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Effects of different HA and NA gene combinations on the growth characteristics of the H3N8 influenza candidate vaccine virus

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ABSTRACT

Since 2022, three human cases of a novel H3N8 avian influenza virus infection have been reported in three provinces in China. Specific vaccines are important means of preparing for the potential influenza pandemic. Thus, H3N8 viruses [A/Henan/cnic410/2022 (HN410) and A/Changsha/1000/2022(CS1000)] were isolated from the infected patients as prototype viruses to develop candidate vaccine viruses (CVVs) using the reverse genetics (RG) technology. Five reassortant viruses with different HA and NA combinations were constructed based on the two viruses to get a high-yield and safe CVV. The results showed that all viruses had similar antigenicity but different growth characteristics. Reassortant viruses carrying NA from CS1000 exhibited better growth ability and NA enzyme activity than the ones carrying HN410 NA. Furthermore, the NA gene of CS1000 had one more potential N-glycosylation site at position 46 compared with HN410. The substitution of position 46 showed that adding or removing N-glycosylation sites to different reassortant viruses had different effects on growth ability. A reassortant virus carrying HN410 HA and CS1000 NA with high growth ability was selected as a CVV, which met the requirements for a CVV. These data suggest that different surface gene combinations and the presence or absence of potential N-glycosylation sites on position 46 in the NA gene affect the growth characteristics of H3N8 CVVs.

1. Introduction

Influenza viruses can infect many hosts, including mammals and birds, causing seasonal epidemics or influenza pandemics. The existing species barrier, humans are mainly infected by subtypes A1 and A3 of the influenza A viruses. However, zoonotic influenza viruses repeatedly cross species barriers and infect humans, causing severe disease or death [1–6].

Since the first H3N8 human infection case in 2022, three human cases of the novel H3N8 avian influenza virus infection, including one death, have been reported in three provinces in China [7,8,9]. These H3N8 viruses are highly identical to the chicken viruses, with six internal genes originating from H9N2 viruses, like prior cross-species avian influenza viruses H7N9 and H10N3[10,11,12]. Although these H3N8 viruses belong to the H3 subtype, they are antigenically distant from contemporary human influenza A (H3N2) viruses. Hence, the seasonal influenza vaccine provides little protection for the human population [13].

These events of human infection with the avian influenza virus have increased global concern and motivated the active development of vaccines and drugs. Vaccines are among the most effective means of preventing influenza. However, the market lacks a mature universal influenza vaccine. Hence, developing specifical, high-yielding, and safe H3N8 candidate vaccine viruses (CVVs) for vaccine production remains crucial. The World Health Organization (WHO) recommends reverse genetics (RG) technology to construct human-infected zoonotic influenza CVV. The (6 + 2) reassortant viruses with hemagglutinin (HA) and neuraminidase (NA) derived from the wild virus in high-yield A/Puerto Rico/8/34 (PR8) virus backbone is the common combination of CVV genes. Although some are sub-standard, most CVVs are high-yielding under this combination [14,15]. Therefore, changing gene combinations remains an effective method to achieve high replication CVVs by replacing or adding other internal genes from wild-type viruses [16,17,18].

Abbreviations: CVV, candidate vaccine virus; WHO, World Health Organization; RG, reverse genetics; HA, hemagglutinin; NA, neuraminidase; PR8, A/Puerto Rico/8/34; HN410, A/Henan/cnic410/2022; CS1000, A/Changsha/1000/2022; SPF, specific pathogen free; NGS, next-generation sequencing; M.O.I, multiplicity of infection; HI, hemagglutination inhibition.

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This study used two viruses [A/Henan/cnic410/2022 (HN410) and A/Changsha/1000/2022(CS1000)], as prototypes to develop CVVs. Reassortant viruses with different HA and NA combinations were analyzed for antigenicity and growth characteristics to determine the effect of different NA on the viral growth capacity. Thus, one reassortant virus carrying HN410 HA and CS1000 NA was selected as the CVV for further pathogenicity evaluation in ferrets.

2. Materials and methods

2.1. Construction of the reassortant virus

The HA and NA genes (Tsingke, China) were synthesized based on the first two human-isolated H3N8 virus (HN410 and CS1000, which carry different motifs (S or G) at position 228 in HA and different numbers of N-glycosylation sites in NA). The genes were cloned into vector pHW2000 plasmid, and the HA 228 site was synthesized as S of HN410 and G of CS1000, respectively. Next, K and N were synthesized at site 46 of the NA gene of HN410 and CS1000, respectively. Reassortant viruses (6 + 2) were rescued in Vero cells, as described previously [19]. These reassortant viruses were cultivated in specific pathogen-free (SPF) eggs and stored at −70 °C until further analyses. All viruses were sequenced using next-generation sequencing (NGS) on the Illumina NextSeq 550 Sequencing System platform (Illumina, CA, USA) to identify their genetic origin and analyzed by the BioEdit (Version 7.1.3.0) software.

2.2. Growth kinetics assay

Here, MDCK cells were used to investigate the growth characteristics as described previously [20]. Reassortant viruses were infected at an multiplicity of infection (M.O.I) of 0.001, and supernatants were collected at specific time points (24, 48, 72, and 96 h post-infection) and stored at -70 °C before processing. The viral infective titer was measured by a hemagglutination assay with 1 % turkey erythrocytes to calculate the $TCID_{50}$ [21]. The data were analyzed by two-way ANOVA using the GraphPad Prism 5 software package (version 5.0).

2.3. Neuraminidase activity assay

The NA-Fluor™ Influenza Neuraminidase Assay Kit (AB, Cat No. 4457091) was used to test the NA activity following the kit manual and previous reports [22]. The NA-Fluor™ buffer solution was the control, and the diluted standard product (Sigma, MI, USA) was detected in parallel to generate the standard curve. The fluorescence intensity was determined using the EnSight Multimode Plate Reader (PE, MA, USA) at an excitation of 360 and an emission of 460 nm, respectively.

2.4. Virus elution assay

The virus elution assay was performed, as described previously [22]. Virus preparations adjusted to a HA agglutination units (HAUs) of 128 and 1 % turkey erythrocytes were used.

2.5. Antigenic analysis assay

Antigenic analyses were performed using Hemagglutination inhibition (HI) tests using 1 % turkey blood cells and following standard protocols [21].

2.6. Ferret test

The serologically negative ferrets for the currently circulating influenza A and B viruses and the test virus were purchased from Wuxi Sangosho Biotechnology Co., Ltd. (Jiangsu, China). Before infection, ferrets were randomly housed individually in isolation units. Four-month-old ferrets (n = 4) were intranasally inoculated with 10^6 $TCID_{50}$ of the test virus in 1 ml PBS following the WHO guidelines. Tissue samples were collected as previously described [23].

2.7. Ethics approval and consent to participate

The animal experiments were conducted following the approved Guidelines for Animal Experiments described by the Animal Health and Welfare Committee of the National Institute for Viral Disease Control and Prevention of China CDC (Approved No.20220314035).

3. Results

3.1. Confirmation of reassortant viruses

The sequence results indicated that all viruses were identical to the original design. All viruses retained their designed amino acid sequence at sites 228 and 46 in HA and NA genes, respectively. Table 1 shows the sources of these viruses. RG-HNHA-CSNA has the same HA gene as RG-HN410 and the same NA gene as RG-CS1000 virus. There is only one amino acid difference between RG-HN410 and RG-HN410-M viruses at position 46 in the NA gene. Moreover, RG-CS1000 and RG-CS1000-M also differ at position 46 in the NA gene with one amino acid.

3.2. Growth characterization of reassortant viruses

The viral titer was tested in MDCK cells to compare the replication efficiency of different viruses from different gene combinations. RG-CS1000 and RG-HNHA-CSNA replication was similar to the high-yield PR8, and both showed significantly better replication than RG-HN410 at all post-infection time points (Fig. 1A). There was no significant difference between RG-CS1000 and RG-HNHA-CSNA (Fig. 1A) at all the time points.

Next, we constructed two mutational reassortant viruses, RG-HN410-M with K to N substitution and RG-CS1000-M with N to K substitution, to learn the influence of the virus replication on the change of position 46 in the NA gene. The growth ability of RG-HN410-M, with one more N- glycosylation site in NA, was significantly improved compared to that of RG-HN410 (Fig. 1B). However, there was no significant difference in the growth ability of RG-CS1000-M and RG-CS1000 (Fig. 1C).

3.3. NA function of different reassortant viruses

The standard curve of NA enzyme activity was obtained following the manufacturer's instructions to deduce the enzyme activities of different viruses. Thus, the NA enzyme activity of RG-HN410 was

Table 1
List of Reassortant Viruses.

Virus Abbreviation	Amino acid motif		Surface genes	Six internal genes
	HA-228	NA-(46–47-48)		
RG[a]-HN410[b]	S	K-E-T	HA and NA from wild virus HN410	PR8[c]
RG-CS1000[d]	G	N-E-T	HA and NA from wild virus CS1000	PR8
RG-HNHA-CSNA	S	N-E-T	HA from wild virus HN410 and NA from wild virus CS1000	PR8
RG-HN410-M	S	N-E-T	HA and NA(46 K→N) from wild virus HN410	PR8
RG-CS1000-M	G	K-E-T	HA and NA(46 N→K) from wild virus CS1000	PR8

Note: a, reverse genetic; b, HN410: A/Henan/cnic410/2022(H3N8); c, PR8: A/Puerto Rico/8/34(H1N1); d, CS1000: A/Changsha/1000/2022(H3N8).

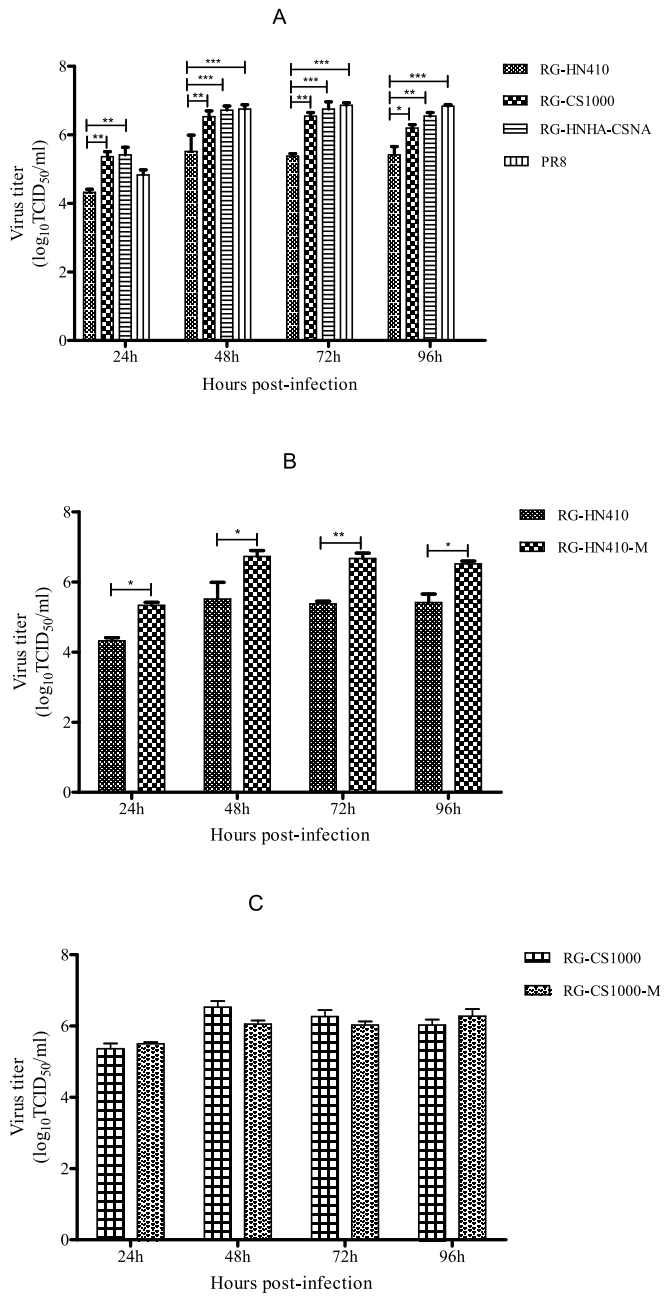

Fig. 1. Growth characteristics of different viruses. (A) Growth kinetics of different combination of viruses in MDCK cells. (B) Comparison of the growth capacity of RG-HN410 and RG-HN410-M viruses. (C) Comparison of the growth capacity of RG-CS1000 and RG-CS1000-M viruses. Cells were infected at a M.O.I. of 0.001. Data were expressed as mean ± SD from the three independent experiments and analyzed by two-way analysis of variance (ANOVA) using the GraphPad Prism 5 software package (version 5.0). (*, $P<0.05$, **, $P<0.01$, ***, $P<0.001$).

significantly lower than that of RG-CS1000 and RG-HNHA-CSNA (Fig. 2A). There was no significant difference in the NA enzyme activity between RG-HN410 and RG-HN410-M viruses, and between RG-CS100 and RG-CS1000-M. RG-CS1000 and RG-HNHA-CSNA viruses exhibited optimal NA enzyme activity. Although viruses contain the same motifs at sites 46–48 in the NA gene, RG-CS1000-M exhibited higher enzyme activity than RG-HN410, and RG-CS1000 exhibited higher enzyme activity than RG-HN410-M (Fig. 2A).

All viruses remained agglutinated for four hours at 37 ℃ in elution assay. RG-CS1000 and RG-HNHA-CSNA reduced from 128 to 64 HAUs from the fifth to sixth hours of observation (Fig. 2B). RG-HN410 remained agglutinated at 128 HAUs during the observation period (Fig. 2B). The elution ability difference among viruses was less than two folds, RG-CS1000 and RG-HNHA-CSNA showed the best elution ability, consistent with the NA enzyme activity.

3.4. Antigenic analysis of the reassortant virus

Hemagglutination inhibition assays were performed using ferret antisera to identify different viruses and their antigenic properties. The antigenic analysis indicated that three antisera could markedly inhibit all the tested viruses. The difference in HI titer was < 4 folds in all the detected viruses (Table 2). The antigenicity of all viruses was consistent, and the difference at position 228 in the HA gene did not affect antigenicity.

3.5. Pathogenicity of the reassortant viruses in the ferret

As a CVV, safety tests on ferrets are needed to verify the virus attenuation. Combined with the above experimental results, RG-HNHA-CSNA was chosen as the CVV for the pathogenicity test. During the two-week observation, the infected animals showed no clinical symptoms. The virus replication was limited to the respiratory tract, including lung and nasal wash, and the viral titer was lower than for the PR8 virus (Table 3) [23]. The virus was not detected in other tissues and organs.

4. Discussion

H3N8 was detected frequently in poultry and wild birds in mainland China and was associated with persistent infection outbreaks in many mammals [24–26]. In 2022, a new subtype, H3N8, was added to the list of avian influenza viruses spreading across species to infect humans in China [7]. In the first two infection cases (HN410 and CS1000), different amino acids were expressed at 228 key sites: HN410 haemagglutinin has a mixed amino acid (G/S), and 228G residues were found in the HA of CS1000 [8]. The appearance of 228S in HA could enhance avian and human receptor-binding capacities, which is critical for viral entry into human epithelial cells [27,28]. The patient in the third case of H3N8 infection in Guangdong, China, died due to immune deficiency [9]. As with other avian influenza cases, those infected with H3N8 influenza had a history of exposure to poultry [7–9]. Whenever a novel influenza virus appears to cross species to infect humans, rapid access to CVV is one of the effective measures to deal with a potential pandemic. Thus, CVVs must be highly productive and safe while keeping the antigens unchanged.

In this study, PR8 was used as a skeleton to construct different combinations of HA and NA gene reassortant viruses to screen high-yield and safe CVVs. The results showed that the growth capacity of RG-HN410 virus was significantly lower than that of RG-CS1000. Further, the growth ability of the 228 site mutation of the RG-HN410 mutant (S228G in HA gene) from S to G was similar to those of the RG-HN410 virus (data not shown), excluding the effects of the 228 sites in HA. Thus, different NAs may affect the growth ability of different combinations of viruses. The experiment confirmed that viruses (RG-HNHA-CSNA and RG-CS1000) carrying the same CS1000 NA gene have similar growth and NA enzyme activity levels. The NA enzyme active site of the influenza virus is considered to be highly conserved. RG-HN410 and RG-CS1000 also remained unchanged and had the same enzyme activity sites. Further analysis showed that the NA of RG-HN410 lacks one potential N-glycosylation site at position 46 compared to that of RG-CS1000. Some studies have shown that N-glycosylation or N-deglycosylation on NA genes affected the biological characteristics of the virus [29–31]. Adding N-glycosylation sites had little effect on the NA enzymatic activity of RG-HN410-M but significantly improved its growth ability. However, removing the N-glycosylation site had no significant impact on the NA enzymatic activity and growth capability of RG-

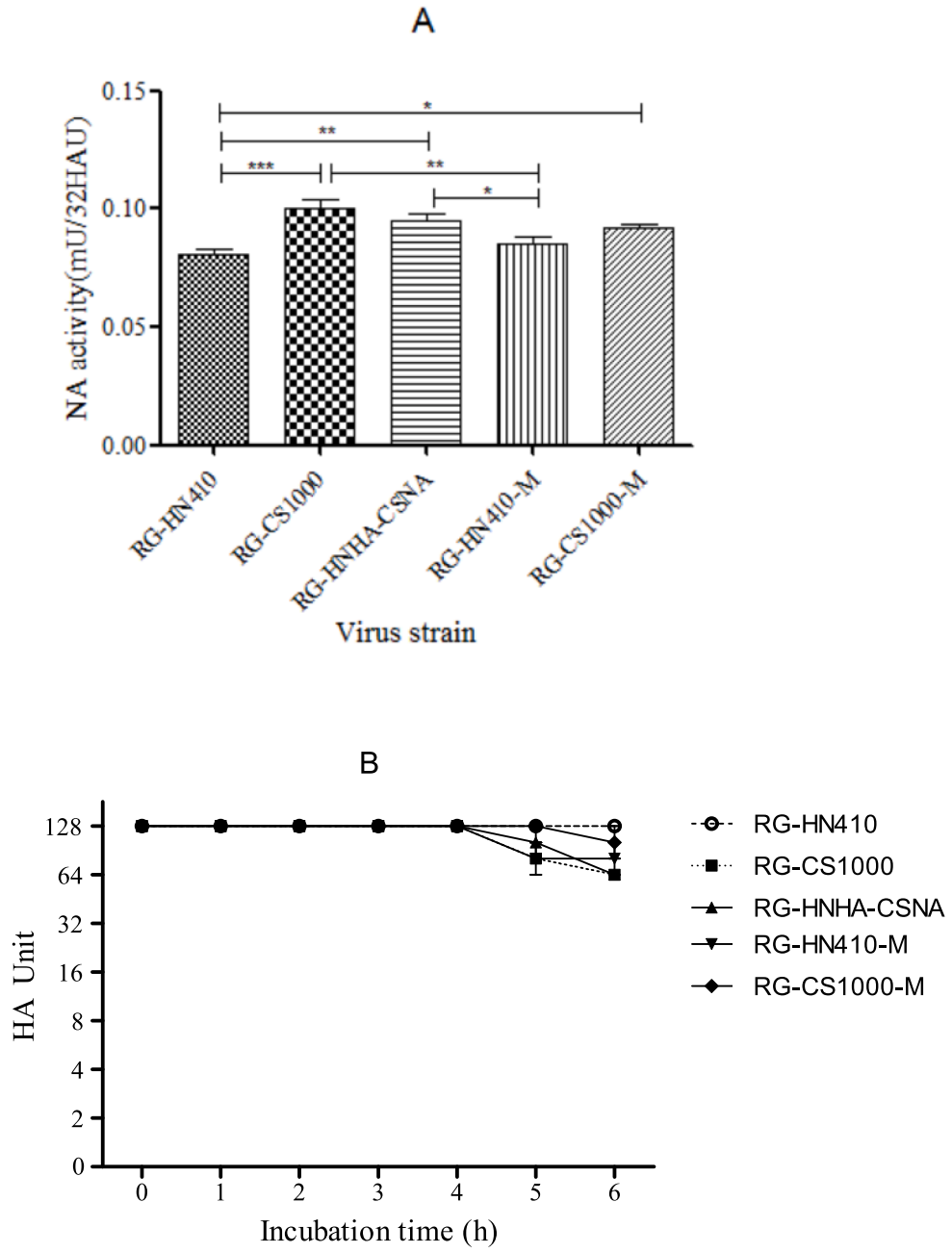

Fig. 2. NA function of different viruses. (A) NA enzyme activity of different viruses was deduced from the standard curve. Data were expressed as mean ± SD from the three independent experiments and analyzed by One-way ANOVA using the GraphPad Prism 5 software package (version 5.0). (*, $P<0.05$, **, $P<0.01$, ***, $P<0.001$) (B) Elution of different viruses from erythrocytes. 1 % turkey erythrocytes were used. Data were expressed as mean ± SD from the three independent experiments.

Table 2
HI titer of Different Viruses.

Viruses [a]	Antisera		
	RG-HN410	RG-CS1000	RG-HNHA-CSNA
RG-HN410	**320**[b]	320	320
RG-CS1000	160	**320**	160
RG-HNHA-CSNA	160	320	**320**
RG-HA410-M	320	320	320
RG-CS1000-M	160	160	320

Note: a. the source of antigen is shown in Table 1.b. values in bold indicated homologous activity.

CS1000-M. These results indicate the strong strain specificity of influenza virus and that the optimal virus fitness results from multiple factors [32].

Besides high yield, the CVV also needs to be low in pathogenicity. In this study, RG-HNHA-CSNA showed no clinical symptoms in animals, and the virus was detected only in the respiratory tract. These results meet the WHO requirements of low pathogenic characteristics of CVV [33].

Therefore, different combinations of HA and NA genes have important effects on the growth characteristics of RG viruses. Although viral growth characteristics result from multiple factors, potential N-glycosylation at the NA 46 site plays an important role. Importantly, this

Table 3
Virus Titer in Different Organs of Ferrets.

Virus	Animal No.	Nasal washes [a]				nasal turbinate [b]	Lung [b]	Other organs [b, c]
		Day 1	Day 3	Day 5	Day7	Day 3	Day 3	Day 3
RG-HNHA-CSNA	1	3.25	3.25	1.76	/			
	2	3.0	3.0	1.89	/			
	3					2.33	1.62	/ [d]
	4					2.68	2.12	/

Note. a, $log_{10}TCID_{50}$/ml. b, $log_{10}TCID_{50}$/g. c, Other organs include brain, spleen, intestine and olfactory bulb of the brain. d, /, denote no virus titer was detected.

study screened a suitable virus, RG-HNHA-CSNA, that meets the requirements for a CVV.

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CRediT authorship contribution statement

Liqi Liu: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Zi Li:** Methodology. **Jia Liu:** Investigation. **Xiyan Li:** Methodology. **Jianfang Zhou:** Methodology. **Ning Xiao:** Methodology. **Lei Yang:** Investigation. **Dayan Wang:** Writing – review & editing, Resources, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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