Original Article

Genetic and clinical assessment of 2009 pandemic influenza in southern China

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Abstract

Introduction: South China has a proven role in the global epidemiology of previous influenza outbreaks due to its dual seasonal pattern. We present the virologic, genetic and clinical characterization of pandemic H1N1 influenza infection (pH1N1) in Shantou and Nanchang, cities in southern China, during the second wave of the 2009-2010 pandemic.

Methodology: Nasopharyngeal swabs were collected from 165 individuals with influenza-like illness (ILI) who presented to the hospitals in Shantou and Nanchang. Laboratory diagnosis and characterization was performed by real-time PCR, virus isolation in embryonated chicken eggs, and sequencing.

Results: pH1N1 activity was sustained in three different temporal patterns throughout the study period. The overall positivity rate of pH1N1 was 50% with major distribution among young adults between the ages of 13 and 30 years. High fever, cough, expectoration, chest pain, myalgia, nasal discharge and efficient viral replication were observed as major clinical markers whereas a substantial number of afebrile cases (17%) was also observed. Rate of hospitalization and disease severity (39%) and recovery (100%) were also high within the region. Furthermore, severe complications were likely to develop in young adults upon pH1N1 infection. Genetic characterization of the HA and NA genes of pH1N1 strains exhibited homogenous spread of pH1N1 strains with 99% identity with prototypic strains; however, minor unique mutations were also observed in the HA gene.

Conclusion: The study illustrates the detailed characteristics of 2009 influenza pandemic in southern parts of China that might help to strategize preparedness for future pandemics and subsequent influenza seasons.

Key Words: Pandemic H1N1 influenza; genetic characterization; clinical presentation; hemagglutinin; neuraminidase

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Introduction

In 2009, the novel strain influenza A (H1N1) virus that carried a unique combination of gene segments from four different lineages emerged and quickly attained pandemic status. With a high attack rate similar to those of contemporary strains, pandemic 2009 influenza H1N1 virus (pH1N1 hereafter) penetrated globally but more specifically in school-aged children, young adults, and patients with underlying co-morbidities [1-3]. The clinical spectrum of the disease ranged from mild to severe illness with increased rate of hospitalization and case fatality. Notably unlike previous pandemics, pH1N1 sustained its infectivity ratio and severity of illness during multiple waves of the pandemic, claiming more than 15,000 mortalities and infecting 1.5 million people in 208 countries (World Health Organization, http://www.who.int/csr/disease/swineflu/en/ date of access 12 December 2010). However, the actual numbers might be much higher due to the unavailability of data from patients with mild sickness and insufficient surveillance in certain areas of the world. Although the transmission of pH1N1 has been demoted to the level of seasonal influenza, the continued circulation of the virus gives it the

potential to mutate. This situation might present more complex challenges to health-care providers and researchers alike as we move into next influenza seasons. Therefore, it is critical to understand the global dynamics of the 2009-2010 pandemic. Although valuable information has been generated, clinico-epidemiological data is still emerging from different parts of the world.

China declared its first few cases of pH1N1 infection in May 2009 [4] and these were followed by a rapid transmission of infection in the community. According to the Chinese Ministry of Health, approximately 197,000 suspected cases of influenza-like illness (ILI) were observed during the 2009-2010 flu season (http://www.cnic.org.cn/eng) with a considerably high proportion of hospitalizations, ICU admissions, and deaths [5-8]. However, available data, mostly based on case reports and retrospective analyses, portray the clinical picture of cases as situated in urban areas of the country, especially in Beijing and Shanghai, which are more likely to have been affected by international travel and do not represent a large portion of the Chinese population [4,6-12]. Southern parts of mainland China, where Shantou and Nanchang are located, represent smaller urban centers more than the typical Chinese demographics. Interestingly, due to unique seasonal variation, influenza activity is sustained in the region throughout the year with a sharp peak in summer and a moderate peak in winter season [13], and thus the area exhibits the gross impact of the influenza epidemiology in the country. Therefore, it merits knowing the epidemiological picture of influenza activity in this region during the 2009 pandemic, which is largely missing in the current body of literature.

In this study we summarize the clinical and virological profile of influenza activity in southern parts of mainland China during the second pandemic wave. To assess the situation, we conducted a hospital-based study on the patients who reported with ILI in two different cities located in southern provinces of China during December 2009 to March 2010. The study period covered the second pandemic wave in the winter season of 2009 as well as the spring festival of 2010, when a large proportion of the population travels inside the country.

Methodology

Patients and inclusion criteria

A hospital-based study was conducted from December 2009 through March 2010 in Shantou and Nanchang, two highly populated cities of Guangdong and Jiangxi provinces respectively, located in the south of China. Following examination by physicians, a total of 172 patients who met the clinical definition of ILI (presence of two or more upper respiratory tract symptoms with/without fever) as defined by the United States Centers for Disease Control and Prevention (CDC) [14], were included in the study. Two nasopharyngeal (NP) swabs were collected in viral transport medium from each patient and immediately transported to the laboratory. Further clinical and demographic details including age, sex, height, weight, history of infection, travel and antiviral treatment, co-morbidities, and clinical signs were obtained by clinical examination and direct interviews. Hospitalized cases remained under observation for disease progression, complications, treatment outcome, and case mortality, if any. Complications that developed in hospitalized cases included pneumonia, shortness of breath, acute respiratory distress (ARD), sinus tachycardia, pleural effusion and respiratory failure. Pneumonia was defined as the presence of increased alveolar marking observed by chest X ray.

The study was approved by the ethical board of Infectious Disease Hospital, Nanchang University, Nanchang 9th Hospital, China. Written consents were obtained from all participants involved in the study.

Virus isolation

Presence of pH1N1 virus was also evaluated by virus isolation. Samples were inoculated in 9- to 11-day-old embryonated eggs as described previously [16] with the exception of incubation at 33^{o}C for 48 hours. Samples with hemagglutination titer > 1:2 were considered positive, which was further confirmed by real-time RT-PCR for pH1N1 virus according to World Health Organization (WHO) and US CDC protocols [15]. Egg Infectious $Dose_{50}$ (EID_{50}) of positive samples was further determined by standard method for future virological studies [16].

Molecular diagnosis and virus typing

Molecular diagnostic procedures were performed according to WHO and US CDC protocols [15]. Briefly, RNA was extracted from NP swabs and

Table 1. List of primers used for Influenza virus typing

Primer pair	Sequences	Product size
MP-FluA	F -5′_ AAGACCAATCCTGTCACCTCTGA 3′ R- 5′_ CAAAGCGTCTACGCTGCAGTCC_3′	94
HA-H1[$]	F-5′_GTAAAACGACGGCCAGTTATAAACAGCAGTCTTC_3′ R- 5′_GGAAACAGCTATGACCATGATATGTCCAAATGTCTA_3′	444
HA-H3	F-5′_ GTAAAACGACGGCCAGTC TGTATGCTCAAGCATCAGG _3′ R-5′_ GGAAACAGCTATGACCATG ATTTGCCAATGGGTGTATCT _3′	286
HA-FluB	F-5′_GTAAAACGACGGCCAGTAATCTTCTCAGAGGATA_3′ R- 5′_GGAAACAGCTATGACCATGTTCTCCTGTGTAGTAAG_3′	647
β-actin (human)	F-5′-ATG GGT CAG AAG GAT TCC TAT GTG-3′ R- 5′-CTT CAT GAG GTA GTC AGT CAG GTC-3′	359

$ HA of A/Brisbane/59/2007 like virus

subjected to real-time RT-PCR for the detection of pH1N1 virus using a commercial kit (Liferiver, Shanghai, China).

Real-time RT-PCR assays were also developed to detect contemporary flu strains including Influenza A- H3N2, H1N1 (A/Brisbane/59/2009 lineage, the then seasonal strain) and Influenza B. For this purpose, RNA samples were reverse transcribed by using a high-capacity cDNA RT kit (Applied Biosystems, Foster City, USA) followed by amplification in a MyCycler PCR machine (BioRad, Shanghai, China) using SYBR Green master mix (Invitrogen, Guangzhou, China) and 0.5pmol/µl of forward and reverse primers as listed in Table 1. Positive and negative controls were processed with each run. Amplification of human *β-actin* gene served as housekeeping control.

Sequencing

A total of 10 pH1N1^{+} samples were selected for the sequencing of hemeagglutinin (HA) and neuraminidase (NA) genes. Four primer pairs covering 400 to 500 bp length with 100bp overlapping sequence were designed for each gene. RNA were reverse transcribed and subjected to PCR amplifications in a final volume of 180µl. Purified PCR preps (Promega, Madison, USA) were sequenced from Invitrogen (Guanzhou, China). Sequences were aligned and assessed by NCBI nBLAST and ClustalW multiple alignment tools. Phylograms were constructed by MEGA version 4 using the neighbor joining (NJ) method in the nucleotide model of Kimura two-parameters, with 1000 replicate bookstrapping. Evolutionary distances at amino acid and nucleotide levels were assessed by pairwise p-distances.

Statistics

Statistical analyses were performed using PSAW Statistics 18 (SPSS Inc., Chicago, IL, USA). Fisher's exact and Chi square tests were used for comparison of categorical data, and the two-tailed *t*-test was applied in case of continuous variables. Relative risks and odds ratios (OR) were estimated with 95% confidence intervals (CI) by multivariate analysis.

Results

Characterization of influenza activity

From December 2009 to March 2010, NP swabs were collected from 172 patients (116 from Shantou and 56 from Nanchang) who reported with ILI symptoms. Of these, 165 were included in the study on the basis of complete clinical information and sample quality. The pandemic 2009 H1N1 virus was present in 82 (50%) out of 165 ILI cases with the co-circulation of seasonal strains as follows: influenza A H1N1 (A/Brisbane/59/2007-lineage), 4 cases (2%); A (H3N2), 1 case (0.6%); type B influenza, 2 cases (1%); and unsubtyped influenza A, 26 cases (16%). Laboratory investigation revealed that influenza activity occurred in three different temporal patterns during this period. As shown in Figure 1, pH1N1 cases peaked during the first five weeks with a 55% (43%-73%) detection rate per week followed by an ebb of 22% in February 2010. In the last two weeks of March 2010, although ILI cases were fewer, the virus resumed a positivity rate of 55%. The median age of pH1N1 infected cases was 21 ± 13.8 years

Table 2. Clinical information of patients infected with 2009 pandemic H1N1 influenza virus

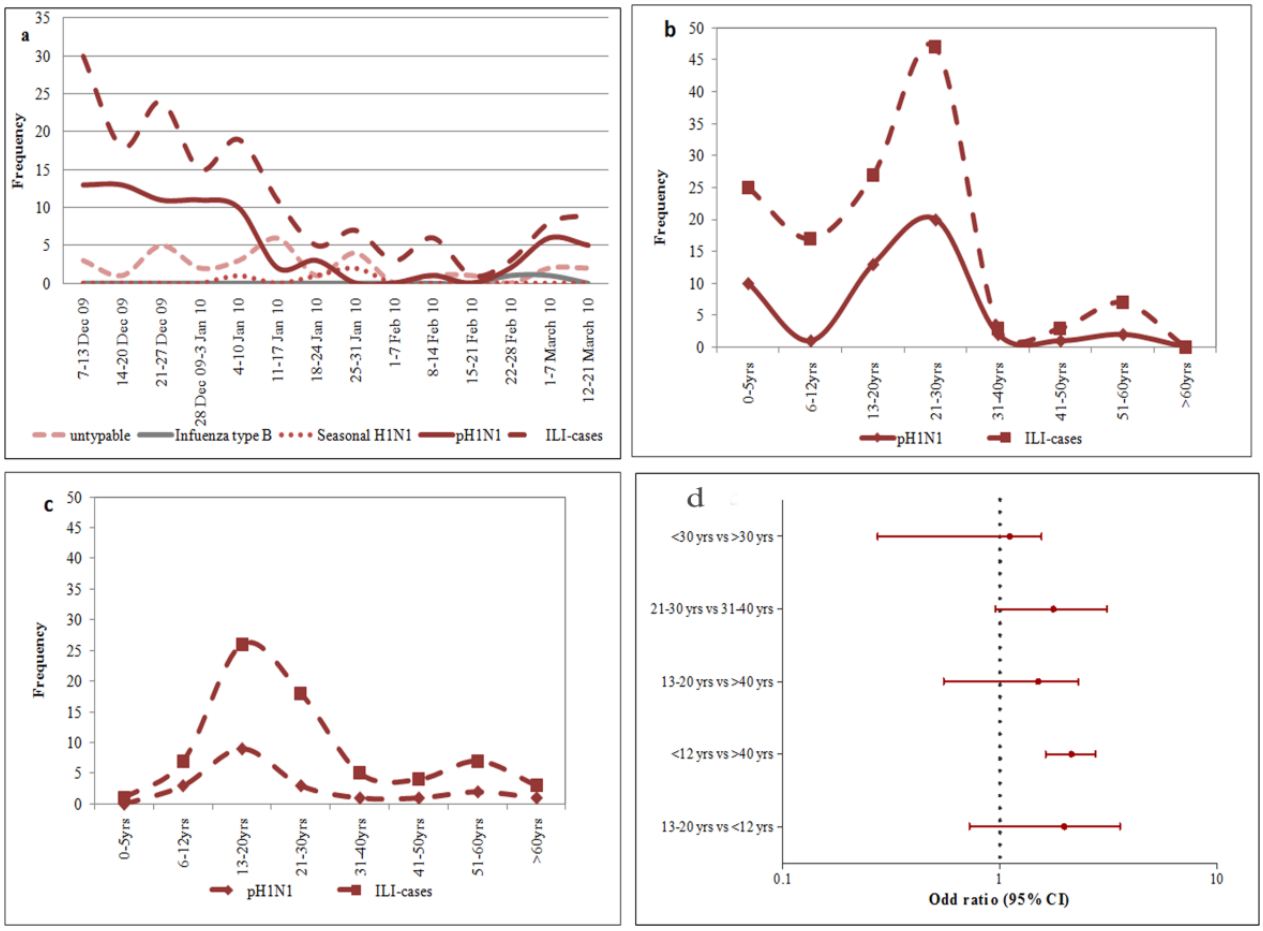
Parameters		H1N1 confirmed cases (n = 82)		Other ILI cases (n = 83)	
		n	%	n	%
Symptoms					
	Fever	62	76	48	58
	Cough	52	63	46	55
	Nasal discharge	36	44	26	31
	Myalgia	22	27	18	22
	Fatigue	20	24	17	20
	Headache	14	17	30	36
	Conjunctivitis	9	11	6	7
	Expectoration	5	6	0	0
	Chest pain	2	2	0	0
	Dyspnea	2	2	0	0
	Diarrhea	0	0	1	1
Hospitalization		32	39	21	25
Co-morbidities					
	Pregnancy	3	4	2	2
	Others	6	7	27	32
Disease Progression					
	Pneumonia with other complications	21	26	25	30
Deaths		0	0	0	0
Antiviral treatment		9	11	0	0

with a male:female ratio of 1:1.2; however, 55% were between the ages of 13 and 30 years. There was no difference in the positivity rate of samples collected from Shantou and Nanchang, but the respective mean age, 18.37 and 27 years, differed significantly ($P < 0.05$). A total of 14 (17%) of the pH1N1 infected cases were children under the age of 12 years. Patients younger than 30 years of age were more likely to be infected with pH1N1 virus than those over 30 years, with an odds ratio (OR) of 1.556 (0.27-1.56 95% CI) (Figure 1). The observation confirmed the sustained influenza activity in the region with the predominance of pH1N1 throughout the study period. It also indicated that, as expected, young healthy people were the primary population affected during the second pandemic wave.

Clinical picture

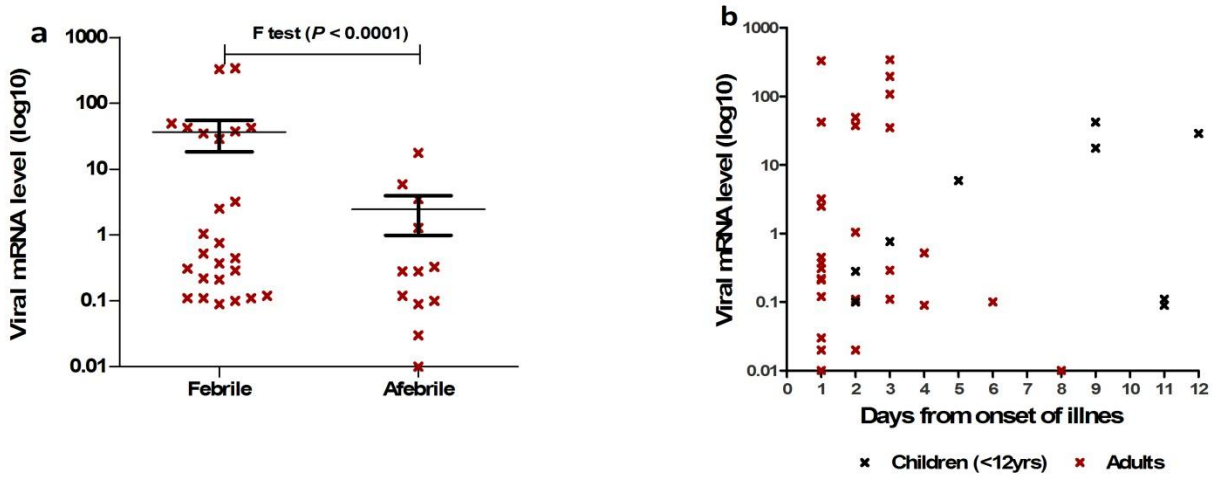
Relative comparison between pH1N1 infected and uninfected patients showed that cough (63% vs 55%, $P < 0.05$), nasal discharge (44% vs 31%, $P < 0.001$) and myalgia (27% vs 22%, $P < 0.05$) were more frequently associated with pH1N1 infection (Table 2). Other less frequently observed symptoms included fatigue, expectoration, chest pain, conjunctivitis, headache, and dyspnea whereas no case of diarrhea or vomiting was seen. It is also observed that a considerable number (24%, n = 40) of ILI cases were afebrile at the time of sample collection while 66% (n = 110) had fever. Temperature records were missing for 15 cases. Among pH1N1 infected patients, 62 (76%) were febrile and 14 (17%) were afebrile (Table 2). No significant difference in average temperature was found between pH1N1 infected and uninfected patients ($P = 0.9287$). To determine the difference between febrile and afebrile patients, we further examined the viral replication in nasopharyngeal (NP) swabs. Not surprisingly, median viral loads were significantly higher in febrile compared with afebrile patients ($P<0.05$) as shown in Figure 2. Further analysis revealed that samples from 11 febrile cases that were collected between 7 and 12 days post-onset of illness had detectable pH1N1 RNA levels, while none of the samples from afebrile patients was found positive with pH1N1 if sampling was late with the course of illness. Age specific characterization showed that the former group mainly consisted of children younger than 12 years of age. To summarize, febrile and afebrile pH1N1 infected cases were simultaneously present in the community and

Figure 1. Demographic description of ILI cases and pH1N1 infected cases



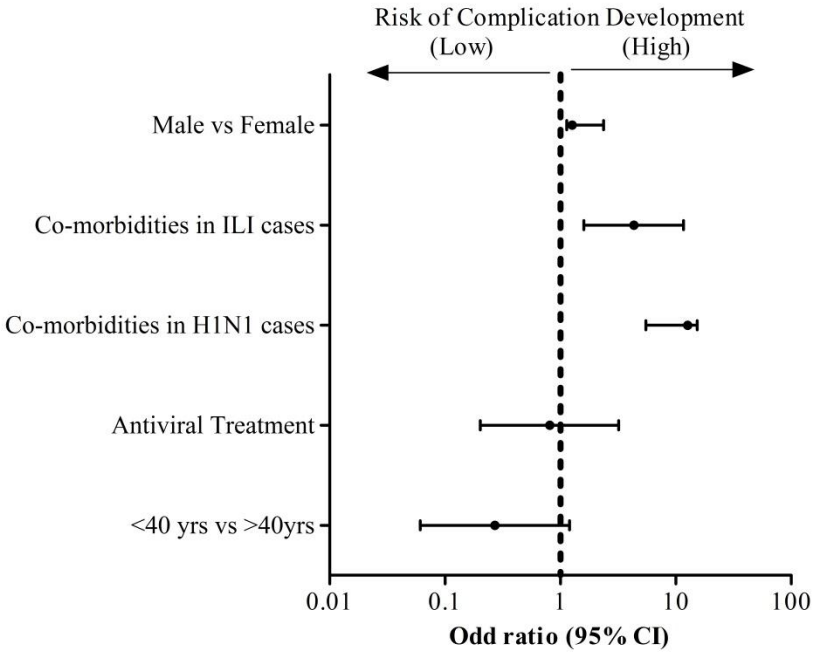
(a): Weekly distribution of pH1N1 and other seasonal influenza viruses in ILI cases included in this study (n = 165). Age distribution of ILI and pH1N1 infected patients in (b) Shantou (c) Nanchang. (d) Univariate analysis correlates likelihood of pH1N1 infection in different age groups.

Figure 2. Analysis of viral replication



(a) Scatter plot shows comparison between the viral loads of febrile and afebrile patients. Results are expressed as median mRNA levels with interquartile range. Mann Whitney U two-tailed *t* test was applied to analyze the groups and F test compares variances in each group. **(b)** Evaluation of viral loads between different age groups was analyzed according to day post-onset of illness. Linear regression was applied for curve analyses.

Figure 3. Multivariate analysis illustrating factors increasing the risk of complication development in pH1N1 cases.



viral replication actively contributed to the clinical course of infection.

Disease progression in pH1N1 infected patients

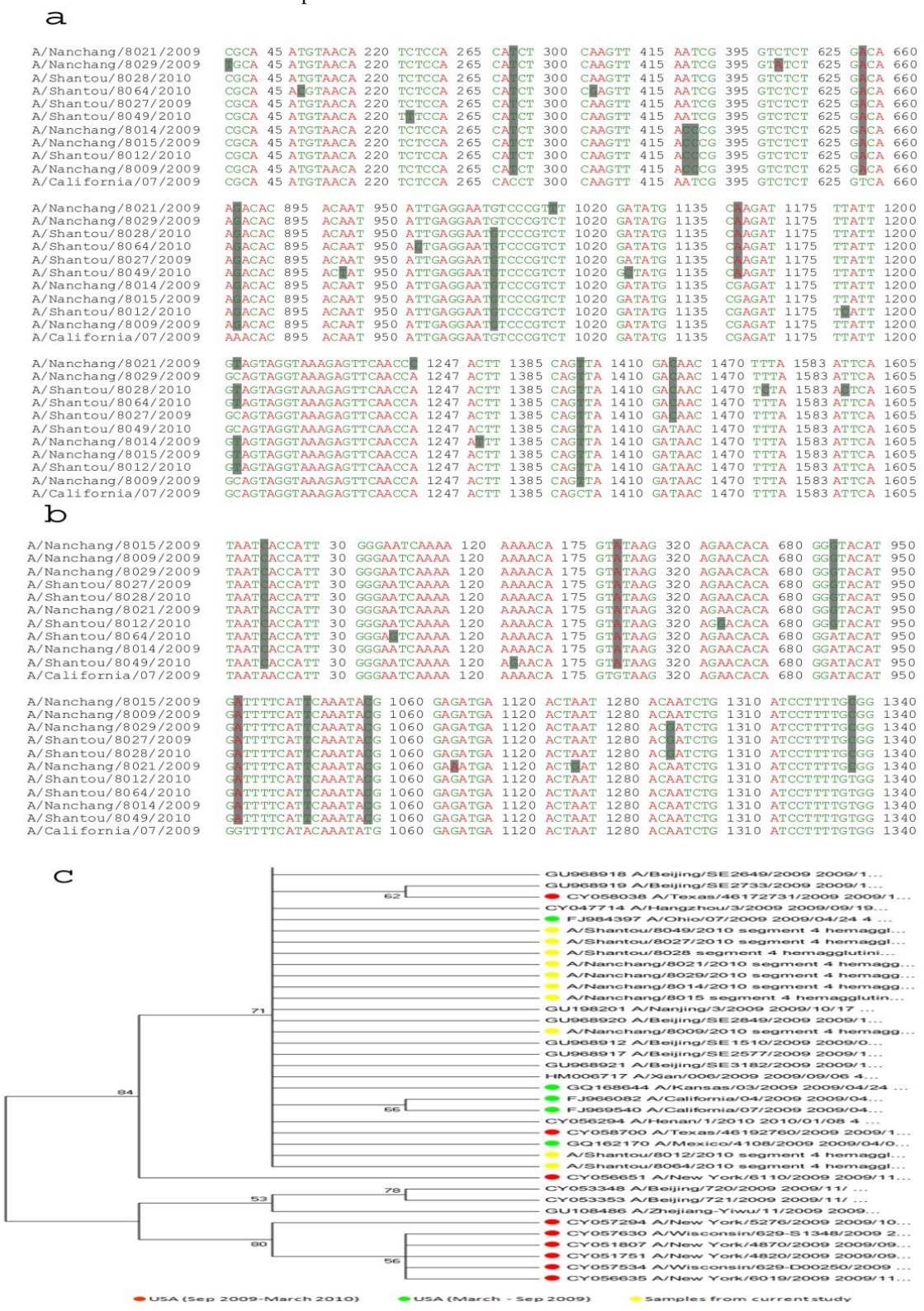
We also noticed increased hospitalization rates among patients with laboratory-confirmed pH1N1 infection. Out of 82 patients, 32 (39%) pH1N1 infected cases were hospitalized because of the development of intense respiratory tract symptoms while 12 (15%) such patients refused to be hospitalized. Out of 32 hospitalized cases, 21 developed pneumonia while other severe complications such as shortness of breath, acute respiratory distress (ARD), sinus tachycardia, pleural effusion and respiratory failure were observed in a few cases. According to multivariate analysis, patients with underlying co-morbidities were more prone to develop complications. In addition, complications were less likely to occur among those patients who were less than 40 years of age and those who were treated with antiviral drugs (Figure 3). The inverse correlation of antiviral treatment and age with the development of complications was perhaps due to the low prevalence of underlying illnesses in such cases. Median viral loads of hospitalized cases were not significantly different from non-hospitalized and non-complicated cases. Antiviral treatment with oseltamivir was given to 9 (28%) of the hospitalized cases. There were no cases with fatalities in this study.

Genetic characterization of HA gene

HA genes of 10 pH1N1 strains were sequenced. BLAST analysis based on the number of nucleotides present in HA gene exhibited 99% identity with the prototypic A/California/07/2009 H1N1 strain. It also indicated a close identity (> 99%) with A/Nanjing/3/2009 and other Chinese isolates, indicating the spread of homogenous strains in China.

Multiple alignments revealed several nucleotide substitutions in our strains as shown in Figure 4. Several mutations such as E391K, P100S, I208L, T214A, S220T and I338V were also present in our strains as they were in other representative global isolates. Our samples also contained unique mutations such as A409V, D144E, K71N, S340F and H416P; however, no correlation with respect to demography, disease progression, and clinical findings was found (Table 3).

Figure 4. Genetic characterization of 2009 pandemic H1N1 influenza



Sequences of (a) HA and (b) NA genes were aligned with prototype pH1N1 influenza strains using ClustalW multiple alignment tools. Nucleotide substitutions are marked as grey, (c) Phylogenetic analysis of HA gene with other representative isolates from China (unlabelled) and United States by neighbor joining method. Bookstrap values represent 1000 replicates**.**

Table 3. Mutation analysis of selected strains of pH1N1 influenza virus

Strain		**A/Nanchang/8014/2009**	**A/Nanchang/8015/2009**	**A/Nanchang/8021/2009**	**A/Shantou/8027/2009**	**A/Shantou/8028/2010**	**A/Nanchang/8029/2009**	**A/Nanchang/8009/2009**	**A/Shantou/8049/2010**	**A/Shantou/8012/2010**	**A/Shantou/8064/2010**
Sample collection		Dec7 2009	Dec12 2009	Dec08 2009	Dec13 2009	Jan5 2010	Dec16 2009	Dec18 2009	Jan5 2010	Jan6 2010	Jan2 2010
Age (yrs)		32	21	19	6	25	20	8	21	21	NA
Gender		F	M	F	M	M	F	M	F	M	NA
Temp. (^{o}C)		39.1	38.5	39.2	38.8	37	39.1	38.5	37	37	37
Hospitalization		✓	✓	✓			✓	✓			
Complication			✓		✓						
Pregnancy/ Co-morbidities		✓					✓			✓	
HA	Genbank ID	CY080247	CY080249	CY080251	CY080253	CY080255	CY080257	CY080259	CY080261	CY080263	CY080265
	K71N										✓
	P100S	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	D144E			✓			✓				
	S145P	✓	✓					✓		✓	
	I208L	✓	✓	✓	✓	✓		✓	✓	✓	✓
	T214A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	S220T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	I338V	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	S340F			✓							
	E391K			✓	✓	✓	✓		✓		✓
	A409V	✓	✓	✓		✓				✓	✓
	H416P			✓							
NA	Genbank ID	CY080248	CY080250	CY080252	CY080254	CY080256	CY080258	CY080260	CY080262	CY080264	CY080266
	N42S										✓
	V106I	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	N248D	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

mutations are compared with prototypic strain: A/California/07/2009 H1N1, NA – not available, ND – Not done

To analyze the pattern of the pandemic in two different pandemic waves in China, we performed HA based phylogenetic analysis of pH1N1 strains with other Chinese isolates that are available on the NCBI influenza database. In addition, HA-pH1 sequences of Chinese strains which were isolated during: 1) first pandemic wave (from March to August 2009,n = 29); 2) second pandemic wave (from September 2009 to March 2010, n = 49) were compared with HA-pH1 sequences reported from the United States of America (USA) during the same time periods: 1) first pandemic wave (March to August 2009, n = 347); and 2) second pandemic wave (September 2009 to March 2010, n = 336). Sequences were randomly selected from the NCBI influenza resource database. Phylogenetic analysis revealed that dominant homogenous strains in China were closely related to the prototypic global strains A/California/04/2009 and A/California/07/2009 irrespective of flu season (Figure 4). The results also indicated the minor epidemiological differences between pH1N1 strains that circulated in China and the USA during the second pandemic wave.

Genetic characterization of NA gene

Sequence analysis of the NA gene exhibited several mutations in the nucleotide sequence leading to amino acid substitutions N248D and V106I, which previously have been identified in other Chinese isolates including A/Nanjing/3/2009 and A/Shanghai/1/2009. No H274Y substitution targeting oseltamivir resistance was observed. An additional substitution of Asparagine to Serine at residue 42 was found in one strain (Figure 4).

Discussion

In this study, the sample collection took place from December 2009 to March 2010, thereby covering the second pandemic wave that occurred during the winter of 2009-2010 in the northern hemisphere. Interestingly, the initial wave of influenza activity faded and it was followed by a modest resurgence of the disease, which may have been caused by the large movement of people that takes place during the Chinese Spring Festival.

During the previous influenza seasons, severe respiratory complications were more prevalent among the elderly; however, a shift in the age distribution towards younger patients has previously been observed in the 2009 influenza pandemic, although the reasons for this phenomenon remain unclear. In our study the median age was 21 years, which is comparable to those of other studies done in China that reported median ages of 24 years [7] and 21 years [17] and also studies in North America with 20 years [18], Spain with 29 years [19] and Russia with 18 years [20]. We found that during the pandemic period 17% of overall pH1N1 infected cases were children (< 12 years). This observation agrees with the published data from China and neighboring countries [7,21] but is in contrast with that of North America where pediatric infections were more prevalent [22,23].

Our results confirm previous reports indicating that young and middle-aged individuals infected with pH1N1 are more prone to develop severe complications, as opposed to patients with seasonal influenza where the elderly was the most affected group [9,24]. In the areas of southern China covered in this study, human Infection with pH1N1 virus culminated in 50% of ILI cases with higher rates of hospitalization (39%) and recovery (100%) compared with other cohorts from the United States, Mexico, Canada, and Beijing [3,10,25,26], illustrating a slightly different trend of pandemic infection in this region.

High fever is often associated with increased viral loads and prolonged shedding that provides an extended opportunity for disease establishment; therefore, it is often considered a key determinant of disease severity. In our studied population, clinical (febrile) and subclinical (afebrile) cases were simultaneously present with efficient viral replication in the former group as reported elsewhere [27]. Interestingly, unlike other reports, we observed a higher rate of subclinical infection [7,17,28] that probably contributed toward the overall low mortality rate and may have acted as a silent source of infection in the community. One possible reason for the difference in observations is population characteristic because south China is dominated by the Han ethnic group so it may be possible that host factor contributes towards the susceptibility and clinical course of disease.

Genetic analysis indicated the presence of several mutations in NA and HA genes including HA-E391K, P100S, T214A, 1338V, I208L, S220T and NA- N248D, V106I, which have already been observed in various strains globally [29,30]. Phylogenetic analysis of HA sequences reported by us and other Chinese labs showed that, during first pandemic wave, H1N1 virus was predominantly homogenous with prototypic strains with some minor mutations, unlike reports from the USA which

showed distinct heterogeneities at that time [31]. Thus we speculate that in the second pandemic wave from September 2009 onward, China and the United States might have experienced different patterns of viral circulation. However, this observation is based only on HA gene sequences available in the NCBI influenza resource database. Genome-wide analysis is necessary to draw final conclusions.

This study provides the description of pandemic influenza activity in southern parts of China showing the sustained pH1N1 infection, less frequent pediatric infection, simultaneous presence of febrile and afebrile cases, as well as higher rate of hospitalization and recovery as unique features that contribute valuable information to the current understanding of the 2009 influenza pandemic.

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