

# Research Note: Effects of *Escherichia coli* co-infection on the protective efficacy assessment of two common infectious bronchitis vaccines

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**ABSTRACT** Avian infectious bronchitis (IB), a highly contagious disease hazardous to the poultry industry, is caused by an etiological agent called the infectious bronchitis virus (IBV). Some IBV strains (IBVs) alone usually do not cause high mortality in field conditions if not with secondary pathogens including *Escherichia coli* (*E. coli*). Herein, we established an IBV and *E. coli* co-infection model to evaluate the protective efficacy of two IBV vaccine strains against a new emerging genotype GVI-1 with mild virulence in experimental conditions. Chickens were inoculated with IBV field isolate ZQX (genotype GVI-1) and challenged 4

days later with the *E. coli* strain MS160427 (serotype O8). Subsequently, these chickens were euthanized at seven days postchallenge (d.p.c.) with *E. coli*. An autopsy revealed that lesions in the IBV plus *E. coli* co-infection group were more severe than those in the IBV-infected group. This pathological model was used to assess the protective effect of two commonly used vaccine strains (H120 and 4/91) against the IBV ZQX strain, and a significantly better protective efficacy was observed for 4/91 compared with H120. Thus, IBV and *E. coli* co-infection could be employed in assessing the protective efficacy of IBV vaccines.

**Key words:** avian infectious bronchitis, *E. coli*, vaccine, pathological model, co-infection

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## INTRODUCTION

Infectious bronchitis (IB) is an acute, highly contagious, and globally distributed respiratory disease affecting poultry industry (Jackwood and de Wit, 2013). In etiology, infectious bronchitis virus (IBV) is an enveloped virus with a single-stranded positive-sense non-segmented RNA genome and is approximately 27.6 kb in length. This virus belongs to the *Gammacoronavirus* genus. Because of the incomplete proofreading mechanism of coronavirus RNA polymerase and gene recombination during viral replication, new genotypes and serotypes of IBV variant strains appear continuously and often cause immune failure from IB (Xia et al, 2016). IBV is introduced by the respiratory route and then colonizes the inner part of the respiratory tract, followed by the renal region, reproductive system, and gastrointestinal tract, inducing respiratory distress, kidney damage, and reduced egg production.

Vaccination has been proved to be an effective strategy to prevent IBV outbreaks. However, the methods for evaluating the protective efficacy of the IBV vaccine vary in different countries. In the United States, the effectiveness of the IBV vaccine is tested only by IBV re-isolation via embryo passage from tracheal swabs five days post-challenge (d.p.c.), but virus re-isolation cannot distinguish the IBV vaccine from the challenge strains. In Europe, the efficacy of IBV vaccines is, in most cases, evaluated using the ciliostasis test (De Wit and Cook, 2014), but this test essentially requires a strict experimental operation. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) has also been adopted, but it requires specific experimental apparatus (Tucciarone et al., 2018). However, not so many IBV strains are able in experimental conditions to cause clear clinical symptoms. Virulent IBV strains may be associated with severe respiratory symptoms and/or nephritis in flocks. Yet, some field isolates potentially cause mild or unobservable symptoms in laboratory conditions. This makes it difficult to evaluate the protective efficacy of vaccines against mildly virulent isolates (Chhabra et al., 2015). Of interest, co-infection, such as *E. coli* and avian influenza H9N2 virus, could significantly increase the mortality of H9N2 isolates (Mosleh et al., 2017).

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Genotype GVI-1 is a recently emerged genotype of IBV (Ren et al., 2019). It shows mild virulence in experimental settings but can cause high morbidity and mortality in field conditions in some cases. Thus, the present study aimed to establish a co-infection model of IBV (genotype GVI-1) and avian pathogenic *E. coli* (APEC), and then use this model to evaluate the protective efficacy of 2 commercial vaccine strains H120 and 4/91. Findings from this work may provide an insightful reference for the protective efficacy evaluation of the IBV vaccine against mild IBV isolates in the future.

## MATERIALS AND METHODS

### Viruses, Vaccines, Eggs, and Chickens

Specific pathogen-free (SPF) embryos were obtained from the Beijing Merial Vital Laboratory Animal Technology Co., Ltd (Beijing, China). Healthy 1-day-old unvaccinated Sanhuang chickens were obtained from De-Kang agricultural and livestock technology Co., Ltd (Sichuan, China). The H120 strain was obtained from the China Institute of Veterinary Drug Control (Beijing, China), and the 4/91 strain was supplied by Internet International B.V. (Boxmeer, NL). The new genotype GVI-1-like isolate, China/Sichuan/ZQX/201604 (hereafter referred to as ZQX, GenBank JF732903) was isolated from Sichuan Province, China, in 2016, which has caused approximately 10% of mortalities in field conditions. H120, 4/91, and ZQX strains were propagated by injecting them into the allantoic cavities of 9- to 11-day-old SPF chicken embryos followed by 60 h incubation at 37°C. We harvested the allantoic solution and assessed median embryo infectious doses (EID<sub>50</sub>) of IBVs following the method described by Reed and Muench (Reed and Muench, 1938).

### Optimum Strain and Dose for E.coli Infection

A total of 35 Avian pathogenic *E. coli* strains were isolated from diseased chickens in Sichuan province during 2015–2017, after which we explored the serotypes and pathogenicity. A total of 40% of serotyped *E. coli* isolates (14/35) belonged to serotype O8, and a highly pathogenic *E. coli* strain MS160427 (serotype O8) was chosen to establish the co-infection model. To test the optimal dose of infection with a single *E. coli* strain, Forty 38-day-old commercially unvaccinated Partridge Shank broilers were randomly categorized into four groups with 10 chickens per group. Birds from the 4 groups were inoculated intratracheally with  $5 \times 10^6$  colony forming unit (CFU),  $1 \times 10^7$  CFU,  $2 \times 10^7$  CFU, and  $1 \times 10^8$  CFU of MS160427 *E. coli*, respectively. Birds were monitored daily, and those who died naturally were immediately necropsied. All surviving birds were euthanized at 7 d.p.c. for gross observation. A bird with fibrinous pericarditis, perihepatitis, or airsacculitis was considered as a colibacillosis case. Optimal dosage was defined as a dosage that can induce colibacillosis in 10 to

20% of birds, but cannot cause death during the experimental period.

### Establishing a Co-infection Model of IBV and E. coli

The 34-day-old commercial unvaccinated Partridge Shank broilers were randomly categorized into 6 groups (I–VI) with 12 chickens per group. Birds in groups I and III were inoculated with 0.1 mL/10<sup>5</sup> EID<sub>50</sub> of ZQX virus; birds in groups II and IV were inoculated with 0.1 mL/10<sup>6</sup> EID<sub>50</sub> of ZQX virus; birds in group V and VI were inoculated with 0.1 mL PBS. Four days post-infection (38 days old), birds in groups III, IV, and V were inoculated with an optimal dose of *E. coli* MS160427 per bird, whereas birds in groups I, II, and VI were inoculated with 0.1 mL PBS. All inoculations were via the intratracheal route.

We monitored the birds daily, and those who died naturally were immediately necropsied. All surviving birds were euthanized at 7 d.p.c. with *E. coli* (45 days old) for gross and histological analyses. The percentages of pericarditis, airsacculitis, hemorrhage in the trachea, and histological lesion in the trachea were recorded, and mean lesion scores (MLSs) of pericarditis, hemorrhage in the trachea, and histological lesion in the trachea were calculated for each group. The group displaying obvious fibrinous pericarditis, tracheal hemorrhage, and histological lesion in the trachea was defined as the optimal co-infection model.

### Evaluating the Immune Efficacy of Two Commercial Vaccine Strains

Using the optimal co-infection model, we evaluated the protective effect of H120 and 4/91 strains against the mild IBV isolate ZQX. In all, 72 twenty-day-old commercial unvaccinated Partridge Shank broilers were randomly categorized into six groups, A–F, with 12 birds per group. Birds in groups A and C were vaccinated with 0.1 mL/10<sup>5.0</sup> EID<sub>50</sub> of the H120 strain, birds in groups B and D were vaccinated with 0.1 mL/10<sup>5.0</sup> EID<sub>50</sub> of the 4/91 strain, and birds in groups E and F were inoculated with 0.1 mL PBS. The vaccine or PBS was inoculated as an eye drop. At 14 days postimmunization (dpi., 34 days old), birds in group A, B, C, D, and E were challenged with  $1 \times 10^6$  EID<sub>50</sub> of IBV-ZQX via the intra-tracheal route, whereas birds in group F were inoculated with 0.1 mL of PBS in the same manner. Four days later (38 days old), birds in groups A, B, and E were challenged with 0.1 ml of  $2 \times 10^7$  CFU *E. coli* MS160427 intratracheally, whereas birds in groups C, D, and F were inoculated with 0.1 mL of PBS via the same route. Birds were monitored daily, and those who died naturally were immediately necropsied. The clinical signs, morbidities, and mortality were recorded. All surviving birds were euthanized at 25 dpi (45 days old) for gross observation. The percentages of pericarditis, airsacculitis, and hemorrhage in the trachea of the tested

birds were recorded, and the MLSs of fibrinous pericarditis and tracheal hemorrhage were calculated following the method described above.

## Statistical Analysis

The MLSs of fibrinous serositis, tracheal hemorrhage, and microscopic lesions in the trachea were analyzed using Mann-Whitney U test. Morbidity and mortality were compared by Chi-square test using the IBM SPSS Statistics 24 software (IBM Corp, Armonk, NY). A  $P$ -value  $< 0.05$  was considered statistically significant.

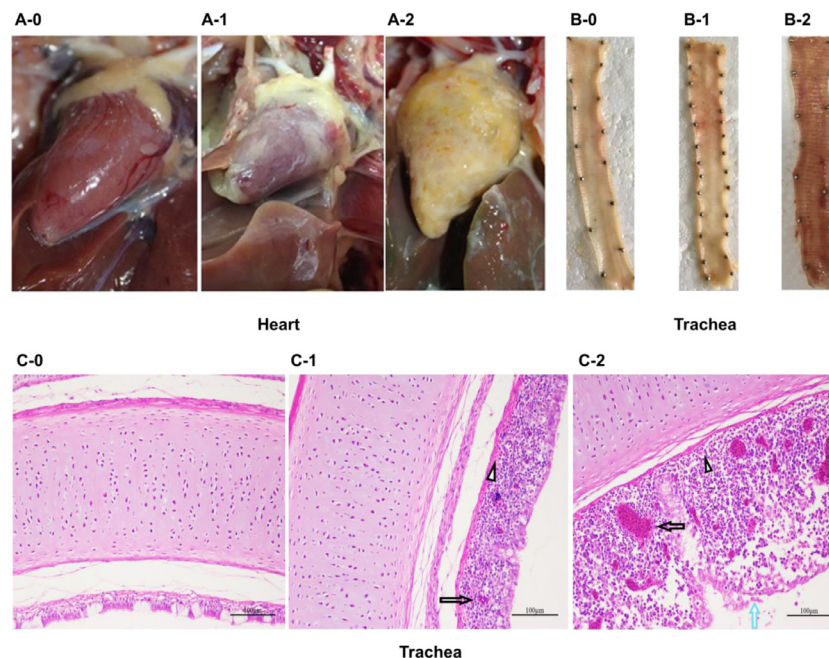
## RESULTS AND DISCUSSION

In the *E. coli* infection test, a representative virulent *E. coli* strain MS160427 from the prevalent serotype O8 was selected to establish the co-infection model. The result showed that 20 and 30% of chickens in groups challenged with  $2 \times 10^7$  CFU and  $1 \times 10^8$  CFU of MS160427 were identified as colibacillosis case after gross dissection, respectively. There was no apparent fibrinous pericarditis, perihepatitis, or airsacculitis observed in chickens from the other 2 challenged groups. The  $2 \times 10^7$  CFU was identified as the optimal dose of *E. coli* for the following co-infection test.

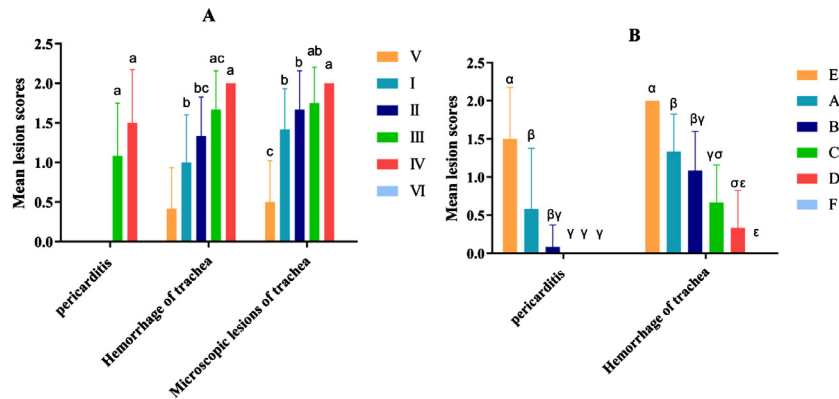
For the establishing of co-infection model of IBV and *E. coli*, chickens inoculated intratracheally with  $10^5$  or  $10^6$  EID<sub>50</sub> of ZQX virus showed clinical signs such as gasping, coughing, sneezing, and depression as early as 2 to 3 d.p.c. The severity of clinical signs reached its peaks

at 6 d post-IBV challenge and alleviate gradually later (group I and II), whereas the respiratory symptoms in chickens with a subsequent *E. coli* challenge became more severe (group III and IV). Notably, no chicken died in any group during the experimental period. With regard to gross lesions, chickens infected solely with IBV were presented with only diverse hemorrhage and a small amount of mucus in the trachea, whereas chickens co-infected with IBV and *E. coli* displayed more severe tissue damage (Figure 1). Fibrinous pericarditis was observed only in groups III (83.33%) and IV (91.67%), and the MLSs of pericarditis in group IV were significantly higher than those in group III ( $P < 0.05$ ); airsacculitis was observed in groups III (100%), IV (100%), and V (20%); tracheal hemorrhage was observed in groups I (83.33%), II (100%), III (100%), IV (100%), and V (50%), and the MLSs of tracheal hemorrhage can be arranged from high to low as follows: IV  $>$  III  $>$  II  $>$  I  $>$  V. These showed significant differences in pairwise comparison; microscopic damage in the trachea could be observed in 100% of chickens in groups I to IV, whereas it was observed in only 60% of chickens in group V. The highest MLS for microscopic tracheal damage was seen in group IV ( $P < 0.05$ ) and the lowest in V group. The group IV was defined as the optimal co-infection model (Figure 2).

The respiratory tract particularly trachea plays an essential role in innate immunity and prevention of pathogen infection; lesions in the respiratory tract may promote secondary bacterial infections (Jackwood et al., 2015). In the present study, the trachea of chickens infected with IBV (ZQX) alone showed a mild



**Figure 1.** (A) Macroscopic lesion observation in the heart. The heart lesions were scored as follows: (A-0) 0 = no lesions, (A-1) 1 = a small amount of cellulosic exudation (slightly opaque), (A-2) 2 = thick or a large amount of cellulosic exudation; (B) macroscopic lesion observation in the trachea. Tracheal lesions were scored as follows: (B-0) 0 = no lesions, (B-1) 1 = only minor punctate hemorrhages, (B-2) 2 = diffuse hemorrhage; (C) microscopic lesion of the trachea. These lesions were scored as follows: (C-0) 0 = no obvious tracheal lesions were found in the control group; (C-1) 1 = massive amounts of infiltration of inflammatory cells (black triangle), small amount of red blood cell infiltration (black arrow), lamina propria hyperplasia, and losses of cilia; (C-2) 2 = losses of epithelial cells and cilia, glandular degeneration, massive amount of infiltration of red blood cells (black arrow) and inflammatory cells (black triangle).



**Figure 2.** (A) MLS for pericarditis, tracheal hemorrhage, and microscopic lesions of trachea in each group ( $n = 12$  chickens/group) while establishing a model with IBV and *E. coli* co-infection. (B) MLS for pericarditis and tracheal hemorrhage in each group ( $n = 12$  chickens/group) in the test for evaluating the immune efficacy of H120 and 4/91 vaccine strains. Statistical differences between different groups were determined by the analysis of Mann–Whitney U test. <sup>abc</sup>, <sup>αβγδϵ</sup> Different letters indicate significant differences of the data between different groups; the same letter indicates no significant difference of the data between different groups.

inflammatory response and the epithelial cells showed no shedding. In co-infection model, respiratory lesions were more serious, the epithelial cells of the trachea were exfoliated and necrotic, and the lamina propria became more thickened; furthermore, a large number of lymphocytes and red blood cells were infiltrated. Therefore, *E. coli* could significantly enhance the pathological injury caused by IBV.

Using the IBV and *E. coli* co-infection model established above, we evaluated the protective efficacy of H120 and 4/91 vaccine strains against mild IBV ZQX strain. The result showed that both vaccines could provide effective clinical protection against the GVI-1 genotype, but the protective effect of 4/91 vaccine strains was better than that of H120 vaccine strains. Immunized chickens showed mild respiratory lesions as early as 3 d. p.c. with ZQX strain (groups A–D), and the respiratory symptoms intensified following *E. coli* inoculation. In contrast, the respiratory signs alleviated gradually in immunized chickens with no further challenge with *E. coli*. For gross lesions, pericarditis could only be observed in group A (41.67%), group B (8.33%), and group E (91.67%), and the MLSs of pericarditis from high to low were as follows: group E > group A > group B. The difference was statistically significant ( $P < 0.05$ ); airsacculitis could only be observed in group A (75%), group B (66.67%), and group E (100%); tracheal hemorrhage could be observed in groups A–E; the percentage of which was 100%, 91.67%, 66.67%, 33.33%, and 100%, respectively, and the order of MLSs for tracheal hemorrhage from high to low was as follows: group E > group A > group B > group C > group D (Figure 2).

Clinical protection indices, such as morbidity, mortality, and pathological change, are not more reliable than other laboratory tests like virus isolation, ciliastasis, and qRT-PCR, but are easier to judge in field conditions. It is easier to perform the protective efficacy test against virulent IBV than against mildly virulent IBV as it cannot cause death in experimental conditions. However, IBV infection may increase the sensitivity to other pathogens such as *E. coli* or H9 AIV (Belkasmi et al.,

2020). In this study, a preliminary infection of IBV increased the pathogenicity of *E. coli*, thus making the protective efficacy evaluation of IBV vaccine more intuitive. The co-infection model could be adopted to assess the protective efficacy of the IBV vaccine against field isolates, especially against mild virulent IBV.

## DISCLOSURES

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, “Effects of Escherichia coli co-infection on the protective efficacy assessment of two common IBV vaccines”

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2021.101324>.

## REFERENCES

- Belkasmi, S. F. Z., S. Fellahi, C. D. Touzani, F. Z. Faraji, I. Maaroufi, M. Delverdier, J. L. Guérin, O. F. Fihri, M. El Houadfi, and M. F. Ducatez. 2020. Co-infections of chickens with avian influenza virus H9N2 and Moroccan Italy 02 infectious bronchitis virus: effect on pathogenesis and protection conferred by different vaccination programmes. *Avian Pathol.* 49:21–28.
- Chhabra, R., J. Chantrey, and K. Ganapathy. 2015. Immune responses to virulent and vaccine strains of infectious bronchitis viruses in chickens. *Viral Immunol.* 28:478–488.
- De Wit, J. J., and J. K. Cook. 2014. Factors influencing the outcome of infectious bronchitis vaccination and challenge experiments. *Avian Pathol.* 43:485–497.
- Jackwood, M. W., B. J. Jordan, H. J. Roh, D. A. Hilt, and S. M. Williams. 2015. Evaluating protection against infectious bronchitis virus by clinical signs, ciliostasis, challenge virus detection, and histopathology. *Avian Dis.* 59:368–374.

- Jackwood M. W. and de Wit J. J., Infectious bronchitis, In: Swayne D. E., Glisson J. R., McDougald L. R., Nolan L. K. and Suarez D. L., *Diseases of Poultry*, 13th ed., 2013, John Wiley and Sons; Ames, IA, 139–159.
- Mosleh, N., H. Dadras, K. Asasi, M. J. Taebipour, S. S. Tohidifar, and G. Farjanikish. 2017. Evaluation of the timing of the *Escherichia coli* co-infection on pathogenicity of H9N2 avian influenza virus in broiler chickens. *Iran J. Vet. Res.* 18:86–91.
- Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoint. *Am. J. Hyg.* 27:493–497.
- Ren, M., J. Sheng, T. Ma, L. Xu, Z. Han, H. Li, Y. Zhao, J. Sun, and S. Liu. 2019. Molecular and biological characteristics of the infectious bronchitis virus GVI-1/GVI-1 lineage isolated in China. *Infect. Genet. Evol.* 75:103942.
- Tucciarone, C. M., G. Franzo, G. Berto, M. Drigo, G. Ramon, K. C. Koutoulis, E. Catelli, and M. Cecchinato. 2018. Evaluation of 793/B-like and Mass-like vaccine strain kinetics in experimental and field conditions by real-time RT-PCR quantification. *Poult. Sci.* 97:303–312.
- Xia, J., X. He, K. C. Yao, L. J. Du, P. Liu, Q. G. Yan, Y. P. Wen, S. J. Cao, X. F. Han, and Y. Huang. 2016. Phylogenetic and antigenic analysis of avian infectious bronchitis virus in southwestern China, 2012–2016. *Infect. Genet. Evol.* 45:11–19.