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CLINICO-PATHOLOGICAL STUDIES ON CO-INFECTION BETWEEN LOW PATHOGENIC AVIAN INFLUENZA VIRUS H9N2 AND HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS H5N1

By

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ABSTRACT

Egypt is a hotspot for both H5 and H9 subtype avian influenza (AI) A virus infections and the continued evolution/circulation of both viruses and their spread across Egypt since 2012 raise the concern that prior H9N2 virus infection may limit the detection of subsequent H5N1 infection in gallinaceous poultry through attenuating the severity of disease. Recent animal studies have contributed to the growing evidence that temporary non-specific innate immune responses may lead to interference among avian influenza viruses (AIV). In the case of antigenically similar variants of a pathogen such as low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI), interference may be similarly manifested at the host scale through immunological cross-protection(CP);the prevention or partial inhibition of an individual host to future infections may lead to altered transmission dynamics during pathogen co-circulating.In the case of influenza,cross-protection as a consequence of adaptive immunity is expected since neutralising antibodies and cross-reactive cell-mediated immunity are known to act across homo and hetero sub typically diverse viruses.The present results showed that there is cross-protection in the co-infected groups as shown in the decreasing percentage of morbidity and mortality and increasing the percentage of survivability in co-infected groups that challenged with LPAI H5N1 virus and HPAI H5N1 virus at different time intervals than the mono-infected group that challenged with HPAI H5N1 virus. It can be concluded that, the partial cross-protection can lead to change in the epizootiological pattern of HPAI H5N1 in the field. That is related to circulation of LPAI H9N2, beside the

different vaccination strategies used and application of different levels of biosecurity. The current findings can be taken in consideration for the control of HPAI in poultry considering frequent co-circulation of both LPAI and HPAI.

Keywords:

Avian influenza virus, highly pathogenic avian influenza, low pathogenic avian influenza, cross-protection.

INTRODUCTION

Poultry industry in Egypt has been experiencing an endemic HPAI-H5N1 subtype since 2006 **(Peyre *et al.* 2009).** The situation has been aggravated by the emergence of H9N2, H5N8, and H5N2 in 2011, 2016, and 2019; respectively **(Salaheldin *et al.* 2018, Hagag *et al.* 2019 and El-Zoghby *et al.* 2012).**

Although vaccination campaigns were implemented,to date AIVs are still evolving, spreading and causing severe problems to poultry industry. Besides the negative economic drawback, the identification of LPAI-H9N2 subtype in a country that is endemic for HPAI-H5N1 avian influenza raises concerns on its control and on the public health implications of such co-circulation **(Arafa *et al.* 2012 and Marinova *et al.* 2016).**

The global concern about the H9N2 viruses is not only associated with their ability to infect avian species and sporadically, mammals such as pigs and humans **(Butt *et al.* 2005 and Peiris *et al.* 2001),** but also associated with their ability to donate their genes to other AIV giving rise to high and low pathogenic AIVs that might therapy achieve the capability to efficiently cross the species barrier and infect humans such as the LPAIV H7N9 reported in China since 2013 **(Liu *et al.* 2014, Wang *et al.* 2017, FAO 2017, Li *et al.* 2014 and GAO *et al.* 2013).** So, the co-existence of these avian influenza subtypes in the same susceptible poultry population may result in the emergence of natural reassortants, similar to what has occurred in Pakistan and Southern China in the recent past **(Iqbal *et al.* 2009 and Dong *et al.* 2011).**

In Egypt the detection of HPAIV H5N1 in poultry flocks previously infected with H9N2 in Egypt under natural conditions has been frequently reported in individual hosts **(Monne *et al.* 2013 and Kayali *et al.* 2016).** In Egypt, LPAIV H9N2 and HPAIV H5N1 viruses continue to co-circulate intensivelyin poultry**(Monne *et al.*2013,Arafa *et al.*2016andYoung *et al.* 2016)** and vaccination of poultry against both viruses using inactivated monovalent or bivalent H5/H9 vaccines is common **(Abdelwhab *et al.*2016)** to control infection by both viruses.

Consequently, the co-circulation of both viruses HPAI and LPAI can result in two scenarios; the first scenario is resulting in a Novel AIV genotype as a result of frequent Reassortment between LPAIV and HPAIV with enhanced zoonotic potential **(Chang *et al.* 2018, Aamir *et al.* 2007,Guan *et al.* 1999 and Gerloff *et al.*2014).**The second scenario is the probability of limitation of detection of H5N1 infection circulating with H9N2 viruses by reducing its lethality and pathogenicity resulting in a decreased incidence of H5N1 outbreaks **(Arafa *et al.* 2016).**

To better validate this potential role of co-circulation of LPAI H9N2 viruses in the spread of HPAI H5N1 viruses without the effect of different vaccination campaigns used or presence of other co-circulating pathogens, the present study was set to experimentally explain the Clinicopathological infection between LPAI H9N2 and HPAI H5N1 in specific pathogen free (SPF) non-vaccinated chickens.

MATERIAL AND METHODS

Ethics statement:

The present experiment was carried out in a biosafety level-3 (BSL-3) laboratory and according to the ReferenceLaboratory for Veterinary QualityControl on Poultry Production (RLQP) guidelines for research ethics.

Virus origin and propagation:

Both LPAIV H9N2 and HPAI H5N1 are Egyptian stains which were isolated in NLQP and Animal Health Research Institute in Egypt: LPAIV H9N2 (A/Chicken/Egypt/114940V/2011/ H9N2) and HPAIV H5N1(A/ Chicken/Egypt/ S175/NL QP/2015/H5N1). The two viruses were subjected to propagation using specific-pathogen-free (SPF) embryonated chicken eggs (ECE) as described by **Villegas and Purchase (1990)**, Titration for calculation of EID50 according to the method of **Reed and Muench(1938)**, evaluation of HA titer and MDT and recording IVPI for each virus.Virus-containing allantoic fluids were diluted using phosphate buffer saline (PBS) to obtain an inoculum with titer of 10^6 EID_{50}/bird.

Chickens:

Specific Pathogen Free (SPF) chickens, four-weeks-old,were obtained from Kom Usheim farm, Fayoum, Egypt.The birds were housed in biosecurity level-3 (BSL3) isolators with feed and water provided *ad libitum.*

Experimental design:

In vivo co-infection experiment:

A total of 70 SPF chickens were divided into 7 equal groups; ten birds each. Seven groups were used to clarify the hypothesis that LPAI H9N2virus in chicken provides cross-protective immunity to HPAI H5N1 virus infection at dose 100 µl of 10^6 EID_{50}/bird via intranasal inoculation, as shown in (Table 1).

Table (1): Chicken groups inoculated with 100 µl of 10^6 EID_{50} per bird via intranasal inoculation used for the in vivo experiment.

Groups	No. of birds	Challenge Virus/ Age/ Week		Remarks
		LPAI H9N2	HPAI H5N1	
1	10	No	No	Negative control
2	10	4	No	H9 positive control
3	10	No	4	H5 positive control
4	10	4	4	Co-infection groups
5	10	4	5	
6	10	4	6	
7	10	4	7	

Group 1 served as a negative control group, group 2 served as positive control for LPAI H9N2 virus, group 3 served as positive control for HPAI H5N1 virus, group 4 were inoculated with 100 LPAI H9N2 and HPAI H5N1 at the same time at 4 weeks of age, group 5 were inoculated with the LPAI H9N2 firstly and then inoculated with the HPAI H5N1 with 1 week interval in between. Group 6 were inoculated with the LPAI H9N2 firstly at 4 weeks of age and then inoculated with the HPAI H5N1 with 2 week interval in between and group 7 were inoculated with the LPAI H9N2 firstly at 4 weeks of age and then inoculated with the HPAI H5N1 with 3 week interval in between.

Monitoring and observation rate:

Control groups (1, 2 and 3) were monitored till the end of experiments, while birds in the co-infection groups (4, 5, 6 and 7) were monitored and observed for 14 days post 2nd challenge according to each group to record clinical score and survival rate. Morbidity and mortality rates were calculated according to the OIE regulations for the Intravenous Pathogenicity Index (IVPI).

Sampling:

Bloodsampleswereweeklycollectedfrom10birds/groups before the infectionphase.

Post-infection phase samples were collected from all survival at the time of testing as shown in Table (2).

Table (2): Total Number of blood samples collected for serological monitoring during the study.

Time on start	Study groups						
	G1	G2	G3	G4	G5	G6	G7
DAY 1	10	10	10	10	10	10	10
W1	10	10	NA	3	10	10	10
W2	10	10	NA	3	3	10	10
W3	10	10	NA	NA	2	3	10
W4	10	10	NA	NA	NA	2	5
W5	10	10	NA	NA	NA	NA	4
Total Samples collected/group	60	60	10	16	25	35	49

NA: not applicable due to bird mortalities.

Cloacal and oropharyngeal swabs were collected at 2nd,4th and 6th day post challenge (dpi) for H9N2, and at 3rd, 6th, 10th and 14th dpi for H5N1 to assess virus shedding via the respiratory and digestive tracts for each virus.

Tissue specimens of lungs, trachea, pancreas, kidneys and small intestine (duodenum) were collected from 2 chickens from all groups after 48 ^{hrs} from each experimental infection.

Serology:

Serum samples were investigated for AIV-specific antibodies using an indirect influenza A antibody ELISA kit (The ProFlock plus AIV ELISA kit) according to the manufacturer's instructions. Positive samples were further examined by a hemagglutination (HI) assay using the homologous H9N2 ang H5N1 viruses and performed according to the standard protocols described by the World Organization for Animal Health (OIE, 2015) to evaluate post-infection humoral immune response.

PCR:

Oropharyngeal and cloacal swabs were collected at indicated time points post-H5N1 and post-H9N2 challenge, to compare the virus shedding patterns via the respiratory or digestive tracts. Viral RNA was extracted using the QIAamp Viral RNA Mini Kit (QIAGEN) catalogue No. 52904 following the manufacturer's instructions **(Ben *et al.* 2010, Lòndt *et al.* 2008).**

Histopathology:

To study tissue tropism; selected tissues and organs from chickens (lungs, trachea, kidneys, pancreas, small intestine"duodenum")were fixed in 10%neutral bufferedformalinand processed for hematoxylin and eosin staining **(Bancroft *et al.* 2012).** The severity of organs and tissue lesions were scored as ordinaryon a4-step scale(- = unchanged,+= mild,++ = moderate, +++ = severe).

Statistical analyses:

Kruskal-Wallis one way analysis of variance was used to compare viral loads between groups. Viral load was defined as the average of Log 10 (PCR copies) per bird during the first week after H5 challenge. Multiple comparisons between groups were conducted by Bonferroni post-hoc test. Data are represented in the significant difference between them.

A linear regression analysis was used to examine whether antibody titers were associated with the interval between H9 and H5 infection in the co-infected groups. Age-adjusted antibodies titers were calculated to exclude the effect of age. They were calculated by subtracting antibody titers of co-infected groups from the average antibody titer of the H9 control group at the corresponding age.Then,age-adjusted antibody titers were regressed against the interval between H9 and H5 infection.

All statistical analysis and graphs were performed using RStudiov 1.3.1093 **R. Core Team. (2020), RStudio Team (2020).**

RESULTS

In order to mimic the natural evidence of co-infection between LPAIV and HPAIV for evaluating the possibilities of cross protection between them;7 groups of ten 4-week-old specific pathogen free (SPF) chickens were co-inoculated with HPAI and LPAI viruses at different time interval of intranasal inoculation (dose: 100 µl of 10^6 EID_{50}/bird of each virus).

In vivo experiment:

Clinical score and survival rate of chickens reveal interference of LPAI and HPAI viruses' infection at different time intervals.

Neither morbidity (quantitative measure; clinical score; Fig.1) nor mortality (quantitative measures; survival probability, Fig.2) was observed in any bird of group 1 (control negative group), but all birds were morbid and dead at the 4th dpi in mono-infected group 3 (H5N1 control group). Time-dependent morbidity and mortality was evident in co-infection groups that received an inoculum of LPAI H9N2 at the 4th week of age in all groups and another inoculum of HPAI H5N1 at different time intervals (same time,1 week,2 weeks, and 3 weeks in between the 2 inoculum). In the co-infection groups; the morbidity was distributed along the days of experiment as it begins at 3rd dpi in most groups except group 5 delayed to 4th dpi (co-infection group with 1 week interval); Fig. (3) and ends at 7th dpi in most groups except group 4 (co-infected at the same day) extend to the 8th dpi. Fig. (5). Also mortality decreased and was distributed all over the days Fig. (2); birds started to die at the 4th dpi to 8th dpi not as the control mono-infected group with HPAI H5N1; all birds died at the 4th dpi; but group 5 deaths were delayed to 5th dpi Fig. (4) and group 4 deaths were stopped at 7th dpi Fig. (6). the best results of cross-protection and survivability were shown in group 7 (co-infection group with 3 weeks interval); 30% and 40 % respectively Fig. (7, 8).

Average clinical scores of co-infection groups decreased with increasing the time interval between H9 and H5 inoculation. However, mortality did not reach 100% in co-infection groups as occurred in group 3 (H5N1 control group) and thus the survival probability in groups co-infected with both viruses was significant.

Serological response following challenge:

Serum samples were collected from the surviving chickens at day 1, and weekly post infection at wk 1, wk 2, wk 3, wk 4 and wk 5 as shown in (Table 2). Presence of serologic significance immunity, as a linear regression analysis was used to examine whether antibody titers were associated with the interval between H9 and H5 in the co-infected groups(p-value=0.002, 0.096)at1 week and2 weeks post H5 infection respectively. Fig. (11).Further analysis by HI was done to measure immunity against H5N1 which decreased with the long interval between H9N2 infection and H5N1 challenge. The best result for immunity was found at the 2 weeks interval between the H9N2 & H5N1 challenge (p-value= 0.038) as shown in Fig. (12)

HP H5N1 virus shedding is impeded by LP H9N2 co-infection (PCR):

Oropharyngeal (OP) and cloacal (CL) viral shedding patterns was detected by amplification of the extracted RNA materials by reverse transcriptase PCR (RT-PCR) targeting generic AIV-M-specific (Common Gene) and pathotype-specific H9 LP and H5 HP RT-PCR. The total number of positive swabs, the viral loads and the duration of virus shedding varied among the co-infected groups (Table 3). Relating the M-specific values to viral infectivity viral RNA was extracted from diluted HPAIV H5N1 suspensions with a known infectivity titre, and Relating the M-specific values to viral infectivity viral RNA was extracted from diluted LPAIV H9N2 suspensions with a known infectivity titre,the values from these extracts were used to generate a standard curve linking infectivity with Cq values based on M-gene-specific RT-PCR **R.Core Team. (2020), RStudio Team.(2020).** the resulting values were figured in Fig. (9, 10). All virus-inoculated chickens excreted AIV RNA in OP and/or CL swabs. Chickens of the LPAIV H9N2 mono-infected group 2 shed virus orally and cloacally starting from 2nd dpi, minor amounts of viral RNA were found at 4th dpi and not found viral RNA excreted either orally or cloacally at 6th dpi. Virus excretion in the HPAI-H5N1 mono-infected chickens of group (3) showed a steady increase of virus shedding until the death of all birds within 4 days. In summary, highly differences between the mono-infected group HP-H5N1 control group (3) and the co-infected group (7) in survival and clinical score were observed (P.Value < 0.05) Fig. (10).No statistical differences were observed in the remaining co-infected groups (4, 5 and 6) than the mono-infected H5N1 HPAI control group (G 3, 4).

Table (3): Total number of positive birds that shed the virus/ total infected birds.

Parameters	G1	G2	G3	G4	G5	G6	G7
Total no. of shedder birds (%)	**0**	**0**	**10 (100%)**	**8 (80%)**	**10 (100%)**	**10 (100%)**	**10 (100%)**
Shedder birds that died	**0**	**0**	**10**	**7**	**8**	**8**	**6**
Shedder birds that recovered	**0**	**0**	**0**	**1**	**2**	**2**	**4**
Total no. of birds died without shedding	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Mean virus load/ shedder birds that died	**NA**	**NA**	**5.5±0.5**	**5.4±0.54**	**5.4±0.23**	**5.5±0.4**	**5.2±0.39**

NA: not applicable due to bird mortalities.

Histopathology:

In general, at 2nd day post-infection two birds from each group were humanely euthanized and different organ samples were collected.

Chickens in group (2) mono-infected with LPAI-H9N2 revealed mild tracheal inflammation, deciliation, alveolar congestion with cellular exudate, and only focal necrosis in renal tubules.

In contrast,HPAI-H5N1mono-infected chickensrevealed a clear picture of acute inflammation in all internal organs starting from trachea subepithelial congestion and hemorrhage, deciliation and slough of epithelium lining. The lungs were showing severe alveolar hemorrhage and extracellular exudation same as the bronchial and peribronchial tissues. Pancreas showed severe necrosis and hemorrhages on the pancreatic acini, moreover, the small intestine showed necrotic lesions and severe inflammation of the epithelium part of the duodenum.The kidneys have also shown severe necrosis in the renal tubules and interstitial nephritis. In co-infected group (G4) has shown the same severe picture as group 3 Fig. (13), while the histopathological pictures of all other 3 co-infected groups (G5, G6 and G7) have shown less severe pictures ranges from mild to moderate when compared to G3 and G4 in the different examined organs that were ranged from moderate to severe. The histopathological scoring decreased gradually, while the least histopathological score; mild picture; was observed in co-infected group 6 (2 weeks interval between H9N2 and H5N1 challenge) Fig. (15), but also histopathological scoring raised again to moderate picture in co-infected group 7 (3 weeks interval between H9N2 and H5N1 challenge) Fig. (16).

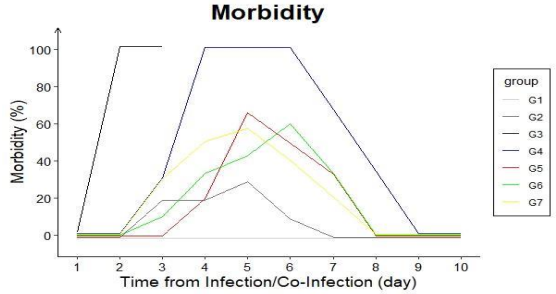
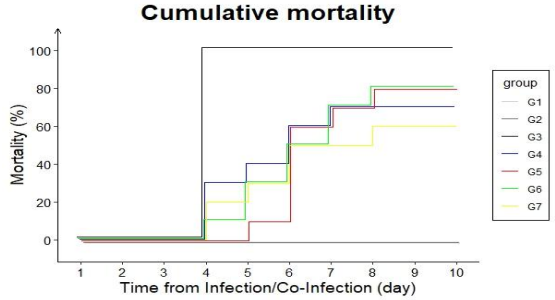


Fig. (1)



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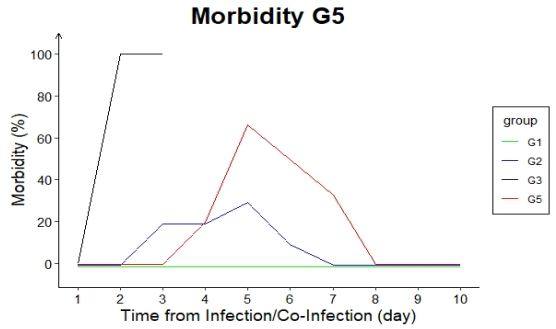

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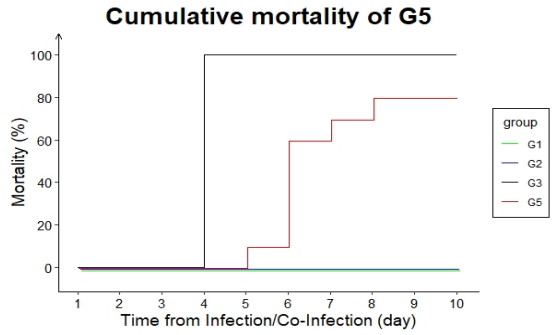

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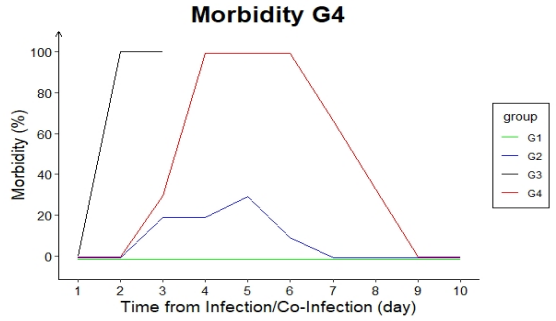

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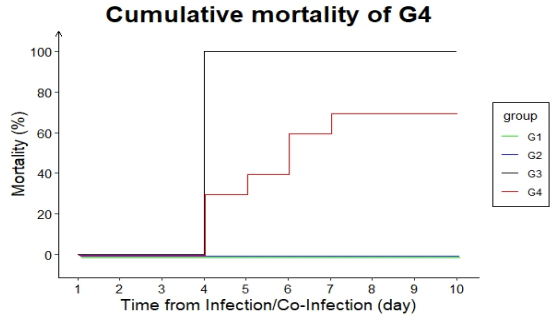

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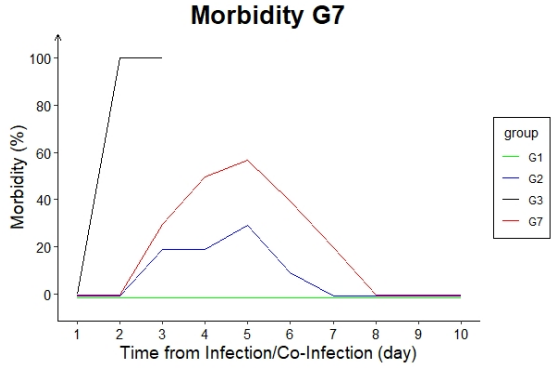

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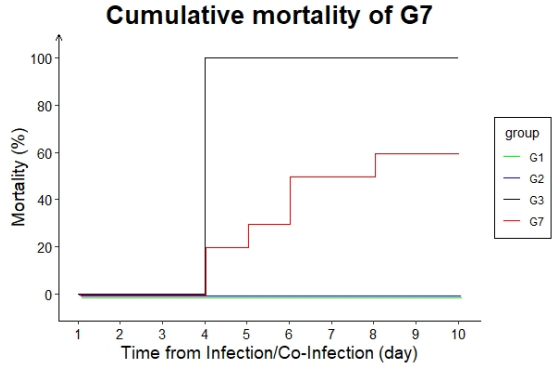

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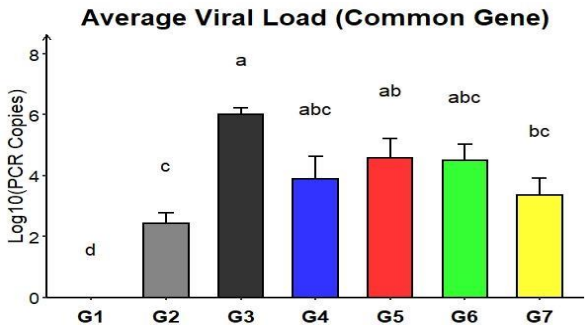

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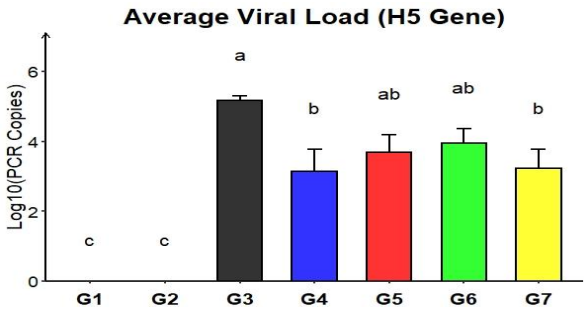

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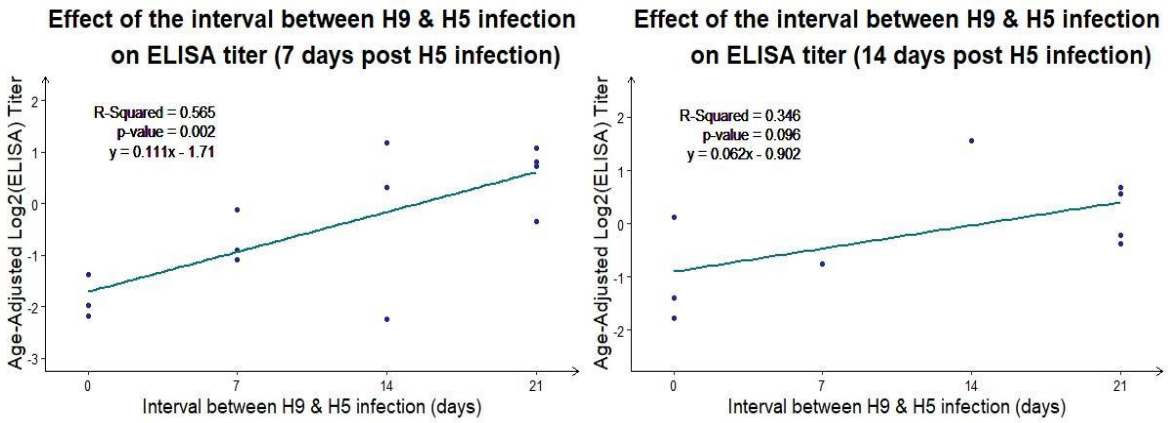

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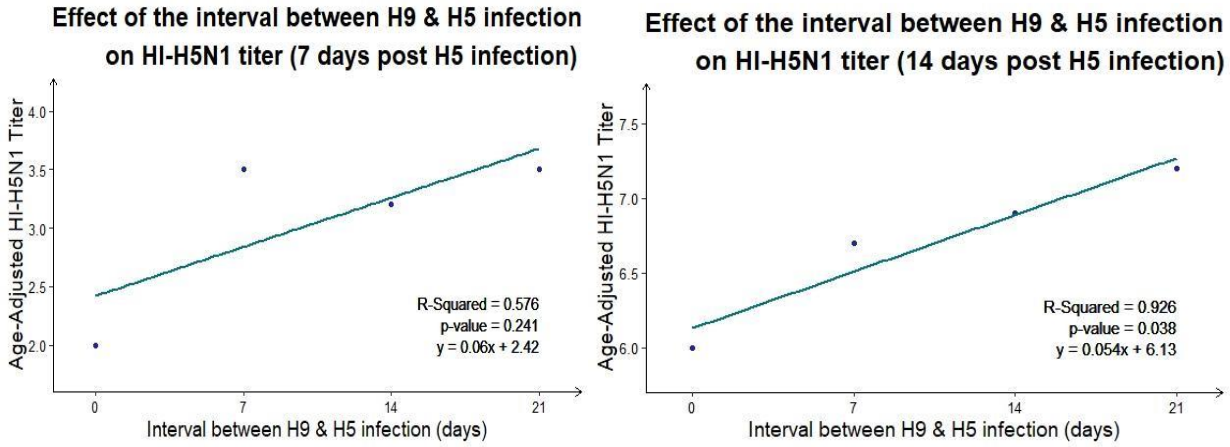

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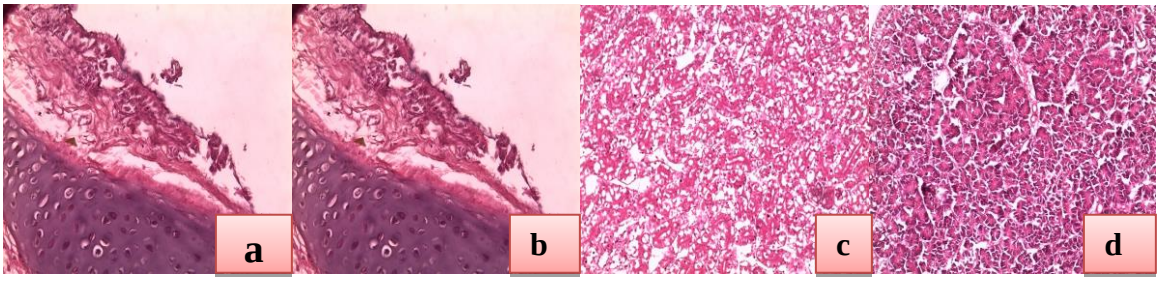

Fig. (13): Group 4 (LP H9N2 & HP H5N1 challenged at the same day) **a.** Trachea showed sloughed epithelial lining **(H&E x400). b.** Lung showed severe pneumonia, air capillaries and parabronchus filled with exudates **(H&E x200). c.** Kidney showed severe nephritis and necrosis of renal tubules **(H&Ex200)**. **d.** Pancreas showed focal necrosis of pancreatic acini **(H&E x200)**.

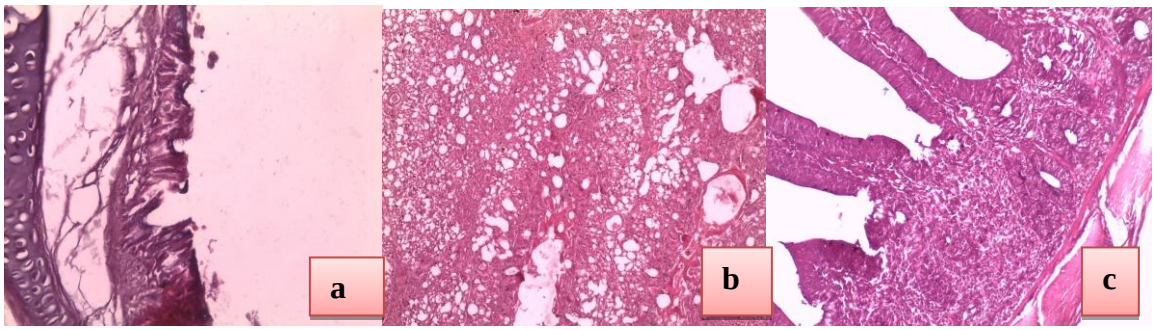

Fig. (15): Group 6(HP H5N1 challenged after 2 weeks from LP H9N2 challenge) **a.** Trachea showed hyperplasia of epithelial lining and necrosis of glands, submucosal edema **(H&E x400), b.** Lung showed air capillaries were congested, filled with exudates **(H&E x200), c.** Intestine showed mononuclear cell infiltration in the mucosal layer **(H&E x200).**

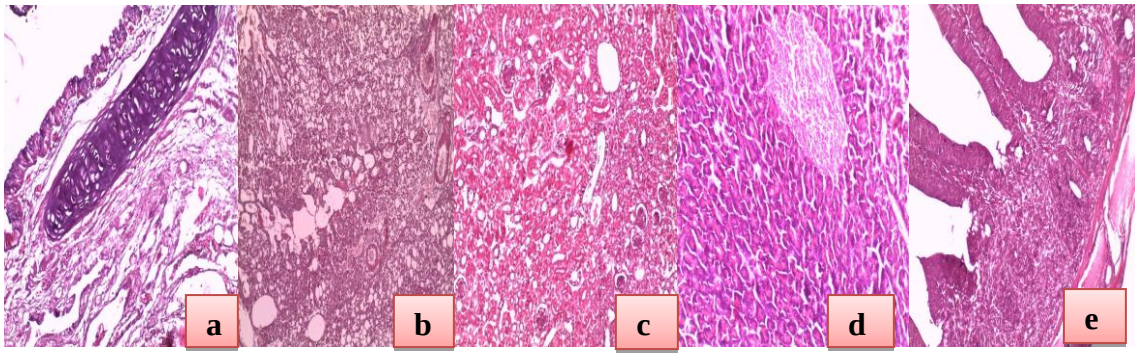

Fig. (16): Group 7 (HP H5N1 challenged after 3 weeks from LP H9N2 challenge) **a.** Trachea showed congestion of submucosa **(H&E x200), b.** Lung showed air capillaries were congested and filled with exudates and parabronchi were filled with cellular exudates. **(H&E x200), c.** Kidney showed degenerative changes and necrosis of renal tubules **H&Ex200, d.** Pancreas showed focal necrosis **(H&E x200) e.** Intestine showed mononuclear cell infiltration in the mucosal layer **(H&E x200).**

DISCUSSION

HPAI-H5N1 virus is endemic in Poultry in Egypt and has posed a public health hazard since 2006. Moreover, the widespread occurrence of LPAI-H9N2 in commercial chickens was reported to be accompanied by a decreased incidence of HPAI-H5N1 outbreaks **(Arafa *et al.* 2012).** The detection of HPAI-H5N1 in poultry flocks previously infected with LPAI-H9N2 in Egypt under natural conditions has been reported **(Monne *et al.* 2013).** The exposure to H9N2 viruses possibly provoked cell-mediated immunity against H5N1 due to their similar internal genes. Therefore, partial protection by what is called "protected window" after H9N2 infection can mask the lethal infection of H5N1 that may go unnoticed while the virus is shed by infected birds.This is a potentially important problem for developing countries such as Egypt that use vaccination programs against HPAIV H5 **(Arafa *et al.* 2016 and Marinova-Petkova *et al.* 2016)** and cull flocks only when clinical disease is clear **(Khalenkov *et al.* 2009).** Although co-circulation of H5N1 and H9N2 has been suggested to limit the spread and the epizootiologic pattern of the infections for both subtypes **(Arafa *et al.* 2016)**, their co-circulation in susceptible host populations can increase the likelihood of generating novel reassortant viruses with public implications and zoonotic concern **(Guan *et al.* 1999).**

However, data on the impact of prior infection of chickens with LPAI-H9N2 on the virulence and excretion of HPAI-H5N1 without the effect different vaccination campaigns used are still lacking. To study this hypothesis, 10 SPF chickens were inoculated with LPAI-H9N2 strain (A/Chicken/Egypt/114940V/NLQP/2011) and challenged them 1, 2, and 3 weeks later with HPAI-H5N1 virus (A/Chicken/Egypt/S175/NLQP/2015) at dose 100 µl of 10^6 EID_{50}/bird via intranasal inoculation.

Our findings showed that pre-existing immunity induced by infection with LPAIV-H9N2 modifies the course of an experimental challenge infection with HPAI-H5N1 in SPF non-vaccinated chickens. Morbidity as well as mortality and titer of virus shedding were affected. Prior infection of chickens with LPAI-H9N2 virus modulated clinical signs in all co-infected groups and the number of birds that survived the infection with HPAI-H5N1 increased gradually by time (Table3). Partial protection against lethal challenge for some HPAIVs due to prior infection with some LPAIVs has been previously described **(Jones *et al.* 2004, Khalenkov *et al.* 2009 and Seo *et al.* 2001),** while mono-infected control group with HPAI-H5N1 (G3) showed generalized hemorrhage and rapid death of all chicken at 4^{th} dpi

(van *et al.* 2003, Swayne *et al.* 2003 and Van *et al.* 2005)

Interestingly, prior infection of chickens with LPAI-H9N2 virus and then challenged with HPAI-H5N1 at 2 Weeks interval responded with a significantly higher (*P-value* =0.038) humoral immune response than other co-infected groups Fig. (12). Infection of chickens with H9N2 and H5N1 at the same time (G4) decreased the percentage of birds that shed the virus to 80%, while prior-infection with H9N2 3 weeks earlier than H5N1 (G7) resulted in decrease the mean virus load comparable to those of mono-infected control group with HPAI-H5N1(G3) (Table3).Theimmunologicalmechanismsmediating the delayed development of clinical signs and decreased virus excretion are likely based on cross-reactive cellular immunity, possibly induced by the internal proteins of LPAI-H9N2 as described before by (**Seo and Webster2001)** which demonstrated that cross-protection between the A/Quail/Hong Kong/H1/97 (H9N2) variant isolated in 1997 and the lethal H5N1 virus circulating at that time **(Seo *et al.* 2001).** Similarly, **(Kolthoff 2008)** showed that naïve mute swans died shortly after infection with HPAI H5N1 but those with previous exposure to LPAI viruses were able to survive without apparent symptoms **(Kalthoff *et al.* 2008).** Moreover, **(Khalenkov 2009)** suggested that sustained LPAI-H9N2 virus replication to high titers and consistent transmission are necessary for the potential induction of cross-reactive cellular immunity mechanisms **(Khalenkov *et al.* 2009)**. In this study, which attempted to mimic the Egyptian situation as simply as possible to clarify the role of LPAI-H9N2 on the consequences of HPAI-H5N1 course without the field complications, a single LPAI-H9N2 virus isolate was used, which clearly replicated vigorously enough to induce such cross-protection. In spite of that using other LPAIVs and/or other HPAIVs as H5N8 which recently undergo the transition of dominance from H5N1 across countries since March 2017 **(Amer *et al.*2021);** might result in different protection rates, higher or lower, cannot currently be excluded.

The possibility that HPAI exposure may yield a mild infection due to the partial cross-immunity conferred by LPAI has been well documented in laboratory experiments with caged chickens **(Van *et al.* 2003,2005)**; these studies show that LPAI-positive birds survived a HPAI infection. In addition, despite prior-infection with LPAI-H9N2 and challenged with HPAI-H5N1 afterwards at different time intervals excreted viruses up to 6 dpi, although at markedly lower titers compared to those mono-infected with HPAI-H5N1(G3). In Egypt, where currently no "stamping out" policy is known to be applied, the prolonged survival and virus shedding of HPAI- H5N1-infected chickens, either after prior-LPAI-H9N2 infection or

following homologous vaccination with inactivated H5 vaccines, may foster the endemic status of the virus and its continuous evolution in partially immunized birds **(Naguib *et al.* 2017).**

CONCLUSION

Many subtypes of AIVs have been detected in farm birds, live-bird markets and backyard birds. So far, HPAI-H5N1 and LPAI-H9N2 have been responsible for economically important clinical AI outbreaks in poultry. The co-circulation of these viruses with the presence of HPAI-H5N8 in the field may foster further genetic modifications resulting in possible emergence of a pandemic influenza virus of zoonotic public health importance that has the ability to sustain human-to-human transmission, or result in cross-protection through cross-reactive cellular immunity which can change the outcome of AIV infection in birds especially in the live-bird markets and create a situation for the perpetuation of HPAI viruses.

In conclusion, chickens previously infected with an Egyptian LPAI-H9N2 virus developed a delayed course of infection with less viral load following a challenge with a lethal dose of Egyptian HPAI-H5N1. H9 pre-infections didn't conceal the clinical signs and so didn't interfere with syndromic surveillance against HPAI-H5N1, as the clinical signs developed but in a slightly delayed manner.This study helps us to understand the possible effects of prior LPAI-H9N2 infection on the infection of chickens with HPAI-H5N1 in Egypt, where it is endemic in poultry. It is recommended that further experimental studies be conducted to identify the potential impact of co-circulation of both LPAI-H9N2 and HPAI-H5N8 on course of HPAI-H5N1 in the field. Therefore; raising awareness of all poultry farmers, enhanced strict maintenance biosecurity measures in commercial farms and backyard poultry sectors, and gradual phasing out of live-bird markets are the key measures for controlling the spread of AIVs. Vaccination also needs to be planned, well-regulated with comprehensive coverage and revalidate matching between the vaccine strains and currently circulating viruses. Finally, active and passive systematic continuing surveillance of AIVs at farms, live-bird markets, backyard, and environment must be complemented for AIV control and for early detection of new emerging viruses.

Declaration:

The authors declare no conflict of interest.

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