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The effect of using several infectious bronchitis vaccines on the expression of TLR3 in the broiler trachea and kidney

N.B. Kareem and A.M. Al-Aalim

Department of Microbiology, College of Veterinary Medicine, University of Mosul, Mosul, Iraq

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Correspondence:
A.M. Al-Aalim
ammarmahmmod@uomosul.edu.iq

Abstract

The poultry sector is a relatively recent yet rapidly growing industry, having been established in the early twentieth century, and it provides a protein source for the human population. Infectious bronchitis virus is the most rife virus in the poultry sector, which causes titanic economic loss with mortality rates reaching up to 90%, resulting from severe nephritis. During IBV infection, TLR3, TLR7 are believed to recognize viral RNA, triggering immune responses that restrict viral replication and dissemination. To protect poultry from IBV, inactivated or live-attenuated vaccines are administered. However, the presence of diverse mutant serotypes poses challenges in vaccine design and efficacy. Our study aimed to evaluate the protective efficacy of different IBV vaccines by assessing TLR3 gene expression following immunization with either variant or classical IBV vaccine strains. This was achieved through the use of 160 one-day-old Ross 308 broiler chicks divided into four different groups (N=40) and vaccinated with either the variant or classical IBV vaccine or with both at distenic vaccination programs. Then the trachea and kidney were collected 1, 3, 7, 14, 16, and 21 days post-vaccination to assess TLR3 gene expression by using qPCR with specific primers. The results show that all vaccinated groups increased TLR3 gene expression on the third and sixteenth day post-vaccinationthis lead to concluded that the use of variant or classical IBV vaccine strains in broilers causes an increase in TlRgene expression in the kidney and the trachea, and the combination of variant or classical IBV can increase the efficacy of vaccination due to increased TLR3 expression in broilers.

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Introduction

The poultry industry is a critical economic sector in many countries, serving as a major source of high-protein food at a relatively low cost, particularly in developed nations (1). In our country, the poultry sector is a relatively recent yet rapidly growing industry, having been established only in the early twentieth century (2). This sector has expanded significantly, with the number of operational poultry farms increasing from 2,830 in 2001 to 3,012 in 2022. Concurrently, poultry meat and egg production reached 162.5 tons and 5,010.5 million eggs, respectively. The Nineveh province ranks first among Iraqi provinces in poultry farming, housing 499 operational farms that contribute 24,877 tons of meat and 152,178 million eggs to the local market (3-5).

Infectious bronchitis virus (IBV) is an enveloped, single-stranded and positive-sense RNA virus, belonging to the genus Gamma-coronavirus, within the family Coronaviridae and the subfamily Orthocoronavirinae, order Nidovirales (6). The IBV genome encodes four essential structural proteins: membrane protein (M), envelope protein (E), nucleocapsid protein (N), and spike protein (S) (7). While IBV primarily targets the respiratory system, several field isolates and variants have been associated with infections affecting the kidneys, digestive tract, and reproductive system. The

disease manifestation varies depending on the IBV strain and the affected organ system, the virus-induced symptoms and lesions result from its replication in mucosal cell membranes, particularly in the respiratory and gastrointestinal tracts, leading to macro- and microscopic tissue alterations (8,9). Nephropathogenic IBV strains can induce severe nephritis with mortality rates reaching up to 90%, often with less pronounced respiratory symptoms (10).

The pathogen recognition receptors (PRRs), which are part of the innate immune system, such as Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs), are responsible for detecting viral components (11). TLRs play a crucial role in avian innate immunity by recognising pathogen-associated molecular patterns (PAMPs). In chickens, approximately ten distinct TLRs have been identified, each involved in recognizing different viral ligands and participating in TLR-mediated signalling pathways (12). TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are surface-expressed receptors that detect structural components of pathogens, including proteins, lipids, and lipoproteins. In contrast, TLR3, TLR7, TLR8, and TLR9 are intracellular receptors that primarily recognize viral nucleic acids (13,14).

During IBV infection, TLR3, TLR7, and TLR8 are believed to recognize viral RNA, triggering immune responses that restrict viral replication and dissemination (15). Activation of these TLRs induces the production of interferons and other cytokines, which regulate infection and prime the adaptive immune system. Specifically, TLR3 and TLR7 activate the NF-κB signalling pathway, leading to the release of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which enhance immune cell recruitment and antiviral defences (16). The expression of TLR3 in chickens is comparable to that in mammals and is primarily found in immune cells such as macrophages and dendritic cells, as well as epithelial cells of the respiratory tract (17). Although IBV is a single-stranded RNA (ssRNA) virus, double-stranded RNA (dsRNA) intermediates form during replication, which TLR3 recognizes, similar to other coronaviruses (18-20).

To protect poultry from IBV, inactivated or live-attenuated vaccines are administered. However, the presence of diverse mutant serotypes poses challenges in vaccine design and efficacy. Identifying predominant IBV serotypes and assessing the cross-protective potential of available vaccines are crucial for effective immunization strategies. In Iraq, several regions have reported IBV outbreaks in both layer and broiler flocks (21). Since current vaccines do not provide cross-protection against all IBV genotypes, the disease continues to present significant challenges to the poultry industry (22). Despite the use of inactivated and live-attenuated vaccines (e.g., H120 and 491 strains) in Iraqi poultry farms, IBV outbreaks persist in broiler farms (23,24). This study aims to evaluate the protective efficacy of different IBV vaccines by assessing TLR3 gene expression following immunization with either variant or classical IBV vaccine strains.

Materials and methods

Ethical Approval

All animals were treated and euthanised humanely, and tissue collected according to the instructions provided by the Institutional Animal Care and Use Committee of the College of Veterinary Medicine, under the certification No. UM.VET.2024.029 on 7/15/2024.

Vaccine

Two types of live IBV vaccine (Nobilis IB Ma5 (classical) and 4/91(variant serotype) strain supplied from MSD®/Netherlands were used to study its effect on TLR3 expression after vaccination.

Experimental study

In this study, we used a total of 160 one-day-old Ross 308 broiler chicks. The chicks were housed in separate floor cages within the poultry hall at the College of Veterinary Medicine, Mosul University. Water and feed were provided throughout the experiment. A 100-watt continuous illumination system was maintained for 24 hours (25). The chicks were randomly assigned to four experimental groups (n=40). The first group received distilled water and served as the control, while the second, third, and fourth groups were administered the classical IB vaccine, the variant IB vaccine, and a combination of both classical and variant IB vaccines, respectively. The vaccination protocol included two doses administered via the eye-drop method, with the first dose given on day one and a booster dose on day 14. The animals were euthanised, and tissue samples (kidney and trachea) were collected from each group at 1, 3, 7, 14, 16, and 21 days post-vaccination to assess TLR3 expression. All tissue samples were immediately frozen at -20°C until RNA extraction (26).

RNA Extraction and Amplification

The RNA extraction from both trachea and kidney samples was performed using the SV Total RNA Isolation System (Promega, USA), similar to (27). The Oligo(dT)15 primer was used to synthesize the first strand of cDNA using the GoScript™ Reverse Transcription System (Promega, USA). The amplification of TLR3 and β-actin (Table 1) was conducted using the GoTaq® qPCR Master Mix (Promega, USA). The amplification mixture consisted of 5 µl of cDNA, 10 µl reaction mixture, 2.5 µl each of forward and reverse primers, and 5 µl nuclease-free water, bringing the total volume to 20 µl.

Gene expression was estimated using the Tower 3 qPCR machine (Analytik Jena, Germany). The reaction conditions included an initial denaturation at 95°C for 300 seconds, followed by 40 cycles of denaturation at 95°C for 30

seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The β-actin gene was used as a housekeeping gene to normalise TLR3 gene expression, and the Livak ΔΔCT method was employed to calculate the fold changes in TLR3 gene expression (28).

Statistical analysis

All results were statistically analysed using the GraphPad Prism statistical program Version 8.0.2. The two-way ANOVA analysis explained significant differences between and within the groups (29).

Table 1: The primer sequence in this study

Primer	Sequence	References
TLR3 F	TCA GTA CAT TTG TAA CAC CCC GCC	30
R	GGC GTC ATA ATC AAA CAC TCC	
β-actin F	CAA CAC AGT GCT GTC TGG TGG TA	
R	ATC GTA CTC CTG CTT GCT GAT CC	

Results

The mRNA expression of TLR3 in kidney samples on the first day of the trial show no significant changes (P<0.05); while all groups show significant elevation on TLR3 expression in all vaccinated groups compared to the unvaccinated control group at 3,7,14,16, and 21 days' post vaccination, except G2 (Classical Nobilis IB Mas5) and G4 (classical and variant vaccine) at 7, 16, and 21 days, which have no significant difference.We can see that TLR3 gene expression was elevated at two main points: the third and sixteenth days. The G4 (classical and variant vaccine) showed the highest TLR3 expression level, reaching 2.24 and 2.96 on the third- and sixteenth-day post-vaccination, as seen in figures 1 and table 2.

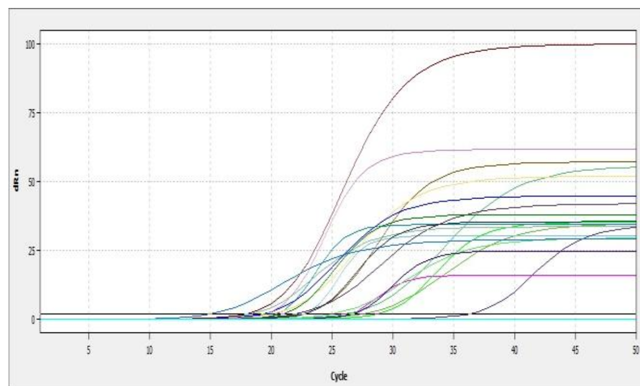

Figure 1: Amplification curve of TLR3 in kidney samples using qPCR.

Also, we recorded no significant differences between all days in the first group at a value of (P<0.05).; while the second Group (Classical Nobilis IB Mas5), the third group (Variant Nobilis 4/91 Strain Vaccine), and the fourth group (Classical Nobilis IB Mas5 and Variant Nobilis 4/91 Strain Vaccine) recorded significant differences within their days, with some exceptions found in the second and third groups, as seen in figure 2 and table 2.

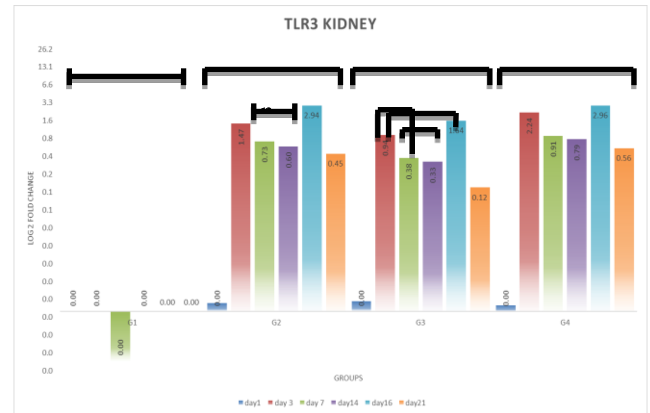

Figure 2: The kidney TLR3 gene expression between days within the groups of vaccinated animals.

The result of the TLR3 mRNA expression levels in trachea samples showed significant changes between all groups at (P<0.05), on the first days of experiment but no significant differences were found between the (G2 vs. G4, G3 vs. G4) groups. All groups show significant elevation on TLR3 expression in all vaccinated groups compared to control group at 3,7,14,16, 21 days' post vaccination, except G2 and G3 at 16 days, which have no significant difference. In our study, we can see that there was an elevation of TLR3 gene expression in two main points at the third and sixteenth and the G4 (classical and variant vaccine) shows the highest TLR3 expression level, reaching 20.0 and 9.61 on the third- and sixteenth-days post-vaccination, as seen in figures 3 and table 3.

The results recorded that there were no significant differences between all days in the first group, except (D3 vs. D14, D14 vs. D21), which showed significant differences at (P<0.05). The significant differences in results recorded between all days in the second, third, and fourth groups, respectively, except for some days that showed no significant differences at (P<0.05). As seen in figures 4 and table 3.

Table 2: The kidney TLR3 gene expression using qPCR between and within days in groups

Days	Groups			
	G1 (No Vaccine)	G2 (Classical Nobilis IB Mas5)	G3 (Variant 4/91 Strain Vaccine)	G4 (Classical And Variant Vaccine)
D1	0.950 ±0.14 A	1.295±0.10	1.495 ±0.08	1.180 ±0.01
D3	0.950 ±0.22 A	1474±0.86	962.1 ±14.91 C	2236 ±0.46
D7	0.325 ±0.22 A	739.1±3.025 B,a	372.7 ± 0.29 CD	905.1 ±0.05,a
D14	0.950 ±0.00 A	593.4±1.55 B	331.6 ±0.17 D	786.2 ± 1.03
D16	0.950 ±0.00 A	2959±20.1b	1630 ±3.29C	2944 ±8.44 b
D21	0.950 ±1.88 A	453.9±3.32 c	121.4 ±3.74	555.1 ±3.74 c

All data represented the Mean ± SD of TLR3 mRNA gene expression. The similar Capital letter indicates no significant difference between days within groups at P<0.05. The similar small letter indicates no significant difference between groups at P<0.05.

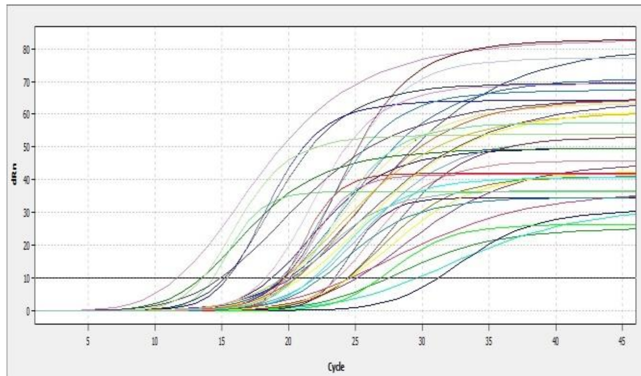

Figure 3: Amplification curve of TLR3 in trachea samples using qPCR.

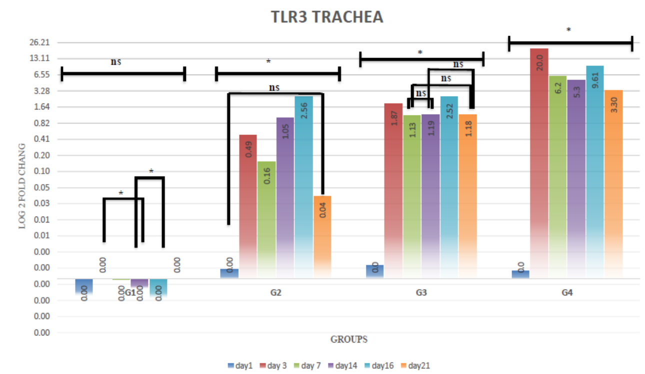

Figure 4: The trachea TLR3 gene expression between days within the groups of vaccinated animals.

Table 3: The Trachea TLR3 gene expression using qPCR between and within days in groups

Days	Groups			
	G1 (No Vaccine)	G2 (Classical Nobilis IB Mas5)	G3 (Variant 4/91 Strain Vaccine)	G4 (Classical And Variant Vaccine)
D1	0.6 ±0.1 A	1.465±5.5 F,a	1.765±5.7 b	1.30±0.35 ab
D3	1± 0.08 AB	485.6±2.80	1866±1.59	20026±1.68
D7	0.885 ± 0.09 A	152±0.05	1124±2.83 G	6204±0.26
D14	0.69 ± 0.06 AC	1043±0.5	1191±5.15 GF	5256±0.45
D16	0.525 ± A	2553±1.9 p	2525±3.87GF,p	9604±3.93
D21	1±0.01 AE	34.37±1.3 F	1167±11.46	3304±3.47

All data represented the Mean ± SD of TLR3 mRNA gene expression. The similar Capital letter indicates no significant difference between days within groups at P<0.05. The similar small letter indicates no significant difference between groups at P<0.05.

Discussion

The role of TLR3 in viral immunology is well established, and it is well known for its ability to recognize pathogen-associated molecular patterns (PAMPs) encoded by RNA viruses (31,32). TLRs (Toll-like receptors) are the primary PRRs that are considered to participate in the identification of components of viruses. These findings are comparable to those seen in human beings (12). The expression of chicken TLR3 appears to be comparable to that of mammals (33). Toll-like receptor 3 (TLR3) expression in the kidneys of chickens vaccinated with classical IBV Ma5, variant IBV 4/91, and a mixture of both strains was evaluated in this work. When viral double-stranded RNA is detected by TLR3, a major pattern recognition receptor (PRR), it triggers innate immune responses that are essential for

antiviral defence (34). No significant alterations in TLR3 expression were observed in the control group (G1) during the study duration, confirming the absence of viral stimulation and establishing the concept of unchallenged birds maintaining stable baseline TLR3 levels. On the other hand, all groups that received the vaccine (G2, G3, and G4) showed upregulation of TLR3 expression, with most prominent changes occurring after the booster dose. Maximum TLR3 expression was reached on Day 16 (two days post-booster), suggesting that the additional immunogenicity of the booster given on Day 14 improves and maintains innate immune responses significantly. This observation is consistent with what is already known about the dynamics of TLR activation, where repeated exposure to an antigen yields faster and stronger TLR3 immune responses (35). The findings demonstrate that group G4, the combined vaccination cohort, exhibited the highest TLR3 expression among all groups. This was probably due to the simultaneous administration of the Ma5 and 4/91 strains, which provided enhanced relative PAMPs (36). Evidence suggests that heterologous prime-boost employing antigenically different strains can enhance both the innate and adaptive immune responses (37). In the comparison of single-strain vaccine groups, G2 (Ma5) had a stronger and earlier TLR3 response than G3 (4/91). This may indicate inherent changes in the viral replication ability and antigenic properties of the Ma5 strain compared to the 4/91 variety. Ma5 is a traditional strain recognized for its strong immunogenicity, although variant strains such as 4/91, despite being antigenically distinct, may provoke a delayed innate response (38). The significant elevation of TLR3 in the kidneys and respiratory tract indicates a systemic innate immune response following IBV immunization. Given that IBV exhibits a tropism for renal tissue and respiratory epithelium (39), our findings further substantiate the systemic nature of IBV infection and immunity. Taken together, vaccination with IBV strains promoted the TLR3-mediated innate immune response in the kidney and the most potent response was induced by the combined vaccine (Ma5 + 4/91). The booster immunization additionally enhanced TLR3 expression, indicating the need for a booster injection for optimal activation of the innate and possibly adaptive immune system (40).

The expression of TLR3 in the tracheal tissue of chickens post-vaccination with various Infectious Bronchitis Virus (IBV) strains: classical MA5, variation 4/91, and a combination MA5 + 4/91 approach. Represented an essential element of the innate immune system, TLR3 particularly identifies viral double-stranded RNA (dsRNA) and triggers antiviral signalling pathways, which include the synthesis of type I interferons and pro-inflammatory cytokines (18,41). The absence of innate immune stimulation was confirmed by the fact that TLR3 expression in the unvaccinated control group (G1) remained at baseline (0.00 log2 fold change) across all time points. This is in accordance with research that indicates that TLR3 expression in avian respiratory tissues remains low in the absence of pathogenic exposure (42). These birds function as a negative control and emphasize that the observed gene expression changes in vaccinated groups are vaccine-induced.In chickens vaccinated with the classical MA5 strain (G2), TLR3 expression increased slightly, reaching a peak at day 16 (2.56) before experiencing a significant decline by day 7 (0.16) and nearly returning to baseline levels by day 21 (0.04). The initial increase indicates a moderate activation of innate immune signalling, presumably resulting from the recognition of viral RNA following vaccination. The swift decrease following the booster suggests a temporary immune response, which may be inadequate for long-lasting protection. Classical IBV strains have been shown to induce weaker innate immune activation in comparison to variant strains (43). The group vaccinated with the variant 4/91 strain (G3) demonstrated elevated and prolonged TLR3 expression. A moderate response was recorded on day 3 (1.87) and day 7 (1.13), with expression reaching its peak on day 16 (2.52) after the booster administration. The sustained increase in TLR3 levels indicates that the 4/91 variant elicits a more robust antiviral response, potentially attributable to variations in replication efficiency or dsRNA presentation that more effectively activate TLR3 (44). The enhanced innate activation may facilitate improved mucosal immunity within the respiratory tract.The combined vaccine group (G4) exhibited the most pronounced and sustained upregulation of TLR3, with peak values recorded at day 3 (20.0) and a maximum response at day 16 (9.61) following the booster administration. Expression was still elevated on day 21, measuring (3.30). The results suggest a synergistic effect of the combined vaccine, likely attributable to broader antigenic stimulation and increased dsRNA production, both of which enhance TLR3 activation. This aligns with earlier studies indicating that multivalent or combination vaccines can generate stronger and more enduring innate and adaptive immune responses (45). The observed biphasic peak pattern—early (day 3) and late (day 16)—in vaccinated groups corresponds to the timing of primary and booster vaccinations administered on day 1 and day 14, respectively. This timing corresponds with established TLR kinetics, wherein initial TLR activation primes immune responses, and subsequent exposure enhances gene expression through memory-like innate pathways (46). The delayed peak observed on day 16 in G4 indicates effective recall responses, which is a favourable characteristic of vaccine-induced immunity.

Conclusion

This study concludedthat the use of variant or classical IBV vaccine strains in broilers causes an increase in TLR3 gene expression in the kidney and the trachea, and the combination of variant or classical IBV can increase the

efficacy of vaccination due to increased TLR3 expression in broilers.

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Conflict of interest

No conflict of interestwhere found

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تاثير استخدام أنواع مختلفة من لقاحات اتهاب القصبات المتعدد على التعبير الجيني لمستضدات التول ٣ في الرغامي والكلية في دجاج التسمين

نشوان برهان كريم و عمار محمود احمد العالم

فرع الأحياء المجهرية، كلية الطب البيطري، جامعة الموصل، الموصل، العراق

الخلاصة

يُعد قطاع الدواجن من الصناعات الحديثة نسبيًا وسريعة النمو، إذ بدأ تطوره في أوائل القرن العشرين، ويُشكّل مصدرًا مهمًا للبروتين للإنسان. يُعتبر فيروس التهاب الشعب الهوائية المعدي (IBV) من أكثر الفيروسات شيوعًا في هذا القطاع، حيث يُسبب خسائر اقتصادية جسيمة، مع معدلات نفوق قد تصل إلى ٩٠% نتيجة الإصابة بالتهاب كلوي حاد. يُعتقد أن المستقبلات الشبيهة بالتول TLR3) و (TLR7تتعرف على الحمض النووي الريبي الفيروسي أثناء العدوى بـIBV ، مما يؤدي إلى تفعيل الاستجابات المناعية التي تحد من تكاثر الفيروس وانتشاره. ولغرض الحماية من فيروسIBV ، تُستخدم لقاحات معطلة أو حية مُضعفة. ومع ذلك، فإن وجود أنماط مصلية متحورة ومتنوعة يُشكل تحديًا في تصميم اللقاحات وفعاليتها.هدفت دراستنا إلى تقييم الكفاءة الوقائية لأنواع مختلفة من لقاحات IBV من خلال تحليل تعبير جين TLR3 بعد التمنيع بسلالات لقاحية كلاسيكية أو متحورة. تم تنفيذ الدراسة باستخدام ١٦٠ فرخًا من سلالة روس ٣٠٨ بعمر يوم واحد، قُسمت إلى أربع مجموعات(N=40) ، وتم تلقيحها إما بلقاح متحور أو كلاسيكي أو بكليهما وفق برامج تلقيح مختلفة. ثم تم جمع عينات من القصبة الهوائية والكلى في الأيام ١، ٣، ٧، ١٤، ١٦، و٢١ بعد التلقيح لتحليل تعبير جين TLR3 باستخدام تقنية qPCRوباستعمال بادئات نوعية.أظهرت النتائج أن جميع المجموعات المُلقحة سجلت زيادة في تعبير جين TLR3 في اليومين الثالث والسادس عشر بعد التلقيح. ويُمكن الاستنتاج من ذلك أن استخدام سلالات لقاحية كلاسيكية أو متحورة من فيروس IBV في دجاج اللحم يؤدي إلى زيادة في تعبير جينات TLR في الكلى والقصبة الهوائية، وأن الجمع بين اللقاحين قد يُحسّن من فعالية التحصين نتيجة زيادة التعبير الجيني لـ TLR3في دجاج التسمين.