*Epidemiol. Infect.* (2013), **141**, 1061–1069. © Cambridge University Press 2012
doi:10.1017/S0950268812001318

# Phylogenetic, molecular and drug-sensitivity analysis of HA and NA genes of human H3N2 influenza A viruses in Guangdong, China, 2007–2011

P. HUANG[1,2]*, L.-J. LIANG[1,2], N.-M. HOU[1], X. ZHANG[1], W.-Z. SU[1], S.-Y. YU[3], Y.-H. ZHANG[1], J. WU[1] AND W. Q. CHEN[2]

[1] *Key Laboratory for Emergency Pathogen Detection, Guangdong Provincial Center for Disease Control and Prevention, Guangzhou, China*
[2] *Department of Epidemiology, School of Public Health, Sun Yat-Sen University, Guangzhou, China*
[3] *Department of Epidemiology, School of Public Health and Tropic Medicine, Southern Medical University, Guangzhou, China*

*Received 11 April 2012; Final revision 23 May 2012; Accepted 30 May 2012; first published online 25 July 2012*

## SUMMARY

Annual H3N2 subtype influenza outbreaks in Guangdong, China are a severe public health issue and require ongoing monitoring of emerging viral variants. The variation and evolution of haemagglutinin (HA) and neuraminidase (NA) genes of influenza isolates from Guangdong during 2007–2011 and others from GenBank were analysed using Lasergene 7.1 and MEGA 5.05, and serological analysis of antigens was determined by haemagglutination inhibition (HI). Susceptibility to antiviral drugs was correlated with genetic mutations. Phylogenetic analysis and alignment of HA and NA genes were performed on 18 Guangdong isolates and 26 global reference strains. The non-synonymous (dN) evolutionary rate of HA1 was 3·13 times that of HA2. Compared with the A/Perth/16/2009 vaccine HA gene, homologies of Guangdong isolates were between 98·8–99·7% and 98·0–98·4% in 2009 and 2010, respectively. Amino-acid substitutions were found in five epitopes of HA1 from Guangdong isolates between 2007 and 2011, especially in epitopes B (N160K) and D (K174R/N). The K189E/N/Q and T228A mutations in the receptor-binding site (RBS) occurred in the 2010 strains, which affected the antigenicity of HA1. The antigenicity of the epidemic H3N2 isolates in 2010 was somewhat different from that of A/Perth/16/2009. The Guangdong H3N2 isolates were determined to be oseltamivir-resistant with $IC_{50}$ of $0{\cdot}396 \pm 0{\cdot}085$ nmol/l ($n = 17$) and zanamivir-resistant with $IC_{50}$ of $0{\cdot}477 \pm 0{\cdot}149$ nmol/l ($n = 18$). Variations were present in epitopes B and D, two sites in the RBS and two glycosylation sites in the Guangdong H3N2 HA1 gene. The majority of the Guangdong H3N2 isolates were sensitive to oseltamivir and zanamivir. Compared to the World Health Organization 2012 vaccine strains, Guangdong H3N2 strains varied genetically and antigenically to some degree.

**Key words**: Drug sensitivity, epitope, influenza, H3N2, phylogenetic analysis, molecular epidemiology.

* Author for correspondence: Dr P. Huang, Key Laboratory for Emergency Pathogen Detection, Guangdong Provincial Center for Disease Control and Prevention, 176 Xingang Road West, Guangzhou 510300, China.
(Email: pphuang1@163.com)

https://doi.org/10.1017/S0950268812001318 Published online by Cambridge University Press

## INTRODUCTION

Influenza A viruses cause annual global epidemics resulting in severe morbidity and mortality. With the exception of the emergence of the pandemic H1N1 virus in 2009, the dominant circulating viruses had been of the H3N2 subtype since it first emerged in 1968, which is defined by two surface glycoproteins: H3 haemagglutinin (HA) and N2 neuraminidase (NA) [1–3]. The HA protein is encoded by influenza gene segment 4, while NA is encoded by segment 6, both of which are subtype-specific. HA binds to sialic acid-containing receptors on target cells to initiate virus infection, whereas NA cleaves sialic acids from cellular receptors to facilitate progeny virus release and to promote the spread of the infection to neighbouring cells [4].

HA is a trimer of identical subunits, each of which contains two polypeptides (HA1 and HA2) that result from proteolytic cleavage of a single precursor (HA). Cleavage of the precursor is essential for activation of the membrane fusion potential and hence infectivity. Each HA monomer contains a receptor-binding site (RBS) at its membrane-distal tip. HA proteins can combine with a variety of animal red blood cell surface glycoprotein receptors, resulting in red blood cell (RBC) agglutination and host cell infection by fusing with the cell membrane [5]. Thus, this ability to cause RBC agglutination is often used as the basis for viral characterization.

NA is a mushroom-shaped tetramer of identical subunits, with the head of the mushroom suspended from the virus membrane on a thin 60-Å-long stalk, the length of which is variable in virus strains. Each of the subunits that form the head of the mushroom is made up of a six-bladed propeller-like structure, the blade of which is formed by four anti-parallel strands of $\beta$-structure. The enzyme active site, containing a number of conserved charged amino-acid residues, is located roughly at the centre of each subunit [6].

Influenza A infection stimulates the production of virus-specific antibodies in the host humoral immune system. While these antibodies potentially block viral re-infection, they may also contribute to mutation in the virus or antigenic escape that would decrease its recognition by antibodies in subsequent generations. Once new variants occur, the new epidemic season with an increased likelihood of a pandemic requires an update in the vaccine strain. The influenza outbreak in Guangdong, China in 1996 was caused by an influenza virus with HA1 gene mutations in antigenic domains A, B, C, D and E, mainly those of A, C and E [7]. A phylogenetic analysis of influenza HA1 genes from Japanese strains found two evolutionary clusters with antigenic conversions between them, where basic amino acids tended to substitute uncharged polar amino acids, such as K135T, K145N, H155T, K156Q, R189S and R197Q [8]. Substitutions in relatively conserved NA active sites will lead to modification of enzymatic activity. Therefore, NA inhibitors (NAIs) are designed to target the conserved structure of the NA active site [9]. The findings of this study highlight the continuing antigenic drift of viral influenza antigens in China.

## MATERIALS AND METHODS

### Strains

H3N2 strains isolated in Guangdong from 2007 to 2011 were identified by our laboratory. The isolates were selected based on space–time sampling [10] (early isolates were selected in the same region in the same epidemic season) from different years in different regions (administrative cities) and different seasons in Guangdong, including four isolates in 2007, four in 2008, five in 2009, four in 2010 and one in 2011 (another three strains in 2011 were used only for sensitivity to antiviral drug assays) for a total of 18 isolates (GenBank nos. CY091825–CY091858, CY099953–CY099954). Sample numbers from cities in Guangdong included: Zhuhai ($n=6$), Yunfu ($n=2$), Zhaoqing ($n=2$), Foshan ($n=1$), Shaoguan ($n=5$) and Shantou ($n=2$) (Fig. 1). The HA and NA gene sequences of H3N2 reference strains were obtained from GenBank.

### Primer synthesis

Based on the worldwide HA and NA genes of human H3N2 isolates during 1968–2010, a series of primers were designed using Primer Premier 5.0 (Premier Biosoft International, USA) and synthesized by Life Technologies (Shanghai Branch). The primers were as follows: PHAF1 (gcaaaagcaggggataattc), PHAR1 (ttgtttggcatrgtcacgttc); PHAF2 (tatgcctcccttaggtcactag), PHAR2 (tcattggraatgcttccatttgg); PHAF3 (agcacmgggaayctaattgctc), PHAR3 (tgtcytcaacatatttctcgagg); PHAF4 (ttcaggacctcgagaaatayg), PHAR4 (agtagaaacaagggtgtttt); PNAF1 (agcaaagcaggagt), PNAR1 (tcgtgacaacttgagctggac); PNAF2 (tatcaatttgcmcttggrcagg), PNAR2 (tcccatccacacrtcatttcc);

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**Fig. 1.** Strains sampled from different cities in Guangdong. Zhuhai ($n=6$), Shaoguan ($n=5$), Foshan ($n=1$), Yunfu ($n=2$), Zhaoqing ($n=2$) and Shantou ($n=2$).

PNAF3 (agacaacggaaaggctccaatag), PNAR3 (agtagaaacaaggagttttt).

### Sequence detection

Viral RNA was extracted with a Qiagen QIAamp Viral RNA mini kit (Qiagen, UK), according to the manufacturer's instructions, for RT–PCR amplification using Qiagen Sensiscript Reverse Transcriptase and TaKaRa PyroBest Taq. The PCR fragments were extracted and purified with a Qiagen Gel Extraction kit. Briefly, total viral RNA was extracted from 140 $\mu$l of viral suspension (QIAamp Viral RNA kit) and added to a total reaction volume of 50 $\mu$l containing 5× Qiagen OneStep RT–PCR buffer 10 $\mu$l (0·5 mM), 1 $\mu$l dNTP mix, 1 $\mu$l enzyme mix, 1 $\mu$l Primer F, 1 $\mu$l Primer R (25 nmol/ml), 2 $\mu$l RNA and 34 $\mu$l $H_2O$-RNAase-free. The reaction was incubated at 50 °C for 30 min and 94 °C for 5 min, followed by a total of 35 cycles at 94 °C for 30 s, 45 °C for 30 s and 72 °C for 90 s (ABI GeneAmp PCR System 2700, Applied Biosystems, Singapore). PCR fragments were detected with an ABI PRISM BigDye Terminator v. 3.0 Ready Reaction Cycle Sequence kit (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, USA). The PCR sequences were analysed using Lasergene 7.1 to splice the fragment sequences [11].

### Phylogenetic analysis

Nucleotide sequences were aligned, and dendrograms were constructed using the neighbour-joining (NJ) approach implemented in MEGA 5.05 [12]. The reliability of the tree was estimated using 1000 bootstrap replications. Genetic distances based on NJ phylogenetic trees were calculated applying Kimura's two-parameter method in MEGA 5.05.

### Antigenic analysis

The cross-reactivity of the isolated viruses was investigated by the haemagglutination inhibition (HI) assay, using polyclonal antibodies to H3N2 viruses [13]. The antisera were derived by vaccinating New Zealand rabbits with four booster doses of $\beta$-propiolactone-inactivated flu virus.

### Sensitivity to antiviral drugs

Susceptibility to antivirals was measured using 50 $\mu$l of 100 $\mu$M methyl-umbelliferyl-*N*-acetyl neuraminic acid (MU-NANA, Sigma, USA) substrate as described previously [14]. The NAI susceptibility of influenza virus was expressed as the concentration of NAI required to inhibit the NA enzyme activity by 50% (50% inhibitory concentration, $IC_{50}$). An amount of virus that resulted in a signal of approximately $1\times10^5$ relative fluorescence units (RFU) was added to serial dilutions of oseltamivir or zanamivir (GlaxoSmithKline, USA), respectively, in a 96-well plate format. Guangdong/03/2005 was used as the control for sensitivity to these two drugs. The $IC_{50}$ values indicative of enzyme susceptibility to the drugs were analysed using Microsoft Excel 2003 (Microsoft

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Corporation, USA). The mean $IC_{50}$ value +3 S.D. criterion selected as a cut-off value was calculated for each NAI. In this study, extreme outliers (isolates with $IC_{50}$s more than tenfold higher than the mean $IC_{50}$ for drug) were excluded from statistical analysis. A one-way analysis of variance was performed to compare the $IC_{50}$s among the years for each drug, using SPSS v. 16.0 (SPSS Inc., USA) [15]. From a subset of viruses, the phenotypic assay-based drug susceptibility was confirmed by analysis of NA gene mutation.

## RESULTS

### Evolutionary rate and homology

With the A/Wyoming/03/2003 HA gene as a reference, there were 245 nucleotide substitutions in the HA gene analysed in 43 strains from 2003 to 2011; the overall ratio of synonymous (dS) to non-synonymous (dN) substitutions was 1·88:1 (160:85). In analysing the HA subunits separately, the dS:dN ratio of HA1 (328 amino acids) was 1·28:1 (89:69) and for HA2 (238 amino acids) it was 4·44:1 (71:16). This difference in dS:dN ratios between the HA1 and HA2 subunit genes was statistically significant ($\chi^2 = 15{\cdot}826$, $P < 0{\cdot}001$). The dN evolutionary rate of the HA1 was 3·13 times that of HA2 (69/328 *vs.* 16/238).

Alignment of the 43 HA gene sequences revealed the least homology (86·1%) between the A/Hong Kong/1/1968 and A/Denmark/21/2011 strains and the highest homology (87·9%) between the A/Hong Kong/1/1968 and A/Wyoming/03/2003 strains. As shown in Figure 2*a*, there were high homologies between the A/Brisbane/10/2007 vaccine HA gene and 2007 Guangdong isolates (four strains; 99·4–99·7%) or the 2008 Guangdong isolates (four strains; 98·6–99·0%); as well as between the HA gene of the vaccine strain A/Perth/16/2009 and the 2009 Guangdong isolates (five strains; 98·8–99·7%) or 2010–2011 Guangdong isolates (five strains; 98·0–8·4%) [12]. Notably, however, the most recent Guangdong isolates showed lower homologies with the vaccine strains than did the earlier Guangdong isolates, indicating antigenic drift.

By alignment of NA genes between vaccine strains (A/Brisbane/10/2007, A/Boston/4/2008, A/Perth/16/2009) and Guangdong isolates of the corresponding years, the homologies were 97·7–98·3%, 97·6–98·3% and 97·7–98·2%, respectively. The homologies between the A/Perth/16/2009 vaccine strain and 2010–2011 Guangdong isolates were 97·6–97·7%, which were even lower than those observed with the HA alignment above (Fig. 2*b*).

### Phylogenic diversity

With the A/Wyoming/03/2003 vaccine strain as a reference, all HA genes in the 43 Guangdong (GD) strains from 2003 to 2011 included the substitutions of S16A/T, T377I/M/R and E495G, while most of them showed changes of G66E/K, A144T, K161N, Y175F, V202G, S205N/K, S209F, Y235S, S243P, D241N, N391D and R466K. Phylogenic analysis of diversity revealed clustering of substitutions by year. For example, the common substitution sites of the 2008– 2011 isolates were S140N, K174R/N and K189E/N/Q, those of 2009–2010 isolates were E078K and N160K, and those of 2010–2011 isolates were D69N, Y110H, T228A, I246V and E296A. Strains from every year showed unique features; for instance, C12S only occurred in the HA gene 2008 isolates, while F95L and V229A were found only in 2009 isolates, and D69N, Y110H, P237L/T, I246V and E296A only in 2010 isolates. Compared with HA of the A/Perth/16/2009 vaccine strain, mutations of the 2009 isolates were D069N, Y110H, N160K, T228A, P237L/T and E296A (Fig. 2*a*).

With the NA gene of the A/Wyoming/03/2003 strain as a reference, 174 nucleotides substitutions were found, resulting in 63 amino-acid changes in the 43 Guangdong strains from 2003 to 2011. Substitutions E199K and Q432E took place in NA genes of all strains, while substitutions N93D, D147N, H150R, V194I, I215V, K221E, Y310H, L370S, N372K, S387L and Q432E occurred in NA genes in the majority of strains. As with the HA genes, clustering by year was evident in the phylogenic relationships and amino-acid substitutions in the Guangdong isolate NA genes, such as D147N and I215V in the 2008–2011 isolates, and S367N and I467L in the 2010–2011 isolates.

There were 11 potential glycosylation domains in the HA gene of the A/GD/15/2007 isolate, including $NST_{024-026}$, $NGT_{038-040}$, $NAT_{054-056}$, $NCT_{079-081}$, $NES_{138-140}$, $NWT_{142-144}$, $NGT_{149-151}$, $NNS_{160-162}$, $NVT_{181-183}$, $NST_{262-264}$ and $NGS_{301-303}$. Compared with the HA gene of Hong Kong/1/1968, substitutions included $NST_{024-026}$, $NAT_{054-056}$, $NSS_{061-063}$, $NES_{138-140}$, $NWT_{142-144}$ and $NNS_{160-162}$. HA genes of some strains gained glycosylation sites, such as $NSS_{061-063}$ in A/GD/30/2007, while some lost glycosylation sites,

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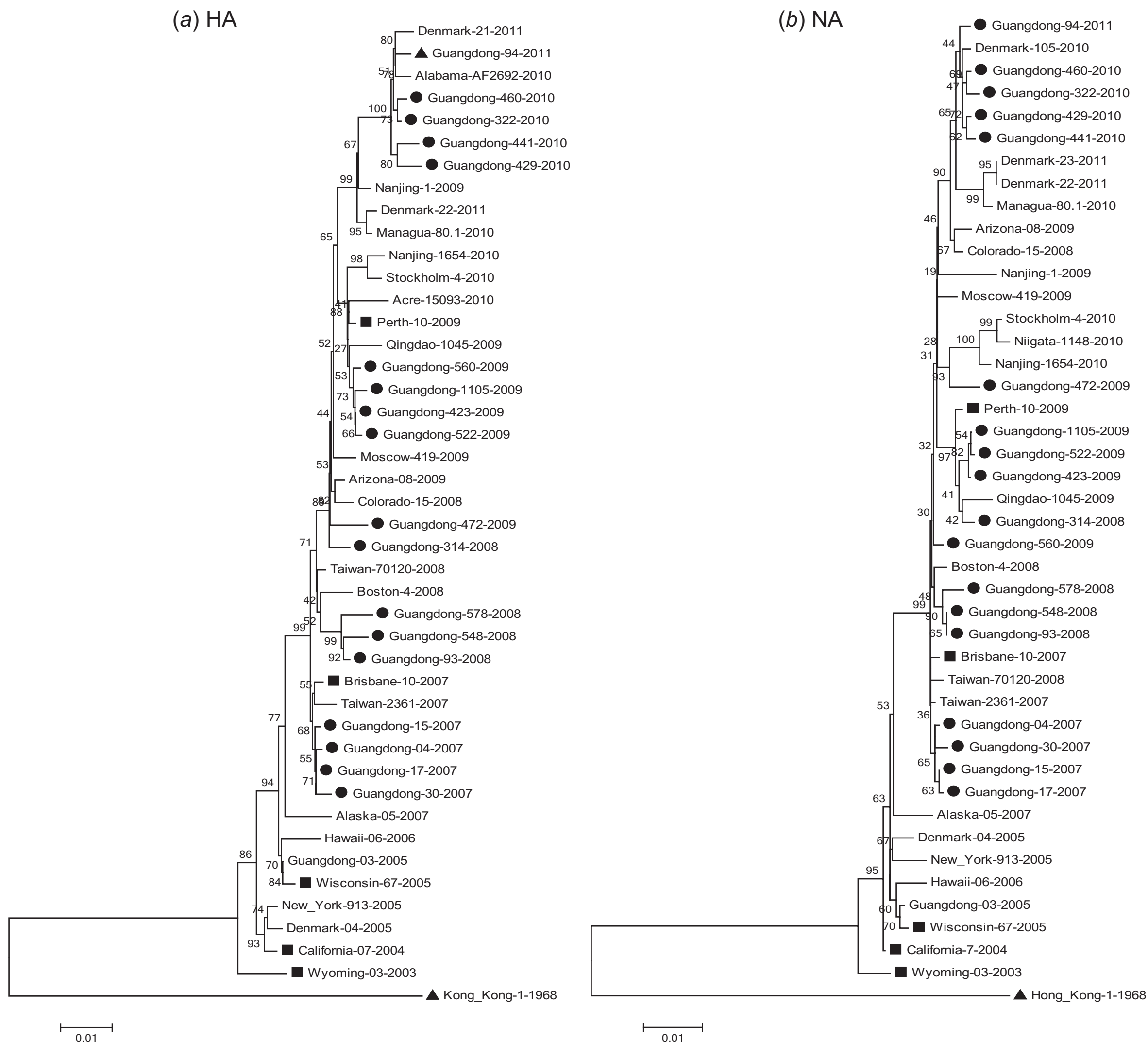


**Fig. 2.** Phylogenic trees were generated using HA and NA genes of 44 H3N2 strains. The nucleotide sequences included Guangdong isolates (●), the vaccine strains (■), most recent common ancestor (▲) and others in (*a*) HA genes and (*b*) NA genes. Phylogenetic trees were generated by neighbour-joining analysis with 1000 bootstrapped replicates and the percent bootstrap values for each note are shown in each tree.

such as $NES_{138-140}$ in A/GD/93/2008, A/GD/548/2008, A/GD/578/2008 and A/GD/441/2010, and $NNS_{160-162}$ in all 2009 isolates and some 2010 isolates. The HA gene of the A/GD/15/2007 strain possessed 18 cysteines, and only the 2008 isolates contained $C_{012}$ substitutions.

Eight potential glycosylation sites were found in the NA gene of A/GD/15/2007, including $NIT_{61-63}$, $NTT_{70-72}$, $NWS_{86-88}$, $NDT_{145-147}$, $NAT_{200-202}$, $NGT_{234-236}$, $NDS_{329-331}$ and $NRS_{402-404}$. The potential glycosylation site $NIT_{93-95}$ emerged in NA genes of A/Wyoming/03/2003, A/Wisconsin/67/2005, A/GD/03/2005 and A/Hawaii/6/2006. There were 21 cysteines in the A/GD/15/2007 NA protein, but no cysteine substitutions occurred in the Guangdong isolates.

## Serological antigenic analysis

HI assays were performed using antisera of rabbits infected with Guangdong H3N2 viruses isolated from 2007 to 2011. Compared to A/GD/15/2007, both A/GD/441/2010 and A/GD/94/2011 showed fourfold lower titres (1:640 *vs.* 1:2560 HI units), and compared to A/GD/94/2011, both A/GD15/2007 and A/GD/578/2008 showed twofold lower titres (1:640 *vs.* 1:1280 HI units) (Table 1). This result indicated that the A/GD/94/2011 strain was remarkably

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Table 1. *Haemagglutination inhibition (HI) reaction of influenza H3N2 viruses*

| Virus | Reciprocal HI titre for antiserum | | | | |
|---|---|---|---|---|---|
| | A/GD/ 15/2007 | A/GD/ 578/2008 | A/GD/ 1105/2009 | A/GD/ 441/2010 | A/GD/ 94/2011 |
| A/GD/15/2007 | 1:2560 | 1:1280 | 1:5120 | 1:1280 | 1:640 |
| A/GD/578/2008 | 1:1280 | 1:1280 | 1:5120 | 1:1280 | 1:640 |
| A/GD/1105/2009 | 1:1280 | 1:1280 | 1:5120 | 1:2560 | 1:1280 |
| A/GD/441/2010 | 1:640 | 1:640 | 1:2560 | 1:2560 | 1:1280 |
| A/GD/94/2011 | 1:640 | 1:640 | 1:2560 | 1:1280 | 1:1280 |

antigenically distinct from A/GD/15/2007 and A/GD/578/2008 and antigenically distinct to some degree from A/GD/1105/2009.

#### Susceptibility to antiviral drugs

By using a NA activity assay to determine the $IC_{50}$ of NAIs [14], the Guangdong isolates were determined to be oseltamivir-resistant with $IC_{50}$ of $0{\cdot}395 \pm 0{\cdot}083$ nmol/l ($n=20$) and zanamivir-resistant with $IC_{50}$ of $0{\cdot}460 \pm 0{\cdot}154$ nmol/l ($n=21$) (Table 2). The oseltamivir $IC_{50}$ of 6·82 nmol/l for A/GD/548/2008 was excluded, as it was higher than the cut-off value ($IC_{50}$ +3 S.D.) and was an extreme outlier (more than tenfold higher than the mean $IC_{50}$). The high oseltamivir $IC_{50}$, together with the observation of the D151V substitution of the Guangdong/548/2008 NA gene in this study ($IC_{50}=6{\cdot}820$ nmol/l), suggested that this mutation changed the sensitivity to oseltamivir but seemed to have no effect on zanamivir resistance ($IC_{50}=0{\cdot}467$ nmol/l). Except for the outlier, $IC_{50}$ values with oseltamivir or zanamivir against isolates of each year were not statistically significantly different. There was 100% correlation between the drug-sensitive phenotype and the presence of R292 and E119. The codons AGA of R292 and GAA of E119 apparently co-existed in the 43 Guangdong NA genes since this subtype emerged in 1968.

## DISCUSSION

Since the first outbreak of Hong Kong influenza in 1968, the H3N2 subtype influenza virus causing that pandemic has circulated for 43 years as the major subtype in the population. Without an RNA-dependent RNA polymerase (RDRP), influenza virus genes have a very high frequency of mutation without correction [16]. In this study, the dN rate of HA1 genes was greater than that of HA2 genes, indicating that HA1 proteins bear higher immunological pressure than HA2 proteins.

From an X-ray crystal structure, five epitope regions located on the HA1 protein of the H3N2 subtype determine viral antigenicity. Of the five epitope regions, A and B are at the centre of the main binding sites close to the neutralizing antibody [17]. Based on the reference HA1 sequence of the Wyoming/03/2003 vaccine strain, specific amino-acid substitutions were found: S140N in epitope A in 2008 isolates, N160K in epitope B in 2009–2010 isolates, R277Q in epitope C in 2007 isolates, K174R/N in epitope D in 2007–2010 isolates and E78K in epitope D in 2007–2010 isolates. Similarly to results in a Korean study, Guangdong isolate HA gene substitutions occurred in epitopes A (K140I), B (K158R) and E (K173N/Q and S262N) [13]. The variable sites in the Japan 1989–2006 H3N2 subtype HA1 genes were similar to those in the A/Beijing/352/89 and A/Sydney/5/97 strains, including K135T, K145N, H155T, K156Q, R189S and R197Q [8]. Meanwhile, phylogenetic analysis of the Thailand 2006–2009 H3N2 subtype HA genes revealed higher genetic variation than reference genes, and H3N2 clusters were closely related to the World Health Organization (WHO)-recommended vaccine strains in each season [1]. Substitutions in the pocket-shaped RBS at the top of the HA1 globular head domain, which plays an important role in host specificity [18], included K189E/N/Q of Guangdong 2008–2010 isolates and T228A in Guangdong 2010 isolates. These two variable sites have influenced the current influenza epidemic, especially the T228A substitution which emerged in 2010.

Seven antigenic epitopes of the N2 subtype NA protein have been located at positions 153, 197–199, 328–336, 339–347, 367–370, 400–403 and 431–434 and the enzyme active site of NA protein was found to include amino-acid sites 119, 156, 178, 179, 198, 222, 227, 274, 277, 294 and 425 [15, 19]. The six epitopes

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Table 2. *NA inhibitor (NAI) data analysis of $IC_{50}$ grouped according to year*

| Year | Drug [$IC_{50}$ (nM) per year] | | | | | |
|---|---|---|---|---|---|---|
| | Oseltamivir | | | Zanamivir | | |
| | Range (*n*) | Mean ± S.D. | Cut-off | Range (*n*) | Mean ± S.D | Cut-off |
| 2007 | 0·434–0·501 (4) | 0·469 ± 0·030 | | 0·370–0·424 (4) | 0·402 ± 0·023 | |
| 2008 | 0·299–0·355 (3) | 0·327 ± 0·028 | | 0·402–0·712 (4) | 0·514 ± 0·136 | |
| 2009 | 0·333–0·536 (5) | 0·457 ± 0·079 | | 0·362–0·798 (5) | 0·559 ± 0·163 | |
| 2010 | 0·249–0·382 (4) | 0·310 ± 0·055 | | 0·325–0·795 (4) | 0·481 ± 0·212 | |
| 2011 | 0·325–0·423 (4) | 0·376 ± 0·048 | | 0·200–0·413 (4) | 0·321 ± 0·090 | |
| Total | 0·249–0·536 (20) | 0·395 ± 0·083 | 0·644 | 0·200–0·795 (21) | 0·460 ± 0·154 | 0·922 |
| *P* value | 0·02 | | | 0·161 | | |

The NA inhibition assay was performed with viruses standardized to NA activity and incubated with NAIs. $IC_{50}$ determined by plotting the dose–response curve of inhibition of NA activity as function of the compound concentration. Values are from three independent determinations. A total of 18 isolates were tested and only one extreme outlier in oseltamivir was excluded from statistical analysis. The $IC_{50}$ and mean $IC_{50}$ ranges of oseltamivir and zanamivir were computed for isolates in different years. The cut-off was determined based on the elected criterion of the mean $IC_{50}$ + 3 S.D. *P* value was determined by one-way analysis of variance ($\alpha = 0{\cdot}05$).

(peptides 151–156, 221–228, 292–300, 368–374, 398–405, 428–435) were determined that covered previously reported epitope sites (153, 222, 294, 367–370, 400–403, 431–434, respectively), but had changes in length and peptide sequence to some degree [20, 21]. In the alignment of the NA genes from the 43 strains isolated from 2003 to 2011 in this study, substitutions were found at amino-acid sites 199, 370 and 432. Distinct from the Wyoming/03/2003 vaccine strain, the NA proteins of Guangdong isolates contained the E199K, S367N, K369T, L370S, G401D and Q432E substitutions. Residues in the influenza H3N2 NA catalytic site (R118, D151, R152, R224, E276, R292, R371, Y406) directly interact with the substrate and supporting framework sites (E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294, E425) [15]. While NAIs interact with multiple residues in the conserved structure of the NA active site, escape mutations generated *in vitro* or *in vivo* experiments have been limited to several conserved or semi-conserved residues, e.g. R292K and E119G/A/D/V in N2 subtypes. Although the escape mutations for NAIs of the N2 protein usually involve substitutions of E119V, R152K, H275Y, R293K and N295S, only the D151V change was found in the A/Guangdong/548/2008 isolate (oseltamivir-sensitive outlier) in this study.

Antigenic drift in HA1 has been shown to result in structural changes to escape antibody neutralization [22]. Of the five HA1 epitopes, A and B are close to the RBS, which is an important target of neutralizing antibodies. Global vaccine strains selected by the WHO/US Center for Disease Control (CDC) are often the dominant strains of influenza seasons. Historically, changes in epitopes A and B often drove antigenic drift and could be used to explain the selections of 24 WHO vaccine strains, but it has been proposed that using predicted epitope mutations would be a better choice in favour of whole influenza vaccine strains [22]. Using the Markov chain theory and the maximum-likelihood prediction model, it was found that the number of amino acids in HA1 epitope B has increased since the emergence of H3N2 in 1968 [23]. Interestingly, the number of charged residues in the dominant epitope of the dominant circulating strain is never fewer than that in the vaccine strain, suggesting that the selective pressure on charged amino acids increases the hydrophilicity of the viral epitope while decreasing its affinity for host antibodies.

A single amino-acid substitution in a glycosylation site was shown to affect antigenic drift of the global influenza viruses in a recent study of the H3N2 subtype HA gene [24]. Substitutions in the glycosylation site $NES_{138-140}$ influenced epitope A of Guangdong 2008 isolates, as did $NNS_{160-162}$ substitutions in epitope B of Guangdong 2009 isolates, which was undoubtedly of epidemiological significance. What is not yet clear is the biological significance of $C_{12}$ replacement of the Guangdong 2008 isolates. The Guangdong isolates from 2007 to 2011 were found to be susceptible to both NA inhibitors oseltamivir

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and zanamivir, except for the outlier of oseltamivir-sensitive A/GD/548/2008 [14].

Up to 2012, influenza H3N2 viruses as well as B viruses were the prevalent strains in Guangdong, China. The WHO/CDC-recommended A/Perth/16/2009 strain for 2011–2012 global use vaccine [25] might almost cover the HA antigenicity of global viruses but did not seem to completely cover the HA antigenicity of Guangdong 2010–2011 isolates and other strains (Fig. 2). The serological antigenic analysis showed antigenic changes to some degree in the 2010–2011 strains, although no WHO reference strains were included in the assay (Table 1). Several epitopes were different between the Guangdong 2010–2011 strains and the A/Perth/16/2009 strain, including K78E in epitope E, K160N in epitope B and T288A in the RBS. Based on these findings and the risk of the global H3N2 strains evolving further antigenically, WHO/CDC should consider a revision, an update and the inclusion of new 2012 vaccine strains that more closely match the circulating H3N2 strains.

## ACKNOWLEDGEMENTS

This project was financially supported by the National Natural Science Foundation of China (Grant no. 30972757). The authors thank Hanzhong Ni, Hui Li, Xingfen Yang and Changwen Ke (Guangdong Provincial Center for Disease Control and Prevention) and Mengfeng Li (Sun Yat-Sen University).

## DECLARATION OF INTEREST

None.

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