histopathological change in lymphoid organs 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, both vaccination programs are commonly used in vaccinating chickens in Iraq. ELISA have been employed for the detection of antibodies against varies pathogens due to ELISA technique is more accurate, sensitive and rapid as confirmed by (Mao *et al*., 2022) [45].

The titers of 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.

Maternal antibodies an importance in protecting chicks, especially during the first few weeks of life when their immune system is still not fully functional (Hamal *et al*., 2006) [34].

during the rearing period, parent flocks are vaccinated several times with live and killed vaccines against the AI, ND, IB and IBD viruses this will result in a high concentration of IgY in her blood, which will be transferred to the blood of their off springs after hatching via the yolk sac (Gharaibeh and Mahmoud, 2013) [31]. The titer of IgY in chicks blood is proportional to the IgY titer in blood of their mothers (Hamal *et al*., 2006) [34].

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) [9, 19, 31, 43, 26]. The result of this 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) [31] confirmed that there is 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 (4).

Table 4: Explain 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%.

The results of current study shows there are significant difference in level ($p\leq0.01$) 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 ELISA antibodies titer against IBD between chicks in G1 and G3 as shown in table 4. 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 4. 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 4. 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 4.

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) [43, 13, 70, 11, 21, 44]; 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*., 2021) [22] 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) [28], 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) [12, 61], 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)) [44] concluded that the inactivated Lasota vaccine against 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) [42] and Deka *et al*., (2020) [26] 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. This 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) [62] 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) [52] 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) [46] 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) [46] 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 4.

However, the results of Allaoui *et al*., (2022) [6] 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 4, 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) [57] 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) [54], 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) [48, 4, 38, 15, 65] 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.

The histopathological findings of this study of lymphoid tissues were summarized as follows: The histopathological lesions in the bursa of Fabricius in 35-day-old group 1 chicks were moderate hyperplasia and dilatation of the bursa follicles, characterized by a thick cortex and moderate intramedullary lymphoid hyperplasia as shown in (Figure 1-A). These changes suggest an active immune response, likely stimulated by the comprehensive vaccination program administered on day one**,** These observations are consistent with findings reported by (Starciuc, 2007) [58] and (Nishizawa *et al*., 2007) [49] and (Wegner *et al*., 2025) [66] and (Balqis *et al*., 2019; Sultan *et al*., 2016) [18, 60], who demonstrated that vaccination as IBD, ND protocols promote follicular hyperplasia and cortical thickening in the bursa, reflecting enhanced immunostimulation and B-cell proliferation.

While as, histopathological lesions of the bursa of Fabricius in chickens in G2 at day 35 showed well-preserved follicular architecture with minimal lymphocyte depletion.

This aligns with findings by Starciuc *et al*., (2011) [59], Goud and Sreedevi, (2009) [32] and Al-Zubeady, *et al*., (2018) [68] whom reported that intermediate IBD vaccine strains cause only mild, reversible changes in bursal tissue. Additionally, (Balqis *et al*., 2019) [18] and (Igwe *et al*., 2020) [36] whom demonstrated that chickens vaccinated with LaSota ND vaccine does not significantly alteration in follicular morphology at day 28 as shown in (figure1-B).

While as, histopathological lesions of the bursa of Fabricius in chickens in G3 (control group) at day 35 showed at 35 days of age revealed normal follicular architecture, with clearly defined cortical and medullary zones. The cortex was densely populated with B lymphocytes, reflecting active immune development at this age. No signs of degeneration, inflammation, or fibrosis were observed as shown in (figure 1-C). These findings are consistent with Sharma, (1991) confirmed that unvaccinated birds show organized bursal structure with no pathological alterations. Also, (Yunis *et al*. (2000) [67] and Villanueva & Bernardo, (2016) [64] who emphasized the stability of immune tissue architecture in the absence of external antigenic stimulation.

Histopathological examination of the thymus in chickens in the G1 at 35-day-old chickens showed marked lymphocyte (thymocyte) proliferation in the cortex, along with numerous mononuclear cells and eosinophils as shown in (figure 1-D). These findings align with Jia *et al*., (2014) [39], Treesh *et al*., (2014) [63], Ayman *et al*., (2020) [16] and Gewaily *et al*., (2023) [30] who reported increased thymic lymphocyte proliferation after vaccinations, reflecting enhanced immune activation. Which vary from one chicken to another based on factors such as age, health status, and the type of vaccine used.

Histological analysis of the thymus in chickens in G2 at 35-day-old revealed well-preserved cortical and medullary regions, with dense lymphocyte populations in the cortex as shown in (figure 1-E). These findings are consistent with Jia *et al*., (2014) [39], Hasan & Ali, (2015) [35], Zahid *et al*., (2017) [69], Kajal *et al*., (2023) [40] and Gewaily *et al*., (2023) [30] whom reported increased thymic lymphocyte density but mild hyperplasia following vaccination with ND and IBD vaccines, reflecting active T-cell development, indicating an immunological response. No histopathological lesions were observed in thymus of chickens in G3 (control group) at 35-day-old as shown in (figure 1-F)

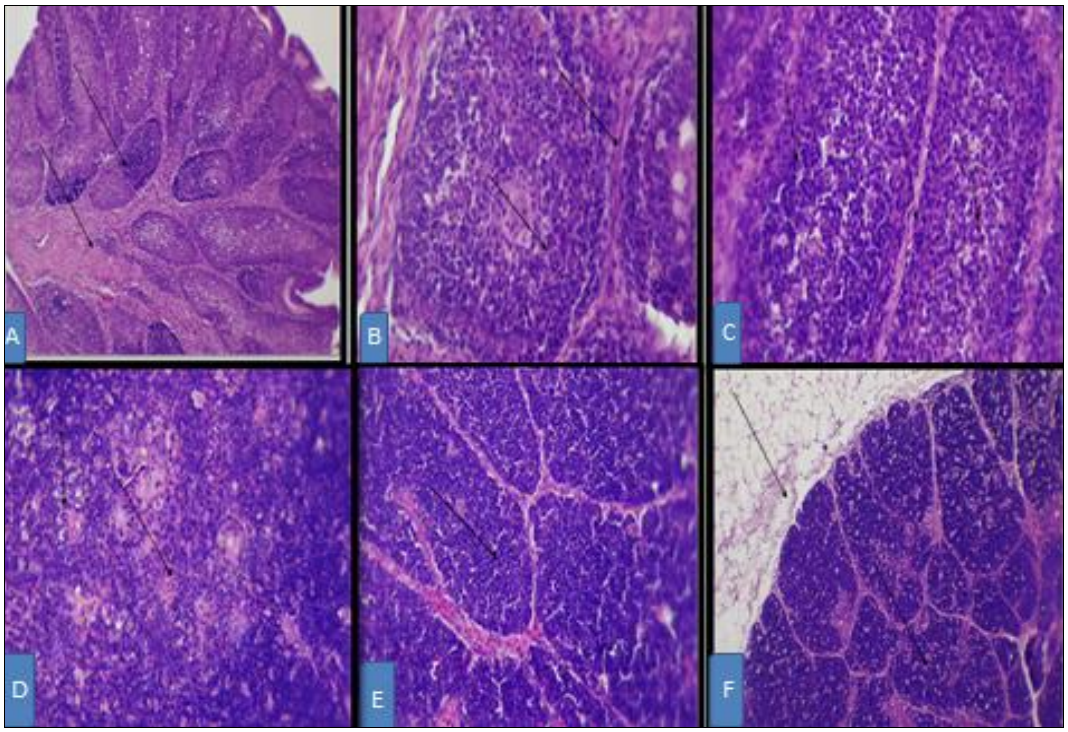

Fig 1: Histopathological sections of lymphoid organs explain: A: Bursa of Fabricius of broiler in G 1 shows hyperplasia in bursal follicles and widening of bursal follicles and thickening cortex and moderate lymphcytic hyperplasia within the medulla of bursal follicles 10 × (H & E). B: Bursa of Fabricius of broiler in G 2 shows depletion of lymphocytes in medulla and interfollicular fibrous connective tissue proliferation 40 × (H & E). C: Bursa of Fabricius of broiler in control group shows bursal follicles formed marked cortex and medulla with thin septum between follicles 40 × (H & E). D: Thymus in G1 shown thicking intrfollicular septa within congestion blood vessels and prolferation lymphocyts (thymocyte) and numerous mononuclear and esoinphils in cortex 10 × (H & E). E: Thymus in G 2 shows prolferation lymphocyts in thymus lobular and thicking intrlobular septa 40 × (H & E). F: Thymus in control G3 showS normal architecture as encapsulated by a capsule and the lobules separated by septa inside the gland, the cells organized and distributed in the cortex and medulla 10 × (H & E).

The histopathological findings were observed in spleen of experimental groups at 35-day-old of chickens were: The histopathological lesions were seen in spleen of chickens in G1 were moderate white pulp hyperplasia and mild depletion of blood sinusoids in the red pulp of the spleen from indicate an active immune response as shown in (figure 2-A). White pulp hyperplasia may be reflect lymphoid proliferation due to antigenic stimulation, while sinusoid depletion may be due to increased immune cell trafficking or vascular changes**.** This response aligns with the intensive vaccination program administered on day one, which included IBV strains (Ma5, Clone 30, IB 491), IBD, and combined H9N2 and ND LaSota vaccines. Such a regimen is known to provoke robust immune activation**.** Similar findings were reported by (Reshag and Hamza, 2017) [53], Igwe *et al*., (2020) [36], Budnik and Guralska,

(2022) [20], Khalaf and Ali, (2023) [41] and Gewaily *et al*., (2023) [30], whom observed that white pulp dilatation with hyperplasia and red pulp alterations following vaccination.
While as the histopathological lesions were observed in spleen of chickens in G2 were of the moderate white pulp hyperplasia, reactive germinal centers, and mild perivascular necrosis (figure 2-B) indicating a stimulated yet non-pathological immune responseas shown in**.** Similar splenic responses have been reported by (Azhar *et al*., 2012) [17], who observed increased lymphoid activity and preserved splenic architecture in broilers vaccinated with both intermediate and mild IBDV vaccine strains. While as, the chickens in G3 (control group) revealed a normal splenic structure, with well-defined white pulp and intact red pulp as shown in (figure 2-C).

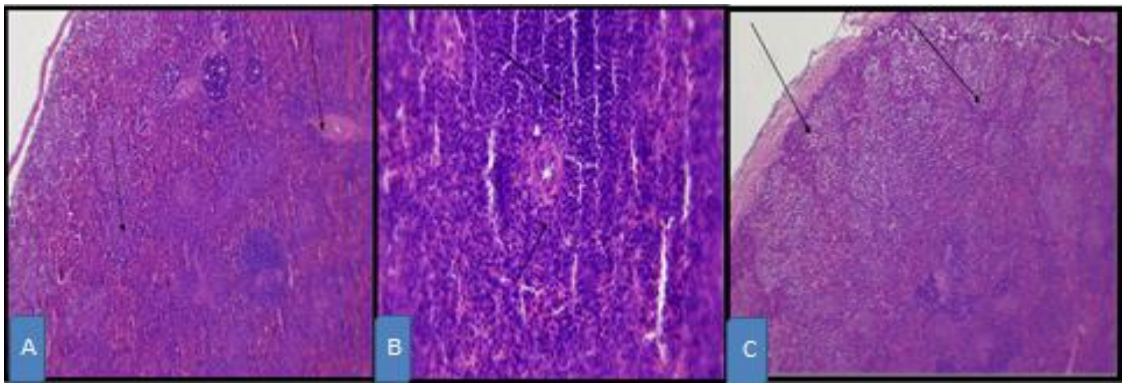

Fig 2: Histopathological sections of spleen of broiler chickens at day 35 of age amonge experimental groups show. A-Spleen of chickens in G 1 show moderate hyperplasia in white pulp and congestion blood vessels and depletion of blood sinusoid of red pulp 10 × (H & E). B-Spleen of chickens in G 2 show hyperplasia in white pulp and proliferation lymphocytes around of blood vessels in red pulp 40 × (H & E). C-Spleen of chickens in G3 (control group) show normal appearance capsule and normal white pulp (lymphocytes) surround the central vein with red pulp formed the bulk of the splenic parenchyma 10 × (H & E).

Conclusions
From the results of this study, we conclude that both vaccination programs induce good a humoral immune response. the vaccination programs (time of vaccination, type of vaccine, routs of administration) applied in poultry houses lead to histopathogical changes in lymphoid organ may be used in estimated immune response in chickens. Further future studies need on vaccination programs designs until reach to suitable program can protecting chickens against infectious diseases.

Ethics clearance
All animal samples in the study were managed and processed in accordance with the necessary biosafety and security protocols. Before starting this study, the Ethics and Scientific Committee in the Department of Pathology and Poultry Diseases at the College of Veterinary Medicine, Al-Qasim Green University, Ministry of Higher Education and Scientific Research, Iraq, granted approval for the research protocol (No. 2650 on 21/10/2024). The guidelines for the Care and Use of Laboratory Animals, along with the specific guide for broiler chickens, were strictly followed during the study.

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

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