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LETTER

# Inactivated H9N2 vaccines developed with early strains do not protect against recent H9N2 viruses: Call for a change in H9N2 control policy

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H9N2 virus has been widely distributed in wild birds and poultry around the world since its first emergence in the United States of America in 1966 (Gu *et al.* 2017; Carnaccini and Perez 2020). The virus appeared in chickens in China in the early 1990s, and over the last two decades has gradually become the dominant epidemic subtype (Sun and Liu 2015; Bi *et al.* 2020). Although H9N2 virus infection alone cannot cause severe disease or death in poultry, H9N2 virus-infected birds experience a degree of egg production drop and can be easily infected by other pathogens, thus causing economic losses for poultry industry. Studies have indicated that certain avian influenza viruses, including H7N9, H10N8, and H3N8, are more likely to infect humans after acquiring the internal genes of the H9N2 virus (Gao *et al.* 2013; Shi *et al.* 2013; Liu *et al.* 2015; Yang *et al.* 2022; Xu *et al.* 2023). More importantly, H9N2 viruses have acquired the ability to exclusively bind to human-type receptors (Li *et al.* 2014) and have infected 114 humans in nine countries according to the sequence information available in Global Initiative on Sharing Avian Influenza Data (GISAID); 103 of these infections occurred in China. Therefore, efficient control of H9N2 virus is important not only for poultry industry but also for human public health.

Inactivated vaccines against H9N2 virus have been widely used in chickens in China since 1998. Currently, 54 kinds of vaccines, including 31 multivalent vaccines, are used in China for H9N2 influenza control (Dong *et al.* 2022). A previous study by Li *et al.* (2019) indicated that chickens that had high levels of hemagglutinin inhibition (HI) antibodies could still be infected by H9N2 virus in the live poultry markets, suggesting that the widely used poultry H9N2 vaccines may not protect against the currently circulating H9N2 viruses.

To gain insight into the protective efficacy of the H9N2 vaccines to H9N2 viruses circulating in recent years, we selected and evaluated a commercial vaccine produced with a virus isolated in 1990s (to avoid unnecessary

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trouble, we have hidden the strain information of the vaccine in this article, and hereafter referred to as “early-virus”) as the seed virus, which was approved for use in the early 2000s. The homology of the HA gene of “early-virus” with that of seven representative viruses detected in 2019–2021 ranged from 92.5 to 93.9% at the nucleotide level and from 92.7 to 95.0% at the amino acids level (Fig. 1-A). To understand the antigenic relevance of “early-virus” to these viruses, we evaluated the cross-reactivity of “early-virus” antiserum against these viruses

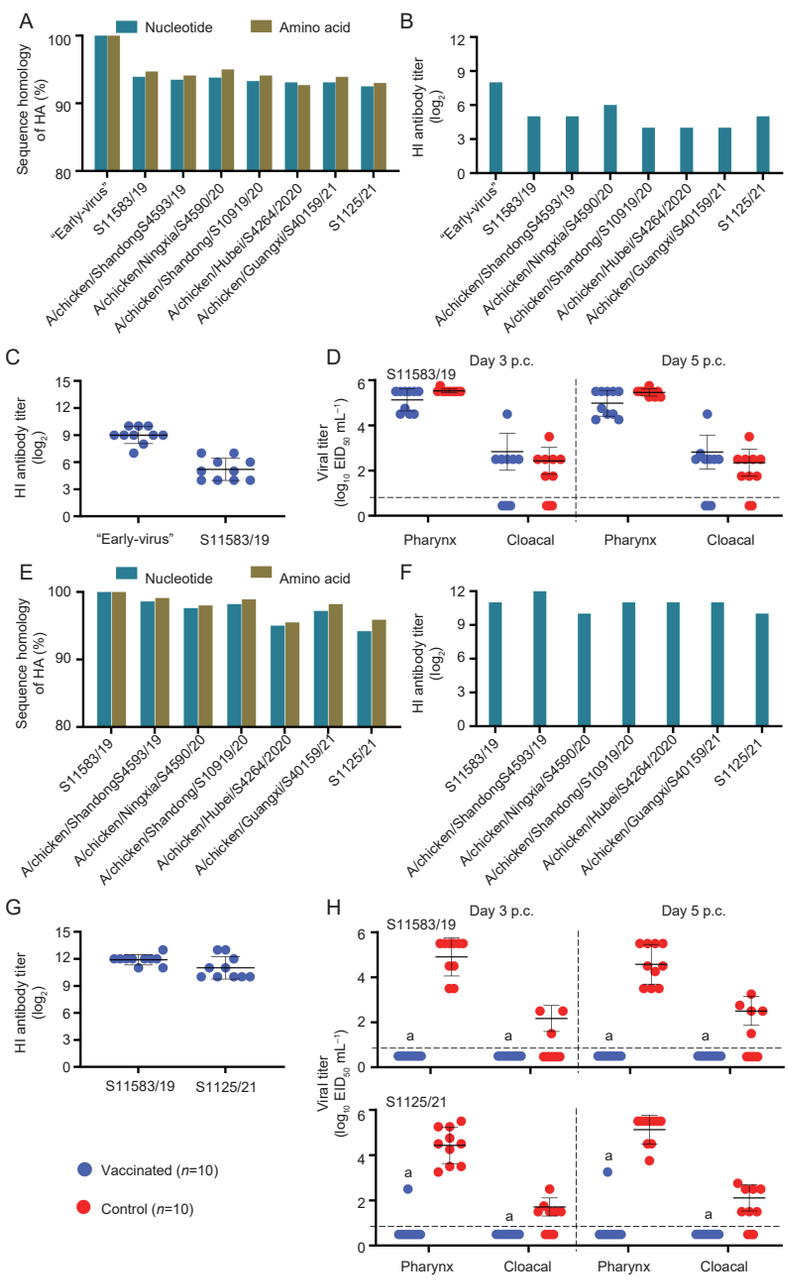


**Fig. 1** Protective efficacy of two different H9N2 vaccines against recently circulating H9N2 viruses. A, the homology of the HA of “early-virus” with that of recently circulating H9N2 viruses at the nucleotide level and at the amino acids level. B, cross-reactivity of “early-virus” antiserum against different H9N2 viruses. C, HI antibody titers of “early-virus” vaccine-inoculated chickens against the vaccine seed virus and the indicated challenge virus. D, virus shedding titers of “early-virus” vaccine inoculated chickens after challenge with S11583/19. The error bars represent the standard deviations. E, the homology of the HA of S11583/19 with that of recently circulating H9N2 viruses at the nucleotide level and at the amino acids level. F, cross-reactivity of S11583/19 antiserum against different H9N2 viruses. G, HI antibody titers of S11583/19 vaccine-inoculated chickens against the vaccine seed virus and the indicated challenge virus. H, virus shedding titers of S11583/19 vaccine inoculated chickens after challenge with the indicated viruses. The error bars represent the standard deviations. The dashed lines in panels D and H indicate the lower limit of virus detection. The letter ‘‘a’’ indicates $P<0.001$ compared with the corresponding titers of control birds.

in an HI assay. The "early-virus" antiserum antibody titer against the homologous virus was 256, whereas the HI antibody titers against the seven viruses ranged from 16 to 64, which were 4- to 16-fold lower than that to the homologous titer (Fig. 1-B).

To evaluate the protective efficacy of the "early-virus" vaccine, groups of 10 3-week-old white leghorn specific pathogen free (SPF) chickens were injected intramuscularly with 0.3 mL of the vaccine or with PBS as a control. Three weeks post-vaccination (p.v.), sera were collected from the chickens and assessed for HI antibody titers against the vaccine strain and the representative virus A/chicken/Guangxi/S11583/2019(H9N2) (hereafter referred to as S11583/19). The chickens were then challenged intranasally with 10 50% chicken infectious dose ($CID_{50}$) of S11583/19. Oropharyngeal and cloacal swabs of all chickens were collected on day 3 and 5 post-challenge (p.c.) to detect the virus in eggs. Birds were observed for disease and death for 2 weeks p.c.

At 3 weeks p.v., the mean HI antibody titers in chickens against the vaccine seed virus and S11583/19 were 8.8 $log_2$, and 5.2 $log_2$ respectively (Fig. 1-C). After challenge with S11583/19, all 10 chickens in the control group shed high titers of virus through the pharynx on day 3 and day 5 p.c., respectively, and seven and eight of the 10 chickens shed virus through the cloaca on days 3 and day 5 p.c., respectively (Fig. 1-D); all 10 chickens in the vaccinated group also shed high titers of virus through the pharynx on day 3 and day 5 p.c., respectively, and six and seven of the 10 chickens shed virus through the cloaca on day 3 and day 5 p.c., respectively (Fig. 1-D). All of the birds in the vaccinated groups and control groups were apparently healthy and survived during the 14-day observation period. These results indicate that the "early-virus" vaccine is immunogenic in chickens, but cannot prevent the replication and shedding of the current H9N2 virus.

To investigate whether a vaccine produced with a more recent strain could provide better protection, we selected the S11583/19 as the seed virus for the vaccine preparation. The homology of the HA gene of the S11583/19 to that of the other six representative viruses detected in 2019–2021 ranged from 94.2 to 98.6% at the nucleotide level and from 95.5 to 99.1% at the amino acids level (Fig. 1-E). The S11583/19 antiserum reacted well with the six other viruses detected in 2019–2021, with a titer difference of less than 2-fold compared with that to the homologous titer (Fig. 1-F).

To evaluate the protective efficacy of the S11583/19 vaccine, we used two viruses for the challenge study: S11583/19 and the 2021 virus A/chicken/Jilin/S1125/2021 (hereafter referred to as S1125/21). At three weeks p.v. with the S11583/19 vaccine, the mean HI antibody titers in chickens against S11583/19 and S1125/21 were 11.9 $log_2$ and 11 $log_2$, respectively (Fig. 1-G). All of the 10 chickens in the control group that were challenged with S11583/19 shed high titers of virus through the pharynx on day 3 and day 5 p.c., respectively, and three and five of the 10 chickens shed virus through the cloaca on day 3 and day 5 p.c., respectively (Fig. 1-H). The 10 chickens in the vaccinated group did not shed virus at any timepoint after the S11583/19 challenge (Fig. 1-H). All 10 chickens in the control group that were challenged with S1125/21 shed virus through the pharynx on day 3 and day 5 p.c., respectively, and six and seven of the 10 chickens shed virus through the cloaca on day 3 and day 5 p.c., respectively (Fig. 1-H). However, in the vaccinated group, virus shedding was only detected from the pharynx of one chicken (Fig. 1-H). All of the birds were apparently healthy and survived during the 14-day observation period. These results indicate that S11583/19 vaccine provides better protection than the "early-virus" vaccine against recent H9N2 virus infection.

Different subtypes of avian influenza viruses have been detected in nature, and control of highly pathogenic avian influenza is always a high priority; however, the low pathogenic avian influenza viruses, such as H9N2, are somewhat forgotten or "neglected" (Gu *et al.* 2017). Influenza virus easily mutates, and mutations in the HA gene often alter the antigenicity of influenza viruses, allowing the virus to escape vaccine-induced host immunity (Liu *et al.* 2022; Zhang *et al.* 2023). The key to a successful influenza vaccination strategy is to ensure that the vaccine seed virus is always antigenically matched to the target virus.

In China, the vaccines used for H5 and H7 avian influenza control are carefully evaluated and updated in a timely manner to ensure they provide solid protection against viruses that pose a risk (Zeng *et al.* 2018, 2020, 2022). Ten different H5 vaccine seed viruses have been generated and successively used to control the H5 viruses bearing different clades of HA gene that were introduced into China over the past two decades (Shi *et al.* 2023). Every H5 vaccine used in China specifically targets strains that pose a direct threat to Chinese poultry. When the virus is eliminated, the vaccine against this particular strain is no longer used (Zeng *et al.* 2024). Similarly, the vaccine seed virus has been updated three times for H7N9 avian influenza control since September 2017 (Shi *et al.* 2018; Yin *et al.* 2021; Hou *et al.* 2024). However, none of the H9N2 vaccines have been updated, even though some of them were developed with seed viruses that were isolated more than 20 years ago (Dong *et al.* 2022). In this study, we tested one of the H9N2

vaccines that were developed 20 years ago and found that it did not prevent chickens from being infected by the current H9N2 virus, and we speculate that many of the other H9N2 vaccines currently in use may not provide better protection than the "early-virus" vaccine against the current H9N2 strains. The S11583/19 vaccine provided complete protection against homologous virus challenge and prevented 90% of the chickens from being infected with S1125/21, suggesting that an antigenically well-matched vaccine can provide sound protection against H9N2 virus challenge.

In summary, our study suggests that the H9N2 influenza vaccines currently used in China cannot protect chickens from being infected with the currently circulating H9N2 virus, and a strain-surveillance-based vaccine update strategy and policy, such as that used for highly pathogenic avian influenza vaccines, should be established for H9N2 vaccine development and application.

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## Declaration of competing interest

The authors declare that they have no conflict of interest.

## Ethical approval

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The protocol for the animal studies was approved by the Committee on the Ethics of Animal Experiments of Harbin Veterinary Research Institute, CAAS (230224-03-GR).

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