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BRIEF REPORT

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Neutralization potency of the 2023-24 seasonal influenza vaccine against circulating influenza H3N2 strains

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ABSTRACT
Seasonal influenza is a severe disease that significantly impacts public health, causing millions of infections and hundreds of thousands of deaths each year. Seasonal influenza viruses, particularly the H3N2 subtype, exhibit high antigenic variability, often leading to mismatch between vaccine strains and circulating strains. Therefore, rapidly assessing the alignment between existing seasonal influenza vaccine and circulating strains is crucial for enhancing vaccine efficacy. This study, based on a pseudovirus platform, evaluated the match between current influenza H3N2 vaccine strains and circulating strains through cross-neutralization assays using clinical human immune sera against globally circulating influenza virus strains. The research results show that although mutations are present in the circulating strains, the current H3N2 vaccine strain still imparting effective protection, providing a scientific basis for encouraging influenza vaccination. This research methodology can be sustainably applied for the neutralization potency assessment of subsequent circulating strains, establishing a persistent methodological framework.

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Introduction

Seasonal influenza, caused by influenza viruses, is a highly contagious respiratory disease. As per the World Health Organization (WHO) records, there are approximately 3 to 5 million severe influenza cases and 290,000 to 650,000 influenza-related deaths each year worldwide.[1,2] Influenza virus can infect individuals across all age groups, among whom the elderly, young children, and individuals with underlying health conditions are high-risk populations with severe disease after infection. Presently, with the lifting of COVID-19 restrictions, there is a notable uptick in respiratory diseases in winter caused by various pathogens, including influenza. In China, an unprecedented outbreak of influenza occurred in the winter of 2023, of which a total of 2,120 influenza-like illness (ILI) outbreaks have been reported from October to December revealed by the Chinese National Influenza Center. The predominant circulating subtype was influenza A (H3N2), followed by influenza B (Victoria) lineage. Getting influenza vaccination annually stands out as a pivotal measure for preventing influenza, as well as mitigating influenza outbreaks.[3] However, seasonal influenza viruses, particularly the H3N2 subtype, exhibit a remarkable evolving nature, also known as "antigenic drift." To maintain vaccine efficacy, the WHO routinely updates the compositions of influenza vaccine to align with most circulating influenza strains.[4]

Repeated outbreaks of influenza have raised concerns regarding the effectiveness of influenza vaccine. For the 2023–24 northern hemisphere influenza season, the WHO recommends A/Darwin/9/2021(H3N2)-like virus as the H3N2 strain of egg-based vaccines and A/Darwin/6/2021 as cell culture-based vaccines. These two strains exhibit similar antigenicity. Questions emerge that how well does this vaccine strain aligns with the circulating influenza H3N2 strains and does the influenza vaccination still offer protective effects? To address these concerns, we conducted this study.

Using a pseudovirus platform as a foundation, this study evaluated the alignment between the 2023 and 2024 seasonal influenza vaccine H3N2 strain (A/Darwin/6/2021) and currently circulating influenza strains. The results emphasize that the WHO-recommended vaccine strain could confer effective protection against influenza, offering a scientific basis for advocating influenza vaccination.

Materials and methods

Cell lines

HEK293T (American Type Culture Collection, CRL-3216) and MDCK (American Type Culture Collection, CCL-34)

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cells were cultured in a 5% CO_2 environment at 37°C in high-glucose DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin–streptomycin solution (100 U/ml), and 20 mM *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Gibco). About 0.25% Trypsin-EDTA (Gibco) was used to detach cells for subculture at intervals of 2–3 days.

Generation and production of H3N2-fluc pseudovirus

The generation of pseudoviruses was conducted as described previously.[5,6] In brief, the H3N2 influenza virus hemagglutinin (HA) protein expression plasmids were constructed by inserting HA genes into the pcDNA3.1. The HA genes use in this study were sourced from Global Initiative for Sharing All Influenza Data (GISAID), including A/Hebei-Cixian/1181/2023 (EPI_ISL_17465752), A/NARA/21/2023 (EPI_ISL_17952529), A/Wisconsin/20/2023 (EPI_ISL_17100843), A/New York/IVYG43Q07S9/2023 (EPI_ISL_18005250), A/South Africa/R04355/2023 (EPI_ISL_17891586), A/YOKOHAMA/31/2023 (EPI_ISL_17801740), A/Mauritius/P08894/2023 (EPI_ISL_17223808), A/Sedbury/2992/2023 (EPI_ISL_17200366), A/Massachusetts/08/2023 (EPI_ISL_17101153) and A/Ashgabad/40S/2023 (EPI_ISL_16970447). Subsequently, HEK293T cells were co-transfected with HA protein expression plasmid and human immunodeficiency virus (HIV) backbone plasmid (pSG3.Δenv-FlucΔnef) using transfection reagent Lipofectamine 3000 (Invitrogen) at a ratio of 1:2. After 18 h of transfection, neuraminidase from Clostridium (Aladdin) was added to release the newly packaged pseudotyped viruses. The supernatant containing the pseudovirus was harvested 48 h later. Add TPCK-trypsin (Sigma-Aldrich) to the collected pseudovirus for enzymatic cleavage at a concentration of 40 μg/ml and then incubate it at 37°C in a 5% CO_2 incubator for 30 min. After centrifugation at 4000×g for 10 min, filter the mixture through a 0.45 μm filter membrane. Aliquot the samples and store them at −80°C.

Titration of the influenza H3N2 pseudotyped viruses

The pseudovirus titer was assessed by infecting MDCK cells with threefold serial dilutions of the pseudovirus. Trypsin-treated MDCK cells were added to 96-well culture plates (100 μl per well). After 24 h of incubation at 37°C with 5% CO_2, chemiluminescence signals were detected using the Britelite plus reporter gene assay system (PerkinElmer). The 50% tissue culture infectious dose ($TCID_{50}$) was calculated using the Reed-Muench method, as described previously.[7,8]

Serum sample

Fifty pairs of serum samples were collected from human subjects both before and 4 weeks after trivalent inactivated split-virion influenza vaccine vaccination. Prior exposure to seasonal influenza in these donors was unknown. These samples were obtained from the Shaanxi Provincial Centre for Disease Control and Prevention, China. The study protocol was conducted in accordance with the "Guidelines for the laboratory management of Biological samples analysis in Drug clinical trials" issued by Chinese Food and Drug Administration in 2011 and approved by the Ethics Committee of the Shaanxi Provincial Centre for Disease Control and Prevention in China. Written informed consent was obtained from all volunteers prior to blood collection.

Pseudovirus-based neutralizing assay (PBNA) in vitro

Neutralization was assessed by measuring the reduction in luciferase gene expression. Serum samples (initially diluted at 1:20, then serially diluted three-fold) were mixed with 1.3 × 10^4 $TCID_{50}$ of influenza H3N2 pseudoviruses in duplicate wells of 96-well flat-bottom culture plates and incubated for 1 h at 37°C. This setup included virus control and cell control wells, each in six replicates. Subsequently, freshly trypsinized MDCK cells were added to each well and incubated for 24 h. Infectivity was determined by measuring bioluminescence, as described previously.[9] The 50% effective dilution (ED_{50}) was calculated using the Reed-Muench method. Results were recorded as the mean of three replicates.

Statistical analysis

Statistical analysis was performed using Prism 8 software (GraphPad, San Diego, CA). Results were calculated and presented as means ± standard error of the mean (SEM). Unpaired two-tailed Student's *t*-tests were employed for comparing two groups of data. Significance thresholds were set as follows: *$p < .05$, **$p < .01$, ***$p < .005$, and ****$p < .0001$.

Results

The selection and analysis of representative circulating influenza H3N2 strains

Utilizing phylodynamic data on influenza viruses provided by the GISAID database, we identified a total of 10 major clades of circulating H3N2 as of December 2023 (Figure 1). Within these 10 clades, we selected 10 strains with the highest frequency of circulation as the representatives for each clade (Table 1). Analysis reveals that these representative H3N2 strains exhibit varying degrees of amino acid mutation on HA as compared with the vaccine strain (A/Darwin/6/2021) (Figure 2), raising concerns regarding the influenza vaccine efficacy.

Neutralization potency of the influenza vaccine against circulating H3N2 strains

Pseudoviruses serve as valuable virological tools in the study of viruses due to their safety and versatility.[10] Utilizing an HIV pseudotyped virus production system, we developed a pseudovirus-based neutralization assay targeting the influenza H3N2 virus. HA amino acid sequences of representative H3N2 strains were synthesized, and pcDNA3.1-HA expression plasmids were generated. Based on the HIV pseudovirus packaging system, we successfully constructed nine pseudoviruses, including eight circulating strains representing the 2023–24 seasonal influenza H3N2 strains and the vaccine H3N2 strain (A/Darwin/6/2021).

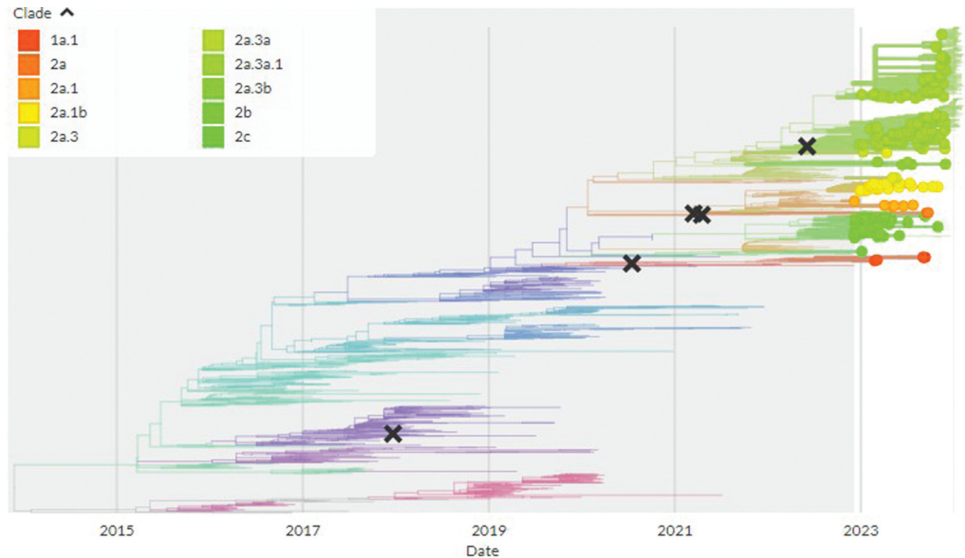

Figure 1. The global phylodynamics of influenza H3N2 virus (cited from GISAID).

Table 1. The representative strain of influenza H3N2 virus in 2023.

Clade	Isolate ID	Isolate name	Frequency
3C.2a1b.2a.1a.1	EPI_ISL_17465752	A/Hebei-Cixian/1181/2023	2
3C.2a1b.2a.2a.1	EPI_ISL_17952529	A/NARA/21/2023	62
3C.2a1b.2a.2a.1b	EPI_ISL_17100843	A/Wisconsin/20/2023	628
3C.2a1b.2a.2a.3	EPI_ISL_18005250	A/New York/IVYG43Q07S9/2023	30
3C.2a1b.2a.2a.3a.1	EPI_ISL_17891586	A/South Africa/R04355/2023	596
3C.2a1b.2a.2a.3a	EPI_ISL_17801740	A/YOKOHAMA/31/2023	59
3C.2a1b.2a.2a.3b	EPI_ISL_17223808	A/Mauritius/P08894/2023	26
3C.2a1b.2a.2a	EPI_ISL_17200366	A/Sedbury/2992/2023	3
3C.2a1b.2a.2c	EPI_ISL_16970447	A/Ashgabad/40S/2023	5
3C.2a1b.2a.2b	EPI_ISL_17101153	A/Massachusetts/08/2023	387

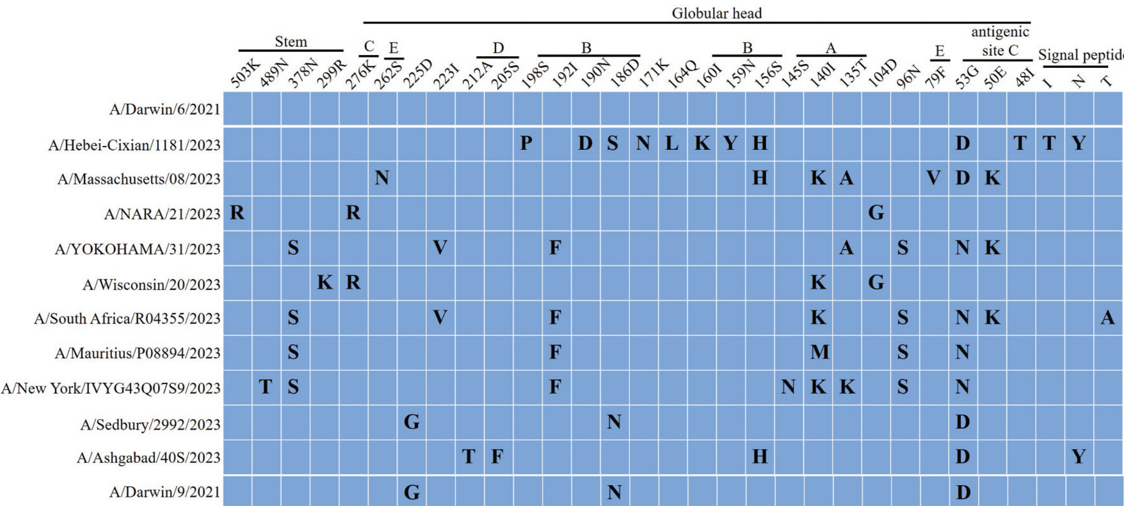

Figure 2. Amino acid mutation sites in HA of representative circulating H3N2 strains as compared with the vaccine strain.

However, attempts to package pseudoviruses for A/Sedbury/2992/2023 and A/Ashgabad/40S/2023 were unsuccessful.

Blood samples were collected from 50 volunteers before and 28 days after influenza vaccination. Serums obtained from these samples was then tested for neutralizing antibody levels against nine different influenza H3N2 viruses generated from the pseudovirus platform, providing insights into the matching status between the vaccine strain and circulating strains.

As depicted in Figure 3, we observed that the neutralizing antibody levels post-vaccination increased significantly against all nine strains. The mean ED_{50} values of neutralizing

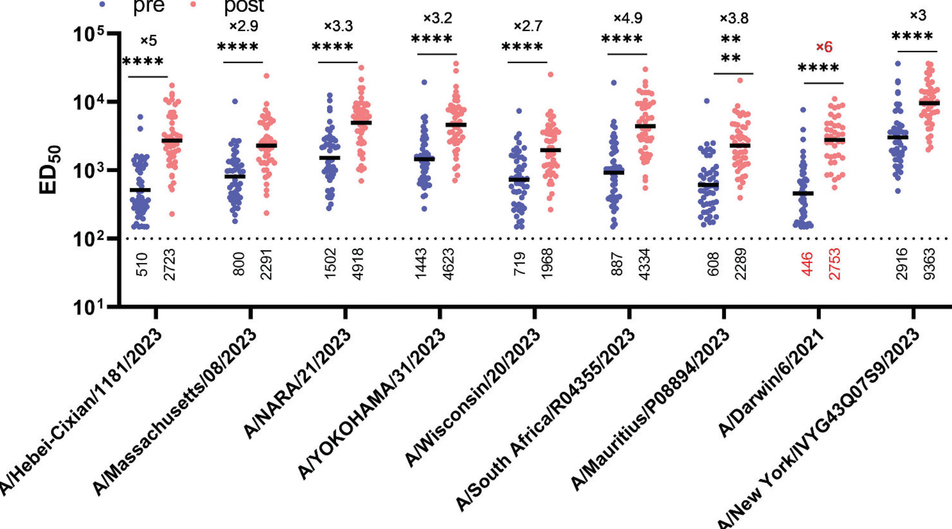

Figure 3. Neutralizing antibody levels against the pseudovirus of 2023-24 seasonal influenza H3N2 strains and influenza vaccine H3N2 strain. Blue dots represent pre-vaccination, and red dots represent post-vaccination. The bold black line represents the geometric mean titer of the data in each strain group. The thin black lines on the top of the graph indicate the multiples of geometric mean titer before and after vaccination, as well as the significant levels. The sample size of each group is around 40–50. The data are presented as geometric means, and statistical analyses were paired *t*-tests.

antibodies pre-vaccination ranged from 446 to 2916, while the ED_{50} values post-vaccination elevated into a range of 1968 to 9363. Notably, the neutralizing antibody levels of the circulating strains A/Hebei Cixian/1181/2023 increased 5-fold after vaccination, which is equivalent to the increase in antibody levels of the original strain (6-fold). However, the site mutation on its HA is the most common among the 10 circulating strains (Figure 2). We know that the amino acid mutations associated with antigenic variations are primarily located in five major antigenic sites (A to E), especially in sites A and B, where seven residues are near the receptor-binding sites (residue 145 at site A, and residues 155, 156, 158, 159, 189, and 193 at site B).[11] Although the circulating strain A/Hebei-Cixian /1181/2023 has many mutation sites, only two of these mutations are near the receptor-binding sites, as shown in Figure 2, so their impact on antigenicity is minimal. Furthermore, mutations on the HA protein may not only aim to evade host immunity but also enhance the strain's fitness during replication and transmission processes, aspects not detectable in pseudovirus assays. The neutralizing antibody levels post-vaccination exhibited variations against different circulating strains, which could be linked to the evolution of influenza strains, as despite the high neutralizing antibody response post-vaccination against all strains, discernible differentiations are still emerging among distinct influenza strains. Additionally, the sampled population may have been previously exposed to the circulating strain A/New York/ IVYG43Q07S9/2023, leading to preexisting immunity and consequently higher neutralizing antibody levels both before and after immunization. According to serological diagnostic criteria, a patient's serum antibody titers in the acute and convalescent phases must increase by at least 4-fold to diagnose an infectious disease. The antibody titers must significantly exceed those of the normal population or increase progressively with the course of the disease to hold diagnostic value. As illustrated in Figure 3, the increase in neutralizing antibody titers against different circulating strains compared to the vaccine strain is less than 4-fold. Therefore, the differences between circulating strains and the vaccine strain are insignificant. In brief, the result suggests that the vaccine continues to offer clinical protection against these circulating strains.

In conclusion, this study demonstrates that despite mutations in circulating H3N2 influenza strains, the current vaccine strain matches the circulating strains, imparting protective effects against influenza and providing a scientific basis for encouraging influenza vaccination.

Discussion

The frequent occurrence of sporadic influenza outbreak and epidemic can be attributed to its susceptibility of antigenic drift and shift, challenging the prediction of circulating antigenic variants.[12,13] This inherent characteristic enables the influenza virus to evade immune responses elicited by prior infections or vaccinations.[14,15] While influenza vaccines have demonstrated a certain protective effect on mitigating the spread of seasonal influenza, the constant mutation of influenza viruses makes it necessary to update strains regularly, aiming to match the vaccine strains with the circulating strains well.[16,17] For example, vaccine H3N2 strains have undergone 15 replacements between 2000 and 2023. Seasonal Flu Vaccine Effectiveness studies from the Centers for Disease Control and Prevention (CDC) indicate that the average effectiveness of current influenza vaccines is estimated to be around 60%, with occasional years showing effectiveness below 20%. Therefore, evaluating the effectiveness of influenza vaccines is extremely important.

Numerous candidate vaccines are currently being evaluated.[18,19] In the clinical evaluation of seasonal influenza vaccines, *in vitro* assays such as hemagglutination inhibition (HI) and microneutralization (MN) assays are used to

determine antibody titers in individuals immunized with seasonal influenza vaccines. However, these *in vitro* assays require the use of live influenza viruses, which can hinder vaccine research and development. To circumvent the need for live viruses, pseudoviruses have been explored to detect specific antibodies against H1N1, H5N1 and so on.[20,21] Our laboratory has previously produced H7N9 pseudovirus and compared the correlation between PBNA and the traditional HI and MN antibody assays. The results of PBNA were found to correlate strongly with those obtained from both HI and MN assays.[5] Additionally, similar results were observed with other influenza pseudoviruses, such as H1N1 and H5N1.[20,21] Moreover, we assessed serum neutralizing antibody levels against vaccine strain using the HI assay in this study. The results show that the agreements between PBNA and HI are good; the correlation coefficients were 0.79 (pre-vaccination) and 0.73 (post-vaccination), respectively (Figure 4). These data suggest a good correlation between PBNA and the conventional neutralization method, indicating that PBNA can replace the traditional method.

On the basis of a pseudovirus platform, this study assessed the alignment between the 2023 and 2024 seasonal influenza vaccine H3N2 strain (A/Darwin/6/2021) and circulating influenza H3N2 strains. The findings shows that the WHO-recommended vaccine strains remain well matched with the circulating strains, serving as a reference for influenza vaccination. However, due to the frequent antigenic drift of influenza H3N2 viruses, not all influenza strains can produce pseudoviruses successfully. In this study, the circulating strains A/Sedbury/2992/2023 and A/Ashgabad/40S/2023 failed to produce pseudoviruses. The failure may be due to the lack of HA protein expression during pseudovirus production or conformational changes in the protein, resulting in HA proteins that cannot effectively bind to receptors. As shown in Figure 2, A/Ashgabad/40S/2023 has a mutation S156H, which is located within antigenic site B and also near the receptor-binding sites. Previous studies have indicated that mutations at this site affect receptor binding and viral replication fitness.[22] The A/Sedbury/2992/2023 strain may have altered antigen expression due to mutations. Additionally, the HA mutation sites of A/Sedbury/2992/2023 are identical to those of the egg-based vaccine strain A/Darwin/9/2021. It is well known that egg adaptation can induce mutations. Although there are many successful examples of using egg-based strains, this process also increases the likelihood of unsuccessful outcomes. The existing data do not yet support a direct relationship between the failure to construct pseudoviruses and whether mutations limit pseudovirus functionality, and further research is needed.

Over the past 50 years, H3N2 viruses have undergone frequent antigenic drift, with at least 18 antigenic variants reported.[23] In this study, through sequence alignment with the vaccine strain A/Darwin/6/2021, the mutation sites in the selected circulating strains are as follows: antigenic site A: T135A/K, I140K/M, S145N; antigenic site B: S156H, N159Y, I160K, D186S/N, N190D, I192F, S198P; antigenic site C: I48T, E50K, G53D/N, K276R; antigenic site D: S205F, A212; and antigenic site E: F79V, S262N (Figure 2). Studies have shown that antigenic site B is immunodominant in the H3N2 influenza virus strain included in the vaccine preparations.[24] Among the representative circulating strains selected for this study, only site 192 in antigenic site B appears in multiple circulating strains, with the exception of the A/Hebei-Caixian/1181/2023 strain. Most mutations are not located at these critical amino acid sites, so antigenic drift is not significant, which is consistent with the similar levels of neutralizing antibody increase observed across different strains (a difference is generally considered significant if it is 4-fold or greater). This also partially explains why the current vaccine strains still provide protective effects against the circulating strains. Most antigenic drift events involve amino acid substitutions at multiple positions within the same site or across different sites. For instance, during the 2014–15 influenza season, multiple genetic clades (3C.2a and 3C.3a) emerged from A/Texas/50/2012 (H3N2) (TX/12)-like viruses and co-circulated. Among them, two subclades (3C.3a, represented by A/Switzerland/9715293/2013 [SWZ/13], and 3C.2a, represented by A/Hong Kong/4801/2014 [HK/14]) were antigenically distinct from TX/12-like viruses. The amino acid substitutions N145S(A)-N225D(near D)-A138S(A)-F159S(B) and N145S(A)-N225D(near D)-N144S(A)-F159Y(B)-Q311H(C)-K160T(B) drove the emergence of SWZ/13 and HK/14 from TX/12, with all these substitutions located in receptor-binding sites A or B. Thus, viruses with N145S-N225D likely served as intermediate precursors for SWZ/13 and HK/14 viruses.[25] In the following 2015–16 season, the majority of circulating strains were HK/14-like viruses, with SWZ/13-like viruses co-circulating at a lower proportion. Numerous antigenic drift events have occurred around these conserved antigenic sites,

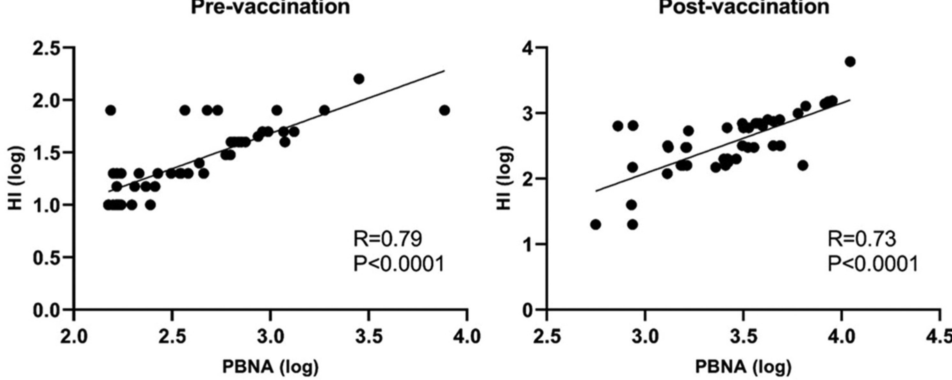

Figure 4. Comparison between PBNA and HI: Pearson's correlation analysis was utilized to evaluate their correlation.

involving amino acid substitutions such as D53K(C)-E82K(E), K156E(B), G135E(A)-N145K(A)-N193S(B), and K145N(A)-Y159F(B). These events drove the emergence of A/Texas/1/77 from A/Victoria/3/1975, A/Bangkok/01/1979 from A/Texas/1/77, A/Beijing/352/1989 from A/Sichuan/2/1987, and A/California/07/2004 from A/California/07/2004.

We conducted cross-neutralization assays utilizing clinical human immune sera to evaluate the cross-reactivity against globally circulating influenza strains. This approach serves as a complementary method to the WHO's animal serum-based monitoring system. Moreover, this research methodology offers a sustainable framework for continued assessment of the neutralizing capacity of subsequent H3N2 circulating strains, contributing to the establishment of a persistent and robust methodological foundation.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Informed consent statement

Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Institutional review board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Shaanxi Provincial Centre for Disease Control and Prevention (Ethics number: 2023-002-02-01).

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