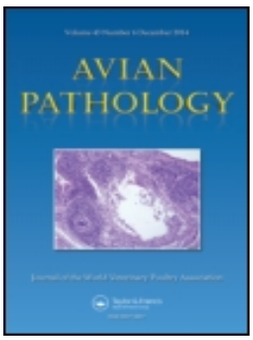

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Identification of antigenic epitopes in the hemagglutinin protein of H7 avian influenza virus

Lu Yao[a,c,d#], Yuqing Chen[a,c,d#], Xingbo Wang[b,e], Zhenwei Bi[a,c,d], Qian Xiao[a,c,d], Jing Lei[a,c,d], Yan Yan[b,e], Jiyong Zhou[b,e], Liping Yan[a,c,d*]

Address

[a] MOE Joint International Research Laboratory of Animal Health and Food Safety, Institute of Immunology, Nanjing Agricultural University, Nanjing 210095, PR China

[b] Key Laboratory of Animal Virology, Ministry of Agriculture, Zhejiang University, Hangzhou 310058, PR China

[c] Jiangsu Engineering Laboratory of Animal Immunology, Institute of Immunology, Nanjing Agricultural University, Nanjing 210095, PR China

[d] Jiangsu Detection Center of Terrestrial Wildlife Disease, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, PR China

[e] Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, Zhejiang University, Hangzhou 310058, PR China

[#]Y.L. and C.Y.Q. contributed equally to this work.

[*]Corresponding author

E-mail address:

Lu Yao: 382227992@qq.com

Yuqing Chen: 2017107074@njau.edu.cn

Xingbo Wang: 1196992101@qq.com

Zhenwei Bi: 582936410@qq.com

Qian Xiao: 892920925@qq.com

Jing Lei: leijing@njau.edu.cn

Yan Yan: smy201711@yeah.net

Jiyong Zhou:52768356@qq.com

Liping Yan: yanliping@njau.edu.cn

ABSTRACT

The H7 subtype avian influenza virus (AIV) has been reported to infect not only poultry but also humans. The hemagglutinin (HA) protein is the major surface antigen of AIV and plays an important role in viral infection. In this study, five monoclonal antibodies (mAbs) against the HA protein of H7 virus, 2F8, 3F6, 5C11, 5E2 and 5C12, were produced and characterized. Epitope mapping indicated that ^{103}RESGSS^{107} was the minimal linear epitope recognized by the mAbs 2F8/3F6/5C11, and the other mAbs, 5E2/5C12, recognized the epitope 103-145aa. The protein sequence alignment of HA indicated that the two epitopes were not found in other subtypes of AIV, and all five mAbs did not cross-react with other subtypes, suggesting these mAbs are specific to H7 virus. The epitope ^{103}RESGSS^{107} was highly conserved among Eurasian lineage strains of H7 AIV, whereas three amino acid substitutions (E104R, E104K and E104G) in the epitope occurred in 98.44% of North-American lineage strains. Any of these single mutations led the mutated epitope not to be recognized by mAbs 2F8/3F6/5C11;

thus, these mAbs can distinguish between Eurasian and North-American lineages of H7 strains. Furthermore, the mAbs 2F8, 3F6 and 5C11 could be highly blocked with H7-positive serum in blocking assays, revealing that ^{103}RESGSS^{107} may be a dominant epitope stimulating the production of antibodies during viral infection. These results may facilitate future investigations into the structure and function of HA protein, as well as surveillance and detection of H7 virus.

- **RESEARCH HIGHLIGHTS**
- Five mAbs against HA protein of H7 AIV were generated and characterized
- Two novel epitopes ^{103}RESGSS^{107} and 103-145aa were identified
- The epitope ^{103}RESGSS^{107} differs between Eurasian and North-American lineage
- The mAbs 2F8, 3F6 and 5C11 could distinguish two lineages of H7 strains

Keywords: Avian influenza virus; monoclonal antibody; H7; epitope; hemagglutinin; identification

Introduction

Avian influenza (AI), a highly contagious acute infectious disease, causes epidemics among various poultry species, as well as many host species, such as dogs, pigs, horses, marine mammals and humans (Smith *et al*., 2009). The disease has caused a large number of deaths worldwide and has resulted in huge losses to the global economy (WHO, 2003; Yoon *et al*., 2014). Initially, the H7 subtype virus was mainly found in birds and occasionally caused human infections with mild illness. Simultaneously, the H7 low pathogenicity avian influenza virus (LPAIV) circulating in poultry constantly increased the chances of new recombination or mutations that may

alter viral characteristics, resulting in severe disease and high mortality (Hu *et al*., 2014). In fact, H7N9 virus has triggered five waves since 2013, and humans that were infected with H7N9 showed serious symptoms ranging from conjunctivitis and upper respiratory tract disease to pneumonia and multiorgan failure.

Avian influenza virus (AIV) belongs to the genus *Orthomyxovirus* and contains eight single-stranded negative-sense RNA segments (PB2, PB1, PA, HA, NA, M, and NS). The hemagglutinin (HA) protein presents as a homotrimer in which each monomer consists of two di-sulfide-linked HA1 and HA2 subunits after the cleavage of the HA0 precursor. The HA protein, which is responsible for receptor binding and membrane fusion, plays a key role in the influenza virus entry pathway (Luo, 2012). Some studies have confirmed that the removal of the N-linked glycosylation sequence of HA contributes to the increase in HA affinities to an α-2,6-linked sialyl receptor and a reduction in virus growth and spread (Wagner R, 2000; Wang *et al*., 2007). Differences in viral shedding and the transmission of various AIV strains are dependent upon specific HA and/or NA proteins (Ortigoza *et al*., 2018). In the host immune defense response, the HA protein of HPAI H5N1 viruses can directly activate γδ T cells, which play an important role in the defense against HPAI H5N1 infection (Dong *et al*., 2018).

Four distinct antigenic sites (site Sa, Sb, Ca and Cb) and five antigenic sites (site A, B, C, D and E) have been described as conserved epitopes on HA protein using influenza H1 and H3, respectively (Brownlee *et al*., 2001; Goff *et al*., 2013; Wiley DC, 1981). Each antigenic site contains many different epitopes, and the neutralizing mAb response focuses on the immunodominant regions of HA1 (Temoltzin-Palacios and

Thomas, 1994). Crystal structures was widely used to identify the antigenic sites. For example, the detailed structural and biochemical analysis of the surface antigens of H5N6 and H5N8 viruses showed that these viruses have a strict avian receptor binding preference (Yang *et al*., 2016). Structures of three neutralizing and protective antibodies in complex with the H7 hemagglutinin revealed that they recognize overlapping residues surrounding the receptor-binding site of hemagglutinin (Huang *et al*., 2019). The neutralizing mAbs were also used to map the antigenic sites in the HA protein of AIV from the characterization of escape mutants. In H7 strains, the R131G mutation located in site A (RRSGSS), the G189E mutation near antigenic site B and many other mutations (G119E, K157E, R247H and S150L/G151E) have led the mutant viruses to escape neutralization of the mAbs, which is indicative of antigenic regions related to immune protection (Schmeisser *et al*., 2015; Tan *et al*., 2016; Vasudevan *et al*., 2018). In another study, mAbs were divided into different groups utilizing epitope maping: neutralizing mAbs mainly recognized the epitopes located at major antigenic sites A and D, while non-neutralizing mAbs that cross-reacted with several HA subtypes possibly recognized the HA stem (Ito *et al*., 2019). However, until now, the precise epitopes of mAbs against the HA protein of H7 AIV are scant. The purpose of this study was to generate mAbs against the HA protein of H7 AIV and to subsequently define the precise epitopes recognized by the mAbs.

Materials and methods

Virus strains, cells, serum and animals

Sp2/0 myeloma cells were grown in RPMI-1640 medium supplemented with 10%

fetal calf serum (Gibco-BRL, USA). MDCK cells (Madin-Darby Canine Kidney cells) and 293T cells were cultured with DMEM medium supplemented with 10% fetal calf serum (Biological Industries). All cell lines were cultured at 37°C in 5% CO_2. The different subtypes of influenza virus (H1N1, H2N2, H3N8, H4N6, H5N1, H6N5, H7N1, H7N2, H7N3, H7N9, H8N4, H9N2, H10N7, H11N9, H12N5 and H13N6) used in this study were obtained from Key Animal Virology Laboratories of the Ministry of Agriculture of China (Table S1). All viruses were propagated in 9-day-old embryonated chicken eggs and were stored at -80°C. Positive chicken serum of H7 AIV were prepared from SPF chickens in isolators. The six-week-old female BALB/C mice were purchased from the Sino-British SIPPR/BK Lab Animal Ltd. (Shanghai).

Expression of recombinant proteins

The HA1 gene of H7N3 was amplified by RT-PCR using the specific primers F1 (Table 1). Briefly, the viral RNAs of H7N3 was extracted by using the RNeasy mini kit (Qiagen Inc, CA) and first-strand cDNA was synthesized using AMV reverse transcriptase (TaKaRa Biotechnology, China) according to the manufacturer's instructions. Then the fragment was amplified with the specific program as follows: 5 min at 95°C for predenaturation; 35 cycles of 30 s at 95°C for denaturation, 30 s at 54°C for annealing, 1 min at 72°C for elongation; and finally, 10 min at 72°C for overall elongation. The obtained HA1 fragment was digested with *Xho*I and *Xba*I and subcloned into the prokaryotic expression vector pCold I (Takara, Japan). The recombinant vector was verified by restriction analysis and nucleotide sequencing and was then transformed into *E. coli* BL21 cells for expression of the HA1 gene. The

expression of His-H7-HA1 protein was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside, and the protein was purified using a Ni-NTA agarose (Thermo, USA) according to the manufacturer's instructions. The purified fusion protein was identified with SDS-PAGE and western blot. A series of truncated fragments of the HA1 gene were synthesized using specific primers (Table 1) and were subcloned into the expression vector pGEX-4T- I as a fusion protein with a GST tag. The concrete protocols were the same as described above. All variants of the epitope among H7 strains were expressed as the GST-fusion protein to implement cross-reaction examination (Table S2). In addition, the HA genes of H7N3 and North-American strain A/goose/Nebraska/17097-4/2011(H7N9) were cloned into the eukaryotic expression vector pCAGGS, then the recombinant vector was transfected into 293T cells with Cellfectin® Reagent (Thermo, USA) for expression of HA protein.

Preparation of anti-HA protein monoclonal antibodies

The mAbs were prepared as described in a previous study (Xie *et al*., 2018). The recombinant H7-HA1 protein was used as the immunogen for the development of mAbs in this study. Briefly, six-week-old female BALB/c mice were injected subcutaneously with the mixture of 50 μg of the purified recombinant HA protein and an equal volume of complete Freund's adjuvant (Sigma-Aldrich, USA), and the mice were again injected intraperitoneally with the mixture of the purified recombinant HA protein and an equal volume of incomplete Freund's adjuvant at 14 dpi. and 28 dpi. Three days after the last immunization with 100 μg of the protein, the spleen cells of the best responder animals were harvested and fused with SP2/0 myeloma cells using polyethylene glycol 2000

(Sigma-Aldrich, USA) according to protocols. The fused cells were selected in hypoxanthine, aminopterin and thymidine (HAT) medium. The positive clones were screened by ELISA using the recombinant HA protein and were subcloned 4 times by limiting dilution, then the hybridomas secreting mAbs against H7-HA were acquired. The mouse ascites were obtained by injecting the positive hybridomas into the paraffine primed BALB/c mice. The isotypes of the mAbs were identified by a mouse monoclonal antibody isotyping kit (Beijing Biodragon Immunotechnologies Co., Ltd, China) according to the manufacturer's protocol.

Enzyme linked immunosorbent assay

To screen the mAbs against HA protein, ELISA plates were coated with 2 μg/ml of purified H7-HA1 protein in carbonate-bicarbonate buffer at 4°C overnight. After washing with 200 μl PBS containing 0.1% Tween-20 (PBST) three times, the plates were blocked with 200 μl blocking buffer (PBS containing 5% skimmed milk) at 37°C for 1 h. Then, the wells were washed again and incubated with culture supernatants for 1 h at 37°C; the supernatant of SP2/0 myeloma cells was used as a negative control. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (HRP-YKS) (Sigma-Aldrich, USA) was added to detect the specific antibodies. After reaction at 37°C for 1 h, the plates were incubated with the tetramethylbenzidine (TMB) substrate for approximately 10-15 min, and the enzyme reaction was terminated with 2 M H_2SO_4. The OD value was measured at 450 nm in a multimode reader (Tecan, Switzerland). All the samples whose OD_{450} value was 2.1 times higher than the negative control were considered to be positive (Bi *et al*., 2017).

Immunofluorescence assay

MDCK cells were seeded at a concentration of 2×10^5 cells/mL in 96-well plates and incubated at 37°C for 10h. Four different H7 stains (H7N1, H7N2, H7N3 and H7N9) and twelve subtype stains of AIV (H1-H13, except for H7) were diluted in 1:1000 with PBS. The cells infected with virus suspension supplemented with 2% fetal calf serum for 48-72 h. Then the cells were fixed using fixative solution (the same dose of methanol and acetone) for 20 min at -20°C. After washing three times, the mAbs were added and incubated for 1 h at 37°C. Then, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (FITC-YKS) (Sigma-Aldrich, USA) diluted in 1:300 for 1 h at 37°C. After washing, the cells were observed under an OLYMPUS microscope connected to a Leica DFC 490 digital color camera.

Western blot

The recombinant protein samples were mixed with an equal volume of sample loading buffer, and the mixture was boiled for 10 min and centrifuged at 12000×rpm for 5 min. Then, the proteins were separated by 12% SDS-PAGE and transferred onto the nitrocellulose (NC) membranes for western blot. After washing with PBST three times, the membranes were blocked with the 5% skimmed milk in PBS for 30 min at 37°C. Then the membranes were incubated with hybridoma supernatants or ascites overnight at 4°C. The mAbs were removed, and the membranes were incubated with HRP-YKS for 30 min at 37°C and the substrate 3,3'-diaminobenzidine (Vazyme, China). Detection of the immunoreactive bands was performed using an Amersham Imager 600 (GE, USA).

Neutralization assay

The 96-well plates containing monolayer MDCK cells were infected with tenfold serial dilutions of the H7N3 virus as descript above. The cytopathic effect (CPE) was observed after 48 h and, the number of cytopathic wells was recorded. The 50% tissue culture infectious dose ($TCID_{50}$) titers of H7N3 virus were calculated by the Reed–Muench method, as described previously (Lee *et al*., 2018). The mixture of twofold serial dilutions of the mAbs and virus suspension containing 100 $TCID_{50}$ of H7N3 was reacted for 1 h at 37°C, then the mixture was added into MDCK cells. After adsorbing for 2 h, virus inoculum was removed, and the cells were cultured with DMEM medium containing 5% fetal calf serum. Neutralization titers are presented as reciprocals of the highest mAb dilutions causing a reduction of the virus over 50%.

Hemagglutinin inhibition assay

The HA titer of the H7N3 was measured as described previously (Stadlbauer *et al*., 2018). Briefly, two-fold serial dilutions of virus were incubated with 1% chicken red blood cells. The HA titer was expressed as the reciprocal of the highest ascetic dilution that agglutinated the red blood cells. The hemagglutinin inhibition (HI) assay was performed using four HA units of H7N3 virus. Twenty-five microliters of two-fold serial dilutions of ascites reacted with four HA units of virus in 96-well plates at 37°C for 30 min. The 1% chicken red blood cells were added and incubated for another 30 min. The HI titer was reported as the reciprocal of the maximum ascetic dilution that completely inhibited hemagglutinin of four HA units of the virus.

Blocking assay

First, the protein chip containing H7-HA protein was developed as previously described (Yan *et al*., 2018). The H7 subtype specific chicken serum diluted with wash buffer (TBST) was added to the microarray chamber and incubated for 1 h at 37°C; washing buffer without serum was included as a negative control. After washing three times with TBST, the chip was incubated with mAbs at 37°C for 1 h. Then, the blocking activity of mAbs was detected by HRP-YKS. Finally, the chip was incubated with the chemiluminescent substrate, and the absorbance was measured at 645 nm with Amersham Imager 600. Chemiluminescent signals were acquired using GenePix Pro 6.0 software, and the inhibition rate was calculated using the formula: Inhibition rate = (1 - SI of sample / SI of negative) × 100%, (SI of sample = signal intensity of sample - signal intensity of background; SI of negative = signal intensity of negative control - signal intensity of background).

Homology analysis and alignment of epitope sequences

To identify the similarities of epitopes with other subtypes of influenza A virus, HA protein sequences of all influenza A viruses were downloaded from the Influenza Research Database (IRD, http://www.fludb.org, 02.09.2018) for the alignment of epitope sequences. The amino acid sequences of different lineages of H7 strains were chosen to evaluate the conservation of the epitopes among H7 strains. The full names of H7 strains used in the study were displayed in Table S3. The nucleotide sequences were edited using the Seqman module of the DNAStar package, and the amino acids sequences were aligned using Clustal W.

Molecular modeling for H7-HA

To locate the epitopes in the structure of the HA protein, the HA structure of H7N3, applied as the template, was obtained from the Protein Databank (PDB accession number, 4LN3), and the 3-D structure was mapped using Pymol (http://www.pymol.org/). The positions of sites A to E, the epitopes recognized by the mAbs and the amino acid substitutions were marked in different patterns.

Ethics statement

The mice were handled humanely according to the rules described by the Animal Ethics Procedures and Guidelines of the People's Republic of China and the Institutional Animal Care and Use Committee of Nanjing Agricultural University [SYXK (Su) 2017-0007].

Results

Expression and identification of recombinant H7-HA1 protein

The HA1 gene was amplified by RT-PCR, with the size being 984 bp (Figure S1(a)). Restriction analysis and nucleotide sequencing indicated the HA1 gene was correctly inserted into the vector pCold Ⅰ. SDS-PAGE showed that the His-HA1 fusion protein was primarily expressed in precipitation at 37°C and had a molecular weight of 38 KDa and purified His-HA protein could be detected by H7-positive serum in western blot (Figure S1(b)).

Generation and characterization of mAbs against the HA protein

Five hybridoma cells (2F8, 3F6, 5C11, 5E2, and 5C12) stably secreting mAbs against H7-HA1 protein were screened in indirect ELISA, and mouse ascites were obtained by injecting the hybridomas into paraffin-treated BALB/c mice. The hybridoma supernatants and ascites of mice were gathered for further characterization.

The ELISA titers of the five mAbs ranged from $1:2^{11}$ to $1:2^{14}$ in hybridoma supernatants and from $1:10^5$ to $1:10^9$ in ascites (Table 2). The isotype identification revealed that all five mAbs had a kappa light chain, while the heavy chain of 2F8/3F6/5C11, 5E2 and 5C12 was subclass IgG2a, IgG2b and IgG1, respectively (Table 2). Western blot and IFA results showed that the five mAbs could react with the recombinant H7-HA1 protein and native HA protein in H7N3-infected MDCK cells (Figure 1). The MDCK cells were respectively infected with three H7 strains (H7N1, H7N2 and H7N9) and twelve subtypes of AIV (H1-H13 subtype, except for H7), and were then used to detect the reactivity of the five mAbs with different viruses. The result showed that the five mAbs could react with three different H7 viruses (H7N1, H7N2 and H7N9) but not with other subtypes of AIV (Figure 2). The results of HI assay showed that the HI titers of mAbs 2F8, 3F6, and 5C11 were 1:320, 1:160, and 1:160, respectively (Table 2), while mAbs 5E2 and 5C12 did not display HI activities. All of the mAbs had no neutralization activities in the NT assay.

The blocking activity of mAbs against H7

The purified HA protein of H7 virus was spotted on the chip as the antigen; after incubating with the H7 positive chicken serum, the mAbs were added, and the blocking activity was detected using HRP-YKS. When the H7-positive serum was added, the binding of the mAbs 2F8, 3F6, 5C11 and 5C12 to the antigen was inhibited, which resulted in a decrease in chemiluminescent signals (Figure 3), indicating that these mAbs could be blocked by the H7-positive chicken serum. The inhibition rates of mAbs calculated using the formula were 87.80%, 97.96%, 92.44%, 3.66% and 48.67% for

2F8, 3F6, 5C11, 5E2 and 5C12, respectively (Table 3).

Mapping of the epitopes of HA protein

To map the epitopes recognized by the mAbs, a series of truncated fragments were produced using the primers (Table 1). Firstly, the mAbs 2F8, 3F6 and 5C11 recognized the fragment F3 (75-172aa) separated from HA1. Following further truncation, the mAbs 2F8, 3F6 and 5C11 were able to recognize three fragments F6 (75-118aa), F7 (103-145aa) and F8 (130-172aa) (Figure S2(a)). Moreover, these mAbs could react with the fragment F9 (103-118aa), which was the overlap of F6 and F7 and further bind to the fragment F10 (103-114aa), which was derived from F9 (Figure S2(a)). The gradual reduction of single amino acids from both terminals of F10 (103-114aa) was performed to locate the precise epitope recognized by mAbs 2F8, 3F6 and 5C11. The fragments containing five amino acids ^{103}RESGG^{107} were recognized by all three mAbs 2F8, 3F6 and 5C11, while the smaller fragments with the deletion of any one of the five amino acids eliminated their reactivity (Figure 4 and Figure 5(a)), suggesting that ^{103}RESGG^{107} was the minimal epitope recognized by the mAbs 2F8, 3F6 and 5C11. To identify how these mAbs could recognize another fragment, F8 (130-172aa), the sequences were aligned, and the segment ^{130}RRSGS^{134}, with the highest similarity to the epitope ^{103}RESGG^{107}, was constructed into the vector pGEX-4T-1, but none of the mAbs reacted with the expressed fragment of interest (Figure S3(a)). This suggested that the fragment F8 may have antigenicity similar to that of the epitope ^{103}RESGG^{107}.

In the same way, the mAbs 5E2 and 5C12 showed strong reactivity with the fragment F7 (103-145aa) (Figure S2(b)), and none of the four fragments with the

gradual deletion of amino acids could be bound by the mAbs (Figure 4 and Figure 5(b)). These results showed that the mAbs 5E2 and 5C12 recognized the epitope (103-145aa).

Homology and alignment of epitope sequences

The alignment of the amino acid sequences of the HA protein revealed that two amino acid substitution sites (104E and 107G) appeared in the epitope ^{103}RESGG^{107}, and the epitope 103-145aa was variable among different H7 strains (Figure 6(a)). A total of 73908 strains of influenza virus from 1902 to 2018 were downloaded and analyzed for similarities with epitopes, and the alignment analysis indicated that the epitope sequences were not found within the H1-H18 subtypes of influenza A virus except for H7 strains (Table 4).

Among the 895 strains of H7N9 virus, the epitope ^{103}RESGG^{107} appeared in 777 strains (86.82%). Except for H7N9, 1826 of the other H7 strains with different NA subtypes were also analyzed, while 767 strains (42.00%) contained the epitope ^{103}RESGG^{107} (Table 4). Phylogenetic analysis of the HA proteins of different influenza viruses revealed they were grouped into two lineages, namely, the Eurasian and North-American lineages. Of the 1631 analyzed strains of Eurasian lineage, 1527 strains (93.62%) contained the sequence ^{103}RESGG^{107}, and five substitutions (E104G, E104K, E104N, E104A and G107T) occurred in these strains (Figure 6(b)). In contrast, only 17 among the 1091 strains (1.56%) of North-American lineage had the same sequence, and the substitution always occurred in site 104. The substitution E104G was the most common, present in 65.60% in North-American lineage strains; the second-most-common was E104R, which accounted for 31.11%; and the least common was E104K

(Figure 6(b)). The distribution of the epitope and different variants in Eurasian and North-American lineages was displayed (Table S4). All variants were constructed and expressed to detect the reactivity with the mAbs 2F8, 3F6 and 5C11, and the result showed that none of variants could be recognized by the mAbs 2F8, 3F6 and 5C11 (Figure S3(a)). As shown in Figure S3(b), the mAbs 2F8, 3F6 and 5C11 could efficiently recognize the HA protein with RESGG sequence expressed in 293T cells but not with RGSGG or RRSGG sequence.

Molecular modeling for H7-HA

The Pymol software was used to model the location of epitopes of 2F8/3F6/5C11 and 5E2/5C12 on the HA protein. The epitope of ^{103}RESGG^{107} was positioned in the depression under site E, and it was close to the 130-loop and the bottom of the HA1. The amino acid residues 104E and 107G were exposed at the surface of HA, and these two amino acids might be significant for the antibody binding capability of the epitope. The epitope 103-145aa was located at the surface of head part of the HA protein, with the six amino acid residues ^{123}RRSGSS^{128} covering site A and with the four amino acid residues ^{142}WLLS^{145} overlapping site B (Figure 7).

Discussion

Since the novel H7N9 influenza virus emerged in China in spring of 2013 (Gao *et al*., 2013), the virus has triggered five outbreak waves that have caused a large number of deaths (Quan *et al*., 2018). The HA protein, the major viral surface glycoprotein, was responsible for receptor binding and membrane fusion and played a significant role in immunity and pathogenicity (He *et al*., 2014; Kaur *et al*., 2011). Therefore, elucidating

the B-cell epitopes in the HA protein will help to clarify the molecular mechanisms underlying viral immunity. In this study, five mAbs (2F8, 3F6, 5C11, 5E2 and 5C12) against the HA protein of the H7 subtype were generated and characterized. The five mAbs could react with the recombinant HA protein and native HA protein of H7 AIVs, such as H7N1, H7N2, H7N3 and H7N9, but did not cross-react with other subtypes of AIV (H1-H13 subtypes), which meant that these mAbs are specific to H7 influenza virus. The protein chip method was established to detect the antibodies against infectious bronchitis virus in our laboratory previously (Yan *et al*., 2018). In this study, the protein chip assay was used to detect the blocking activity of mAbs, and the result showed that the mAbs 2F8, 3F6 and 5C11 could be highly blocked by the H7-specific serum, with 87.80%, 97.96% and 92.44% inhibition rate, respectively. This illustrated that the epitope recognized by these mAbs is a dominant antigenic epitope during virus infection. The characteristics of the mAbs aroused our interest in locating the precise epitopes on the HA protein recognized by these mAbs.

The epitopes represent the antigenic moiety and provide a valuable diagnostic tool for most immune populations. Currently, there are some studies in which the antigenic epitopes on the HA protein were predicted using mAbs, and site A was identified as the epitope of H7 virus on the HA protein by which the virus can escape with the R149G mutation (Schmeisser *et al*., 2015; Stadlbauer *et al*., 2018; Tan *et al*., 2016). Epitopes recognized by several broadly neutralizing mAbs across HA subtypes have be identified to be located in the fusion peptide and helix A region from the HA stem, suggesting these regions are related to immunity protection (Ekiert *et al*., 2011; Henry Dunand *et*

al., 2015). Recently, a novel linear epitope, TAADYKSTQSAIDQITGKLN, crossing group 1 and group 2 influenza A viruses, was identified in the C terminus of helix A of HA2 (Li *et al*., 2019). However, the precise linear epitopes of mAbs specific to H7 have yet to be mapped. In this research, the 29 truncated HA protein segments of H7 were used to identify the critical and precise epitopes recognized by these mAbs. Epitope mapping showed that the mAbs 2F8, 3F6 and 5C11 recognized the epitope ^{103}RESGG^{107}, while the mAbs 5E2 and 5C12 bound to 103-145aa.

The epitopes recognized by these mAbs were found in H7 strains but not in other subtypes, supporting our results that these mAbs only reacted with H7. Influenza A viruses can be divided into Eurasian and Northern American lineages based on phylogenetic analysis of HA, and strains common in China were mainly derived from the Eurasian lineage. Among the 1631 analyzed Eurasian lineage strains, the epitope recognized by the mAbs 2F8, 3F6 and 5C11 was present in 93.62% strains. In contrast, only 1.56% strains of the Northern American lineage contained the epitope sequence, and amino acid substitutions (E104R, E104G and E104K) existed in most strains. Further, any single amino acid substitution in the epitope (103-107aa) eliminated the reactivity of the mutant epitope with the mAbs 2F8, 3F6 and 5C11. This phenomenon was also displayed in 293T cells transiently expressed the whole HA protein of Eurasian or North American strains. These indicated that the mAbs 2F8, 3F6 and 5C11 might be used for differentiating Eurasian and Northern American lineage strains. The other epitope 103-145aa recognized by the mAbs 5E2/5C12 and had a certain degree of variation, with mutations predominantly within E104, D109, A112, S118 and R130.

In the 3-D structure of HA protein, the position of ^{103}RESGG^{107} seems inaccessible in the native prefusion conformation of HA, suggesting that either mAbs 2F8/3F6/5C11 bind after conformational changes during the fusion process or that the mAbs induce small conformational changes in nearby residues. At the same time, the absence of neutralizing activity and low HI activity of the mAbs 2F8, 3F6 and 5C11 indicated that the epitope ^{103}RESGG^{107} under site E could not replace the neutralizing site located in HA surface. B-cell epitopes are linear or conformational, the linear epitope was generally short and could be recognized directly by immunoglobulin. The epitope 103-145aa covered neutralizing antigenic site (site A and site B), but mAbs 5E2 and 5C12 have no HI activity or neutralizing activity. Given the amino acid differences between different strains and the IFA results, the precise epitope bound by the mAbs 5E2 and 5C12 is likely a three-dimensional conformational arrangement of the longer sequence. Moreover, this three-dimensional conformation may affect the effective binding of neutralizing antigenic sites to mAbs.

In summary, in this study five mAbs against the HA protein of H7 AIV were generated, and two epitopes (103-107aa and 103-145aa) were first defined. The epitope 103-107aa was conserved in the Eurasian lineage but was variable in the North-American lineage, so the mAbs 2F8, 3F6 and 5C11 could distinguish between H7 strains of Eurasian and North-American lineage. The results have potential to help elucidate the structure, function and antigenicity of the HA protein to develop a rapid diagnosis method for H7 virus infection.

Disclosure statement

No potential conflict of interest was reported by the authors.

Acknowledgments

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Table

Table 1. Primers set used to amplify the overlapping and truncated segments of the HA gene.

Fragment	Location	Primer sequence
F1	-2-326	5'CCGCTCGAGAATGCAGACAAAATCTGCCTCG3' 5'GCTCTAGATTAAGCACCAAATAGGCCTCTTCC3'
F2	-2-95	5'CTGGTTCCGCGTGGATCCCCGGAATTCAATGCAGACAAAATCTGCCTC3' 5'TCAGTCACGATGCGGCCGCTCGAGTTCATTCACGAACTTCCCAG3'
F3	75-172	5'CTGGTTCCGCGTGGATCCCCGGAATTCTTAATTATTGAGAGGCGAGAAG3' 5'TCAGTCACGATGCGGCCGCTCGAGCCCCCATATTATTAGAGCTGG3'
F4	153-249	5'CTGGTTCCGCGTGGATCCCCGGAATTCCCACAGATGACTAAGTCAT3' 5'TCAGTCACGATGCGGCCGCTCGAGGCTTGCACGGTCTGGAGCTATGA3'
F5	230-326	5'CTGGTTCCGCGTGGATCCCCGGAATTCCCCAATGATACAGTCACCTTC3' 5'TCAGTCACGATGCGGCCGCTCGAGAGCACCAAATAGGCCTCTT3'
F6	75-118	5'CTGGTTCCGCGTGGATCCCCGGAATTCTTAATTATTGAGAGGCGAGAA3' 5'TCAGTCACGATGCGGCCGCTCGAGGCTGTATGTGAATCCCATTGC3'
F7	103-145	5'CTGGTTCCGCGTGGATCCCCGGAATTCAGGGAATCAGGCGGGATTGAC3' 5'TCAGTCACGATGCGGCCGCTCGAGTGACAGGAGCCATTTCATTTCT3'
F8	130-172	5'CTGGTTCCGCGTGGATCCCCGGAATTCAGGAGATCAGGATCTTCATTC3' 5'TCAGTCACGATGCGGCCGCTCGAGCCCCCATATTATTAGAGCTGG3'
F9	103-118	5'AATTCAGGGAATCAGGCGGGATTGACAAGGAAGCAATGGGATTCACATACAGCC3' 5'TCGAGGCTGTATGTGAATCCCATTGCTTCCTTGTCAATCCCGCCTGATTCCCTG3'
F10	103-114	5'AATTCAGGGAATCAGGCGGGATTGACAAGGAAGCAATGGGAC3' 5'TCGAGTCCCATTGCTTCCTTGTCAATCCCGCCTGATTCCCTG3'
F11	107-118	5'AATTCGGGATTGACAAGGAAGCAATGGGATTCACATACAGCC3' 5'TCGAGGCTGTATGTGAATCCCATTGCTTCCTTGTCAATCCCG3'
F12	130-145	5'AATTCAGGAGATCAGGATCTTCATTCTATGCAGAAATGAAATGGCTCCTGTCAC3' 5'TCGAGTGACAGGAGCCATTTCATTTCTGCATAGAATGAAGATCCTGATCTCCTG3'
F13	104-114	5'AATTCGAATCAGGCGGGATTGACAAGGAAGCAATGGGAC3' 5'TCGAGTCCCATTGCTTCCTTGTCAATCCCGCCTGATTCG3'
F14	105-114	5'AATTCTCAGGCGGGATTGACAAGGAAGCAATGGGAC3' 5'TCGAGTCCCATTGCTTCCTTGTCAATCCCGCCTGAG3'
F15	106-114	5'AATTCGGCGGGATTGACAAGGAAGCAATGGGAC3' 5'TCGAGTCCCATTGCTTCCTTGTCAATCCCGCCG3'

F16	103-113	5’AATTCAGGGAATCAGGCGGGATTGACAAGGAAGCAATGC3’ 5’TCGAGCATTGCTTCCTTGTCAATCCCGCCTGATTCCCTG3’
F17	103-112	5’AATTCAGGGAATCAGGCGGGATTGACAAGGAAGCAC3’ 5’TCGAGTGCTTCCTTGTCAATCCCGCCTGATTCCCTG3’
F18	103-111	5’AATTCAGGGAATCAGGCGGGATTGACAAGGAAC3’ 5’TCGAGTTCCTTGTCAATCCCGCCTGATTCCCTG3’
F19	103-110	5’AATTCAGGGAATCAGGCGGGATTGACAAGC3’ 5’TCGAGCTTGTCAATCCCGCCTGATTCCCTG3’
F20	103-109	5’AATTCAGGGAATCAGGCGGGATTGACC3’ 5’TCGAGGTCAATCCCGCCTGATTCCCTG3’
F21	103-108	5’AATTCAGGGAATCAGGCGGGATTC3’ 5’TCGAGAATCCCGCCTGATTCCCTG3’
F22	103-107	5’AATTCAGGGAATCAGGCGGGC3 5’TCGAGCCCGCCTGATTCCCTG3’
F23	103-106	5’AATTCAGGGAATCAGGCC3’ 5’TCGAGGCCTGATTCCCTG3’
F24	103-140	5’CTGGTTCCGCGTGGATCCCCGGAATTCAGGGAATCAGGCGGGATTGA3’ 5’TCAGTCACGATGCGGCCGCTCGAGATTTCTGCATAGAATGAAGATCCT3’
F25	110-145	5’CTGGTTCCGCGTGGATCCCCGGAATTCAAGGAAGCAATGGGATTCAC3’ 5‘TCAGTCACGATGCGGCCGCTCGAGTGACAGGAGCCATTTCATTTC3’
F26	110-144	5’CTGGTTCCGCGTGGATCCCCGGAATTCAAGGAAGCAATGGGATTC3’ 5’TCAGTCACGATGCGGCCGCTCGAGCAGGAGCCATTTCATTTC3’
F27	110-143	5’CTGGTTCCGCGTGGATCCCCGGAATTCAAGGAAGCAATGGGATTC3’ 5’TCAGTCACGATGCGGCCGCTCGAGGAGCCATTTCATTTCTGC3’
F28	111-145	5’CTGGTTCCGCGTGGATCCCCGGAATTCGAAGCAATGGGATTCACA3’ 5’TCAGTCACGATGCGGCCGCTCGAGTGACAGGAGCCATTTCATTTC3’
F29	113-145	5’CTGGTTCCGCGTGGATCCCCGGAATTCATGGGATTCACATACAGC3’ 5’TCAGTCACGATGCGGCCGCTCGAGTGACAGGAGCCATTTCATTTC3’

Table 2. Characterization of five mAbs against the HA protein of H7 AIV.

mAbs	ELISA titers of supernatant	ELISA titers of ascites	HI titers of ascites	Isotype	Light chain
2F8	2^{11}	10^{9}	320	lgG2a	Kappa
3F6	2^{14}	10^{7}	160	lgG2a	Kappa
5C11	2^{12}	10^{6}	160	lgG2a	Kappa
5E2	2^{12}	10^{5}	-	lgG2b	Kappa
5C12	2^{12}	10^{6}	-	lgG1	Kappa

Table 3. The inhibition rate of the five mAbs.

mAbs	2F8	3F6	5C11	5E2	5C12
SI of sample	7334	1297	4771	54829	24476
SI of negative	60105	63461	63087	56910	47680
Inhibition rate（%）	87.80	97.96	92.44	3.66	48.67

Table 4. Conservation of the epitope ^{103}RESGG^{107} recognized by mAbs 2F8, 3F6 and 5C11 within HA sequence of different subtypes.

mAb	Epitope	Relative conservation (%)					
		H7N9	H7NX	H1NX	H3NX	H5NX	H9NX
2F8,3F6, 5C11	RESGG	86.82[a] (777/895)	42.00 (767/1826)	0 (0/28024)	0 (0/27958)	0 (0/6121)	0 (0/6689)

[a] A total of 73908 HA sequences of influenza virus available in Influenza Research Database (as of September 2, 2018) were analyzed; numbers in parentheses represent the sequences screened and positive for each subtype.

Figure legends

Figure 1 Reactivity of mAbs with recombinant H7-HA1 protein and H7N3 strains in MDCK cells. (a) Monoclonal antibodies in the supernatant of the hybridomas cells were detected by western blot. M: protein molecular weight marker; lane 1: recombinant H7-HA protein; lane 2: lyses of pCold Ⅰ-transformed *E. Coli* (BL21) as a negative control. (b) Reactivity of the five mAbs with the H7N3-infected MDCK cells and the uninfected MDCK cells was detected by IFA assay. Cells were fixed at 48 h after infection with H7N3. Immunofluorescence was detected in the cells infected with H7N3 by the five mAbs. No immunofluorescence was detected in normal cells.

Figure 2 Specificity of mAbs with different avian influenza virus strains in MDCK cells by IFA. MDCK cells were fixed at 48 h postinfection with different avian influenza virus. MAb 2F8 reacted with four H7 strains, but not with other subtype strains (data from other viruses not shown). MAbs 3F6, 5C11, 5E2 and 5C12 had the same reactivity as the mAb 2F8; data not shown.

Figure 3 Blocking activity of mAbs against H7. (a) Schematic illustration of the

protein chip. The spot in blue is the H7-HA1 protein; the spot in white is background control; (b) Chip reacted with mAbs without serum; (c) Chip reacted with mAbs and serum; The washing buffer was used as non-blocking control.

Figure 4 Schematic diagram of the HA protein. The expression constructs of the HA fragments and their reactivity with mAbs in western blot. The lines are drawn to scale. '+': Positive; '-': Negative.

Figure 5 Epitope mapping of mAbs by western blot. (a) MAb 2F8 specifically reacted with the fragments which contained the ^{103}RESGG^{107} sequence; mAbs 3F6 and 5C11 recognized the same epitope (data not shown); (b) MAbs 5E2 specifically reacted with the fragments that contained the 103-145aa sequence; mAbs 5C12 recognized the same epitope (data not shown). Epitopes are shown in bold.

Figure 6 Analysis of epitopes. (a) Amino acid sequence alignment of the epitopes among different H7 strains using Clustal W. The red rectangle indicates amino acid residues 103-107, which are recognized by mAbs 2F8, 3F6 and 5C11; the blue rectangle indicates amino acid residues 103-145, which are recognized by mAbs 5E2 and 5C12. (b) The percentage of each mutant epitope is represented by pie charts, and the sequences are labeled for large components.

Figure 7 Molecular modeling of HA and the epitopes of mAbs. The epitopes recognized by mAbs 2F8/3F6/5C11 and 5E2/5C11 are labeled in purple and blue, respectively. The epitope (103-107aa) is located under site E, while the epitope (103-146aa) is located on the surface of the HA molecule. Five antigenic sites A-E are located on the HA1 surface based on the structure of H3N2 influenza viruses. The image was

generated with Pymol software (Delano Scientific).

Figure S1 Preparation of immune antigen. (a) The amplification of the H7HA fragment. M: DL2000 Maker; 1-2: Amplification of the H7HA fragment; 3: Negative control. (b) The purified H7HA protein was detected by western blot. M: protein maker; 1-4: the purified protein; 5: the negative control.

Figure S2 Epitope mapping of mAbs by western blot. Truncated fragments were detected with the mAb 2F8 (a) and the mAb 5E2 (b). F1: H7-HA1; F2: -2-95aa; F3: 75-172aa; F4: 153-249aa; F5: 230-326aa; F6: 75-118aa; F7: 103-145aa; F8: 130-172aa; F9: 103-118aa; F10: 103-114aa; F11: 107-118aa; F12: 130-145aa; F24: 103-140aa; F25: 110-145aa; mAbs 3F6 and 5C11 recognized the same fragments as 2F8, mAbs 5C12 recognized the same fragments as 5E2 (data not shown).

Figure S3 Epitope mapping of mAbs by western blot. (a) Mutant fragments were detected with the mAb 2F8. F30: 103-107aa (E104R); F31: 103-107aa (E104G); F32: 103-107aa (E104K); F33: 103-107aa (E104N); F34: 103-107aa (E104A); F35: 103-107aa (G107T); F36: 103-107aa (E104R and G107S); F1 as positive control; (b) Western blotting were performed for the mAb 2F8 using 293 T cells transfected with different HA protein. 1: The lysates of 293 T cells transfected with the empty vector; 2-3: The lysates of 293 T cells transfected with HA protein of Eurasian strain (RESGG) and North-American strain (RGSGG), respectively. The mAbs 3F6 and 5C11 showed the same data (data not shown).

Figure 1

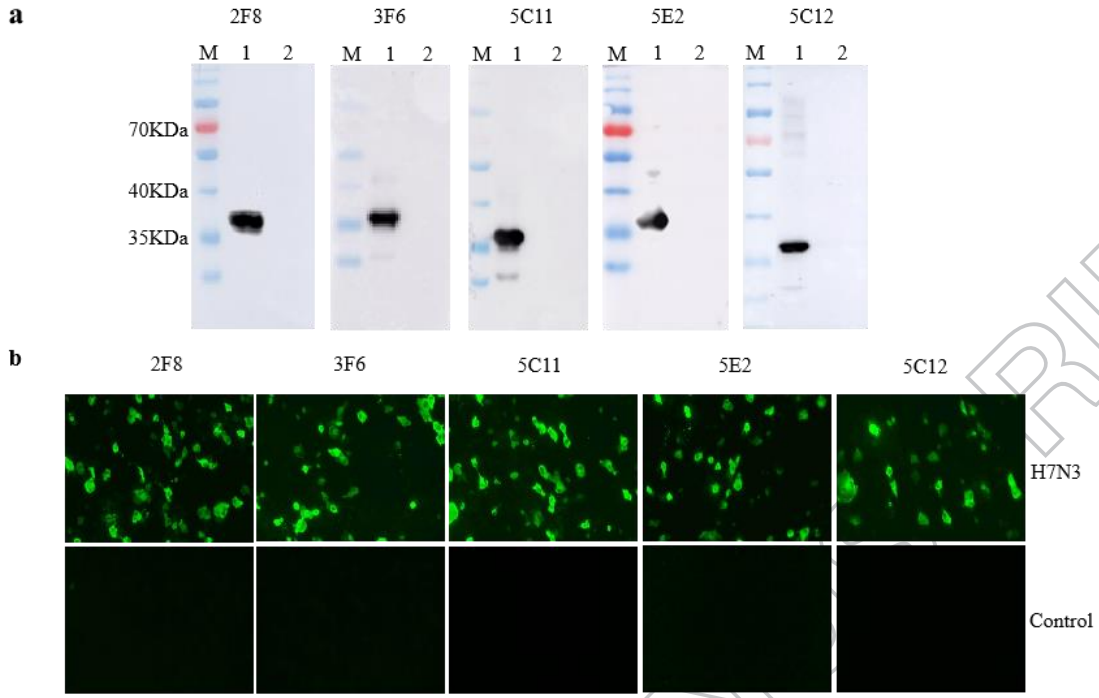

Figure 2

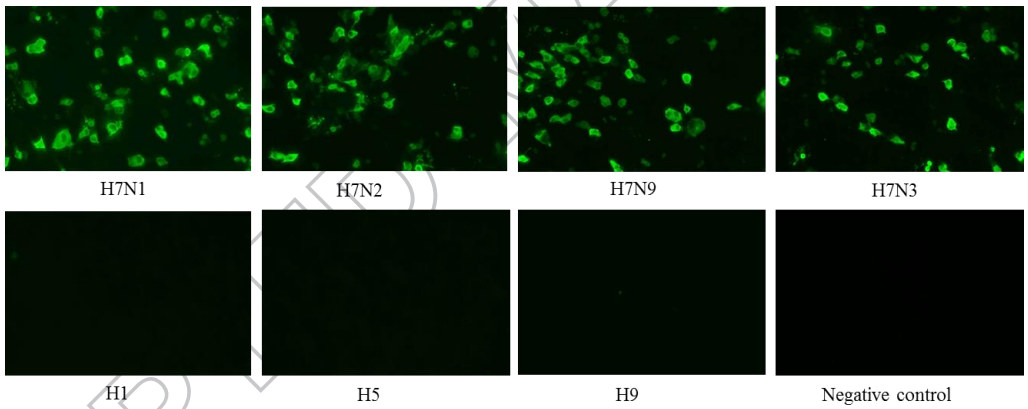

Figure 3

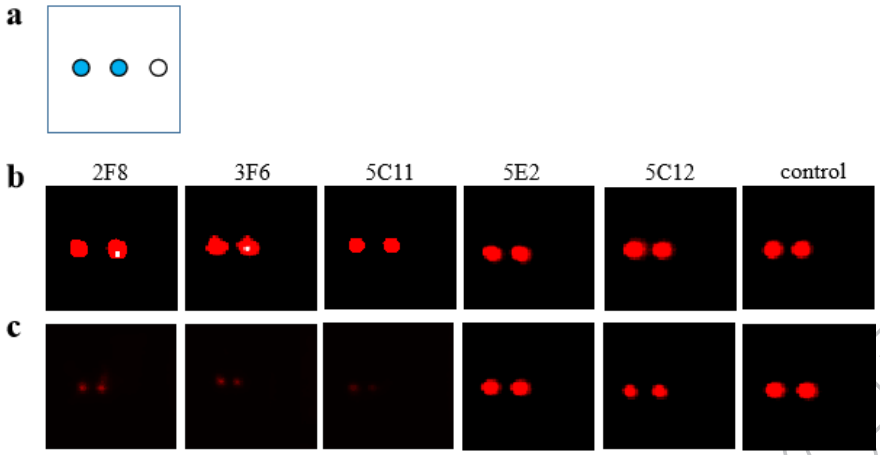

Figure 4

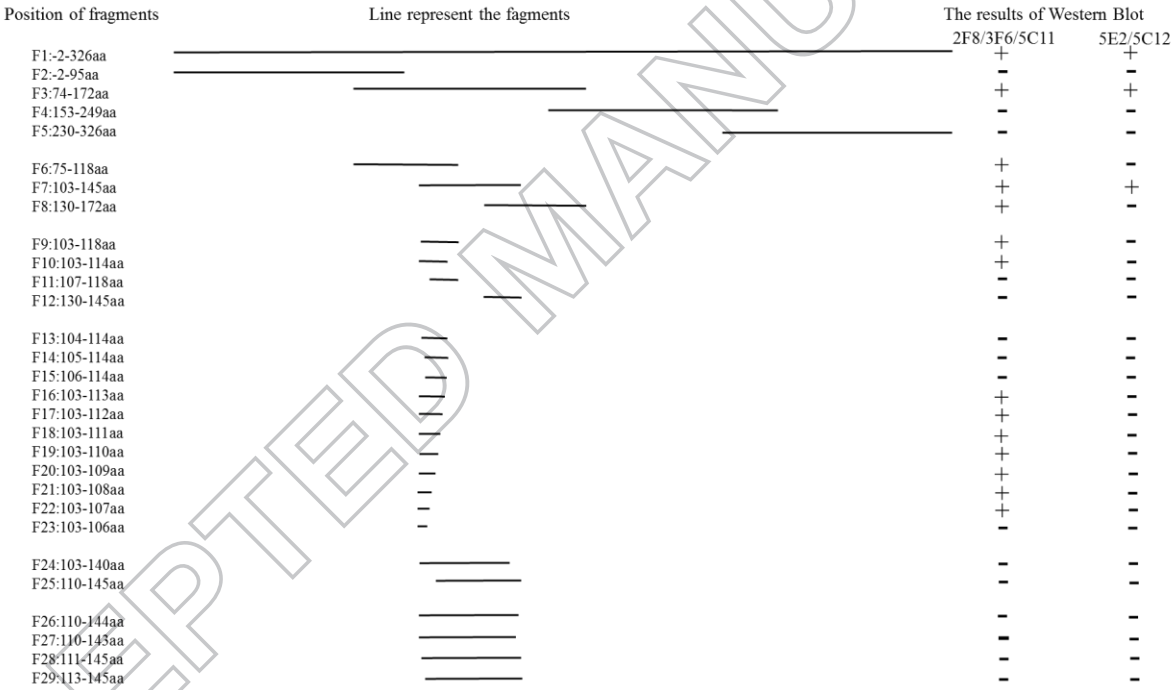

Figure 5

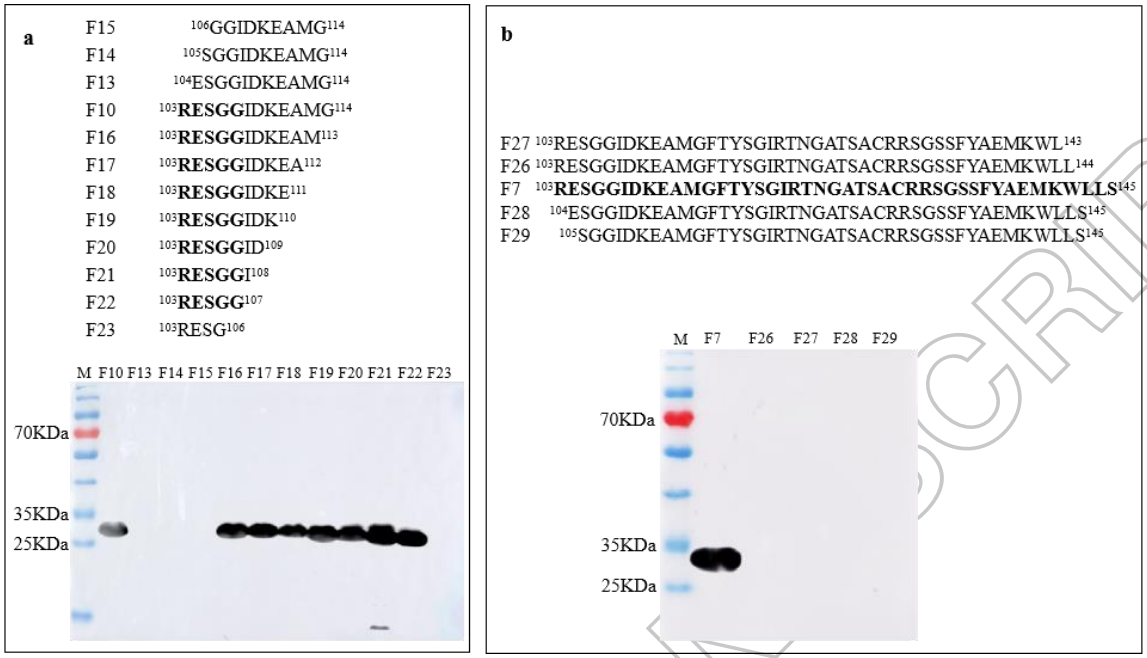

Figure 6

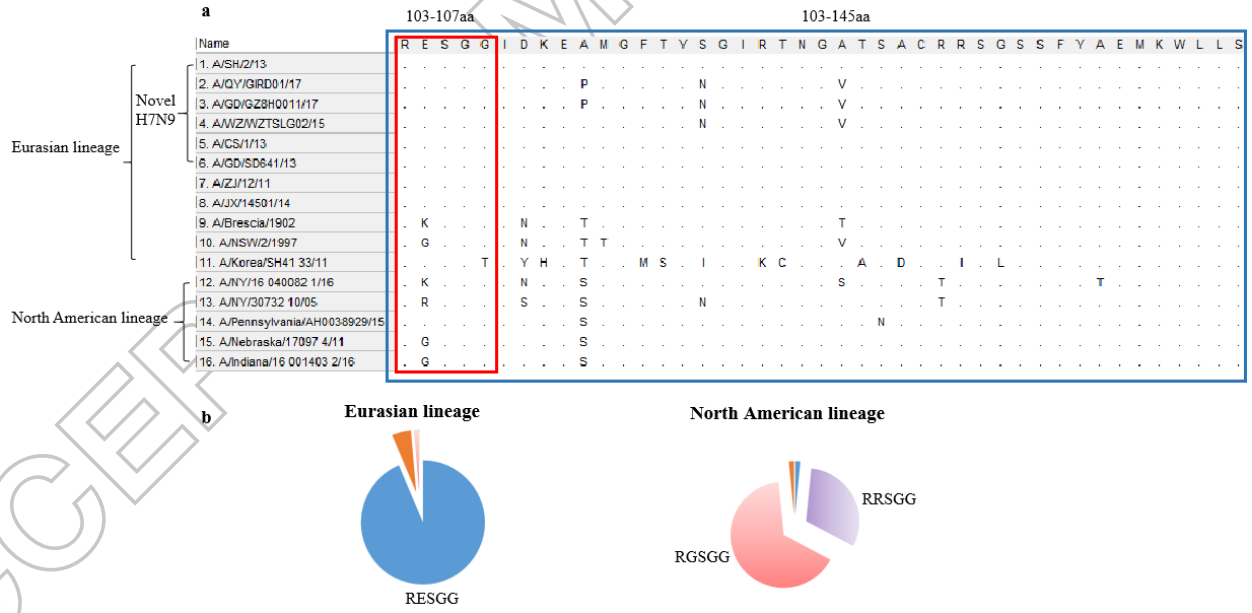

Figure 7

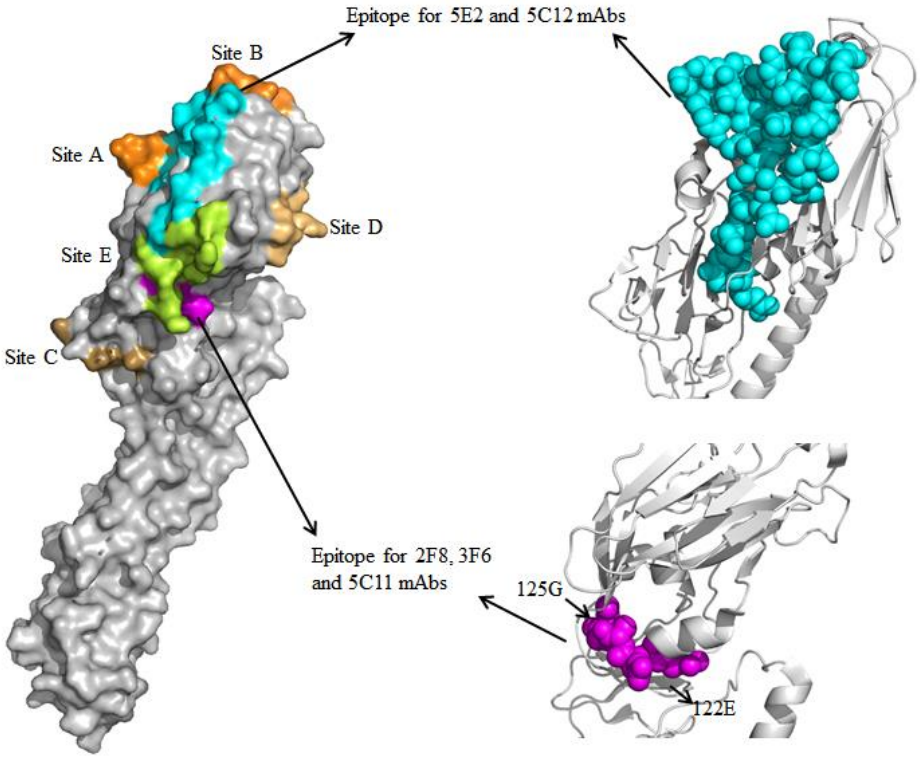

Figure S1

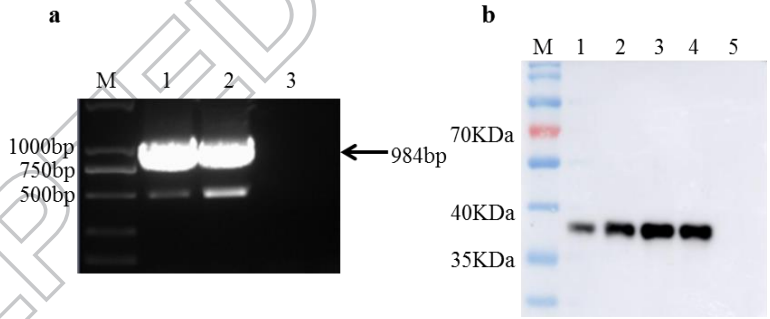

Figure S2

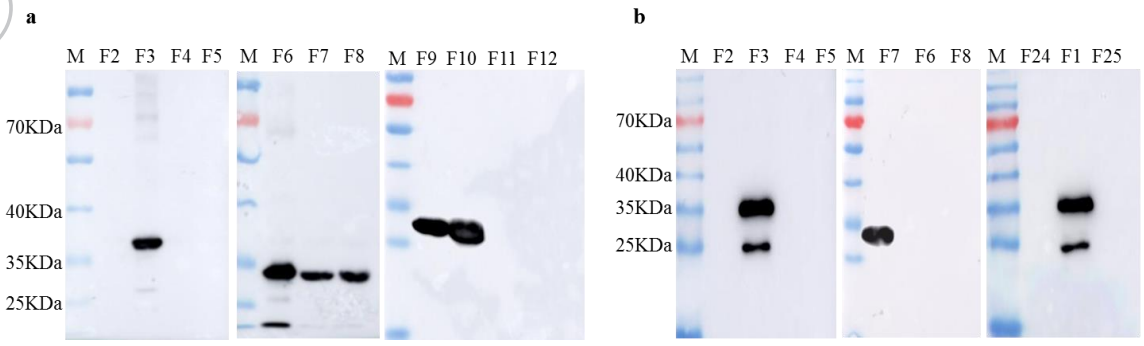

Figure S3

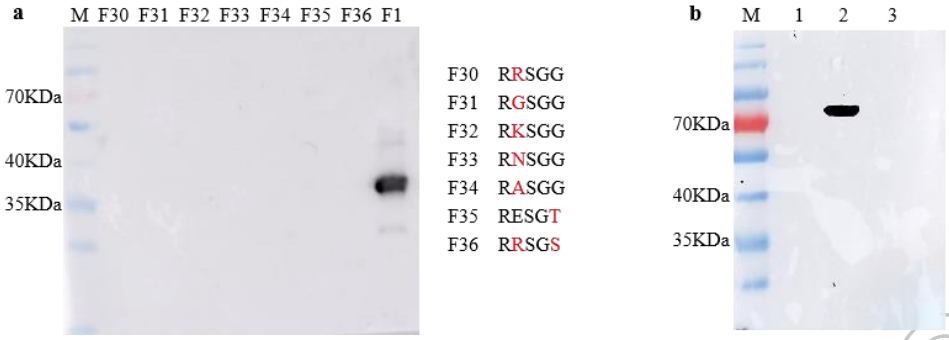

Table S1. The information of different subtypes of influenza virus.

Subtype	Name of isolate	HA titer	Highly similar sequences[a] (⩾99%)
H1N1	AIV-H1N1 P2009	2^9	MH061695.1
H2N2	AIV-H2N2 21103	2^5	L11134.1
H3N8	AIV-H3N8 11102	2^8	CY005816.1
H4N6	AIV-H4N6 20411	2^7	GU052381.1
H5N1	AIV-H5N1 060315	2^5	JX565019.1
H6N5	AIV-H6N5 20411	2^7	CY014656.1
H7N1	AIV-H7N1	2^8	DQ003216.1
H7N2	AIV-H7N2	2^7	AB302789.1
H7N3	AIV-H7N3 201369	2^7	JQ906576.1
H7N9	AIV-H7N9 A181	2^{11}	MF510879.1
H8N4	AIV-H8N4 20413	2^9	CY014659.1
H9N2	AIV-H9N2 201313	2^9	KF059279.1
H10N7	AIV-H10N7 20410	2^7	CY014671.1
H11N9	AIV-H11N9 21103	2^9	CY014687.1
H12N5	AIV-H12N5 11103	2^7	GU052216.1
H13N6	AIV-H13N6 11103	2^6	CY014694.1

[a] Accession number from the GenBank databases.

Table S2 . Oligonucleotides encoding mutant sequences of epitope ^{121}RESGG^{125}.

Fragment	variant	Primer sequence
F30	E122R	5'AATTCCGAAGGTCAGGAGGAC3' 5'TCGAGTCCTCCTGACCTTCGG3'
F31	E122G	5'AATTCCGAGGGTCAGGAGGAC3' 5'TCGAGTCCTCCTGACCCTCGG3'
F32	E122K	5'AATTCAGGAAATCAGGCGGAC3' 5'TCGAGTCCGCCTGATTTCCTG3'
F33	E122N	5'AATTC AGAAACTCAGGCGGAC3' 5'TCGAGTCCGCCTGAGTTTCTG3'
F34	E122A	5'AATTCAGAGCATCAGGCGGAC3' 5'TCGAGTCCGCCTGATGCTCTG3'
F35	G125T	5'AATTCCGGGAGTCTGGCACAC3' 5'TCGAGTGTGCCAGACTCCCGG3'
F36	E122R&G125S	5'AATTCAGGAGATCAGGATCTC3' 5'TCGAGAGATCCTGATCTCCTG3'

Table S3. The full name corresponding to the strains used for alignment.

Abbreviation	Full name
A/SH/2/13	A/Shanghai/2/2013(H7N9)
A/QY/GIRD01/17	A/Qingyuan/GIRD01/2017(H7N9)
A/GD/GZ8H0011/17	A/Guangdong/GZ8H001/2017(H7N9)
A/WZ/WZTSLG02/15	A/chicken/Wenzhou/WZTSLG02/2015(H7N9)
A/CS/1/13	A/Changsha/1/2013(H7N9)
A/GD/SD641/13	A/chicken/Guangdong/SD641/2013(H7N9)
A/ZJ/12/11	A/duck/Zhejiang/12/2011(H7N3)
A/JX/14501/14	A/chicken/Jiangxi/14501/2014(H7N6)
A/Brescia/1902	A/chicken/Brescia/1902(H7N7)
A/NSW/2/1997	A/chicken/New_South_Wales/2/1997(H7N4)
A/Korea/SH41_33/11	A/wild_duck/Korea/SH41_33/2011(H7N7)
A/NY/16_040082_1/16	A/feline/New_York/16_040082_1/2016(H7N2)
A/NY/30732_10/05	A/chicken/New York/30732-10/2005(H7N2)
A/Pennsylvania/AH0038929/15	A/mallard/Pennsylvania/AH0038929/2015(H7N7)
A/Nebraska/17097_4/11	A/goose/Nebraska/17097_4/2011(H7N9)
A/Indiana/16_001403_2/16	A/turkey/Indiana/16-001403-2/2016(H7N8)

Table S4. Natural mutations of the epitope [121]RESGG[125] in H7 strains.

HA amino acid position[a]		
Eurasian lineage		North American lineage
122	125	122
E(93.62)[b]	T(0.06)	E(1.56)
G(1.23)		R(31.11)
K(4.97)		G(65.60)
N(0.06)		K(1.56)
A(0.06)		

[a] H7 numbering.

[b] A total of 2,722 HA sequences of H7 strains available in Influenza Research Database (as of September 2, 2018) were analyzed; numbers in parentheses represent the percentage of H7 strains bearing each residue in the HA.