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

Molecular Investigation of Interference of *E. coli* and *Mycoplasma gallisepticum* with Immune Response of Vaccinated Broiler Chicken by Newcastle Disease and Infectious Bronchitis Vaccines

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Abstract | This study aims to investigate the molecular interactions and interference of *E. coli* and *Mycoplasma gallisepticum* infections with the immune response of broiler chickens vaccinated with Newcastle Disease (ND) and Infectious Bronchitis (IB) vaccines. A total of 200 day-1 old broiler chicken was divided into five groups. Each group contains (Forty birds) as follows: G1: vaccinated with Newcastle disease (ND) vaccine via injection, spray, and drinking water on day 1, day 7, and day 17, respectively. G2: vaccinated by ND injection on 1 day, ND via drinking water + IB via spray on day 7, and ND vaccine via drinking water and IB via spray at 17 days old. G3: vaccinated with ND via injection and ND eye drop on day 1, IB via spray on day 7, and ND via spray in 17 days. G4: vaccinated with ND + IB (mixed) via eye drop, spray, and drinking water on day 1, day 7, and day 17, respectively. G5 was kept as control. The results for bacterial identification of *E. coli* cultured were positive, the MPN/g for counting of *E. coli*, and *M. gallisepticum* as gene expression, titer antibody response to IB, and ND virus were significantly increased in the mixed vaccinated group (G4), in contrast to other treated groups. In conclusion, the mixed live IB and ND vaccination program induced a stronger antibody response compared to single vaccinations, which contributed to significantly higher levels of secondary bacterial infections in the mixed-vaccine group.

Keywords | Newcastle and infectious bronchitis, *E. coli* and *M. gallisepticum*, Secondary infections, vaccination, Broiler chickens

Received | November 05, 2025; **Accepted** | December 10, 2025; **Published** | December 22, 2025
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Citation | Fajer OA, Abdulmaged SH (2025). Molecular investigation of interference of *E. coli and Mycoplasma gallisepticum* with immune response of vaccinated broiler chicken by Newcastle disease and infectious bronchitis vaccines. J. Anim. Health Prod. 13(s1): 954-961.
DOI | https://dx.doi.org/10.17582/journal.jahp/2025/13.s1.954.961
ISSN (Online) | 2308-2801

INTRODUCTION

The growth of the poultry industry is severely hampered by the elevated prevalence of poultry diseases, which calls for immunising hens before the onset of sickness (Bodman-Harris *et al*., 2024). At the global, national, and farm levels, several tactics can be used to successfully prevent and control the introduction and spread of poultry illnesses. Vaccination is frequently a part of poultry disease management plans (Alqazlan *et al*., 2022; Abdelaziz *et al*., 2024). Therefore, vaccinations and their administration techniques might be viewed as protection against certain infectious illnesses. Numerous elements, including vaccine handling, vaccine quality and type, use of local antigens, immunogenic action inside the bird's body, and adherence to company recommendations, are necessary for an effective vaccination program (Raji *et al*., 2024; Al-Zuhariy, 2023). Even in flocks that have received vaccinations, the possibility of disease outbreaks cannot be totally eliminated because of vaccine defects (Gloanec *et al*., 2025).

The poultry industry faces significant challenges from infectious diseases, particularly respiratory illnesses caused by pathogens like *Mycoplasma gallisepticum* (*M. gallisepticum*) and *Escherichia coli* (*E. coli*) (Mohammed, 2008; Yehia *et al*., 2023). These bacteria not only pose direct threats to broiler health but also complicate the efficacy of vaccination programs designed to protect against viral illnesses, including Infectious Bronchitis (IB) and Newcastle Disease (ND). The interplay between these bacterial infections and viral vaccinations is crucial for understanding overall flock health and productivity (Śmiałek *et al*., 2020; Fasina *et al*., 2024). When broiler chickens are vaccinated against ND or IB, their immune systems are primed to respond to viral antigens; however, concurrent infections with *E. coli* can lead to an immune response that is skewed or diminished, potentially reducing vaccine efficacy (Huang *et al*., 2020; Bagheri *et al*., 2023).

M. gallisepticum is a widespread cause of respiratory infections that have led to higher mortality rates and significant financial losses for the global poultry industry. Because *M. gallisepticum* infections downregulate the host immune response, they are frequently linked to co-infection outbreaks of other pathogens, including *E. coli,* that alter the immunological landscape in vaccinated birds (Ali et al., 2024; Liu *et al*., 2024). Vaccination against ND stimulates both humoral and cellular immune responses, but the presence of *M. gallisepticum* and *E. coli* can lead to immunosuppression or a dysregulated immune response. This can result in a decreased ability to mount an effective response to viral antigens, leading to increased susceptibility to viral diseases (Feberwee *et al*., 2025; Kamal *et al*., 2025).

This study investigates the effect of Infectious Bronchitis and Newcastle disease vaccinations on the immune response and disease pathogenesis in broilers following a challenge with *E. coli* and *M. gallisepticum*, a scenario designed to mimic viral vaccine-induced immunological stress.

MATERIALS AND METHODS

This study was conducted at the University of Baghdad's College of Veterinary Medicine, were kept in the College animal house total 200 day-1 old broiler chicken were divided into five groups each group contains (Forty birds) and vaccinated with ND and IB vaccines (Newcastle disease vaccine: MO5+CLONE 30/ Intervet International B.V. (MSD Animal Health)/ Holland), (Infectious bronchitis virus: PROVAC™ IB/ Kompharm Ltd. – Biofactory/ Serbia) (Mixed vaccine: Mo5 + Clone 30/ Intervet International B.V. (MSD Animal Health)/ Holland). as following: G1 group: This group will be vaccinated on day 1 with Newcastle disease vaccine via injection, on 7-day Newcastle disease via spray and on day 17 Newcastle disease via drinking water. G2 group: This group will be vaccinated on day 1 with Newcastle disease injection, on 7-day Newcastle disease via drinking water + Infectious Bronchitis via spray, and on 17-day-old Newcastle disease vaccine via drinking water and Infectious Bronchitis via spray. G3 group: This group will be vaccinated in one day with Newcastle disease via injection and Newcastle disease eye drop, on 7 days, Infectious Bronchitis via spray, and on 17 days Newcastle disease via spray. G4 group: This group will be vaccinated on day 1 with Newcastle disease + Infectious Bronchitis (mixed) via eye drop, on 7 days Newcastle disease + Infectious Bronchitis (mixed) via spray, and on 17 days Newcastle disease + Infectious Bronchitis (mixed) via drinking water. G5 group: as Control (negative).

Blood samples were collected at day 1 to check and measure maternal immunity using the ELISA test for IB and ND. Antibody responses were measured at days 7, 14, and 28 using the ELISA test for IB and ND. To Bacterial detection, swabs were taken from trachea, lung and intestine on day 7, 17 and repeat on day 25 as following: detection of *E. coli* by culture media (Eosin Methylene Blue and MacConkey agar/ (Himedia/ India)) method and via PCR (ThermoFisher/ USA) for molecular detection of 16S rRNA gene for *E. coli* (27F: 5'- AGAGTTTGATCCTGGCTCAG- 3' and 1492R: 5'-GGTTACCTTGTTACGACTT-3') at 17 and 35-days old, counting in feces using the most probable number (MPN/g) approach the G4 group were highly significant than other groups and the G5 (control) showed zero MPN/g and detection of *M. gallisepticum* by Real-Time PCR using RNA extraction kit (Geneaid Biotech Ltd. Kit, New Taipei City/ Taiwan) and The cDNA was synthesized by using Geneaid™ DNA Isolation Kit, First-Strand cDNA Synthesis SuperMix kit (New Taipei City/ Taiwan) at 7, 17 and 25-days old of chickens.

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyse the data, with a significance threshold of $P<0.05$. As explained by Snedecor and Cochran (1983), the least significant differences (LSD) were used to identify specific group differences.

RESULTS AND DISCUSSION

Counting of *E. coli* in feces using the most probable number (MPN/g) approach

The Most Probable Number (MPN/g) of *E. coli* in chicken feces is shown in Figure 1, which is especially helpful for low organism concentrations (<100/g), comparing five groups (G1–G5) at two time points: 17 days and 35 days, Groups G1–G4 show varying bacterial loads, while G5 has 0 MPN/g (negative control). The G4 group showed a

significant (P<0.05) increase in the MPN/g for counting of *E. coli* as compared to other groups (G1, G2, G3, G5 groups). All groups except G5 showed an increase in *E. coli* counts by 35 days, besides the G4 group also showed a significant (P<0.05) increase in the MPN/g in this duration than the other infected groups.

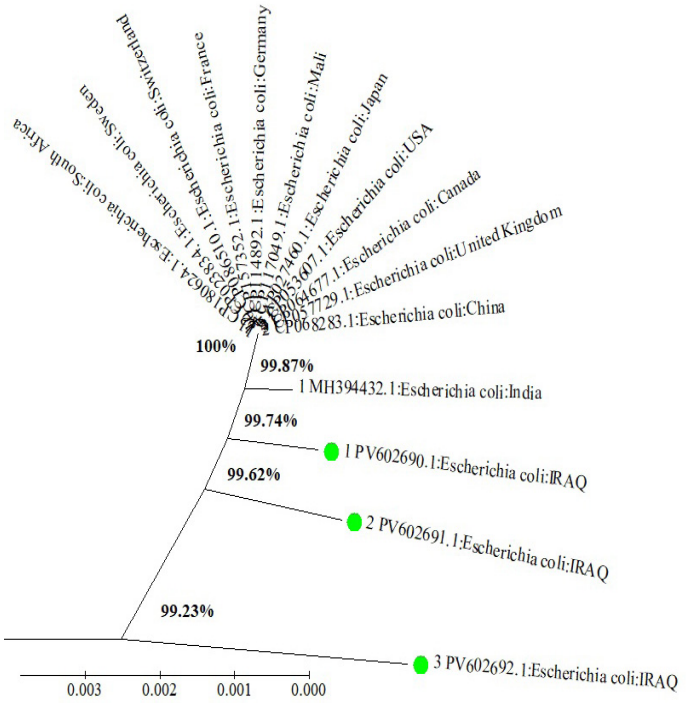

Figure 1: Phylogenetic Tree constructed by neighbor-joining.

Molecular analysis by using the 16S rRNA gene for *E. coli*

E. coli isolates were molecularly identified by comparing their 16S rRNA gene sequences to reference bacterial strains included in the NCBI GenBank database, based on percentage identity (Table 1). The amplification results using the 16S rRNA gene for *E. coli* produced full sequences of 1,250 bp, as shown in Figure 2, for three isolates. The *E. coli* strains have been registered in GenBank under the accession number Iraqi isolates of *E. coli* strain ID: PV602690.1, as illustrated in Figure 3-4. Additionally, the 16S rRNA gene alignment of the *E. coli* strains with a few National Center for Biotechnology Information (NCBI) databases revealed that this isolate, OR142657.1, is 99% similar to the Indian isolate MH394432.1.

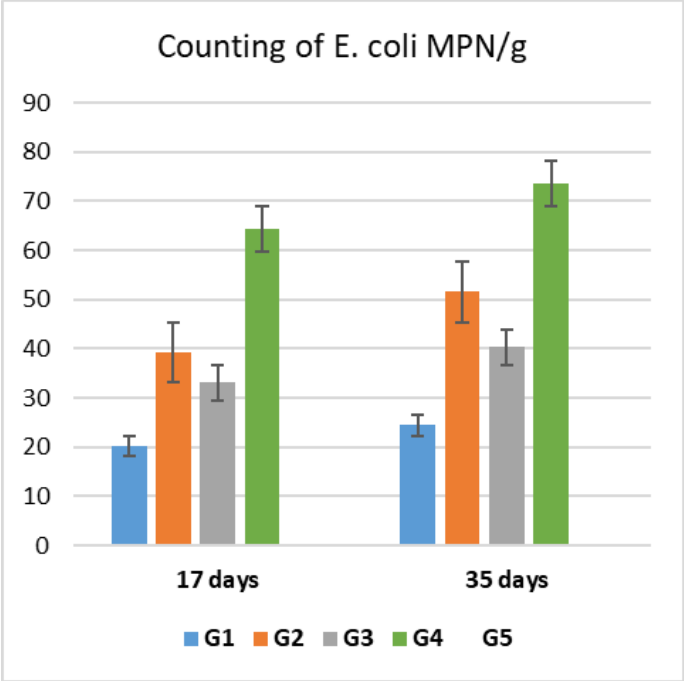

Figure 2: *E. coli* in feces counted using the most probable number (MPN/g) approach.

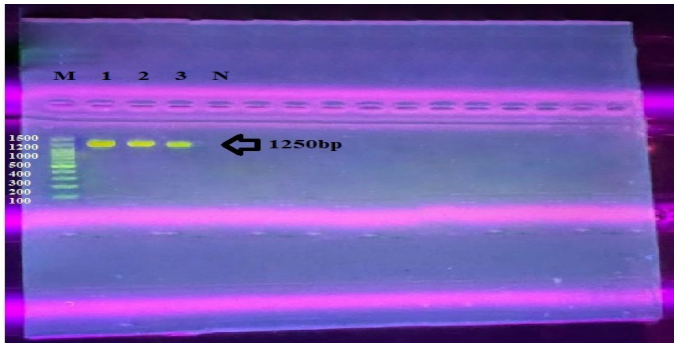

Figure 3: PCR product: the band size. The product was electrophoresed on 1.5% agarose at 5 volts/cm2. 1x TBE buffer for 1:30 hours. M: DNA ladder (100).

Table 1: Molecular identification of *E. coli* isolates was conducted by comparing the 16S rRNA gene sequences with reference bacterial strains in the NCBI GenBank database, based on percentage identity.

16S ribosomal RNA gene					
Ident-ities	**Sequence ID with submission**	**Sequence ID with compare**	**Source**	**Nucleoti-de**	**Type of substitution**
99%	ID: PV602690.1	ID: MH394432.1	*E. coli*	T\A	Transvertion
99%	ID: PV602691.1	ID: MH394432.1	*E. coli*	C\T	Transition
				C\A	Transvertion
99%	ID: PV602692.1	ID: MH394432.1	*E. coli*	C\T	Transition
				A\G	Transition
				T\G	Transvertion
				C\T	Transition
				C\G	Transvertion

Molecular detection of *M. gallisepticum*

Molecular detection of *M. gallisepticum,* as gene expression changed significantly over 25 days, is shown in Figure 4 as follows: Day 7: Expression decreased in the control group (G5) and increased in G4, while groups G1-G3 showed intermediate levels. Day 17: Expression surged in all treated groups (G1-G4), peaking in the vaccinated group (G4). The control group (G5) remained stable and significantly lower, and on Day 25, Expression increased significantly again in G4. Groups G2 and G3 remained elevated with no significant difference between them, while G1 and the control (G5) stayed consistently and significantly low.

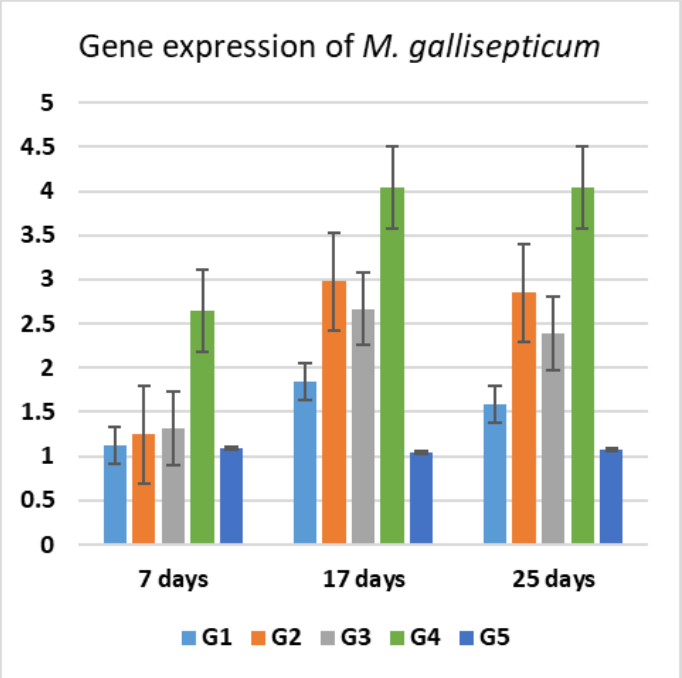

Figure 4: Gene expression of *M. gallisepticum* in broiler chickens in different periods by RT.PCR.

Measuring the antibody titer in serum against NDV by the ELISA test

The result of the titer antibody response against Newcastle virus, as shown in Figure 5 varied significantly by group and time: day 7: G5 group had the highest titer, significantly exceeding all others, G3 and G4 followed, while G1 and G2 were significantly lower, in period of day 14: A significant increase was detected in G1, while G5 decreased significantly. Groups G2 and G4 showed intermediate levels, and in period of day 28, the mean antibody titer peaked significantly in G1, followed by G4 and G3. Group 5 (G5) showed a significant decrease, indicating a transient immune response.

Measuring the antibody titer in serum against IBV by the ELISA test

The result in Figure 6 shows that the mean value of antibody titer response against infectious bronchitis; at 7 Days period: There is no a significant difference between G1, G2, G3 and G5 vaccinated groups as compared with G4 group at the same period, a significant increase in the antibody titer was detected during day 14 and day 28 periods in G4 group as compared to G2, G3, G1, and G5 vaccinated groups, respectively.

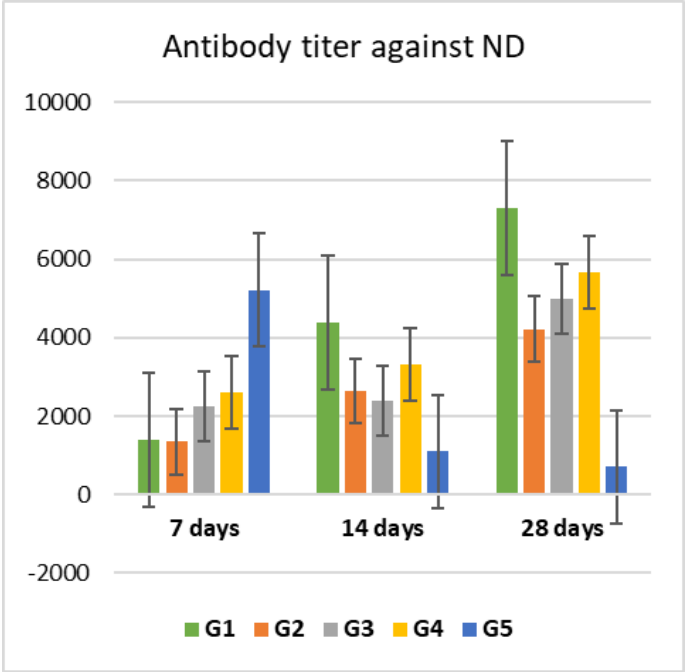

Figure 5: Antibody titer in serum against NDV of broiler chicken in different periods by ELISA test, (pg/ml).

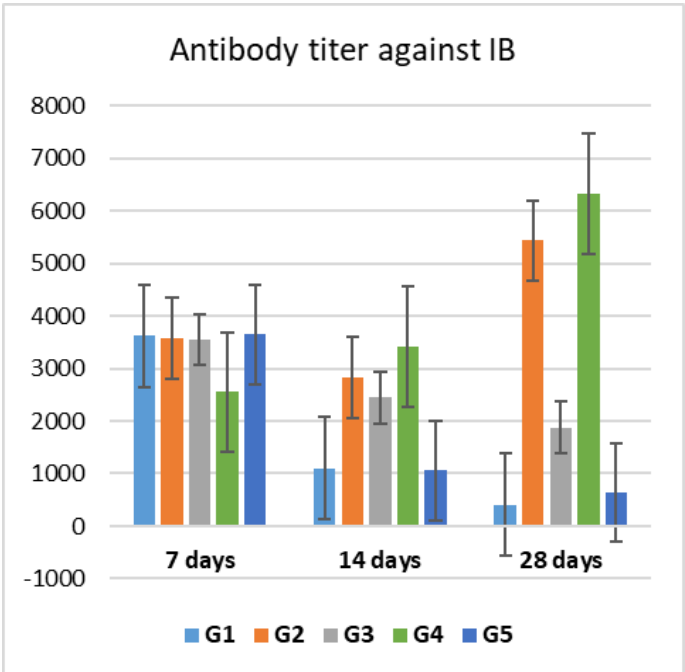

Figure 6: Antibody titer in serum against IBV of broiler chicken in different periods by ELISA test, (pg/ml).

Culture tests showed the presence of pathogens *E. coli* all isolates of bacteria in produced colonies when grown on Eosin Methylene Blue with a green metallic sheen after 24 hours of incubation at 37 °C and a pink color on

MacConkey (MAC) agar, indicating their ability to ferment lactose, which is a characteristic feature for identifying this bacterium, these growth characteristics further support the identity of these isolates as *E. coli* this study's findings of *E. coli* colony morphology on MAC and EMB agar were consistent with those made by (Islam *et al*., 2024; Zarif *et al*., 2025). The results in Figure 1 exhibit the identification of *E. coli* contaminated and shading from chickens through the MPN method revealed that broiler chickens in the G4 group a significantly higher levels than the other groups at day 17 and day 25 (Wardhana *et al*., 2021) suggested the samples were considered positive if the MPN was higher than 1×101 CFU/g. The presence of bacteria in the fecal sample of broiler chicken and associated surfaces may also contribute to infection of the flock (Martínez-Chávez *et al*., 2015).

Table 1 presents the molecular identification of bacterial species, including the percentage similarity between the diagnosed isolates and reference strains in the NCBI GenBank. Given that these isolates were frequently detected in mixed infections, they are likely opportunistic rather than highly pathogenic in poultry. This observation aligns with the findings of (Kravik *et al*., 2023), who reported a high prevalence of these serotypes in healthy poultry flocks during non-outbreak conditions. However, these results contrast with (Alkurtany and Sarhan, 2024), who identified NCBI-registered bacterial strains with a 99.17–99.26% match rate. Since none exhibited a 100% match, these isolates can be considered genetically distinct. This divergence may result from genetic variations induced by environmental factors, including pollution, which can drive mutations and subsequent physiological changes in bacterial species (Hassan and Ajaj, 2021). The high prevalence of *E. coli* (99%) identified in this study is in agreement with findings from a previous thesis (M'sadeq, 2019; Huda, 2024), which reported that *E. coli* has been frequently associated with yolk sac infections in poultry. The prevalence observed in this study agrees with these earlier findings, strengthening the widespread nature of *E. coli* contamination in chick yolk sacs and its significant role in causing early mortality in poultry (Ulmer Franco, 2011).

M. gallisepticum is one of chronic respiratory diseases and regarded as one of the primary diseases that impair chicken production and result in significant financial losses, particularly when they coexist and contribute to subsequent viral infections (Chandhar, 2018; Basit *et al*., 2021; Abtisam *et al*., 2024), so the vaccination of broiler with NDV and IB vaccine result in immunosuppression to flock and allow to infected by the secondary infection such as *M. gallisepticum* which is responsible for respiratory problems and the annual global economic losses incurred by these organisms to the poultry industry. The samples in all groups (G1, G2, G3, G4, and G5) that were used to isolate *M. gallisepticum* were taken from the lungs, trachea, and intestine because it was found to be feasible and beneficial according to the previous study of (Mahmmoud *et al*., 2022), who isolated *M. gallisepticum* from these organs and identified it by PCR. Based on gene expression of the five groups the result showed the G4 vaccinated group is a highly affected by secondary infection with *M. gallisepticum* than the other groups because this group (G4) vaccinated by mixed vaccine by NDV and IB which is exhibited high expression levels of gene expression in three periods (7, 17 and 25 days) and decreased gradually into G1 group that is vaccinated by one methods and one vaccine in each period. This finding agrees with (Wu *et al*., 2019) reported among these pathogens, because *M. gallisepticum* infections down-regulate the human immune response, and they are frequently linked to co-infection outbreaks of other diseases. As intensive farming has expanded, the risk of co-infection has increased. Co-infection with *M. gallisepticum* and *E. coli*, however, is not well documented (Sid *et al*., 2016; Aljoburi, 2024). Additionally, the findings support the findings from (Bakaletz, 2017; Ali and Ali, 2019) that viruses induce secondary bacterial co-infection through a variety of pathways across the airway. These results concur with (Wu *et al*., 2019), who shows that simultaneous infection with *M. gallisepticum* and *E. coli* leads to more severe inflammatory damage in chickens compared to single-pathogen infections, resulting in bronchial cilia loss and excessive mucus buildup in the lungs.

This experiment investigates the impact of vaccinations against viral diseases, Newcastle (ND) and Infectious Bronchitis (IB), on the immune response and disease pathology in broilers that are later exposed to bacterial infections by *E. coli* and *M. gallisepticum*. Figure 5 shows the antibody titer (pg/ml) against Newcastle Disease Virus (NDV) in broiler chickens across five groups (G1–G5) at three time points (7, 14, and 28 days) using the ELISA technique. The antibody titers at 7 days revealed a reduction in G1, G2, G3, and G4 (which were vaccinated with both NDV and IB) compared with G5 (negative control). This result is consistent with (Sanz-Muñoz *et al*., 2025), who state that using live vaccines during the first week of life to protect chicks against diseases, while maternal antibodies are present, can neutralize the antigen, causing the vaccine not to provide active immunity because high maternal antibody levels hinder vaccine growth and reduce the immunity generated in chicks (Karami *et al*., 2024; Sanz-Muñoz *et al*., 2025).

G1 group of broilers, later exposed to bacterial infections by *E. coli* and *M. gallisepticum*, received a vaccine against Newcastle disease virus (NDV) using different methods. Serological analysis showed a progressive increase in

antibody titers (pg/ml) at 14- and 28-days post-vaccination in these groups. This aligns with (Abdoshah *et al*., 2022; Mahmmoud *et al*., 2022), who demonstrated that re-vaccinating with the ND vaccine after 14 and 20 days could induce both specific and non-specific immune responses against NDV, which correlates with the development of protective NDV antibody titers. Additionally, a sufficient number of non-specific substances, such as cytokines like interferon gamma, might be produced, promoting the formation of natural killer T-lymphocytes, macrophages, cytotoxic T-lymphocytes, and antigen-stimulated B-lymphocytes (Riaz *et al*., 2021). Furthermore, the G4 group, vaccinated with NDV and IB at three different periods, showed an increase in antibody titers between 14 and 28 days compared to G2, which received both vaccines but separately, in different ways and times. G2 exhibited lower antibody titers. The G3 group, vaccinated only with the ND vaccine in two periods (1 and 17 days), while administering the IB vaccine via spray at 7 days old, showed different results. IBV and NDV interact because both initially infect the respiratory tract's epithelial cells before replicating in the cytoplasm (Gelb *et al*., 2004; Talib and Ulaiwi 2025). Using a combination vaccine is better than administering two different vaccines simultaneously, as the Infectious Bronchitis vaccine may influence the response to the Newcastle Disease vaccine (Kang *et al*., 2019).

CONCLUSIONS

The mixed live IB and ND vaccination program (G4) induced a stronger antibody response compared to single vaccinations, but caused more severe pathological lesions in the trachea, lungs, and intestines. These lesions likely contributed to significantly higher levels of secondary bacterial infections (*E. coli* and *M. gallisepticum*) in the mixed-vaccine group. Despite the improved immune response, the mixed vaccine is not recommended for field use due to the unacceptable trade-off between enhanced immunity and increased tissue damage and disease susceptibility.

ACKNOWLEDGEMENT

Sincere appreciation is extended to the Department of Pathology and Poultry Diseases, College of Veterinary Medicine, University of Baghdad, for providing laboratory and animal housing facilities and general support during the research.

NOVELTY STATEMENT

This study is the first to evaluate the combined effect of *E. coli* and *Mycoplasma gallisepticum* infection on the immune response of broilers vaccinated with a mixed Newcastle disease and infectious bronchitis vaccination program, suggesting that mixed vaccination improves the antibody response but may increase susceptibility to secondary bacterial infections.

AUTHOR'S CONTRIBUTION

The supervisor came up with the idea and design of the study, gave ongoing advice and edited the article critically. The researcher performed the experiments, collected and examined the data, interpreted the findings, and wrote the first draft of the article.

Declaration of fund

The authors declare that they have not received funding.

Generative AI and AI-assisted technology statement

Artificial intelligence tools were used only for grammar correction and sentnece editing.

Conflict of interest

The authors have declared no conflict of interest.

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