

Retrospective immunogenicity analysis of seasonal flu H3N2 vaccines recommended in the past ten years using immunized animal sera



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Summary

Background Influenza A (H3N2) virus (A/H3N2) has complex antigenic evolution, resulting in frequent vaccine strain updates. We aimed to evaluate the protective effect of the vaccine strains on the circulating strains from past ten years and provide a basis for finding a broader and more efficient A/H3N2 vaccine strain.

Methods Eighty-four representative circulating A/H3N2 strains were selected from 65,791 deposited sequences in 2011–2020 and pseudotyped viruses were constructed with the VSV vector. We immunized guinea pigs with DNA vaccine containing the A/H3N2 components of the vaccine strains from 2011 to 2021 and tested neutralizing antibody against the pseudotyped viruses. We used a hierarchical clustering method to classify the eighty-four representative strains into different antigenic clusters. We also immunized animals with monovalent vaccine stock of the vaccine strains for the 2020–2021 and 2021–2022 seasons and tested neutralizing antibody against the pseudotyped viruses.

Findings The vaccine strains PE/09, VI/11 and TE/12 induced higher levels of neutralizing antibody against representative strains circulating in recommended year and the year immediately prior whereas vaccine strains HK/14, HK/19 and CA/20 induced poor neutralization against all representative strains. The representative strains were divided into five antigenic clusters (AgV), which were not identical to gene clades. The AgV5 strains were most difficult to be protected among the five clusters. Compared with single-dose immunization, three doses of monovalent vaccine stock (HK/19 or CA/20) could induce stronger and broader neutralizing antibodies against strains in each of the antigenic clusters.

Interpretation The protective effect of vaccine strains indicated that the accurate selection of A/H3N2 vaccine strains must remain a top priority. By increasing the frequency of immunization, stronger and broader neutralizing antibodies against strains in all antigenic clusters were induced, which provides direction for a new immunization strategy.

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Introduction

After the first strain of influenza A virus H3N2 (A/H3N2), A/Hong Kong/1/1968, was identified in Hong Kong in

1968, this virus has spread rapidly.¹ Since its initial spread, A/H3N2 strains have undergone extensive genetic and antigenic evolution. Hemagglutinin (HA)

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Research in context

Evidence before this study

We searched the PubMed database on September 16, 2020, with no restrictions, using the terms “seasonal influenza A/H3N2” and “vaccine effectiveness”. Most of the results we retrieved were clinical studies; many were limited to specific regions or years, and lacked data about vaccine protection against influenza A/H3N2 circulating viruses during the past ten years.

Added value of this study

In this study, a pseudotyped virus library of 84 globally representative influenza A/H3N2 strains circulating between 2011 and 2020 was successfully constructed, and guinea pigs were immunized with DNA vaccines constructed from recommended vaccine strains. By measuring the neutralization of these pseudotyped viruses by sera from immunized animals, we estimated the protective effect of vaccine strains against circulating influenza strains. Additionally, a cluster analysis was performed on the representative circulating strains, yielding five antigenic clusters. From these clustering results, we concluded that antigenic clusters are not identical to gene clades. We further studied the effect of booster immunization on neutralization,

and found that using three doses for immunization significantly increased the level of neutralizing antibody in sera; the resulting protective effect against all representative strains was stronger and more extensive than immunizing with a single dose.

Implications of all the available evidence

Our results indicate that the vaccine strains recommended after 2015 do not protect effectively against circulating influenza strains. This suggests that establishing the antigenicity of influenza A/H3N2 virus remains difficult, and that the determination of appropriate vaccine strains should still be a top priority. Although we were not able to verify these findings using human immunized sera, the data from animal sera indicate that three doses of influenza vaccine induce significantly stronger and broader neutralizing antibodies against different antigenic strains than a single dose. This clearly supports the application of a new immunization strategy. Our research highlights the importance of selecting the most appropriate influenza vaccine strains to achieve a high protective efficacy of the overall seasonal influenza vaccine.

and neuraminidase (NA) are two major surface glycoproteins of influenza virus (IFV). The rapid evolution and accumulation of changes in HA and NA result in antigenic drift, which is driven by escape from host immune responses.^{2,3} Antigenic drift is achieved via changes in the amino acids at five antigenic sites.^{4–6} The A/H3N2 virus HA proteins have accumulated at least 75 substitutions over the past half century (13% of whole protein).⁷ Many HA mutations alter both the antigenicity and receptor-binding properties of IFV.^{8,9}

To increase the effectiveness of the influenza vaccine, World Health Organization (WHO) Global Influenza Surveillance and Response System (GISRS) continuously monitors the IFVs circulating in humans and evaluates the recommended influenza vaccine composition twice yearly ([https://www.who.int/en/news-room/fact-sheets/detail/influenza-\(seasonal\)](https://www.who.int/en/news-room/fact-sheets/detail/influenza-(seasonal))). Consequently, the influenza vaccine has been updated every 2–5 years.¹⁰ New vaccine strains should be: significantly different antigenically from the current vaccine strains, currently the dominant influenza virus or likely to become it during the next influenza season, and suitable for vaccine strain preparation.¹¹ In the past decade, influenza vaccine strains have been changed seven times.

Many studies have evaluated influenza vaccine efficacy (VE). In the 2014–2015 influenza season, the prevalent clade 3C.2a, characterized by the mutations F159Y and K160T, had a new potential glycosylation site, and the recommended influenza vaccine had particularly low VE against this clade¹²; in the 2016–2017 season, seasonal influenza vaccine VE was only approximately 43%.¹³

A meta-analysis of 56 studies using a test-negative design, which has emerged as a valid method for estimating VE,¹⁴ reported a pooled VE for A/H3N2 of 33%.¹⁴

Here, we selected influenza vaccine strains and representative circulating strains from the past decade and used them to construct pseudotyped viruses with the vesicular stomatitis virus (VSV) vector. The levels of neutralization activity against the representative strains of sera from animals that received influenza vaccine were tested. This study aimed to evaluate the protective effect of the influenza vaccine strains against the circulating strains from the past ten years, analyze the antigenic differences among the epidemic strains from the past ten years, and provide a basis for selecting a broader and more efficient influenza vaccine strain.

Methods

Ethics

All animal procedures were performed in accordance with guidelines for the ethical review of laboratory animal welfare of National Institutes for Food and Drug Control (Number 2020(B) 001), and accept the guidance and supervision and inspection of the laboratory animal welfare ethics review committee.

Cells

Huh-7 (human, liver, RRID: CVCL_0336) and 293T (human, kidney, RRID: CVCL_0063) cell lines were obtained from the Japan Research Biological Resources

Collection and American Type Culture Collection, respectively. The cell line has been checked by the cell identification assay and is free from other cellular contamination. In addition, exogenous virus factor test was also performed, showing no exogenous virus contamination. Sterility testing and mycoplasma testing have been performed recently, and all meet the requirements. All cell lines were cultured in Dulbecco's modified Eagle's medium (HyClone, Logan, UT) with 100 U/ml of penicillin-streptomycin solution (GIBCO, Grand Island, NY), 20 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (GIBCO), and 10% fetal bovine serum (PAN-Biotech, Adenbach, Germany) at 37 °C in a humidified atmosphere with 5% CO₂.

Representative strain selection

All the HA amino acid sequences were downloaded from the GISAID EpiFlu™ database. Because the HA1 domain contains all the HA protein antigenic sites, only the HA1 region is considered below. One of the identical sequences in the HA1 region was selected for sequence alignment, and the number of repetitions of the same sequence in the total sequence library was calculated. We used Clustal-omega for protein sequence alignment. We constructed evolutionary trees by year because of the large number of sequences. Phylogenetic trees were constructed using a phym/IFV-specific amino acid substitution rate matrix. The evolutionary tree was clustered using ClusterTree (<https://biit.cs.ut.ee/clustvis/>). The lower the threshold is set, the finer the segmentation of the resulting evolutionary tree, so we set the threshold at 0.05, which can completely separate the vaccine strains of 2011–2020.

Phylogenetic tree

A maximum likelihood phylogenetic tree was constructed for 84 representative sequences using MEGA. The resulting phylogenetic tree was further modified using the software Adobe Illustrator CS6.

A/H3N2 pseudotyped virus production

The HA protein sequences listed in Table 2 were downloaded from GISAID. The HA protein expression plasmid pcDNA3.1-HA was constructed from the GenBank sequence MN908947; the entire sequence was synthesized on the backbone plasmid pcDNA3.1(+) using General Biological System (Anhui, China). The replication-defective G*ΔG-VSV (Kerafast, Boston, MA) was used as the backbone virus. 293T cells were transfected with pcDNA3.1-HA using Lipofectamine 3000 (Invitrogen, Carlsbad, CA), in accordance with the manufacturer's instructions, and simultaneously infected with G*ΔG-VSV. At 4 h post-transfection, the cells were given fresh medium containing 1% fetal bovine serum and 7 MU/ml of *Clostridium* neuraminidase

(Aladdin, Shanghai, China) to induce the release of HA-pseudotyped viruses from the surface of the producer cells. The supernatant containing the pseudotyped viruses was harvested 24 h and 48 h later. Pseudotyped viruses were filtered out with 0.45-μm mixed cellulose ester membrane filters (Millipore, Boston, MA). For HA cleavage, pseudotyped viruses were activated by adding 40 μg/ml TPCK-trypsin (Sigma-Aldrich, Saint Louis, MO). After being incubated at 37 °C in a humidified atmosphere with 5% CO₂ for 30 minutes, they were aliquoted and stored at –80 °C for further use.

Titration of A/H3N2 pseudotyped viruses

The pseudotyped virus titers were evaluated by transducing Huh-7 cells with three-fold serial dilutions of pseudotyped viruses. The 96-well titration plate was incubated at 37 °C with 5% CO₂; after 1 h, 2 × 10⁴ Huh-7 cells were added to each well. After a 24 h incubation at 37 °C with 5% CO₂, chemiluminescence signals were detected using the Britelite plus reporter gene assay system (PerkinElmer, Waltham, MA), and the 50% tissue culture infectious dose (TCID₅₀) was calculated as described previously.¹⁵

Neutralization assay

Immunized sera were diluted to an appropriate initial concentration, which was then used in a three-fold serial dilution. The resulting dilutions were mixed with pseudotyped viruses and incubated at 37 °C for 1 h. The mixtures were then added to a 96-well cell culture plate containing 2 × 10⁴ Huh-7 cells in 100 μl/well. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 24 h, after which the chemiluminescence signals were detected as described above. The virus neutralization titer was calculated using the Reed-Muench method with PerkinElmer Ensign software.

Immunized sera production

Forty-four female Hartley guinea pigs, weighing 200–220g, were obtained from National Institutes for Food and Drug Control (NIFDC, Beijing, China) and randomly divided into eleven groups (four animals/group). The sample size was designed with traditional way and agreed with ethical committee. Their feeding environment, immunization methods and immunization doses are the same. Nine groups of guinea pigs were inoculated with one of the following plasmids: pcDNA3.1-A/Cambodia/e0826360/2020 HA, pcDNA3.1-A/Hong Kong/2671/2019 HA, pcDNA3.1-A/Kansas/14/2017 HA, pcDNA3.1-A/Singapore/INFIMH-16-0019/2016 HA, pcDNA3.1-A/Hong Kong/4801/2014 HA, pcDNA3.1-A/Switzerland/9715293/2013 HA, pcDNA3.1-A/Texas/50/2012 HA, pcDNA3.1-A/Victoria/361/2011 HA, pcDNA3.1-A/Perth/16/2009 HA. Guinea pigs were electroporated with 200 μg plasmid at day 0, day 14, day 28.

Immunization was repeated twice at two-week intervals, and serum samples were obtained two weeks after the third immunization.

Two groups were immunized with 1/5 human dose of the influenza vaccine monovalent stock solution recommended for the 2020–2021 or 2021–2022 season (3 µg of HA protein/dose), respectively. Immunization was repeated twice at two-week intervals, and serum samples obtained two weeks after the first, second, and third immunizations were stored at –80 °C and thawed and heat-inactivated at 56 °C for 0.5 h before use.

The Animal Care and Use Committee at the NIFDC approved the study protocol. The animals were kept in plastic cages (two animals per cage) and maintained in an environmentally controlled room (temperature 25 °C, relative humidity 60%) with free access to fresh solid pellet diet and water. The animal could be placed quietly for five minutes before continuing the experiment when it was restless due to pain. At the end of the experiment, sacrificed by carbon dioxide inhalation.

Processing of serum neutralization data

GraphPad Prism 8.0 was used to analyze the neutralization activity of the immunized sera against the representative strains. When a dimensionality reduction analysis was performed on the serum neutralization data of the strains, we used the hierarchical clustering method to cluster the strains. Heatmap Illustrator (HemI) software was used to display the clustering results.

Statistical analysis

For the vaccine-elicited sera neutralization assay, statistical significance was determined using SPSS 20.0. The data distribution was firstly analyzed by using Shapiro–Wilk test. If they do not conform to a normal distribution, Kruskal–Wallis test should be applied to analyze nonparametric multiple group comparisons. *P*-values <0.05 were considered to indicate significant differences. *, *P* < 0.05, **, *P* < 0.01, ***, *P* < 0.005, ****, *P* < 0.0001.

Role of the funding source

The funder had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Construction of an A/H3N2 pseudotyped virus library

All “host-human” HA amino acid sequences (65,791 sequences) of A/H3N2 from 2011 to 2020 were retrieved from the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu™ database, and an

HA1 domain-based phylogenetic tree was created based on those sequences. To select representative sequences from the constructed phylogenetic tree, we set a threshold of 0.05, which can separate the A/H3N2 vaccine strains of 2011–2020. Using this threshold, the retrieved sequences formed 91 clusters. One sequence was selected from each cluster, yielding 91 representative IFV strain sequences (Fig. 1A and B). The selected sequences were evenly distributed throughout the phylogenetic tree (Fig. 1C, a clear original image can be found in Supplementary Fig. S1.), suggesting that the sequences selected by this algorithm are representative. Higher diversity levels are indicated by higher numbers of clusters rather than higher numbers of deposited sequences. The results indicate that the sequences from 2017 to 2019 are relatively conserved whereas those deposited from 2013 to 2015 are more diverse (Fig. 1A and B).

VSV-based pseudotyped viruses were successfully constructed from 84 of the 91 representative strains. For the seven representative strains, we tried repeatedly, including changing plasmid transfection doses and optimizing packaging conditions, but failed to construct these strains, and as they all appeared only once in the library of 65,791 sequences, we did not consider them to be important. We also constructed pseudotyped viruses for all nine A/H3N2 vaccine strains recommended by WHO from 2011 to 2021 (Table 1). Thus, a pseudotyped virus library containing 84 representative strains and 9 A/H3N2 vaccine strains was obtained (Table 2).

Antigenic analysis of representative strains

To investigate the level of protection provided by the WHO-recommended A/H3N2 vaccine strains against the representative circulating strains, the neutralization levels of sera from animals immunized with DNA from each of the nine A/H3N2 vaccine strains against the 93 pseudotyped IFVs were assessed. Although all immunized sera were able to neutralize most of the tested strains, immunized sera generated with different A/H3N2 vaccine strains exhibited different neutralization trends, by which they can be divided into three categories (Fig. 2A). The sera in category one from animals immunized with PE/09, VI/11, TE/12, or SING/16 had variable neutralization levels against the representative IFV strains, with EC₅₀ ranging from 10²–10⁵. The sera in category two from KA/17 or SZ/13 immunization and the sera in category three from HK/14, HK/19, or CA/20 immunization had concentrated neutralization levels, showing that the neutralization levels of category two for most IFV strains were close to the mean level (Fig. 2A). Notably, the sera in category three were the least protective, with most IFV strains having an EC₅₀ in the range of 10²–10³.

To analyze the antigenic evolution of A/H3N2 in the last decade, we used a hierarchical clustering method to cluster the 84 representative strains according to their

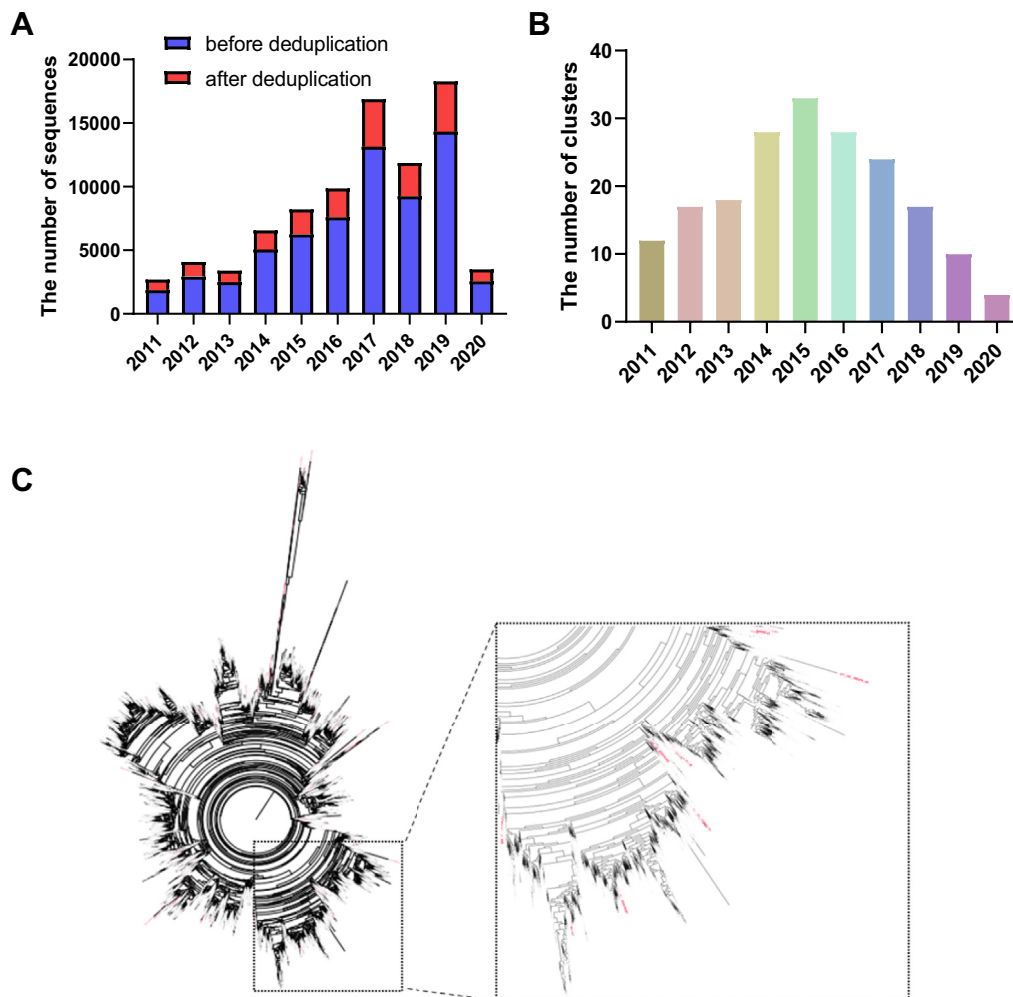


Fig. 1: Selection of representative IFV strains A): Total sequence statistics for each year from 2011 to 2020, and sequence statistics after deduplication. B): The number of phylogenetic tree clusters by year from 2011 to 2020, i.e., the number of selected representative IFV strains (because some strains were prevalent for many years, they were counted every year in which they appeared). C): The phylogenetic tree constructed after deduplication of all IFV sequences in ten years; the selected sequences are marked in red. In order to make the picture clearer, we have enlarged the part as shown on the right.

neutralization value EC_{50} . This approach produced five antigenic clusters, which we named AgV1–AgV5 (Fig. 2B), that contained 24, 10, 6, 23, and 21 strains, respectively (Table 3). Among the 84 representative IFV strains, 50 strains were “high frequency”, i.e., each strain represented >10 circulating strains, while 34 strains had low frequency, i.e., each represented ≤ 10 circulating strains. The 50 high frequency strains were mainly distributed in AgV1 and AgV4, while the 34 low frequency strains accounted for the largest proportion of AgV5. The proportion of high frequency strains was higher than the proportion of low frequency strains for all antigenic clusters, except AgV5.

We further analyzed the neutralization level in immunized sera generated from each A/H3N2 vaccine

strain against the five antigenic clusters. After the Shapiro–Wilk test, it was judged that the data of this study did not conform to the normal distribution. Therefore, differences in the immune responses against the strains in the five antigenic clusters were analyzed by using Kruskal–Wallis test. Eight of the nine vaccine strains that induced sera showed statistically significant differences in the levels of neutralization against the five antigenic clusters (HK/19, $p < 0.005$; others, $p < 0.0001$, Fig. 2C). Significant differences were not observed with CA/20-immunized sera because the neutralization level of this sera against most strains was poor. For multiple testing between different antigenic clusters, see Supplementary Table S1 for details. Most of the immunized sera had their best neutralizing activity

vaccine strains	seasons
A/Perth/16/2009(PE/09)	2011-2012
A/Victoria/361/2011 (VI/11)	2012-2013, 2013-2014
A/Texas/50/2012(TX/12)	2014-2015
A/Switzerland/9715293/2013(SZ/13)	2015-2016
A/Hong Kong/4801/2014(HK/14)	2016-2017, 2017-2018
A/Singapore/INFIMH160019/2016(SING/16)	2018-2019
A/Kansas/14/2017(KA/17)	2019-2020
A/Hong Kong/2671/2019(HK/19)	2020-2021
A/Cambodia/e0826360/2020(CA/20)	2021-2022

Table 1: The nine A/H3N2 vaccine strains recommended by WHO from 2011 to 2021.

Isolate ID	Protein accession ID	Number of replicates	Popular years	Popular areas	Note
EPI_ISL_186598	EPI607841	2619	2014 2015 2016	North America,Europe,Asia	
EPI_ISL_193307	EPI631490	2	2015	North America	
EPI_ISL_202918	EPI677422	2	2015	North America	
EPI_ISL_193035	EPI630467	235	2014 2015 2016	North America, Asia	
EPI_ISL_160201	EPI520419	16	2014 2015	North America	
EPI_ISL_159512	EPI516676	102	2014 2015 2016	North America,Europe,Asia	
EPI_ISL_200031	EPI663225	1	2015	South America	
EPI_ISL_240500	EPI872931	20	2012 2013	Oceania,North America	
EPI_ISL_168713	EPI550975	235	2014 2015 2016	North America,Asia	
EPI_ISL_200017	EPI663211	1	2015	North America	
EPI_ISL_272703	EPI1034399	1	2015	Asia	
EPI_ISL_192791	EPI629917	1	2012	North America	
EPI_ISL_160165	EPI520324	74	2013 2014	North America,Asia	
EPI_ISL_160594	EPI522992	1	2013	North America	
EPI_ISL_136690	EPI427231	806	2013 2014	North America,Asia	
EPI_ISL_288656	EPI1125084	129	2017 2018 2019	North America,Asia	
EPI_ISL_164262	EPI536412	17	2013 2014	Asia	
EPI_ISL_300385	EPI1183643	4	2017 2018	Asia	
EPI_ISL_255574	EPI957233	139	2017 2018	North America,Europe,Asia	
EPI_ISL_277579	EPI1058718	38	2017	Africa	
EPI_ISL_320961	EPI1272581	3	2018	Oceania	
EPI_ISL_278635	EPI1062682	3	2012 2016	Europe	
EPI_ISL_314618	EPI1255052	2	2018	Oceania	
EPI_ISL_281229	EPI1076101	81	2017 2018	North America,Europe,Asia	
EPI_ISL_322097	EPI1278774	1	2018	Asia	
EPI_ISL_129773	EPI398030	145	2012 2013 2014	North America,Europe	
EPI_ISL_357863	EPI1448323	786	2017 2018 2019	North America,Europe,Oceania	
EPI_ISL_101595	EPI349371	17	2012	North America	
EPI_ISL_157764	EPI509981	1138	2012 2013 2014 2015	North America,Asia	
EPI_ISL_106342	EPI356897	2	2011	Africa	
EPI_ISL_161279	EPI526687	16	2012 2013 2014	North America	
EPI_ISL_135837	EPI422660	5	2011	South America	
EPI_ISL_172781	EPI565771	147	2014 2015 2016	North America,Europe,Asia	
EPI_ISL_86087	EPI302276	44	2011	North America	
EPI_ISL_156582	EPI507253	3	2013 2016	Europe	
EPI_ISL_130285	EPI399809	1	2011	North America	
EPI_ISL_394110	EPI1604866	1	2015	Africa	
EPI_ISL_229169	EPI806326	1	2015	Asia	
EPI_ISL_367161	EPI1503952	1	2019	Asia	
EPI_ISL_378745	EPI1551462	2	2017	Asia	
EPI_ISL_229137	EPI806294	1	2014	Asia	
EPI_ISL_229154	EPI806311	2	2015	Asia	
EPI_ISL_151118	EPI489605	1	2013	Europe	
EPI_ISL_501166	EPI1766950	3	2020	North America	
EPI_ISL_341299	EPI1370001	2	2018	Oceania	
EPI_ISL_239893	EPI869119	1	2016	North America	

EPI_ISL_176912	EPI580916	1	2014	Asia	
EPI_ISL_165317	EPI539159	2	2014	Oceania	
EPI_ISL_362161	EPI1479073	947	2017 2018 2019	North America,Europe,Asia	
EPI_ISL_87911	EPI309455	271	2011 2012 2013	North America,Europe	
EPI_ISL_172779	EPI565755	426	2013 2014 2015 2016	North America,Europe,Asia,Africa	
EPI_ISL_96031	EPI335720	13	2011	Africa	
EPI_ISL_357867	EPI1448355	276	2019 2020	North America,Europe,Asia	
EPI_ISL_400618	EPI1640359	824	2019 2020	Africa,Oceania,North America	
EPI_ISL_239687	EPI868313	2	2016	North America	
EPI_ISL_90056	EPI316883	112	2011 2012	Europe,Africa	
EPI_ISL_168734	EPI551101	60	2014 2015 2016	Asia	
EPI_ISL_419619	EPI1722138	3727	16 2017 2018 2019 20	North America,Europe	
EPI_ISL_199573	EPI662767	127	2015 2016	North America,Europe,Oceania	
EPI_ISL_275815	EPI1048432	505	2016 2017	Europe,Asia	
EPI_ISL_169397	EPI553239	68	2014 2015	Asia	
EPI_ISL_248141	EPI911584	2792	2016 2017 2018	North America,Europe,Asia	
EPI_ISL_244866	EPI896806	21	2015 2016	North America	
EPI_ISL_170439	EPI557617	25	2014 2015	Asia	
EPI_ISL_225949	EPI780912	342	2016 2017	North America,Europe,Asia	
EPI_ISL_237572	EPI858882	985	2014 2015 2016 2017	North America,Europe,Asia	
EPI_ISL_230293	EPI813865	331	2014 2015 2016	North America,Oceania,Asia	
EPI_ISL_352951	EPI1425421	10	2018	Asia	
EPI_ISL_257427	EPI967451	461	2016 2017	North America,Europe	
EPI_ISL_104013	EPI353928	12	2012	Europe	
EPI_ISL_249975	EPI924983	545	2016 2017	North America,Europe	
EPI_ISL_94713	EPI331069	408	2011 2012	North America,Europe,Asia	
EPI_ISL_369552	EPI1514983	271	2011 2012 2013	Asia	
EPI_ISL_277535	EPI1058561	92	2017 2018	North America,Asia	
EPI_ISL_171767	EPI561813	286	2014 2015	North America,Europe	
EPI_ISL_192806	EPI629948	30	2013 2014	North America	
EPI_ISL_234127	EPI836825	560	2016 2017	North America,Europe	
EPI_ISL_207685	EPI697069	1	2015	Asia	
EPI_ISL_225105	EPI776028	267	2015 2016 2017	North America	
EPI_ISL_122344	EPI378502	73	2012	North America	
EPI_ISL_177907	EPI682763	4	2014	Asia	
EPI_ISL_277242	EPI1056717	46	2017	North America	
EPI_ISL_229165	EPI806322	1	2015	Asia	
EPI_ISL_263392	EPI997817	1	2015	Europe	
EPI_ISL_2404688	EPI1868486	/	/	/	PE/09
EPI_ISL_2454696	EPI1868575	/	/	/	VI/11
EPI_ISL_2454729	EPI1868581	/	/	/	TX/12
EPI_ISL_2454697	EPI1868576	/	/	/	SZ/13
EPI_ISL_2443866	EPI1868553	/	/	/	HK/14
EPI_ISL_1655936	EPI1858150	/	/	/	SING/16
EPI_ISL_2454700	EPI1868579	/	/	/	KA/17
EPI_ISL_3205461	EPI1884455	/	/	/	HK/19
EPI_ISL_2378896	EPI1868379	/	/	/	CA/20

The number of replicates refers to the number of occurrences of identical sequences in the HA1 domain among a total of 65,791 sequences. The isolate ID and protein accession ID represent one of the replicates. The last nine are the vaccine strains and their abbreviations are shown in the notes.

Table 2: Details of the 93 IFV strains in our library.

against AgV4 and worst neutralizing activity against AgV5. The neutralization pattern of the four immunized sera PE/09, VI/11, TE/12, and SING/16 was similar against different antigenic clusters, i.e., their best neutralizing effect was against the strains from AgV4, followed by AgV3, then AgV2, AgV1, and finally AgV5. KA/17, HK/14, and SZ/13 immunized sera had nearly the same neutralization levels against IFV strains from AgV2 and AgV4, then progressively less against strains

from AgV1, AgV3, and AgV5. CA/20 and HK/19 immunized sera had little difference in their neutralization levels against members of the five antigenic clusters.

Correlation between the genetic relatedness and antigenicity of A/H3N2 strains

To investigate whether there is a correlation between the genetic relatedness and antigenicity of the 84

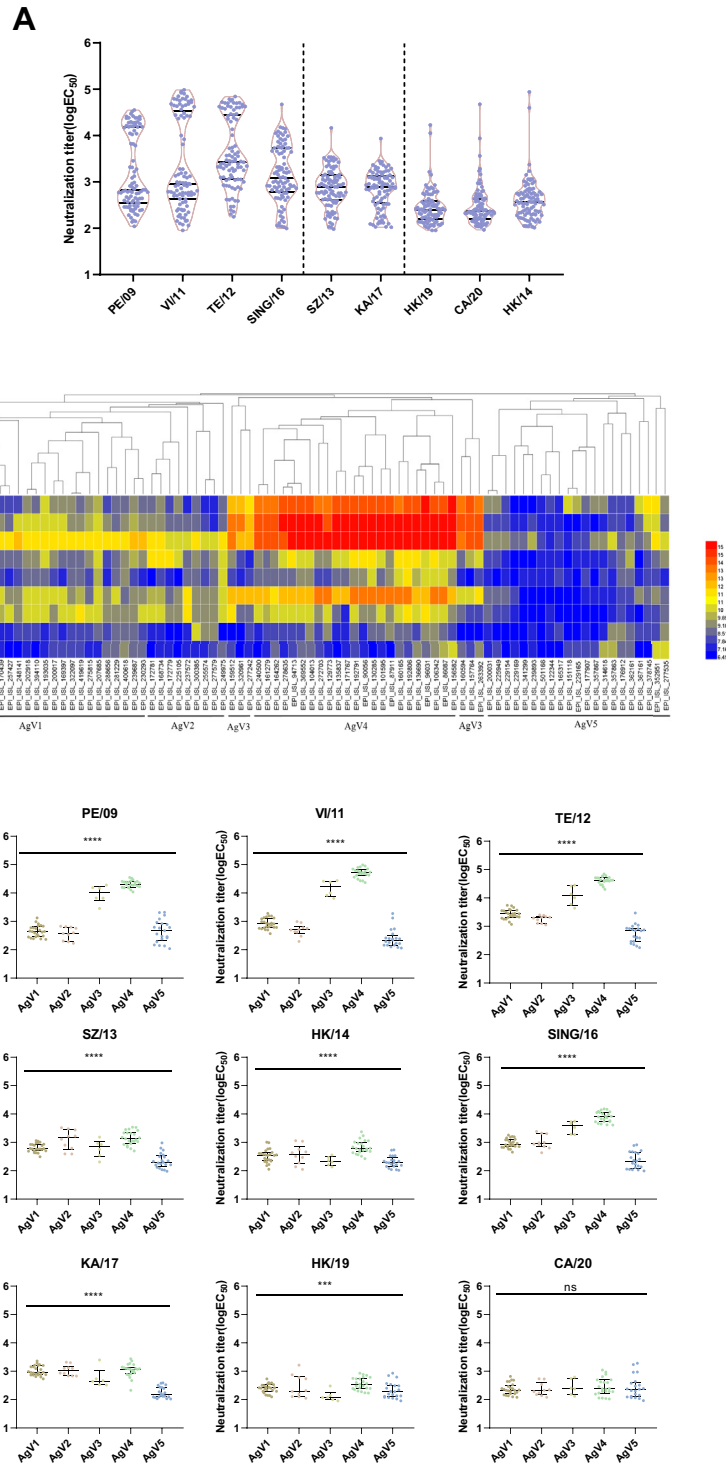


Fig. 2: Antigenicity analysis of representative IFV strains A): Neutralization by nine immunized sera against all IFV strains. Purple indicates the log (EC₅₀) values of different IFV strains. The black line represents the median value with interquartile range. The results shown are based on 3–5 assay repetitions. B) Antigenic cluster of A/H3N2 viruses in our library. Using Heatmap Illustrator (HemI) we established a heatmap, based on the log₂ (EC₅₀) value and then used the hierarchical clustering method to divide these strains. The top shows the clustering tree, and the bottom shows the antigenic cluster information. C): Neutralization of nine immunized sera against IFV strains in different antigenic clusters.

Antigenic cluster	Number of strains	Number of high frequency strains	Number of low frequency strains
AgV1	24	16	8
AgV2	10	9	1
AgV3	6	3	3
AgV4	23	16	7
AgV5	21	6	15

Table 3: Statistics on the number of high frequency strains and low frequency strains in different antigenic clusters.

representative strains, we constructed a phylogenetic tree with them using the maximum likelihood method (Fig. 3). These strains belonged mainly to the following clades: 3C, 3C.3, 3C.2a, 3C.3a, 3C.2a1, 3C.2a2, and 3C.2a3. We found that all the strains in AgV4 distributed in clades 3C.3. For AgV3, three strains belonged to clade 3C.3, while the rest were distributed in clades 3C.2a1, 3C.2a, and 3C. The AgV1 strains were distributed in multiple clades: 11 strains belonged to clade 3C.2a, 7 strains belonged to clade 3C.2a1, two strains each belonged to clades 3C.2a2 and 3C.2a3, and some belonged to clade 3C. For AgV2, most of the strains belonged to clade 3C.2a1, and the remaining three strains belonged to clades 3C.2a and 3C.3a. Unlike the other antigenic clusters, AgV5 contained seven strains that belong to an unknown clade; because their amino acid sequences are quite different from those of other sequences, their gene clade is unknown. Further study on these strains is needed. Fortunately, although none of the immunized sera were able to neutralize these strains, they all appeared only once or twice. The remaining IFV strains in AgV5 belonged to clades 3C.2a1, 3C.2a, and 3C. We found that although the genetic clades were related with the antigenic clusters, they are not identical.

Year distribution of different antigenic clusters

To understand the evolution of antigenic clusters, we further analyzed the distribution of antigenic clusters by year. AgV4 strains were prevalent mainly from 2011 to 2014. AgV1 and AgV2 strains were prevalent mostly from 2015 to 2018, but some also appeared in 2019 and 2020. Strains from the remaining two antigenic clusters, AgV3 and AgV5, appeared every year (Fig. 4A). The AgV5 strains, if the 13 low frequency strains are not considered, were observed mainly in 2017–2020. However, the strains circulating in 2015–2020 were basically distributed in each antigenic cluster, indicating that they had antigenic diversity.

To compare the protective effect of the immunized sera against representative IFV strains of each year, we analyzed their neutralization levels. PE/09, VI/11, TE/12, and SING/16 immunized sera all exhibited good neutralization levels for the representative strains from 2011 to 2013 but poor neutralization levels for representative strains from other years (Fig. 4B). PE/09, VI/11, and TE/12 were recommended for use in the 2011–2012, 2012–2014 and 2014–2015 northern hemisphere influenza seasons, respectively. The neutralization levels of immunized sera generated from these three A/H3N2 strains against representative strains from 2011 to 2013 were basically the same as the levels against their immunization strains, but their neutralization levels to representative IFV strains from other years were almost 100 times worse. Immunized sera generated from SING/16, recommended for use in 2018–2019, was not the best for neutralizing the IFV strains that circulated in 2018, but it successfully neutralized the IFV strains that circulated in 2011–2013 (Fig. 4B). Notably, immunized sera generated from the remaining five vaccine strains, SZ/13, HK/14, KA/17, HK/19, and CA/20, which were recommended for use in 2015–2016, 2016–2018, 2019–2020, 2020–2021, and 2021–2022, respectively, had poor neutralization and no significant differences in their neutralization levels against the representative IFV strains from different years. Almost all the immunized sera had a gradual decrease in their neutralization levels for the representative IFV strains from 2011 to 2020.

Neutralizing antibody induced by three A/H3N2 vaccine doses broadly neutralized various IFV strains

Because the immunized sera generated from vaccination with DNA from HK/19 or CA/20 had relatively low levels of neutralization against all tested circulating strains, but had relatively broad neutralization compared to other vaccine strains, we immunized guinea pigs with

The y-axis indicates the EC₅₀ values of different IFV strains and represents the median with the interquartile range. Neutralization of different cluster strains was compared by the rank sum test; $p < 0.05$ was considered statistically significant. ns, not significant, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0001$.

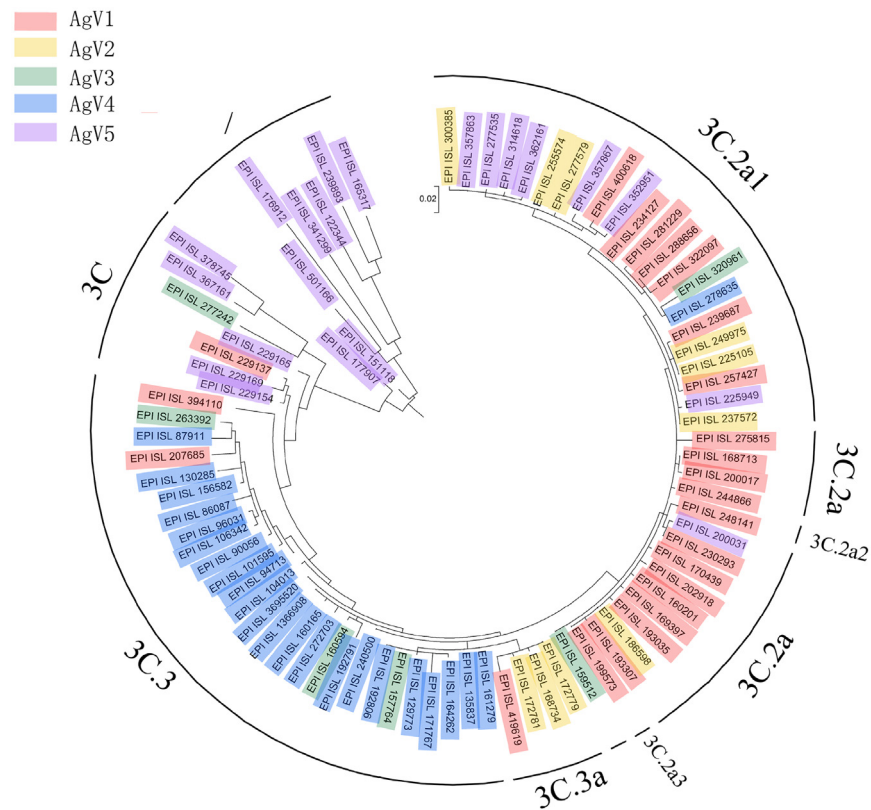


Fig. 3: The correlation between A/H3N2 genetics and antigenicity. A phylogenetic tree of the IFV strains was constructed from the HA1 domain sequences. Both the gene clade and antigenic cluster are marked on the tree.

one, two, or three doses of monovalent stock of HK/19 or CA/20 vaccine strains. Compared with the first-injection dose, the neutralization titer of HK/19 and CA/20 for its own strain was increased by almost 100 times with the third-injection dose. The neutralization mean titer of HK/19 against all representative strains was increased by nearly 5 times and 20 times with the two and third dose, respectively, and for CA/20, 4 times and 8 times respectively. The sera after three doses were able to broadly neutralize all representative strains with high titers (Fig. 5A and B).

To verify whether the two vaccine strains could induce broad-spectrum neutralizing antibodies against all strains, we analyzed the neutralization levels of these immunized sera for different years and different antigenic cluster strains. The results showed that the neutralization levels of all the sera against strains in different years were not significantly different (Fig. 5C). However, the neutralization levels of all the immunized sera against different antigenic clusters were statistically different (HK/19-two and three doses, $p < 0.005$; others, $p < 0.0001$, Fig. 5D). Further analysis indicated that AgV5 caused this difference. There was no difference in the neutralization of three-dose

immunization against AgV1–AgV4 (Fig. 5D). For AgV5, consistent with the DNA-immunized sera, the neutralization level was still the lowest. The level of neutralization of AgV5 was also enhanced by three doses of immunization.

Discussion

IFVs remain one of the most pressing global public health concerns owing to their widespread distribution, rapid evolution, and recombination potential.¹⁶ At present, the most effective measure against IFV infection is vaccination. The effectiveness of vaccines is dependent on close antigen matching between the circulating strains and vaccine strains.¹⁷

This study analyzed the antigenic differences of representative A/H3N2 strains and the protective effect of recommended influenza vaccine strains from the past ten years. We found that the neutralization levels of immunized sera against representative strains vary. According to the neutralization titer results, the nine influenza vaccine strains were divided into three categories: 1) PE/09, VI/11, TE/12 and SING/16; 2) SZ/13 and KA/17; and 3) HK/14, HK/19, and CA/20. Different

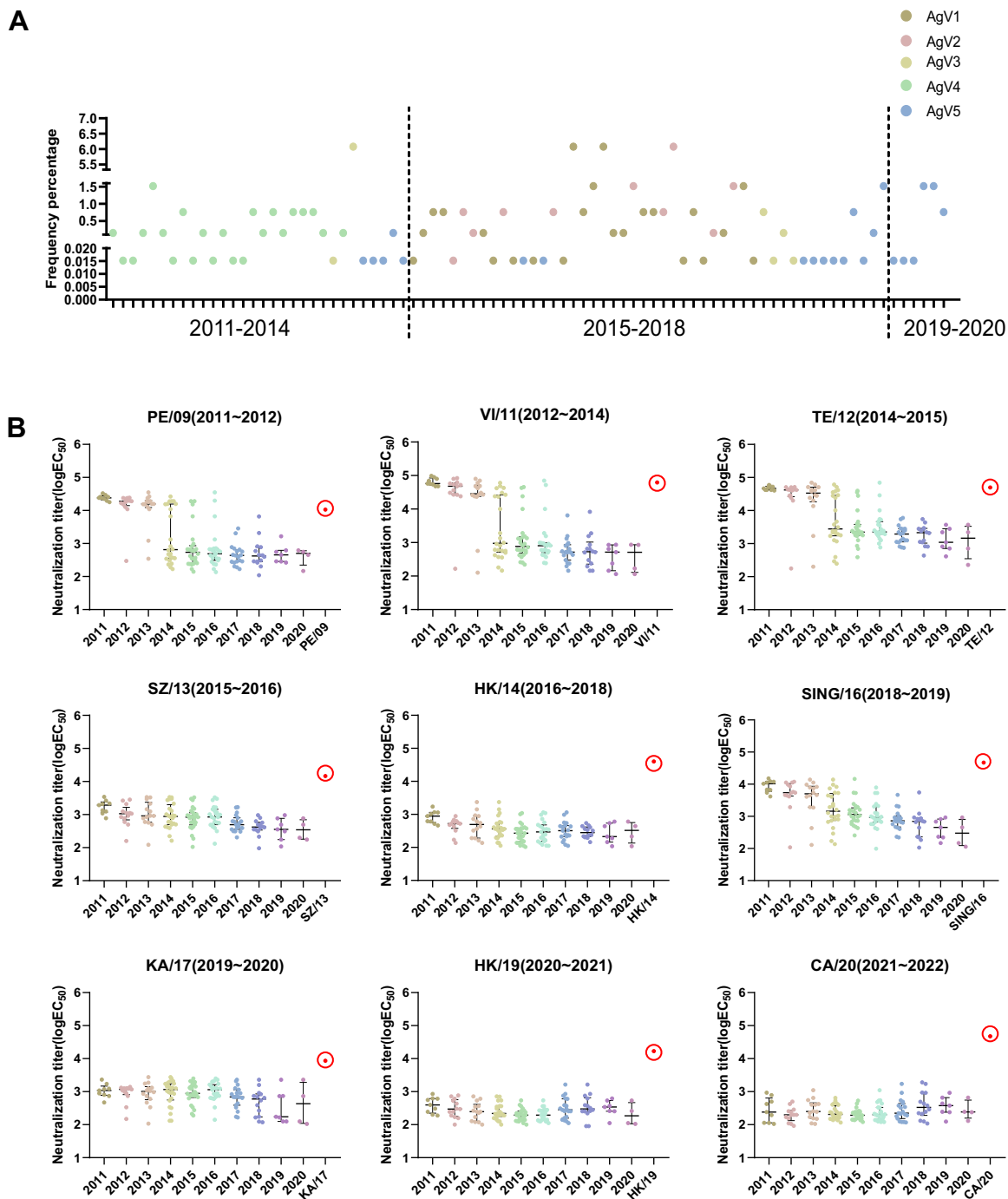


Fig. 4: Year distribution of IFV members of different antigenic clusters. A) Distribution of different antigenic clusters in different years. Because most IFV strains were circulating in multiple years, we calculated the time period rather than determining the year specifically. We counted the values of 0–10 as 10, 10–100 as 100, 100–500 as 500, 500–1000 as 1000, and 1000–4000 as 4,000, and calculated the frequency of these sequences among the 65,791 sequences in our library. B): Neutralization levels of nine immunized sera against circulating IFV strains in different years. Most IFV strains were circulating in multiple years; these strains were counted every year. For convenience of comparison, the EC₅₀ of each immunized sera against its own vaccine strain are listed on the right side in red. The black line represents the median value with interquartile range.

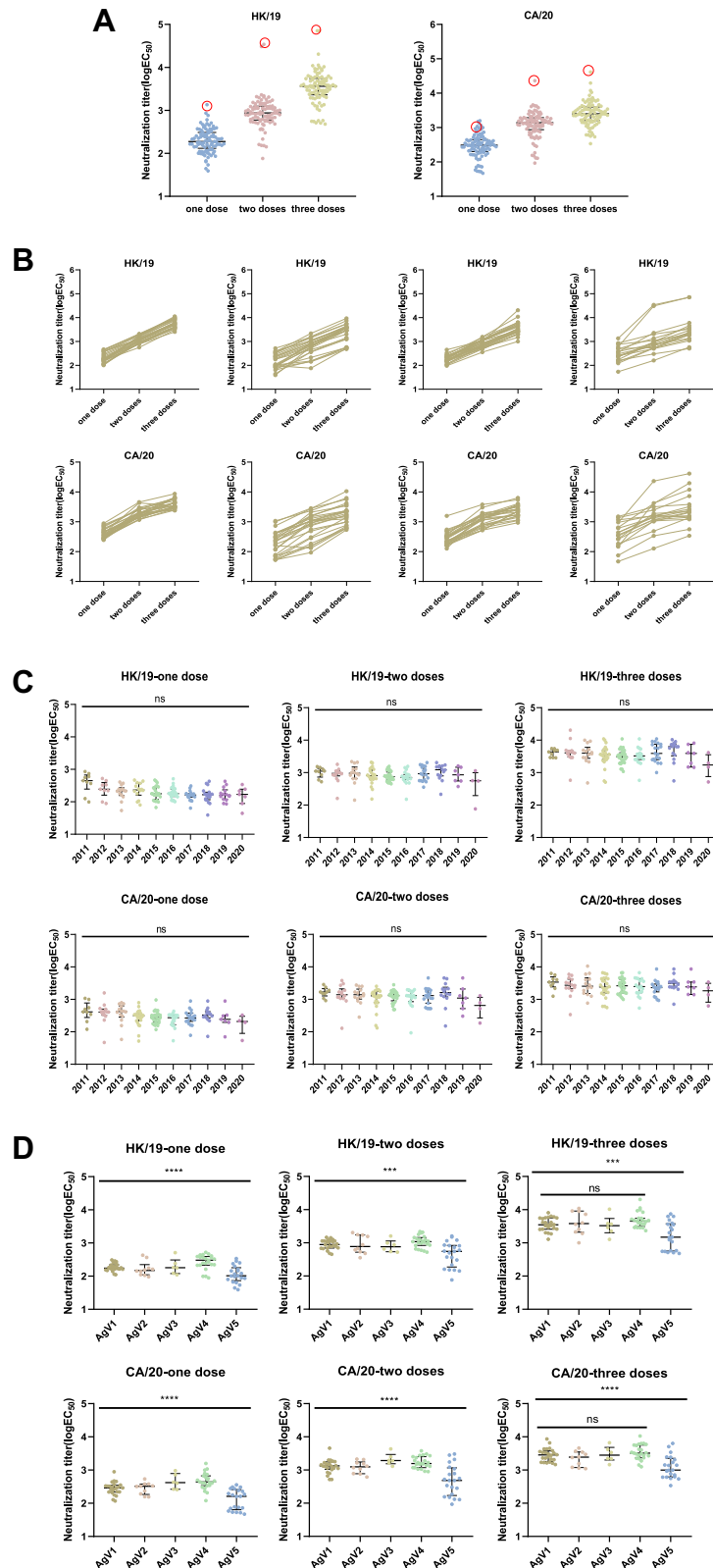


Fig. 5: Neutralization of monovalent vaccine stock-immunized sera against different IFV strains. A) Neutralization of two monovalent vaccine stock-immunized sera against all the IFV strains. The red circle indicates the titer of monovalent vaccine stock-immunized sera against

categories had different neutralizing tendencies for representative strains. Representative strains from the past ten years were divided into five antigenic clusters. Among these antigenic clusters, AgV4 members were relatively sensitive to PE/09, VI/11, and TE/12 immunized sera, while AgV5 members were basically insensitive to all immunized sera. The prevalence of antigenic clusters in different years was also variable. The strains in AgV4 were prevalent in 2011–2014, those in AgV1 or AgV2 were prevalent mainly in 2015–2018, those in AgV3 were found partly in 2011–2014 and partly in 2015–2018, and those in AgV5 were prevalent mainly in 2017–2020.

The neutralizing effects of different A/H3N2 vaccines on the circulating IFV strains in the past ten years also differed. PE/09 vaccine strain was recommended for the 2011–2012, VI/11 for the 2012–2013 and 2013–2014, and TE/12 for the 2014–2015.¹⁸ These three A/H3N2 vaccine strains were a better match with the circulating strains in the recommended years, although the genes were constantly evolving with minor antigenic changes. In contrast, the effect of immunized sera generated from the recommended vaccine strains from later years was not satisfactory, with no difference between their protection against the strains circulating in their recommendation year and those circulating in other years. The genetic diversity of the circulating strains from 2017 to 2020 is relatively minor, but the protection against these strains provided by the immunized sera was poor. In particular, the neutralization levels of HK/19 and CA/20 immunized sera were low and indistinguishable for each antigenic cluster. From these results, the IFV strains appeared to fall into two categories: pre-2015 and post-2015. For the IFV strains circulating before 2015, their antigenicity was similar to that of the corresponding vaccine strains, i.e., they can be well neutralized by immunized sera generated from the vaccine strain recommended for these years, and they can also cross-protect against each other. However, for the IFV strains circulating after 2015, their antigenicity may be quite different from that of the vaccine strains, and immunized sera generated from the recommended influenza vaccine strains cannot provide a high level of protection.

Annual influenza vaccines do not provide protection against antigenically drifted variants or durable protection extending beyond the next influenza season.¹⁹ Thus, the highest priority is to develop a universal influenza vaccine that would provide long-lasting protection against multiple strains of the virus, including strains with the potential to cause a pandemic. Several recent

studies have focused on conserved epitopes, HA stem region, NA and matrix protein 2 (M2)^{19–22} for the development of universal vaccine. Here, we found that the induction of high titer of neutralizing antibody can also provide broad protection with increasing doses of influenza vaccine. Although our study did not provide protection against other subtypes of IFV, our results show that the three-dose immunization can protect strains that belong to different clades have emerged over the past decade at a relatively high level. Whether higher doses or adjuvanted vaccines can induce stronger and broader immunogenicity needs to be investigated. This gives a new direction of immunization without having to change vaccine strains every year.

The main advantage of our study was that a large number of circulating strains were analyzed for HA protein antigenicity, but there are also certain limitations with the study. First, the commonly used experimental animal to study the immunogenicity of the influenza virus is the ferret. However, ferrets were difficult to obtain in our laboratory. Because a large number of sera samples were required for testing 93 pseudotyped viruses, guinea pigs were selected for immunization to obtain sera in this study. Guinea pigs are also one of the susceptible animals used to study the transmissibility of influenza virus.^{23–25} Second, from the current research results, we concluded that three-dose immunization resulted in relatively broad-spectrum neutralizing antibodies in guinea pigs. However, the results were not linked with human immunized sera, which need to be investigated in clinical trials.

For animal research, the design of sample size is more important but complex. Charan et al.²⁶ provided rational methods, especially “resource equation” method, to design the sample size. According to this method, the sample size in this study was suitable.

In summary, the method successfully established here provides a new auxiliary tool for monitoring the degree of matching between influenza vaccine strains and circulating strains. This work also further monitors the antigenic changes of HA protein, thus providing reference for the recommendation and screening of future influenza vaccine strains.

Contributors

Y.W., C.Z., and W.H. conceived, designed, and supervised the experiments; M.Z., Y.A., X.W., M.C., X.Z., C.Y., J.T., Z.C., and X.L. performed the neutralization assays; Y.W. and M.Z. analyzed the experimental data. Y.A. distributed and immunized the animals. X.W. performed the sequence alignment and phylogenetic tree analysis. M.Z. and Y.W. wrote the manuscript. All authors approved the final manuscript.

its own vaccine strain. The results shown are based on 3–5 assay repetitions. B) Neutralization trend of different doses of immunization for each IFV strains. Owing to the high number of IFV strains, we divided these 93 strains into four graphs. C) Neutralization of monovalent vaccine stock-immunized sera against representative IFV strains circulating in different years. D) Neutralization of monovalent vaccine stock-immunized sera against IFV strains in different antigenic clusters. The black line represents the median value with interquartile range. ns, not significant, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0001$.

Data sharing statement

Data generated by this study is available upon request to the corresponding author.

Declaration of interests

All authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ebiom.2022.104350>.

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