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Immunological assessment and homogeneity of commercial inactivated bivalent Newcastle and avian influenza H9N2 vaccines in broilers

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Abstract

Our study aimed to evaluate the immune response of 7 types of bivalent inactivated vaccines. 225 chicks were distributed into 9 groups, groups from 1 to 7; vaccinated on 1 day with different doses of these vaccines against Newcastle Disease Virus-Avian Influenza Virus-H9N2 subcutaneously according to the direction of producers as follows: G1 vaccinated by PRO-VAC^{TM} AINK. G2 immunized by Poul Shot® flu H9N2+ ND. G3 received QVAC ND-H9. G4 injected with MEFLUVAC^{TM} H9ND7. G5 was immunized with CEVAC® NEW FLU H9 K. G6 received Nobilis N9H2+ND P. G7 was vaccinated with Gallimune. G8 was considered as the positive control group, while G9 was a negative control group. Subsequently, all groups of the experiment except (G9) were vaccinated by eye drop with the live attenuated vaccine (Nobilis® ND Clone-30) at 1 and 14-day-old chicks. The results of the ELISA and HI tests of antibodies titer to ND antigen have varied between groups, and the high titer was observed in G3 and G2 at 28 and 35 days. The lowest and highest titers were detected in G6 and G4 when these titers were measured by general and specific ELISA for AI. Most of the vaccinated groups (G1 to G7) revealed high to moderate expression of CD19 memory B-cells by immunohistochemical staining. In conclusion, most of the tested vaccines in this research gave a sufficient number of antibodies to ND antigen, whereas, in contrast to the AI antigen, many of these vaccines demonstrated a weak antibody response, except with the G6 vaccine.

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Introduction

Newcastle disease has a wide host range and global spread. It has been regarded as one of the most prevalent and threatening diseases of poultry, causing significant financial losses in domestic poultry, especially in chickens (1). The typical form of virulent NDV infection is associated with high mortality, depression, hemorrhage in several organs, and dyspnea (2). Newcastle disease virus belongs to the Avulavirus genus among the Paramyxoviridae. Paramyxoviruses have a single-stranded RNA. The genome is approximately 15 kb in size and comprises six polypeptide-coding genes (3). In Iraq, and according to many studies, NDV is a prevalent disease affecting broilers and layers (4). Numerous avian species have been infected with the H9N2 as low pathogenic avian influenza virus (LPAIV), which has caused significant economic losses because of decreased egg production or high mortality brought on by co-infection with other pathogenic agents (5). Avian influenza virus AIV is a negative-sense, single-stranded RNA virus with eight segments in its genome, which is a member of the type A influenza virus (family: Orthomyxoviridae). LPAI has frequently resulted in mild to severe mortality with clinical symptoms that include depression, respiratory symptoms, and a decrease in egg production (6). As a result, low levels of antibodies or failure of vaccination processes due to several causes in many breeds of poultry make NDV a

continuing concern to poultry producers. NDV control aims to reduce or eradicate the disease through adequate injection of effective vaccines to diminish the clinical illness (7). Therefore, Commercial poultry and backyard chickens must be vaccinated against NDV in the endemic areas, including Iraq. Vaccination with commercially available vaccines using live vaccines with low-virulence strains, including B1 and LaSota, has been widely applied. Most parts of the nation have also utilized inactivated oil-emulsified vaccines as routine vaccines for poultry, but high death rates and financial losses despite widespread immunization demonstrate that vaccination campaigns were unable to totally stop outbreaks of the viruses that are currently in circulation. Antigen varieties and other emulsion adjuvants are being investigated to improve the vaccines (8). Furthermore, to avoid safety-related issues, (9) highlights the need to select excellent chemicals and natural oils when implementing them. Also, vaccination is recommended to control avian diseases as part of a coordinated approach, along with biosecurity principles and surveillance systems, and a beneficial effect in reducing the risk of infection and associated sequelae has been shown (10). Annually, many inactivated NDV oil-adjuvant mono or bivalent, along with H9N2 commercial vaccines, were used in the vaccination of poultry. In terms of antibody response, the kind of inactivated vaccines, the species of birds bred on farms, and the recommended dose of the vaccine are crucial (11). In poultry projects, vaccination against AIV and NDV is a crucial means of preventing and controlling these diseases (12). CD19 is a cell surface glycoprotein that is mainly present on the surface of B-cells from early development to maturity. It regulates B-cell development activation and differentiation. CD19 is a member of the immunoglobulin superfamily that acts as a co-receptor with the B-cell receptor (BCR) to control B-cell activity. It is also involved in the amplification of signals essential to B-cell activation after antigen binding (13).

Consequently, the present research was designed to determine the immunological response, uniformity, body performance, and immunohistochemical study of commercially available inactivated bivalent vaccines against Newcastle and avian influenza H9N2 vaccines in broilers.

Materials and methods

Ethical approval

The Committee of Scientific Morals issued the endorsement certificate with the number UM.VET.2024.035 on September 2024, and gave the College of Veterinary Medicine the moral authority to perform this planned work.

Experimental groups

250-day-old chicks (ROSS 308) were allocated randomly to 9 groups (each of 25 chicks) in separated pens; groups from 1 to 8 were vaccinated at one day old with different doses of bivalent oily inactivated vaccines against NDV-AIV-H9 by subcutaneous route according to the direction of producers as follows: G1 vaccinated by PRO-VAC™ AINK Which produced by Komipharm International Co./Korea (0.25 ml/sc). G2 was immunized with Poul Shot® flu H9N2+ ND, which was produced by CAVAC /Korea (0.2 ml/sc). G3 received the QVAC ND-H9 vaccine, which was manufactured by QYH BIOTECH COMPANY LIMITED/China (0.25ml/sc). G4 was injected with MEFLUVAC™ H9ND7, which is produced by MEVAC/ Egypt (0.3 ml/S.C.). G5 was immunized with CEVAC® NEW FLU H9 K, which was produced by Ceva-Phylaxia Veterinary Biologicals Co. Ltd. /France (0.2 ml/sc). G6 received NOBILIS N9H2+ND P, which was manufactured by MSD Animal Health/ Netherland (0.25ml/sc). G7 was vaccinated with Gallimune, which was produced by Boehringer Ingelheim/ France (0.3 ml/sc). G8 vaccinated only with live attenuated vaccine (positive control group). G9 represents a negative control group (non-vaccinated). Subsequently, all groups of the experiment except (G9) were vaccinated by eye drop with the live attenuated vaccine (Nobilis® ND Clone 30- MSD animal health) (dose=10^6 EID_{50}) at 1 and 14-day-old chicks.

Samples of blood

In order to obtain the serum, about 2 ml of blood were taken from the chicks' wing or jugular vein at 1, 7, 14, 21, 28, and 35 days of age. The samples of blood were centrifuged at 2500 rpm for 20 min., and they were subsequently kept in Eppendorf-labeled vials at -20°C for further analysis (14-16).

Indirect ELISA test

All serum samples were examined for the presence of antibodies. Antibodies level against NDV, AI- -type A Ag, and AI-H9 Ag with the aid of the following ELISA kits (Biochek- catalog No. CK116-NDV) for NDV antibodies. General AI-Type A is used to detect antibodies for all type A influenza strains in chickens (Biochek- catalog No. CK121-AI). Lastly (ID.vet- ID Screen® Influenza H9 Indirect ELISA) (17). A specific kit was used for the estimation of anti-H9 antibodies in the serum of all groups just at 21,28 and 35 days. These tests were done according to the manufacturer's instructions.

Hemagglutination inhibition test

In a 96-well micro-titer plate, 50 ml of each serum sample was then diluted (2-fold dilution) with 50 µL of PBS. After that, 50 µL of NDV antigen (4HAU) (GD Academy/Netherlands) was added and properly mixed. After 25 min. of room temperature incubation, 50 µL of a 2% chicken RBC solution was added to the microtiter plate and thoroughly mixed. The microtiter plate was incubated at room temperature for 45 min. Before the HI test, the antibodies' titer (18).

Growth performance

Chicks of this study were fed on a basal diet manufactured according to the usual requirements of the broiler (19). Weekly observations of the primary and final body weight, total feed consumed per bird, and food conversion ratio (FCR) were carried out to look for variations among the experiment's groups (20-22).

Immunohistochemical staining

Sections for immunohistochemical analysis were obtained from the Bursa of Fabricius and thymus at the end of the experiment (35 days). The sections were rehydrated in a distinct dropping ethanol series after being deparaffinized using a series of xylene. For paraffin-embedded tissues, the CD19 primary antibodies used in the immunohistochemical method were taken into consideration. The Poly-HRP detection tool (Elabscience, USA) was utilized to stain the CD19 rabbit polyclonal antibody. The slides were heated in an oven for ten minutes before the addition of the primary antibodies. The slides were then incubated with the primary antibody and secondary Poly-HRP anti-rabbit antibodies (Elabscience, USA) for an entire night in a kept cool room. The slides were counterstained with hematoxylin after being developed with DAB (23). Using a digital camera and the ImageJ program, the digital photos were collected and examined. Through the analysis of CD19 protein in the bursa of Fabricius and Thymus, the degree of immunostaining was computed as a percentage of memory B cell expression.

Statistical analysis

Data was analyzed using SPSS version 22.0. Duncan's test was used to compare the calculated values of antibodies, weight gain, and FCR variables. The findings were displayed as mean values ±Standard Error (SE). The relationship between the antibody's titer, ELISA, and HI was performed using correlation factors, such as the R-factor and person test (24,25).

Results

The results of table 1 show that the humoral immune response by antibodies against the ND vaccine antigen has fluctuated between groups since the beginning of the experiment. However, the difference in antibody titer was clear with the advanced ages starting from the age of 21 days; the maximal titer of antibodies in groups 3 and 5 was 4255.2±1023.8, 5442±2407.6 at 21 days of age. At 28 and 35 days, group 3 had the highest antibody titer, 8369.2±1415, 9991±767.3, indicating significant differences from G1, G4, G6, G7, G8 and G9. However, the titer of antibodies in most of the vaccinated groups with oily inactivated vaccines was fair, except in the fourth and eighth groups, respectively 1810.2±565, 366.7±508.8. At the end of the trial (day 35), G3 had a good coefficient of variation value of 15%. Also, the values of CV% varied between groups of experiments, and the highest values were distributed among different ages and groups.

As can be seen from table 2, group 6 had the highest titer of antibodies against AI-antigen type A since day 21 of the experiment, 3235.7±1967.5. This significant difference of group 6 persisted until the end of the experiment when group antibodies titer at age 35 days was 7744.2±1326.2; furthermore, the value of CV was 34% in this group was less than 50% at 35 days of age, indicating satisfactory findings.

Table 1: Antibodies titer for NDV by ELISA

Groups	1 day	7 days	14 days	21 days	28 days	35 days
G1	5029.2±16993.75 59a	204.1±2485.2 16b	836.5±2469.5 68a	805.9±3622 44 abc	1064.6±4257.5 50 b	1513±6158.2 49 b
G2	5029.2±16993.75 59a	268.7±2207.2 24 b	203.4±885.7 46 b	1049.8±3300.7 64 abc	1098±6293.2 35 ab	742.1±9115.7 16 ab
G3	5029.2±16993.75 59a	880.6±3155 56 ab	271±1333.5 41 ab	1023.8±4255.2 48 a	1415±8369.2 34 a	767.3±9991 15 a
G4	5029.2±16993.75 59a	512.6±2963.5 35 ab	183.3±1593.7 23 ab	180.4±767.5 47 bc	1397.1±2985 94 bc	565±1810.2 62 c
G5	5029.2±16993.75 59a	437.1±1808 48 b	226.4±1556.5 29 ab	2407.6±5442 88 a	1490±3742.7 80 b	1193.9±7883.2 30 ab
G6	5029.2±16993.75 59a	636.2±3809.5 33 ab	401.2±1617.2 50 ab	614.4±3776.5 33 ab	1078.1±5711.2 38 ab	1631.7±6412.2 51 b
G7	5029.2±16993.75 59a	116.5±2915.7 77 ab	689±1637.5 84 ab	187±2078.7 18 abc	357.5±3847 19 b	1413.2±6338 45 b
G8	5029.2±16993.75 59a	656.3±4609.5 28 a	250±906.5 55 b	892.6±2101 85 abc	120.4±266.5 90 c	508.8±366.7 73 c
G9	5029.2±16993.75 59a	401.3±2231.7 36 b	295±969.5 61 b	84.2±302.7 57 c	10.9±49.2 43 c	60.2±136 88 c

A significant difference at $P<0.05$ is displayed by values in the same row that have different letter superscripts. Data expressed as mean±SE, CV%.

Table 2: Antibodies titer for AIV by ELISA

Groups	1 day	7 days	14 days	21 days	28 days	35 days
G1	427.18±764.3 111 a	33±82.5 80 b	13.6±21 129 b	798.1±875.2 182 b	207.3±452.7 92 b	310.4±982.5 63 b
G2	427.18±764.3 111 a	73.8±206 71 ab	9.6±15.2 127 b	33.7±34.7 197 b	96±97 198 b	884.4±904.7 196 b
G3	427.18±764.3 111 a	29.9±152 39 b	74.6±92.7 162 ab	10.2±11.2 182 b	213.5±254.2 168 b	69.5±110.2 126 b
G4	427.18±764.3 111 a	118.3±325.2 73 ab	15±43 70 ab	0.0±1.0 0 b	4.5±5.5 180 b	0.0±1.0 0 b
G5	427.18±764.3 111 a	40.3±93.5 86 b	14.1±34.2 82 b	6.3±11.7 109 b	0.0±1.0 0 b	86.1±127.7 135 b
G6	427.18±764.3 111 a	217.7±523.5 83 a	102.8±183 112 a	1967.5±3235.7 122 a	1660.4±2590 128 a	1326.2±7744.2 34 a
G7	427.18±764.3 111 a	95.6±196 97 ab	4.7±5.7 180 b	93.5±241.5 78 b	34.6±180.2 38 b	13.9±106.7 25 b
G8	427.18±764.3 111 a	108.6±245.5 89 ab	34±58.2 117 ab	2.2±3.2 133 b	0.0±1.0 0 b	0.0±1.0 0 b
G9	427.18±764.3 111 a	66.1±207.7 64 ab	1.5±2.5 50 b	0.0±1.0 0 b	0.0±1.0 0 b	0.0±1.0 0 b

A significant difference at P<0.05 is displayed by values in the same row that have different letter superscripts. Data expressed as mean±SE, CV%.

According to table 3, the results demonstrated that there was a significant difference in antibody titer against H9 antigen between groups that were measured by specialized indirect ELISA kit for H9 antigen. The sixth and seventh groups gave the highest titer of antibodies at ages 21, 28, and 35, while the sixth group gave its highest titer at ages 28 and 35 days, 14779.3±5684.7, 20835±2221.1, respectively. Table 6 also explains that the sixth and seventh groups started to provide a detectable titer of antibodies at 21 days post-vaccination, whereas the first, second, third, and fifth groups did not show detectable antibodies until 35 days of age. Finally, the antibody titer remains significantly low at the same age, 44.7±19. At the end of the trial, we looked at the CV% of vaccinated groups that displayed high titer of antibodies G1, G5, G6, and G7 and found that G6 had the lowest and excellent value of this parameter, 2%.

Table 4 represents the mean (Log^2±SE) of serum antibody titers for the NDV antigen by HI data. At 7, 21, and 35 days, accordingly, these titers were higher in G6 4.25±0.25, G5 5.25±0.25, and G2 9.25±0.47. As displayed in the mentioned table, the CV% values varied between groups.

The r-value (correlation factor) alters between the ELISA and HI tests when correlation is determined regarding them, and positive correlation values were observed at the end of the experiment when compared to at the beginning of it (Figure 1).

Table 3: Antibodies titer for H9 by ELISA

Groups	21 days	28 days	35 days
G1	282±332, 147 b	819.2±1528.3, 93 bc	2118.8±5710.2, 74 b
G2	47.1±77, 106 b	15.6±68, 40 c	3901.4±3985.7, 196 b
G3	42±87.3, 84 b	87.1±144.6, 104 c	1491.8±2863.7, 104 b
G4	22.8±79, 51 b	2.9±27.3, 19 c	19±44.7, 84 c
G5	24.1±100, 42 b	6.2±64.6, 17 c	3626.7±5221.5, 139 b
G6	3761.8±8161, 80 a	5684.7±14779.3, 67 a	222.1±20835, 2 a
G7	5869.1±7134.6, 142 ab	956.6±7031.6, 24 b	4337.3±7179.7, 121 b
G8	39±106, 64 b	20.1±57, 61 c	8.1±80, 20 c
G9	25.3±110.3, 40 b	48.7±120.6, 70 c	10.8±47, 47 c

A significant difference at P<0.05 is displayed by values in the same row that have different letter superscripts. Data expressed as mean±SE, CV%.

Table 4: Antibodies titer for NDV by HI test

Groups	21 days	28 days	35 days
G1	0.25±3.25, 15.38 ab	0.62±4.75, 26.31 ab	0.85±7.75, 22.07 a
G2	0.47±3.25, 29.23 ab	0.25 ±4.25, 11.76 ab	0.47 ±9.25, 10.27 a
G3	0.00±4, 0 a	0.70±5, 28.2 ab	0.62±8.75, 14.28 a
G4	0.47±3.75, 25.33 a	0.64±1.50, 86 c	0.62±2.75, 45.45 b
G5	0.28±2.5, 23.08 b	0.25±5.25, 9.52 a	0.28±7.50, 7.6 a
G6	0.25 ±4.25, 11.76 a	0.25±4.75, 10.52 ab	0.62 ±7.25, 17.24 a
G7	0.00±4, 0 a	0.40±4, 20.25 ab	1.19±7.50, 31.73 a
G8	0.25 ±4.25, 11.76 a	0.64±3.50, 36.85 b	0.64 ±1.50, 86 bc
G9	0.62 ±2.25, 5.55 b	0.50±0.50, 200 c	0.28±0.50, 114 c

A significant difference at P<0.05 is displayed by values in the same row that have different letter superscripts. Data expressed as mean±SE, CV%.

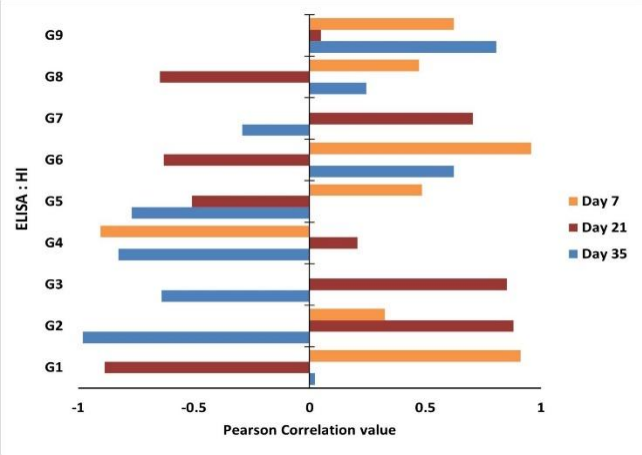

Figure 1: The correlation (r) of antibodies titer between ELISA and HI tests.

Table 5 illustrates how the average body weight of the birds varied by group and by age. The observations showed that groups 1 and 9 had the highest weights at 7 and 14 days, while group 7 had the highest weight at 21 days, 985.7±27.7. At 28 days, groups 3, 5, and 6 had lower body weights than the first and seventh groups at the significant level, and at 35 days, group 5 had the lowest rate, 1978.5±56.5, while group 9 had the best body weight, 2281±991.

Table 6 shows that at 28 days, the feed conversion factor in groups 9 and 6 was at its best level of 0.814±0.004 and 0.838±0.015, respectively, while at 35 days, group 6 had the best value of 1.412±0.11 in comparison to the other groups that obtained different inactivated vaccines.

We estimated the distribution of memory B cells in the bursa of Fabricius and thymus sections after chicks of the experiment received live attenuated and different bivalent inactivated vaccines by using immunohistochemical Image J software. Positive and negative allocation of memory B cells in the tissue is valuable to qualify the degree of CD19 expression. In all sections, memory B cells were noticed at the cortico-medullary junction of the bursa of Fabricius and thymus (Figures 2 and 3). In the bursa of Fabricius, the immunostaining of the CD19 was a high expression in group 3, moderate expression in groups 1, 2, 5, 6, and 7, weak expression in group 4, and negative expression in groups 8 and 9 after vaccination, while G8 that not received an inactivated vaccine explained no expression of CD19. In the thymus, the immunostaining of CD19 was a high expression in group 3, moderate expression in groups 1, 2, 4, 5, 6, and 7, and negative expression in groups 8 and 9 after vaccination.

Table 5: Average of body weight (gram) at 7, 14, 21, 28, and 35 days in experimental groups

Groups	7 days	14 days	21 days	28 days	35 days
G1	184.4±4.7 a	476.6±8.0 a	938.1±17.3 ab	1625.4±43.8 a	2237.3 ±61.1 ab
G2	176.1±4.5 abc	463.6±9.9 ab	938.5±24.7 ab	1521±44.7 ab	2168.3±66.8 abc
G3	3.7±170.2 bc	436.3±7.9 b	18.4±888.7 ab	26.4±1439.4 b	54.6±2053.2 abc
G4	169.2±2.8 bc	458.6±12.4 ab	950.8±34.3 ab	1580.2±54.6 ab	2107.9±69.8 abc
G5	174.8±5.9 abc	461.3±8.4 ab	891.1±37.3 ab	1441.6±53.9 b	1978.5±56.9 c
G6	164.3±4.1 c	450.1±13.4 ab	868.3±30.4b	1463±36.8 b	2003.5±49.8 bc
G7	182.2±3.9vab	465.4±8.7 ab	958.7±27.7 a	1627.2±50.5 a	2225.5±78.1 ab
G8	174.8±4.9 abc	448.2±6.8 ab	936.2±20.5 ab	1544.7±43.6.6 ab	2139.2±91.3 abc
G9	184.5±4 a	473.6±9.4 a	936.7±22 ab	1585±65.4 ab	2281±99.1 a

A significant difference at P<0.05 is displayed by values in the same row that have different letter superscripts. Data expressed as mean±SE.

Table 6: Values of feed conversion ratio at 7, 14,21,28, and 35 days in experimental groups

Groups	7 days	14 days	21 days	28 days	35 days
G1	0.864 b	0.756 c	1.255 b	1.236 f	1.634 d
G2	0.967 d	0.743 bc	1.151 a	1.254 f	1.544 c
G3	0.938 cd	0.721 b	1.169 a	1.133 e	1.662 de
G4	0.593 a	0.744 bc	1.147 a	0.995 c	1.838 f
G5	1.033 e	0.719 b	1.270 bc	1.089 d	1.552 c
G6	1.056 e	0.786 d	1.329 c	0.838 a	1.44 b
G7	0.965 d	0.599 a	1.152 a	0.936 b	1.689 e
G8	0.941 cd	0.787 d	1.154 a	0.997 c	1.261 a
G9	0.888 bc	0.950 e	1.114 a	0.814 a	1.436 b

A significant difference at P<0.05 is displayed by values in the same row that have different letter superscripts. The lowest value represents the high feed conversion factor.

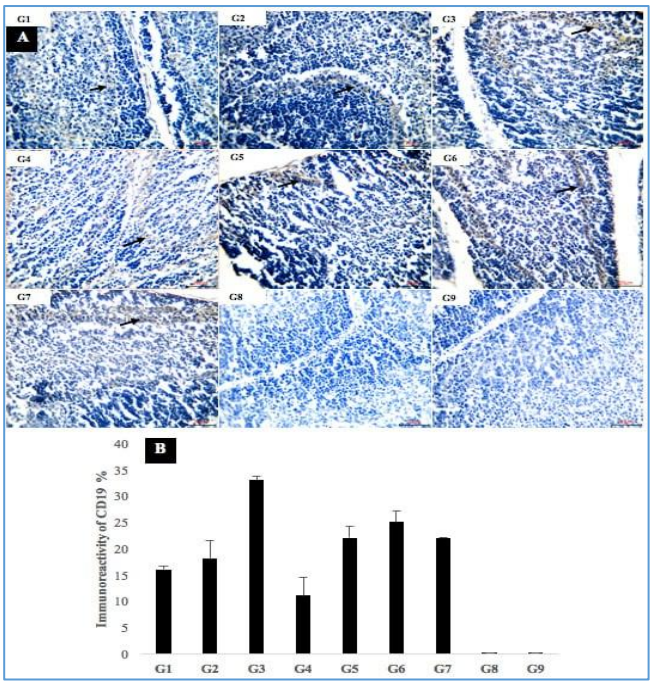

Figure 2: Immunohistochemical detection of CD19 in bursa of fabricius. A. The expression of CD19 in the bursa of Fabricius from vaccinated groups with live attenuated and bivalent inactivated vaccine. The CD19 is expressed only in the memory B cells as a dark stain mainly located at the cytoplasm of memory B cells at the cortico-medullary junction (black arrows). G1 (live and PRO-VAC^{TM} AINK vaccine), G2 (live and Poul Shot flu H9N2+ND vaccine), G3 (live and QVAC ND-H9 vaccine), G4 (live and MEFLUVAC^{TM} H9ND7 vaccine), G5 (live and CEVAC NEW FLU H9 K vaccine), G6 (live and NOBILS N9H2+ND P vaccine), G7 (live and Gallimune vaccine), neither the live attenuated vaccine (G8) nor the unvaccinated G9 exhibited any CD19 expression (20µm IHC). B. Quantification of the percentage of CD19 immunostaining in the positive stain memory B cells in the bursa of Fabricius in different groups by using Image J software.

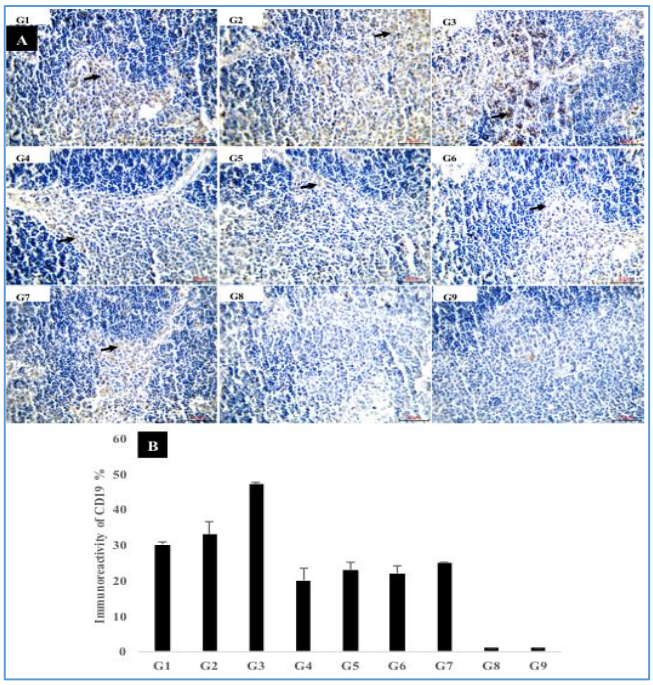

Figure 3: Immunohistochemical detection of CD19 in thymus. A. The expression of CD19 in the thymus from vaccinated groups with live attenuated and bivalent inactivated vaccine. The CD19 is expressed only in the memory B cells as a dark stain mainly located at the cytoplasm of memory B cells at the cortico-medullary junction (black arrows). G1 (live and PRO-VACTM AINK vaccine), G2 (live and Poul Shot flu H9N2+ND vaccine), G3 (live and QVAC ND-H9 vaccine), G4 (live and MEFLUVACTM H9ND7 vaccine), G5 (live and CEVAC NEW FLU H9 K vaccine), G6 (live and NOBILS N9H2+ND P vaccine), G7 (live and Gallimune vaccine), G8 (live attenuated vaccine), G9 (unvaccinated). (20µm IHC). B. Quantification of the percentage of CD19 immunostaining in the positive stain memory B cells in the thymus in different groups by using Image J software.

Discussion

One of the essential variables in determining the degree of protection afforded by the administered vaccine is the serological evaluation of vaccinated birds. Presently, ELISA kits to identify NDV antibodies are readily available because of several clear benefits that include superior sensitivity, specificity, and result replication, as well as the ability to use minimal quantities of biological fluid samples under study, reagent stability and availability, ease of use and quick reaction, instrumental reporting of the results, and automating of nearly each ELISA stages (26). Thus, our results in table 1 explained that most tested inactivated vaccines have a good protective level of antibodies, particularly at 21, 28, and 35 days post-vaccination, because of the activity of adjuvant content in the composition of these vaccines, and these data supported by the findings of Liu *et al.* (27), who determined that in addition to high-quality antigens, a powerful vaccine required a carefully chosen adjuvant to boost the antigen's humoral and cellular immune responses. Many vaccines develop a high number of antibodies based on an inactivation process that should prevent the virus from replicating while also preserving its external structural elements to stimulate the immune response properly (28). In addition, these results are similar to the finding of AL-Jumaili *et al.* (28), when they mentioned that vaccination groups receiving a combination of live and inactivated vaccines typically showed higher antibody titers, and this method of eliciting immunity involves entirely inactivating the virus without altering its surface glycoprotein structure. A relatively low post-vaccination antibody titer resulted from the high maternal antibody against ND at 7 days of age; these outcomes fit with our data at 7 days of the experiment (29). The CV% is used mostly to evaluate the effectiveness of vaccines and to assess the humoral immune response in chicks post-vaccination. Hence, the CV% values in the study's findings highlight the effectiveness of the previously demonstrated inactivated vaccines.

The findings in Table 2 demonstrate the lowest antibody titer in all treated groups except G6, which is the opposite of the outcomes of El-fadl *et al.* (30), who stated that the MEVAC inactivated H9N2 vaccine showed a protective antibodies titer with the HI test. These differences may arise because a variety of factors influence vaccine immunogenicity, such as antigen mass, formulation, and vaccination age, which are crucial for eliciting an adequate immune response (31). These findings also disagree with those of Talat *et al*. (32), who reported that MEFLUVAC-H9ND-16 exhibited a high and protective antibodies titer by HI test due to the high antigen concentration in the vaccine (350 HAU units/dose). As a result of their unvaccinated parents, the one-day-old chicks' maternal antibodies titer was insufficient. Therefore, it is advised that chicks with deficient maternal protective antibodies obtain a vaccination right away. Because there was no interference from maternal antibodies against vaccination antigens, the immune response exists normally in this case (29).

The developed ELISA has a biosafety score because it may be used in a regular laboratory without necessitating a cabinet for biological security. The high titer of antibodies appears in G6 and G7, but G6 is more potent, especially at 28 and 35 days, with an excellent CV% value (table 3). Since the initial vaccination requires antigen recognition and memory cell generation, AI antibody titer formation was slower than ND (29). Consequently, some compounds must be included in vaccine technology, especially with AI, to improve immunity. Thus, the highest and earlier titer in G6 is due to the effect of pathogen-associated molecular patterns by the influence of these components on the improvement of humoral immunity via the formation of antibodies, which is what produces the significant rise in the antibody's titer (33). The amount of vaccine used in poultry vaccination is highly valuable despite the fact that it is clear that the type and quantity of antigen in the inactivated ND and AI vaccines are the main indicators of vaccine immunogenicity (34). Additionally, the variation in antibody titer with each vaccine may be explained by differences in adjuvant quality, quantity, and characteristics; likewise, the duration of the immune response and the CV% in the vaccinated flock may be impacted by the homogeneity of the antigen particles among the vaccine components.

In the words of Tang *et al.* (35), the HI test has been proposed to be the most advantageous serological approach to determine an immune response to the AIV and NDV vaccines. High levels of antibodies were found mainly in G2 and G3 of our serological data, illustrating the significance of immunological response for determining vaccine efficacy (24). These results agreed with (36), who suggested that administrating inactivated vaccine with live vaccine produces higher immunity for a longer period. In agreement with the conclusions of Boven *et al.* (37), vaccination with effective vaccines elicited significant HI antibody titer in the vaccinated groups. Our results with G6 are parallel with Sarcheshmei *et al.* (38), who declare that administering live B1 vaccine by ocular route along with an oil-emulsified vaccine at 8 days of age and boosting vaccination with a lentogenic live virus vaccine may be capable of inducing efficient humoral and local immunity responses, while a lack of vaccine efficacy will result in inadequate immune stimulation, which is similar to G4 findings.

Around 30% and 50% is an acceptable coefficient of variability (39), indicating that flock immunization is good, and the high score of %CV demonstrates that the antibodies are diverse; this variation could result from inconsistent antigen particle composition or defective in vaccine manufacturing procedures at any phase of the process.

As pointed out by Alexender (40), certain researchers have shown a clear correlation between HI and ELISA titers, while others have noticed variation in both. Our findings are

contrary to those of Ojasvita *et al.* (41), who identified no relationship between HI and ELISA when the HI test is likely limited to antibodies against HN protein solely, while ELISA may detect antibodies to multiple antigens. The statistical examination of the concurrent HI and ELISA test results shows a positive linear correlation between the two techniques. The variations in the test results, particularly in the first two weeks of the trial, demonstrated that the various test methodologies were the cause of this mechanism: Particles of the virus can be agglutinated by IgG and IgM, which are predominant during the first two weeks of a primary immune response (42). The correlation between these two tests is somewhat in agreement with Chaka *et al*. (43), whose findings of the indirect ELISA test varied remarkably from those of the two other tests, with 92% of the samples showing positive as compared with less than 15% for the blocking ELISA and HI.

Among the effects of vaccination on body weight, some vaccines of the experiment showed a significant decrease in this parameter (44), as they mentioned that NDV vaccination decreased BWG and feed efficiency when compared to the control group after 21 days. Eventually, there were no apparent differences in body weight between some groups at the end of the experiment when compared to the control group, indicating that compensating growth might occur between 21 and 35 days (45).

Based on the research, stress brought on by vaccinations raised blood levels of cortisol and adrenocorticotropic hormone and had a negative impact on growth parameters like mean daily increment, mean daily food intake, and coefficients of feed conversion (46). This clarifies why the unvaccinated group (G9) had brilliant FCR values, followed by the group (G6) with good FCR, suggesting that a particular vaccine induced the least amount of stress in this group, but contrary to our outcomes, the vaccine had no noticeable effect on the productive parameters (47).

There are no or very few investigations about CD19 B-cell expression in chickens, but a study of immune response to COVID-19 mRNA vaccines in human using flow cytometry technique mentioned the association between humoral immune response and CD19 B-cells indicating their actual efficacy in triggering a humoral immunity (48). Another study also showed a significantly high expression of memory B cells expressing IgG (CD19+CD20+CD27+IgG+) after one and three months of vaccination against COVID-19 (48), so the high expression of CD19 in our findings with different inactivated vaccines may be due to stimulation of vaccinial antigens to B-cells as a crucial element of humoral immune response.

Conclusions

The majority of the bivalent vaccines included in this study demonstrated an adequate immune response to ND antigen; however, unlike AI antigen, most of the tested vaccines exhibited a weak antibodies titer except N9H2+ND P (MSD) vaccine as well as FCR.

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Conflict of interest

Regarding the publication of the current study, the researcher states that there are no conflicts of interest.

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التقييم المناعي والتجانسي للقاحات التجارية الثنائية المعطلة ضد مرض النيوكاسل وإنفلونزا الطيور ج٩ن٢ في الدجاج اللاحم

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الخلاصة

هدفت هذه الدراسة إلى تقييم الاستجابة المناعية لسبعة أنواع من اللقاحات المعطلة الثنائية ضد مرضي النيوكاسل وأنفلونزا الطيور النوع اتش-٩. تم توزيع ٢٢٥ فرخ إلى تسعة مجاميع، تم تحصينها ابتداء من الأولى الى السابعة بعمر يوم واحد بجرعات مختلفة من اللقاحات بالحقن تحت الجلد وفقا لتعليمات الشركات المنتجة لها وكما يلي: تم تحصين المجموعة الأولى بلقاح بروفاك. تم تحصين المجموعة الثانية بلقاح بول شوت. حصنت المجموعة الثالثة بلقاح كفاك. تم حقن المجموعة الرابعة بلقاح مفلوفاك. تم تحصين المجموعة الخامسة بلقاح سيفاك. حقنت المجموعة السادسة بلقاح نوبيليس بامب. تم تحصين المجموعة السابعة بلقاح كالي اميون تم اعتبار المجموعة الثامنة كسيطرة موجبة، بينما المجموعة التاسعة كسيطرة سالبة. بعد ذلك تم تحصين جميع أفراخ التجربة باستثناء المجموعة التاسعة بواسطة التقطير بالعين بلقاح حي مضعف نوبيليس كلون-٣٠ بعمر يوم واحد وأربعة عشر يوما. وقد تباينت نتائج اختبارات الاليزا وتثبيط التلازن الدموي بالنسبة للأجسام المضادة لفيروس مرض النيوكاسل بين المجاميع، ولوحظ أعلى نسبة لها في المجموعتين الثالثة والثانية عند عمر ثمانية وعشرون وخمسة وثلاثون يوما. بينما تم الكشف عن أدنى وأعلى معيار لأضداد الإنفلونزا في المجموعتين السادسة والرابعة عندما تم قياس هذا المعيار بواسطة الاليزا نوع أي والنوع المتخصص اتش-٩ عند عمر خمسة وثلاثون يوما، معظم المجاميع المحصنة أظهرت تعبير نسجي عالي الى متوسط لعنقود التمايز ١٩ الخاص بخلايا الذاكرة البائية بتقنية الكيمياء المناعية النسجية. وأخيرا، فإن معظم اللقاحات قيد الدراسة والتي تم اختبارها أعطت كمية كافية من الأجسام المضادة لمستضد مرض النيوكاسل، بينما كانت النتائج ضعيفة بالنسبة لمستضد فيروس الإنفلونزا، حيث أظهرت العديد من هذه اللقاحات أجساما مناعية ضعيفة باستثناء لقاح المجموعة السادسة.