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Effect of co-infection with Low Pathogenic Avian Influenza H9N2 subtype and Infectious Bronchitis viruses on their replication in emboryonated chicken egg and tissue culture cells.

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Abstract
Field observation in Egypt indicates that low pathogenic Avian Influenza (LPAI) H9N2 subtypes is very common in poultry flocks. H9N2 virus can pass in the farm silently without noticed clinical outcome but detected accidentally in testing of broiler for AI prior slaughter. When co-infection occur by other respiratory virus like Infectious Bronchitis virus (IBV) marked clinical outcome (high mortality and sever drop in egg production) noticed. In this study we evaluate the effect of co- infection of (LPAI) H9N2 subtype followed by IBV by using quantitative Real Time Polymerase Chain Reaction (qrt-PCR) with 24 hours interval with challenge dose 10^4EID_{50} for 96 hours post inoculation (HPI) and vice versa in embryonated chicken egg (ECE) and chicken embryo fibroblast (CEF) tissue culture cells. We concluded that H9N2, IBV co-infection will cause interference and reduce the viral replication of both viruses for 72 hrs. which may attributed to competition for virus receptors. This interference is followed by replication phase by both viruses with less titers as compared to single virus replication.

Keywords: *H9N2, qrT, PCR, IBV, ECE, CEF.*

Introduction

In the recent decades, major increase in the universal poultry meat and egg production industry has been occurred **(Mottet and Tempio, 2017).**

Infectious poultry diseases still one of the major problems of the poultry industry, and can lead to extensive economic losses, as well as zoonotic problem to industry staff **(Ellstrom *et al.,* 2014; Samy and Naguib, 2018).**

The major threatening agents are viral diseases, as avian influenza virus (AIV), infectious bronchitis virus (IBV), Newcastle disease virus (NDV), infectious bursal disease, and Marek's disease; also, many bacterial agents can negatively affect poultry **(Agunos *et al.,* 2016).**

Such diseases often occur mixed with other pathogenic agents, and/or managemental faults **(Samy and Naguib, 2018; Wang *et al.,* 2017).**

Furthermore, numerous co-infections/ interferences (virus-virus or virus-bacteria) among the poultry population have recently been detected all over the world **(Samy and Naguib, 2018).**

LPAIV and IBV are main causes of economic losses in the poultry industry, due to increased mortality, retarded growth rate, and reduced egg and meat productions.

Both viruses are affecting the respiratory and the reproductive tracts in addition to the renal system of chickens, causing respiratory signs, impaired weight-gain and usually permanent decrease of egg laying performances **(Cavanagh, 2003; Liu *et al.,* 2004).**

Both AIV and IBV are RNA viruses of birds, belonging to the Orthomyxoviridae and the Coronaviridae families, respectively, and hav-

ing numerous clinical features in common **(Carstens, 2009).**

The AIV genome has eight linear segments of negative-sense ssRNA. The IBV genome has a non-segmented positive-sense ssRNA. The AIV type A viruses are classified as low pathogenic (LP) and high pathogenic (HP) viruses, based on their virulence for chickens and the presence of multiple basic amino acids at the cleavage site of the hemagglutinin (HA), the precursor protein **(Capua *et al.,* 2009; Boursnell *et al.,* 1987).**

The receptor of AIV on the cell surface is the N-acetyl neuraminic acid (α2, 3-sialic acid-galactose) **(Fernandez-Siurob *et al.,* 2014).**
However, the Corona viruses have evolved with a variety of initiation of infection strategies for both the attachment stage and the consequent membrane fusion.
They have even need the presence of definite cell surface proteins for infection; the N-acetyl neuraminic acid (α 2,3-sialic acid) being the receptor protein determinant for primary attachment for group 3 Corona virus to which belongs the IBV **(Delmas *et al.,* 1992; Wickramasinghe *et al.,* 2011; Winter *et al.,* 2001).**
Perhaps, IBV may bind to only very specific subset of α 2,3 -sialylated glycans, present only on chicken cell surfaces **(Wickramasinghe *et al.,* 2011).**

Studying the influence of infection by one virus on another virus is important to understand the interactions between respiratory viruses and the impact of the control measures applied such as vaccination. Multiple infections with AIV and IBV were rarely studied.

On the other hand, none of these studies have quantitatively estimated the degree of *in vitro* and *in ovo* interferences take occurred **(Seifi *et al.,* 2012).**

Due to the prevalence of both viruses itsimportant to study the viral interference by evaluating AIV and IBV multiplication , using the recently developed multiplex real-time RT-PCR (rRT-PCR) **(Nacira *et al*. 2018).**

Materials and Methods

Viruses strains:

Four standard titrated viruses (of 10^4EID$_{50}$ titer) were obtained from the repository of the Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Egypt. Purified Egyptian virus **A/Chicken/Egypt/ V1527/2018** Avian Influenza sub type H9N2 live virus with Gen Bank Accession No **MK212394 and**/or **IBV-Eg-15170F-SP** as IBV Variant II live virus with Gen Bank Accession No**KY119259.** Virus strains were ten-fold serially diluted to get the applicated inoculums concentrations (10^4 EID$_{50}$).

SPF inoculation:

Specific pathogen free eggs (SPF) 9-11 day old embryonated chicken eggs (ECE) were used for isolation and propagation of virus isolates and pathogenicity testing. They were obtained from (Koum-shiem SPF, Fayoum, Egypt). ECEs were inoculated via the allantoic sac according to the OIE guidelines.

Tissue culture cell:

Chicken Embryo Fibroblast **(CEF)** the CEL cultures were prepared from 18-day-old ECE. Thus, the muscles of the embryos were collected, washed with PBS, cell were maintained in DMEM containing 10% fetal bovine serum (FBS) and antibiotic concentration according to stander procedure in 6 well micro plates. The confluent cells were washed twice with PBS and infected with 100 ul of 10^4 EID$_{50}$ from each virus and incubated 1 h at 37°C with 5% CO2 and after that discard the supernatant fluid and adding 1 ml MD containing 2% serum and incubated at 37°C with 5% CO2 environment. Supernatant fluid collected at different time point 24 h, 48 h, 72 h and 96 h post infection and stored at -70°C until titration by HA test. One uninfected well in each plate was considered as negative control.

Haemagglutination assay

The haemagglutination assay was performed according to **OIE 2015** guide line.

Quantitative Real Time PCR (qrt-PCR):

Viral RNA was extracted by using QIAamp viral RNA mini kit **(Qiagen, Germany)** in accordance with manufacturer's instructions. Ag

Path ID One-Step RT-PCR **(Thermo scientific, USA)** and specific oligonucleotide primers and taqman probes assays were used for detection and quantification of AIV-H9N2 **(Ben Shabat *et al.*, 2010)**. and nucleoprotein gene of IBV **(Meir *et al.*, 2010)**. To determine AIV-H9N2 and IBV viruses titers, a standard curve for each virus was generated using titrated viruses in SPF-ECE. The qrt-PCR reaction volume was 25 µl containing 4.5 µl of extracted RNA, 12.5 µl 2 × One-step RT-PCR ready mix, 1 µl RT enzyme, 0.5 µl of 50 pmol of both forward and reverse primers, 0.125 µl of specific probe of 30 pmol conc. And 6 nuclease free water. The thermal profile included a reverse transcription step at 50 °C for 30 min followed denaturation step at 95°C for 15 min. The PCR cycling was performed in 40 cycles of denaturation at 95°C for 15 s, annealing (60°C for IBV) and (54°C for AIV-H9) for 30 s and extension at 72°C for 10 s. QRT-PCR was performed using StepOnePlus Real-Time PCR machine **(Applied Biosystem Thermo scientific, USA).**

Results

Table (1). Results of HA activity of ECE by log^2:

Hours post inoculation (HPI)	H9N2 Control	IBV Control	H9N2+ IBV	IBV+ H9N2
24	0	NON	0	0
48	5	NON	4	4
72	7	NON	6	5
96	8	NON	0	6

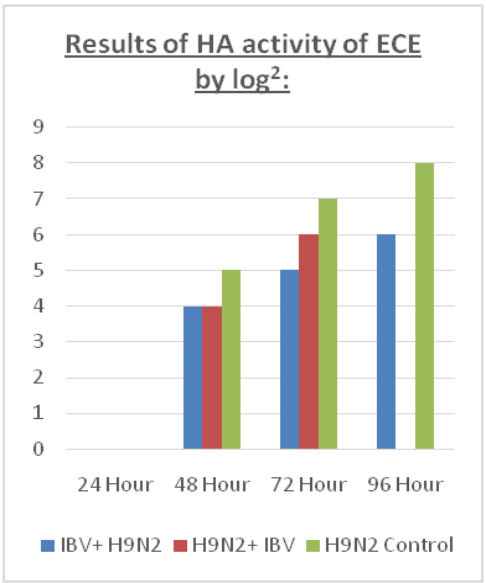
*NON=not Haemagglutinating virus

Table (2). Results of HA activity of CEF by log^2:

Hours post inoculation (HPI)	H9N2 Control	IBV Control	H9N2+ IBV	IBV+ H9N2
24	0	NON	0	0
48	4	NON	3	3
72	6	NON	5	4
96	7	NON	0	5

*NON=not Haemagglutinating virus

Flu chart (1) HA activityby log^2:



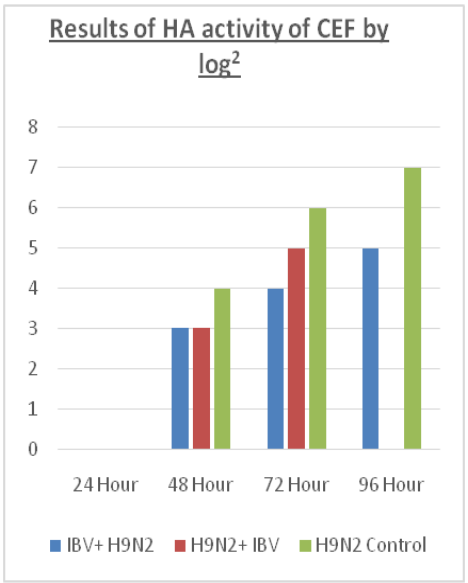


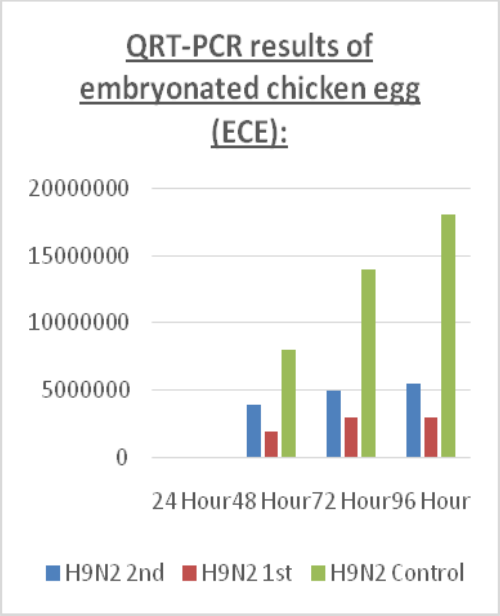
Table (3). NO. of ECE daily deaths out of five egg inoculated each time:

Hours post inoculation (HPI)	H9N2 Control	IBV Control	H9N2+ IBV	IBV+ H9N2
24	0/5	0/5	1/5	0/5
48	1/5	1/5	2/5	1/5
72	2/5	2/5	2/5	2/5
96	2/5	2/5	0/5	2/5

Table (4). qrt-PCR results of embryonated chicken egg (ECE):

Hours post inoculation (HPI)	H9N2			IBV		
	Control	1st	2nd	Control	1st	2nd
24	0	$1.6*10^6$	0	$0.3*10^3$	$0.4*10^3$	$0.2*10^3$
48	$8*10^6$	$2*10^6$	$4*10^6$	$1*10^3$	$0.8*10^3$	$0.3*10^3$
72	$14*10^6$	$3*10^6$	$5*10^6$	$2*10^3$	$2*10^3$	$0.3*10^3$
96	$18*10^6$	$3*10^6$	$5.5*10^6$	$3*10^3$	$3*10^3$	$2*10^3$

Flu chart (2) qrt-PCR results of embryonated chicken egg (ECE):



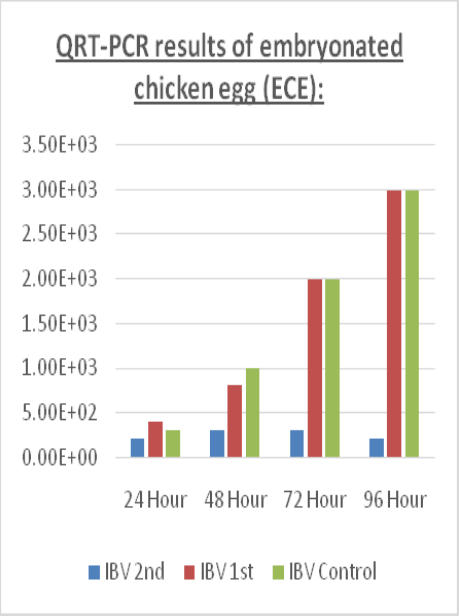
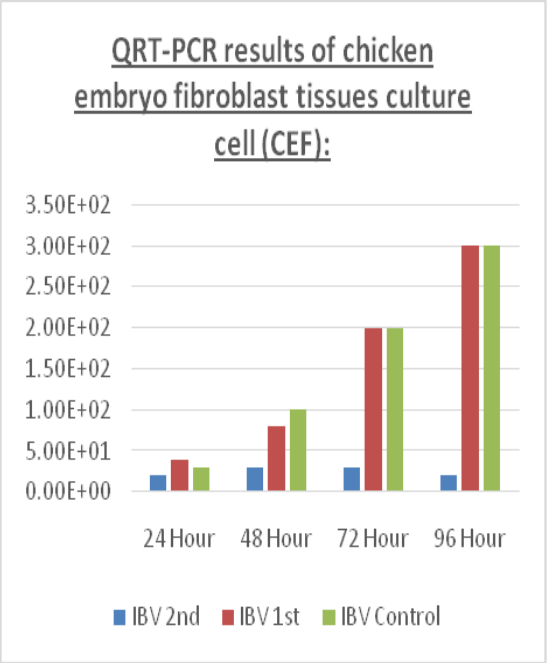
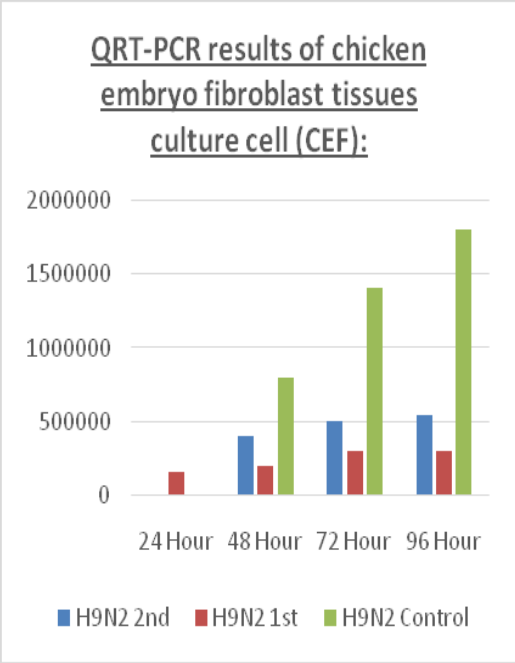


Table (5). qrt-PCR results of chicken embryo fibroblast cell line (CEF):

Hours post inoculation (HPI)	H9N2			IBV		
	Control	1st	2nd	Control	1st	2nd
24	0	$1.6*10^5$	0	$0.3*10^2$	$0.4*10^2$	$0.2*10^2$
48	$8*10^5$	$2*10^5$	$4*10^5$	$1*10^2$	$0.8*10^2$	$0.3*10^2$
72	$14*10^5$	$3*10^5$	$5*10^5$	$2*10^2$	$2*10^2$	$0.3*10^2$
96	$18*10^5$	$3*10^5$	$5.5*10^5$	$3*10^2$	$3*10^2$	$2*10^2$

Flu churt (3) qrt-PCR results chicken embryo fibroblast cell line (CEF):





Discussion

The universal trend in broiler poultry industry all-over the world is reduction of number of vaccine administration to avoid stress on birds in vaccination period to gain maximum growth rate in very short time.

In Egypt from our field experience, the infection by H9N2 or IBV viruses singly is very weak infection lead to about 10% mortality and can pass in the farm without any notice (silent passage and carrier). But in case of dual infection occur there was exacerbating of mortality rate lead to 60% and these observation explained by **(Seifi *et al.*, 2010)** as it seems that H9N2 viruses cause severe mortality in the infected birds when they co-infected with vaccinal or virulent IBVs.

Infection of a bird with dual viruses lead to viral interference which is a phenomenon in which virus-infected cells and hinder the multiplication of a second entered virus, viral interference can be explained by many mechanisms including (1) fight for cell receptors in attachment stage of viral replication (2) intracellular host machinery competition, and (3) virus-mediate interferon interference. **(Samy and Naguib, 2018).**

Obvious changes that are noticed as being combined with viral co-infection include changes in viral multiplication patterns, tissue tropism, pathological and immunological response so we conduct this study to evaluate the effect of co- infection of (LPAI) H9N2 subtype followed by (IBV) with 24 hours interval for 96 HPI with challenge dose 10^4 for 96 hours post inoculation (HPI) and vice versa in embryonated chicken egg (ECE) and chicken embryo fibroblast (CEF) tissue culture cells and detect the number of both viruses copies by Qrt -pcr in comparison with the control.

The results of **tables (1, 2 and 3)** and **flu chart (1)** stated that in case of (LPAI) H9N2 the HA titer is decreased by1 log titer at 48 HPI while showed 2 log titer at 96HPI. When daily deaths of ECE showed that H9+IBVinoculated die 1 out of 5 in 48 HPI but die 2 out of 5 in 72 HPI.

We find in both ECE & CEF cell line that the amount of viral growth in ECE is higher than CEF cell line almost one log **as shown inall results** due to there were Many factors effect on ECE and cell culture system for virus isolation such as molecular genetics properties of the virus, receptor binding properties of the host cell and some other host related factors lead to successful and efficient propagation **(Azab *et al.*, 2017).**

All results of Qrt-pcr showed that the first introduced virus either H9N2 or IBV was inter-

fere the growth of each other expressed by low viral copies yield if compared with control due to competition on host cell receptors and these results declared for 72 HPI then at 96 HPI the both viruses had the capability to begun to grow again but still less than control one and these finding was matched with **(Aouini *et al.*, 2018)** who stated that the yields of AIV and IBV during dual infection with H9N2 and IBV were drastically lower than those gotten during single infections. When both AIV (H9N2) and IBV were mixed together, signs of interference between the two infecting viruses were noticed with an interfering effect detected by reduction of the growth of both viruses when AIV and IBV were infected at 1h interval, interference phenomenon was diverse from simultaneous infection.

In fact, there was a strong reduction of the growth of AIV (H9N2) after IBV superinfection and vice versa, within the first three days. It was obviously shown that AIV was able to hinder the replication of IBV and IBV was also able to reduce the growth of AIV multiplication. However, on the fourth day of infection, both viruses were capable to grow again, even in existence of the enemy virus but less than the level of single infections growth rate.

Finally we concluded that either LPAIV (H9N2) subtype or IBV has adverse effect on the other virus growth when they are inoculated sequentially for first three days post inoculation but in the fourth day post inoculation the both viruses began to grow again but still less than control one. The results showed that infection with both viruses may result in impermanent competition for cell receptors or capable cells for replication for first three days.

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