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Interim estimates of divergence date and vaccine strain match of human influenza A(H3N2) virus from systematic influenza surveillance (2010–2015) in Hangzhou, southeast of China

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SUMMARY

Objectives: In the post-pandemic period 2010–2015, seasonal influenza A(H3N2) virus predominated in Hangzhou, southeast of China, with an increased activity and semi-annual seasons. This study utilized *HA* virus gene segment sequences to analyze the divergence date and vaccine strain match of human influenza A(H3N2) virus from systematic influenza surveillance in Hangzhou.

Methods: Virological and serological analyses of 124 representative A(H3N2) viruses from prospective studies of systematic surveillance samples were conducted to quantify the genetic and antigenic characteristics and their vaccine strain match.

Results: Bayesian phylogenetic inference showed that two separate subgroups 3C.3 and 3C.2 probably diverged from group 3C in early 2012 and then evolved into groups 3C.3a and 3C.2a, respectively, in the 2014/15 influenza season. Furthermore, high amino acid substitution rates of the HA1 subunit were found in A(H3N2) group 3C.2a variants, indicating that increased antigenic drift of A(H3N2) group 3C.2a virus is associated with a vaccine mismatch to the 2015/16 vaccine reference strain Switzerland/9715293/2013 (group 3C.3a).

Conclusions: A portion of the group 3C.2a isolates are not covered by the current A(H3N2) vaccine strain. These findings offer insights into the emergence of group 3C.2a variants with epidemic potential in the imminent influenza seasons.

1. Introduction

Influenza viruses are important causative pathogens of respiratory tract infections in humans and animals globally and occasionally give rise to human pandemics. Currently circulating subtypes of human seasonal influenza viruses are A(H1N1)pdm09, A(H3N2), and B-Yamagata lineage, which are the three components of the annual trivalent vaccine recommended by the World Health Organization (WHO) Collaborating Centers for Reference and Research on Influenza (WHOCCs).[1] A(H3N2) evolves significantly faster than the other subtypes,[2] and thus could spread more quickly; it usually causes more severe outcomes among risk groups.[3] Season by season, continuous antigenic drift occurs in these viruses, altering their ability to cause infection and be transmitted among hosts. The circulating viruses escape the human immune response by having differences from the vaccine strains, and this can lead to vaccine failure.[4] In response to the threats posed by variants, the recommended seasonal A(H3N2) vaccine strain for use in the northern hemisphere seasons has been replaced by the WHO four times in the last 5 years.[1]

Hangzhou is a city of national tourism with a registered population of 8.9 million. It is located on the south wing of the Yangtze River Delta and has a humid, subtropical climate, facilitating the airborne survival and transmission of influenza viruses.[5] A(H3N2) viruses emerged in the post-pandemic period and have predominated in Hangzhou since August 2010. Previous genetic analysis of the very recent Hangzhou A(H3N2) strains indicated the emergence of escape variants with antigenic drift in

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the HA1 domain.[6] However, the lack of a comprehensive comparison of prevalent isolates with vaccine strains has hampered attempts to estimate the potential vaccine efficacy (VE) and to reconstruct the origins of these epidemics in the locality. This study was performed to quantify the genetic diversity and antigenic dynamics of seasonal A(H3N2) viruses in Hangzhou using a dataset of 124 isolates from more than 60 months of systematic surveillance. This was done in order to determine the possibility of more severe outbreaks in the near future.

2. Materials and methods

2.1. Systematic influenza surveillance

The surveillance was conducted from January 2010 to March 2015 among patients with an influenza-like illness (ILI) admitted to two tertiary hospitals in Hangzhou, China. Nasal swabs, oropharyngeal swabs, and/or tracheal aspirate samples were collected (N = 8958) with the informed consent of the patients or their spouses (Figure 1A). These were sent to the Hangzhou Center for Disease Control and Prevention for diagnosis within 24 h.

2.2. Virus detection and subtype identification

Viral RNA was extracted directly from the supernatant of the clinical sample using the RNeasy Mini Kit, as per the manufacturer's instructions (Qiagen, Germany). This was tested for the presence of seasonal influenza virus using a diagnostic real-time reverse transcription PCR (RT-PCR) on an ABI 7500 instrument (Applied Biosystems, USA) following the WHO guidelines.[7] Swab materials of positive samples were then inoculated into Madin–Darby canine kidney (MDCK) cells for virus isolation.

2.3. Hemagglutinin (HA) gene sequencing

Of 664 clinical specimens from laboratory-confirmed A(H3N2) infections, 124 (18.7%) with a high viral load (cycle threshold (Ct) value <30) were selected randomly from each epidemic for sequencing (E1, n = 8; E2, n = 9; E3, n = 8; E4, n = 5; E5, n = 54; E6, n = 22; E7, n = 18). The HA segment was subsequently amplified by RT-PCR with the primers described previously, using a PrimeScript One Step RT-PCR Kit Ver.2 (TaKaRa, Japan).[8,9] The amplicons were sequenced in both directions at Sangon (Shanghai, China). All sequences were assembled and edited with Lasergene v. 7.1.0 (DNASTAR). The full-length sequences obtained were then deposited in Global Initiative on Sharing Avian Influenza Data (GISAID) databases under accession numbers **EPI622591**–**EPI622714**.

2.4. Phylogenetic analysis

Multiple sequence alignment was conducted using Clustal X v. 1.8 combined with reference sequences of A(H3N2) viruses available in the GISAID databases (the accession numbers are listed in Table 1). Phylogenetic trees were generated by maximum likelihood method with bootstrap analysis (1000 replicates) using the MEGA v. 5.0 program.[10] The group numbers were assigned to achieve consistency with earlier studies.[11]

In order to assess the extent of divergence between different groups or clusters, these data were then used to infer the temporal phylogenies, viral evolution rates, and divergence dates of A(H3N2) viruses employing a relaxed clock model with uncorrelated log-normal rate distribution in a Bayesian Markov chain Monte Carlo (MCMC) framework implemented in BEAST package v. 1.7.4[12] and Tracer v. 1.5.0 software. Bayesian phylogenetic inference was presented graphically using FigTree v. 1.4.2 application.

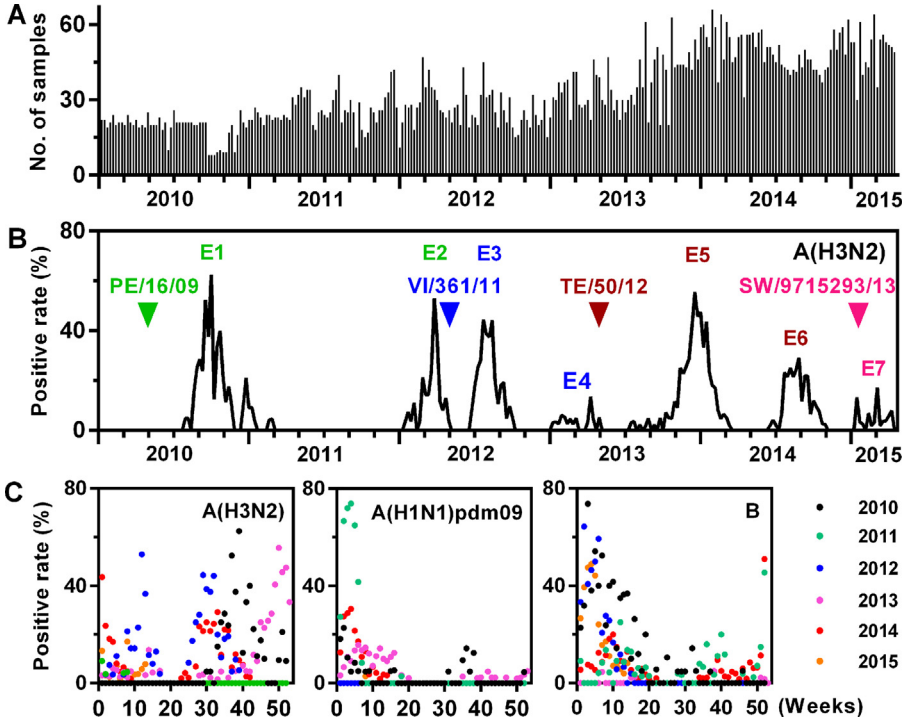

Figure 1. The prevalence of seasonal influenza virus in Hangzhou, China. (A) The number of patients with an influenza-like illness (ILI) sampled weekly during a 5-year surveillance period, from January 2010 to March 2015. (B) The positive rate of seasonal influenza A(H3N2) virus; seven A(H3N2) epidemics are seen (E1, August 2010 to December 2010; E2, January 2012 to March 2012; E3, July 2012 to September 2012; E4, January 2013 to March 2013; E5, July 2013 to February 2014; E6, July 2014 to October 2014; E7, January 2015 to March 2015). The annual vaccine candidates recommended by the WHO during these seasons are also shown, in the same color as the associated epidemics. (C) Overlapping images of the positive rates indicate the seasonal distribution of the currently circulating subtypes of human seasonal influenza viruses.

Table 1
Origin of HA sequence information of reference A(H3N2) strains included in the phylogenetic analyses[a]

Isolate name	Group	Accession number	Collection date	Originating laboratory	Submitting laboratory	Authors
A/Stockholm/18/2011	3A	**EPI326139**	2011-Feb-21	Swedish Institute for Infectious Disease Control	National Institute for Medical Research	
A/England/691/2010	3A	**EPI319221**	2010-Dec-13	Health Protection Agency	National Institute for Medical Research	
A/England/259/2011	3B	**EPI346607**	2011-Nov-16	Health Protection Agency	National Institute for Medical Research	
A/Maryland/02/2012	3B	**EPI358041**	2012-Jan-18	Maryland Department of Health and Mental Hygiene	Centers for Disease Control and Prevention	
A/Victoria/361/2011	3C	**EPI349106**	2011-Oct-24	Melbourne Pathology	WHO Collaborating Centre for Reference and Research on Influenza	Deng YM, Caldwell N, Iannello P, Komadina N
A/Texas/50/2012	3C.1	**EPI391247**	2012-Apr-15	Texas Department of State Health Services–Laboratory Services	Centers for Disease Control and Prevention	
A/Norway/1903/2014	3C.2	**EPI539623**	2014-May-20	WHO National Influenza Centre	National Institute for Medical Research	
A/Hong_Kong/5738/ 2014	3C.2a	**EPI539806**	2014-Apr-30	Government Virus Unit	National Institute for Medical Research	
A/Finland/385/2013	3C.3	**EPI502957**	2013-Dec-11	National Institute for Health and Welfare	National Institute for Health and Welfare	Ikonen N, Haanpaa M
A/Switzerland/ 9715293/2013	3C.3a	**EPI530687**	2013-Dec-6	Hôpital Cantonal Universitaire de Genève	National Institute for Medical Research	
A/Quebec/44/2014	3C.3x	**EPI573951**	2014-Dec-8		Other database import	Skowronski D, Chambers C, Sabaiduc S, De Serres G, Dickinson J, Winter A, Drews S, Fonseca K, Charest H, Gubbay J, Petric M, Krajden M, Kwindt T, Martineau C, Eshaghi A, Bastien N, Li Y, Skowronski DM, Dickinson JA, Winter AL, Gubbay JB, Kwindt TL
A/Serbia/71/2011	4	**EPI326115**	2011-Jan-6	Institute of Immunology and Virology Torlak	National Institute for Medical Research	
A/Alabama/05/2010	5	**EPI278805**	2010-Jul-13	US Air Force School of Aerospace Medicine	Centers for Disease Control and Prevention	
A/Victoria/802/2012		**EPI379585**	2012-May-9	Austin Health	WHO Collaborating Centre for Reference and Research on Influenza	Deng YM, Iannello P, Caldwell N, Jelley L, Komadina N
A/Perth/10/2010		**EPI390117**	2010-May-25		Other database import	Wentworth DE, Dugan V, Halpin R, Lin X, Bera J, Ghedin E, Fedorova N, Overton L, Tsitrin T, Stockwell T, Amedeo P, Bishop B, Chen H, Edworthy P, Gupta N, Katzel D, Li K, Schobel S, Shrivastava S, Thovarai V, Wang S, Ramanunninair M, Silverman J, Devis R, Phan L, Le J, Pokorny BA, Onodera S, Fulvini AA, He Y, Kilbourne ED, Bucher D, Bao Y, Sanders R, Dernovoy D, Kiryutin B, Lipman DJ, Tatusova T
A/Victoria/208/2009		**EPI232453**	2009-Jun-2	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	
A/IOWA/19/2010	6	**EPI335923**	2010-Dec-30	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	
A/Maine/07/2012	7	**EPI376509**	2012-May-21	Maine Health and Environmental Testing Laboratory	Centers for Disease Control and Prevention	
A/Perth/16/2009		**EPI211334**	2009-Apr-7	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	
A/Norway/1330/2010	2	**EPI302231**	2010-Dec-3	WHO National Influenza Centre	National Institute for Medical Research	
A/Norway/1186/2011	1	**EPI326137**	2011-Mar-16	Norwegian Institute of Public Health	National Institute for Medical Research	
A/California/ VRDL277/2009		**EPI274218**	2009-Apr-30		Other Database Import	The NIAID Influenza Genome Sequencing Consortium
A/Brisbane/10/2007		**EPI165489**	2007-Feb-6		Other Database Import	
A/Wisconsin/67/2005		**EPI160218**	2005-Aug-21		Other Database Import	

Table 1 (*Continued*)

Isolate name	Group	Accession number	Collection date	Originating laboratory	Submitting laboratory	Authors
A/California/7/2004		**EPI367105**	2004-Sep-16		Other Database Import	Wentworth DE, Dugan V, Halpin R, Lin X, Bera J, Ghedin E, Fedorova N, Overton L, Tsitrin T, Stockwell T, Amedeo P, Bishop B, Chen H, Edworthy P, Gupta N, Katzel D, Li K, Schobel S, Shrivastava S, Thovarai V, Wang S, Ramanunninair M, Silverman J, Devis R, Phan L, Le J, Pokorny BA, Onodera S, Fulvini AA, He Y, Kilbourne ED, Bucher D, Bao Y, Sanders R, Dernovoy D, Kiryutin B, Lipman DJ, Tatusova T
A/Fujian/411/2002		**EPI358784**	2002-Aug-11		Other database import	Galiano M, Johnson BF, Myers R, Ellis J, Daniels R, Zambon M

WHO, World Health Organization; NIAID, National Institute of Allergy and Infectious Diseases; GISAID, Global Initiative on Sharing Avian Influenza Data.
[a] All reference sequences were downloaded from the GISAID database.

Potential recombinant events and breakpoints were also scanned amongst aligned sequences by different recombination detection algorithms in RDP4 v. 4.46, such as RDP, GENECONV, Chimaera, MaxChi, BootScan, SiScan, and 3Seq.[13]

2.5. Genetic and antigenic analysis

The HA nucleotide and deduced amino acid sequences were compared within all of the A(H3N2) strains from 2010 to 2015. The pair-wise sequence identities among 124 Hangzhou A(H3N2) strains were calculated and plotted on a heat map drawn with ggplot2 v. 1.0.1 in R package.

An estimate of the relative genetic diversity was obtained by calculating the *p*-distance based on the equation $p = n_d/n$.[14] The numbers of amino acid substitutions on the HA1 subunit of A(H3N2) variants were counted. The potential VE was estimated using the formula $E = 0.47 - 2.47 \times P_{epitope}$; the parameter $P_{epitope}$ was calculated using a previously established method.[4] Further antigenic characteristics of isolates were confirmed by hemagglutination inhibition (HI) assay with anti-HA sera of seasonal A(H3N2) northern hemisphere vaccine components. Serum HI antibody titers were measured for each isolate using guinea pig red blood cells and standard HA sera (anti-A/Perth/16/2009-like HA serum for the 2010–2012 seasons, anti-A/Victoria/361/2011-like HA serum for the 2012–2013 season, anti-A/Texas/50/2012-like HA serum for the 2013–2015 seasons, and anti-A/Switzerland/9715293/2013-like HA serum for the 2015–2016 season; National Institute for Biological Standards and Control, UK) following WHO standard procedures.[15] To conduct the assay, two-fold serial dilutions of the sera were prepared and mixed with a specific amount of the influenza virus and then the red blood cells. The HI test was repeated three times, and the geometric mean titers were then calculated.

3. Results

3.1. The prevalence of influenza A(H3N2) viruses

From January 2010 through March 2015, the presence of influenza virus RNA in respiratory samples of ILI patients in Hangzhou was 17.7% (1583/8958): 60.4% (n = 956) were typed as influenza A and 39.6% (n = 627) as influenza B. Of the subtyped influenza A viruses, 69.5% (n = 664) were A(H3N2) viruses making up seven influenza activity peaks (Figure 1B). The first A(H3N2) epidemic came after the end of the A(H1N1)pdm09 pandemic and continued for nearly 6 months until the reemergence and transient domination of A(H1N1)pdm09 variants in the 2010/11 season.[16] In comparison to subtype A(H1N1)pdm09 and type B viruses, A(H3N2) has entered its semi-annual seasons with increased activity since early 2012 (Figure 1C).

3.2. The phylogeny and divergence dates

Comprehensive phylogenetic analysis of the gene encoding surface antigen HA of A(H3N2) isolates revealed that all of the Hangzhou strains clustered within the Victoria/208/2009 clade (Figure 2). The recently circulating viruses were mainly with genetic group 3, except for the 2010/11 season viruses belonging to group 5 or 6.

Much greater HA diversity was observed after 2012. The A(H3N2) viruses formed a large group 3C, but were scattered into different kinds of clusters, indicating dynamic genetic evolution and their adaptation to the hosts. Divergence events depicted on the Bayesian phylogenetic tree show that the group 3C.2 and 3C.3 viruses probably diverged from group 3C in early 2012 (Figure 3), and thus constituted two distinct clusters respectively.

The emergence of these novel clusters was confirmed by the initial detection of Hangzhou/A379/2012 (3C.2) and Hangzhou/A289/2012 (3C.3) strains during epidemic E3 in July 2012 (Figure 2). Following this, group 3C.3 viruses became the predominant strains, constituting nearly 100% of A(H3N2) viruses in E4 and 96.3% in E5. The evolution of group 3C.3 into 3C.3a at the beginning of 2014 might represent a viral adaptation for continuous circulation in E6 (100%) and E7 (77.8%). On the other side, 22.2% (4/18) of the early 2015 strains clustered within group 3C.2a, which is considered genetically and antigenically distinct from group 3C.3a viruses. These typically bear 3 to 7 amino acid differences at the key antigenic sites of the HA1 domain from the 2015/16 vaccine reference strain Switzerland/9715293/2013 (group 3C.3a) (Figure 2). However, no recombinant event was found by RDP scanning during this period of seasons.

3.3. The genetic and antigenic characteristics and vaccine strain match

The results of pair-wise analysis showed that the nucleotide and amino acid identities were 96.8–100% and 95.6–100%, with the peak frequency around 99.1% and 98.4%, respectively (Figure 4A). Significant differences in sequence identities among seasons were

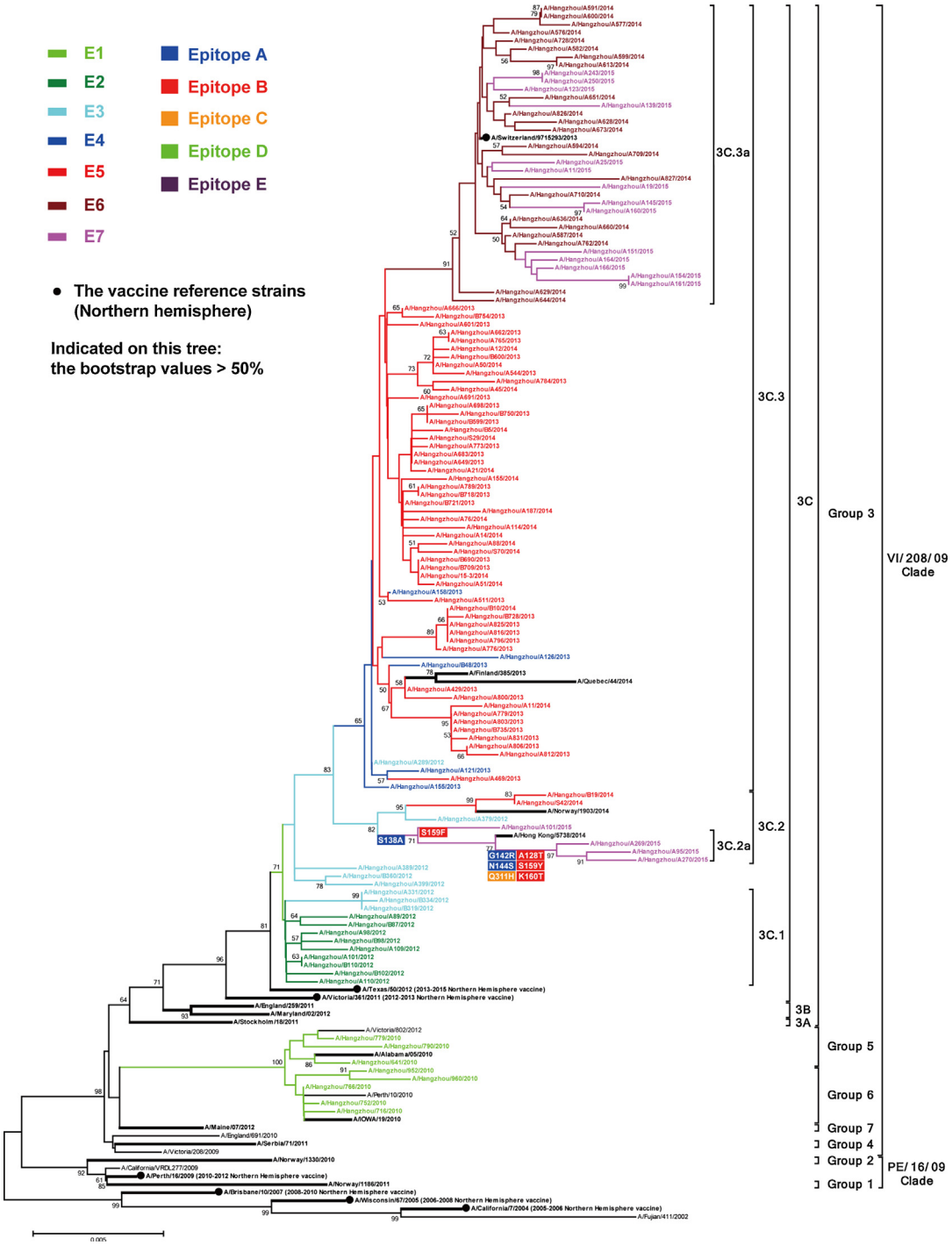

Figure 2. Phylogenetic relationships of A(H3N2) viruses on the *HA* gene. The names of the isolates are indicated with different colors according to the season they belong to, and the genetic clusters to which group they are clustered are also marked. Signature amino acid substitutions are annotated at the node of group 3C.2a in different colors according to their positions in the epitopes. Only bootstrap values >50% are shown.

observed through a heat map of the numeric matrix of the identity values (Figure 4B). Note that the identities of amino acid sequences were sometimes a bit lower.

The genetic and antigenic characteristics of 124 A(H3N2) strains were estimated by *p*-distance and number of amino acid substitutions, and the potential VE was calculated. Three different models gave out consistent results (Figure 5). The data indicated that the replacement of vaccine candidate pushed the vaccine strain genetically and antigenically closer to the circulating viruses each time. However, in the following epidemic, the genetic distance became longer and the predicted VE decreased quickly because of the high substitution rates on the HA1 subunit of A(H3N2) variants.

During E7, several outlying dots were observed, indicating a significant divergence and antigenic differences to the recommended vaccine reference strain. Due to the probable biases of predicted VE caused by the principal limitations, the results were confirmed with the HI assay (Figure 5D). Most of the A(H3N2) viruses emerging in the 2014/15 season reacted well against antiserum raised to Switzerland/9715293/2013. However, the four outlying group 3C.2a isolates cross-reacted weakly, representing an antigenically distinct group 3C.2a from group 3C.3a.

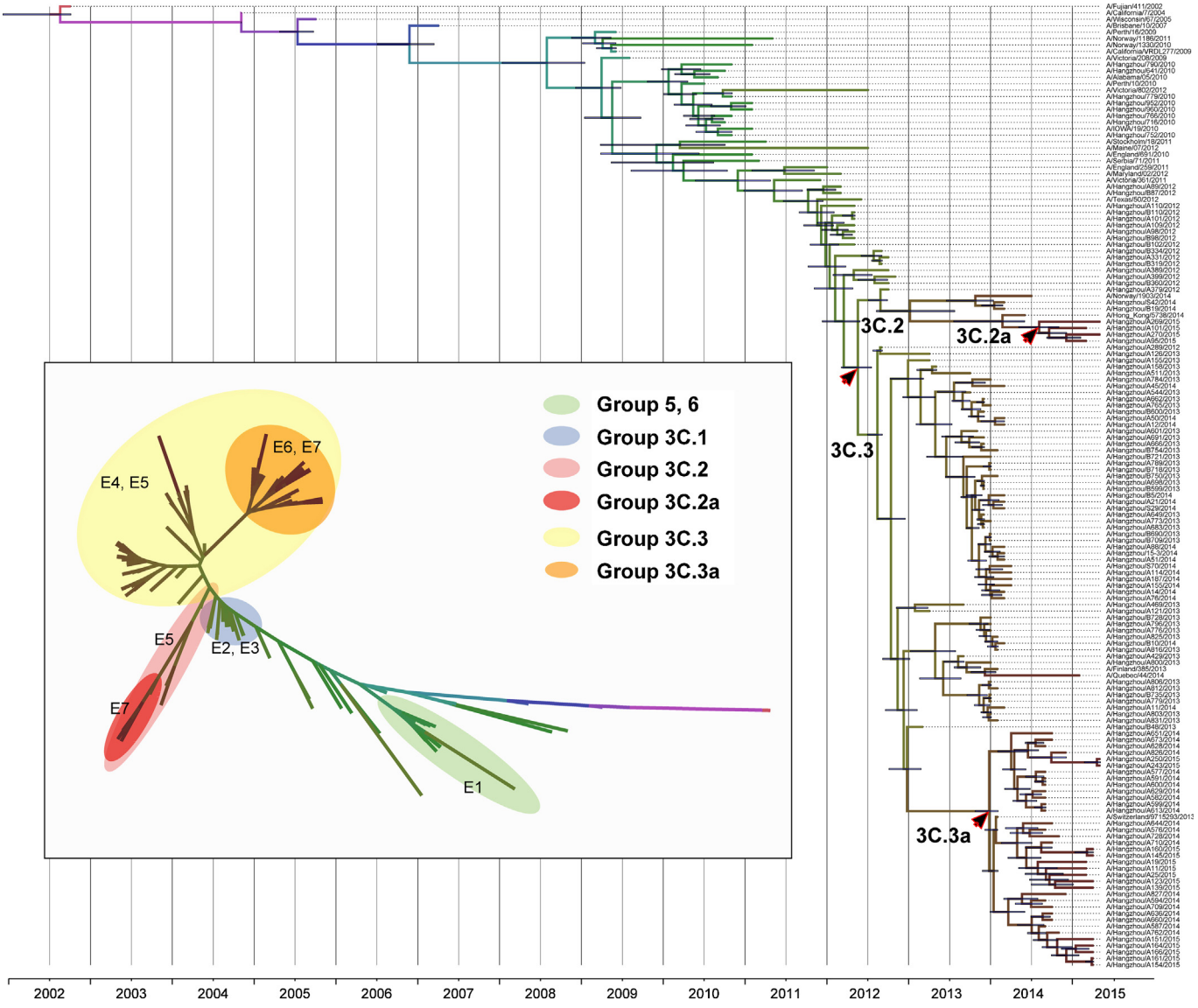

Figure 3. Divergence events based on the *HA* genes of seasonal influenza A(H3N2) viruses. The pertinent representative viruses were downloaded from the GISAID database, including the sequences of WHO-recommended vaccine strains for the northern hemisphere. Arrows indicate the divergence dates when novel subgroups of A(H3N2) emerged. Node bars represent 95% credible intervals of divergence times. The groups of A(H3N2) variants are differentiated by color in a simple phylogenetic tree.

4. Discussion

Influenza A(H3N2) viruses accounted for 69.5% of influenza A-positive cases during the post-pandemic seasons (2010–2015) in Hangzhou, China. Viral transmission control requires a close antigenic match between recently circulating viruses and the vaccine reference strain.[17] In China, annual vaccination against seasonal influenza viruses is recommended for all persons before the influenza season, especially for certain populations at risk of severe outcomes, including pregnant women, children, and the elderly.[18] Ahead of the next season, to address the lack of genetic and antigenic characteristics of the epidemic isolates in comparison to vaccine strains, virological and serological analyses of A(H3N2) viruses from prospective studies of systematic surveillance samples in Hangzhou during the post-pandemic period were conducted.

The monitoring data analysis showed that A(H3N2) has entered its semi-annual seasons with increased activity since 2012, while the predominant group 3C viruses have diverged into two separate paths. Novel subgroups of A(H3N2) emerged following these two evolution paths in the 2014/15 season. One was group 3C.3a, including predominant circulating strains in the recent season, and the other was subgroup 3C.2a, which were occasionally detected at the beginning of 2015. Viruses with similar sequences in the *HA* gene have also been detected sporadically in other countries,[19] and are considered antigenically different from the 2014/15 A/Texas/50/2012(H3N2)-like clade 3C.1 vaccine reference strain.[20,21] However, the complete characteristics of antigenic drift of A(H3N2) variants in the southeast of China and their vaccine match have not been reported to date.

The HA protein is one of the major glycoproteins present on the viral surface and facilitates proper attachment to the host cellular receptors.[22] The HA1 domain contains the five major antigenic sites (A–E) acquired for annual evaluation of vaccine effectiveness.[4] Mutations causing antigenic drift of HA are efficient in evading the host immune response, and are always closely scrutinized for signs of variation and evolution. In this study, the VE of the vaccine strains against circulating viruses in the contemporary period was complicated by antigenic dynamics. Switzerland/9715293/2013, a group 3C.3a strain, is the 2015/16

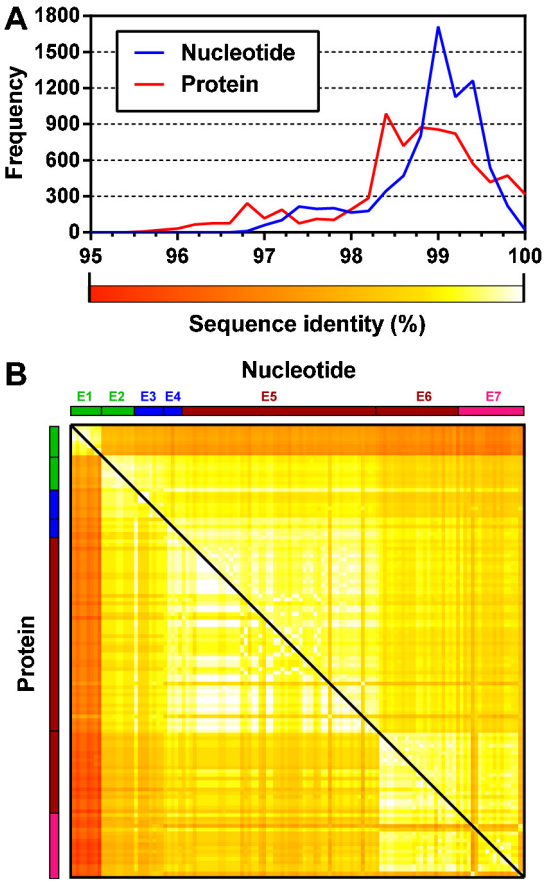

Figure 4. A The pair-wise identities of nucleotide and deduced amino acid sequences among 124 Hangzhou A(H3N2) strains from 2010 to 2015. (B) The numeric matrix of the identity values of nucleotide (upper triangle) and amino acid (lower triangle) are plotted on a heat map drawn with ggplot2 v. 1.0.1 in R package.

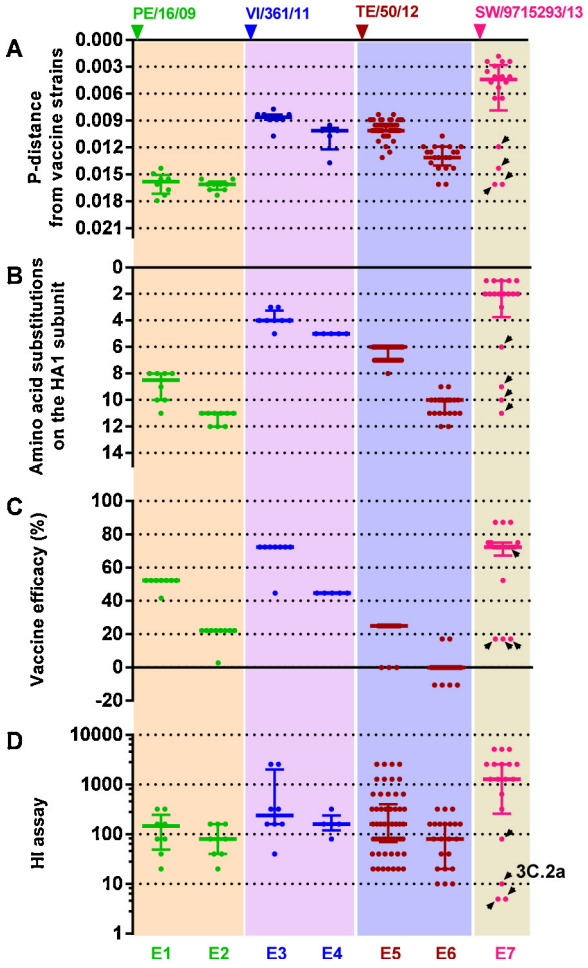

Figure 5. Genetic and antigenic characterization of A(H3N2) strains from different seasons. The annual vaccine candidates are listed in the same color as the corresponding epidemics. (A) Rates of *p*-distance of the *HA* gene from vaccine strains. (B) Amino acid substitutions calculated on the HA1 domain. (C) An estimate of potential vaccine efficacy (VE) against the circulating strains. (D) Antigenicity measured by hemagglutination inhibition (HI) assay. Arrows indicate the immune escape variants of A(H3N2) group 3C.2a strains. Error bars for observations: median with interquartile range.

vaccine candidate; this only covers portions of the antigenic types of circulating A(H3N2) viruses, which may lead to vaccinated people or those who have previously been infected by the 3C.3a strain easily being infected by a group 3C.2a variant with distinct antigenic form. The HI assay was also conducted to confirm that the VE drop was not an artifact of statistical biases. Consistent with predicted vaccine mismatch, little cross-reaction was observed with serum HI antibody titer against A(H3N2) group 3C.2a variants. Although Hammond et al. have suggested that antigenic relatedness between clade 3C.2a and 3C.3a viruses is good,[23] the present study showed that at least a portion of subgroup 3C.2a viruses are not covered by the 2015/16 vaccine reference strain. This potential challenge requires urgent further study.

Based on the present analysis, little antigenic cross-reaction was found between group 3C.2a and 3C.3a strains. Thus the 2015/16 A/Switzerland/9715293/2013-like clade 3C.3a vaccine reference strain may not offer the desired protection against the circulating group 3C.2a A(H3N2) viruses in the southeast of China. Given these findings, further in-depth investigations should be considered to determine the increased potential of group 3C.2a prevalence in the upcoming influenza seasons. If and when such an epidemic occurs, people who have been infected by a group 3C.3a strain in the past will lack immunity to group 3C.2a viruses, and the 2015/16 vaccines against the circulating viruses will also become less effective.

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Ethical approval: Signed informed consent was obtained from the patients or their spouses.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

Authors' contributions: LJ designed the study and wrote the manuscript. ZZB and YXF were responsible for influenza surveillance data maintenance. YXH was involved in the sample collection. LJ and ZZB did the detection and subtype identification. KY and ZYY isolated the viruses. LJ and ZYY participated in the antigenic analysis. LJ and WHQ performed the data analysis.

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