Characteristics of Avian Influenza H9N2 Virus Isolated from Humans and its Seroprevalence Among Occupationally Exposed Populations in China

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Abstract

Background: The first human-infected H9N2 influenza case can be traced back to 1998. Although the H9N2 influenza virus has low pathogenicity in animals, it donated partial or whole cassettes of internal genes to reassort novel viruses, such as H7N9, H10N8 and H5N6 viruses, that caused human infections with high fatality. Since 2013, sporadic but increasingly frequent human cases caused by H9N2 influenza virus have been confirmed globally, and most of them were from China.

Methods: Information on human infections with H9N2 influenza virus was collected. Viral molecular determinants were determined by deep sequencing, and phylogenetic analysis was performed using MEGA 6.06. Antigenic analysis was performed by a hemagglutination inhibition (HI) assay. Receptor binding preference analysis was conducted based on a solid-phase binding assay with synthetic sialylglycopolymers. Antiviral susceptibility was determined by a fluorescence-based neuraminidase (NA) inhibition assay. Serological study of occupationally exposed populations was performed by HI assay screening and confirmed by microneutralization assay.

Results: From 2013 to 2018, 33 human H9N2 cases were reported in China, among them 75.7% were children under 10 years old. The 22 viruses were isolated and concentrated in the Y280/G9 lineage of the HA and NA genes. All human H9N2 viruses belonged to the Y280/G9 antigenic lineage, presented a human-like receptor binding preference and remained susceptible to NA inhibitors, but most demonstrated resistance to M2 inhibitors. The seroprevalence of occupationally exposed populations was 2.15%, 3.17%, 2.93% and 1.54% from 2015 to 2018, respectively. A significant difference in seroprevalence was shown between provinces with human cases (3.66%) and provinces without human cases (2.18%).

Conclusions: The continuous antigenic drift and human-like receptor binding preference of the H9N2 virus enable it to have a high risk of causing human infections. The status of the seropositivity in occupationally exposed populations implies a substantial threat to public health. Research on human infection with H9N2 influenza virus should be strengthened to monitor the emergence of sustainable human-to-human transmission and the possibility of an endemic or a pandemic related to it.

Background

The earliest published avian influenza outbreak caused by H9N2 influenza virus in China can be traced to 1992 [1]. Since 1998, H9N2 had been a predominating subtype of avian influenza virus affecting the poultry industry through the beginning of the 21st century. Even in current times, H9N2 avian influenza is still endemic, imposing an animal health threat and causing economic losses.

Apart from its impact on animal health, the public health threat to humans is also critical. As documented in the first human cases infected with avian influenza H9N2 virus, which were reported by Dr. Guo's team in 1998 in China, the H9N2 influenza virus could traverse the species barrier and infect humans directly. Next, five virus strains were isolated from respiratory tract specimens of outpatients and inpatients in the southern region of China [2]. Then, 2 children in Hong Kong [3] and 1 infant from southern China were reported infected in 1999 [4]. The second human H9N2 infection in Hong Kong was reported in December 2003. However, recently, sporadic human infections of H9N2 influenza virus have been confirmed and reported to the World Health Organization (WHO); these infections were from China, Bangladesh, Egypt, Oman and Pakistan [5], and the majority was from China.

The strong ability of influenza H9N2 virus adaptation and evolution in poultry populations makes it a great threat to public health due to its supporting role in the emergence of novel lethal influenza viruses, such as H7N9, H10N8 and H5N6 [6–8]. Enhanced surveillance of human infection with avian influenza has occurred, including for the abovementioned novel influenza and influenza H9N2 itself.

Systematic studies on the genetic and antigenic profiles, receptor binding properties, and antiviral susceptibility of H9N2 viruses isolated from humans and the seroprevalence of the H9N2 virus in occupationally exposed populations are essential for global risk assessment. This study is the first general analysis of all available human-isolated H9N2 influenza viruses in China till to 2018 and the serological study among occupationally exposed populations nationwide in China.

Methods

Viruses and cells

The H9N2 viruses used in this study were isolated from humans and were proliferated in specific pathogen-free (SPF) embryonated chicken eggs. Madin-Darby canine kidney (MDCK) cells were used for virus titration. MDCK cells were cultured in DMEM with 5% fetal bovine serum (FBS).

Hemagglutination assay with different types of erythrocytes

A 1% erythrocyte suspension was prepared with chicken, turkey, guinea pig and horse red blood cells (RBCs). The HA titers were determined as described previously [9].

Gene sequencing and phylogenetic analysis

A MagMAXTM CORE Nucleic Acid Purification Kit was used to extract viral RNA, which was amplified using an Invitrogen Superscript One-step RT-PCR Platinum Taq HiFi Kit. The primers and probes used were previously described. A Qiagen MinElute Reaction Cleaning Kit was used to purify the PCR products. NGS (next-generation sequencing) sample preparation processed 1 ng of purified DNA product with an Illumina Nextera XT DNA sample preparation kit (96 samples). Sequencing was performed using a MiSeq v2 kit as previously described [10]. The full genome sequence was assembled by the CLC platform.

Homology analysis of nucleic acids and amino acids was performed on the NCBI website with BLAST. Phylogenetic analysis was performed on MEGA 6.06 using the Maximum Likelihood method. The reliability of the tree topology was assessed by bootstrapping with 1000 replications.

Receptor binding preference analysis

A solid-phase binding assay was used as described previously [11]. Synthetic sialylglycopolymers, including 3'-SLN, 6'-SLN, 3'-SL, and 6'-SL (Neu5Ac α 2,3Gal β 1-4GlcNAc, Neu5Ac α 2,6Gal β 1-4GlcNAc, Neu5Ac α 2,3Gal β 1-4Glc, and Neu5Ac α 2,6Gal β 1-4Glc, respectively), were coated on plates. Thirty-two hemagglutination units (HAUs) of each virus were added to each well. An ELISA was then used. The primary antibody was a universal monoclonal antibody against group 1 hemagglutinin (HA), and goat anti-human IgG-HRP was chosen as the secondary antibody. Tetramethylbenzidine (TMB) substrate solution (BD Biosciences) was used to visualize the results. The optical density was read at 450/630 nm.

Antigenic analysis

A HI assay was performed to indicate the antigenicity difference. The paired viruses and relevant polyclonal ferret antisera were used as references, including representative viruses of the G1 and Y280/G9 lineage, such as A/quail/HongKong/G1/1997, A/chicken/HongKong/G9/1997, and A/HongKong/308/2014. All sera were pretreated and were diluted with PBS to a final dilution fold of 1:10. An equal volume of antigen (8 HAUs/50 μ l) was added to the sera at a serial 2-fold dilution. The HI titers were determined by adding 50 μ l/well 1% Turkey RBCs.

Fluorescence-based neuraminidase inhibition assay

A NA-Fluor™ Influenza Neuraminidase Assay Kit (Applied Biosystems) was used to test the susceptibility of influenza virus to neuraminidase (NA) inhibitors, zanamivir (kindly provided by GlaxoSmithKline) and oseltamivir carboxylate (kindly provided by Hoffmann-La Roche), as described previously [12]. A/Beijing-Haidian/1942/2014 (H3N2) (E119) and A/Texas/12/2007 (H3N2) (E119V) were used as NA inhibitor-sensitive and NA inhibitor-resistant references, respectively. GraphPad Prism 5 software was used to calculate the IC₅₀ (drug concentration required to inhibit NA enzyme activity by 50%).

Serological study

Routine serological surveillance of occupationally exposed populations for avian influenza virus has been implemented since 2008 in mainland China. According to the regulatory policy of the national pandemic preparedness plan in China, informed consent was exempted. Serum samples were collected from poultry workers who are working in live poultry markets, large-scale poultry farms, backyard poultry farms, poultry slaughter factories, or wild bird habitats. Sera were pretreated in 4 volumes of RDE at 37°C for 18 hours followed by 56°C for 30 minutes. The HI assay was performed as described in the WHO manual [9]. An HI titer $\geq$ 40 was considered a suspected positive sample, followed by a microneutralization (MN) test for confirmation which was performed as previously described [13]. An MN titer $\geq$ 40 was considered seropositive for H9N2 infection.

Results

H9N2 human cases in mainland China

A total of 33 H9N2-infected human cases were documented from 2013 to 2018 in mainland China. Almost all were mild cases except one fatal case, who suffered from an underlying chronic medical condition. Of the 33 cases, the proportion of males and females was 17:16, and the age ranged from 2 months to 84 years. The median age was 4 years old. Most of the individuals were children under 10 years old (25/33, 75.7%). The cases were distributed in 9 provinces and one municipality, which were mostly concentrated in central southern China, and two were located in northern China, including Gansu Province and Beijing municipality. Field investigation indicated that 15 of 31 (48.4%) of the cases had clear poultry exposure histories, whereas 8 of 31 (25.8%) had no poultry exposure histories. Furthermore, the exposure history of 8/31 (25.8%) cases was unclear. Available viruses and their abbreviation names are listed. In addition to mainland China, the Hong Kong Special Administrative Region (SAR) also reported a total of 8 cases: 2 cases each year in 1999, 2009 and 1 case each year in 2003, 2007, 2008 and 2013 [5]. Laboratory-confirmed human H9N2 cases were reported by other countries, such as Bangladesh and Egypt, to the WHO; 2 cases were from Bangladesh, and 4 were from Egypt from 2011 to 2016 [5] (Table1).

Genetic characterization

A total of 29 human H9N2 virus sequences are available for analysis, including 24 human viruses (2013-2018), one virus (1999) in mainland China, 3 human viruses from Hong Kong and one from Bangladesh. We also chose 4 animal H9N2 viruses and one environment-related G1-like virus for this analysis. E627K and D701N mutations in the PB2 protein were confirmed for the virulence and transmission of H5N1, H7N9 and H9N2 viruses in mammals [14]. We found that 23/29 viruses contained E627 and that 28/29 contained D701. Only A/Sichuan-Bazhou/1453/2014 presented the change E627K. The other 5 Hunan H9N2 isolates, A/Hunan/34179/2018, A/Hunan/42088/2017, A/Hunan/37286/2017, A/Hunan-Chenzhou/45789/2015 and A/Anhui-Luijiang/39/2018, presented E627V substitutions. The E627V mutation has been studied in the H7N9 virus and confirmed to not compromised fitness and transmissibility in both avian and mammalian species [15]. A/Hong Kong/33982/2009 had a D701N substitution.

The amino acid sequence of the HA cleavage site is PSRSSR↓GLF in all tested viruses since 2013, while motifs with more diversity were present in viruses isolated before 2013. All of which indicated molecular markers of low pathogenicity to avian. After 2014, all human H9N2 viruses had acquired Q226L, H183N, A190V and I155T mutations, which were confirmed to be related to human receptor binding preference [16]. Before 2014, human H9N2 viruses contained 2-3 of the 4 abovementioned amino acid site substitutions.

All H9N2 viruses isolated from humans acquired 3 amino acid deletions (positions 63-65) at the NA stalk region after 2013, which were supposed to increase virulence in chickens and mice [17]. NA position 274, 292, 294 and 119 substitutions that supported NA inhibitor resistance were not identified in all human

H9N2 viruses [18].

The amantadine and rimantadine resistance mutation S31N/G in M2 was present in most H9N2 viruses [19]. Several amino acid changes in internal genes related to increased pathogenicity or virulence in H5N1 avian influenza virus were not identified in H9N2 viruses in this study (Table 2).

Phylogenetic analysis

All human H9N2 influenza viruses isolated in mainland China in this study belonged to the Y280/G9 lineage. Viruses isolated from 2013 to 2018 were clustered and represented by the viruses A/Hong Kong/308/2014 and A/Anhui-Luijiang/39/2018, candidate vaccine viruses (CVVs). However, A/Guangdong/333/99 was more similar than the CVVs to previous circulating viruses of the Y280/G9 lineage, such as BJ/94. The NA gene phylogenetic tree of H9N2 viruses showed the same model as the HA gene (Figure 1).

Antigenic analysis

HI titers against the homologous viruses ranged from 1:160-1:5120. A one-way HI assay showed that all the tested human H9N2 influenza viruses reacted well to at least one of the Y280 lineage reference antisera. A/Hong Kong/308/2014 (abbr. HK308) recommended as a vaccine strain in 2014, reacted well with most of the Y280 lineage viruses (12/17) before 2018. However, none of the 5 viruses isolated in 2018 were well inhibited by ferret antisera raised against the reference viruses before 2018. But all the viruses in 2018 reacted well with A/Anhui-Luijiang/39/2018 (abbr. AHLJ39), which was the representative strain in China and was recommended by the WHO as a new candidate vaccine (Table 3).

Receptor binding preference

All tested viruses were able to bind α 2,6 sialylglycopolymers, although two viruses, A/Hong Kong/33982/2009 and A/environment/Guangdong/14883/2016, also bound to α 2,3 sialylglycopolymers with moderate affinity. These two viruses presented dual receptor binding features. All human infected H9N2 viruses demonstrated the capacity to bind human receptor-like sialylglycopolymers, but different binding profiles existed. Some viruses (3/18), such as HN37286, JX47249 and GD333, had similar high affinities for 6'-SLN and 6'-SL, and some viruses (2/18), such as BJ58064 and GSJYG1397, had a preference for high affinity for 6'-SLN and lower affinity for 6'-SL. Most viruses (13/18) had a receptor binding preference only for 6'-SLN (Figure 2).

We further tested the binding capacity of the tested H9N2 viruses with different types of erythrocytes (Supplementary Table 1). All viruses presented no hemagglutination with horse erythrocytes, which express almost exclusively α 2,3 Gal linkages. Several studies have shown that human, guinea pig, chicken and turkey erythrocytes express both linkages. In addition, chicken erythrocytes display greater SA α 2,3 Gal linkages, while turkey erythrocytes display greater SA α 2,6 Gal linkages [20]. This is a possible reason why H9N2 virus titers in turkey RBCs were 0-1024-fold higher than those in chicken RBCs in our study.

Antiviral susceptibility of influenza H9N2 viruses

All 26 tested viruses were fully susceptible to the two NA inhibitors oseltamivir and zanamivir, with mean IC₅₀ values ranging from 0.09-0.78 nM. These IC₅₀ values were similar to those of circulating NA inhibitor-susceptible human H3N2 viruses (0.1 nM for oseltamivir, 0.45 nM for zanamivir). The fold change of H9N2 viruses IC₅₀ with NA inhibitor-sensitive reference virus's IC₅₀ is less than 4 (Supplementary Table 2).

Seroprevalence of H9N2 viruses in occupationally exposed populations

Serum samples were collected from occupationally exposed populations from 2015 to 2018, with 15779, 14395, 15863 and 15523 samples, respectively. The seroprevalence rates based on the MN test were 2.15% (340/15779), 3.17% (456/14395), 2.93% (464/15863) and 1.54% (239/15523) each year. We further compared the positive rate of provinces with H9N2 human case occurrences and of those provinces without H9N2 human case occurrences on a yearly basis. The seroprevalence of provinces with human cases was significantly higher than that of provinces without human cases ($P < 0.05$) (Table 4). We defined a seropositive rate of 2% as the baseline (average of seropositive rate is 2.4%), and the positive rates of 8 provinces in 2015, 18 provinces in 2016, 15 provinces in 2017 and 8 provinces in 2018 were above the baseline.

Discussion

H9N2 influenza virus is usually called a panzootic virus since this subtype of virus has been shown to be a donor of internal genes to generate zoonotic influenza viruses with pandemic potential. A prior study revealed that H9N2 influenza virus provided its internal genes to generate a highly pathogenic avian influenza H5N1 virus, which caused the human H5N1 outbreak in Hong Kong in 1997 [21]. Recent studies also supported the understanding of the emergence of novel influenza H7N9, H10N8 and H5N6 viruses from reassortment with six internal genes of circulating H9N2 virus [6–8]. All facts indicated that this subtype of influenza virus had acquired strong abilities of adaptation and spread among the avian population and exhibited high risks to humans after 2013. This situation may have caused human H9N2 influenza cases to be concentrated after 2013.

H9N2 influenza virus has been one of the predominant subtypes affecting poultry health since the end of the twentieth century. It is stably established in chicken flocks and causes endemic outbreaks in vast areas in China. Moreover, this virus can be easily isolated from wild birds, live poultry markets, backyard flocks and the environment in China, even in air samples in live poultry markets [22]. This prevalence is the reason why humans infected by this virus are frequently reported.

The evolution of the H9N2 influenza genotype was comprehensive. Multiple H9N2 genotypes were cocirculating over 10 years before the G57 genotype predominated in vaccinated farm chickens in China. G57 genotype-like viruses finally facilitated the genesis of novel H7N9, H10N8 and H5N6 viruses [23]. Our study showed that recently isolated human H9N2 viruses clustered with G57-like viruses in phylogenetic trees of the HA and NA genes.

HA of H9N2 influenza viruses continues to evolve into two primary lineages (Y280/G9 and G1), which circulate in the poultry of some countries in Asia and the Middle East, such as China, Vietnam, Egypt and Bangladesh. In mainland China, the Y280/G9 virus has a broad range of hosts, such as chickens, ducks, minor poultry species, swine and humans. However, G1-like viruses have been isolated only from minor poultry and humans in southern China [24]. This result was consistent with our surveillance data of poultry-related environmental samples.

Receptor binding preference has important implications for influenza replication and transmission [25]. Influenza H9N2 viruses have acquired the ability to bind human receptors while circulating in avian species and mammalian hosts. Although this ability was recently demonstrated by most H9N2 viruses, efficient human-to-human transmission was not present. This finding revealed that virus spread among humans needs a more complicated mechanism. Receptor binding preference was also revealed via the hemagglutination phenomenon of H9N2 viruses with different types of erythrocytes.

Clinical signs in chickens with H9N2 virus infection were quite mild. Chickens became more susceptible to secondary infection, especially *Escherichia coli* infections, with a mortality rate of at least 10%. The virulence of the H9N2 virus was higher in laying hens, and the morbidity of H9N2-infected laying hens was approximately 10% [26]. Human symptoms induced by the H9N2 virus are analogous to seasonal flu. The outcomes of patients were quick recovery and no lethality. To date, only one fatal case was found in 2016, which occurred in an individual who suffered from a chronic underlying condition. Most human H9N2 influenza cases were found through an influenza-like illness (ILI) surveillance system rather than the unknown pneumonia surveillance system.

Human infection with influenza H9N2 is often unnoticed since it generally results in mild or asymptomatic illness. Serological surveys are an optimal approach to identify subclinical infections and assess the risk of transmission to humans. A number of serological surveys or studies were carried out in some countries of Asia, Africa, the Middle East and North America [27]. Seroprevalence ranged from 1%-43% by HI and 0.6%-9% by MN, in which the results varied depending upon the infection definition (cut-offs of antibody titer), viruses (circulating or previously circulated), and methods (HI or MN) used for testing. Selection of low titers as cut-offs can lead to overestimation of influenza infection. Cross reaction with circulating seasonal influenza viruses by HI assay usually results in a low sensitivity to influenza infection determination. Few serological studies of influenza H9N2 among the human population, especially for occupationally exposed populations, have been performed in some areas, such as Guangzhou, Jiangsu, Shandong, Beijing, and Shanghai, in China or nationwide [28–30]. These studies were based on designated populations, small sample sizes or short time durations and can reflect a partial view of the infection status of H9N2 influenza in China. Seroprevalence ranged from 0.7–15.5%, which was similar to our study, in which 4-year continual surveillance data were analyzed from 2015 to 2018.

At present, the influenza H9N2 virus has acquired a stronger ability to adapt to avian species and has led to more concern for public health, either directly infecting humans or generating novel influenza viruses with pandemic potential. Serological surveillance also supported that subclinical infection existence. Efforts should be strengthened to monitor virus changes and infection status in terms of providing prepandemic warnings in a timely manner.

Conclusion

Human influenza H9N2 cases occurred sporadically in China. There no human-to-human transmission has been identified to date. The continuous antigenic drift and human-like receptor binding preference of the H9N2 virus enable it to have a high risk of causing human infections. NA inhibitors are still working as the antiviral against the H9N2 influenza virus. The seropositivity rate in occupationally exposed populations is variable among different regions, implying a substantial threat to public health. We should strengthen the surveillance of human infection with H9N2 influenza virus and the research on virological characteristics.

Abbreviations

HA: Hemagglutinin

NA: Neuraminidase

NGS: Next generation sequencing

MN: Microneutralization

HI: Hemagglutination inhibition

ILI: Influenza-like illness

CVVs: Candidate vaccine viruses

Declarations

Ethical Approval and Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declared that they have no conflicts of interest.

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Authors' contributions

Writing: review and editing: DW. Funding acquisition: LD; JD. Supervision: DW; YS. Conceptualization: DW. Methodology: JD, HB, YZ, LD, WH, SZ, XL, LY, XZ, JL, TC. Writing: original draft preparation: JD. All authors read and approved the final manuscript.

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Tables

Table 1. Human-isolated influenza A H9N2 viruses

No.	Onset time	Province	Sex	Age	Outcome	Poultry exposure history	Virus isolate	Virus abbreviation
GD-1	1998	Guangdong	Male	4y	Recovered	Unknown	A/Shantou/217/98	
GD-2	1998	Guangdong	Female	1y	Recovered	Unknown	A/Shantou/239/98	
GD-3	1998	Guangdong	Female	36y	Recovered	Yes	A/Shantou/252/98	
GD-4	1998	Guangdong	Male	14y	Recovered	Yes	A/Shaoquan/402/98	
GD-5	1998	Guangdong	Female	75y	Recovered	Yes	A/Shaoquan/408/98	
GD-6	1999	Guangdong	Female	1 y10 m	Recovered	None	A/Guangzhou/333/99	
case 1	2013.11.19	Hunan	Male	7y	Recovered	Yes	A/Hunan-Lengshuitan/11197/2013	HNLST11197
case 2	2014.12.21	Guangxi	Male	35y	Recovered	-	A/Guangxi-Jiangzhou/11021/2014	GXJZ11021
case 3	2014.12.5	Sichuan	Male	2y	Recovered	-	A/Sichuan-Bazhou/1453/2014	SCBZ1453
case 4	2014.12.24	Guangdong	Male	3y	Recovered	Yes	A/Guangdong/01747/2014	GD01747
case 5	2015.2.4	Guangdong	Female	84y	Recovered	Yes	NA	
case 6	2015.4.14	Anhui	Female	4y	Recovered	Yes	A/Anhui/33329/2015	AH33329
case 7	2015.9.6	Anhui	Male	6y	Recovered	Unknown	A/Anhui/43269/2015	AH43269
case 8	2015.9.1	Hunan	Male	2y	Recovered	Unknown	A/Hunan/44558/2015	HN44558
case 9	2015.9.15	Hunan	Female	15y	Recovered	Yes	A/Hunan/44557/2015	HN44557
case 10	2015.10.27	Hunan	Female	1y	Recovered	Unknown	A/Hunan-Chenzhou/45789/2015	HNCZ45789
case 11	2016.2.1	Henan	Female	5y	Recovered	None	A/Henan/10867/2016	HeN10867
case 12	2016.2.9	Sichuan	Female	57y	Fatal	Unknown	NA	
case 13	2016.6.10	Guangdong	Female	4y	Recovered	Yes	A/Guangdong/SF058/2016	GDSF058
case 14	2016.7.9	Guangdong	Male	2 y11 m	Recovered	Yes	A/Guangdong-Yuexiu/1822/2016	GDYX1822
case 15	2016.8.2	Jiangxi	Female	4y	Recovered	None	A/Jiangxi/47249/2016	JX47249
case 16	2016.8.7	Yunnan	Male	9 m	Recovered	Yes	NA	
case 17	2016.8.8	Guangdong	Female	29	Recovered	Yes	NA	
case 18	2016.8.25	Gansu	Male	4y	Recovered	None	A/Gansu-Jiayuguan/1397/2016	GSJYG1397
case 19	2016.12.11	Beijing	Male	3y	Recovered	Yes	A/Beijing/58604/2016	BJ58604
case 20	2016.12.11	Guangdong	Female	7 m	Recovered	Yes	NA	
case 21	2016.12.5	Guangxi	Male	1 y 9 m	Recovered	None	NA	
case 22	2017.2.6	Gansu	Male	11 m	Recovered	Yes	NA	
case 23	2017.4.28	Beijing	Male	32y	Recovered	None	NA	
case 24	2017.4.28	Guangdong	Female	2 m	Recovered	Unknown	NA	
case 25	2017.9.18	Hunan	Male	9 m	Recovered	None	A/Hunan/37286/2017	HN37286
case 26	2017.12.01	Hunan	Female	1 y 8 m	Recovered	Yes	A/Hunan/42088/2017	HN42088
case 27	2017.12.29	Anhui	Female	9 y	Recovered	Unknown	A/Anhui-Luijiang/39/2018	AHLJ39
case 28	2018.1.21	Guangdong	Female	3 y	Recovered	Unknown	A/Guangdong/18SF003/2018	GD18SF003
case 29	2018.2.13	Beijing	Female	51 y	Recovered	Yes	NA	
case 30	2018.7.21	Guangdong	Female	24 y	Recovered	Unknown	NA	
case 31	2018.10.16	Guangdong	Male	3 y	Recovered	Yes	A/Guangdong/18SF064/2018	GD18SF064

case 32	2018.10.10	Guangxi	Male	3 y	Recovered	None	A/Guangxi-Xiangshan/11522/2018	GXXS11522
case 33	2018.11.27	Hunan	Male	2 y	Recovered	None	A/Hunan/34179/2018	HN34179
Outside mainland China								
1	1999	HK SAR	Female	13 m	Recovered	-		
2	1999	HK SAR	Female	4y	Recovered	-		
3	2003	HK SAR	Male	5y	Recovered	None		
4	2007	HK SAR	Female	9 m	Recovered	Unknown		
5	2008.12	Shenzhen	Female	3 m	Recovered	Yes		
6	2009	HK SAR	Female	35 m	Recovered	-		
7	2009.10	HKSAR	Female	47y	Recovered	Unknown		
8	2013.12.28	HK SAR	Male	86y	Recovered	None		
9	2011	Bangladesh			Recovered	-		
10	2015.01.16	Egypt	Male	3y	Recovered	Healthy poultry		
11	2015.02.01	Bangladesh	Female	3.5y	Recovered	Poultry		
12	2015.06	Egypt	Female	7y	Recovered	Poultry		
13	2015.06	Egypt	Female	9 m	Recovered	Poultry waste		
14	2016.04.10	Egypt	Male	18 m	Recovered	Live poultry market		

Note: -, indicates that information is not available.

Table 2. Key molecular markers of influenza A H9N2 viruses in this study

Virus	HA					NA					M		PB2	
	155	183	190	226	HA cleavage site (333-341)	63-65 deletion	274	294	292	119	31	27	627	70
A/chicken/Hongkong/G9/1997	T	N	A	L	PARSSRGLF	NO	H	N	R	E	S	V	E	D
A/duck/Hongkong/Y280/97	T	N	T	L	PARSSRGLF	Y	H	N	R	E			E	D
A/guinea fowl/HK/NT101/2003(G1)	T	N	A	L	PARSSRGLF	Y	H	N	R	E	S	V	E	D
A/Hong Kong/1073/99(G1)	T	H	E	L	PARSSRGLF	NO	H	N	R	E	S	V	E	D
A/quail/Hong Kong/G1/97	T	H	E	L	PARSSRGLF	NO	H	N	R	E	S	V	E	D
A/Bangladesh/0994/2011(G1)	T	H	A	L	PAKSSRGLF	NO	H	N	R	E	N	V	E	D
A/Hunan/34179/2018	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	V	D
A/Guangdong/18SF064/2018	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/GXXS/11522/2018	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/AHLJ/39/2018	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	V	D
A/Guangdong/18SF003/2018	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/Hunan/42088/2017	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	V	D
A/Hunan/37286/2017	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	V	D
A/Beijing/58604/2016	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/GSJYG/1397/2016	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/Jiangxi/47249/2016	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/GDYX/1822/2016	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/Guangdong/SF058/2016	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/Henan/10867/2016	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/Zhongshan/201501/2015	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/HNCZ/45789/2015	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	V	D
A/Hunan/44557/2015	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/Hunan/44558/2015	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/Anhui/43269/2015	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/Anhui/33329/2015	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/GXJZ/11021/2014	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	S	V	E	D
A/Guangdong/01747/2014	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/HNLST/11197/2013	T	N	A	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/SCBZ/1453/2014	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	K	D
A/Hongkong/308/2014	T	N	T	L	PSRSSRGLF	Y	H	N	R	E	N	V	E	D
A/Hongkong/33982/2009	T	H	D	Q	PARSNRGLF	NO	H	N	R	E			E	N
A/Guangdong/333/99	T	N	T	L	PSRSSRGLF	NO	H	N	R	E	S	V	E	D
A/environment/Guangdong/14883/2016(G1)	T	H	A	Q	PARSSRGLF	NO	H	N	R	E	S	V	E	D

Table 3. HI titers from antigenic analysis of influenza A H9N2 viruses

No.	HA Clade	Reference virus	Y280						G1		
			GD333	HK-G9	HK308	HN11197	GD01747	SCBZ1453	AHLJ39	BJ/94	HK-G1
1	Y280	A/Guangdong/333/99 E6	1280	640	<40	1280	<40	<40	80	320	<40
2	Y280	A/Chicken/HK/G9/1997 E3	320	1280	<40	640	<40	<40	40	320	<40
3	Y280	A/HK/308/2014 E5	<10	80	5120	640	5120	5120	640	<10	<40
4	Y280	A/HNLSG/11197/2013 C1E2	320	640	640	5120	160	640	320	320	<40
5	Y280	A/Guangdong/01747/2014 E2	<10	160	5120	320	5120	5120	1280	<10	<40
6	Y280	A/Sichuan-Bazhou/1453/2014 E2	<10	40	5120	160	5120	5120	5120	<10	<40
7	Y280	A/Anhui-Luijiang/39/2018	80	<40	640	160	1280	640	5120	ND	ND
8	G1	A/Chicken/BJ/1/1994 E3	<10	40	<40	160	<40	<40	<40	160	<40
9	G1	A/Quail/Hong Kong/G1/97 E7	<10	<40	<40	<40	<40	<40	<40	<10	1280
Tested virus											
1	Y280	A/Hunan/34179/2018 E3	80	40	640	160	640	320	1280	<20	<40
2	Y280	A/Guangdong/18SF064/2018 E2	80	<40	640	80	640	320	2560	<20	<40
3	Y280	A/GXXS/11522/2018 C1E2	80	<40	640	160	640	640	2560	<20	<40
4	Y280	A/AHLJ/39/2018 E3	80	<40	640	160	1280	640	5120	20	<40
5	Y280	A/Guangdong/18SF003/2018 C1E1	80	<40	640	80	640	640	2560	40	<40
6	Y280	A/Hunan/42088/2017 E1	<10	80	2560	320	2560	2560	ND	<10	<40
7	Y280	A/Hunan/37286/2017 E2	<10	80	2560	640	5120	5120	ND	<10	<40
8	Y280	A/Beijing/58604/2016 E3	<10	80	5120	160	5120	5120	ND	<10	<40
9	Y280	A/GSJYG/1397/2016 C2E1	<10	80	5120	320	5120	5120	ND	<10	<40
10	Y280	A/Jiangxi/47249/2016 E2	<10	80	2560	320	2560	2560	ND	<10	<40
11	Y280	A/GDYX/1822/2016 C2E2	<10	40	640	640	2560	2560	ND	<10	<40
12	Y280	A/GD/SF058/2016 E2	<10	<40	640	320	1280	640	ND	<10	<40
13	Y280	A/Henan/10867/2016 E2	<10	<40	640	160	2560	2560	ND	<10	<40
14	Y280	A/HNCZ/45789/2015 C2E1	80	320	5120	640	5120	5120	ND	<10	<40
15	Y280	A/Hunan/44557/2015 E2	<10	40	1280	160	1280	1280	ND	<10	<40
16	Y280	A/Hunan/44558/2015 E3	<10	40	2560	320	5120	5120	ND	<10	<40
17	Y280	A/Anhui/43269/2015 E2	<10	40	1280	160	2560	2560	ND	<10	<40
18	Y280	A/Anhui/33329/2015 E2	<10	<40	640	640	2560	2560	ND	<10	40
19	Y280	A/GXJZ/11021/2014 E3	<10	80	5120	320	5120	5120	ND	<10	<40
20	Y280	A/GD/01747/2014 E3	<10	40	5120	320	5120	5120	ND	<10	<40
21	Y280	A/SCBZ/1453/2014 E3	<10	80	5120	320	5120	5120	ND	<10	<40
22	Y280	A/HNLSG/11197/2013 C1E2	320	640	640	5120	160	640	320	320	<40
23	G1	A/HK/33982/2009 V1E5	<10	<40	<40	<40	<40	<40	ND	<10	320
24	G1	A/Environment/GD/14883/2016 E2	<10	<40	<40	<40	<40	<40	ND	40	80

Note: _ indicates HI titers against the homologous viruses

Red letters indicate the HI titers > 4 fold difference with the HI titers against the homologous viruses

ND indicates not done

Table 4. Comparison of influenza H9N2 seroprevalence between provinces with and without human cases

Year	Provinces with human cases		Provinces without human cases		χ^2	P
	positive	negative	positive	negative		
2015	117	3837	223	11602	16.187	P<0.05
2016	146	3435	310	10504	12.849	P<0.05
2017	137	2561	327	12838	53.06	P<0.05
2018	68	2966	171	12318	12.246	P<0.05

Figures

Figure 1

Phylogenetic trees of HA and NA genes from influenza A H9N2 viruses. Phylogenetic tree was constructed using the Maximum Likelihood method in MEGA 6.06. The bootstrap value was tested with 1000 replications. Viruses in black square indicated being selected as the human H9N2 vaccine candidate recommended by WHO.

Figure 2

Receptor binding properties of Influenza A H9N2 viruses. Horizontal coordinates: glycans concentration, Vertical coordinates: Absorbance 450nm. Binding affinity of the viruses to four different biotinylated glycans (6'-SLN, 3'-SLN and 6'-SL, 3'-SL). 6'-SLN and 6'-SL were used to represent as α 2,6- linked sialic acid, 3'-SLN and 3'-SL were used to represent as α 2,3- linked sialic acid. glycans at the concentration of 0, 0.3125, 0.625, 1.25, 2.5 and 5 μ g/ml. The data shown were the means of two separate assays performed induplicate and absorbance was read at 450 nm. The pandemic H1N1 virus (A/California/04/2009) and H5N1 virus (A/Anhui/01/2005 RG) were used as α 2,6 and α 2,3 receptor binding control.

Supplementary Files

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