Evaluation of immunity and protection in chicken administered a developed vaccine against predominant Middle Eastern strains of genotypes VI and VII of velogenic Newcastle Disease Virus

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Abstract

Background The velogenic-Newcastle Disease Virus (v-NDV) causes an important disease in chicken, associated with serious economic losses to the global poultry industry. This research evaluated the immunity in broilers administered a developed bivalent vaccine, aiming at protection against predominant Middle Eastern strains of genotypes VI and VII of v-NDV. The completely randomized design implemented in this evaluation included eight treatments, differing in birds being administered or deprived of the developed vaccine, with a difference in type of challenge, either by v-NDV strain(s) of genotype VI, VII, or both. Vaccination was administered subcutaneously at 6 and 21 d of age, followed by an intra-pectoral challenge at the age of 28 d.

Results The acquired humoral immunity by vaccinated and challenged birds to Hemagglutinin (H) protein was the highest at market age of 40 d, compared to challenged birds deprived of vaccination, and to vaccinates deprived of challenge ($P < 0.05$). The same statistical difference pattern was obtained by the cell-mediated immunity (CMI), represented by birds' level of serum IFN- γ . The type of challenge by either strain(s) of genotype VI, VII, or VI+VII did affect statistically the cross reactivity of acquired humoral immunity specific to H protein of homologous versus heterologous strains. The absence of humoral immunity and the low IFN- γ levels at 28 d of age in challenged birds deprived of vaccination lead to highest mortality, and lowest performance compared to vaccinates and challenged, vaccinates and deprived of challenge, and unvaccinated-unchallenged birds ($P < 0.05$).

Conclusions The developed bivalent vaccine was able to induce enough humoral and CMI responses, enabling protection of the broilers against production losses by each of the three types of v-NDV challenges. It is recommended to conduct future studies to evaluate such types of vaccines in chicken breeders and commercial layers, reared in various world's zones with existing endemicity of v-NDV.

Background

The impact of v-NDV on poultry health and economy of its global industry is highly significant [1–3]. The mortality losses in poultry due to v-NDV could reach to 100% [4], with significant negative impact on production performance of a few surviving birds [2, 5].

The predominant v-NDV strains in poultry of the Middle Eastern countries, including Israel, United Arab Emirates, Iraq, Jordan, Egypt, Lebanon, and Kingdom of Saudi Arabia belong mainly to genotypes VI and VII [6–8]. The genotype VI strains have affinity to respiratory, nervous, and gastrointestinal (GI) tract of the birds, causing apparent gross lesions of bluish ulcers in the intestine and petechial hemorrhage in the proventriculus [7]; however, the genotype VII strains have affinity to tissues of the respiratory and nervous system [9] causing apparent torticollis and pneumonic signs [7].

Global reports are pointing at frequent failure of NDV-commercial vaccines in induction of acceptable immunity and protection against emerging strains of NDV [10]. Reports from the Middle East countries included also documentations of failure of various imported commercial vaccines in protection of poultry

against v-NDV [7, 11]. Other workers documented the persistent shedding of the NDV, in spite of the administration to poultry of the available vaccines in their market [12–14].

The hypothesis of this research predicted that a development of a bivalent autogenous vaccine constructed from predominant v-NDV strains of genotypes VI and VII will acquire enough immunity for protection against velogenic NewCastle Disease.

Results

Pathological and molecular characteristics of v-NDV

The pathological and molecular characteristics of v-NDV vaccine seed and challenge strains, included in this study, are shown in Table 1. The intracerebral pathogenicity indices of the genotypes VI and VII strains were 1.86 and 1.92, respectively. This high pathogenicity is confirmed by the high mean broiler’s mortality percent, observed in the three field outbreaks caused by each strain of genotypes VI and VII, resulting in respective mean percent losses of 96.5 and 100.0 ($P > 0.05$). The velogenic nature of the two strains of genotypes VI and VII is molecularly demonstrated at their Fusion Protein Cleavage Site (FPCS), in which the amino acid Glutamine (Q) is found at position 114, surrounded by dibasic arginine (R) at position 113 and lysine (K) at position 115.

Table 1

Pathogenicity and sequencing of fusion protein cleavage sites (FPCS) of predominant Middle Eastern v-NDV strains of genotype VI and VII

Genotype	ICPI ^a	Mean Mortality % ^b	FPCS ^c
VI	1.86	96.5 ¹	RRQKRF
VII	1.92	100.0 ¹	RRQKRF
^a ICPI is the Intracerebral Pathogenicity Index, determined in 10 birds/genotype strain, according to protocol of OIE [15].			
^b The Mean Mortality % is recorded from three field outbreaks in broiler flocks caused by each genotype strain.			
^c The amplification and sequencing at FPCS were performed according to previously documented protocol by Seal et al [16].			
¹ Means in a column, followed by the same numerical superscript, are insignificantly different ($P > 0.05$).			

Acquired Humoral Immunity By Vaccine And Challenge

The completely randomized design (CRD) of this work is presented in Table 2, including eight treatments (TRTs) differing in birds being administered or deprived of the developed vaccine, and in type of challenge, either by strain(s) of genotype VI, VII, or VI + VII, in which the vaccination was performed at 6 and 21 d of age, and challenge administered at an age of 28 d. The acquired humoral immunity, specific to either the H-protein of strains belonging to genotype VI or VII, was boosted similarly at 7 days post the second vaccination of birds allocated to TRTs 1, 2, 3, and 7. The different challenges by either the strain of genotypes VI, VII, or combined VI + VII resulted in higher significant humoral immunity to H-protein of their homologous strains compared to the heterologous ones ($P < 0.05$); this significant difference in response is indicated by the numerical superscripts following the means presented in the last column of Table 3. The deprivation from vaccination, in the presence of either one of the three different challenges in birds of TRTs 4, 5, and 6 did result in significantly lower acquired immunity to the H-protein of strains used in the challenge at market age of 40 d. The decay in acquired humoral immunity by the vaccine administered to birds of TRT 7 at 6 and 21 d of age, in absence of a challenge at 28 d, led to a drop in HI titer, specific to H-protein of each of the two strains belonging respectively to genotypes VI and VII, by the market age of 40 d. The HI titer at 40 d of age in vaccinates that were deprived of challenge was significantly lower than the maintained titers obtained by vaccinates and challenged birds of TRTs 1, 2 and 3 ($P < 0.05$). It is worth noting that the negative control birds of TRT 8 had a complete absence of HI titer to H protein of genotypes VI and VII strains, an indication of an absence of exposure to such pathogens.

Table 2

A brief presentation of the completely randomized design (CRD) adopted in this study for evaluation in broilers of the immunity and protection provided by the developed autogenous vaccine against v-NDV strains of genotypes VI and VII

Treatment ^a (TRT)	Vaccination ^b	Strain(s)' genotype used In challenge	
		VI	VII
1	+	+	-
2	+	-	+
3	+	+	+
4	-	+	-
5	-	-	+
6	-	+	+
7	+	-	-
8	-	-	-
^a Each TRT is comprised of four replicate isolation rooms, with 25 birds/room			
^b Vaccination is administered at 6 and 21 d of age, subcutaneously in the neck, with a dose volume of 0.5 ml/bird			

Table 3

Acquired humoral immunity in broilers by the developed v-NDV vaccine^a and challenge^b

TRT	Vaccination	Strain(s)' genotype used In challenge		Mean serum HI titer ^c to H protein of genotype VI/VII strains at different ages (d)		
		VI	VII	21	28	40
1	+	+	-	42.5 ¹ /43.7 ¹	75.7 ¹ /79.8 ¹	114.6 ^{1,e} /96.5 ^{1,f}
2	+	-	+	40.8 ¹ /42.7 ¹	73.8 ¹ /75.2 ¹	92.3 ^{2,e} /122.0 ^{2,f}
3	+	+	+	44.2 ¹ /43.0 ¹	70.8 ¹ /72.2 ¹	122.0 ^{1,e} /111.2 ^{1,e}
4	-	+	-	0.0 ² /0.0 ²	0.0 ² /0.0 ²	52.7 ^{3,e} /25.2 ^{3,f}
5	-	-	+	0.0 ² /0.0 ²	0.0 ² /0.0 ²	20.7 ^{4,e} /48.0 ^{4,f}
6	-	+	+	0.0 ² /0.0 ²	0.0 ² /0.0 ²	NA ^d
7	+	-	-	44.5 ¹ /41.7 ¹	73.8 ¹ /75.2 ¹	58.2 ^{3,e} /55.0 ^{4,e}
8	-	-	-	0.0 ² /0.0 ²	0.0 ² /0.0 ²	0.0 ^{5,e} /0.0 ^{5,e}
^a Vaccination is administered at 6 and 21 d of age, subcutaneously in the neck, with a vaccine dose equivalent to 0.5 ml/bird						
^b Challenge by either the genotype VI or VII strain, or by both, at an age of 28 d						
^c The serum HI titer is the Hemaagglutination - Inhibition titer quantifying the humoral antibodies specific to the H protein present in the 4 HA units/50 µL of the genotype VI or VII strains used in the test against 0.1% of c-RBC						
^d NA stands for not applicable, due to the 100% mortality obtained by birds of TRT 6, by the age of 40 days.						
¹⁻⁵ Means of HI titer in a column, followed by different numerical superscripts, are significantly different at P < 0.05.						
^{e,f} Means of HI titer in numerator and denominator of a fraction located in a row of the last column, followed by different alphabet superscripts, are significantly different at P < 0.05.						

Acquired CMI By Developed Vaccine And Challenges

The acquired CMI to vaccination and to challenge, quantified by the IFN-γ levels in sera of the differently treated birds, is shown in Table 4. The pattern of quantified INF-γ is similar to that obtained by the humoral responses to H-protein of the two genotype-strains of v-NDV; more specifically, the highest IFN-γ

levels, post the vaccine booster delivered at 28 d of age, was obtained by the vaccinated birds of TRTs 1, 2, 3, and 7, with mean levels that were significantly higher than that obtained by all unvaccinated birds ($P < 0.05$). This acquiring of higher CMI response at challenge age of 28 days in birds of TRTs 1, 2, and 3 helped to further rise significantly their INF- γ levels at 12 d post challenge (40 d of age), compared to unvaccinated-challenged birds of TRTs 4, 5, and 6 ($P < 0.05$). It is worth noting that vaccinated birds, deprived of challenge (TRT 7), had a decay in INF- γ level by the market age of 40 d. In addition, the negative control birds of TRT 8 had a similar background levels of INF- γ at the three ages of 21, 28, and 40 d.

Table 4
Acquired cell-mediated immunity (INF-Y) in broilers by the vaccine^a and challenge^b

TRT	Vaccination	Strain(s)' genotype used In challenge		Mean serum INF-Y of broilers at an age (d)		
		VI	VII	21 d	28 d	40 d
1	+	+	-	720 ¹	853 ¹	978 ¹
2	+	-	+	713 ¹	847 ¹	1005 ¹
3	+	+	+	719 ¹	850 ¹	1012 ¹
4	-	+	-	612 ²	607 ²	804 ²
5	-	-	+	593 ²	601 ²	797 ²
6	-	+	+	606 ²	610 ²	NA ^c
7	+	-	-	725 ¹	817 ¹	732 ³
8	-	-	-	598 ¹	590 ²	594 ⁴
^a Vaccination is administered at 6 and 21 d of age, subcutaneously in the neck, with a dose volume of vaccine equivalent to 0.5 ml/bird						
^b Challenge by either the genotype VI or VII strain, or by both at an age of 28 d						
^c NA stands for not applicable, due to the 100% mortality obtained by birds of TRT 6 by the age of 40 days						
¹⁻⁴ Means of INF-Y in a column, followed by different numerical superscripts, are significantly different at $P < 0.05$						

Protection By Developed Vaccine

The evaluation of the acquired protection in differently treated broilers by the developed vaccine, against different challenges, is based on birds' cumulative mean of mortality, feed conversion, and live body weight by the market age of 40 d. The feed conversion is calculated by the division of the consumed feed/total body weight of broilers in each isolation room, and the mean was calculated from the feed conversion of birds in the four replicate rooms/treatment.

Discussion

The obtained intracerebral pathogenicity indices of the genotypes VI and VII strains were skewed towards the high scale of pathogenicity, as documented by the World Animal Health Organization [15]. In addition, the high mortality observed in field outbreaks caused by each genotype strain is in agreement with previous literature related to performance of broilers infected by similar strains [7, 16, 17]. The FPCS sequence of the two isolates is confirmative of the velogenic nature of the two genotypes of NDV [18].

The acquired humoral response to the second vaccination in TRTs 1, 2, 3, and 7 is most likely due to class switch from IgM to IgG [19]; the high level of IgG isotype is expected to react efficiently against the different nature of challenges, administered at 28 d of age to birds of TRTs 1, 2, and 3. The specific higher significant immune response to H protein of the homologous compared to heterologous strains used in the challenge indicates most likely a difference in the configuration of H-protein epitopes present in strains of genotype VI and VII, in spite of the typical sequence of their FPCS [20, 21]. Differently challenged birds of TRTs 4, 5, and 6, that were deprived of vaccination, had lower immune conversion by the market age, an in agreement data with a documentation confirming that a challenge by v-NDV in absence of sensitization by vaccines will lead to highly virulent challenge due to absence or presence of an unsubstantial immunity [22, 23]. The fact that the vaccine-HI titer, at 40 d of age in vaccinates that were deprived of challenge (TRT 7), was significantly lower than the maintained titers obtained by vaccinated and challenged birds of TRTs 1, 2 and 3, presents a proof of the ability of developed vaccine to prime the immune cells, enabling them to respond efficiently to the different challenges [24].

The data of Table 4, related to acquired CMI to vaccination and to challenge, quantified by the IFN- γ levels in sera of the differently treated birds, document the impact of administering the developed vaccine at two different ages on inducing higher levels of IFN- γ ; this response is indispensably needed for protection against v-NDV challenges administered at 28 d of age [25, 26]. The data of INF- γ levels post challenge is in agreement with documentations confirming an improvement in INF- γ level of vaccinated - challenged birds compared to unvaccinated - challenged ones [27, 28]. In addition, a previous report showed that INF- γ decays by time in vaccinated birds that are deprived of a secondary antigen exposure (challenge), as observed in birds of TRT 7, while the negative controls maintain similar low background levels of INF- γ at different ages [28].

The significant higher performance of the vaccinated-challenged broilers (TRTs 1, 2, and 3), in comparison to unvaccinated-challenged ones (TRTS 4, 5, and 6) (Table 5) is most likely due to the significant difference in the vaccine-acquired humoral and CMI, an obtained data that is in agreement

with previous documentations [29, 30, 31, 32, 33]. The similar high production parameters of lower mortality percent, lower feed conversion, and high live body weight in birds of TRT 7 and those of the negative controls reflect the safety of the developed killed vaccine on production [34], pending avoidance of stress, by careful handling of the birds during the subcutaneous administration of the vaccine.

Table 5 Performance^a of broilers administered the developed v-NDV vaccine^b and challenged^c compared to other differently treated broilers

TRT	Vaccination	Strain(s)' genotype used In challenge		Performance of broilers up to 40 d of age		
		VI	VII	Mean Mortality %	Mean FCR	Mean live body weight (g)
1	+	+	-	6.2 ¹	1.87 ¹	2350 ¹
2	+	-	+	5.4 ¹	1.83 ¹	2370 ¹
3	+	+	+	5.8 ¹	1.84 ¹	2367 ¹
4	-	+	-	98.3 ²	3.34 ²	1630 ²
5	-	-	+	98.7 ²	3.53 ²	1598 ²
6	-	+	+	100.0 ²	NA ^d	NA
7	+	-	-	1.5 ³	1.75 ³	2395 ³
8	-	-	-	1.8 ³	1.68 ³	2407 ³

^aCumulative performance up to market age of 40 d

^bVaccination is administered at 6 and 21 d of age, subcutaneously in the neck, with a dose volume of equivalent to 0.5 ml/bird

^cChallenge by either the genotype VI or VII strain, or by both at an age of 28 d

^dNA stands for not applicable, due to the 100 % mortality obtained by birds of TRT 6 by the age of 40 days

¹⁻³Means of each performance parameter in a column, followed by different numerical superscripts, are significantly different at P<0.05

Conclusions

This completely randomized design (CRD) allowed to analyze the obtained data statistically, evaluating the efficiency in broilers of the developed vaccine in its induction of protective humoral immunity to H-protein of the v-NDV genotypes VI and VII, and in acquiring significant levels of IFN- γ that provided an

acceptable protection against the different nature of challenges, reflected by the high performance in vaccinated and challenged birds.

Methods

The experimental approach included a determination of the pathological and molecular characteristics of v-NDV strains involved in field outbreaks, preparation of autogenous bivalent v-NDV vaccine from the characterized predominant strains, preparation of different challenges, establishment of a completely randomized experimental design, and evaluation of the immunity and protection provided by the developed vaccine against the different challenges by v-NDV strains. The completely randomized experimental design enabled to address the scientific objectives, aiming at evaluation of the immunity and protection by the vaccine against predominant v-NDV strains.

Pathological and molecular characteristics of v-NDV strains

The intracerebral pathogenicity Index (ICPI) of v-NDV strains was determined by the protocol of Office Internationale des Epizooties (OIE), Manual of Standards for Diagnostic Tests and Vaccines [15]. Briefly, it involved the individual inoculation of each strain of genotype VI or VII into the brain of 10-day-old chicks, offsprings of NDV - free parents, obtained from our institution's hatchery, and monitoring their mortality at specific times. The reverse transcription – polymerase chain reaction (RT-PCR) was implemented to amplify the fusion protein cleavage site (FPCS) and its surrounding dibasic amino acids (35), while the sequencing of the generated amplicons was accomplished by Big-Dye terminator v.3.1 kit (Applied Systems, USA). The mean mortality percent, presented in Table 1, is obtained from field outbreaks in three respective broiler flocks, caused by each strain of genotype VI and VII.

Preparation of autogenous bivalent v-NDV vaccine

The preparation of an autogenous bivalent v-NDV vaccine, containing the pathologically and molecularly characterized strains of genotypes VI and VII, included the growth of each strain in chicken embryos of 10 day-old, obtained from our institution's hatchery. The inoculum applied of each strain through the allantoic membrane route was of 100 particles/100 ul/embryo. The harvesting of the propagated virus was at the day of mortality of the embryo (averaging 48 hr post inoculation). Each harvested strain was adjusted to a strength of 128 HA units against 0.1 % of chicken-red blood cells (c-RBC) suspension. The adjusted harvest of each strain was inactivated by an overnight contact with 0.3 % formalin; the absence of viral viability, post this contact, was checked by individual inoculation of each of the two formalized strains in 10 d - old embryos, followed by incubation for 3 d, and testing the allantoic fluid of embryos for absence of agglutination against a 0.1 % of c-RBC suspension. Equal volume of each adjusted strength of the two inactivated strains were pooled and emulsified with equal volume of mineral oil (viscometer reading of 40 SUS), with an inclusion of Arlacel C (Emulsifier) and Tween 20 (Surfactant), at the level recommended by the manufacturer (TCI Development, Shanghai, China). The emulsified preparation was

passed in a colloidal mill at the following stator gap of 0.002', resulting in water-in-oil micelles with average diameter of 1.2 µm.

Preparation of different challenges

Three different challenges were prepared from the harvested allantoic fluid, deprived of inactivation by formalin. The harvested genotype VI and VII strains was each adjusted to 10^4 x Embryo Infectious Dose₅₀ (EID₅₀). Each bird assigned to specific challenge-treatment received genotype VI strain (TRTS 1 and 4) or VII strain (TRTS 2 and 5), in 50 µl/bird, through the left pectoral muscle. Each challenged bird in TRTs 3 and 6 received 50 µl of each genotype strain in the left pectoral muscle.

Completely Randomized Experimental design

The completely randomized experimental design is comprised of 8 Treatments (TRTs), with four replicate isolation rooms per treatment, in which each room contained 25 meat birds (Table 2), obtained at one-day old from our institution's hatchery. The day-old birds had a mean weight of 38 g, and weight range of 37-40 g. The inclusion of this number of birds is indispensable for providing unbiased data that will allow the implementation of proper statistical analysis for comparison of assessed parameter means among the 8 different treatments. The day-old birds were randomly distributed in their pens, reared on the floor that is previously disinfected and covered with wood shaving; the birds were provided with continuous lighting, controlled temperature and feed formulation, following the instructions of Aviagen Co for Ross 308 broiler management. The birds had a continuous access to feeders and drinkers, while abiding by a welfare-related assessment to the broiler's environment throughout the whole trial. The specie of the experimental birds was *Gallus gallus* domesticus, Ross 308 strain, with ratio of males to females equivalent to 1:1. Birds of TRT 1 were vaccinated each with 0.5 ml of the developed vaccine, delivered subcutaneously in the neck at 6 and 21 d of age, followed by challenge with genotype VI strain at 28 d of age. Birds of TRT 2 were similarly vaccinated but challenged with strain of genotype VII. Birds of TRT 3 were similarly vaccinated but challenged with strains of both genotype strains. Birds of TRTs 4, 5, and 6 were the positive controls, deprived of vaccination but respectively challenged with strains of genotype VI, VII, and VI + VII. Birds of TRT 7 were similarly vaccinated, but deprived of all types of challenge, while birds of TRT 8 were the negative controls, deprived of vaccination and challenge. The experiment was terminated at the market age of 40 days by euthanization, using an injection via the pectoral muscle, equivalent to 20 mg/Kg of tiletamine/zolazepam.

Acquired humoral Immunity by Vaccine and Challenge

The acquired humoral immunity in broilers, specific to the hemagglutinin protein of v-NDV present in the vaccine and challenge, was assessed by hemagglutination-inhibition (HI) test [17]. Blood was collected from the brachial vein of each surviving bird at the ages of 21, 28, and 40 d. Sera were separated from the

collected coagulated blood; the HI titer of each serum was obtained against an antigen of 4 HA units/50 µl of allantoic fluid containing the individual strain of genotype VI or genotype VII, and including in the test a 0.1% of c-RBC suspension [36]. The HI titer of each sample detected the maximum dilution of the collected serum that still contained enough antibodies specific to H protein in the antigen, preventing this protein from binding to its receptor on the c-RBC, and by that inhibiting agglutination phenomenon.

Acquired CMI to developed vaccine and challenges

The same collected chicken sera were also analyzed for the level of IFN- γ at the ages of 21, 28, and 40 d., reflecting the acquired CMI to developed vaccine and challenges in these birds [37, 38]. The analysis of sera IFN- γ was performed by sandwich-ELISA system, using a commercial kit by Shanhai CrystalDay Biotech Co., Yangpu district, Shanghai, China [39, 40].

Protection by developed vaccine

The evaluation of the acquired protection in differently treated broilers by the developed vaccine, against different challenges, is based on birds' cumulative mean of mortality, feed conversion, and live body weight by the market age of 40 d. The feed conversion is calculated by the division of the consumed feed/total body weight of broilers in each isolation room, and the mean was calculated from the feed conversion of birds in the four replicate rooms/treatment.

Statistics

The One Way Analysis of Variance (ANOVA) was used to compare the means of HI titers, IFN- γ, feed conversion, and live body weight, among the eight different treatments; however, the means of cumulative mortality percent, up to market age of 40 d, were compared among the 8 treatments by CHI Square (χ^2). The significant difference among means of each statistically analyzed parameter is reported at $P < 0.05$ [41].

Abbreviations

ANOVA: Analysis of variance; CMI: Cell mediated immunity; c-RBC: Chicken red blood cell; CRD: Completely randomized design; d: day; EID₅₀: Embryo infectious dose₅₀; FPCS: Fusion protein cleavage site; g: gram; GI: Gastrointestinal; H protein: Hemagglutinin protein; HA: Hemagglutination test; HI test: Hemagglutination-Inhibition test; ICPI: Intracerebral pathogenicity index; IFN-γ: Interferon gamma; Ig: Immunoglobulin; K: Lysine; χ^2 : Chi Square; µL: Microliter; µm: Micrometer; OIE: Office Internationale des Epizooties; Q: Glutamine; R: Arginine; RT-PCR: Reverse transcription-polymerase chain reaction; SUS: Sabolt universal seconds; TRT: Treatment; TRTS: Treatments

Declarations

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Authors' contributions

SSY, TAK, and EKB designed the studies. EKB, SSY, TAK, and OAB performed the animal and laboratory work. SSY and EKB wrote the project proposal and study protocols. EKB and SSY recorded the data, analyzed statistically, and reported. TAK and OAB aided in data interpretation and discussion. All authors contributed to writing, proof reading, and approval of the manuscript submission.

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Availability of data and materials

The datasets and analysis are available upon requests made to the E-mail address of the manuscript's corresponding author.

Ethics approval and consent to participate

The experimentation on the broilers complied with the guide of the National Institutes of Health for the care and use of laboratory animals (NIH publication no.8023); this compliance was in agreement with the fundamental ethical principles for animal use, set by Basel Declaration Society, and the ethical guidelines of the International Council for Laboratory Animal Science. In addition, the procedures of this work were approved by the Research and Development Office (RDO) at the Ministry of Education, Kingdom of Saudi Arabia, pending a complete exclusion of all sorts of human experimentation.

Consent for Publication

Not applicable. The manuscript does not contain any individual person's data.

Competing interests

The authors declare that they have no conflict of interests among the authors or any other parties; in addition, the authors declare that they have no financial or non-financial interests that undermine, by any way, the objectivity, integrity, and value of this publication.

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