Contribution of the amino acid mutations in the HA gene to antigenic variation and immune escape of H9N2 avian influenza virus

Huoying Shi[1], Rui Zhu[1], Shunshun Xu[1], Wangyangji Sun[1], Quan Li[1], Shifeng Wang[2], and Xiufan Liu[1]

[1]Yangzhou University
[2]University of Florida

April 16, 2024

Abstract

H9N2 influenza virus has been clustered into multiple lineages because of antigenic drift. The continuous rapid evolution of H9N2 virus increases the difficulties of the prevention and control programs. As a major antigenic protein, hemagglutinin (HA) protein has always been of interest, especially amino acid mutations altering viral antigenicity. It has been well-documented that some amino acid mutations in HA of H9N2 avian influenza virus (H9N2 virus) alter the viral antigenicity, but little is reported about how these antibody escape mutations affect antigenic variation. Herein, we identified 15 HA mutations which were potentially relevant to viral antigenic drift, and found that a key amino acid mutation A198V at position 198 in HA, the only nonconservative site in the receptor binding sites, was directly responsible for viral antigenic variation. Furthermore, the rF/HA $_{\mathrm{A198V}}$ virus showed poor cross-reactivity to immune sera from animals immunized with the viruses F/98 (A198), SD/SS/94 (A198), JS/Y618/12 (T198), and rF/HA $_{\mathrm{A198V}}$ (V198) by microneutralization (MN) assay. The A198V substitution in parent virus caused significantly decreased cross-MN titers by enhancing the receptor binding activity, but did not prevent antibody (Ab) binding physically. Additionally, the strong receptor binding avidity increased the NA activity significantly, while prevented the viral release from cells. Moreover, A198V substitution promoted H9N2 virus escape from pAbs-neutralizing reaction *in vitro*, and slightly affected cross-protection *in vivo*. Our results suggested that the A198V mutation with strong receptor binding avidity contributed to viral antigenicity and immune escape, and played a key role in the process of adaptive evolution of H9N2 virus.

Contribution of the amino acid mutations in the HA gene to antigenic variation and immune escape of H9N2 avian influenza virus

Rui Zhu[a,b*], Shunshun Xu[a,b], Wangyangji Sun[a,b], Quan Li[a,b], Shifeng Wang[c], Huoying Shi[a,b,d +], Xiufan Liu[a,b]

[a]College of Veterinary Medicine, Yangzhou University, Yangzhou, 225009, Jiangsu, China; [b]Jiangsu Co-Innovation Center for the Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, 225009, China;[c]Department of Infectious Diseases and Immunology, College of Veterinary Medicine, University of Florida, Gainesville, FL, 32611-0880, USA; [d]The International Joint Research Laboratory of Agricultural and Agri-product Safety, Yangzhou University, Yangzhou, Jiangsu, China.

[*] Present Address:Jiangsu Agri-animal Husbandry Vocational College, Jiangsu Key Laboratory for High-Tech Research and Development of Veterinary Biopharmaceuticals, Taizhou, Jiangsu 225300, China

[+] Corresponding Author: Huoying Shi, College of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu 225009, People's Republic of China;

Abstract

H9N2 influenza virus has been clustered into multiple lineages because of antigenic drift. The continuous rapid evolution of H9N2 virus increases the difficulties of the prevention and control programs. As a major antigenic protein, hemagglutinin (HA) protein has always been of interest, especially amino acid mutations altering viral antigenicity. It has been well-documented that some amino acid mutations in HA of H9N2 avian influenza virus (H9N2 virus) alter the viral antigenicity, but little is reported about how these antibody escape mutations affect antigenic variation. Herein, we identified 15 HA mutations which were potentially relevant to viral antigenic drift, and found that a key amino acid mutation A198V at position 198 in HA, the only nonconservative site in the receptor binding sites, was directly responsible for viral antigenic variation. Furthermore, the rF/HA_{A198V} virus showed poor cross-reactivity to immune sera from animals immunized with the viruses F/98 (A198), SD/SS/94 (A198), JS/Y618/12 (T198), and rF/HA_{A198V} (V198) by microneutralization (MN) assay. The A198V substitution in parent virus caused significantly decreased cross-MN titers by enhancing the receptor binding activity, but did not prevent antibody (Ab) binding physically. Additionally, the strong receptor binding avidity increased the NA activity significantly, while prevented the viral release from cells. Moreover, A198V substitution promoted H9N2 virus escape from pAbs-neutralizing reaction *in vitro* , and slightly affected cross-protection *in vivo* . Our results suggested that the A198V mutation with strong receptor binding avidity contributed to viral antigenicity and immune escape, and played a key role in the process of adaptive evolution of H9N2 virus.

Keywords

H9N2, avian influenza virus, haemagglutinin, mutations, antigenic variation, immune escape

Introduction

H9N2 avian influenza virus (AIV) spread rapidly and infected more than 90% of chicken flocks since its breakout in Hebei province, China in 1998. It became one of the most important epidemics in poultry industry in China (Gu,Xu,Wang, & Liu, 2017). Since then, vaccination strategy of inactivated vaccine for control of H9N2 avian influenza had been extensively executed, and worked well (Li et al., 2005). However, H9N2 virus is undergoing adaptive evolution under the vaccine immune pressure. As a major antigen and receptor binding protein of H9N2 virus, the haemagglutinin (HA) from the circulating field strains were clustered into three lineages before 2007, A/Chicken/Beijing/1/94-like (BJ/94-like), A/Quail/Hong Kong/G1/97-like (G1-like), and A/Duck/Hong Kong/Y439/97-like (Y439/97-like) (Sun & Liu, 2015). In 2013, G57 was emerged as the predominant genotype of H9N2 virus. A new genotype G118 was discovered in 2015 (Jin et al., 2020). With the evolution of H9N2 virus, the specific antibodies induced by inactivated vaccines could not effectively block the attachment of HA of the circulating virus to the target cells (Chambers,Kawaoka, & Webster, 1988). This resulted in the decrease in the protection efficacy of the existing vaccines and isolation of breakthrough H9N2 viruses in vaccinated chicken flocks with high antibody titer (Li et al., 2019). Therefore, it is important to monitor antigenic mutation of the HA from H9N2 virus.

Currently, over 30 antigenic sites of H9N2 virus have been reported, most of which were mapped by monoclonal antibody (mAb) precisely (Peacock et al., 2016; Jin et al., 2019; Kaverin et al., 2004; Okamatsu, Sakoda, Kishida,Isoda, & Kida, 2008; Ping et al., 2008; Wan et al., 2014; Zhu et al., 2015). The viral evolution could promote virus escaping from the neutralization of antibody by adding N-linked glycosylation (NLG) to shield the antigenic sites (An et al., 2019; Cherry,Lipman,Nikolskaya, & Wolf, 2009), changing virus-antibody binding property (Li et al., 2013), or altering receptor-binding specificity (Wan & Perez, 2007; Peacock et al., 2017; Teng et al., 2016; Yang et al., 2017).

We previously reported that the H9N2 vaccine representative strain A/Chicken/Shanghai/F/1998 (F/98, H9N2), which belonged to BJ/94-like lineage, occurred antigenic variation continually when passaged in specific pathogen-free (SPF) chicken embryos or SPF chickens with or without homologous vaccine antibodies (Jin et al., 2018; Su et al., 2020). In this study, we generated recombinant F/98 viruses containing single HA mutation from the passaged viruses occurring antigenic drift, and identified the contribution of these HA mutations to the antigenic variation using HI assay. We made a comprehensive analysis of the role of

the key mutation A198V in antigenic variation or immune escape by cross-microneutralization (MN) assay and cross-protection *in vivo* . Our results showed that A198V substitution caused the decreased readouts of cross-MN titers significantly by enhancing the receptor binding activity though it did not prevent Abs binding physically. In addition, the strong receptor binding avidity increased the NA activity, prevented viral release from cells, and slightly affected cross-protection *in vivo* .

Materials and methods

Ethical compliance

The SPF chickens and chicken embryos used in this study were purchased from Nanjing Biology Medical Factory, Qian Yuan-hao Biological Co, Ltd.. Procedures involving the care and use of animals were approved by the Jiangsu Administrative Committee for Laboratory Animals (permission number SYXK 2016-0020) and performed in accordance with the Jiangsu Laboratory Animal Welfare and Ethics guidelines of the Jiangsu Administrative Committee of Laboratory Animals.

Viruses and cells

The H9N2 virus F/98 was isolated in Shanghai in 1998, stored at - 70 °C at the Animal Infectious Disease Laboratory, School of Veterinary Medicine, Yangzhou University. The GenBank accession numbers of the sequence of the F/98 strain are AY253750-AY253756 and AF461532 (Lu et al., 2005). Human embryonic kidney cells (293T) and Madin-Darby canine kidney (MDCK) cells, purchased from ATCC (Manassas, VA, USA), were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Hyclone, South Logan, UT, USA) and were incubated at 37 degC with 5% CO_2.

Generation of H9N2 AIVs by reverse genetics

The primers, synthesized by Tsingke Biological Technology (Nanjing, China), used to amplify the DNA sequence to add the single mutation in HA protein of F/98 virus were designed using Primer 5.0 software (Primer-E Ltd., Plymouth, UK) based on the HA gene sequence of the F/98 H9N2 avian influenza virus. The full-length HA genes containing the single mutation was amplified by PCR, and inserted into a transcriptional/expression vector pHW2000 (Hoffmann, Neumann, Kawaoka, Hobom, & Webster, 2000) by using ClonExpress II One Step Cloning Kit (Vazyme Biotech Co., Ltd., Nanjing, China), resulting in the plasmids pHW204-HAmutations. Seven dual-promoter plasmids, including pHW201-PB2, pHW202-PB1, pHW203-PA, pHW205-NP, pHW206-NA, pHW207-M, and pHW208-NS, from the F/98 virus strain, were stocked in our lab at -70 degC (Shi, Ashraf, Gao,Lu, & Liu, 2010). The recombinant viruses were rescued by transfection in the 293T cell as previously described (Peng et al., 2019). Briefly, a total weight of 2.4 ng of the eight plasmids mixture with a rate of 1:1 was mixed with 100 μL Opti-MEM medium (GIBCO, BRL, Grand Island, USA). Next, 7 μL of PolyFect transfection reagent (QIAGEN, Duesseldorf, Germany) was added. The samples were incubated at room temperature for 10 min and then added to the 70-80% confluent monolayers of 293T cell in 24-well plates. After incubation at 37°C with 5% CO_2 for 6 h, 2 μg/mL of TPCK-trypsin (Sigma, St. Louis, MO, USA) was added to the wells. Thirty hours after transfection, the supernatants were harvested and inoculated into 10-day-old SPF embryonated chicken eggs for virus propagation. The rescued virus was analyzed with a hemagglutinin assay, and the HA genes from the rescued virus was sequenced by Tsingke Biological Technology (Nanjing, China) to confirm the accuracy of the designed mutation.

Determination of the 50% tissue cell infectious dose ($TCID_{50}$)

The $TCID_{50}$ assay was performed as we described previously (Peng et al., 2019). Briefly, the viruses were diluted in DMEM without serum to a concentration of 10^{-1} to 10^{-11} and then added to MDCK cells in 96-well plates, respectively. After incubation at 37 °C with 5% CO_2 for 1 h, the supernatants were removed. The plates were washed twice with PBS, and then 100 μL of DMEM was added to each well. After incubation at 37°C with 5% CO_2 for 72 h, the HA titers of the cell supernatants were analyzed. The virus titers were calculated according to the Reed-Muench formula (Reed & Muench, 1937).

Anti-sera

As described previously (Zhu et al., 2018), six three-week-old SPF chickens were immunized twice by subcutaneous injection of 0.3 mL of oil-emulsion of inactivated whole virus vaccines of the viruses F/98 and rF/HA_{A198V}, which were inactivated by adding 0.2% formalin (v /v) for 24 h at 37 °C, respectively. The antisera were collected and pooled from the vaccinated SPF chickens at three weeks after the vaccination.

Hemagglutinin-inhibition (HI) assay and Microneutralization (MN) assay

Antisera were treated with cholera filtrate (Sigma-Aldrich, St. Louis, MO, USA) to remove nonspecific hemagglutination inhibitors before HI assay. HI assay was performed using 4 hemagglutination units (HAU) of H9N2 and 1% (v /v) chicken erythrocytes as we described previously (Peng et al., 2019).

MN assay was performed as previously described (H. Zhu et al., 2018). Briefly, the sera were serially diluted with 100 $TCID_{50}$virus and incubated at 37 degC with 5% CO_2 for 1 h. The serum-virus mixtures were added to MDCK cells and incubated for 1 h. After incubation, the serum-virus mixtures were removed. Serum-free DMEM containing 2 μg/mL TPCK- trypsin was added to each cell and incubated at 37°C and 5% CO_2. After 72 h of incubation, culture supernatant was mixed with equal volume of 1% (v /v) chicken erythrocytes to confirm the existence of hemagglutination by virus. The MN titer was defined as the highest dilution of serum with absence of hemagglutination.

Enzyme-linked immunosorbent assay (ELISA)

ELISA assay was performed as we described previously (Peng et al., 2019). Sucrose gradient-purified viruses were diluted in PBS and added to Nunc-Immuno MaxiSop 96-well plates (Corning, NY, USA) at 16 HAU per well. After incubation overnight at 4degC, samples in wells were blocked with PBS-nonfat dry milk. Antisera against the F/98 virus, the rF/HA_{S145N} virus or the rF/HA_{A198V} virus in chickens were then added in serial twofold dilutions with PBS containing 0.05% Tween 20, respectively, and incubated for 3 h at 37degC. After washing, goat anti chicken horseradish peroxidase antibody (Abcam, Cambridge, MA) was added and allowed to incubate for 1.5 h at 37 degC. After washing, TMB (3,3',5,5' Tetramethylbenzidine) (Sigma, St. Louis, MO, USA) substrate was added, and the reaction was stopped by adding H_2SO_4. Absorbance was recorded at 450 nm using an automated ELISA plate reader (model EL311SX; Biotek, Winooski, VT). The area under curve (AUC) of either virus was assessed for virus-Abs binding with GraphPad Prism 8 software (San Diego, CA) above that of the corresponding negative control.

Receptor binding assay

Receptor binding assay was performed as previously described (Chambers,Parkhouse,Ross,Alby, & Hensley, 2015). Briefly, the chicken erythrocytes were pretreated with different amounts of α2-3,6,8 neuraminidase (New England Biolabs, Beverly, MA, USA) for 1 h at 37 °C. The chicken erythrocytes were washed with PBS and added (as 1% (v /v) solutions) to 4 HAU of each virus (as determined using nontreated chicken erythrocytes). Agglutination was measured after incubation for 1 hour. Virus with higher receptor binding avidity is able to bind to chicken erythrocytes that are treated with high amounts of α2-3,6,8 neuraminidases.

Neuraminidase (NA) activity assay

NA activity assays of F/98 virus and recombinant virus rF/HA_{S145N} were analyzed using a Neuraminidase Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China) as previously described (Qin et al., 2018). Each virus was calibrated with the same $TCID_{50}$ titer by diluting the virus sample. Then, 10 μL was mixed with 70 μL detection buffer, 10 μL NA fluorogenic substrate, and 10 μL double-distilled water. Fluorescence was monitored using a multifunctional microplate reader (Tecan). NA activities are shown as the fluorescence intensity above the background values for allantoic fluid without virus.

Virus elution assay

Virus elution assay was performed as previously described (Qin et al., 2018). In brief, F/98 and rF/HA_{S145N} viruses were diluted serially in PBS, and 50 μL aliquots of these two-fold dilutions were incubated with 50 μL

1% (*v* /v) chicken erythrocytes in V-bottom microtiter plates. The plates were placed at 4°C for 30 min and then transferred to 37°C. The decrease in HA titer was monitored for 6 h, which reflects the NA-mediated virus elution from chicken erythrocytes.

Virus release assay

MDCK cells were divided into 2 treated groups including approximately 10^6 cells per group, and the group without cells was as control, then the 3 groups were treated with 1,000 $TCID_{50}$ H9N2 virus in ice bath for 1 h. The first group and the control group were incubated at 37degC for 30 min; and the second group was incubated in ice. The supernatant was collected after centrifugation, then viral total RNA was extracted from the supernatant using the TIANamp Virus RNA kit (Tiangen, Beijing, China). Reverse transcription into cDNA was performed by HiScript(r) II Reverse Transcriptase (Vazyme Biotech Co., Ltd., Nanjing, China) (U12 A/G: AGC/AAAAGCAGG). The copies of M gene were determined by quantitative real-time reverse transcriptase PCR (qRT-PCR) according to the instructions provided in the ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China)(Spackman et al., 2002). Each group had 3 replications. The data were analyzed using absolute quantitative method. Data of the relative viral infection was (the copies of M gene in the control group – the copies of M gene in ice group) / (the copies of M gene in the control group), and the data of the relative viral release was (the copies of M gene in the control group – the copies of M gene in ice group) / (the copies of M gene in the 37 degC group – the copies of M gene in ice group).

Chicken experiments

A total of 24 three-week-old SPF chickens were divided into three groups: the F/98 vaccine group including 12 chickens, and the rF/HA_{A198V} vaccine group including 12 chickens, which were immunized with the emulsion vaccine of the F/98 virus, and the rF/HA_{A198V} virus, respectively. At day 21 post-vaccination, chickens were bled from the wing vein for sera, and HI reactions against the F/98 virus, or the rF/HA_{A198V}virus was performed, respectively. Then, six chickens from each group were challenged intranasally and intratracheally with 10^6 EID_{50} of the F/98 virus, or the rF/HA_{A198V} virus. Chickens were monitored daily for morbidity and mortality after challenge. At days 3 and 5 post-challenge, tracheal and cloacal swabs from challenged chickens were collected in 1 mL of PBS containing antibiotics. After one freeze-thaw cycle, the swabs were centrifuged at 3000 rpm for 10 min. A 0.2 mL supernatant was taken to inoculate 10-day-old SPF chicken eggs. Viral shedding in the trachea and cloacal was evaluated via HA titers of the allantoic cavity of SPF chicken eggs at day 5 post-inoculated according to the standard of HA[?]2^3.

Statistics analysis

Data were shown as the mean +- *SD* for all assays. The Student's t test analysis was used to compare between different groups and analyzed with GraphPad Prism 8 software. Differences were considered statistically significant when a P value was <0.05.

Results

A198V HA mutation causes HI titer decrease significantly

We previously reported that the 15 mutations (as seen in Figure 1A) on the HA protein from the passaged virus occurred when the F/98 strain passaged continuously in SPF embryonated chicken eggs or in SPF chickens (Jin et al., 2018; Su et al., 2020). In order to evaluate the role of these mutations, 15 recombinant viruses, each containing a single HA mutation from the passaged viruses occurring antigenic drift in F/98 backbone, were generated. These viruses are rF/HA_{K131R}, rF/HA_{Q133H}, rF/HA_{S145N}, rF/HA_{Q164L}, rF/HA_{A168T}, rF/HA_{G181E}, rF/HA_{A198V}, rF/HA_{M224K}, rF/HA_{Q234L}, rF/HA_{Y264H}, rF/HA_{G270R}, rF/HA_{G274R}, rF/HA_{K278E}, rF/HA_{I386V}, and rF/HA_{K399N}. The serum against the paternal virus F/98 in chickens was used as the reference serum to analyze the antigenicity of the recombinant viruses by HI assay. Compared with the paternal virus F/98, the mutations S145N, Q164L, A168T, M224K, and Q234L slightly reduced HI titers. The mutation A198V exhibited 6.67-fold lower HI titers, which was antigenically distinct from F/98 (Figure 1B).

To further confirm the contribution of HA A198V mutation to the antigenic change of F/98 strain, 5 recombinant viruses possessing multiple mutations from HA protein of the 47thgeneration of F/98 strain (containing K131R+S145N+G181E+A198V) passaged in SPF embryonated chicken eggs (Jin et al., 2018) in F/98 backbone were generated, respectively, including rF/HA348 (K131R+S145N+G181E), rF/HA349 (K131R+S145N+A198V), rF/HA389 (K131R+G181E+A198V), rF/HA489 (S145N+G181E+A198V), and rF/HA47 (K131R+S145N+G181E+A198V). The results showed that the viruses possessing A198V mutation, including rF/HA349, rF/HA389, rF/HA489, and rF/HA47, displayed the same 6.67-fold lower HI titers as rF/HA$_{\mathrm{A198V}}$ (Figure 1C). These results suggested that the HA mutation A198V is the key change related to the antigenicity of H9N2 virus.

A198V HA mutation may contribute to escape from neutralizing-antibodies

As the Figure 2A shown, amino acid position 198 is located in the sialic acid binding domain as one of the receptor binding sites, suggesting that A198V substitution might play a key role in the evolution of H9N2 viruses. In order to study the role of the mutation A198V in escaping from neutralizing antibodies, microneutralization (MN) assay was performed, which is more sensitive than HI assay (Segovia,Franca,Bahnson,Latorre-Margalef, & Stallknecht, 2019). In comparison to the parental F/98 strain, the rF/HA$_{\mathrm{A198V}}$virus exhibited 8-fold reduction in MN titers to anti-F/98 serum (Figure 2B), and 8-fold reduction to homologous anti-rF/HA$_{\mathrm{A198V}}$serum (Figure 2C), which suggested that the A198V mutation promoted the rF/HA$_{\mathrm{A198V}}$ virus escape from anti-F/98 or anti-rF/HA$_{\mathrm{A198V}}$ serum. Further, we comprehensively compared the serological cross-reactivity induced by the H9N2 viruses A/Chicken/Guangdong/SS/1994 (GD/SS/94, original HA A198), A/Chicken/Jiangsu/YZ618/2012 (JS/YZ618/12, original HA T198) (R. Zhu et al., 2018), and rF/HA$_{\mathrm{A198V}}$ in chickens. Results showed that the rF/HA$_{\mathrm{A198V}}$ virus displayed 4-fold reduction of MN titers to anti-GD/SS/94 serum compared to the GD/SS/94 virus (Figure 2D); the rF/HA$_{\mathrm{A198V}}$ virus displayed 8-fold reduction of MN titers to anti-JS/YZ618/12 serum (Figure 2E) compared to the JS/YZ618/12 virus. However, compared to the viruses GD/SS/94 and JS/YZ618/12, the rF/HA$_{\mathrm{A198V}}$ virus displayed 4- and 8-fold reductions of HI titers to homologous anti-rF/HA$_{\mathrm{A198V}}$ serum, respectively (Figure 2F). These results showed that HA A198V mutation could decrease the readout of MN titers to anti-H9N2 sera significantly, even to anti- rF/HA$_{\mathrm{A198V}}$ serum, indicating that this substitution could promote viral escape from neutralizing-antibodies.

A198V HA substitution increases receptor binding avidity of F/98 virus, and does not prevent antibody binding physically

HI or MN titers can be decreased by the reduction of antibody binding to virus or/and increasing receptor binding avidity (Hensley et al., 2009; Li et al., 2013b). To clear the molecular mechanisms of A198V mutation for escape from neutralizing-antibodies, the interactions between virus and receptors on red blood cell surface and antibody binding ELISA were performed, respectively. The results showed that the virus rF/HA$_{\mathrm{A198V}}$ bound to chicken erythrocytes treated with 32-fold higher α2-3,6,8 neuraminidase concentrations than the F/98 strain. Compared to the virus F/98, rF/HA$_{\mathrm{K131R}}$, rF/HA$_{\mathrm{S145N}}$, rF/HA$_{\mathrm{G181E}}$, or rF/HA348 possessing HA A198, the viruses rF/HA349, rF/HA389, rF/HA489, or rF/HA47 carrying HA V198 bound to chicken erythrocytes treated with at least 4-fold higher α2-3,6,8 neuraminidase concentrations (Figure 3A), indicating A198V mutation increased receptor binding avidity of F/98 virus significantly. Antibody binding ELISA confirmed that serum generated against the F/98 virus bound similarly to either F/98 virus or rF/HA$_{\mathrm{A198V}}$ virus (Figure 3B); serum generated against the rF/HA$_{\mathrm{A198V}}$ virus also bound similarly to either F/98 virus or rF/HA$_{\mathrm{A198V}}$ virus (Figure 3C), which suggested that HA A198V substitution did not cause a significant antigenic change. Taken together, these data indicated that the A198V mutation promoted escape from pAb response by increasing viral receptor binding avidity, but not by preventing antibody binding physically.

HA A198V substitutionelute from cells at a slower rate

The sialic acid receptor is the target receptor shared with HA and neuraminidase (NA). We postulated that the residue substitution with high receptor binding avidity at the position 198 located in the sialic acid binding domain of HA might affect the viral NA activity. The NA activity assay was performed, and the

result showed that the NA activity of the rF/HA_{A198V} virus was more strong than that of the F/98 virus (P ¡0.05) (Figure 4A).

To further evaluate the effect of the A198V HA mutation on the functional balances of HA and NA, we measured the elution of virus from chicken red blood cells (RBCs). rF/HA_{A198V} virus bound tightly to RBCs as indicated by slow blood elution within 3 h. However, 3 h later, the elution F/98 virus from RBCs was at a similar rate to that of rF/HA_{A198V} virus with a strong positive correlation (r =0.9944, R^2=0.9887,P ¡0.01) (Figure 4B).

The HA A198V resulted in a slower release of virus from the RBCs, and we speculated that the strong receptor binding avidity could affect the viral release from cells. To investigate whether the rF/HA_{A198V} virus impacted on the viral infection and release from cell surface, we detected the amounts of viruses by detecting M genes with real-time reverse transcription (RT)-PCR assay in MDCK cells. The results showed that the A198V mutation had no influence on the viral infection (Figure 4C), and the F/98 virus released from MDCK cells surface faster than the rF/HA_{A198V} virus (P ¡0.01) (Figure 4D). These results indicated that although A198V mutation enhanced NA activity, the strong receptor binding avidity was not conducive to the virus release from the cell surface in the early stages of H9N2 virus infection.

HA A198V substitution reduce the protective efficiency of inactivated H9N2 avian influenza virus vaccine

To assess the role of HA A198V substitution in immune escape *in vivo* , we performed the immunogenic test. The anti-sera in SPF chickens immunized with the whole inactivated vaccine of the virus F/98 or rF/HA_{A198V} were collected at 21d after immunization for the HI assay. The anti-F/98 serum against the F/98 virus was 15.3-fold higher than that against the rF/HA_{A198V} virus (P ¡0.001). The anti-rF/HA_{A198V} serum against the F/98 virus was 14.4-fold higher than that against the rF/HA_{A198V} virus (P ¡0.001). The HI titer of serum from the chickens vaccinated with rF/HA_{A198V} virus were lower than that from the chickens vaccinated with F/98 virus (Figure 5). These data demonstrated that the specific-antibody in serum induced by the inactivated vaccine of the rF/HA_{A198V}virus was lower than that induced by the inactivated vaccine of the F/98 virus. Moreover, the protection efficiency test showed that the antibody induced by the F/98 vaccine could provide 100% protection against the challenge by the F/98 virus, and 83.3% protection against the challenge by the rF/HA_{A198V} virus; while the antibody induced by the rF/HA_{A198V} vaccine could provide 83.3% protection against the challenge by either the F/98 virus or the rF/HA_{A198V} virus (Table 1). These data reveal that the single A198V mutation contributes to immune escape.

Discussion

HA is the most important antigenic protein of H9N2 virus, which stimulates host chicken to product the HA-specific neutralizing antibodies. HA mutations in antigenic sites promoted the virus to escape from antibody-based immune responses in host. Several studies reported HA mutations that affect the antigenic variation of H9N2 virus. In these studies, HA mutations from different H9N2 antigenic variants are mainly located at or near the receptor binding sites in HA, most of which were selected with HA-specific mAbs *in vitro* (Peacock et al., 2016; Jin et al., 2019; Kaverin et al., 2004; Okamatsu,Sakoda,Kishida,Isoda, & Kida, 2008; Ping et al., 2008; Wan et al., 2014; Zhu et al., 2015). Few studies on the HA mutations selected with pAbs driving from inactivated vaccine *in vivo* and the contribution of single HA mutation in H9N2 virus to antigenic variation or immune escape were reported. We previously identified 15 antigenic variants in HA gene passaged with or without selection pressure of H9N2 inactivated vaccine (Su et al., 2020; Jin et al., 2018). Here, the contribution of the 15 HA mutations to antigenic variation and immune escape were studied with a [?]4-fold change in HI titers of standard antiserum as significant antigenic change, which result in escape from antibodies (Klingen,Reimering,Guzmán, & McHardy, 2018; Wang et al., 2017).

The amino acids located at or around receptor binding sites of HA are the major determinants of the antigenicity of influenza virus, which were confirmed in A/H3N2 virus, avian-origin A/H5N1, swine-origin A/H3N2, and horse-origin A/H3N8(Lewis et al., 2011; Hensley et al., 2009; Lewis et al., 2016). The mutations S145N, Q164L, A168T and M224K are near the receptor binding site. The mutations A198V and Q234L

are at the receptor binding site of HA (Figure 1A). In particular, the positions 145, 164, 168, and 234 on H9 were mapped as the antigenic sites by mAbs, where the mutations S145N, Q164K, A168T, and L234Q occurred (Ping et al., 2008; Kaverin et al., 2004; Wan et al., 2014; Zhu et al., 2015). All the above mutations, including S145N, Q164L, A168T, M224K, A198V, and Q234L, had the potential to alter the antigenicity of H9N2 virus. Of these potential mutations, only A198V HA mutation caused [?]4-fold decreased readouts of HI titers or cross-MN titers. The receptor binding site in HA of H9N2 virus includes the residues at positions 109, 161, 163, 191, 198, 202 and 203, of which all are conservative except the residues at the position 198. About 90% of H9N2 wild viruses in China possess V or T at position 198 in HA (R. Zhu et al., 2018). Additionally, the A198V mutation occurred in all of the passaged viruses occurring antigenic variation in SPF chicken embryos or SPF chickens with or without homologous vaccine antibodies (Su et al., 2020; Jin et al., 2018), suggesting that the HA mutation A198V played a key role in the process of adaptive evolution of F/98 strain.

HI or MN titers were determined by viral antigenically distinct or viral receptor binding avidity. Li et al. (Li et al., 2013c) found single N145K mutation in H3 increased viral receptor binding, and did not prevent anti-145N sera binding. Single K145N mutation in H3 reduced reactivity to anti-145K sera, which consistent with the report of Doud et al. (Doud,Hensley, & Bloom, 2017) that only some specific amino acid residue at antigenic sites can alter the viral antigenicity. In our study, A198V substitution promoted viral escape from pAbs-neutralizing reaction by enhancing the receptor binding activity significantly while A or V at the position 198 in HA did not prevent virus-antibody binding. In virus elution assay, we found that the mutation A198V initially inhibited the virus elution from the red blood cell surface, and subsequently the elution of the virus rF/HA_{A198V} was similar to that of the F/98 strain. As further data shown, the mutation A198V with high receptor avidity in HA also led to an increase in NA activity significantly, whereas the enhanced NA activity did not enough maintain the functional balance between HA and NA. The strong receptor binding avidity prevented the release of the virus rF/HA_{A198V} from cells, but did not affect viral entry into host cells and infectivity, which was unexpected. These results suggested that the strong receptor binding avidity is not conducive to maintain the functional balance between HA receptor binding affinity and NA activity or the viral release from cell surface. Naturally, to restore the viral fitness, the compensatory mutations that increase avidity or modulate NA function are required (Das et al., 2011). That is why HA receptor binding avidity drove AIV antigenic drift (Hensley et al., 2009b), which might be a potential biological significance of the mutation A198V in the evolution of H9N2 virus.

Hensley et al. reported that a positive correlation was shown between receptor binding avidity and escape from antiserum (Hensley et al., 2009b). Sealy et al. characterized H9N2 viruses isolated in Pakistan during 2014-2016 and found association of increased receptor binding avidity with escape from antibody-based immunity (Sealy et al., 2018). So we postulated that A198V mutation should contribute viral immune escape *in vivo* . Expectedly, we found that the antibody induced by the F/98 vaccine could provide 100% protection against the challenge by the F/98 virus, and 83.3% protection against the challenge by the rF/HA_{A198V} virus, and the antibody induced by the rF/HA_{A198V} vaccine could provide 83.3% protection against the challenge by either the F/98 virus or the rF/HA_{A198V} virus. Thus, although single A198V mutation slightly affected cross-protection *in vivo* , it broke down the full protective efficiency of the inactivated vaccine of paternal F/98 virus, suggesting that single A198V mutation with strong receptor binding avidity facilitated the virus rF/HA_{A198V} to escape from the antibodies induced by the vaccine of the paternal virus F/98 or its own. The slight contribution of A198V to viral immune escape might be related to the phenomenon that homologous vaccine antibodies could not provide acceptable protection for vaccinated chicken flocks in recent years (Li et al., 2019b).

In summary, our finding revealed that the mechanism of A198V mutation promoting virus escape from pAbs *in vitro* , and the contribution to escape from the antibody-based immunity. A198V substitution promoted H9N2 virus escape from pAbs-neutralizing reaction by enhancing the receptor binding activity and did not prevent Abs binding physically, which led to the decreased readouts of cross-MN titers significantly whereas slightly affected cross-protection *in vivo* . These data remind us that the HA receptor binding avidity, antibody-virus binding and cross-protection should be taken into account synthetically when we monitor the

new emerging of the antigenic variation.

Funding: This research was funded by the National Natural Science Foundation of China (32172802, 31672516, 31172300, 30670079), supported by the Grant No. BE2016343 from Jiangsu province, the Jiangsu University and College Natural Science Foundation (12KJA230002), the Doctoral Program of Higher Education of China (20133250110002) and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Conflicts of Interest: All the authors have declared that no conflicts of interest exist regarding to the publication of the data in this manuscript.

References:

An, Y., Parsons, L. M., Jankowska, E., Melnyk, D., Joshi, M., & Cipollo, J. F. (2019). N-glycosylation of seasonal influenza vaccine hemagglutinins: implication for potency testing and immune processing.

Chambers, B. S., Parkhouse, K., Ross, T. M., Alby, K., & Hensley, S. E. (2015). Identification of hemagglutinin residues responsible for H3N2 antigenic drift during the 2014-2015 influenza season. *Cell Reports,* 12(1)**,** 1-6.doi:10.1016/j.celrep.2015.06.005

Chambers, T. M., Kawaoka, Y., & Webster, R. G. (1988). Protection of chickens from lethal influenza infection by vaccinia-expressed hemagglutinin. *Virology,* 167(2)**,** 414-421

Cherry, J. L., Lipman, D. J., Nikolskaya, A., & Wolf, Y. I. (2009). Evolutionary dynamics of N-glycosylation sites of influenza virus hemagglutinin. *PLoS Curr,* 1**,**N1001.doi:10.1371/currents.rrn1001

Das, S. R., Hensley, S. E., David, A., Schmidt, L., Gibbs, J. S., Puigbo, P.,... Yewdell, J. W. (2011). Fitness costs limit influenza A virus hemagglutinin glycosylation as an immune evasion strategy. *Proc Natl Acad Sci U S A,* 108(51)**,**E1417-E1422.doi:10.1073/pnas.1108754108

Doud, M. B., Hensley, S. E., & Bloom, J. D. (2017). Complete mapping of viral escape from neutralizing antibodies. *PLoS Pathogens,*13(3)**,** e1006271.doi:10.1371/journal.ppat.1006271

Gu, M., Xu, L., Wang, X., & Liu, X. (2017). Current situation of H9N2 subtype avian influenza in China. *Veterinary Research,*48(1)**,** 49.doi:10.1186/s13567-017-0453-2

Hensley, S. E., Das, S. R., Bailey, A. L., Schmidt, L. M., Hickman, H. D., Jayaraman, A.,... Yewdell, J. W. (2009a). Hemagglutinin receptor binding avidity drives influenza A virus antigenic drift.*Science,* 326(5953)**,** 734-736.doi:10.1126/science.1178258

Hensley, S. E., Das, S. R., Bailey, A. L., Schmidt, L. M., Hickman, H. D., Jayaraman, A.,... Yewdell, J. W. (2009b). Hemagglutinin receptor binding avidity drives influenza A virus antigenic drift.*Science,* 326(5953)**,** 734-736.doi:10.1126/science.1178258

Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G., & Webster, R. G. (2000). A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc Natl Acad Sci U S A,* 97(11)**,**6108-6113.doi:10.1073/pnas.100133697

Jin, F., Dong, X., Wan, Z., Ren, D., Liu, M., Geng, T.,... Ye, J. (2019). A single mutation N166D in hemagglutinin affects antigenicity and pathogenesis of H9N2 avian influenza virus. *Viruses,*11(8).doi:10.3390/v11080709

Jin, H., Wang, W., Yang, X., Su, H., Fan, J., Zhu, R.,... Liu, X. (2018). Evolution of H9N2 avian influenza virus in embryonated chicken eggs with or without homologous vaccine antibodies. *BMC Veterinary Research,* 14(1)**,** 71.doi:10.1186/s12917-018-1391-6

Jin, X., Zha, Y., Hu, J., Li, X., Chen, J., Xie, S.,... Jia, W. (2020). New molecular evolutionary characteristics of H9N2 avian influenza virus in Guangdong Province, China. *Infection Genetics and Evolution,*77**,** 104064.doi:10.1016/j.meegid.2019.104064

Kaverin, N. V., Rudneva, I. A., Ilyushina, N. A., Lipatov, A. S., Krauss, S., & Webster, R. G. (2004). Structural differences among hemagglutinins of influenza A virus subtypes are reflected in their antigenic architecture: analysis of H9 escape mutants. *Journal of Virology,*78(1)**,** 240-249.doi:10.1128/jvi.78.1.240-249.2004

Klingen, T. R., Reimering, S., Guzman, C. A., & McHardy, A. C. (2018). In silico vaccine strain prediction for human influenza viruses.*Trends in microbiology (Regular ed.),* 26(2)**,**119-131.doi:10.1016/j.tim.2017.09.001

Lewis, N. S., Daly, J. M., Russell, C. A., Horton, D. L., Skepner, E., Bryant, N. A.,... Smith, D. J. (2011). Antigenic and genetic evolution of equine influenza A (H3N8) virus from 1968 to 2007. *Journal of Virology,* 85(23)**,** 12742-12749.doi:10.1128/JVI.05319-11

Lewis, N. S., Russell, C. A., Langat, P., Anderson, T. K., Berger, K., Bielejec, F.,... Vincent, A. L. (2016). The global antigenic diversity of swine influenza A viruses. *ELife,* 5**,**e12217.doi:10.7554/eLife.12217

Li, C., Yu, K., Tian, G., Yu, D., Liu, L., Jing, B.,... Chen, H. (2005). Evolution of H9N2 influenza viruses from domestic poultry in Mainland China. *Virology,* 340(1)**,**70-83.doi:10.1016/j.virol.2005.06.025

Li, Y., Bostick, D. L., Sullivan, C. B., Myers, J. L., Griesemer, S. B., Stgeorge, K.,... Hensley, S. E. (2013a). Single hemagglutinin mutations that alter both antigenicity and receptor binding avidity influence influenza virus antigenic clustering. *Journal of Virology,*87(17)**,** 9904-9910.doi:10.1128/JVI.01023-13

Li, Y., Bostick, D. L., Sullivan, C. B., Myers, J. L., Griesemer, S. B., Stgeorge, K.,... Hensley, S. E. (2013b). Single hemagglutinin mutations that alter both antigenicity and receptor binding avidity influence influenza virus antigenic clustering.

Li, Y., Bostick, D. L., Sullivan, C. B., Myers, J. L., Griesemer, S. B., Stgeorge, K.,... Hensley, S. E. (2013c). Single hemagglutinin mutations that alter both antigenicity and receptor binding avidity influence influenza virus antigenic clustering. *Journal of Virology,*87(17)**,** 9904-9910.doi:10.1128/JVI.01023-13

Li, Y., Liu, M., Sun, Q., Zhang, H., Zhang, H., Jiang, S.,... Huang, Y. (2019a). Genotypic evolution and epidemiological characteristics of H9N2 influenza virus in Shandong Province, China. *Poult Sci,*98(9)**,** 3488-3495.doi:10.3382/ps/pez151

Li, Y., Liu, M., Sun, Q., Zhang, H., Zhang, H., Jiang, S.,... Huang, Y. (2019b). Genotypic evolution and epidemiological characteristics of H9N2 influenza virus in Shandong Province, China. *Poult Sci,*98(9)**,** 3488-3495.doi:10.3382/ps/pez151

Lu, J. H., Liu, X. F., Shao, W. X., Liu, Y. L., Wei, D. P., & Liu, H. Q. (2005). Phylogenetic analysis of eight genes of H9N2 subtype influenza virus: a mainland China strain possessing early isolates' genes that have been circulating. *Virus Genes,* 31(2)**,**163-169.doi:10.1007/s11262-005-1790-1

Okamatsu, M., Sakoda, Y., Kishida, N., Isoda, N., & Kida, H. (2008). Antigenic structure of the hemagglutinin of H9N2 influenza viruses. *Archives of Virology,* 153(12)**,** 2189-2195.doi:10.1007/s00705-008-0243-2

Peacock, T. P., Benton, D. J., Sadeyen, J. R., Chang, P., Sealy, J. E., Bryant, J. E.,... Iqbal, M. (2017). Variability in H9N2 haemagglutinin receptor-binding preference and the pH of fusion. *Emerg Microbes Infect,* 6(3)**,** e11.doi:10.1038/emi.2016.139

Peacock, T., Reddy, K., James, J., Adamiak, B., Barclay, W., Shelton, H.,... Iqbal, M. (2016). Antigenic mapping of an H9N2 avian influenza virus reveals two discrete antigenic sites and a novel mechanism of immune escape. *Sci Rep,* 6**,** 18745.doi:10.1038/srep18745

Peng, Q., Zhu, R., Wang, X., Shi, H., Bellefleur, M., Wang, S.,... Liu, X. (2019). Impact of the variations in potential glycosylation sites of the hemagglutinin of H9N2 influenza virus. *Virus Genes,*55(2)**,** 182-190.doi:10.1007/s11262-018-1623-7

Ping, J., Li, C., Deng, G., Jiang, Y., Tian, G., Zhang, S.,... Chen, H. (2008). Single-amino-acid mutation in the HA alters the recognition of H9N2 influenza virus by a monoclonal antibody. *Biochem Biophys Res*

Commun, 371(1), 168-171.doi:10.1016/j.bbrc.2008.04.045

Qin, T., Zhu, J., Ma, R., Yin, Y., Chen, S., Peng, D.,... Liu, X. (2018). Compatibility between haemagglutinin and neuraminidase drives the recent emergence of novel clade 2.3.4.4 H5Nx avian influenza viruses in China. *Transboundary and Emerging Diseases,* 65(6),1757-1769.doi:10.1111/tbed.12949

Reed, L. J., & Muench, H. (1937). A Simple Method of Estimating Fifty Per Cent Endpoints. *American Journal of Epidemiology*

Sealy, J. E., Yaqub, T., Peacock, T. P., Chang, P., Ermetal, B., Clements, A.,... Iqbal, M. (2018). Association of increased receptor-binding avidity of influenza A(H9N2) viruses with escape from antibody-based immunity and enhanced zoonotic potential. *Emerging Infectious Diseases,* 25(1), 63-72.doi:10.3201/eid2501.180616

Segovia, K. M., Franca, M. S., Bahnson, C. S., Latorre-Margalef, N., & Stallknecht, D. E. (2019). Are microneutralization and hemagglutination inhibition assays comparable? Serological results from influenza experimentally infected mallard ducks.

Shi, H., Ashraf, S., Gao, S., Lu, J., & Liu, X. (2010). Evaluation of transmission route and replication efficiency of H9N2 avian influenza virus. *Avian Diseases,* 54(1),22-27.doi:10.1637/8937-052809-Reg.1

Spackman, E., Senne, D. A., Myers, T. J., Bulaga, L. L., Garber, L. P., Perdue, M. L.,... Suarez, D. L. (2002). Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *Journal of Clinical Microbiology,*40(9), 3256-3260.doi:10.1128/JCM.40.9.3256-3260.2002

Su, H., Zhao, Y., Zheng, L., Wang, S., Shi, H., & Liu, X. (2020). Effect of the selection pressure of vaccine antibodies on evolution of H9N2 avian influenza virus in chickens. *AMB Express,*10(1), 98.doi:10.1186/s13568-020-01036-0

Sun, Y., & Liu, J. (2015). H9N2 influenza virus in China: a cause of concern. *Protein & Cell,* 6(1),18-25.doi:10.1007/s13238-014-0111-7

Teng, Q., Xu, D., Shen, W., Liu, Q., Rong, G., Li, X.,... Li, Z. (2016). A single mutation at position 190 in hemagglutinin enhances binding affinity for human type sialic acid receptor and replication of H9N2 avian influenza virus in mice. *Journal of Virology,*90(21), 9806-9825.doi:10.1128/JVI.01141-16

Wan, H., & Perez, D. R. (2007). Amino acid 226 in the hemagglutinin of H9N2 influenza viruses determines cell tropism and replication in human airway epithelial cells. *Journal of Virology,* 81(10),5181-5191.doi:10.1128/JVI.02827-06

Wan, Z., Ye, J., Xu, L., Shao, H., Jin, W., Qian, K.,... Qin, A. (2014). Antigenic mapping of the hemagglutinin of an H9N2 avian influenza virus reveals novel critical amino acid positions in antigenic sites.*Journal of Virology,* 88(7),3898-3901.doi:10.1128/JVI.03440-13

Wang, X., Ilyushina, N. A., Lugovtsev, V. Y., Bovin, N. V., Couzens, L. K., Gao, J.,... Wan, H. (2017). Amino acids in hemagglutinin antigenic site B determine antigenic and receptor binding differences between A(H3N2)v and ancestral seasonal H3N2 influenza viruses. *Journal of Virology,* 91(2).doi:10.1128/JVI.01512-16

Yang, W., Punyadarsaniya, D., Lambertz, R. L. O., Lee, D. C. C., Liang, C. H., Hoper, D.,... Herrler, G. (2017). Mutations during the adaptation of H9N2 avian influenza virus to the respiratory epithelium of pigs enhance sialic acid binding activity and virulence in mice.*Journal of Virology,* 91(8).doi:10.1128/JVI.02125-16

Zhu, H., Lee, A., Li, C., Mak, W., Chen, Y. Y., Chan, K. H.,... To, K. (2018). Low population serum microneutralization antibody titer against the predominating influenza A(H3N2) N121K virus during the

severe influenza summer peak of Hong Kong in 2017. *Emerg Microbes Infect,* 7(1)**,** 23.doi:10.1038/s41426-018-0041-1

Zhu, R., Xu, D., Yang, X., Zhang, J., Wang, S., Shi, H.,... Liu, X. (2018). Genetic and biological characterization of H9N2 avian influenza viruses isolated in China from 2011 to 2014. *PLoS One,* 13(7)**,**e199260.doi:10.1371/journal.pone.0199260

Zhu, Y., Yang, D., Ren, Q., Yang, Y., Liu, X., Xu, X.,... Liu, X. (2015). Identification and characterization of a novel antigenic epitope in the hemagglutinin of the escape mutants of H9N2 avian influenza viruses. *Veterinary Microbiology,* 178(1-2)**,**144-149.doi:10.1016/j.vetmic.2015.04.012

Table 1 Virus shedding from the swabs on days 3 and 5 post challenged with F/98, or rF/HA_{A198V} viruses

Group	Virus shedding# D3	Virus shedding# D5	Protection (%)
Chickens vaccinated the F/98 inactive vaccine			
Challenged with F/98 virus	$0/6^a$	$0/6^a$	100
Challenged with rF/HA_{A198V} virus	$1/6^a$	$0/6^a$	83.3
Chickens vaccinated the rF/HA_{A198V} inactive vaccine			
Challenged with F/98 virus	$1/6^a$	$0/6^a$	83.3
Challenged with rF/HA_{A198V} virus	$1/6^a$	$0/6^a$	83.3

Data are numbers of chickens shedding virus/total number of chickens at days 3 and 5.

Positive sample of chickens shedding virus indicated higher than the detection limit of 2^2 HA titer. Protection was calculated with data on day 3. The appearance of the same letter means that there is no marked difference among the groups under the condition of $P > 0.05$.

Figure legends:

Figure 1. (A) The location of amino acid mutations in the three-dimensional structure of HA protein of H9N2 subtype avian influenza virus. Yellow color indicates the locations of the HA receptor binding sites including the positions 146-150, 109, 161, 163, 191, 198, 202, 203, and 232-237. Pink color (the positions 133, 145, 164, 168, 198 and 234) indicates mutations on antigenic sites that have been reported. Red color (the positions 131, 181, 264, 270, 274, 278, 386 and 399) indicates mutations that have not been previously reported as antigenic sites. (B) HI titers of F/98 immune sera from chickens (n=8) to each recombinant virus with single mutants in HA from the passaged viruses occurring antigenic drift in embryonated chicken eggs or chickens. (C) HI titers of F/98 immune sera from chickens (n=8) to each recombinant virus with multiple mutants in HA from the passaged viruses occurred in the 47th generation in embryonated chicken eggs under selective pressure on antibodies. A [?]4-fold change in HI titers of standard antiserum was considered as significant antigenic change.

Figure 2. (A) The location of amino acid mutations in the three-dimensional structure of HA protein of H9N2 subtype avian influenza virus. Blue color indicates the locations of the position 198 in the HA receptor binding site. Yellow color indicates the locations of the HA receptor binding sites including the positions 109, 161, 163, 191, 198 (blue color), 202, 203; orange color indicates the locations of the right edge of the receptor binding pocket 232-237 and the left edge of the receptor binding pocket 146-150. (B) MN titers of F/98 immune sera from chickens (n=8) to the viruses F/98 or rF/HA_{A198V}. (C) MN titers of rF/HA_{A198V}immune sera from chickens (n=8) to the viruses F/98 or rF/HA_{A198V}. (D) MN titers of GD/SS/94 immune sera from chickens (n=6) to the viruses F/98, rF/HA_{A198V} or GD/SS/94. (E) MN titers of JS/YZ618/12 immune sera from chickens (n=6) to the viruses F/98, rF/HA_{A198V} or JS/YZ618/12. (F) MN titers of rF/HA_{A198V} immune sera from chickens (n=6) to the viruses F/98, rF/HA_{A198V}, GD/SS/94, or JS/YZ618/12. A [?]4-fold change in MN titers of standard antiserum was considered as significant antigenic change. These data indicated the fold change was relative to the virus rF/HA_{A198V}.

Figure 3. (A) HA mutations A198V increase receptor binding avidity of H9N2 virus F/98, whereas S145N decreases receptor binding avidity. Relative viral receptor binding avidities were determined by hemagglutination of red blood cells pretreated with increasing amounts of α2-3,6,8 neuraminidase. Data are expressed as the maximal amount of neuraminidase that allowed full agglutination. The data are representative of three independent experiments. (B, C) Single A198V mutation does not affect Ab binding. Direct antibody binding to F/98 or rF/HA_{A198V} viruses were determined by ELISA using sera collected from chickens vaccinated with inactivated F/98 (B) or rF/HA_{A198V} (C). The AUC of ELISA was calculated for virus-Abs binding by GraphPad Prism 8 software above the value of the corresponding negative control, which was performed under the same conditions. Means and SD from three independent experiments. Statistical significance was based on student's t test (**$P < 0.01$; *** $P < 0.001$). O.D., optical density.

Figure 4. (A) Neuraminidase activities of F/98 and rF/HA_{A198V} viruses, which were determined in the fluorescence intensity of NA fluorogenic substrate. (B) Virus elution from chicken erythrocytes. Two-fold dilutions of F/98 and rF/HA_{A198V} viruses were incubated with equal volumes of chicken erythrocytes at 4 for 30 min, and the HA titers at 37 representing virus elution from chicken erythrocytes was monitored for 6 hours. The result was presented as the percentage of the initial HA titer at 4. (C, D) Virus release assay. A198V HA mutation does not affect viral infection (C), and prevents virus released from MDCK cells surface significantly (D). The results were displayed as mean±$SD$of three independent experiments. The comparisons were performed with t test. **Indicates very significant difference between groups (P <0.01).

Figure 5. The cross-HI reactions between F/98 and rF/HA_{A198V} viruses. Three-week-old SPF chickens were vaccinated once by subcutaneous injection of 0.3 mL of oil-emulsion of inactivated whole virus vaccines of the F/98 and rF/HA_{A198V} viruses, which were inactivated by adding 0.2% formalin (v /v) for 24 h at 37 °C, respectively. At 21 d.p.v., 12 chickens from each group (the F/98 vaccine group and the rF/HA_{A198V} vaccine group) were bled to analyze cross-HI titers against the F/98 and rF/HA_{A198V} viruses. Statistical significance was based on student's t test (*P< 0.05).