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Generation of a reassortant avian influenza virus H5N2 vaccine strain capable of protecting chickens against infection with Egyptian H5N1 and H9N2 viruses

Ahmed Kandeil¹, Yassmin Moatasim¹, Mokhtar R. Gomaa¹, Mahmoud M. Shehata¹, Rabeh El-Shesheny¹, Ahmed Barakat³, Richard J. Webby², Mohamed A. Ali^{1,*}, and Ghazi Kayali^{2,*}

¹Center of Scientific Excellence for Influenza Viruses, National Research Centre, Giza, Egypt

²Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN

³Microbiology Department, Ain Shams University, Egypt

Abstract

Background—Avian influenza H5N1 viruses have been enzootic in Egyptian poultry since 2006. Avian influenza H9N2 viruses which have been circulating in Egyptian poultry since 2011, showed high replication rates in embryonated chicken eggs and mammalian cells.

Methods—To investigate which gene segment was responsible for increasing replication, we constructed reassortant influenza viruses using the low pathogenic H1N1 PR8 virus as backbone and included individual genes from A/chicken/Egypt/S4456B/2011(H9N2) virus. Then, we invested this finding to improve a PR8-derived H5N1 influenza vaccine strain by incorporation of the NA segment of H9N2 virus instead of the NA of H5N1. The growth properties of this virus and several other forms of reassortant H5 viruses were compared. Finally, we tested the efficacy of this reassortant vaccine strain in chickens.

Results—We observed an increase in replication for a reassortant virus expressing the neuraminidase gene (N2) of H9N2 virus relative to that of either parental viruses or reassortant PR8 viruses expressing other genes. Then, we generated an H5N2 vaccine strain based on the H5 from an Egyptian H5N1 virus and the N2 from an Egyptian H9N2 virus on a PR8 backbone. This strain had better replication rates than an H5N2 reassortant strain on an H9N2 backbone and an H5N1 reassortant on a PR8 backbone. This virus was then used to develop a killed, oil-emulsion vaccine and tested for efficacy against H5N1 and H9N2 viruses in chickens. Results showed that this vaccine was immunogenic and reduced mortality and shedding.

* To whom correspondence may be addressed, Mohamed Ahmed Ali, PhD, Center of Scientific Excellence for Influenza Viruses, National Research Centre, El-Buhouth Street, Dokki, Giza 12311, Egypt. Phone: +20233322446, Fax: +20237481483; mohamedahmedali2004@yahoo.com, Address correspondence Ghazi Kayali, PhD, MPH, Department of Infectious Diseases, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105. Phone: (901) 595-2555, Fax: (901) 595-8559. ghazi.kayali@stjude.org.

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Discussion—Our findings suggest that an inactivated PR8-derived H5N2 influenza vaccine is efficacious in poultry against H5N1 and H9N2 viruses and the vaccine seed replicates at a high rate thus improving vaccine production.

Keywords

Avian influenza virus; Reverse genetics; Vaccine

Introduction

The epidemiology of avian influenza (AI) infections has changed over the last two decades due to the spread of highly pathogenic avian influenza (HPAI) H5N1 viruses in domestic poultry [1]. In Egypt, clade 2.2 HPAI H5N1 viruses have been enzootic in poultry since 2006. Low pathogenic avian influenza (LPAI) H9N2 viruses, circulating in Egyptian poultry since 2011, added additional burden to the Egyptian poultry industry [2, 3]. The co-circulation and co-infection of both subtypes, H5N1 and H9N2, was observed [4].

Vaccination is a major aspect of the AI control strategy in Egypt and several commercial inactivated vaccines were licensed to control H5N1 and H9N2 in poultry. Most vaccines are based on adjuvanted, whole, inactivated virions prepared using wild-type or reverse-genetics viruses. Plasmid-based reverse genetics is a powerful tool that allows the removal of virulence factors from reassortant vaccine strains such as the multibasic amino acid motif at the HA cleavage site in HPAI subtypes. Most reverse genetics-based AI vaccines utilize six internal genes from A/Puerto Rico/8/34(H1N1) strain (PR8) and the hemagglutinin (HA) and neuraminidase (NA) glycoproteins from circulating influenza viruses to prepare human [5-7] and poultry vaccines [8-13]. These vaccines are safe and provide protective immunity [5, 11]. Reassortant vaccine strains containing the modified HA from A/Vietnam/1194/2004(H5N1) and 7 segments of PR8 grew better than those containing 6 segments of PR8 and modified HA and NA segments from H5N1 (9.5 and 8.8 EID₅₀/mL respectively). This increased the virus antigen content in the candidate influenza vaccine strains [14].

AI H9N2 viruses isolated from Egypt grew efficiently in embryonated chicken eggs and mammalian cells [4]. Hence, we investigated whether a certain gene segment was responsible for this replication and consequently whether this segment will increase the replication rate of a reassortant H5 vaccine strain if introduced through reverse genetics. We then assessed the immunogenicity and protection of the resulting vaccine in chickens.

1. Materials and methods

2.1. Viruses

The LPAI A/chicken/Egypt/S4456B/2011(H9N2) and HPAI A/duck/Egypt/M2583D/2010(H5N1), representative of viruses circulating in Egypt, were propagated in allantoic cavities of 11 day old embryonated chicken eggs for 48 hrs.

2.2. Plasmids and reverse genetics

The multibasic amino acid sequence (EKRRKKR/GLF) at the cleavage site of the H5N1 virus was transformed into a monobasic form (ETR/GLF) as described previously [15]. All eight gene segments of H9N2, 8 segments of PR8, and the full length altered HA and NA segments of H5N1 were amplified by RT-PCR, cloned in pHW2000, sequenced, and subsequently used to generate reassortant viruses (Fig. 1.) as previously described [16, 17].

2.3. Viral titration by plaque assay

Viruses were 10-fold serially diluted in infection medium (DMEM with 4% BSA, 1% antibiotic-antimycotic mixture, and 0.5 µg/ml TPCK-treated trypsin). A monolayer of MDCK cells in 6-well plates was inoculated with 100 µl of each dilution and 400 µl infection medium. The viruses were allowed to adsorb to the cells for 1 hr. The inocula were replaced with DMEM overlay medium containing 1% agarose, 4 % BSA, 1% antibiotic-antimycotic mixture, and 1µg/ml TPCK-treated trypsin. The plates were incubated at 37 °C with 5% CO₂ for 2 days. The plaques were visualized by staining the monolayer by crystal violet.

2.4. Growth kinetics of rescued viruses in MDCK and eggs

Rescued reassortant viruses were inoculated onto a monolayer of MDCK cells at multiplicity of infection (MOI) of 0.005. The supernatants from the infected cells were collected at specific time points and titrated by hemagglutination (HA) and TCID₅₀. In eggs, equal HA titer (4 HAU) of each virus was inoculated into 5 eggs. The allantoic fluids were harvested at 24 and 48 hrs post infection and titrated by TCID₅₀ and HA. Reassortant viruses that were used as vaccine strains were titrated by HA and quantified by TaqMan qRTPCR (egg grown)[18] or by HA and TCID₅₀ (MDCK grown).

2.5. Vaccine preparation, immunization of chickens, and serological assays

Equal virus antigen titer of each vaccine strain (256 HAU) was inactivated using 0.1% formalin and mixed with Montanide ISA 70 VG (Seppic, France) in the ratio recommended by manufacturer (30 antigen/70 adjuvant). Six one-day-old Lohmann-White chickens with low maternal antibodies (< 3 Log₂ HI titer against H5N1 and H9N2) were used per vaccine. At age 7 days, 0.25 ml of each experimental vaccine was administered intramuscularly. Blood samples were obtained weekly (up to week 9 post vaccination) from each group and tested for antibodies against H5N1 and H9N2 viruses using hemagglutination inhibition (HI) and virus neutralization (VN) assays as previously described [19]. Titers were reported using a log₂ scale.

To evaluate the anti-NA antibodies, we used an enzyme-linked lectin-based NA inhibition assay (ELLA) as described previously [20, 21]. We generated reassortant viruses containing N1 of H5N1 (NA-H5N1/PR8) and N2 of H9N2 (NA-H9N2/PR8) viruses on a PR8 backbone to avoid non-specific inhibition by H5 and H9-specific antibodies (Fig. 1.).

2.6. Challenge of vaccinated chickens with HPAI H5N1 and LPAI H9N2

Groups of 6 immunized chickens, were infected with 0.5 ml of challenge viruses at a dose of 50 CLD₅₀/0.5ml for H5N1 and 0.5 ml of 7.5 log₁₀ EID₅₀/ml for H9N2 via the natural route (i.e., intranasal, intraocular, and intratracheal), at 4 weeks post vaccination (wpv). Chickens were monitored daily for morbidity and mortality. Cloacal swabs were obtained from each bird at days 1, 3, 5 and 7 post infection for virus titration in eggs. All animal experiments were approved by the Ethics Committee of the National Research Centre, Egypt.

The immunogenicity of the experimental vaccines were tested in 120 1-day old Cobb broiler chickens in a low-biosecurity farm that mimics the majority of poultry farms in Egypt. To monitor the maternal antibody titers, blood samples were taken from 20 chicks at ages 3 and 10 days and tested by HI and VN. At 14 days of age, groups of 20 chickens were vaccinated with 0.5 ml of each experimental vaccine by intramuscular injection and one group served as an unvaccinated control. Groups were separated in different pens. On vaccination day and weekly thereafter (up to 4 wpv), blood samples were collected from 10 chickens per group to monitor antibody titers using HI and VN. Throughout the experiment, tracheal, cloacal, and environmental swabs were collected weekly from each pen to detect AIV infection by RT-PCR [22].

2.8 Statistical analyses

Statistical analyses were done using GraphPad Prism V5 (GraphPad Inc., CA). One-way ANOVA with Tukey post-hoc test was used to compare antibody and virus titers. Differences were considered statistically significant at p-value< 0.05.

2. Results

3.1 Determining the gene segment responsible for increased replication of H9N2 viruses

Two parental (H9N2 and PR8) and 8 reassortant (PB2-H9/PR8, PB1-H9/PR8, PA-H9/PR8, HA-H9/PR8, NP-H9/PR8, NA-H9/PR8, M-H9/PR8, and NS-H9/PR8) viruses were rescued. Those were propagated in eggs for 2 passages and did not cause embryo death. The size of individual plaques was larger in the case of cells infected with H9N2, NA-H9/PR8, PA-H9/PR8 and NS-H9/PR8 viruses than the other viruses (Fig. S1.).

In MDCK cells, the NA-H9/PR8 and NS-H9/PR8 viruses showed higher titers at 24 and 36 hrs post infection as compared to parental viruses (p-value<0.01) (Fig 2A). The NA-H9/PR8, M-H9/PR8, NS-H9/PR8, NP-H9/PR8, HA-H9/PR8, PA-H9/PR8, and PB2-H9/PR8 had significantly higher HA titers compared to parental viruses (p-values<0.001) (Fig 2B). The NA-H9/PR8 virus grown on eggs had a higher HA titer than parental viruses but was statistically significant only when compared to PR8 (p-value<0.05) (Fig. 2C.).

3.2 Replication of reassortant candidate vaccine strains

As the results indicated that the NA of H9N2 is responsible for increased viral replication, we generated 2 reassortant vaccine strains that included the altered HA of H5N1 and the NA of H9N2 on PR8 or H9N2 backbones [H5N2(LP)/PR8 and H5N2(LP)/H9N2 respectively]. We also generated a vaccine strain that included the altered HA and NA of H5N1 on a PR8

backbone [H5N1(LP)/PR8] as a control vaccine. Rescued viruses were propagated in eggs for 2 passages and did not cause embryo death. The sequences of the rescued viruses did not differ from the wild-type viruses.

When propagated in eggs, the H5N2(LP)/PR8 virus showed the highest HA titer and was statistically significant compared to the H5N1(LP)/PR8 and H5N2(LP)/H9N2 at 24 and 48 hrs post infection (p-values<0.05)(Fig.S2A.). No significant differences were detected in viral RNA copy numbers at 24 and 48 hrs post infection indicating that the H5N2(LP)/PR8 virus replicated as efficiently as the H5N1(LP)/PR8 virus (Fig.S2B.).

The MDCK grown H5N2(LP)/PR8 virus had the highest TCID₅₀ titer, was significantly different than the H5N2(LP)/H9N2 at all time points (p-values<0.001), and was significantly different than the H5N1(LP)/PR8 at 36 hrs post infection (p-value<0.001) (Fig. S2C.). As for HA titers of MDCK grown viruses, the H5N2(LP)/H9N2 had the highest titers and was significantly different than the H5N1(LP)/PR8 and H5N2(LP)/PR8 at all time points (p-values<0.01) (Fig. S2D.).

3.3 Efficacy of reassortant candidate vaccine strains

No significant differences were observed among the compared vaccinated chicken groups against H5N1 or H9N2 viruses at 1 and 2 wpv. At 3 wpv, H9N2 vaccinated chickens had significantly higher HI titers than all other groups and the mean HI titer was 8 log₂ when tested against the homologous H9N2 antigen (p-value<0.001). In this group, titers increased weekly to 9.5 log₂ up to 6 wpv. At 6 wpv, the H5N2(LP)/PR8 vaccinated chickens showed significantly higher anti-H9N2 titers than the H5N1(LP)/PR8 and the control group (p-value<0.001) (Fig. 3A./Table S1). When tested against H5N1, H5N1(LP)/PR8 and both H5N2 vaccines provided significantly higher antibody titers (>6 log₂) than the H9N2 vaccinated and control chicken groups as of 3 wpv and onwards (p-value<0.01). Overall, the H5N2(LP)/PR8 was the most immunogenic vaccine followed by H5N1 (LP)/PR8 and H5N2 (LP)/H9N2 vaccines respectively (Fig. 3B. /Table S1).

H9N2 vaccinated chickens showed significantly higher neutralizing antibody titers against H9N2 virus at 2 wpv with mean titer of 7.54 log₂ as compared to the H5N1(LP)/PR8 or control groups (p-values<0.01). The mean titer increased to around 9 log₂ at 4 wpv. H5N2(LP)/PR8 vaccinated chickens showed neutralizing antibody titers as of 3 wpv and peaked at week 7 at 8 log₂ and the titers were significantly higher than the control group (p-values<0.01). A moderate neutralizing activity was observed in H5N2(LP)/H9N2 vaccinated chickens with H9N2 virus (Fig. 3C. /Table S1). We then tested the immunogenicity of our experimental vaccines against H5N1 antigen using VN assay. All H5 based vaccines provided neutralizing titers after 3 wpv (p-values<0.01) as compared to the control and the H9N2 vaccinated chickens (Fig. 3D. /Table S1). Consistent with HI results, H5N2(LP)/PR8 showed the highest VN titers followed by H5N1(LP)/PR8 and H5N2(LP)/H9N2.

ELLA results showed that H5N1 vaccinated chicken group had higher reactivity with the NA-H5N1/PR8 virus than the NA-H9N2/PR8 virus. Conversely, vaccines with N2 showed higher reactivity with H1N2 than H1N1 (Fig. S3.). Consistent with HI and VN, the

H5N2(LP)/PR8 vaccine had higher antibody titers than the H5N2(LP)/H9N2 and the H5N1(LP)/PR8 vaccines.

When challenged with H5N1, unvaccinated chickens died 2 days post infection (dpi) with clinical signs of AI infection including cyanotic combs, subcutaneous edema, and neurological symptoms. All chickens vaccinated with H5N1(LP)/PR8 and H5N2(LP)/PR8 vaccines survived. One chicken vaccinated with H5N2(LP)/H9N2 vaccine died after showing severe disease signs at 3 dpi; the remaining birds survived with no disease signs. 3 H9N2 vaccinated chickens were symptomatic and died on day 2 and the remaining chickens had no symptoms. However, by day 4, the remaining birds had symptoms and died (Fig. 4.).

At 1 dpi, no virus was recovered from the cloaca of all vaccinated groups. At day 3, AIV was isolated from 3 surviving chickens in the H9N2 vaccinated groups ($\text{Log}_{10}\text{EID}_{50}=3.25 \pm 1.44$), 2 of 6 birds vaccinated with H5N2(LP)/PR8 ($\text{Log}_{10}\text{EID}_{50}=2 \pm 0.0$), and two of six chickens vaccinated with H5N1(LP)/PR8 ($\text{Log}_{10}\text{EID}_{50}=3 \pm 0.0$). By day 5, virus was detected in 1 of 5 chickens in the H5N2(LP)/H9N2 group ($\text{Log}_{10}\text{EID}_{50}=3 \pm 0.0$), and 1 of 6 chickens in the H5N1(LP)/PR8 group ($\text{Log}_{10}\text{EID}_{50}=2.5 \pm 0.0$). At day 7, only 1 chicken in the H5N2(LP)/H9N2 group was shedding virus ($\text{Log}_{10}\text{EID}_{50}=2 \pm 0.0$) (Table 1).

When challenged with H9N2, no specific clinical signs were observed except in one bird that was depressed in the control group and died at 7 dpi. None of the chickens shed virus at 1 dpi. Virus was detected in only 1 chicken of the control group ($2 \log_{10}\text{EID}_{50}/\text{ml}$) at day 3. At day 5, 2 of 6 unvaccinated chickens and 1 of 6 H5N2(LP)/H9N2 vaccinated chickens shed virus with a mean titer of $2.5 \log_{10}\text{EID}_{50}/\text{ml}$ and $1.25 \log_{10}\text{EID}_{50}/\text{ml}$ respectively. H9N2 was detected in all unimmunized chickens at day 7 (mean titer $1.83 \log_{10}\text{EID}_{50}/\text{ml}$) and 1 of 6 H5N1(LP)/PR8 vaccinated chickens ($1.25 \log_{10}\text{EID}_{50}/\text{ml}$) (Table 1).

3.4 Immunogenicity of reassortant candidate vaccine strains in the field

During the course of the experiment, there was no evidence of the chickens being exposed to AIV infection as all the swabs were negative by RT-PCR. The maternal immunity antibodies at days 1 and 7 of age cross reacted with H5N1, with $\log_2$ means HI titer of 4.6 and 4.4 respectively. By day 14, no reactivity was detected against H5N1 antigen. All vaccines provided antibody titers higher than $5 \log_2$ by 3 wpv (Fig. 5A./Table S2). At 2 wpv, the H5N2(LP)/PR8 vaccinated chickens showed significantly higher anti-H5N1 HI titers than the H5N1(LP)/PR8, H5N1(LP)/H9N2, and the control group ($p\text{-value} < 0.01$). All vaccinated chicken groups showed significantly higher HI titers than the control group ($p\text{-values} < 0.01$) at 2, 3 and 4 wpv. Against H9N2, all vaccinated chicken groups induced significant HI titers as compared to the control group at 3 wpv ($p\text{-value} < 0.001$) (Fig. 5B/ Table S2). At 2 wpv, the H5N2-based vaccines induced significantly higher HI titers than the H5N1 and control groups ($p\text{-value} < 0.001$).

All vaccines provided neutralizing H5N1 titers at 3 wpv. At that time, H5N2(LP)/PR8 vaccinated chickens had significantly higher titers ($10 \log_2$) than all other groups ($p\text{-value} < 0.05$) (Fig. 5C/ Table S2). H5N1(LP)/PR8 and H5N2(LP)/H9N2 vaccinated chicken groups showed significantly higher antibody titers ($p\text{-value} < 0.001$) after 4 wpv as compared to the controls. When tested against H9N2, none of the tested vaccines provided a detectable

antibody titer by 2 wpv. A slight increase in neutralizing antibody titers against H9N2 virus were observed for both H5N2 vaccines at 3 and 4 wpv (Fig. 5D/Table S2) which were significantly higher than the control group (p-value<0.001).

4. Discussion

Vaccinating chickens against AI has been practiced in Egypt soon after H5N1 was detected in 2006. Several problems were associated with the use of vaccines including improper antigenic matching and low vaccine coverage. In this paper, we tested the efficacy of an H5N2 reassortant vaccine strain capable of protecting chickens from both AI viruses currently circulating in Egyptian poultry.

Previous studies showed that the behaviors and phenotypes of plaque shape of influenza A viruses in cell culture were heterogeneous and could be indicative of variation in growth properties [23]. When grown in cells, reassortant viruses expressing NA, PA and NS genes of H9N2 virus showed large plaques similar to the wild type H9N2 viruses. Many viral protein interactions are known to affect virus assembly and morphology, such as M1-HA, M1-NA, M1-M2, and M1-vRNP interactions [24]. However, it is unclear how the PA and NS genes affected viral plaque morphology and the relation between plaque size and viral replication rates requires further study. We observed an increase in replication rate for a reassortant virus expressing the NA, NS, and M genes of A/chicken/Egypt/S4456B/2011(H9N2) relative to that of either parental viruses or reassortant PR8 expressing other H9N2 genes in both cell and egg cultures. The vaccine strain H5N2/PR8 had higher replication rates than the H5N2/H9N2 showing an advantage in replication of having only the NA from H9N2, in addition to the ability of N2 as a surface protein to generate antibodies. Influenza virus could gain its efficient replication in eggs either by increasing its HA receptor binding affinity or by reducing its NA activity [25].

We constructed H1N1 and H1N2 viruses containing NA gene segments from H5N1 and H9N2 viruses on a PR8 backbone. Growth yield of the reassortant H1N2 virus was higher than H1N1 virus (Fig. S4). The NA enzymatic activity has been reported to vary according to the stalk region of the NA molecule, with the NA species containing a deletion in the stalk having lower activity [26-31]. The N1 NA of A/duck/Egypt/M2583D/2010 (H5N1) virus has a stalk deletion but the N2 NA of A/chicken/Egypt/S4456B/2011(H9N2) virus has no deletion. Previous studies showed that the NA activity of N2 was higher than N1 [26, 32]. A previous study showed that the H5N1 virus had lower NA activity than H1N1-NA-H5N1, even though both strains consisted of the same NA [33].

Our results as well as that of many others suggest that antibodies that inhibit NA activity contribute to protection against influenza infection [34-38]. These antibodies are likely to contribute to vaccine efficacy particularly when the HAs of circulating strains are not antigenically well-matched to vaccine strains. NA-specific antibodies protect against lethal doses of HPAI viruses in chickens [39] and mice [40].

We assessed whether vaccination of chickens with H5N2 vaccine could reduce disease rates of both endemic H5N1 and H9N2 viruses in Egypt. This was evident in reduced mortality

and virus shedding. Unvaccinated chickens challenged with H9N2 shed virus at $2.5 \log_{10}$ EID₅₀/mL similar to other studies using H9N2 viruses [41]. H5N2 vaccinated chickens developed substantial HI and NI antibody titers directed against both viruses upon vaccination with a heterologous H5N2 vaccine, and this provides a level of protection that is generally sufficient to prevent H5N1 and H9N2 infections. Surprisingly, antibody titers were higher for the H5N2(LP)/PR8 vaccine than the H5N1 vaccine. Nevertheless, H5N1 antibodies titers formed were sufficient to give protection against disease. This indicates that cross-protection even between different subtypes may be expected. In the field experiment, antibodies were detected in unvaccinated chickens, this may be due to exposure of the chickens to avian influenza viruses that we did not detect in the swabs we obtained from the farm.

In summary, a vaccine based on a reassortant H5N2 strain was capable of protecting chickens against infection with H5N1 and H9N2 viruses. This strain grew to high titers in embryonated chicken eggs and hence is expected to work well in the commercial production of a vaccine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- We showed that the NA of H9N2 was responsible for the high replication rates of Egyptian H9N2 viruses.
- We generated a reassortant vaccine strain incorporating the HA from H5N1 and NA from H9N2 on a PR8 backbone.
- The H5N2 vaccine was efficacious under laboratory and field conditions.

Rg Virus name	Plasmids used to generate Rg viruses							
H9N2	PB2	PB1	PA	HA	NP	NA	M	NS
PR8	PB2	PB1	PA	HA	NP	NA	M	NS
PB2-H9N2/PR8	PB2	PB1	PA	HA	NP	NA	M	NS
PB1-H9N2/PR8	PB2	PB1	PA	HA	NP	NA	M	NS
PA-H9N2/PR8	PB2	PB1	PA	HA	NP	NA	M	NS
HA-H9N2/PR8	PB2	PB1	PA	HA	NP	NA	M	NS
NP-H9N2/PR8	PB2	PB1	PA	HA	NP	NA	M	NS
NA-H9N2/PR8	PB2	PB1	PA	HA	NP	NA	M	NS
M-H9N2/PR8	PB2	PB1	PA	HA	NP	NA	M	NS
NS-H9N2/PR8	PB2	PB1	PA	HA	NP	NA	M	NS
H5N1(LP)/PR8	PB2	PB1	PA	ΔHA	NP	NA	M	NS
H5N2(LP)/H9N2	PB2	PB1	PA	ΔHA	NP	NA	M	NS
H5N2(LP)/PR8	PB2	PB1	PA	ΔHA	NP	NA	M	NS
NA-H5N1/PR8	PB2	PB1	PA	HA	NP	NA	M	NS
PR8 Segments				H5N1 Segments		H9N2 Segments		

Figure 1.

Summary of reverse genetics derived viruses and plasmids used for generation of rescued viruses. Gray rectangles indicate gene segments of the PR8 virus, orange rectangles indicate gene segments derived from the A/chicken/Egypt/S4456B/2011(H9N2), and red rectangles indicate gene segments of A/duck/Egypt/M2583D/2010(H5N1). Monobasic cleavage site in the HA gene segment of A/duck/Egypt/M2583D/2010(H5N1) is marked with delta symbol.

Figure 2.

Growth kinetics of rescued reassorted (1 gene of H9N2 + 7 genes PR8) and parental H9N2 and PR8 viruses in MDCK cells and eggs. MDCK-grown viruses were titrated by TCID₅₀/ml assay (A) and HA assay (B). Egg grown viruses were titrated by HA (C).

A **B**

Figure 3.

Cross-reactive antibody titers of chickens vaccinated with experimental vaccines as measured by HI assay against H9N2 (A) and H5N1 (B) and VN against H9N2 (C) and H5N1 (D). Data are as of 1 week post vaccination. Maternal antibodies prior to vaccination were less than 3 log₂.

Figure 4. Survival curves of chickens (n = 6 per group) vaccinated with different inactivated vaccines after challenge with an HPAI H5N1 virus (A) and LPAI H9N2 virus (B).

Figure 5.

Weekly antibody titers of vaccinated chickens in field conditions against H5N1 using HI (A) and VN assays (C) and against H9N2 virus using HI (B) and VN assays (D). Arrows indicated time of vaccination.

Titers of A/duck/Egypt/M2583D/2010 (H5N1) and A/chicken/Egypt/S4456B/2011 (H9N2) viruses in coecal samples obtained from immunized and unimmunized chicken groups^a.

Table

Challenge virus	Day post challenge	Control	H9N2	H5N2(LP)/PR8	H5N2(LP)/H9N2	H5N1(LP)/PR8
A/duck/Egypt/M2583D/2010 (H5N1)	1	-	-	-	-	-
	3	*	3.25± 1.44 (3/3) ^b	2±0.0 (2/6)	-	3±0.0 (2/6)
	5	*	*	-	3 ±0.0 (1/5)	2.5±0.0 (1/6)
	7	*	*	-	2±0.0 (1/5)	-
A/chicken/Egypt/S4456B/2011 (H9N2)	1	-	-	-	-	-
	3	2±0.0 (1/6)	-	-	-	-
	5	2.5±0.0 (2/6)	-	-	1.25 ±0.0 (1/6)	-
	7	1.83±0.66 (6/6)	-	-	-	1.25±0.0 (1/6)

-, The titer was below the limit of detection (<1 Log₁₀ EID₅₀/ml).

^aData are the mean Log₁₀ EID₅₀/ml ± SD for positive samples

* Not determined because of death

^bValues in parentheses are number of positive chickens out of each group