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# Chicken anaemia virus enhances and prolongs subsequent avian influenza (H9N2) and infectious bronchitis viral infections

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ABSTRACT

Immunosuppressive viral diseases have a great economic importance in the poultry industry due to the increased susceptibility to secondary infections. Chicken anaemia virus (CAV) is one of the major immunosuppressive diseases in chickens. In addition, low pathogenic avian influenza (LPAI) of subtype H9N2 and infectious bronchitis (IB) viruses are among the most frequently reported respiratory viral diseases in poultry worldwide. In the present study, specific pathogen free chickens were used to understand the impact of CAV on secondary infection with LPAI-H9N2 or IB viruses. Clinical outcomes, viral shedding dynamics, and cytokine levels wereassessed. The results exhibit that chickens previously infected with CAV produceconsiderablyhigher titresof LPAI-H9N2 or IB viruses in the oropharyngeal swabs ($P < 0.05$), tracheas and kidneys. In addition, the immunologic effect of CAV provokedthe development of clinical signs of LPAI-H9N2 and IB virus infections. Moreover, results suggested that pre-infection with CAV directly correlated with elevated levels of IL-6 and IFNγ. These findings underline the importance of CAV pre-infection on LPAI-H9N2 or IB infection in chickens, and indicate that co-circulation of CAV can contribute to the spread and evolution of LPAI H9N2 and IB viruses.

## 1. Introduction

Over the last decades, a significant increase in the global poultry meat and egg production industry has been recorded (Mottet and Tempio, 2017). Infectious poultry diseases remain one of the major threats to the poultry industry, and can lead to substantial economic losses, as well as zoonotic infections in industry staff (Ellstrom et al., 2014; Samy and Naguib, 2018). The most threatening agents are viral diseases, such as avian influenza virus (AIV), infectious bronchitis virus (IBV), Newcastle disease virus (NDV), infectious bursal disease, and Marek's disease; also, several bacterial agents can adversely affect poultry (Agunos et al., 2016). Such diseases often occur in combination with other infectious agents, and/or management problems (Samy and Naguib, 2018; Wang et al., 2017). Additionally, frequent co-infections/interferences (virus-virus or virus-bacteria) among the poultry population have recently been reported from many parts of the world (Samy and Naguib, 2018).

Avian influenza virus, an influenza A virus, is a member of the *Orthomyxoviridae* family (Thomas and Noppenberger, 2007). In poultry, low pathogenic avian influenza viruses (LPAIVs) may cause subclinical infections, mild respiratory symptoms, or drop in egg production (Halvorson, 2008). However, field observations associated with LPAIV H9N2 infections revealed increased mortality rates and drop in egg productivity (Samy and Naguib, 2018). One of the most important reasons for this discrepancy were proved to be mixed infection with other viral and/or bacterial pathogens (Samy and Naguib, 2018). Another important poultry virus, IBV clearly belongs to the most economically important viruses affecting the poultry sector worldwide (Jackwood, 2012). IBV, a member of *Coronaviridae*, is associated with respiratory symptoms and affects the reproductive tract. Chicken anaemia virus (CAV), belonging to *Circoviridae*, is a circular DNA virus, which causes growth retardation and immunosuppression in chickens (MacLachlan and Dubovi, 2017; Rosario et al., 2017). Infection with CAV causes anaemia, lymphoid depletion, and haemorrhages in chickens, that are usually infected before 3weeks of age and lacking maternal antibodies (MacLachlan and Dubovi, 2017).

Viral interference can be elucidated by different mechanisms encompassing: competition for cell receptors attachment, for replication; intracellularly host machinery competition, and virus-induced interferon response (DaPalma et al., 2010). Measurable differences were described associated with mixed viral infections, including changes in viral replication patterns, tissue tropism, pathological, and

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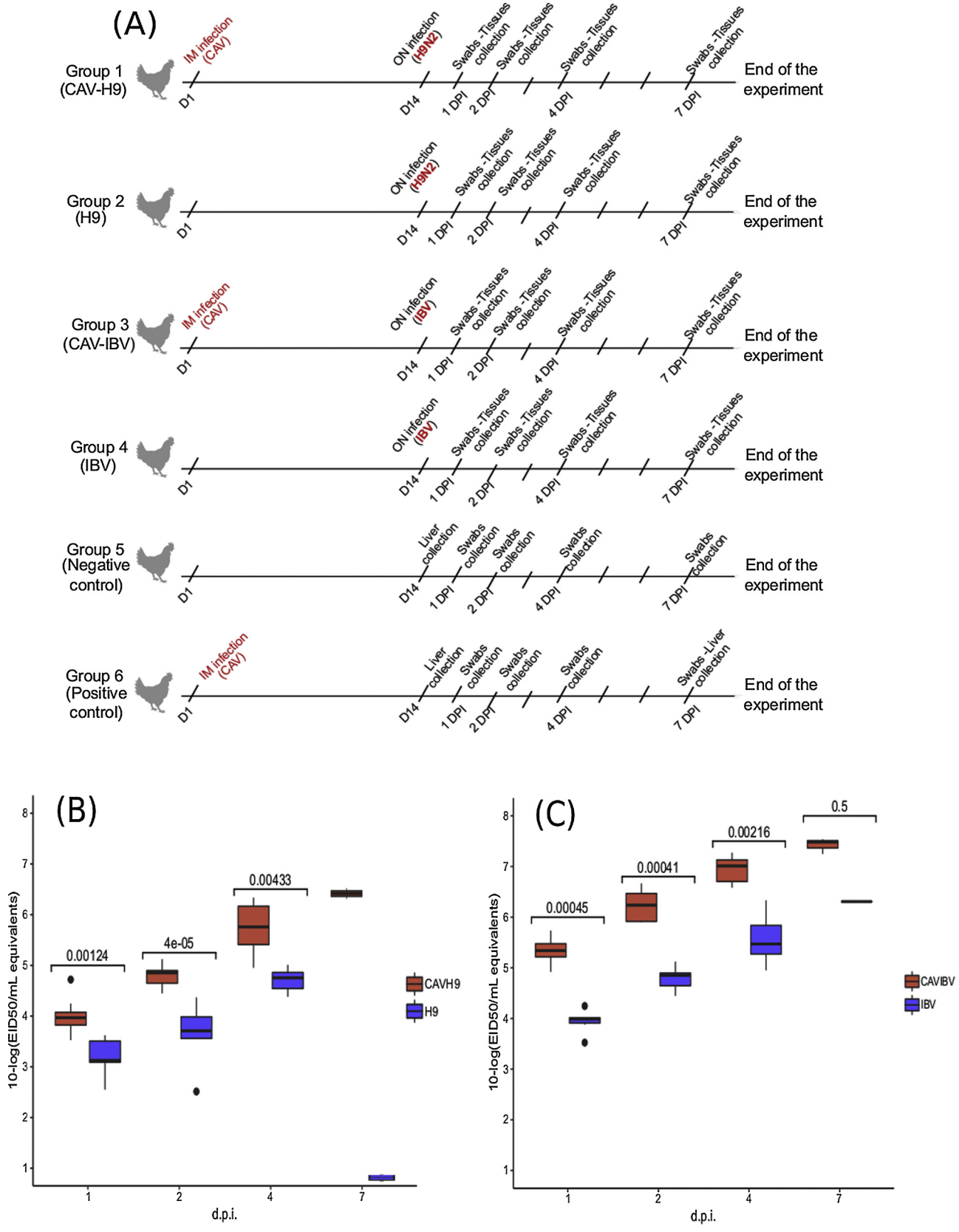


**Fig. 1.** Animal experimental design (a); Oropharyngeal virus shedding in CAV-H9N2 group vs H9N2-only group (b), CAV-IBV group vs IBV-only group (c). (a) Tissue samples are related to lung and kidney for virus titre quantification; and spleen and trachea for cytokines gene expression levels. (b) Boxplot of $\log_{10}$ virus titre in oropharyngeal swabs per DPI; Red (CAV-H9N2 group) and blue (H9N2 group). (c) Boxplot of $\log_{10}$ virus titre in oropharyngeal swabs per DPI; Red (CAV-IBV group) and blue (IBV group). Annotations show p-value for separation calculated by the Wilcoxon/Mann-Whitney method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immunological responses (DaPalma et al., 2010).Worldwide, viral co-infection/interference with other immunosuppressive viruses is common in the poultry industry and has been reported in several studies (De Boer et al., 1994a; Li et al., 2016). In Egypt, LPAIV H9N2 continues to circulate intensively in the Egyptian poultry sectors (Naguib et al., 2017a). In addition, IBV has been frequently recovered from chicken farms in Egypt (Zanaty et al., 2016). Moreover, a previous study has identified widespread CAV infection among Egyptian chicken flocks (Erfan et al., 2018). Co-circulation of IBV and LPAIV H9N2 within the Egyptian poultry population has been described in several earlier studies (Hassan et al., 2016; Naguib et al., 2017b). However, experimental animal studies of the impact of the CAV circulation on the subsequent infection of either LPAIV or IBV are lacking. In this study, Egyptian virus strains were used to assess the impact of CAV infection on the subsequent infection with either LPAIV H9N2 or IBV in specific pathogen free chickens.

## 2. Materials and methods

### 2.1. Viruses

Viruses used in the current study were obtained from the virus repository of the National Laboratory for Veterinary Quality Control on Poultry Production (NLQP), Animal Health Research Institute, Giza, Egypt. LPAIV H9N2: A/chicken/Egypt/15106 V L/2015 (GenBank accession number: KU296207), IBV:IBV-Eg/15170F-SP1 (GenBank accession number: KY119259) and CAV: CAV/Egypt/EG-15/2017 (GenBank accession number: MH001568). Virus titres were determined using specific pathogen free (SPF) embryonated chicken eggs (ECE) (Villegas, 1998). Virus-infected allantoic fluid was diluted in order to obtain an inoculum with a titre of $10^5$of 50% egg infectious dose ($EID_{50}$). Mock inoculum was prepared using non-infected allantoic fluid from SPF–ECE.

### 2.2. Ethical approval

White leghorn chickens were hatched from SPF ECEs that were purchased from Nile SPF Farm, Kom Oshiem, Fayom, Egypt, and raised at NLQP. Birds were housed in isolation units, where feed and water were provided daily. All animal experiments in this study were conducted in accordance with the legally approved protocol (AHRI 04032018) of the Animal Health Research Institute, Giza, Egypt. Infection and immunization experiments were performed in separated cages at animal biosafety level-3 (BSL-3). Oropharyngeal swab samples were obtained with minimum distress. Tissue samples (trachea, lung, kidney and spleen) were obtained from euthanized chickens after euthanization with an intravenous injection of a xylazine-ketamine combination.

### 2.3. Experimental design

Seventy SPF chicks (one day old) were separated into 6 groups (12 chicks for each group except for negative control group that included 10 chicks only) as follows; group 1 and 3 received an intramuscular (IM) dose of 0.1 mL of CAV ($10^5$ $CID_{50}$) at day one followed by intranasal (IN) inoculation of 0.5 mL of $10^5$ $EID_{50}$ of LPAIV-H9N2 or IBV, respectively at day 14 (named hereafter as CAV-H9N2 and CAV-IBV groups). While, groups 2 and 4 received only the same dose of LPAIV-H9N2 or IBV, respectively at day 14 without pre-inoculation of CAV (named H9-only or IBV-only infected group). Moreover, a control group was designed as group5 (negative control) that was IM inoculated with 0.5 mL of the mock inoculum (allantoic fluid of uninfected SPF eggs) at day 1. Additionally, group 6 was kept as a positive control group through intramuscular (IM) dose of 0.1 mL of CAV ($10^5$ $CID_{50}$) at day one to ensure CAV infection in groups 1 and 3. Clinical signs were observed and scored daily for 7 days following the secondary infection. Further, individual oropharyngeal swab samples were collected at 1st, 2nd, 4th, and 7th days post infection (DPI). Lungs and Kidneys) were collected for estimation of virus titre as well as trachea and spleen for quantitation of cytokines genes expression at the same time points from three birds/each secondary infected group. The illustration of this experimental design is presented in Fig. 1a.

### 2.4. Real-time quantitative PCR and reverse transcription PCR

Viral RNA was extracted from oropharyngeal swabs from each infected bird using the QIAamp viral RNA mini kit (Qiagen, Germany, GmbH) following the manufacturer's instructions. Tissue samples were homogenized by the Tissue lyser LT (Qiagen) and then subjected to three successive freeze–thaw cycles, followed by centrifugation at 18,000 × *g* for 10 min to separate the supernatant. Virus shedding was detected by amplification of the extracted RNA by quantitative reverse transcription PCR(RT-qPCR) targeting the hemagglutinin (HA) gene (Ben Shabat et al., 2010) or IBV nucleocapsid (N) gene (Meir et al., 2010) and quantified against standard genomic RNA of H9N2 and IBV, respectively. PCR reactions were performed in 25 µL volumes using AgPath-ID™ One-Step RT-PCR kit (ThermoFisher scientific, Germany) in a Stratagen MX3005 P machine (Agilent, Santa Clara, CA, USA).

Liver samples from the positive control group were collected at day 10 for CAV DNA detection to guarantee CAV virus replication. Total DNA was individually extracted from the supernatant fluid (after homogenization and centrifugation of the tissue samples) using commercial QIAamp DNA Mini Kit (Qiagen, Gmbh, Germany) following the manufacturer's instructions. A 419 bp fragment of VP1 gene was amplified using a specific pair of primers (forward: 5´-CTA AGA TCT GCA ACT GCG GA-3´; reverse: 5´-CCT TGG AAG CGG ATA GTC AT-3´) (Hussein et al., 2002).

### 2.5. Cytokine expression in lungs and spleens

To determine the pro-inflammatory cytokine expression levels following the secondary infection of CAV-H9N2 or CAV-IBV groups versus H9N2-only or IBV-only groups, lungs and spleens from three infected chickens were collected at 1, 2, 4, and 7 DPI. Collected organs were stored in RNA-later in -80$^{O}$C freezer, and tissue homogenates were subsequently assayed for interleukin 6 (IL-6) and interferon gamma (IFNγ). Total RNA was extracted using the RNeasy Mini kit (Qiagen, Germany), including DNase digestion (RNase-free DNase kit; Qiagen). One microgram of total RNA was further used for amplification using the Sensifast™ probe LO-ROX one-step kit (Bioline, UK) and Stratagene MX3005 P real-time PCR machine. Amplification curves and Cq values were determined by the Stratagene MX3005 P software. The data were normalized by28S rRNA gene. Primers and probes (supplied from Metabion, Germany) that were used for IL-6, IFNγ, and 28S ribosomal RNA (28S rRNA) are shown in Supplementary Table 1. To estimate the variation of gene expression in the different samples, the Cq value of each sample was compared with that of the control group according to the "ΔΔCt" method stated by (Yuan et al., 2006). Gene expression data is shown as relative gene expression in relation to that of the non-stimulated sample, in order to calculate the fold change achieved by the stimulation.

### 2.6. Serology

Blood samples were collected one day prior to secondary viral inoculation and tested by enzyme-linked immunosorbent assay (ELISA, Synbiotics Corporation, ProFlock KPL. USA), following the manufacturer's recommendations, for serum sample CAV positivity.

### 2.7. Statistics

Data analysis and visualization was made using RStudio (version

1.0.136) and the ggplot2 R package (R Core Team, 2016; Wickham, 2016). Non-parametric statistical methods were selected based on QQ-plot analysis to analyse virus titres in oropharyngeal swabs and organs (trachea and kidney) at 1, 2, 4 and 7 DPI from the different experimental cohorts (CAVH9N2 vs. H9N2-only and CAVIBV vs. IBV-only). The boxplots illustrate the interquartile range (distance between first and third quartiles) as boxes with the median marked as a black horizontal bar within each box. The whiskers extend 1.5 interquartile range from each box. Data points beyond the end of the whiskers are referred to as outliers and are plotted individually as scatter points. Annotations of boxplots show the p-value as calculated by the Wilcoxon/Mann-Whitney method of the separation of medians. A p-value $< 0.05$ is considered as significant separation. Scatter plots show individual samples. Horizontal bars show the median and whiskers show plus minus one standard deviation. Scatter plots with trend lines show individual data points, horizontal bars illustrating the mean and whiskers are plus minus one standard error of the mean. The grey shaded areas along the added trend lines illustrate the 95% confidence interval of the added trend lines.

## 3. Results

### 3.1. Pre-CAV infection modulates the course of subsequent infection

To monitor the influence of the CAV infection on the subsequent infection by H9N2 or IB viruses, clinical signs were scored daily through 7 successive days following the secondary infection with the following score scheme: 0 = healthy (no abnormal signs); 1 = slightly sick (showing one of the following symptoms: ruffled feathers, respiratory manifestations, depression, facial oedema, cyanosis of comb and wattles or diarrhoea); 2 = sick (showing two signs or more); 3 = dead. CAV inoculated chicks showed anaemic clinical signs starting from day 9–11 post inoculation. Chickens almost lacked clinical signs in experimental infection with LPAIV-H9N2 infected group and no signs have been observed than general depression and decrease feed consumption. The IBV-only inoculated birds showed respiratory clinical signs (gasping, coughing, sneezing, tracheal rales, and nasal discharge). These clinical signs were observed in CAV-H9N2 and greater in the CAV-IBV birds. Symptoms started 3 and 2 DPI for H9N2-only and IBV-only groups, respectively; while it started at day 1 in CAV pre-infected groups. More detailed clinical scoring is shown in Table 1.

### 3.2. Variation in virus shedding patterns

In order to compare virus shedding patterns via the respiratory route, viral RNA loads in oropharyngeal swabs were determined from infected birds at 1, 2, 4, 7 DPI. A significantly higher virus titre was observed in the oropharyngeal excretion within the CAV-H9N2 group vs. the H9N2-only infected group at day 1, 2 and 4 DPI with p-values of 0.00124, 0.0005, and 0.00433 respectively (Fig. 1b).H9N2 virus was detected in 2/3 birds within CAV-H9N2 infected group on 7 DPI, while viral clearance was reported in oropharyngeal swabs of the H9N2-only infected group (Fig. 1b). Likewise, IBV was detected positive in all birds on days 1, 2, 4, and 7 (Fig. 1c). In general, titres of IBV in oropharyngeal swab samples collected on day 1, 2, and 4 were more pronounced within the CAV-IBV group compared to the IBV-only infected group, and were 1–2 $\log_{10}$higher with p-values $< 0.05$ (Fig. 1c). Viral load in the oropharyngeal swabs, within the CAV-IBV group, remained high on 7 DPI, with an increase in viral load where 3/3 birds were positive, but only 1/3 was positive shedder in the IBV-only infected group.

**Table 1**
Degree of severity of clinical signs in all groups.

| Group ID | Clinical scoring/days post H9/IB infection | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| CAV-H9N2 | 1 | 1 | 2 | 2 | 2 | 2 | 2 |
| H9N2-only | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| CAV-IBV | 1 | 2 | 2 | 2 | 2 | 2 | 2 |
| IBV-only | 0 | 1 | 1 | 1 | 2 | 2 | 2 |

### 3.3. Higher virus titre in tissue samples of pre-CAV infected group

To assess the impact of CAV co-infection on H9N2 and IB virus levels in different tissues, three birds from each infected group were scarified and the tracheas and kidneys were tested for their virus load. Tracheal titres of the CAV-H9N2 group were high and comparable with those of the H9N2-only infected group, while kidney titres in both groups were remarkably lower at 1, 2, and 4 DPI. H9N2 virus was also detected in up to 7 DPI in the trachea (2/3) and kidney (1/3) in the CAV-H9N2 group only (Fig. 2a, b). Further, viral loads were 1–2 $\log_{10}$ units higher in trachea and kidney of the CAV-IBV, in contrast to the IBV-only infected group. The highest viral loads (approximately $10^7$ EID50/mL equivalents) were observed in both trachea and kidney in the CAV-IBV chickens at 7 DPI. Interestingly, CAV pre-infection accelerates the virus detection in trachea and kidney, where virus was found in the trachea and kidney of 2/3 chickens within the first 24 h in the CAV-IBV groups while 1/3 in the IBV-only group (data not shown).

### 3.4. Influence of co-infection on cytokine expression levels

To elucidate the association between disease severity either with/without pre-CAV infection and cytokine production, the IL-6 and IFNγ levels in the pre-CAV vs. non-pre-CAV infection was analysed in both lung and spleen. A higher level of IFNγ was noted in lungs and spleens of the CAV-H9N2 group compared to the H9N2-only infected group with a marked increase at 2 and 4 DPI (Fig. 3a, c). The level of IL-6 in lungs of the CAV-H9N2 group at 4 DPI was also elevated compared with levels in the H9N2-only infected group (Fig. 3b). However, no statistical difference was observed in IL-6 levels of spleen samples between the two groups (Fig. 3d). On the other side, a considerable higher level of IFNγ was also observed in lungs and spleens of CAV-IBV group that reached the peak at 4 DPI (Fig. 4a, c). Moreover, the patterns of IL-6 were similar to those previously observed in the H9N2 groups, in respect to marked higher level in lungs and no statistical difference could be seen in spleens (Fig. 4b, d).

### 3.5. Serology

Birds of group 1, 3, and 6 were tested seropositive for CAV one day before the start of the secondary infection experiment; conversely birds from group 2, 4, and 5 were tested negative for CAV antibodies (data not shown).

## 4. Discussion

Avian viral diseases continue to circulate among different poultry populations worldwide and cause devastating economic losses in poultry industries (Pohjola et al., 2017; Samy and Naguib, 2018; Zhuang et al., 2014). Poultry can be exposed to different kinds of immunosuppressive viruses that impair the immune system, which in turn may increase the risk to be infected by other pathogens (Hoerr, 2010; Umar et al., 2016). The interaction between multiple pathogens within the same host population can profoundly alter the spreading dynamics and the pathobiology of infections (Samy and Naguib, 2018).For example, CAV and avian reo virus co-infection enhances reo virus pathogenicity in chickens (McNeilly et al., 1995); this was reported also for CAV and Newcastle disease virus(De Boer et al., 1994b).

Egypt has a thriving poultry industry, where poultry production commonly suffers from different bacterial and viral pathogens.

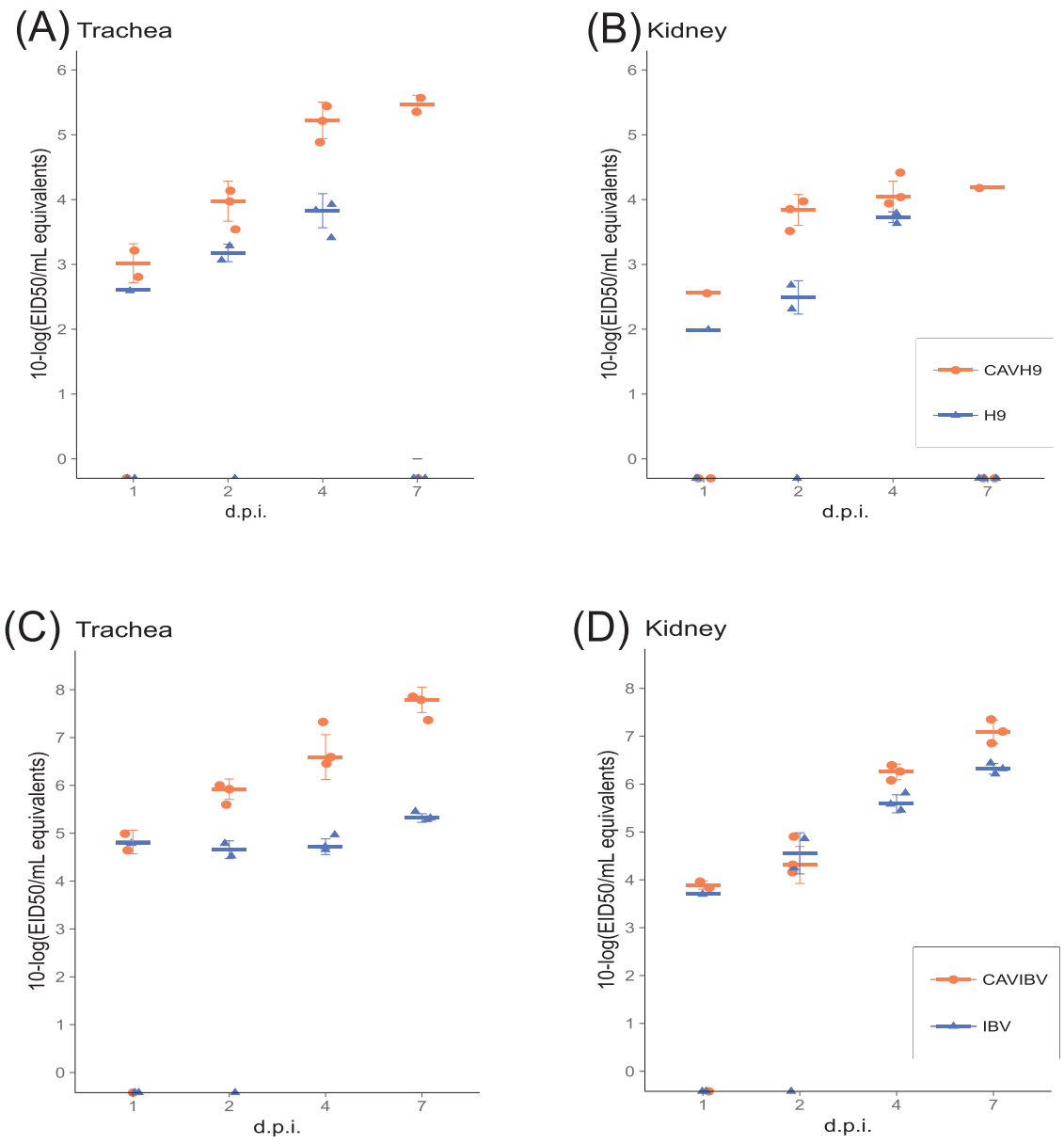


**Fig. 2.** Virus titres in tracheas and kidneys of CAV-H9N2 group vs H9N2-only group (a, b) and CAV-H9N2 group vs IBV-only group (c, d).
Scatter plot of $\log_{10}$ virus titre in tissue samples per DPI with marked median and plus minus one standard deviation. Annotations show p-value for separation calculated by the Wilcoxon/Mann-Whitney method.

Currently, LPAIV-H9N2 and IBV diseases are endemic in Egypt and together pose a huge threat to the poultry industry (Naguib et al., 2017b). Moreover, previous studies have reported a wide geographical spread of CAV among different commercial poultry sectors in Egypt (Abdel-Mawgod et al., 2018; Erfan et al., 2018). CAV infection might play an exacerbating role in H9N2/IBV-associated respiratory disease in chickens. However, experimental studies to understand the impact of such co-circulation is lacking. Hence, in the current study we addressed the severity of CAV coinfection with LPAI-H9N2 or IB virus under experimentally controlled conditions using specific pathogen free chickens as model.

The results of this study revealed that previously CAV infected chickens produced considerably higher virus titres in the oropharyngeal swabs ($p < 0.05$), tracheas and kidneys (Figs. 1 and 2) compared to that of LPAIV-H9N2 or IBV only infected chickens. The immunologic effect of the CAV provoked the development of clinical signs and prolonged oral viral excretion of both LPAI-H9N2 or IB viruses. Spleen, as a principal organ of systemic immunity (John, 1994), and lung, as the main targeted organ for many respiratory viral diseases including LPAIV-H9N2 and IBV (Reese et al., 2006), were selected for assessing the IL-6 and IFNγ levels. Our results suggested that the elevated expression levels of IL-6 and IFNγ in CAV-H9N2 and CAV-IBV groups were related to the pre-infection by CAV. Higher comparable levels of IL-6 in lungs, but interestingly not spleens of both CAV-H9N2 and CAV-IBV groups might account with the respiratory infection responses of LPAIV-H9N2 or IBV indicating a local immune response (Figs. 3 and 4).

In conclusion, this study underlines the importance of prior CAV infection on LPAIV-H9N2 or IBV infection in chickens. Overall, previously CAV infected chickens with exacerbated course of infection, prolonged viral shedding, increased virus titres in oropharyngeal swabs, spleens, and lungs of the CAV-H9N2 and CAV-IBV groups in contrast to that of the H9N2-only and IBV-only infected groups. Indeed, prolonged virus shedding of LPAIV-H9N2 and IBV infected chickens favours virus transmission and may foster the endemic status and continuous evolution of both viruses. Further studies are recommended to identify how such impaired immune response can be invoked by improved vaccines.

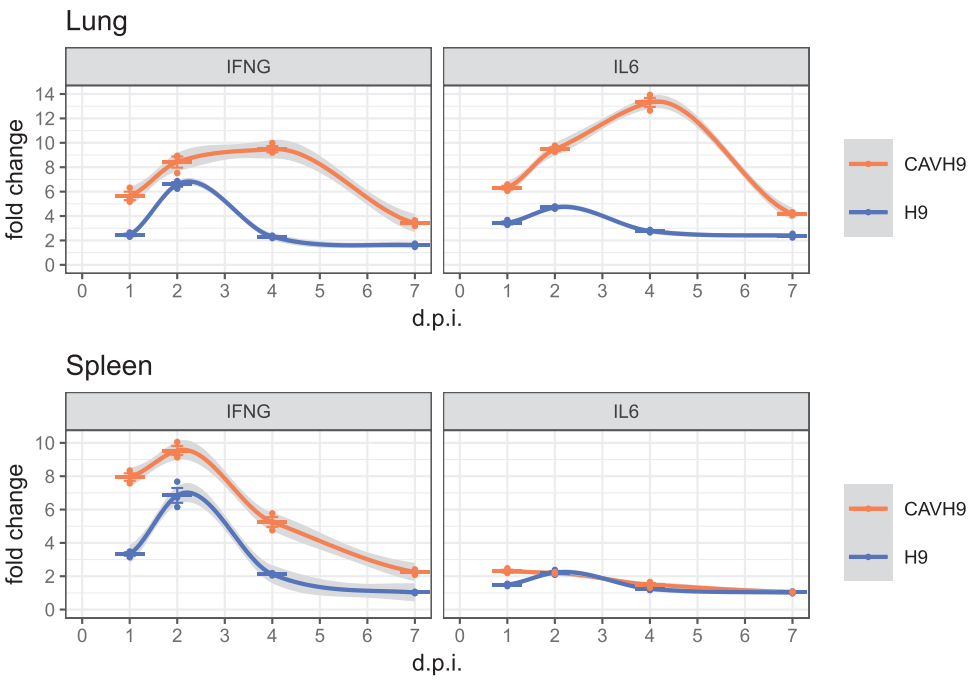


**Fig. 3.** IFNG and IL-6 fold-change in lungs and spleens of CAV-H9N2 group vs H9N2-only group.
Scatter plots with added trend lines for fold change in cytokine gene expression per DPI. Horizontal bar and vertical whiskers show mean and plus minus one standard error of the mean. Grey shaded areas along added trend lines illustrate the 95% confidence interval of the added trend line. Red (CAV-H9 group) and blue (H9 group). Upper left pane (lung IFNG), upper right pane (lung IL6), lower left pane (spleen IFNG) and lower right pane (spleen IL6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In addition, intervention control strategies relying on minimizing stress and reduced exposure to infectious agents through biosecurity are highly recommended.

## Authors contribution

A.E. and M.N. conceived the study. A.S., A.H. conducted the animal experiment. A.E, M.N, and P.E. analysed and interpreted data. A.E and M.N drafted the manuscript. All co-authors critically analysed and revised the manuscript and provided final approval.

## Conflicts of interest

The authors declare no conflict of interest.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.01.024.

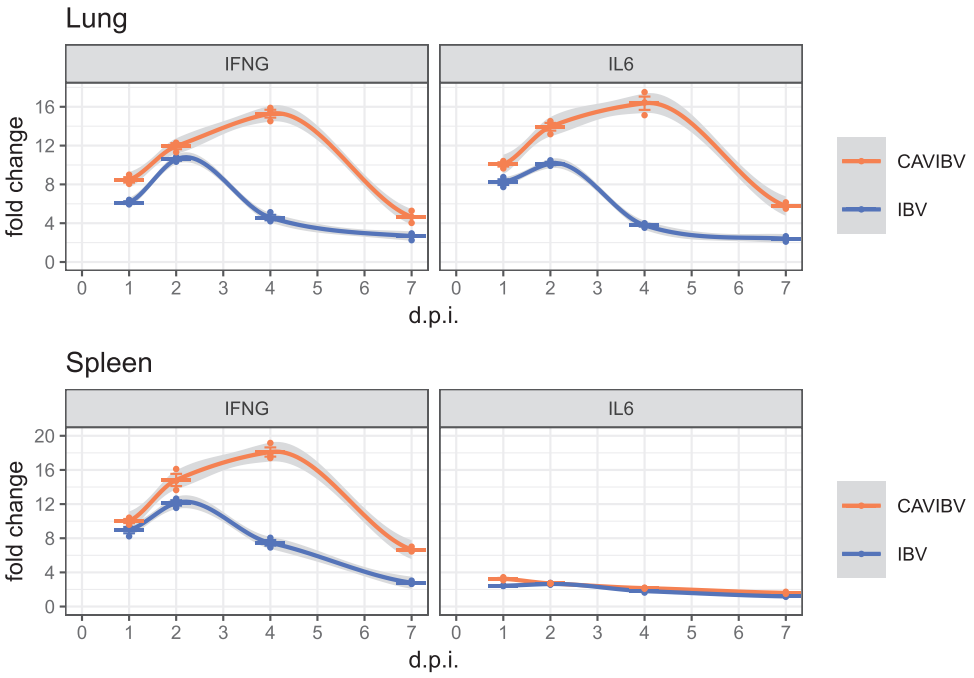


**Fig. 4.** IFNG and IL-6 fold-change in lungs and spleens of CAV-H9N2 group vs IBV-only group.
Scatter plots with added trend lines for fold change in cytokine gene expression per DPI. Horizontal bar and vertical whiskers show mean and plus minus one standard error of the mean. Grey shaded areas along added trend lines illustrate the 95% confidence interval of the added trend line. Red (CAV-IBV group) and blue IBV group. Upper left pane (lung IFNG), upper right pane (lung IL6), lower left pane (spleen IFNG) and lower right pane (spleen IL6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## References


Abdel-Mawgod, S., Adel, A., Arafa, A.-S., Hussein, H.A., 2018. Full genome sequences of chicken anemia virus demonstrate mutations associated with pathogenicity in two different field isolates in Egypt. Virus Dis. 29, 333–341.

Agunos, A., Pierson, F.W., Lungu, B., Dunn, P.A., Tablante, N., 2016. Review of non-foodborne zoonotic and potentially zoonotic poultry diseases. Avian Dis. 60, 553–575.

Ben Shabat, M., Meir, R., Haddas, R., Lapin, E., Shkoda, I., Raibstein, I., Perk, S., Davidson, I., 2010. Development of a real-time TaqMan RT-PCR assay for the detection of H9N2 avian influenza viruses. J. Virol. Methods 168, 72–77.

DaPalma, T., Doonan, B.P., Trager, N.M., Kasman, L.M., 2010. A systematic approach to virus-virus interactions. Virus Res. 149, 1–9.

De Boer, G.F., Van Roozelaar, D.J., Moormann, R.J., Jeurissen, S.H., Wijngaard, J.C., Hilbink, F., Koch, G., 1994a. Interaction between chicken anaemia virus and live Newcastle disease vaccine. Avian Pathol.: J. W.V.P.A 23, 263–275.

De Boer, G.F., Van Roozelaar, D.J., Moormann, R.J., Jeurissen, S.H.M., Van Den Wijngaard, J.C., Hilbink, F., Koch, G., 1994b. Interaction between chicken anaemia virus and live Newcastle disease vaccine. Avian Pathol. 23, 263–275.

Ellstrom, P., Hansson, I., Soderstrom, C., Engvall, E.O., Rautelin, H., 2014. A prospective follow-up study on transmission of campylobacter from poultry to abattoir workers. Foodborne Pathogens Dis. 11, 684–688.

Erfan, A.M., Selim, A.A., Naguib, M.M., 2018. Characterization of full genome sequences of chicken anemia viruses circulating in Egypt reveals distinct genetic diversity and evidence of recombination. Virus Res. 251, 78–85.

Halvorson, D.A., 2008. Control of low pathogenicity avian influenza. In: Swayne, D.E. (Ed.), Avian Influenza. Ames IO Blackwell Publishing, pp. 513–536.

Hassan, K.E., Shany, S.A., Ali, A., Dahshan, A.H., El-Sawah, A.A., El-Kady, M.F., 2016. Prevalence of avian respiratory viruses in broiler flocks in Egypt. Poultr. Sci. 95, 1271–1280.

Hoerr, F.J., 2010. Clinical aspects of immunosuppression in poultry. Avian Dis. 54, 2–15.

Hussein, H.A., Sabry, M.Z., El-Ebiary, E.A., El-Safty, M., Abdel-Hady, A.L., 2002. Chicken infectious anaemia virus in Egypt: molecular diagnosis by PCR and isolation of the virus from infected flocks. Arab J. Biotechnol. 5, 263–274.

Jackwood, M.W., 2012. Review of infectious bronchitis virus around the world. Avian Dis. 56, 634–641.

John, J.L., 1994. The avian spleen: a neglected organ. Q. Rev. Biol. 69, 327–351.

Li, K., Liu, Y., Liu, C., Gao, L., Zhang, Y., Cui, H., Gao, Y., Qi, X., Zhong, L., Wang, X., 2016. Recombinant Marek's disease virus type 1 provides full protection against very virulent Marek's and infectious bursal disease viruses in chickens. Sci. Rep. 6, 39263.

MacLachlan, N.J., Dubovi, E.J., 2017. Chapter 13 - Circoviridae and Anelloviridae. In: MacLachlan, N.J., Dubovi, E.J. (Eds.), Fenner's Veterinary Virology, fifth edition. Academic Press, Boston, pp. 259–268.

McNeilly, F., Smyth, J.A., Adair, B.M., McNulty, M.S., 1995. Synergism between chicken anemia virus (CAV) and avian reovirus following dual infection of 1-day-old chicks by a natural route. Avian Dis. 39, 532–537.

Meir, R., Maharat, O., Farnushi, Y., Simanov, L., 2010. Development of a real-time TaqMan RT-PCR assay for the detection of infectious bronchitis virus in chickens, and comparison of RT-PCR and virus isolation. J. Virol. Methods 163, 190–194.

Mottet, A., Tempio, G., 2017. Global poultry production: current state and future outlook and challenges. World's Poult. Sci. J. 73, 245–256.

Naguib, M.M., Arafa, A.S., Parvin, R., Beer, M., Vahlenkamp, T., Harder, T.C., 2017a. Insights into genetic diversity and biological propensities of potentially zoonotic avian influenza H9N2 viruses circulating in Egypt. Virologys 165–174.

Naguib, M.M., El-Kady, M.F., Luschow, D., Hassan, K.E., Arafa, A.S., El-Zanaty, A., Hassan, M.K., Hafez, H.M., Grund, C., Harder, T.C., 2017b. New real time and conventional RT-PCRs for updated molecular diagnosis of infectious bronchitis virus infection (IBV) in chickens in Egypt associated with frequent co-infections with avian influenza and Newcastle disease viruses. J. Virol. Methods 245, 19–27.

Pohjola, L., Tammiranta, N., Ek-Kommonen, C., Soveri, T., Hanninen, M.L., Fredriksson Ahomaa, M., Huovilainen, A., 2017. A survey for selected avian viral pathogens in backyard chicken farms in Finland. Avian Pathol.: J. W.V.P.A 46, 166–172.

R Core Team, 2016. In: R Core Team (Ed.), R: A Language and Environment for Statistical Computing, 3.3.2 ed. R Foundation for Statistical Computing, Vienna, Austria Sincere Pumpkin Patch.

Reese, S., Dalamani, G., Kaspers, B., 2006. The avian lung-associated immune system: a review. Vet. Res. 37, 311–324.

Rosario, K., Breitbart, M., Harrach, B., Segalés, J., Delwart, E., Biagini, P., Varsani, A., 2017. Revisiting the taxonomy of the family Circoviridae: establishment of the genus Cyclovirus and removal of the genus Gyrovirus. Arch. Virol. 162, 1447–1463.

Samy, A., Naguib, M.M., 2018. avian respiratory coinfection and impact on avian influenza pathogenicity in domestic poultry: field and experimental findings. Vet. Sci. 5.

Thomas, J.K., Noppenberger, J., 2007. Avian influenza: a review. Am. J. Health Syst. Pharm. 64, 149–165.

Umar, S., Munir, M.T., Ahsan, U., Raza, I., Chowdhury, M.R., Ahmed, Z., Shah, M.A.A., 2016. Immunosuppressive interactions of viral diseases in poultry. World's Poult. Sci. J. 73, 121–135.

Villegas, P., 1998. Titration of biological suspensions. In: Swayne, D.E. (Ed.), A Laboratory Manual for the Isolation and Identification of Avian Pathogens. American Association of Avian Pathologists, Kennett Square, Pa, pp. 248–254.

Wang, J., Yue, H., Wu, S., Zhang, H., Qi, G., 2017. Nutritional modulation of health, egg quality and environmental pollution of the layers. Anim. Nutr. 3, 91–96.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.

Yuan, J.S., Reed, A., Chen, F., Stewart Jr., C.N., 2006. Statistical analysis of real-time PCR data. BMC Bioinf. 7, 85.

Zanaty, A., Naguib, M.M., El-Husseiny, M.H., Mady, W., Hagag, N., Arafa, A.S., 2016. The sequence of the full spike S1 glycoprotein of infectious bronchitis virus circulating in Egypt reveals evidence of intra-genotypic recombination. Arch. Virol. 161, 3583–3587.

Zhuang, Q.Y., Wang, S.C., Li, J.P., Liu, D., Liu, S., Jiang, W.M., Chen, J.M., 2014. A clinical survey of common avian infectious diseases in China. Avian Dis. 58, 297–302.