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Original Article

Coexistence of Avian Influenza Virus H10 and H9 Subtypes among Chickens in Live Poultry Markets during an Outbreak of Infection with a Novel H10N8 Virus in Humans in Nanchang, China

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SUMMARY: Infection with the novel H10N8 virus in humans has raised concerns about its pandemic potential worldwide. We report the results of a cross-sectional study of avian influenza viruses (AIVs) in live poultry markets (LPMs) in Nanchang, China, after the first human case of H10N8 virus infection was reported in the city. A total of 201 specimens tested positive for AIVs among 618 samples collected from 24 LPMs in Nanchang from December 2013 to January 2014. We found that the LPMs were heavily contaminated by AIVs, with H9, H10, and H5 being the predominant subtypes and more than half of the LPMs providing samples that were positive for the H10 subtype. Moreover, the coexistence of different subtypes was common in LPMs. Of the 201 positive samples, 20.9% (42/201) had mixed infections with AIVs of different HA subtypes. Of the 42 mixed infections, 50% (21/42) showed the coexistence of the H9 and H10 subtypes, with or without H5, and were from chicken samples. This indicated that the H10N8 virus probably originated from segment reassortment of the H9 and H10 subtypes.

INTRODUCTION

Based on the antigenic properties of the hemagglutinin (HA) and neuraminidase (NA) glycoproteins, influenza A viruses are classified into 18 HA and 11 NA subtypes (1,2). All these viruses have been found in birds, with the exception of H17N10 and H18N11 influenza viruses, which were isolated from fruit bats (2). Occasionally, avian influenza virus (AIV) can break through the species barrier and cause sporadic infections in humans. From 1997 through 2013, infections in humans with avian influenza A viruses such as H5N1 (3) and H7N9 (4) viruses were reported in China, with a severe clinical syndrome and fatal outcomes. In 2013, during the first wave of the H7N9 epidemic, five H7N9 cases were recorded in Nanchang, Jiangxi, China (5). While eastern China was suffering from the second wave of H7N9 infection, however, a newly emerging avian influenza A virus (H10N8 virus) (6) was isolated from a fatal case of severe pneumonia in Nanchang. Subsequently, in the following 2 months, another two H10N8 cases were identified in Nanchang.

Previous studies have demonstrated that live poultry markets (LPMs) may be the source of human AIV infection. Most human cases had previous exposure to LPMs or direct contact with live poultry before the onset of symptoms (3). In addition, several investigations of LPMs following infection in humans with H7N9 virus were conducted, and H7N9 viruses were detected in the poultry markets (7). In addition, mixed infections with viruses of different HA subtypes were identified (8), which might facilitate genetic reassortment, thus contributing to the emergence of new AIVs. As shown by the epidemiological investigation of the 5 patients with the H7N9 virus in Nanchang, the patients also had a history of exposure to LPMs (5). However, the profile of AIVs circulating in LPMs in Nanchang is still unknown. The current cross-sectional investigation was conducted to explore the prevalence of avian influenza A virus in LPMs in Nanchang, China, from December 2013 through January 2014.

MATERIALS AND METHODS

Location: There are 89 LPMs in Nanchang, including 1 wholesale market and 88 retail markets. The wholesale market, which has 46 poultry booths, serves as the major poultry source for the retail markets, while each of the retail markets has at least 2 poultry booths. All these LPMs are located primarily in urban areas characterized by poor sanitation and a humid environment, with the poultry booths often open or partially open to

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the public. Live chickens, ducks, and pigeons are usually kept in one cage or in different stacked cages. Customers can pick any poultry, with direct or indirect contact within one meter of the poultry booth, and the sellers usually provide the services of slaughtering, defeathering, and cleaning.

Sample collection and laboratory testing: A sampling of 24 LPMs, including the 1 wholesale market, was performed to collect samples in five Nanchang districts (Donghu, Xihu, Qingshanhu, Qingyunpu, and Wanli) and four counties (Xinjian, Nanchang, Jinxian, and Anyi). With the exception of the one wholesale market, the other selected markets were selected based upon the following two criteria: (i) the market was identified as a point of exposure during the epidemiological investigation of the H7N9 or H10N8 patients; or (ii) the market was located in the central area of each district or county, had the most consumers of the county, and contained more than 3 poultry booths. Ten poultry booths in the wholesale market and three in each retail market were chosen for collection of swabs from poultry and environmental samples. The chosen booths held at least two species of poultry. Swab samples from different species of poultry (chicken, duck, and pigeon) and environmental samples (fresh poultry feces, cage swabs, and sewage) were collected from each poultry booth. Data collection methods for swab sample collection have been described elsewhere (9). Briefly, a cloacal swab and a tracheal swab from the same bird were placed in the same sample collection tube and counted as one sample, and all of the poultry swab samples were collected from apparently healthy poultry (9).

To monitor the circulating patterns of AIVs in the LPMs, the surveillance was conducted over two periods. From December 11 to December 15, 2013, 261 swab samples and 173 environmental samples were collected. Between January 10 and January 14, 2014, 108 swab samples and 76 environmental samples were collected. All samples were placed in Hank's balanced salt solution containing 0.5% bovine serum albumin, 5 μg/ml amphotericin, and 50 U/ml gentamicin, immediately kept at 4°C with ice packs, then transferred to the laboratory and stored at −70°C (10). Specific real-time reverse transcriptase-polymerase chain reaction assays with self-designed specific primer and probe sets for detecting AIVs were performed to identify subtypes H5, H7, H9, and H10 (11).

Statistical analysis: The prevalences of AIVs were compared among different kinds of samples by using Pearson's chi-square test or Fisher's exact test for categorical variables. The statistical significance level was defined as a P value (P) < 0.05. All statistical analyses were conducted with Epi Info version 3.5.4 <http://wwwn.cdc.gov/epiinfo/html/prevVersion.htm>.

RESULTS

AIV detection: In total 201 AIVs were detected among 618 samples taken across both sampling periods (32.5%). The prevalence of AIVs among the environmental samples was significantly higher than that among the poultry samples (41.8% versus 26.3%, $\chi^2 = 16.2$, $P < 0.01$). In the 201 specimens that tested positive for AIVs, the H9 ($n = 58$), H10 ($n = 55$), and H5 ($n = 54$) subtypes were the predominant subtypes, with a few samples of subtypes H7. And N8 subtypes were also detected (Table 1).

In the first sampling period (December 11–15, 2013), AIVs were detected in 101 of the 434 samples (23.3%). Of these, 18.8% were H5, 29.7% were H9, and 34.7% were H10. The prevalence of AIVs in poultry cage samples (57.9%) was significantly higher than those in sewage (38.9%), fecal (28.1%), and poultry (14.9%) samples ($\chi^2 = 33.8$, $P < 0.01$). Subtype H7 was not found in any samples during the first period. In the second sampling period (January 10–14, 2014), AIVs were detected in 100 of the 184 samples (54.4%). Among these AIVs, 35.0% were H5, 2% were H7, 28% were H9, and 20% were H10. No significant difference was found between the different kinds of samples ($\chi^2 = 7.1$, $P =$

Table 1. Avian influenza A virus subtypes derived from live poultry markets in Nanchang, China, December 2013–January 2014

Date of sample collection	Source of samples	No. of samples	No. of AIV (%)[1)]	Subtype[2)]				
				H5	H7	H9	H10	N8
The first period: December 11–15, 2013	Fresh poultry feces	82	23 (28.1)	6	0	2	5	NA[3)]
	Swab samples of poultry	261	39 (14.9)	8	0	18	19	NA
	Swab samples of poultry cage	19	11 (57.9)	4	0	5	3	NA
	Sewage	72	28 (38.9)	1	0	5	8	NA
	Subtotal	434	101 (23.3)	19	0	30	35	NA
The second period: January 10–14, 2014	Fresh poultry feces	26	12 (46.2)	5	0	5	3	5
	Swab samples of poultry	108	58 (53.7)	17	0	16	16	32
	Swab samples of poultry cage	27	12 (44.4)	4	0	1	0	3
	Sewage	23	18 (78.3)	9	2	6	1	9
	Subtotal	184	100 (54.4)	35	2	28	20	49
Total		618	201 (32.5)	54	2	58	55	49

[1)]: Positive rate of AIVs of the samples.
[2)]: Total number of H5, H7, H9, and H10 subtypes is not equal to the number of AIVs, as not all subtypes were identified, and some subtypes were coexisted in one sample as well.
[3)]: NA, not analyzed.

0.07). The H5 and H9 subtypes occurred more frequently in sewage than in the other samples, and the H7 subtype was found only in the sewage samples; the rates of positivity for H10 subtype were 14.8%, 11.5%, and 4.4% for the poultry swabs, fecal, and sewage samples, respectively. In addition, we detected the N8 subtype in samples collected during this period. As shown in Table 1, 49 samples with subtype N8 were identified, 65.3% of which were from poultry, 18.4% from sewage, 10.2% from fresh poultry feces, and 6.1% from poultry cages. There were 13 markets with the H10 subtype in the survey, and the positive rate of markets with H10 subtype was 54.2% (Fig. 1).

Rate of AIV positivity for poultry in LPMs: As summarized in Table 2, 369 swab samples were collected from poultry, and 97 were positive for AIVs, including 57 specimens from chickens, 34 specimens from ducks, and 6 specimens from pigeons. The overall AIV positivity rate was 25.4% for poultry. The AIV positivity rate for poultry in the second period was significantly higher than that in the first period (53.7% versus 14.9%, $\chi^2 = 59.2$, $P < 0.01$). The distribution of overall rates of AIV positivity among ducks (31.8%), chickens (28.9%), and pigeons (9.2%) was distinctively different when data from both collection periods were combined ($\chi^2 = 12.1$, $P = 0.002$). Among the 97 AIVs isolated from poultry, viruses of subtypes H10 and H9 were dominant in chickens, and those of subtype H5 were dominant in ducks. No subtype H7 viruses were detected in any poultry.

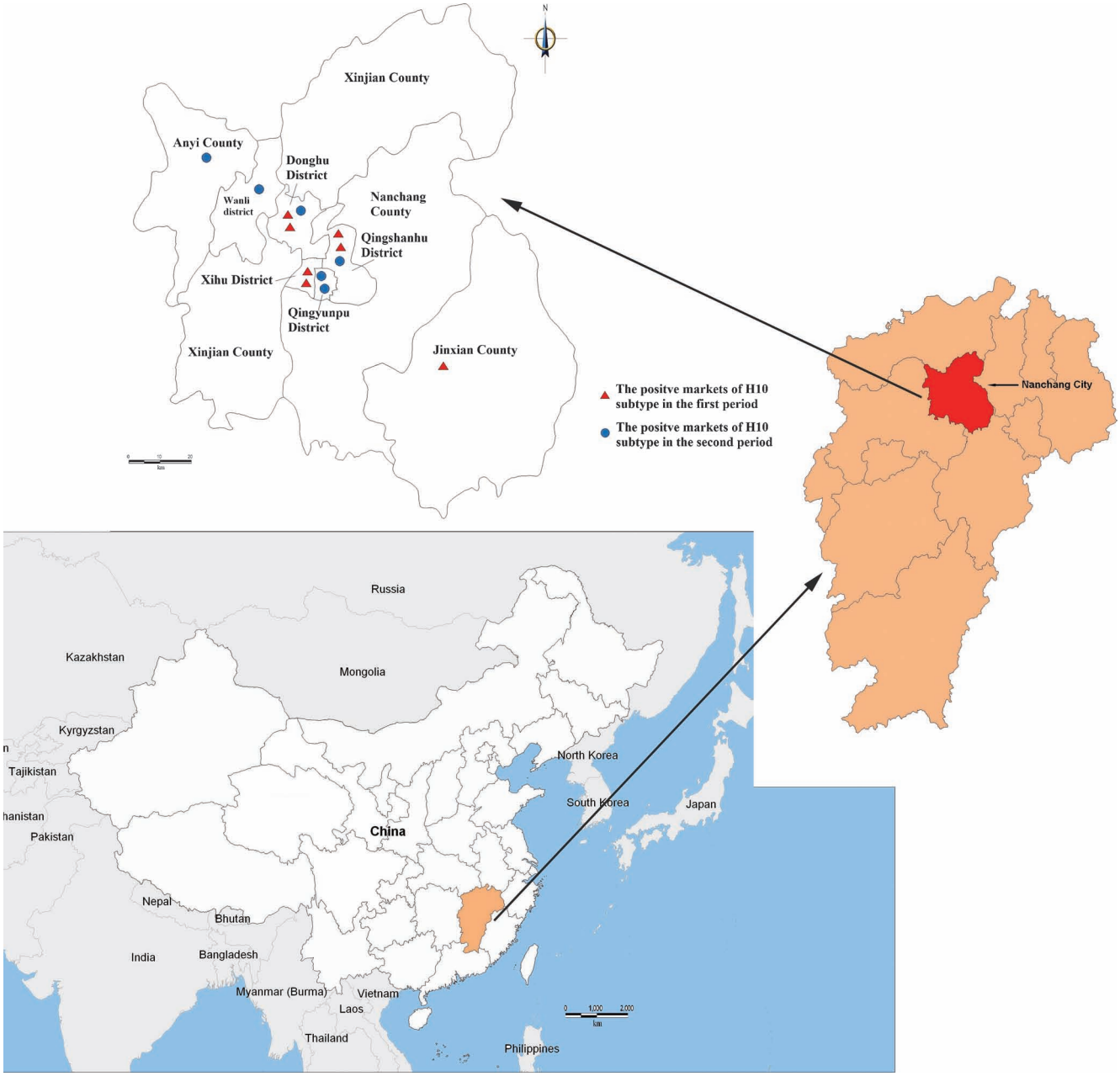

Fig. 1. (Color online) Geographic distribution of avian influenza A virus H10 subtypes in Nanchang, China, December 2013 to January 2014.

Table 2. Avian influenza A virus subtypes detected in live poultry markets in Nanchang, China, December 2013–January 2014

Date of sample collection	Source of samples	No. of samples	No. of AIVs (%)[1)]	Subtype[2)]				
				H5	H7	H9	H10	N8
The first period: December 11–15, 2013	Chickens	145	26 (17.9)	6	0	18	19	NA[3)]
	Ducks	70	12 (17.1)	2	0	0	0	NA
	Pigeons	46	1 (2.3)	0	0	0	0	NA
	Subtotal	261	39 (14.9)	8	0	18	19	NA
The second period: January 10–14, 2014	Chickens	52	31 (59.6)	2	0	12	15	24
	Ducks	37	22 (59.5)	14	0	2	0	8
	Pigeons	19	5 (26.3)	1	0	2	1	0
	Subtotal	108	58 (53.7)	17	0	16	16	32
Total		369	97 (26.3)	25	0	34	35	32

1): Positive rate of AIVs of the samples.
2): The total number of H5, H7, H9, and H10 subtypes is not equal to the number of AIVs, as not all subtypes were identified, and some subtypes were coexisted in a sample as well.
3): NA, not analyzed.

Table 3. Coexistence of different subtypes of avian influenza virus in live poultry markets in Nanchang, China, December 2013–January 2014

Type of coexistence	No. of positive samples						Total
	Chicken	Duck	Pigeon	Poultry cage	Sewage	Poultry feces	
H5 + H9	4	0	0	4	5	4	17
H9 + H10	17	0	0	1	0	1	19
H5 + H9 + H10	4	0	0	0	1	0	5
H5 + H7 + H9	0	0	0	0	1	0	1
Total	25	0	0	5	7	5	42

Coexistence of subtypes H5, H7, H9, H10, and N8: Of the 201 samples positive for AIVs, 42 (20.9%) had mixed infections with AIVs of different HA subtypes (Table 3). Three and four types of mixed infection were identified in the first and second periods, respectively. Among these concomitant infections, 36 were mixed infections with two different HA subtypes (17 mixed infections of subtypes H5 and H9, and 19 of subtypes H9 and H10), and 6 were mixed infections with three different HA subtypes (5 mixed infections of subtypes H5, H9, and H10, and one of subtypes H5, H7, and H9) (Table 3). Among the mixed infections, 59.5% (25/42) were found in the swab samples from chickens, 16.7% (7/42) were from sewage, and none was from samples obtained from ducks or pigeons. Chickens represented 50% (21/42) of the mixed infections of the H9 and H10 subtypes, with or without the H5 subtype. In the second period, we inspected the coexistence of subtype N8 with subtypes H5, H9, and H10. Four different combinations of subtypes were found: two infections were of subtypes H5 and N8, 10 were of subtypes H9 and N8, 9 were of subtypes H9, H10, and N8, and 9 were of subtypes H10 and N8. Almost all the coinfections with viruses of types H10 and N8 were identified from chickens or chicken feces (Table 1 and data not shown).

DISCUSSION

In this study, we conducted a survey of AIVs in 24 LPMs 9 days after the first human case of H10N8 infection reported in Nanchang, China. Among these LPMs, more than half provided at least one sample that tested positive for the H10 subtype. Moreover, we found that the environments of the LPMs were widely contaminated by both AIV subtypes H10 and H9, as well as H5. Furthermore, the H10 subtype coexisted with the H9 subtype among chickens in LPMs in Nanchang during the emergence of the H10N8 virus, which further indicated that the H10N8 virus probably originated from segment reassortment with the H9 subtype (6).

At present, it is generally accepted that LPMs pose an important potential risk for poultry-to-person transmission of AIVs. In Hong Kong in 1997, exposure to live poultry by visiting a poultry market or retail poultry stall was determined to be the risk factor for infection with the H5N1 virus in humans (3). That finding was further confirmed when the mean daily numbers of humans infected with the H7N9 virus was reduced 97%–99% after the closure of LPMs in Shanghai, Hangzhou, Huzhou, and Nanjing in 2013 (12). In the present study, the rate of AIV positivity was 26.3% for poultry in LPMs, which was much higher than the rate in previous reports, such as only 1% in Nanchang in 2000 (13), and 10.8% in Guangxi Province in 2009–2011 (14), suggesting that AIVs were circulating widely in poultry in Nanchang at the time. Moreover, the AIVs were especially prevalent in environmental samples. The LPMs in Nanchang were always characterized by poor sanitation and a wet and humid environment, which might have contributed to the cultivation of AIVs and

led to the creation of the novel H7N9 and H10N8 viruses. Furthermore, the AIV prevalence in the second period was higher than that in the first period, suggesting that the spread of AIVs among poultry increased during the preceding month, with a resulting increased risk of infection in humans in the spring of 2014.

AIVs detected in the present study were circulating predominantly in chickens. Specifically, subtypes H10 and H9 were dominant in chickens, while subtype H5 was dominant in ducks. The composition of AIVs in diverse species was different from that in other live bird markets (13–15). A previous study conducted in Nanchang approximately 10 years ago showed that the H2, H3, H4, and H9 subtypes were isolated most frequently from chickens and ducks in LPMs, but no H7 or H10 subtype viruses were isolated (13). The difference in the isolated subtypes revealed the variation of AIVs over time, while H9 continuously existed in the LPMs in Nanchang. As shown in the second period, the samples with mixed infections with viruses of the H10, H9, and N8 subtypes were all from chickens, which might provide opportunities for genetic reassortment. Previous studies indicated that the H9N2 virus provided genes not only for H7N9 (16) and H5N1 (17) viruses, but also for the H10N8 virus (6). Thus, the coexistence of H10, H9, and N8 as well as H10 and N8 in chickens suggested that chickens might serve as an intermediate host for H10N8 virus reassortment in LPMs and might be the major source of transmission of the influenza virus to humans in Nanchang. The novel H10N8 virus obtained from the first patient (6) was significantly different from the other H10N8 viruses, isolated from a Dongting lake wetland (18) and a live bird market in Guangdong Province (19), which leads to the speculation that the H10N8 virus has a stronger infectivity in chickens and is adapted for infection of humans. Since the H9N2 virus can be shed from healthy infected chickens for 8 days and continues to infect exposed chickens (20), the relationship should be studied further to understand the mechanism of how AIVs, including the H10N8 virus, infect humans.

At the time of this study, the H7N9 virus continued to spread in Zhejiang, Anhui, and Fujian Provinces, which are adjacent to Jiangxi Province, in addition to the Shanghai metro area. Surprisingly, no subtype H7 virus was detected in any poultry and was rarely discovered in environmental samples ($n = 2$), despite the fact that the H7N9 virus had been identified in 5 human cases and was detected in LPMs, 8 months before the survey in Nanchang (5). This suggests that subtype H7 was not the prevalent strain in the winter season. However, the underlying mechanism was still unknown. A long-term surveillance protocol may enable us to understand the variation and evolution of the virus more accurately and in a more timely fashion.

There were several limitations of our study. First, we were unable to identify the neuraminidase subtypes, such as N1 or N2 of the viruses. We believe that the subtype, H5 and H9 viruses detected in this study consisted of H5N1 and H9N2 viruses, as these viruses were the ones mainly circulating among chickens in China in recent years. Second, the sequences of the viruses were not obtained, so we were unable to compare the similarities of the H9 and H10 subtypes between LPMs, and we could not identify the origins of these viruses.

In conclusion, our study provided evidence that mixed infections with AIVs did exist in LPMs in Nanchang City, especially which the H9 subtype coexisted with the H10 subtype in chickens, which might play an important role in the evolution of the H10N8 virus in the winter of 2013. Furthermore, considering the observation of increased circulation of H5N1 and H7N9 viruses among poultry during periods with cooler temperatures, the H10N8 virus may spread further among poultry during the next winter and spring months, which may lead to an increased potential for transmission to exposed persons. Therefore, enhanced and continuing active surveillance for the H10N8 virus among poultry and people, as well as virologic analyses to assess genetic mutations that might suggest an increased transmissibility among humans, is critical for informing prevention and control efforts and evaluating the pandemic potential of the H10N8 virus. Closures of LPMs in areas where new human cases of H10N8 infection are detected may be warranted.

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Conflict of interest None to declare.

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