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Comparison of replication competence between two highly pathogenic avian influenza viruses H5N1 and H5N8 in vitro

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Mixed infections with respiratory viruses have become frequent at poultry farm business in Egypt. The pandemic of highly pathogenic avian influenza virus (HPAIV) H5N1 (clade 2.2.1.2) has been spreading through the country since 2006. In 2016, the Spread of HPAIV H5N8 (clade 2.3.4.4b) to Egypt via wild birds into commercial poultry flocks made overburden on the economic situation of poultry business. The surveillance efforts conducted by the governmental veterinary authority GOVS showed dominating infection of H5N8 with rare spread of H5N1 in poultry market sector. Thus, the current study try to figure out whether a replication competence exhibited between the two highly pathogenic avian influenza viruses HPAIV H5N1 and H5N8 through designation an *in-vitro* induced infection of H5N8 and H5N1 in specific pathogen free embryonated chicken eggs (SPF- ECE).

A concentration of 10^3 EID50 of HPAIV H5N1 and H5N8 were either sequentially or simultaneously inoculated into the allantoic cavity of SPF-ECE at 48 h of incubation, followed by the collection of allantoic fluid. A quantitative reverse transcription real-time polymerase chain reaction was used to determine the quantitative replication of viral RNA copies of neuraminidase gene of both AIV strains.

A replication competence was observed according to which viral strain firstly inoculated. Whereas, H5N1 RNA titers was reduced by $\geq 4\log_{10}$ when H5N8 firstly inoculated. Vice versa H5N8 RNA titers were reduced by $\geq 4\log_{10}$ when H5N1 firstly inoculated. The interference impact of H5N1 was more powerful than that of H5N8 when simultaneous inoculation of both viruses was applied.

In conclusion, quantitative evaluation of mixed inoculation of AIV strains exhibited replication competence of the first virus inoculation on the account of the succeeding one regardless of viral strain. However, H5N8 showed lower replication intensity according to the neuraminidase gene evaluation when both H5N1 and H5N8 were simultaneously inoculated in SPF ECE.

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INTRODUCTION

The poultry business sectors contribute significantly to the national market value by attracting private investment and creating jobs. Poultry meat remains an affordable source of animal nutrition for people of middle or low income (**Abdelwhab & Hafez, 2011**).

Over several years, severe endemic respiratory virus infection resulted in catastrophic mortalities in the farm and backyard sectors. In majority of the afflicted poultry sectors, avian influenza (AI), Newcastle disease (ND), and infectious bronchitis (IB), either single or co-infection have been found; also, bacterial infections can occasionally exacerbate the situation (**Hassan et al. 2016; Radwan et al. 2013; Samy & Naguib, 2018).**

Avian Influenza virus (AIV) has continued to circulate in Egyptian poultry farms and backyard since the first identification of AI virus (AIV) in poultry in 2006, when outbreaks of highly pathogenic (HP) AIV H5N1 of clade 2.2.1 were appeared (**Aly et al. 2008; Peyre et al. 2009).** over the course of years, frequent mutations in the hemagglutinin gene (HA) were occurred resulting in amino acid substitutions, which were linked to clade shifting (2.2.1, 2.2.1.2) and vaccine escape (2.2.1.1) **(Balish et al. 2010; Hassan et al. 2016).** The new clade 2.2.1.2 appeared in 2014–2015 resulted in great number of human infection **(Arafa et al. 2016, WHO, 2019**).

The AI situation in Egypt has been further complicated by the recent arrival of antigenically different strain, HPAIV H5N8 of clade 2.3.4.4b, originated in China and spread across Central Asia into Europe and Africa reaching Egypt in 2016 (**Selim et al. 2017**). As well as, recent studies suggested that multiple introductions of different reassortants HPAI H5N8 viruses of clade 2.3.4.4b occurred in Egypt causing cases in wild birds (**Yehia et al. 2018**). These two subtypes of AIV appeared to be co-circulating in Egyptian poultry farms at the moment which both have been shown zoonotic potential (**Who, 2019**).

In addition to the aforementioned a third co-circulated virus in the Egyptian poultry farms LPAI H9N2 has proven zoonotic potential (**EFSA, 2018**). During 2017 and 2018, a surveillance study found that H9N2 co-circulated with HPAIV H5N8 in 56.8% of flocks with 10 to 12 % sustained infection with HPAI H5N1. Reasons for the relative increase of HPAIV H5N8 infections versus H5N1 remained unclear, but lack of suitable vaccines against clade 2.3.4.4b cannot be excluded (**Hassan et al. 2021**).

Evidence for high incidence of mixed infections of different viral diseases as AIV and Newcastle dis virus was provided by several studies (**Amer et al. 2018 & El-lakany et al. 2018**).The likelihood of viral competence between AIV and Newcastle disease virus (NDV) co-infection was discussed by a previous study **(Soliman et al. 2019)**.The study has provided an evidence of replication competence between AIV H5N1 and H5N8 that revealed higher replication competence of H5N8 against H5N1 as well as both AIV strains showed higher replication competence against NDV that was based on quantitative evaluation of hemagglutinin (HA) gene in-vitro infection in SPF-ECE (**Soliman et al. 2019**). According to previous studies there are lack of information about the effect of neuraminidase NA gene in AIV on replication competence in case of co-infection of different AIV strains

Therefore, the present study aims to figure out the effect of viral competence between H5N1 and H5N8 based on quantitative evaluation of neuraminidase NA gene replication. This study aimed to estimate quantitatively the viral interference of mixed infection between both circulated AIVs H5N1 and H5N8 *in-vitro using qrRT-PCR.*

MATERIALS and METHODS

Ethical approval

the study does not require ethical approval as the study based on SPF-egg model (not living bird model).

Virus strain

Two standard titrated viruses (10^6 EID_{50}

titer) were obtained from the repository of the Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Egypt [HPAIVH5N1(A/chicken/ Egypt /173CAL/2017; HPAIV H5N8 (A/ chicken/Egypt/CA35/2017)] ; GenBank accessions for H5N1 and H5N8 AIVs of the obtained strains are MG192004 and MH762131,respectively.Virus strains were 10-fold serially diluted to select the applied inoculum concentrations (10^2, 10^3, and 10^4 EID_{50}).The viral infectivity of each strain was determined by serial titration in10-11days-old embryonated chicken eggs and was expressed as 50% of the egg infective dose (EID_{50}/ml) using standard methods (**Reed and Muench, 1938**).

SPF-ECE inoculation

SPF-ECEs were purchased from the Egyptian SPF Egg Production Farm (Nile SPF), Kom Oshiem, El-Fayoum Governorate, Egypt. ECEs were inoculated through the allantoic sac according to the OIE guidelines (**OIE, 2014**) and eggs were incubated at 37°C. Inoculated eggs were candled daily for 3-5 successive days. Bacteria-free allantoic fluid was aliquoted and stored at (-80°C) until tested.

Experimental designs for interference study between H5N1 and H5N8

Ten-days-old SPF-ECEs were sequentially infected with two viruses at equal multiplicities.

In **table** (1), a summary of the experimental design was provided. Three groups of 15 eggs per each were inoculated with titers 10^3 EID_{50} of both two viruses. Based on the evaluation of Neuraminidase gene N1 or N8, The first group G1 was designed for sequential inoculation of AIV H5N1 followed by H5N8 and the second group G2 was firstly inoculated with H5N8 followed by H5N1, then incubated for 24 h between the two inoculations. The third group G3, both viruses were inoculated simultaneously. Two positive control groups of 15 eggs each, were designed for a single inoculation of each viral type, while sterile phosphate-buffered saline was injected instead of the second inoculum. One negative control group was also designed. After viral inoculations, ECEs were candled at 12 h intervals. Harvesting of allantoic fluid was done after 48h. All the groups were repeated in triplicates.

Table 1. Experimental design

Groups	G1[a]	G2[a]	G3[a]		Positive control	
1st virus inoculation Then incubation for 24h	H5N1[b]	H5N8	H5N8	H5N1	H5N1	H5N8
2nd virus inoculation Then incubation for 24h	H5N8	H5N1			PBS[c]	PBS
Harvesting of allantoic fluid after incubation period.						
qRT-PCR	Each group has tested for both N1,N8				N1	N8

a: Each group have 15 ECE, all groups were repeated in triplets.
b: H5N1 and H5N8 the inoculated virus concentration was 10^3 EID_{50}
c: Phosphate buffer saline PBS

Quantitative Real Time PCR (qRT-PCR) for Neuraminidase gene N1or N8

Viral RNA was extracted from the harvested allantoic fluids by using QIAamp viral RNA mini kit (Qiagen, Germany) in accordance with manufacturer's instructions. One-Step RT-PCR kit (Thermo scientific, USA) and specific oligonucleotide primers with taqman probes (Metabion, Germany) were used for detection and quantification of AIV-N1 (**Li et al. 2013**) and AIV-N8 (**Hoffman et al. 2016**). To determine AIV-H5N1 and AIV-H5N8 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, 0.125µl of specific probe of 30 pmol conc. And 5.875 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 15s, annealing (55 °C both viruses) for 30s and extension at 72 °C for 10s, QRT-PCR was performed using Step One Plus Real-Time PCR machine (Applied Biosystem Thermo scientific, USA). Samples with a Cq value ≤39 were considered positive.

Statistical analysis

RNA copy titer of each virus was determined using qrRT-PCR. The degree of interference was estimated by comparing AIV (N1 or N8) yields from co-infected ECEs with those of the corresponding controls as measured independently by qrRT-PCR. Statistical variation between the experimental group and control group was determined by ANOVA test where $p<0.05$. This experiment was set up to investigate the effect of the first inoculated virus on the growth of a second inoculated virus in the ECE.

RESULTS

The results of first group (G1) experiment are shown in (figure1), which represent the quantitative measure of AIV-H5N1 and subsequent infection of H5N8. The reduction of H5N8 (N8) was ≥4$\log_{10}$

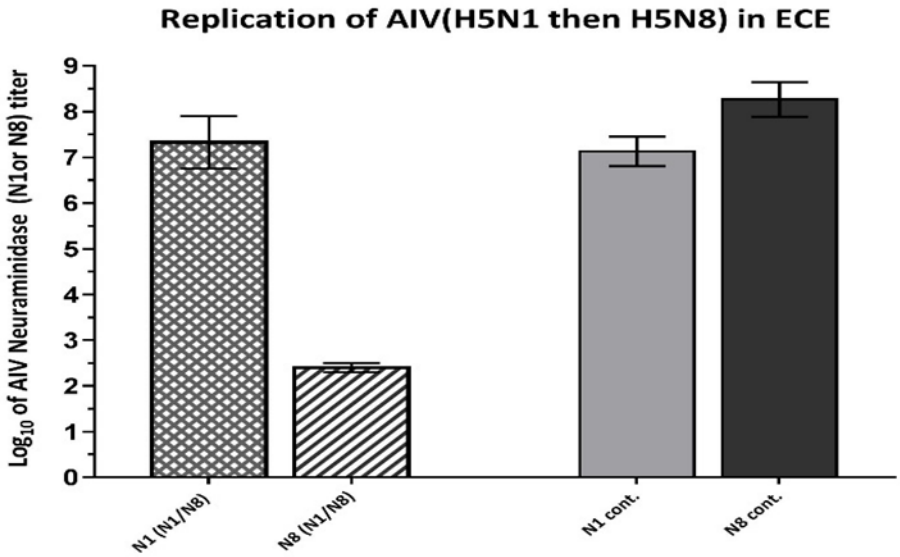

Figure (1). The replication titers of sequential inoculation H5N1 then H5N8. Black and grey columns represent the positive control group (single H5N8 &H5N1 inoculation respectively). Two columns represent N1 and N8 titers of sequential inoculation (H5N1 then H5N8). Bars over the columns represent the error bars of standard deviation to the mean titers of triplicate values. $p<0.05$.

The second group G2 experiment represented the sequential coinfection of first H5N8 then subsequent of H5N1. The AIV (H5N1) RNA titers showed reduction ≥4log_{10} compared with the control group (figure 2).

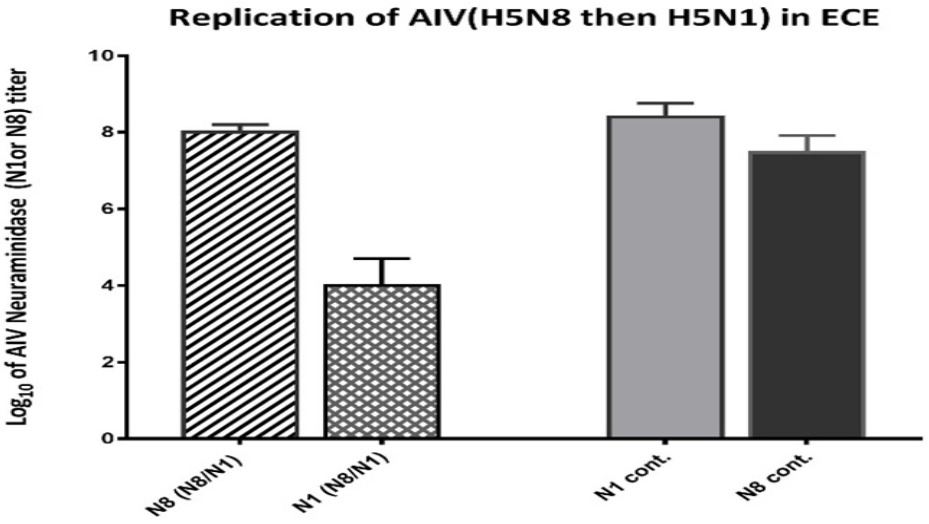

Figure (2). The replication titers of sequential inoculation H5N8 then H5N1 . Black and grey columns represent the positive control group. Two columns represent N8 and N1 titers. Bars over the columns represent the error bars of standard deviation to the mean titers. $p<0.05$

In the third group G3 ,AIV (H5N8) titers showed reduction when ECEs were simultaneously infected with H5N1 (figure-3). The titers of Neuraminidase (N8) of H5N8 RNA showed ≤4log_{10} reduction when co-inoculated with H5N1 compared to the RNA titer of N8 control. The titers of Neuraminidase (N1) of H5N1 RNA showed no reduction in simultaneous coinfection in ECE, as it showed equal titer 7 to 8 log_{10} with the control group.

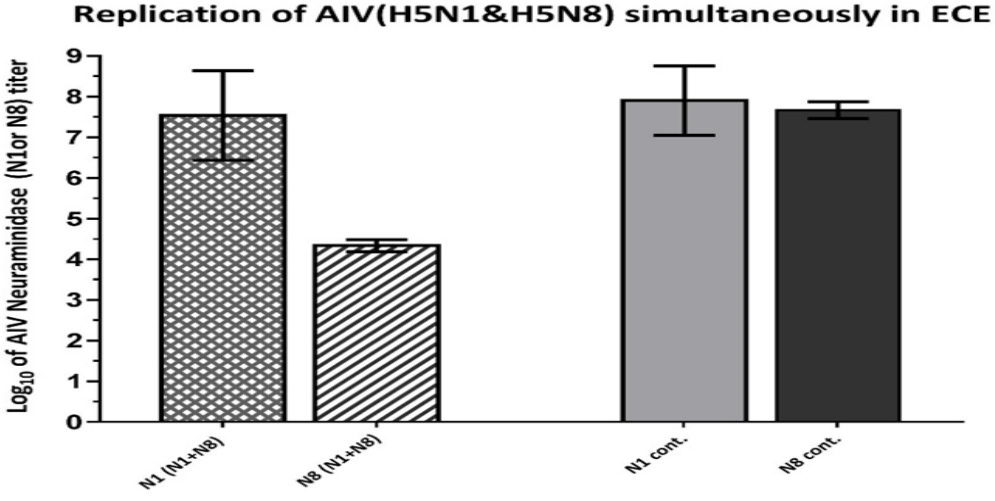

Figure-(3). The replication titers of simultaneous inoculations of H5N1 and H5N8. Black and grey columns represent the positive control group. Two columns represent N1 and N8 titers. Bars over the columns represent the error bars of standard deviation to the mean titers of triplicate values. $p<0.05$

DISCUSSION

Viral interference is a phenomenon where one infected cell with a virus have negative impact to the replication of secondary virus either homologous or heterologous (**Aouini et al. 2018).** In this study, interference of H5N1 and H5N8 HPAIV AIVs with each other was quantitatively evaluated in ECE model using qrRT-PCR. Previous studies have shown that the first virus is capable of inhibiting the growth of another (**Roussan et al. 2008** & **Pantin jackwood et al. 2015**). Previous study was focused on the evaluation of the interference between AIV and NDV using qrRT-PCR to determine the degree of viral competence, also it showed high replication competence for H5N8 against H5N1 after sequential infection of H5N1 and H5N8 based on quantitative evaluation of hemagglutinin (H) gene (**Soliman et al. 2019**).

Such results raise a query whether the competence effect was related to the H gene, neuraminidase (N) or both. In this study evaluation of N gene using qrRT-PCR was applied to determine the viral competence impact of N gene through evaluation of viral replication in SPF-ECE.

Results showed superior competence of first inoculated viral strain. Reduction of AIV-N8 (≥4 log10) compared with the control after first inoculation of AIV-N1. However, the reduction of AIV-N1 after first inoculation of N8 was ≥4 log_{10} compared with control. Interestingly, the effect of N1 is more powerful of N8 in simultaneous infection which showed ≤4 log_{10} reduction of N8.

The obtained results provide that the effect of viral competence was depending on the high replicative first infective strain regardless the type of N gene. The simultaneous infection of both N1 and N8 strains showed greater replication H5N1 than AIV-N8 which was contradictory with pattern of HA gene expression which showed greater expression of H5N8 than H5N1.

In accordance to a related study (**Ge et al. 2012**), the primary infection by NDV virus succeeded to inhibit to a lesser extent (<1-2 log_{10}) the later AIV replication. However, our result was corroborated by the previous research that supported the concept that pre-infection with a H5N1 can minimize virus replication of a later H5N8 and vice versa. A study showed that Quantitative results of AIV and IBV co-infection showed that interferences between the two viruses yielded decreased viral growth. It appears that either AIV or IBV has a negative impact on the other virus growth when they are inoculated simultaneously or sequentially (**Aouini et al. 2018)**.

A query was about the virulence of the viral strains that was important factor that affects interference in the current study. Previous studies **Costa-Hurtado et al. 2014 & Dortmans et al. 2010** reported the direct correlation between NDV strain virulence and the degree of replication. In contrast the virulence was not shown in the study between H5N1 and H5N8, despite of the simultaneous infection showed increased replication of H5N1 over H5N8.

A preceding study (**Liu et al. 2003**) also reported AIV- H9 interference due to NDV replication in ECEs. Another study (**Costa-Hurtado et al. 2015**) was performed in SPF chickens indicated that the previous infection of NDV can decrease the replication of second infection virus HPAIV of H5N2 subtype. These previous studies explained the pattern of replication competence was based on the basic classical theory of receptor competition that depend on the first virus grabbed most of receptors at the expense of second virus replication.

Conclusions

The current study concluded an existence of replication competence between H5N1 and H5N8, and the degree of competence between them based on quantitative evaluation of neuraminidase replication, followed the basic theory of receptor competition which based on the preceding virus inhibit the subsequent virus relatively. The simultaneous infection of both viruses showed higher competence of H5N1 (N1) against H5N8 (N8).

Recommendation

The current search recommends studying the viral interference between two HPAI H5N1 and H5N8 in living bird model.

Competing Interests:

The authors declare that they have no competing interests.

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