

Single gene reassortment of highly pathogenic avian influenza A H5N1 in the low pathogenic H9N2 backbone and its impact on pathogenicity and infectivity of novel reassortant viruses

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Abstract Avian influenza A H5N1 and H9N2 viruses have been extensively circulating in various avian species and frequently infect mammals, including humans. The synchronous circulation of both viruses in Egypt provides an opportunity for possible genetic assortment, posing a probable threat to global public health. To assess the potential risk of the IAV reassortants derived from co-circulation of these two AI subtypes, reverse genetics technology was used to generate a set of IAV reassortants carrying single genetic segments of clade 2.2.1.2 virus A/duck/Egypt/Q4596D/2012 (H5N1), a representative of the most prevalent H5N1 clade in Egypt, in the genetic backbone of A/chicken/Egypt/S4456B/2011 (H9N2), a representative of G1-like H9N2 lineage which is widely circulating in Egypt. Furthermore, the genetic compatibility, growth kinetics and virulence were evaluated *in vitro* in mammalian systems using the MDCK cell line and avian system using SPF embryonated chicken eggs. Pathogenicity and virus shedding were further tested using SPF chickens. Out of the eight desired H9-reassortants, we could rescue only 5 reassortant viruses, either due to

difficulty in cloning (PB1 of H5N1 virus) or genetic incompatibility (NP-H5/H9 and NA-H5/H9). Results revealed higher replication rates for the H9N2 virus having the NS segment of H5N1 virus. The lowest survival rate in both SPF eggs and SPF chickens was associated with the H5N1 parent virus infection, followed by the HA-H5/H9 virus. Our findings also suggest that all other reassortant viruses were of lower pathogenicity than the wild type H5N1 virus.

Introduction

Avian influenza virus (AIV) infections in the veterinary and medical communities have changed during the last two decades due to the emergence of highly pathogenic avian influenza (HPAI) H5N1 virus and low pathogenic avian influenza (LPAI) H9N2 virus in domestic poultry populations in several geographical areas around the world [31, 35, 36]. Egypt has been one of the countries most affected by AI infections during the current decade. Clade 2.2.1.2 HPAI/H5N1 viruses continue to circulate in the Egyptian poultry sector resulting in the deaths of millions of birds. In parallel, hundreds of human cases have occurred due to zoonotic transmission of these AI viruses. As of 19th December 2016, 856 laboratory confirmed HPAI H5N1 human cases including 452 deaths have been reported to the World Health Organization (WHO) across 16 countries with a high case-fatality rate of more than 53% (WHO, Cumulative number of confirmed human cases for avian influenza A (H5N1) reported to WHO, 2003–2016).

H9N2 virus circulation in Egyptian poultry (since 2010) has added additional risk factors to the Egyptian poultry

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industry [14, 23]. Until now, only 3 human infections with H9N2 viruses were reported in Egypt, according to the Egyptian Ministry of Health [6]. Interestingly, the co-circulation of both AIV subtypes, HPAI/H5N1 and LPAI/H9N2 viruses, was identified in several localities during the long term active surveillance programs established in Egypt [18]. This co-circulation in a well-known mixing vessel like poultry could potentially lead to natural genetic re-assortment producing progeny viruses of unknown phenotype. Recently, active surveillance of AIV among poultry in Egypt showed that H5N1/H9N2 co-infection in the same avian host is common [18]. Despite this, unlike reports from Asia, genetic reassortment between both AIVs has not been reported in Egypt [5, 12, 24].

Due to the segmented nature of the viral genome of influenza viruses, the viral segments can be mixed in one host cell generating reassortant viruses with new gene constellations and consequently altered virus characteristics [30]. The recorded influenza pandemics in 1918, 1957, 1968, 1977, and 2009 were caused by simple or even multiple genetic reassortment events between viruses from different origins leading to massive losses among humans. A novel reassortant H7N9 virus currently circulating in China and causing human fatalities, has six internal genes from LPAI/H9N2 virus, the HA gene of an avian H7N3 virus, and the NA gene from an avian H7N9 virus which were all circulating in poultry in China [7]. Previous studies showed that genetic reassortment of influenza viral genome between avian influenza viruses and human influenza viruses generated viruses with high virulence in animal models including reassortment between subtypes H9N2 and H1N1, between H5N1 and H1N1, and between H3N2 and H5N1 [1, 21, 32]. Here, we studied the reassortment potential between AIV H9N2 and H5N1 in Egypt. This was performed by testing the impact of genetic exchange of viral segments from HPAI/H5N1 in the genetic backbone of LPAI/H9N2 on viral replication efficiency and pathogenicity.

Materials and methods

Viruses and cells

The LPAI A/chicken/Egypt/S4456B/2011 (H9N2) (H9N2/S4456B) and HPAI A/duck/Egypt/Q4596D/2012 (H5N1) (H5N1/Q4596D) viruses were propagated in allantoic cavities of 11 day old specific pathogen free (SPF) embryonated chicken eggs for 48 hours post infection (hpi) and stored at -80 °C.

Madin Darby canine kidney (MDCK) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (BioWhittaker, Lonza, Germany). Human embryonic kidney cells (293T) were grown in Opti-MEM medium

(Gibco, Life technologies). All media were supplemented with 5 % inactivated fetal bovine serum (FBS) (BioWhittaker, Lonza) and 1% antibiotic-antimycotic mixture (BioWhittaker, Lonza) and grown at 37 °C under 5% CO₂.

Plasmids and reverse genetics

The eight gene segments of H9N2/S4456B and H5N1/Q4596D viruses were amplified by reverse transcription polymerase chain reaction. The purified PCR product of each viral segment was digested either with BsmBI for PB1, PA, HA, NP, M and NS segments or BsaI for PB2 and NA segments, and then individually ligated with BsmBI -linearized pHW2000 vector in 16 individual ligation reactions. The correct constructs, as confirmed by enzymatic digestion and sequencing, were subsequently used to generate recombinant viruses. Except for the PB1 segment, 7 viral RNA transcribed segments of (H5N1/Q4596D) virus were amplified by reverse transcription polymerase chain reaction (RT-PCR), cloned into pHW2000, sequenced and subsequently used to generate recombinant viruses. Reverse genetics was used to generate a panel of reassortant viruses listed in Fig. 1, as previously described [10, 11].

Growth kinetics of rescued viruses in mammalian cells

Growth properties of rescued reassortant viruses and parental (H9N2/S4456B) and (H5N1/Q4596D) viruses were compared in MDCK cells. Each virus was inoculated onto an MDCK cell monolayer at a multiplicity of infection (MOI) of 0.001. The supernatants of the infected cells were collected in triplicates at specific time points and kept at -80 °C. The titer of each collected sample was determined using TCID₅₀ and HA assays.

Replication rate of rescued viruses in specific pathogen free embryonated chicken eggs

Growth properties of rescued reassortant viruses and parental (H9N2/S4456B) and (H5N1/Q4596D) viruses were compared in specific pathogen-free (SPF) embryonated chicken eggs (SPF-ECE). 10³ EID₅₀ of each virus was inoculated into 5 SPF eggs for each time point. The allantoic fluids were harvested at specific time points and titrated using TCID₅₀.

Pathogenicity examination

The pathogenicity of rescued viruses was examined in both SPF-ECE and SPF chicken as follows:

10-day-old SPF embryonated chicken eggs were inoculated with 100 µl of 10³ EID₅₀ dilutions of each rescued

IAV	Gene constellation								Compatibility
H5N1/Q4596D	PB2	PB1	PA	HA	NP	NA	M	NS	Compatible
PB2 H5/H9	PB2	PB1	PA	HA	NP	NA	M	NS	Compatible
PB1 H5/H9	PB2	PB1	PA	HA	NP	NA	M	NS	ND
PA H5/H9	PB2	PB1	PA	HA	NP	NA	M	NS	Compatible
HA H5/H9	PB2	PB1	PA	HA	NP	NA	M	NS	Compatible
NP H5/H9	PB2	PB1	PA	HA	NP	NA	M	NS	Incompatible
NA H5/H9	PB2	PB1	PA	HA	NP	NA	M	NS	Incompatible
M H5/H9	PB2	PB1	PA	HA	NP	NA	M	NS	Compatible
NS H5/H9	PB2	PB1	PA	HA	NP	NA	M	NS	Compatible
H9N2/S4456B	PB2	PB1	PA	HA	NP	NA	M	NS	Compatible

Fig. 1 List of generated reassortant viruses using reverse genetics, as well as the 2 wild H5N1 and H9N2 parental viruses. A panel of H9N2 based reassortant viruses was generated using 7 *A/chicken/Egypt/*

S4456B/ 2011 (H9N2) (gray) and one *A/duck/Egypt/Q4596D/2012* (H5N1) plasmid (red). The reassortant viruses associated with black colored segments were not generated (color figure online)

and wild viruses. Inoculated eggs were incubated in 37 °C. At each specific time point, 15 eggs were collected for each infected egg group and examined for the viability of embryos on the egg candler at each time point; of which three embryos were examined for viral hemorrhage.

To determine the pathogenicity of reassortant and wild type H5N1 and H9N2 viruses, 8 SPF white Leghorn 4 week-old chickens were infected with 0.5 ml of 10⁶ EID₅₀/ml of each virus. Viral dilutions were prepared in 1X PBS. Infection was done through natural routes (intranasal, conjunctival, intratracheal infection). Six un-infected control chickens (control group) were mock infected and subjected to same conditions.

Organs (liver, spleen, lung and intestine) were collected at day 2 post infection from 3 chickens in each infected group. A half of each organ was fixed in 10% formaldehyde and saved at room temperature. Histopathology was performed on the 3 fixed organs from each viral group to examine the pathological lesions due to viral infection. The second half of each organ was subjected to viral recovery in 2 SPF eggs for each organ, and also to RNA extraction to determine the systemic distribution of generated viruses, when compared to wild type viruses. Oral and cloacal swabs were also collected from each of the 3 chickens at day 2 post infections. Swabs were subjected to real time RT-PCR and EID₅₀ to detect shedding of different viruses. Animal experiments were approved by the Ethics Committee of the National Research Centre, Cairo, Egypt.

Statistical analyses

Statistical analyses were done using GraphPad Prism V5 (GraphPad Inc., CA, USA). One-way ANOVA with Tukey post hoc test was used to compare virus titers.

Results

Cloning of the full genome set of parental H5N1 and H9N2 viruses

Eight cloned segments of H9N2/S4456B virus were amplified and shown to be 100 % homologous to PB2 (Accession number: JX273136.1), PB1 (Accession number: JX273135.1), PA (Accession number: JX273137.1), HA (Accession number: CY110928.1), NP (Accession number: JX273139.1), NA (Accession number: CY110929.1), M (Accession number: JX273134.1), and NS (Accession number: JX273138.1) of *A/chicken/Egypt/S4456B/2011*(H9N2) with no mutations in the cloned genes.

Similarly, BLAST N analysis for the 8 cloned segments of H5N1/Q4596D virus revealed that nucleotide sequences were 100 % homologous to those sequences deposited in GenBank for PB2 (Accession number: KF881587), PA (Accession number: KF8815889), HA (Accession number: JX912994), NP (Accession number: KF881590), NA (Accession number: JX9129943), M (Accession number: KF881591), and NS (Accession number: KF8815902) of *A/duck/Egypt/Q4596D/2012* (H5N1). However, on comparing the cloned PB1 nucleotide sequences with the published sequences in GenBank, deletions in the first 662 bp of the obtained sequence were detected (data not shown). All cloning steps were repeated several times for different amplicons of full-length PB1 gene but the same deletions were detected at the same sites. Neither changing the type of competent cells (TOP10, HB101 and XL1-Blue) nor changing the conditions of transformation rectified the issue. Therefore, the PB1 of H5N1 -in pHW2000 plasmid and the corresponding H9-reassortant was therefore not included in the experiment.

Generation of reassortant influenza viruses

Rescue of the H5/H9-reassortant viruses was attempted using 293T/MDCK co-culture cells. In addition to the parental H9N2 virus, only 5 H9-reassortant viruses, encoding the PB2 (PB2 H5/H9), the PA (PA H5/H9), the HA (HA H5/H9), the M (M H5/H9) and the NS (NS H5/H9) of H5N1/Q4596D were successfully rescued. Sequencing of the genetic material extracted from the generated viruses proved the precise genetic constellation for all of them (Fig. 1). Unexpectedly, the reassortant NP H5/H9 and NA H5/H9 viruses were not rescued after several transfection attempts and after passaging the transfection harvest in SPF eggs and MDCK cells. To validate the generation of NP and NA of H5N1/Q4596D plasmids, each of the 2 plasmids was used successfully to rescue viruses in the genetic backbone of influenza A/Puerto Rico/8/1934 (H1N1, PR8).

Growth kinetics in MDCK

The growth kinetics of the reassortant viruses were determined in comparison with the wild parent H9N2 and H5N1 viruses *in vitro* by infecting MDCK cells at a MOI of 0.001. The 3 culture supernatants at specific time points (12–48 hpi) were titrated by TCID₅₀. No significant difference ($P > 0.05$) was observed between both parent H5N1 and H9N2 viruses after 12 hpi. The PA H5/H9, HA H5/H9 and PB2 H5/H9 reassortant viruses had a significant ($P \leq 0.01$) drop in TCID₅₀ titers after 12 hpi when compared to parent H5N1 and H9N2 viruses as well as other reassortant viruses. However, the TCID₅₀ titers for the NS H5/H9 and M H5/H9 reassortant viruses were comparable to that of the parent viruses (Fig. 2A). Meanwhile, the HA titers for the same time course revealed that the PB2 H5/H9 virus had the highest titers at 36 and 48 hpi, when compared to the rest of the viruses, while NS H5/H9 virus showed the lowest HAU titers during the entire time course. The HA H5/H9, M H5/H9, PA H5/H9, and parental viruses showed no significant differences ($P > 0.05$) at 24, 36, and 48 hpi in their HA titers (Fig. 2C). Viruses with high HA titers could be used as candidates for vaccine preparation.

Growth kinetics in SPF-ECE

To compare the growth kinetics of the reassortant viruses against the parental viruses, the viral titers were assessed at 12 h intervals post inoculation using 5 SPF eggs per time point. All viruses replicated efficiently in eggs with comparable TCID₅₀ titers.

At 24 hpi, the NS H5/H9 reassortant had the highest TCID₅₀ titer among all compared viruses followed by the parental H9N2 virus, with no significant difference between them ($P > 0.05$). The PB2 H5/H9, PA H5/H9,

parental H5N1 and H9N2 viruses also had no significant differences among them at 24 hpi. Both M H5/H9 and HA H5/H9 viruses were significantly different when compared to H9N2, however there was no significant difference between them (Fig. 2B).

No significant differences were observed among viral HA titers at 12 hpi (p -values > 0.05). Differences in growth kinetics between viruses were significant at 24 hpi, when PB2 H5/H9, HA H5/H9 and the parental H5N1 virus showed lower titers. The M H5/H9 reassortant virus showed the highest replication rate at 24 hpi. At 36 and 48 hpi, the HA H5/H9 and the parental H5N1 viruses had lower HAU titers compared to other tested reassortants and the parental H9N2 virus (Fig. 2D).

Pathogenicity in SPF-ECE

The pathogenicity of the reassortants and wild type viruses was assessed in 15 chicken embryos for each time point. The reassortants HA H5/H9 and the parental H5N1 viruses had the highest mortality rate among all compared viruses (Fig. 3A). The onset of embryo mortality started for most reassortants and H5N1 viruses within 36 hpi. The HA H5/H9 reassortant and H5N1 strain led to 100% mortality at 48 hpi. However, the M H5/H9 and NS H5/H9 viruses showed 40% mortality of embryos at 48 hpi, while the PB2 H5/H9 and PA H5/H9 viruses and the parental H9N2 strain led to similar survival rates (73%).

To investigate the pathological signs associated with influenza infection, chicken embryos were examined for hemorrhage at 24 and 48 hpi. Interestingly, only 3 viruses, NSH5/H9, HAH5/H9 and the parental H5N1 induced acute hemorrhage and congestion in the chicken embryos at the predefined time points (Fig. 3B).

Pathogenicity and viral shedding in SPF chickens

The virulence of the reassortant and wild-type viruses were compared for in 4 week-old SPF chickens, with 3 animals being used for each time point. Although SPF chickens infected with H5N1 died at the second day post infection, other chickens infected with the H9N2 virus and the 5 reassortant viruses didn't show any signs of illness until day 4 post infection. To confirm these results, 4 more chickens were infected with the same dose each of HA H5/H9 and NS H5/H9 with H9N2 as control, and survival was checked for 12 days. Only HA H5/H9 caused death of 3 chickens at days 6, 10 and 11 and severe sickness to the fourth chicken at day 12 at the termination of experiment. The survival percentage of chickens infected with NS H5/H9 and/or H9N2 was 100%.

H5N1 virus was detected in all collected organs and in oral and cloacal swabs using RT-PCR (Fig 4). The

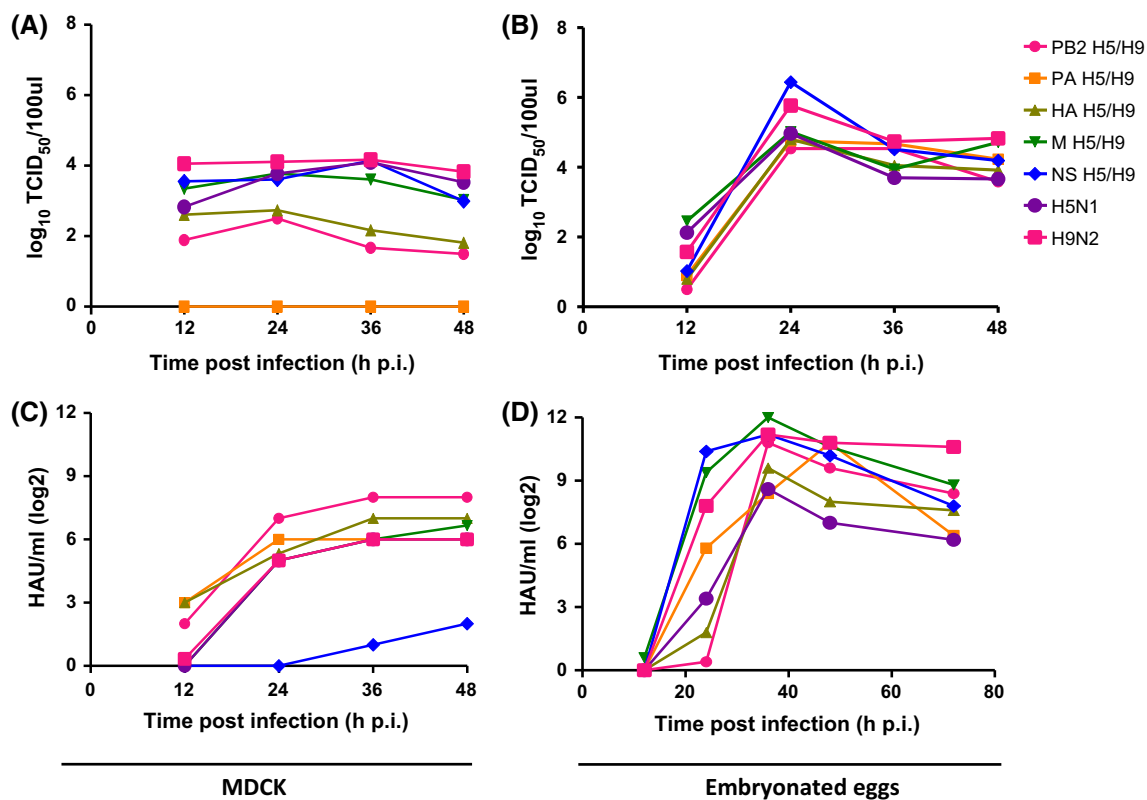


Fig. 2 Growth kinetics of rescued reassortant and parent viruses in MDCK cells and embryonated chicken eggs. MDCK cells were infected with parental and reassortant IAV at an MOI of 0.001. At the time points indicated, supernatant was taken and titrated by TCID₅₀/ml assay on MDCK cells (A) and HA assay (C). Meanwhile, the

growth kinetics of parent and reassortant viruses were assessed in embryonated eggs using 10³ EID₅₀ at 12 hpi. The viral titers in collected allantoic fluids were titrated for their TCID₅₀/ml (B) and HAU/ml (D)

typical signs of HPAI infection including cyanotic combs and wattles, edema of the head and leg shank hemorrhage were observed in all dead chickens of H5N1 infected group. H5N1 virus was recovered from all infected chickens with log₂ EID₅₀/ml titers of 5.08 ± 0.5 and 4.4 ± 0.14 (Table 1) in oral and cloacal swabs, respectively. LPAI H9N2 virus was recovered only from oral swabs in all infected chickens with log₂ EID₅₀/ml titers of 2.9 ± 1.1 (Table 1). H9N2 virus was not detected in any tested organs at day 2 post infection. HA H5/H9 and PB2 H5/H9 viruses were detected in all collected organs but with a lower detection rate than H5N1 viruses. Interestingly, 2 of 3 infected chickens shed HPAI HA H5/H9 virus with log₂ EID₅₀/ml 3 ± 0.7, but with no clinical signs. Although, PA H5/H9 virus was widely detected in all the tested organs in most infected chickens, the virus was not shed in oral and cloacal swabs. In contrast to H5N1 viruses which showed the highest shedding, M H5/H9 and NS H5/H9 showed the least number of positive samples between reassortant viruses (Fig. 4). H5N1 and PB2 H5/H9 viruses were successfully recovered from all organs of infected chickens while HA H5/H9 virus was isolated from all organs except the intestine, with lower isolation rates than H5N1

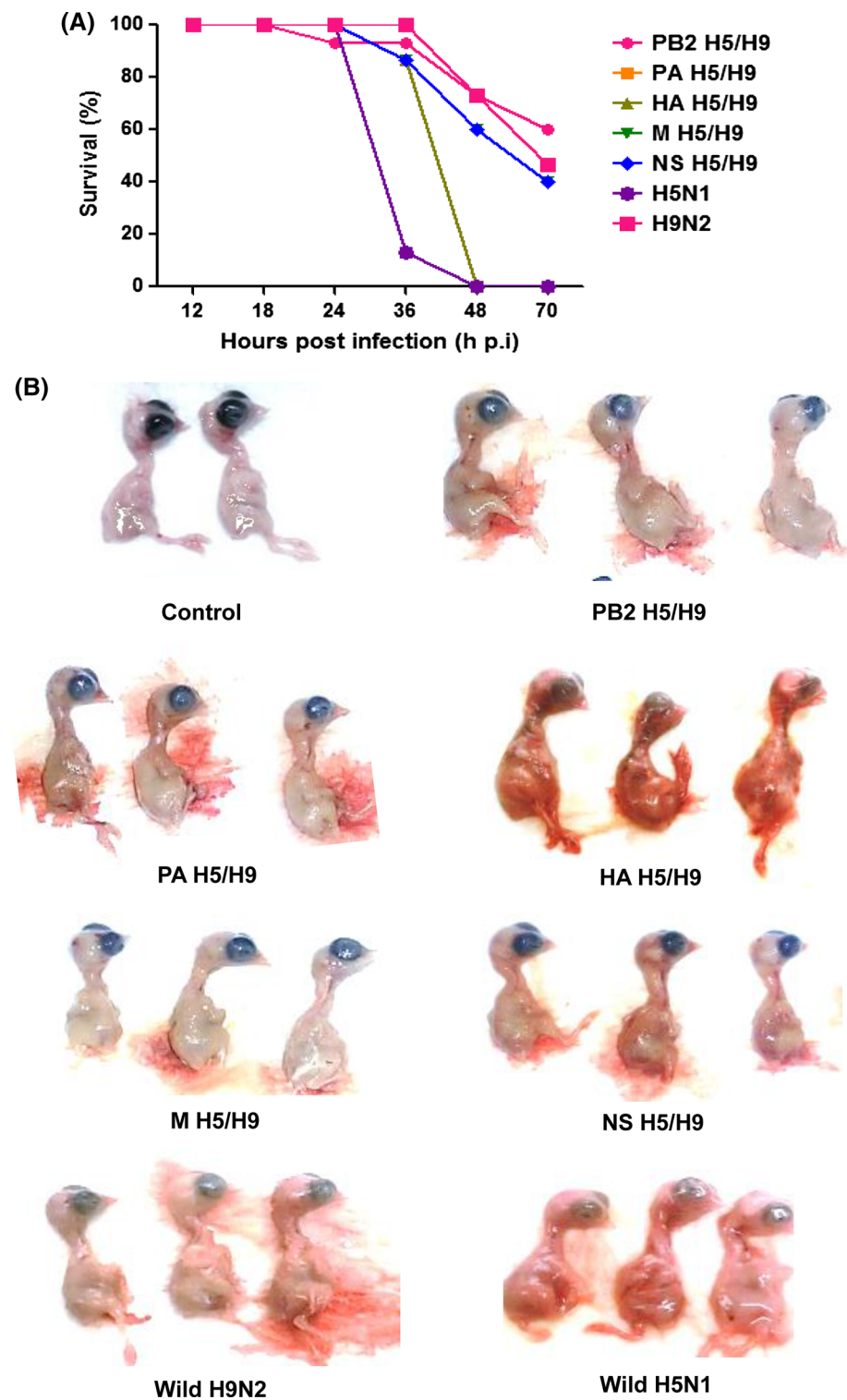
(Supplementary Fig. S1). The PA H5/H9 virus was recovered from the liver and spleen of infected chickens. The M H5/H9 and NS H5/H9 viruses were not isolated from all organs of infected chickens.

Histopathology

A) Lung

Photomicrographs (H&E X40) of lung sections from different experimental groups showed marked degeneration and necrosis of the blood vessel wall with perivascular hemorrhage in parental H5N1-infected chickens (Fig. 5A₂) and severe perivascular hemorrhage with degeneration in the vessel wall associated with thrombus formation in parental H9N2-infected chickens (Supplementary Fig. S2). The PB2 H5/H9 induced a congestion of the vasculature with nuclear and cytoplasmic endothelial cell swelling (Fig. 5A₄). The PA H5/H9 virus led to interstitial pneumonia with nuclear and cytoplasmic swelling of the endothelial cell lining blood vessels which were engorged with leucocytes (Fig. 5A₅). The “HA H5/H9”-infected tissue showed marked cytoplasmic swelling of the endothelial cells lining blood vessels

Fig. 3 Pathogenicity of parent versus reassortant viruses in embryonated chicken eggs. (A) A survival curve for embryos of SPF eggs after infection with different viruses at different time points using 10^3 EID₅₀. (B) Pathological studies of the inoculated chicken embryos at 24 hrs post infection. Only NS H5/H9, HA H5/H9 and H5N1 viruses caused severe acute hemorrhage and congestion in the examined chicken embryos



with pyknosis of their nuclei (Fig. 5A₆). However, both MH5/H9 and NS H5/H9 induced interstitial pneumonia with marked endothelial cell degeneration and thrombosis in the blood vessels (Fig. 5A₇ and 5A₈). These signs were also confirmed through comparison with normal histological structure of the lung (Fig. 5A₁).

B) Spleen

Photomicrographs of histological changes associated with parent and reassortant viruses in spleen are shown in Supplementary Fig. S2. Spleen sections from chickens infected with PB2 H5/H9 virus showed reactive proliferation of the

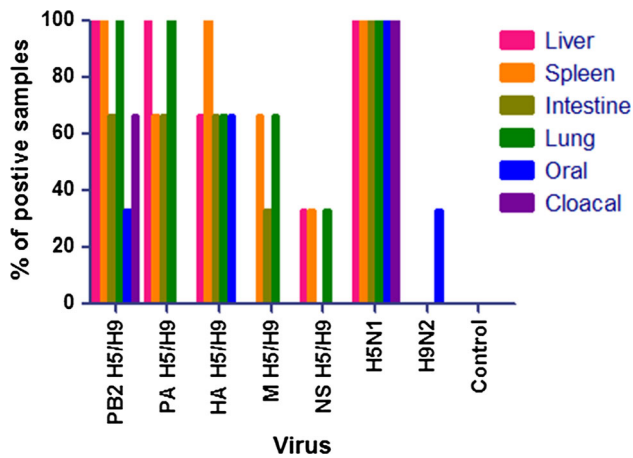


Fig. 4 Systemic distribution of viruses in different organs and collected swabs by RT-PCR at day 2 post infection

Table 1 Titers of recovered parent and reassortant viruses in oral and cloacal swabs obtained from infected chickens^a

Virus	Oral	Cloacal
H5N1	5.08 ± 0.5 (3/3) ^b	4.4 ± 0.14 (3/3) ^b
H9N2	2.9 ± 1.1 (3/3) ^b	-
PB2 H5/H9	2.25 (1/3)	-
PA H5/H9	-	-
HA H5/H9	3 ± 0.7 (2/3)	-
M H5/H9	2.25 (1/3)	-
NS H5/H9	2.25 (1/3)	-

^a Data are the mean log₁₀ EID₅₀/ml ± SD for positive samples
-, The titer was below the limit of detection (<1 log₁₀ EID₅₀/ml)

^b Values in parentheses are the number of positive chickens within each group

lymphocytes in the splenic follicles, while sections of animals in the PA H5/H9 infected group showed scattered pyknotic lymphocytes with focal necrosis and fibrinous exudation, especially at the perivascular lymphocyte sheaths (Fig. 5B₄ and 5B₅). Spleen sections of chicken infected with the HA H5/H9 virus showed marked lymphocytic cell depletion at the white bulb (Fig. 5B₆). Spleen sections from chickens infected with the M H5/H9 virus showed reactive lymphocytic cell proliferation in the splenic follicles (Fig. 5B₇), while in the NS H5/H9 group there was necrosis in the lymphocytes at the perivascular sheath (Fig. 5B₈). The H5N1 wild type virus infected sections showed lymphocytic cell necrosis with fibrinous exudation involving all the splenic follicles (Fig. 5B₂), while the H9N2 group showed mild necrosis of some lymphocytes associated with reactive lymphoid cell proliferation at the periphery of the follicle (Fig. 5B₃). Control non-infected spleen sections showed normal histological structure of the white and red pulp of the spleen (Fig. 5B₁).

C) Intestine

Photomicrographs (H&E X10) of intestinal sections from different experimental groups (Supplementary Fig. S2) showed marked necrobiosis at the tips of the intestinal villi in H5N1-infected chickens (Fig. 5C₂) and necrosis associated with hemorrhage at the intestinal mucosa and lamina propria in H9N2-infected chickens (Fig. 5C₃). In addition, PB2H5/H9 infection induced severe necrosis in the intestinal mucosa lamina propria and submucosa (Fig. 5C₄). The intestinal mucosa was more or less normal in PAH5/H9-infected chicken (Fig. 5C₅); however, mild necrosis in the intestinal mucosa and severe necrosis in the intestinal mucosa and lamina propria were detected the HA H5/H9 and M H5/H9-infected animal tissues, respectively (Fig. 5C₆ and 5C₇). Finally, the NS H5/H9 virus induced mild necrosis at the tips of intestinal villi (Fig. 5C₈). These signs were also confirmed through comparison with normal histological structures in the intestine (Fig. 5C₁).

D) Liver

Photomicrographs (H&E X40) of liver sections from different experimental groups (Supplementary Fig. S2) showed lymphocytic cell reaction in the portal area with endothelial cell degeneration at the blood vessel wall in H5N1-infected chicken (Fig. 5D₂) and a focal area of necrosis infiltrated with monocytic cellular reactions in H9N2-infected chickens (Fig. 5D₃). Degeneration in the endothelial cells lining blood vessels with thrombus formation and perivascular lymphoid cell reaction was observed in PB2 H5/H9 infected chickens (Fig. 5D₄). The PA H5/H9 infection was associated with focal areas of necrosis and hemorrhage with endothelial cell degeneration at the blood vessel wall (Fig. 5D₅). However, the HA H5/H9 infection was associated with minute foci of necrosis infiltrated with mononuclear cells and edema in the Disse spaces (Fig. 5D₆). Focal hepatocellular necrosis without inflammatory cell infiltration along with edema in the Disse spaces in M H5/H9 infected chickens and severe congestion of the vasculature with edema in the Disse spaces and degeneration in the endothelial cells lining blood vessels in NSH5/H9-infected chicken were also observed (Fig. 5D₇ and 5D₈, respectively). These signs were also confirmed through comparison with normal histological structures in the liver (Fig. 5D₁).

Discussion

Although surveillance of AIVs in Egypt showed that H5N1/H9N2 co-infection in the same avian host is common [18, 19] reassortment has not yet been reported, unlike

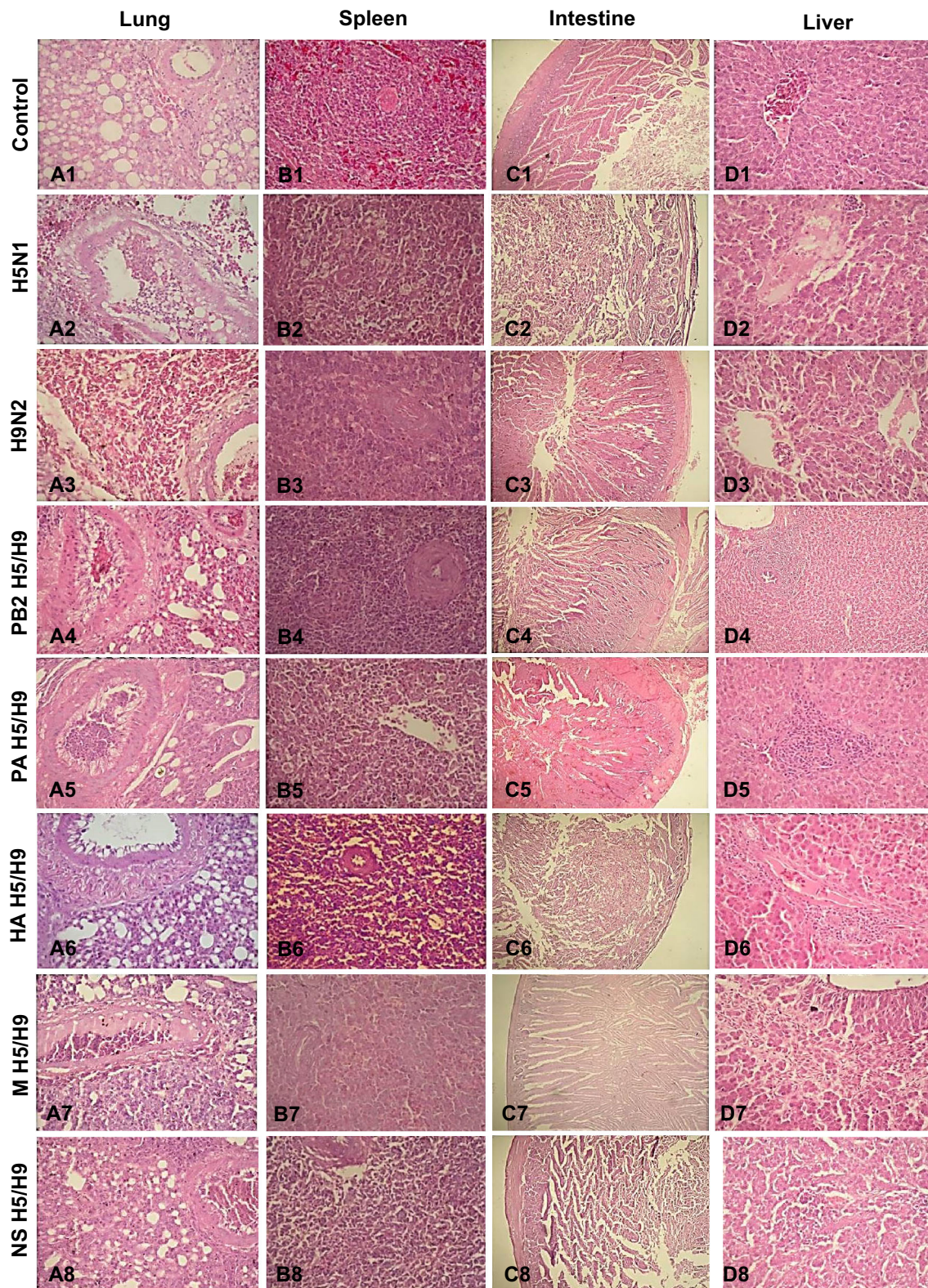


Fig. 5 Histological changes associated with parent and reassorted viruses in different organs of infected chickens; lung (A₁-A₈), spleen (B₁-B₈), intestine (C₁-C₈) and liver (D₁-D₈). The histopathological

changes, magnification and the stain are described briefly at the text and in the supplementary data

in Asia [5, 12, 24]. Recently, two naturally reassortant H9N2 viruses have been detected in pigeons in Egypt that have 5 viral segments from Eurasian AI viruses, where the remaining viral segments are from the subtype H9N2 G1-like lineage [15].

All previous pandemic influenza viruses evolved from reassortment of viruses with low pathogenicity [26]. LPAI H9N2 viruses have undergone extensive reassortment with different AIVs including HPAI H5N1 [5] and H7N3 viruses [3]. Recently, the internal genes of H9N2 viruses have contributed to the genesis of the human-infecting influenza H7N9 [20] and H10N8 [29] viruses.

To evaluate the risk of H5N1/H9N2 reassortants, we studied combinations of experimentally reassortant viruses derived from 7 segments of A/chicken/Egypt/S4456B/2011 (H9N2) virus, and one segment of A/duck/Egypt/Q4596D/2012 (H5N1) virus. These viruses were isolated in the same flu season when co-circulation between H9N2 and H5N1 viruses in the same host was first detected in Egyptian farms [17, 18].

The PB1 of H5N1 virus, when cloned in the pHW2000 plasmid, showed a 662 bp deletion in the 5' region of the PB1 segment, which could be due to the instability of this segment in competent bacterial cells. Similar results were previously observed during the cloning of PB2 from the HPAI A/Turkey/Ontario/7732/1966 (H5N9) virus [37]. Also, genetic instability of cDNAs encoding HA segments in pHW2000 and pHH21 was previously detected when *E. coli* strains (e.g. DH5 α and Top10) were transformed [9]. Therefore, the PB1 segment of H5N1 virus and the corresponding H9-reassortant virus was not included in the experiment.

In the case of NP H5/H9 and NA H5/H9 viruses, both were not rescued. The reason may be due to segment incompatibility with the backbone of H9; indeed these 2 viruses might not be able to form in nature. Viral protein incompatibility among vRNP components is a restricting viral factor for influenza reassortment [2, 27, 33], but little is known about genetic incompatibilities between vRNA segments.

Egyptian AI H9N2 viruses showed impressive replication rates in embryonated chicken eggs and mammalian cells [18]. A previous study showed that the NA of H9N2 was responsible for the high replication rates of Egyptian H9N2 viruses [16].

Our growth kinetic studies of the 5 new reassortant viruses and the wild type H9N2 and H5N1 viruses using TCID₅₀ showed that the PA and HA segments of H5N1 decreased the replication rate of H9N2 viruses on MDCK cells, to a level lower than both wild viruses. A HA titration showed that PB2 reassortant has the highest replication titer. Along with this high replication rate in cells, PB2 H5/H9 virus also caused a high level of systemic shedding in all SPF chickens organs.

Growth kinetic and survival rate analysis in SPF eggs showed that the HA H5/H9 virus is a highly pathogenic virus, due to the presence of multibasic amino acids in the cleavage site of its HA gene, but still less pathogenic than the H5N1 parental virus and with reduced replication rates, when compared to wild type H9N2 viruses. This result was further confirmed in SPF chickens when analysing histopathology and survival rates.

The NS H5/H9 reassortant virus showed high replication titers on mammalian cells (*in vitro*) and in SPF eggs when compared to wild type H5N1 viruses, however it can't be used as a candidate for vaccines as it confers higher pathogenicity than the wild type H9N2 viruses in SPF eggs (according to survival rate and severity of hemorrhaging) and in SPF chickens (as observed in histopathology sections of infected lung, liver and spleen samples).

None of the generated reassortant viruses caused mortality in SPF chickens in a period up to 4 days post infection. The HA H5/H9 virus killed the first infected chicken at day 6 of infection, while all chickens infected with H5N1 virus died at day 2. This delay in mortality rate is not related to maternal antibodies, which were proved to be under the cut-off of $\geq 4 \log_2$ against both H9N2 and H5N1 viruses [4].

Real time PCR of RNA extracted from oral and cloacal swabs showed that unlike the H5N1 virus, none of the five new reassortant viruses could be detected in cloacal samples after 2 days post infection. These results support the results found by Gharaibeh [8] and Kandeil, et al. [16] regarding the late shedding of H9N2 virus in cloacal swabs.

Our study also proved that LP viruses might be systemic, supporting data previously published by others [22, 28]. The systemic distribution of the five new reassortant viruses and the parent H5N1 viruses in SPF chickens' lung, liver, spleen, and intestine was demonstrated using RT-PCR. In contrast to the parent H9N2 virus, the highest systemic distribution between the new reassortant viruses was that of PB2 H5/H9, PA H5/H9 and HA H5/H9 viruses, as confirmed by RT-PCR and by virus recovery from different organs using SPF eggs. Live virus recovery from different organs of infected chickens showed that detection of virus based on virus isolation in SPF eggs was lower than the respective RT-PCR detection levels. A previous study showed that real time RT-PCR was less insensitive to differences in sample storage conditions and freeze-thaw cycles, as opposed to virus isolation [25]. In contrast to the H5N1 parent virus, the H9N2 reassortant virus containing the H5N1 PB2 showed mild or no signs of pathogenicity in all the infected organs, as shown by H&E staining assay. Our experimental infection study indicated that the reassortant viruses were able to replicate in multiple organs of chickens, but the infected chickens did not show any signs of disease. Previous studies indicated that

the PB2, PB1, PA, HA, NP, and NS genes of HPAI H5N1 viruses contributed to viral pathogenicity in ducks and chickens [13, 34]

The genetic complexity of influenza viruses and the co-circulation of these viruses in permissive hosts can create novel viruses with altered reassortant IAV genomes and unknown characteristics.

Despite the high possibility of genetic reassortment between H5N1 and H9N2 viruses in Egyptian poultry, reassortment hasn't happened to date. This might be partially due to the incompatibility of some segments and the low propagation rates of others. In addition other factors aside from genetic compatibility must be involved in the lack of reassortments in field. Our data indicate that reassortment can occur in the wild but the resulting viruses are likely to be less pathogenic than their parental HPAI H5N1 virus. In addition it appears these viruses could go undetected by passive surveillance conducted in the case of severe disease. This finding justifies and warrants continuous surveillance of influenza viruses, especially in areas where multiple influenza subtypes have been reported.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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