Original Article

Epidemiological and viral genome characteristics of the first human H7N9 influenza infection in Guangdong Province, China

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Background: The first H7N9 human case in south of China was confirmed in Guangdong Province on August 2013, outside of the typical influenza season. For investigating the H7N9 virus source and transmission in the local community, we analyze the epidemiology and genome features of the virus isolated from the first human infection detected in Guangdong Province.

Methods: The data including medical records, exposure history and time line of events for the H7N9 patient and close contacts was collected. Variation and genetic signatures of H7N9 virus in Guangdong was analyzed using ClustalW algorithm and comparison with mutations associated with changes in biological characteristics of the virus.

Results: The female patient had a history of poultry exposure, and she was transferred from a local primary hospital to an intensive care unit (ICU) upon deterioration. No additional cases were reported. Similar to previous infections with avian influenza A (H7N9) virus, the patient presented with both upper and lower respiratory tract symptoms. Respiratory failure progressed quickly, and the patient recovered 4 weeks after the onset of symptoms. Genome analysis of the virus indicated that the predicted antigen city and internal genes of the virus are similar to previously reported H7N9 viruses. The isolated virus is susceptible to neuraminidase (NA) inhibitors but resistant to adamantine. Although this virus contains some unique mutations that were only detected in avian or environment-origin avian influenza A (H7N9) viruses, it is still quite similar to other human H7N9 isolates.

Conclusions: The epidemiological features and genome of the first H7N9 virus in Guangdong Province are similar to other human H7N9 infections. This virus may have existed in the environment and live poultry locally; therefore, it is important to be alert of the risk of H7N9 re-emergence in China, including emergence outside the typical influenza season.

Keywords: Avian influenza virus; epidemiology; H7N9; viral genome

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Background

On 19 February 2013, the first avian influenza A (H7N9) virus was identified in humans, initiating an influenza outbreak in Eastern and Northern China in domestic birds and humans. As of 27 July, the case-fatality rate in 135 laboratory confirmed human cases was reported to be 32.6% (1). Phylogenetic analysis revealed that the gene segments of H7N9 virus stemmed from three reassortment events: the H7 gene segment are closest to hemagglutinin (HA) of the H7N3 of wild ducks, the six internal genes clustered with those of poultry H9N2 viruses of China (2), and the N9 gene segment shared the highest similarity with wild ducks in South Korea (3) and Baikal teal from Jiangsu Province in China (4). Because the poultry markets are possible locations for the emergence of new influenza viruses by mutations and/or reassortments (5), more monitoring of poultry markets has occurred recently. As a result, many control policies for prevention and response to H7N9 outbreaks have been reinforced, and surveillance has been increased to find people with symptoms early, even in provinces in China that have not had a human H7N9 case before, such as Guangdong Province.

To date, there have been three stages of the H7N9 epidemic in humans. The first stage consisted of sporadic isolations of virus in February and March, followed by an epidemic in April and May in Eastern and Northern China (6). The third stage is currently ongoing, with sporadic H7N9 virus isolations in China. Avian influenza A (H5N1) and seasonal influenza viruses have shown a seasonal pattern whereby human cases are much more common in winter and autumn than in summer. However, two H7N9 human avian influenza cases recently occurred in the summer; these cases have again attracted global concerns of H7N9 influenza. The first of these summer cases was a 61-year-old female from Hebei Province on July 27, 2013. The second case, from Guangdong Province, where had no reported human H7N9 cases prior to this isolation, was a previously healthy 51-year-old female worker with a long history of contact with live poultry.

Here, we analyze the epidemiology and genome features of the first human infected H7N9 virus in Southern China (Guangdong Province), to investigate the H7N9 virus source and transmission in the local community.

Methods

Epidemiological data collection

Collection of data from the H7N9 patient and her close contacts was required by the National Health and Family Planning Commission and conducted by Guangdong Center for Disease Control and Prevention; it was exempt from ethical review by an institutional review board. The information collected included medical records, exposure history and time line of events for the H7N9 patient and close contacts. All close contacts, as well as the environment in which the patient may have been exposed, were sampled to investigate transmission of H7N9 virus.

RNA extraction, genome sequencing, and phylogenetic analysis

The full genome of the virus was amplified by 12 primer sets (designed by our lab basing on the H7N9 sequences published on Genbank) using OneStep RT-PCR Kit (Qiagen, Hilden, Germany) for sequencing. PCR products were gel purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced by ABI 3130xl automatic DNA analyzer (Life Technologies, Carlsbad, USA). The full genome of the virus was deposited into the GenBank database on 13 September 2013 (GenBank: KF662943-KF66295). Multiple alignments of HA and neuraminidase (NA) genes were conducted using the ClustalW algorithm. Phylogenetic trees were constructed by the maximum likelihood method with 1,000 bootstrap replications in ClustalW. Accession numbers for reference sequences used in the phylogenetic analysis are listed in *Table S1*.

Genetic signatures of A/Guangdong/1/2013(H7N9) (H7N9 Guangdong strain)

To identify the genetic signatures of virus, the full genome of the H7N9 Guangdong strain was compared with sequences of H7N9 viruses previously isolated from humans, birds, and the environment. In addition, key amino acid sequences that have been previously associated with viral characteristics were identified in the H7N9 Guangdong strain.

Results

Epidemiological features

The seasonal distribution of influenza viruses in Guangdong province in 2012-2013 is shown in *Figure 1*. Before epidemic week (EW) 8 the influenza activity was

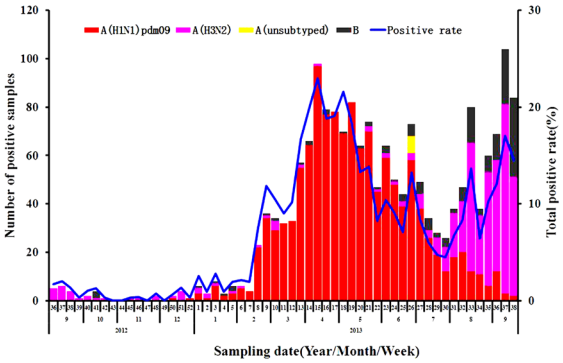

Figure 1 Influenza virus activity in Guangdong Province from September 2012 to September 2013.

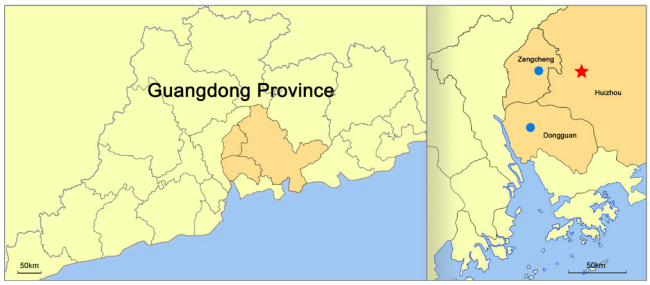

Figure 2 Geographic distribution of H7N9 viruses identified in poultry and humans in Guangdong Province. Blue circles denote identification of H7N9 virus in poultry. The red star represents the confirmed human case of H7N9 virus infection in Huizhou.

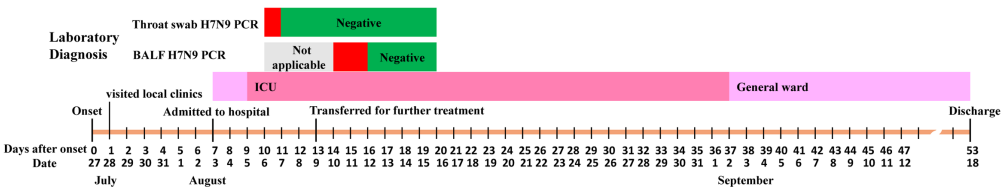

Figure 3 Timeline of disease and treatment of first human infected in Guangdong Province. Positive results of PCR were showed in red.

low. However, after EW8, A (H1N1) pdm09 dramatically increased and predominated until EW30. Prior to August 2013, influenza B and H3N2 viruses were rarely detected, but both became more common in August, with H3N2 predominating. It is particularly intriguing that at EW17 and EW20 two samples from live poultry were found positive for avian influenza A (H7N9) virus in Dongguan and Zengcheng cities both of which located in Guangdong Province (7,8). Despite the fact that human H7N9 cases were distributed across most regions of Eastern or Northern China before July, the most recent animal and human cases detected were concentrated in the cities of the Pearl River delta in Southern China (*Figure 2*).

The H7N9 patient from Guangdong province did not have any recent travel history and lived in a two-story building, of which the ground floor for was used for selling poultry and the second floor for living. This building was located in a wet poultry market of Boluo (a county in Huizhou city). Because of H7N9 outbreaks reported in other regions in China, Guangdong performed emergency surveillance for avian influenza A (H7N9) in wet poultry markets throughout all 21 prefectures from 15 April to 31 May 2013. A total of 3,235 samples were collected from wet market environments, including 120 samples from Huizhou. No sample was found to be H7N9 positive in this surveillance. We tested 65 throat swabs and 125 blood samples from the case's close contacts, including her husbands, son, and daughter, but none tested positive for the A (H7N9) virus, suggesting that this patient did not spread the virus to other humans.

At the onset of disease, the patient developed fever, headache and chills. After receiving medical care in a private clinic for three days without improvement, she visited an outpatient department of a county hospital on day 4, and had watery stools and fatigue on day 6. She was hospitalized at Huizhou central hospital on day 7, her condition became more severe, and she was transferred to the intensive care unit (ICU) because of an infection in the right lung on chest radiograph on day 9. Although an influenza test was negative on day 9, the patient became critically ill and was started on a ventilator. A sample from the patient on day 10 was positive for avian influenza A (H7N9) virus, detected by Huizhou Centers for Disease Control and Prevention (CDC) and confirmed by China CDC. On day 13, the patient was transferred to the Guangzhou Institute of Respiratory Diseases for further treatment. The patient recovered and was discharged uneventfully on day 53 (*Figure 3*).

Table 1 The nucleotide percent identities between A/Guangdong/1/2013, its closest relative and the first three H7N9 human isolates

A/Guangdong/1/2013	Identity (closest relative)	A/Shanghai/1/2013	A/Shanghai/2/2013	A/Anhui/1/2013
PA	99 [A/chicken/Hong Kong/TC18/2011(H9N2)]	99	99	99
PB1	99 [A/duck/QuanhNinh/21/2013(H5N1)]	97	97	97
PB2	99 [A/chicken/Jiangsu/S002/2013(H7N9)]	96	96	96
HA	99 [A/environment/Hangzhou/34/2013(H7N9)]	99	99	99
NP	99 [A/chicken/Hong Kong/TC8/2012(H9N2)]	97	97	97
NA	100 [A/environment/Hangzhou/34/2013(H7N9), A/Hangzhou/2/2013(H7N9)]	99	99	99
M	100 [A/Shanghai/02/2013(H7N9)]	99	100	100
NS	99 [A/chicken/Jiangsu/Q3/2010(H9N2)]	97	97	97

Phylogenetic analysis of A/Guangdong/1/2013 (H7N9)

An H7N9 virus, named A/Guangdong/1/2013 (H7N9), was isolated from day 14 sample (BALF) of the patient using 9-11-day-old embrocated chicken eggs in a BSL-3 laboratory. Multiple sequence alignment showed that the Guangdong strain was 96-100% identical to the first three reported avian influenza A (H7N9) viruses in all eight genes (*Table 1*), including 99% identical in the HA and NA genes. Blast analysis of the viral genome showed that each gene was from avian origin, and no evidence of genetic reassortment with human viruses was found (*Table 1*). Phylogenetic analysis suggested that A/Guangdong/1/2013 (H7N9) has similar antigen city to previous avian influenza A (H7N9) viruses reported in China (*Figure 4*). The HA gene shared the highest identity with A/environment/Hangzhou/34/2013 (H7N9), while the NA gene was most closely related to A/environment/Hangzhou/34/2013 (H7N9) and A/Hangzhou/2/2013 (H7N9) (*Table 1*). The HA and NA genes were 99.6% and 99.9% identical, respectively, with genes of H7N9 viruses detected in the local environment.

Genetic signatures of A/Guangdong/1/2013 (H7N9)

The genome signatures of Guangdong strain were identified, including T160A, V186G, and Q266L in HA, N30D in M1, and E627K in PB2 (*Table 2*). These mutations were observed in most of the avian influenza A (H7N9) viruses isolated from humans in the epidemic of 2013, and appear to have allowed the virus to adapt to humans by increasing binding to the human receptor as well as increasing virus virulence. Although the genome of A/Guangdong/1/2013 (H7N9) was similar to previously isolated H7N9 viruses in China, some unique mutations existed in the PA, PB1, PB2, HA and NS1 genes when compared with other H7N9 viral genes. However, there is a lack of published evidence that these mutations affect the biological properties of H7N9 viruses. Interestingly, 191E in PB2, 65K in HA and 27L, 111V, and 212P in NS1 were observed in A/Guangdong/1/2013 (H7N9) and the viruses from birds and the environment, but were not seen in other H7N9 viruses isolated from humans. This finding implies that the Guangdong H7N9 patient may have been infected from birds or the environment, due to her working in a poultry market.

Discussion

In the 20th century, three of four influenza pandemics were thought to have originated in Southern China (23,24). Guangdong Province has often been the focus of attention in influenza epidemiology. In this study, we analyzed the epidemiology and genetic signatures of the H7N9 virus from the first human H7N9 case in Guangdong Province, and compared the virus to those isolated from birds, the environment, and humans in Eastern and Northern China.

Live poultry markets bring together bird and poultry species from different sources in a concentrated environment, providing an environment for reassortment among avian influenza viruses of different subtypes (25,26). Most human H7N9 infections were diagnosed in poultry workers or visitors to poultry markets, implying that the market environment plays a critical role in the epidemic (27). In this study, the patient who is a poultry worker has been involved in this field for over 10 years. That supports the notion that avian influenza A (H7N9) virus-infected poultry are a transmission source.

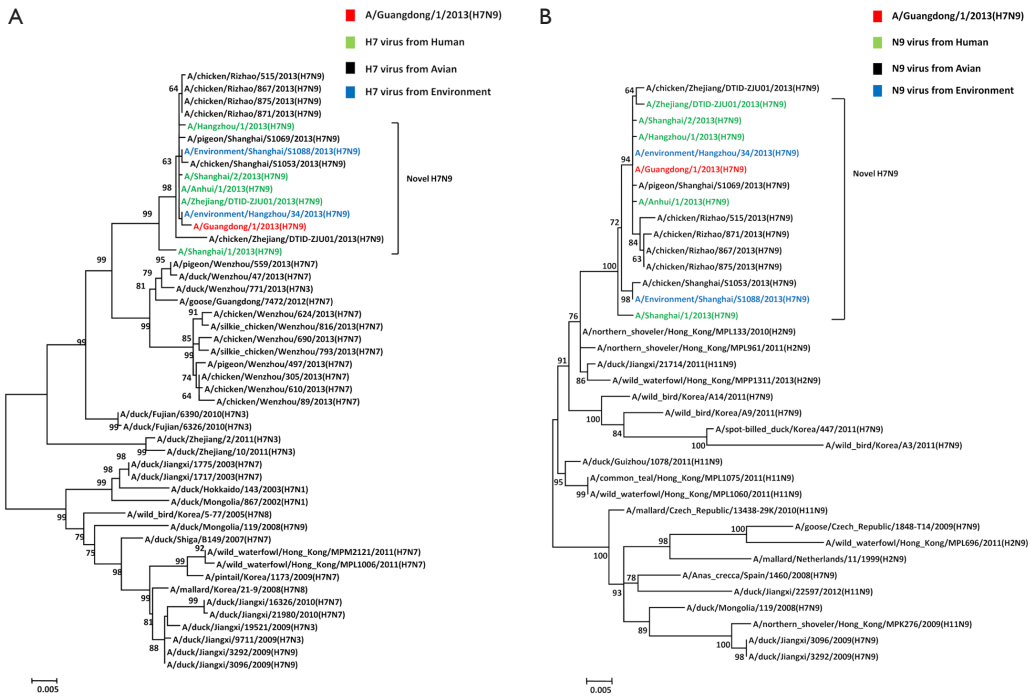

Figure 4 Phylogenetic analysis of antigens in H7N9 virus, (A) hemagglutinin; (B) neuraminidase.

Table 2 Mutations of A/Guangdong/1/2013 compared with H7N9 viruses previously reported in 2013

Viral gene	Amino acid position	A/ Guangdong/1/2013	H7N9 from patients [2013]	H7N9 from birds [2013]	H7N9 from the environment [2013]	Biological feature altered by mutations
PA	343	T	A	A	A	Unknown
	519	T	N	N	N	Unknown
PB1	171	V	M	M	M	Unknown
	368	V	V	V	V	I368V enables droplet transmission in ferrets (9)
	374	V	A	A/T	A	Unknown
	397	M	I	I	I	Unknown
	694	S	N	N	N	Unknown
PB2	89	V	V	V	V	L89V increases polymerase activity (9)
	139	I	V	V	V	Unknown
	191	E	K	K/E/T	K/E	Unknown
	570	I	M	M/I	M/I	M570I may affect RNA cap-binding (10,11)
	627	K	K/E	E	E	E627K increases adaptation to mammals (12)
	676	V	M	M	M	Unknown
	701	D	D/N	D	D	D701N increases adaptation to mammals (9)

Table 2 (*continued*)

Table 2 (*continued*)

Viral gene	Amino acid position	A/ Guangdong/1/2013	H7N9 from patients [2013]	H7N9 from birds [2013]	H7N9 from the environment [2013]	Biological feature altered by mutations
HA	65	K	R/M	R/M/K	R/M/K	Unknown
	138a	A	A/S	A	A	S138A increases virus binding to α-2,6-linked sialic acid receptor (human receptor) (13)
	140	A	T	T	T	Unknown
	160[a]	A	A	A	A	T160A Increases binding to α-2,6-linked sialic acid receptor (human receptor) (14)
	186[a]	V	V/G	V	V	G186V Increases binding to α-2,6-linked sialic acid receptor (human receptor) (15)
	226[a]	L	Q/L	Q/L	Q/L	Q226L increases binding to α-2,6-linked sialic acid receptor (human receptor) (16)
	PEIPKGR*GLF	PEIPKGR*GLF	PEIPKGR*GLF	PEIPKGR*GLF	PEIPKGR*GLF	Cleavage site (9)
NA	294	R	R/K	R	R	R294K is known to confer resistance to oseltamivir and zanamivir (17)
	69-73[b]	deleted	deleted	deleted	deleted	Stalk deletion increases virulence in mammals (18)
M1	30	D	D	D	D	N30D increases virulence in mice (19)
	215	A	A	A	A	T215A increases virulence in mice (19)
M2	31	N	N	N	N	S31N is known to confer resistance to amantadine and rimantadine (20)
NS1	27	L	M/K	M/L/K	M/K	Unknown
	42	S	S	S	S	P42S increases virulence in mice (21)
	80	T	S	S	S	Unknown
	111	V	I	I/V	I/V	Unknown
	152	D	E	E	G	Unknown
	212	P	S	S/Y	S/P	Unknown
	218-230	Deleted	Deleted	Deleted	Deleted	Lack of PDZ-binding motif decrease virulence in mice (22)

[a], H3 numbering; [b], N9 numbering.

Many studies based on retrospective investigations have stated that poultry market exposure is a key risk factor for H7N9 infection. Notably, before the isolation of A/Guangdong/1/2013 (H7N9), avian influenza A (H7N9) viruses were isolated from chickens in live poultry markets of the nearby cities of Dongguan and Zengcheng in Guangdong Province. This finding provides chronological evidence for poultry markets as a source of H7N9 infection. Therefore, it is thought that those who have close contact to live poultry in Southern China have a high risk of infection with avian influenza A (H7N9) virus. Vigilance is needed to continue surveillance of live poultry markets and those who frequent them.

Guangdong Province, located in a subtropical area, has multiple peaks of seasonal influenza activity annually, and the highest peak tends to occur in warmer months (June and July) (28). The activity of avian influenza A (H5N1) is similar to that of seasonal influenza, in that in tropical areas of Asia, avian influenza A (H5N1) has been more common during the warmer months (29). Therefore, this human H7N9 case in Guangdong province occurring in summer season followed the expected seasonality. However, no further positive detection of avian influenza A (H7N9) virus in local live poultry markets and no additional human case has been identified within one month after this isolation in Guangdong or adjacent southern provinces in China. Hence, it does not appear that H7N9 virus is currently moving south.

The avian influenza A (H7N9) virus we identified from the first human case in Guangdong was closely related to A/environment/Hangzhou/34/2013(H7N9) in its surface glycoprotein's (HA and NA), and also has internal genes similar to other N7N9 viruses detected from birds and the environment. We compared the sequences of A/Guangdong/1/2013 (H7N9) with those of previously reported avian influenza A (H7N9) viruses. In the HA protein, A/Guangdong/1/2013 (H7N9) had a Q226L substitution, which increases virus binding to the human receptor; other previous avian influenza A (H7N9) viruses isolated from humans had the same mutation (12,30). In the PB2 protein, A/Guangdong/1/2013 (H7N9) had an E627K mutation; this change is thought to increase the virulence of avian viruses in mice. Avian influenza A (H7N9) viruses from birds and the environment typically have 627E. Interestingly, four previously reported human isolates of H7N9 viruses also had 627E in PB2, while two of them had D701N, which is thought to play a key role in viral adaption to mammals and take the place of the E627K mutation (31). This implies that there is more than one possible PB2 mutation for H7N9 viruses to adapt to mammalian hosts. However, it is worth noting that 191E in PB1, 570I in PB2, 65K in HA, and 27L, 111V, and 212P in NS1 were only observed in A/Guangdong/1/2013 (H7N9) and in H7N9 virus isolates from birds and the environment. Among these mutations, M570I is located in the binding region of PB2 and is reported to possibly alter the secondary structure from a strand to a helix, and may affect RNA cap-binding (10,11). Several unique substitutions were observed in A/Guangdong/1/2013 (H7N9) compared with other avian influenza A (H7N9) viruses, including T343A in PA, which also has been found in A (H1N1) pdm09 viruses (32,33). Whereas effects of them on the fitness of viruses need to be studied further due to unknown biological function. Based on the findings above, to study the virus's virulence more accurately and objectively, it is important that the sequence and virological analyses are considered in combination with the epidemiological findings.

Conclusions

Overall, the epidemiology and genome characteristics of A/Guangdong/1/2013 (H7N9) seemed to be similar with previously reported H7N9 viruses isolated from humans. However, several unique substitutions never reported before need to be investigated, and may already exist in the environment and live poultry locally. Although their impact on the pathogenesis of viruses is unclear, it is necessary to emphasize that agriculture and forestry departments should continue monitoring and sharing animal surveillance information, which can facilitate early warning and intervention.

Acknowledgements

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Authors' contributions: Nan-shan Zhong and Yong-hui

Zhang designed the study. Shi-guan Wu, Xiao-bo Li, Guo-yun Ding and Ji-cheng Huang conducted H7N9 nucleic acid detection and virus isolation in P3 lab. Wen-da Guan and Si-hua Pan sequenced the full genome of virus, did phylogenetic analysis. Run-feng Li, Min Kang, Jie Wu and Yong-ping Li did analysis of genetic features. Wei-qi Pan, Rong Zhou, Ling Chen, Rong-chang Chen, Yi-min Li collected clinical data. Jianfeng He, Chang-wen Ke, Jin-yan Lin and Wen-long Xiao collected and analyzed epidemiological data. Zi-feng Yang wrote the first draft, and all authors contributed to review and revision and have seen and approved the final version.

Disclosure: The authors declare no conflict of interest.

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