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Enhanced cross-lineage protection induced by recombinant H9N2 avian influenza virus inactivated vaccine

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ABSTRACT

Background: Antigenic drift of H9N2 low pathogenic avian influenza viruses (AIV) may result in vaccination failure in the poultry industry and thus a cross-protective vaccine against H9N2 AIV is highly desirable.

Methods: A series of H9N2 recombinant viruses with the internal genes of A/Puerto Rico/8/34 (H1N1, PR8) were generated, based on the compatibility between HA and NA, the effect of HA deglycosylation, and protective antigenic epitopes in HA. After evaluation of their biological and immunological characteristics, three recombinant AIVs with the internal genes of the Y280-like strain SN were selected for protective efficacy studies.

Results: The recombinant viruses $rHA_{SN}NA_3$, rHA_{SN}-△200, rHA_{SN}-△287, and rHA_{SN}-R92G-E93K displayed good cross reactivity and induced higher neutralization antibody titers against both SN and the F98-like strain YZ4. Furthermore, those recombinant viruses had a higher EID_{50} in chicken embryos after the replacement of internal-gene backbone from PR8 to SN. The rSNHA-△200 induced better protection in immunized chickens against challenge of homologous and heterologous H9N2 avian influenza viruses when compared with the wild type strain.

Conclusion: The recombinant virus rSNHA-△200 can be used as a potential broad-spectrum vaccine against H9N2 avian influenza.

1. Introduction

H9N2 avian influenza virus (AIV), which is relatively low in pathogenicity towards birds, mainly causes respiratory symptoms, immunosuppression, and decreased egg production [1]. However, higher mortality can appear in chickens with coinfections [2], which results in huge economic losses in the poultry industry. H9N2 AIVs are also able to transmit across species barriers to cause human infections [3,4]. The segmented genomes of AIVs permits the generation of novel recombinant influenza viruses with pandemic potential, and H9N2 AIVs contribute internal genes to emerging recombinant viruses such as H7N9 [5], which pose severe threats to public health.

In China, several distinct Eurasian avian lineages of H9N2 AIVs including A/chicken/Beijing/94-like (BJ/94-like), A/quail/Hong Kong/G1/97-like (G1-like), A/duck/Hong Kong/Y439/97 (Y439-like) and A/duck/Hong Kong/Y280/97 (Y280-like) were reported [6]. In 1998, A/chicken/Shanghai/F/98-like strains (F98-like, a represented strain of BJ/94-like) spread to most provinces of China within two months, and become the most prevalent subtype for more than ten years [7]. Recent epidemiological studies show that the H9N2 HA genes of isolates from chickens are mainly derived from the Y280-like lineage [5], while G1-like viruses are seldomly isolated from poultry in Southern China. Vaccination is considered as the most effective method of prophylaxis against H9N2 avian influenza, and inactivated vaccines have been most commonly used [8]. However, due to immune escape of H9N2 AIVs [9], some vaccines do not match the antigenic diversity of the prevalent

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H9N2 AIVs, resulting in vaccination failure and outbreaks on chicken farms [10]. Thus, it is crucial to update the vaccine candidates and develop more effective vaccines to prevent and control H9N2 avian influenza.

In this study, we constructed a series of H9N2 recombinant viruses based on three strategies, the compatibility between HA and neuraminidase (NA), the effect of HA deglycosylation, and the substitution of amino acids in epitopes inducing protective humoral immunity. We also used the six internal genes of A/chicken/Fujian/Shengnong/2014 (SN) as the backbone to replace the corresponding genes in A/Puerto Rico/8/34 (H1N1, PR8). After evaluation of the biological and immunological characteristics of these recombinant viruses, three recombinant viruses were selected for determination of protective efficacy.

2. Methods

2.1. Cells and viruses preparation

H9N2 AIVs A/chicken/Fujian/Shengnong/2014 (SN, Y280-like), A/chicken/Jiangsu/YZ4/2012 (YZ4, F98-like), A/chicken/Jiangsu/W2-17/2012 (W2-17, Y280-like), A/chicken/Shandong/QD5/2012 (QD5, Y280-like), A/chicken/Anhui/FY040/2014 (FY2014040, Y280-like), A/chicken/Jiangsu/WJ100/2015 (WJ100, Y280-like) were isolated from live bird markets in Eastern China and propagated in specific-pathogen-free (SPF) embryonated chicken eggs. Human embryonic kidney (293 T) and Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum.

2.2. Construction of plasmids for expressing modified HA and NA

H9N2 AIV strain SN was selected as the parental virus and site-directed mutagenesis in the HA gene of H9N2 AIV strain SN was performed using overlap-PCR with primers shown in Table S1. To generate recombinant viruses with wild-type SN HA and different NA, the SN HA gene and three NA genes from selected viruses were amplified. To generate recombinant viruses with removal of specific glycosylation sites in the HA gene, the glycosylation sites at positions 11, 123, 127, 200, 280, 287, 295, 477, 533 were abolished by substitution of S/T of glycosylation site NXS/T with A, respectively. To generate recombinant viruses with replaced antigenic epitopes, the epitopes of SN HA were modified as R92G-E93K, N145S, and L234Q according to the sequence of amino acids of YZ4 HA. The amplified and modified HA or NA genes were inserted into the pHW2000 vector and confirmed by sequence analysis, and the expression plasmids were named as pHW-SNHA, pHW-NA_1, pHW-NA_2, pHW-NA_3, pHW-SN-HA-△11, pHW-SN-HA-△123, pHW-SN-HA-△127, pHW-SN-HA-△200, pHW-SN-HA-△280, pHW-SN-HA-△287, pHW-SN-HA-△295, pHW-SN-HA-△474, pHW-SN-HA-△533, pHW-SNHA-R92G-E93K, pHW-SNHA-N145S, pHW-SNHA-L234Q, respectively.

2.3. Virus rescue

Viruses were rescued according to previously reported protocols [11,12] with minor modifications. Briefly, the co-cultured 293 T-MDCK cells were transfected with rescued plasmids (pHW-PB2, pHW-PB1, pHW-PA, pHW-NP, pHW-M, pHW-NS), an internal-gene backbone from A/Puerto Rico/8/34 (H1N1, PR8), with/without the substitution of plasmids pHW-SNHA, pHW-SN-HA-△11, pHW-SN-HA-△123, pHW-SN-HA-△127, pHW-SN-HA-△200, pHW-SN-HA-△280, pHW-SN-HA-△287, pHW-SN-HA-△295, pHW-SN-HA-△474, pHW-SN-HA-△533, pHW-SNHA-R92G-E93K, pHW-SNHA-N145S, pHW-SNHA-L234Q, pHW-NA_1, pHW-NA_2, or pHW-NA_3 using Polyfect Transfection Reagent (Qiagen, GmBH, Germany). Also, the internal-gene backbone of PR8 was substituted with the internal-gene backbone of SN. At 48 h post-transfection, the cell supernatant was collected and inoculated into SPF eggs to amplify the rescued viruses, and the genome sequences of the fifth passage recombinant viruses were sequenced to confirm the correction of the rescued viruses [13]. All rescued viruses were titrated for viral infectivity by 50% embryo infective dose (EID_{50}).

2.4. Virus elution from chicken erythrocytes

Virus elution assay was performed as previously described [14–16]. Briefly, the mutant viruses were diluted two-fold in PBS, and 50 μL aliquots were incubated with 50 μL of chicken erythrocytes (1% in PBS) in V-bottomed microtiter plates at 4 °C for 30 min. Afterwards, the plates were transferred to 37 °C, and the precipitation of agglutinated erythrocytes was monitored periodically for the next 10 h [17].

2.5. Thermostability and pH stability

Recombinant viruses were divided into 60 μL aliquots. All aliquots were exposed to 56 °C at 0, 5, 10, 15, 30, 60, 90, 120, 150, 180, and 210 min incubation, and then quickly cooled to 4 °C. Methanol-inactivated recombinant viruses were incubated at 37 °C or 42 °C at 2 h intervals for 18 h. In addition, recombinant viruses were mixed with an equal volume of 100 mM acetate buffer (pH = 4.0 and pH = 5.0), 100 mM phosphate buffer (pH = 6.0), or neutral phosphate buffer (pH = 7.0) and incubated at 37 °C for 10 min. The titers of all samples were determined using hemagglutination assay with 1% chicken red blood cells [18,19].

2.6. Neutralization assay and hemagglutination inhibition assay

For serum preparation, four-week-old SPF chickens (4 chickens each group) were inoculated intramuscularly with 0.2 mL vaccine preparations containing 10^8 EID_{50} of each formalin-inactivated virus mixed with mineral oil adjuvant (SEPPIC, France) and boosted once after two weeks. The serum samples were collected on day 21 after the second immunization and the serum antibody levels against Y280-like strain SN and F98-like strain YZ4 were determined using a neutralization (Nt) assay [20] and hemagglutination inhibition (HI) assay. Appropriate viruses were then chosen as vaccine candidates to carry out immune protection trials [17].

2.7. Vaccination and challenge in chickens

All of the animal studies were approved by the Jiangsu Administrative Committee for Laboratory Animals (Permission number: SYXKSU-2007-0005) according to the guidelines of Jiangsu Laboratory Animal Welfare and Ethical of Jiangsu Administrative Committee of Laboratory Animals.

For protection experiments, six-week-old SPF chickens were randomly divided into five groups (20 chickens each group). Chickens in two groups were immunized intramuscularly with 0.2 mL of the formalin-inactivated viruses mixed with mineral oil adjuvant or 0.2 mL PBS. On day 21 post vaccination, serum samples were collected for determination of the HI titer, and the vaccinated chickens were challenged with H9N2 AIV strain SN and YZ4 intranasally at a dose of 10^6 EID_{50}/0.1 mL, respectively. Viral shedding in the swabs was monitored periodically after the challenge [17].

2.8. Statistical analysis

Comparisons of experimental groups were estimated using *t*-tests with two-tailed analysis and chi square test (χ^2) to determine

significant differences. Differences with a p-value of less than 0.05 were considered to be statistically significant [21].

3. Results

3.1. Construction and characterization of recombinant viruses with SN HA and NA from different strains

The hemagglutination titers of H9N2 AIV strains SN, QD5, W2-17, FY2014040, WJ100, YZ4 were $9\log_2$, $7\log_2$, $7\log_2$, $6\log_2$, $8\log_2$, and $8\log_2$, respectively. The SN strain was selected to construct the plasmid of HA gene because of its best replication in chicken embryonated eggs and prevalence among Y280-like strains.

To determine the NA activity of H9N2 AIV strains, virus elution assays were performed. The elution time of six H9N2 AIV strains ranged from 2 h to more than 10 h. Thereafter, the stains W2-17, SN, and YZ4 were selected as representative strains with fast, moderate, and slow elution speeds (Fig. 1A) to construct the transcription/expression plasmids with each NA gene.

A panel of four recombinant viruses $rHA_{SN}NA_1$ (NA_1 from W2-17), $rHA_{SN}NA_2$ (NA_2 from SN), $rHA_{SN}NA_3$ (NA_3 from YZ4), and $rHA_{SN}NA_4$ (NA_4 from PR8) was rescued. Sequencing results showed that all recombinant viruses were genetically stable without any unwanted mutations after five passages.

Four recombinant viruses were tested in the virus elution assay. The wild-type recombinant virus $rHA_{SN}NA_2$ eluted from erythrocytes in 4 h, which was faster than wild-type SN (Fig. 1B). Similarly, the recombinant virus $rHA_{SN}NA_1$ eluted in 4 h, while $rHA_{SN}NA_3$ eluted in 3 h, indicating that they showed different activities compared to their original parental virus. However, the recombinant virus $rHA_{SN}NA_4$ eluted more slowly (7 h) as compared with $rHA_{SN}NA_2$.

To determine the stability of recombinant viruses, the thermostability and low-pH stability were also tested. Four recombinant viruses were considered stable at 56 °C as the hemagglutination titer decreased by $2\log_2$ after 30 min (Fig. 2A). The inactivated recombinant viruses were thermostable at 37 °C (Fig. 2B) and 42 °C (Fig. 2C).

To evaluate the stability of recombinant virus under low-pH, the hemagglutination titer was determined. The hemagglutination titer of recombinant viruses showed no obvious loss when pH dropped from 6.0 to 5.0, compared with the PBS treated control at pH 7.0 (Fig. S1). The hemagglutination titers of all strains decreased to 0 at pH 4.0.

To evaluate the effect of altered NA genes on the antigenicity of the recombinant viruses, the neutralization titers of sera induced by the HA and NA combined viruses were determined against H9N2 AIV strains SN and YZ4. The recombinant virus $rHA_{SN}NA_3$ induced higher neutralization antibody titers against strain SN and YZ4, compared to the wild-type virus (Table 1), indicating that $rHA_{SN}NA_3$ could be considered as a vaccine candidate strain for further protective efficacy study.

3.2. Construction and characterization of recombinant viruses with removal of specific glycosylation sites in HA

A panel of seven recombinant viruses with 7 rescued plasmids of PR8 and differing in glycosylation sites of HA was rescued, and named as rHA_{SN}-△11, rHA_{SN}-△200, rHA_{SN}-△280, rHA_{SN}-△287, rHA_{SN}-△295, rHA_{SN}-△474, rHA_{SN}-△533, respectively. Sequencing results showed that all recombinant viruses were genetically stable without any unwanted mutations.

To evaluate the effect of deglycosylation on the antigenicity of recombinant viruses, the neutralization titers of sera induced by the viruses with deglycosylation were determined against H9N2 AIV strains SN and YZ4. The recombinant virus rHA_{SN}-△200 induced significantly ($P < 0.01$) higher neutralization antibody titers against strain SN and YZ4, and rHA_{SN}-△287 induced significantly higher ($P < 0.05$) neutralization antibody titers against strain SN and YZ4, while the other viruses showed almost no significant differences when compared with the recombinant wild-type virus (rSN) (Table 2). The result of Nt assay indicated that rHA_{SN}-△200 and rHA_{SN}-△287 could be considered as vaccine candidate strains for further protective efficacy study.

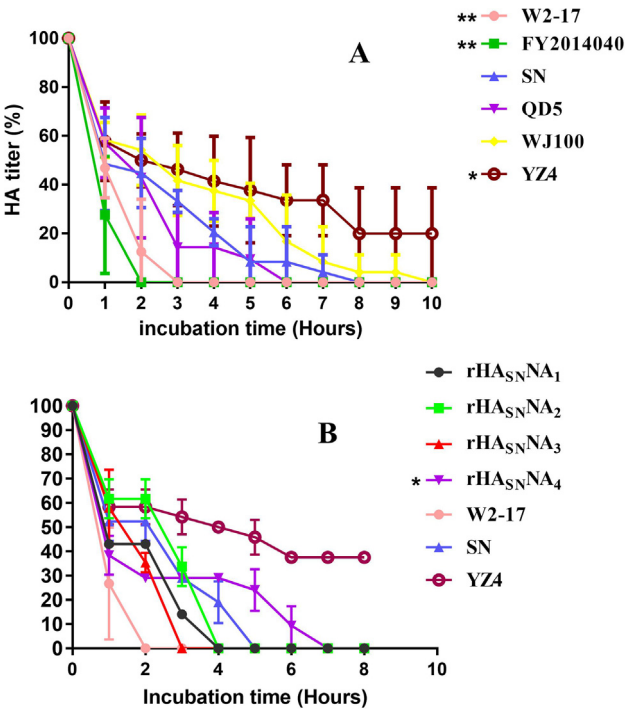

Fig. 1. Viral elution from chicken erythrocytes by wild type strains (A) and the recombinants (B). Two-fold dilutions of viruses were incubated with equal volumes of chicken erythrocytes at 4 °C for 30 min, and the HA titer at 37 °C representing virus elution from chicken erythrocytes was monitored each hour. Results were presented as the percentage of the initial HA titer at 4 °C. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the elution time of strain SN (A) or rHA_{SN} NA_2 (B).

3.3. Construction and characterization of recombinant viruses with replacement of antigenic epitopes in HA

A panel of three recombinant viruses with replacement of target amino acids in HA was rescued, and named as rHA_{SN}-R92G-E93K, rHA_{SN}-N145S, rHA_{SN}-L234Q, respectively. Sequencing results showed that all recombinant viruses were genetically stable without any unwanted mutation.

To determine the antigenicity of recombinant viruses with replaced antigenic epitopes, the HI assay was performed. The HI titers of YZ4, SN, rHA_{SN}-L234Q, rHA_{SN}-N145S, rHA_{SN}-R92G-E93K against SN anti-serum were $7\log_2$, $9\log_2$, $7\log_2$, $9\log_2$, and $10\log_2$, respectively. The HI titers of YZ4, SN, rHA_{SN}-L234Q, rHA_{SN}-N145S, rHA_{SN}-R92G-E93K against YZ4 anti-serum were $9\log_2$, $10\log_2$, $8\log_2$, $10\log_2$, and $12\log_2$, respectively, indicating that the HI titers of rHA_{SN}-R92G-E93K against SN and YZ4 antisera were both higher than those of parental virus SN and YZ4. The HI titer of rHA_{SN}-R92G-E93K was higher than that of other two recombinant viruses. As a result, rHA_{SN}-R92G-E93K was selected to perform further studies.

To evaluate the effect of replacing the antigenic epitope in HA on the antigenicity of recombinant viruses, the neutralization titers of sera were determined against H9N2 AIV strains SN and YZ4. The recombinant virus rHA_{SN}-R92G-E93K induced slightly higher neu-

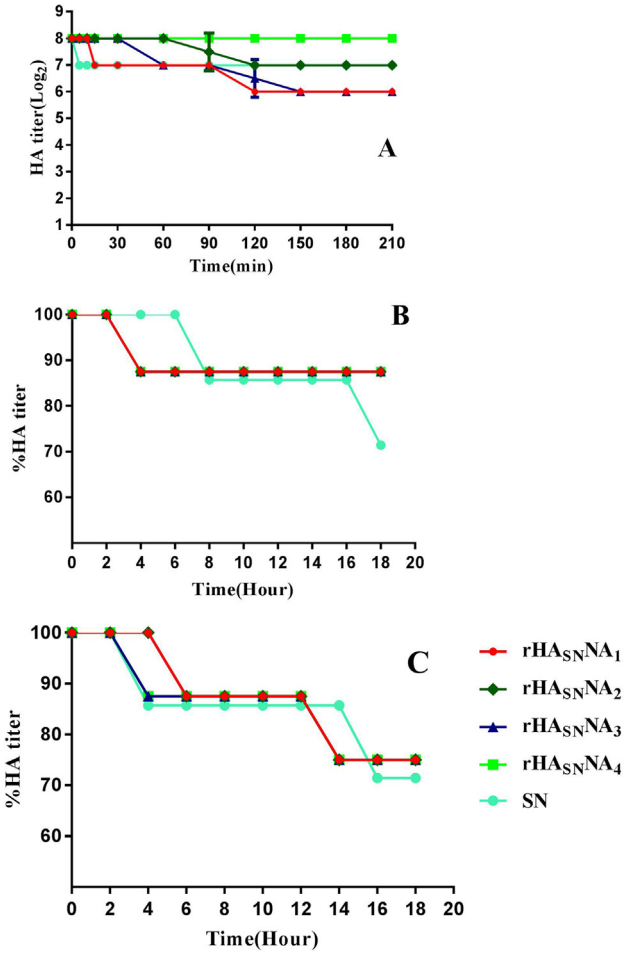

Fig. 2. Thermal stability of the recombinant viruses (A). Sixty-μL aliquots of recombinant viruses were exposed to 56 °C for 150 min. Then inactivated viruses were exposed to 37 °C (B) or 42 °C (C) for 18 h and aliquots were collected every 2 h. The titers of all aliquots were determined using hemagglutination assays.

Table 1
Nt and HI titers of sera induced by the HA and NA combined viruses.

Serum[a]	Nt(HI) titers against different viruses[b]	
	SN	YZ4
$rHA_{SN}NA_1$	93(181)	28(42)
$rHA_{SN}NA_2$	23(338)	20(28)
$rHA_{SN}NA_3$	135(861)	45(108)
$rHA_{SN}NA_4$	56(588)	63(169)
SN	80(861)	32(108)

[a] Chickens were immunized with the inactivated recombinant viruses, and serum samples were collected for the neutralization assay on CEF cells and HI test using chicken RBCs. Values represent reciprocal geometric mean antibody titers (GMT) from five animals.
[b] H9 AIVs from different lineages were used in Nt and HI assays: SN (Y280-like), YZ4 (F98-like).

tralization antibody titers against strain SN and YZ4 when compared with the recombinant wild-type virus, indicating that rHA_{SN}-R92G-E93K could be considered as a vaccine candidate for further protective efficacy study (Table 3).

3.4. Construction and evaluation of H9N2 AIV vaccine candidates with replacement of internal-gene backbone

To enhance viral replication of the vaccine candidate strains in chicken embryos, the internal-gene backbone of above four vaccine candidate strains was changed from PR8 to SN. Four rescued recombinant viruses with replacement of NA gene and/or the internal-gene backbone of SN were generated, and named as rSN-NA_3, rSNHA-△200, rSNHA-△287, rSNHA-9293, respectively. Sequencing results showed that all recombinant viruses were genetically stable without any unwanted mutation.

To evaluate the replication of recombinant viruses in chicken embryos, the EID_{50} of recombinant viruses was determined. The EID_{50}s (log_{10}/0.2 mL) of rSNHA-9293, rSN-NA_3, rSNHA-△200, and rSNHA-△287 with SN internal-gene backbone were 9.00, 8.67, 8.77, and 8.50, respectively, while the EID_{50}s (log_{10}/0.2 mL) of rHA_{SN}-R92G-E93K, $rHA_{SN}NA_3$, rHA_{SN}-△200, and rHA_{SN}-△287 with PR8 internal-gene backbone were 8.00, 7.33, 8.50, and 8.00, respectively, indicating that vaccine candidates with the internal-gene backbone of SN possessed better replication ability in chicken embryos.

The antigenicity of vaccine candidates with the internal-gene backbone of SN were further evaluated by performing neutralization assay. The Nt titers of the sera induced by rSNHA-9293, rSN-NA_3, rSNHA-△200, rSN against SN were 502:1, 631:1, 792:1, and 447:1, respectively. The Nt titers of the sera induced by rSNHA-9293, rSN-NA_3, rSNHA-△200, rSN against YZ4 were 316:1, 252:1, 891:1, and 199:1, respectively. The results showed that all three vaccine candidates induced higher neutralization antibody titers against SN and YZ4 when compared with recombinant wild-type virus rSN. rSNHA-△200 induced the highest neutralization antibody titer among these vaccine candidates against both SN and YZ4.

Table 2
Nt and HI titers of sera induced by deglycosylated viruses.

Serum[a]	Nt(HI) titers against different viruses[b]	
	SN	YZ4
rHA_{SN}-△11	23(64)	10 (16)
rHA_{SN}-△200	447**(2353)	316**(416)
rHA_{SN}-△280	266*(147)	63(39)
rHA_{SN}-△287	320*(1024)	224*(181)
rHA_{SN}-△295	112(362)	56(64)
rHA_{SN}-△474	63(388)	25 (37)
rHA_{SN}-△533	80(446)	10(37)
rSN	186(1218)	40(91)

*$P < 0.05$, **$P < 0.01$, compared with the Nt titers of rSN sera.
[a] Chickens were immunized with the inactivated recombinant viruses, and serum samples were collected for the neutralization assay on CEF cells and HI test using chicken RBCs. Values represent reciprocal geometric mean antibody titers (GMT) from five animals.
[b] H9 AIVs from different lineages were used in Nt and HI assays: SN (Y280-like), YZ4 (F98-like).

Table 3
Nt and HI titers of sera induced by viruses with epitope replacement.

Serum[a]	Nt(HI) titers against different viruses[b]	
	SN	YZ4
rHA_{SN}-R92G-E93K	223(3104)	147(891)
rSN	126(630)	80(128)

[a] Chickens were immunized with the inactivated recombinant viruses, and serum samples were collected for the neutralization assay on CEF cells and HI test using chicken RBCs. Values represent reciprocal geometric mean antibody titers (GMT) from five animals.
[b] H9 AIVs from different lineages were used in Nt and HI assays: SN (Y280-like), YZ4 (F98-like).

3.5. Immune efficacy of vaccine candidates

Six-week-old SPF chickens were immunized with the inactivated recombinant viruses rSNHA-9293, rSN-NA_3, rSNHA-△200,

Table 4
HI titers of sera induced by vaccine candidates.

Strains	HI titers (log2)[a]		
	7 d.p.i[b]	14 d.p.i	21 d.p.i
rSNHA-9293	0.45 ± 0.60	6.45 ± 2.09	9.23 ± 1.38
rSN-NA_3	0.21 ± 0.51	9.08 ± 0.83	9.58 ± 1.28
rSNHA-△200	0.11 ± 0.31	6.82 ± 1.61	9.18 ± 1.06
rSN	0.5 ± 0.59	7.92 ± 1.25	9.33 ± 0.85

[a] Six-week-old chickens were immunized with inactivated vaccine candidates in a 0.2-mL volume. Sera were collected on day 7, 14 and 21 to determine their HI.
[b] d.p.i.: days post immunization.

and wild-type virus SN to evaluate the protective efficacy of vaccine candidates. The HI titers of rSN-NA_3, rSNHA-9293, and rSNHA-△200 were 9.58log_2, 9.23log_2, 9.18log_2, respectively, at 21 days post immunization (Table 4), which was similar to that of wild-type SN.

After challenge, virus shedding in oropharyngeal and cloacal swabs for both SN and YZ4 was determined and protection rates were calculated. The protection rates provided by rSNHA-9293, rSN-NA_3, rSNHA-△200 against SN challenge were 20%, 50%, and 70%, respectively (Table 5). The protection rate provided by the wild type strain was 60%. Moreover, the protection rates provided by rSNHA-9293, rSN-NA_3, and rSNHA-△200 against YZ4 challenge were 80%, 80%, and 90%, respectively, while the protection rate provided by the wild type strain was 70%. Therefore, chickens immunized with rSNHA-△200 showed an enhanced cross protection against both SN and YZ4 challenge.

4. Discussion

HA and NA are two influenza virus glycoproteins located on the viral surface, which contribute to infectivity, transmissibility, virulence, and host specificity. Some studies revealed that the compatibility between HA and NA has a greater impact on viral growth than the HA affinity or the NA activity alone [22]. Also, our previous study suggested that a better match between clade 2.3.4.4 HA and NAx contributes to the promotion of virus growth, which potentially can contribute to an increased prevalence of clade 2.3.4.4 H5Nx AIV since 2010 in China [23]. Therefore, in this study, we constructed recombinant viruses with the HA from SN and NA from different strains. The SN strain was selected as the donor of HA gene for its best replication ability. The NA genes were selected after quantifying NA activity, which was indicated by the elution time in the virus elution assay. The virus elution assay showed that the elution time of $rHA_{SN}NA_1$ and $rHA_{SN}NA_3$ was similar to that of recombinant wild-type virus $rHA_{SN}NA_2$ (rSN), indicating that although NA genes originated from different H9N2 AIVs, the HA gene primarily contributed to the compatibility of HA and NA genes. Previous studies about influenza DNA vaccines revealed that immunization using NA together with HA could provide the most effective protection against influenza. Furthermore, antibodies against HA generally neutralize viral infectivity while antibodies against NA reduced viral replication to a point below the pathogenic threshold [24]. The serum neutralization assay showed that $rHA_{SN}NA_3$ induced higher neutralization antibody titers against SN and YZ4 than the wild-type viruses. Since the four recombinant viruses were comprised of the same HA from SN and distinct NAs from different selected strains, the only difference among these recombinant viruses was the origin of NA. Thus, the higher neutralization antibody titer induced by $rHA_{SN}NA_3$ may suggest that antibodies against NA also play an important role in inducing neutralization antibodies.

The surface glycoprotein HA of AIVs uses glycosylation for a variety of important functions including receptor binding, infectivity, viral release, and virulence [25–27]. AIV recombinant viruses with glycosylation in the HA protein at certain residue sites significantly reduced the reactivity with corresponding vaccine antiserum, causing immunization escape [28]. By removing certain glycosylation sites that shield antigenic epitopes in HA, more conserved epitopes could be exposed to induce a better immune response against HA variants [17]. In this study, we analyzed the HA protein of SN, and identified certain potential glycosylation sites to construct recombinant viruses by removing specific glycosylation sites in HA through site-directed mutagenesis. In the virus rescue, however, the potential recombinant viruses with deglycosylation at sites 123 and 127 failed to be rescued, suggesting that those glycosylation sites may play an essential role in both the formation of HA protein and viral replication. The serum neutralization assay showed that rHA_{SN}-△200 and rHA_{SN}-△287 induced significant higher neutralization antibody titers against SN and YZ4 than the wild-type virus, indicating that the deglycosylation of the 200 or 287 residues in HA enhanced antigenicity. However, high neutralization antibody titers induced by rSNHA-△287 could only be achieved by the boost vaccination of the virus, indicating that rSNHA-△287 had poor immunogenicity and failed to be vaccine candidate.

The HA of AIVs is the major target for humoral protective immune responses [29], since the HA protein is enriched with the neutralizing epitopes capable of eliciting the neutralizing antibodies [30]. The corresponding antibodies against antigenic epitopes in the HA are crucial in vaccination and directly relate to immune efficacy. It has been recently reported that a total of 37 amino-acid positions are antigenically relevant [29–31], among which 20 amino-acid positions (including 90, 92, 133, 145, 147, 149, 153, 155, 163, 164, 166, 167, 168, 197, 200, 201, 206, 207, 234, and 235) show significant effects on HI titer with polyclonal

Table 5
Protective efficacy induced by inactivated vaccine candidates in SPF chickens.

Groups[a]	Challenge viruses	3 d.p.c[b]		5 d.p.c		7 d.p.c	
		O[c]	C[c]	O	C	O	C
rSNHA-9293	SN	8/10	1/10	5/10	1/10	0/10	1/10
rSNHA-9293	YZ4	2/10	0/10	0/10	0/10	0/10	0/10
rSN-NA_3	SN	5/10	1/10	1/10	0/10	0/10	0/10
rSN-NA_3	YZ4	1/10	0/10	2/10	0/10	0/10	0/10
rSNHA-△200	SN	3/10	1/10	1/10	0/10	0/10	0/10
rSNHA-△200	YZ4	1/10	0/10	0/10	0/10	0/10	0/10
rSN	SN	4/10	1/10	3/10	0/10	0/10	0/10
rSN	YZ4	3/10	0/10	0/10	0/10	0/10	0/10
PBS	SN	9/10	8/10	7/10	7/10	4/10	0/10
PBS	YZ4	8/10	7/10	8/10	6/10	2/10	0/10

[a] Chickens in different groups challenged with 10^6 EID_{50} H9 AIVs SN and YZ4, respectively.
[b] d.p.c.: days post challenge.
[c] Oropharyngeal (O) and cloacal (C) swabs.

chicken antisera [9]. In this study, we analyzed the differences in antigenic epitopes between H9 subtypes Y280-Like strain SN and F98-Like strain YZ4 to construct recombinant viruses with replacement of HA antigenic epitopes (92–93, 145, and 234) of strain SN with that of strain YZ4. rHA_{SN}-R92G-E93K induced significantly higher HI antibody titers and slightly higher neutralization antibody titers against SN and YZ4 than rSN, indicating that the replacement of 92 and 93 amino acids in HA displayed enhanced antigenicity and immunogenicity. However, in the protective efficacy assay, the protection rate provided by rSNHA-9293 against SN challenge was 20%, which was significantly lower than that of wild-type virus SN (80%). The rSNHA-9293 with epitopes from YZ4 provided 80% protection against YZ4 challenge, which was slightly higher than that of wild-type virus SN (70%). This result indicates that 92 and 93 amino acids in HA are vital protective antigenic epitopes.

For production purposes, vaccine candidate strains should replicate effectively in embryonated chicken eggs. We found that rHA_{SN}-NA_3, rHA_{SN}-△200, rHA_{SN}-△287 and rHA_{SN}-R92G-E93K with the internal-gene backbone of PR8 showed less productivity than wild-type virus. To enhance viral replication of the vaccine candidate strains, the internal-gene backbone of vaccine candidate strains was replaced from PR8 to SN. All of the vaccine candidate strains with the internal-gene backbone of SN showed an increased EID_{50} when compared with those of PR8, indicating that the replacement of internal-gene backbone from PR8 to SN in vaccine candidates improved their viral growth and productivity in embryonated chicken eggs due to the avian origin of SN and host tropism [32]. rSNHA-9293, rSN-NA_3 and rSNHA-△200 induced higher neutralization antibody titers against SN and YZ4 than the wild-type virus.

In addition to productivity and immunogenicity, the protective efficacy should be primarily evaluated. The result of antibody surveillance showed that 21 days post primary vaccination, the higher HI titers of rSN-NA_3, rSNHA-9293, and rSNHA-△200 were induced by a single immunization. Compared with chickens immunized with the inactivated wild-type SN, chickens immunized with rSNHA-△200 showed a reduced percentage of viral shedding in the oropharyngeal and cloacal swabs for both SN and YZ4 challenge. The rSNHA-△200 provided 70% protection rate against SN challenge and 90% protection rate against YZ4 challenge, respectively, while in the wild type SN groups, 60% and 70% protection rate was provided, indicating that a cross protection was provided by the rSNHA-△200 against H9N2 AIV challenge.

Generally, the result of serum neutralization assay should be consistent with that of protective efficacy assays. However, it is notable that although rSN-NA_3 and rSNHA-9293 induced higher neutralizing antibody titers against wild-type virus, the protection rate provided was lower than that of wild-type virus. Since inactivated vaccines mainly induce humoral antibody responses but lack mucosal and cellular responses to resist natural infection of AIVs [13], it is reasonable that only a significantly higher neutralizing antibody titer can provide a better cross-protection. Some studies revealed that most neutralizing antibodies responded directly against the HA, which were capable of neutralizing HA binding and/or fusion. More importantly, neutralizing antibodies were sufficient for protection against live virus infection and their concentration showed a negative correlation with disease incidence [33]. Therefore, rSNHA-△200 induced significantly higher neutralizing antibodies and the best protection against both homologous and heterologous H9N2 AIVs, and could be used as a cross-protection vaccine candidate to control and prevent H9N2 AIV infections.

Conflicts of interests

All authors declare that they have no competing interests.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.02.012.

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