Distribution of Avian Influenza Viruses According to Environmental Surveillance During 2014-2017, China

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Research Article

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Abstract

Background

Avian influenza viruses persist in animal hosts and continue to cause human infections in China. It is important to analyse the geographic and seasonal distributions of avian influenza viruses and compare the subtypes and their prevalence among sample sites in environment.

Methods

A total of 329,276 environmental samples were collected from locations associated with poultry and wild birds from 2014 to 2017. Viral RNA was extracted from the environmental samples. Real-time PCR assays for influenza A, and the H5, H7, and H9 subtypes were performed on all the samples. Virus isolation was performed on the influenza A-positive samples detected by real-time PCR. Whole-genome sequencing was then performed on an Illumina sequencer.

Results

The proportions of samples that tested positive for total influenza A and the H5, H9 and H7 subtypes varied among different geographical regions and seasons. Significantly higher proportions of influenza A- and H5-, H9-, and H7-positive samples were collected from live poultry markets and poultry slaughtering locations. Influenza A positivity rates in sewage and chopping board swab samples were higher than those in other sample types. Multiple subtypes related to avian influenza viruses, including 9 HA and 7 NA subtypes, were detected in environmental samples.

Conclusions

These findings indicate that multiple subtypes of avian influenza A viruses continuously coexist in environments associated with poultry and increase the risk of reassortment and transmission, highlighting the need for environmental surveillance in China.

Background

Avian influenza viruses (AIVs) were first reported in 1878[1] in Italyand were subsequently isolated from chickens in 1934[2]. AIVs are categorized into two pathotypes according to their virulence in chickens: low-pathogenic (LP) and highly pathogenic (HP) AIVs. AIVs can infect poultry, sometimes causing asymptomatic infections [3, 4]. Of the 16 subtypes of AIVs that have been identified in birds, 10 subtypes (H5N1, H7N2, H7N3, H7N7, H9N2, H7N9, H5N6, H6N1, H10N7, and H10N8) are known to cause human infections[5], among which the H5 subtype has a nearly global distribution in birds. Moreover, the H7N9 subtype widely circulates and had rapidly evolved in live poultry markets (LPMs) in China[6, 7]. Furthermore, continuous reassortments among AIV strains have increased the risk of a pandemic.

Many studies have investigated the demographic and ecological risk factors associated with the effective transmission of AIVs. LPMs play an important role in human infection with AIVs. Bird transport between LPMs affected the emergence of H7N9 in Eastern China, and the closure of LPMs reduced the incidence of human infection with AIVs[8]. Furthermore, one survey found that 80% of households that purchased poultry from LPMs had an increased risk of poultry-to-human infection[9]. LPMs are particularly common in Southern China, and many subtypes, such as H9, H5, and H6, have been enzootic in poultry in China since the mid-1990s[10]. However, environmental factors, such as temperature and breeding conditions, play important roles in AIV survival and infectivity[11].

Avian influenza-related environmental surveillance was established in China in 2009. Samples are collected each month by each province. In the present study, we analysed the environmental samples collected from 2014–2017 as part of this programme to examine the geographic and seasonal distributions of AIVs in China and how these vary with regard to sites and sample types to determine environmental risk factors.

Methods

Environmental sample collection sites

Samples were collected from 2014-2017 by Chinese National Influenza Surveillance Network laboratories. Based on the national surveillance guidelines, at least 40 samples per month were collected from each of the 31 provinces, municipalities, and autonomous regions in China located in seven regions (Eastern, Southern, Central, Northern, Northwest, Southwest, and Northeast China), providing a total of 329,276 samples. These samples were obtained from a range of sites, including LPMs, poultry farms, backyards, slaughterhouses and wild bird habitats. These samples were obtained from a variety of poultry-related materials, including poultry faeces, drinking water, sewage, and swabs from poultry cages, feathers, etc. Each sample was maintained in viral transport medium and immediately transported at a low temperature to the nearest Chinese National Influenza Surveillance Network laboratory.

Environmental sample collection

A 5-ml liquid sample (drinking water, sewage) was collected, and 0.5% BSA, ampicillin (2 x 106 IU/L), streptomycin (200 mg/L), polymyxin B (2 x106 IU/L), gentamicin (250 mg/L), mycin (0.5 x 106 IU/L), oxygen hydrochloride floxacin (60 mg/L), and sulfamethoxazole (200 mg/L) were added. The samples were mixed thoroughly and centrifuged at 3000 rpm for 10 min, and the supernatants were aliquoted into three tubes (each tube contained 1.5 ml). One tube was stored at -80°C, one was designated for nucleic acid identification, and one was designated for virus isolation. Faeces or swab samples were put into 5 ml Hank's medium containing 0.5% BSA, ampicillin (2 x 106 IU/L), streptomycin (200 mg/L), polymyxin B (2 x106 IU/L), gentamicin (250 mg/L), mycin (0.5 x 106 IU/L), oxygen hydrochloride floxacin (60 mg/L), and sulfamethoxazole (200 mg/L). The supernatant was mixed thoroughly, centrifuged at 3000 rpm for 10 min, and aliquoted. The samples were sent to a local network laboratory within 48 hours and stored at 4°C. All the supernatants were aliquoted into three

tubes. Each tube contained 1.5 ml of the sample; one was designated for nucleic acid identification by a local network laboratory, one was designated for virus isolation, and one was transported to the China Center for Disease Control and Prevention (CDC) and stored at- 80℃.

RNA extraction

Viral RNA was extracted from each of the collected samples using a QIAsymphony RNA Kit (931636; Qiagen, Hilden, Germany) with a QIAsymphony SP instrument (Qiagen) according to the manufacturer's instructions.

Real-time PCR

Real-time PCR assays for influenza A, and the H5, H7, and H9 subtypes were performed on all the samples, with primer and probe sets provided in the Chinese National Influenza Surveillance Guidelines (Supplementary Table S1). The reactions were carried out using an AgPath-ID™ One-Step RT-PCR Kit (4387422; Ambion®) under the following cycling conditions: 10 min at 45 °C; 10 min at 95°C; 40 cycles of 15 s at 95 °C; and 45 s at 60°C. The positive control contained all the reaction components and RNA of influenza A, and H5, H7, and H9 subtypes. The negative control contained all the reaction components except for the reverse transcriptase.

Virus isolation

Virus isolation was performed on the influenza A-positive samples detected by real-time PCR. The samples were inoculated into the allantoic cavity of 9- to 10-day-old embryonated chicken eggs; the eggs were incubated at 37℃ for 48 hours and chilled at 4℃ overnight. The allantoic fluid was then harvested, and a haemagglutination assay was performed using 1% turkey red blood cells to detect harvested viruses [24].

Next-generation sequencing

Virus total RNA was extracted by a MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (cat# 42352, Applied Biosystems). The RNA was subjected to reverse transcription and amplification using the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq High Fidelity DNA Polymerase (cat#: 12574035, Invitrogen). The DNA was purified by a MagMax Core Nucleic Acid Purification Kit (cat# 1903031, Thermo Fisher Scientific). The DNA library was prepared using Nextera XT DNA Preparation Kits (cat#FC-131-1096, Illumina). Whole-genome sequencing was then performed on an Illumina sequencer, and the data were analysed using CLC software.

Statistical analysis

A paired t test was performed, and $p < 0.05$ was considered significant.

Results

Geographic distributions of avian influenza viruses

Based on RT-PCR, the overall positivity rate for influenza A was 15.51% (Fig 1-a). The positivity rates were particularly high (17.21-44.94%) in 11 provinces in five of the seven regions tested: Central China (Hunan and Hubei provinces), Eastern China (Fujian, Jiangxi and Zhejiang provinces), Southern China (Guangxi Autonomous Region and Guangdong Province), Southwest China (Si Chuan, Chong Qing municipality and Guizhou), and Northwest China (Gan Su).

Among the influenza A viruses detected, the H5 subtype was detected in an average of 3.17% of samples (Fig 1-b). The positivity rates were significantly high (9.74%-14.66%) in three provinces and one municipality in three regions: Southwest China (Chong Qing municipality), Central China (Hunan and Jiangxi provinces), and Northwest China (Gansu Province).

The mean positivity rate for the H7 subtype was 1.64% (Fig 1-c), with rates of up to 4% being detected in three regions: Eastern China (Jiangsu, Fujian, Zhejiang and Jiangxi provinces), South China (Guangdong Province) and Central China (Hunan Province). During the study period, of the H7 subtypes, only H7N9 was detected.

The highest mean positivity rate for the H9 subtype 9.74% (Fig 1-d). Fifteen provinces, autonomous regions and municipalities displayed high positivity rates (10%-26.82%).

Seasonality of avian influenza viruses in different environments

The monthly nucleic acid positivity rates for influenza A and the subtypes H5, H7, and H9 in the poultry-related environmental samples are shown in Fig 2. The positivity rates in China showed obvious seasonality and were highest in December and January and lowest from May to September.

Variations in total influenza A, H5, H9 and H7 positivity rates among the sampling sites

During 2014-2017, samples with the highest total influenza A and H5, H9 and H7 positivity rates were collected from LPMs (29.91%, 5.19%, 16.85%, 5.21% on average, respectively), followed by slaughterhouses (21.25%, 3.45%, 11.9%, 2.17% on average, respectively). In contrast, poultry farms, backyards, and wild

bird habitats had influenza A positivity rates of 3.26%, 3.36%, and 1.17% on average, respectively, while the H5, H9 and H7 positivity rates were all less than 1%. The statistical analysis indicated that LPMs and slaughterhouses were associated with significantly higher positivity rates for total influenza A and the H5, H9, and H7 subtypes than all other sites during the study period ($p < 0.05$; Table 1).

Influenza A and subtype H5, H9 and H7 positivity rates among different sample types

Environmental samples that were collected from sewage and chopping boards had significantly higher positivity rates for influenza A and the subtypes H5, H7, and H9 than those collected from faeces, cages, and feeding troughs ($p < 0.05$). Furthermore, the positivity rates of subtype H9 in samples originating from sewage and chopping boards were significantly higher than those of subtypes H5 and H7 ($p < 0.05$; Table 2).

Multiple subtypes of influenza A viruses were detected in poultry-related environments

In total, 9 HA subtypes and 7 NA subtypes were detected during the study period, including the HA subtypes H1, H3, H4, H5, H6, H7, H9, H10, and H11 and the NA subtypes N1, N2, N3, N6, N7, N8, and N9. The H5, H7, and H9 subtypes of influenza A virus accounted for the majority of positive samples (Supplemental table S2). The H5 subtype of influenza A virus was present in 23.1-41.52% of the influenza A-positive samples, the H7 subtype was present in 6.86-27.04%, and the H9 subtype was present in 35.19-59.97%. The other subtypes, including H3 (6.3%), H4 (1.04%), and H6 (4.02%), were present in only small proportions of samples (Supplemental table S2).

Based on the virus isolation data, H5, H9 and H7 were the major subtypes. The H5 subtypes comprised H5N1, H5N6, H5N2, H5N8 and H5N9. The proportion of subtype H5N6 increased more than threefold from 11% in 2014 to 34% in 2016 and then decreased to 12.14% in 2017. The H5N1 subtype showed a declining trend from 2014-2017. The proportions of H5N1 were 8.74% (2014), 10.53% (2015), 5.54% (2016) and 5.94% (2017). The H5N2, H5N8 and H5N9 subtype proportions were much lower than the H5N1 and H5N6 subtype proportions. The proportion of H7N9 reached 23% in 2017, which was approximately four times that in 2014 (6%), while the proportion of H9N2 decreased from 2014 to 2017, with proportions of 59%, 51%, 35% and 36%, respectively (Fig 3 and supplementary tableS2). GraphPad Prism 5 was used to construct the Fig 3.

In approximately 90% of the samples, only a single subtype of influenza A virus was detected. A small proportion of samples (1.5%) contained a combination of different subtypes; for example, subtype H9 was detected with H7, H5 and other subtypes in the same sample, further proving the co-circulation of multiple subtypes.

Discussion

Based on four-year environmental surveillance data, we detected the geographical distributions of total influenza A and the H5, H9, and H7 subtypes in the environment. The geographical distribution of H7 occurred in only the Yangtze and Pearl River deltas and a few adjacent provinces. Some studies reported that these regions were the sources of newly emerged H7N9 human infections during 2013–2017[10]. Poultry is considered to be the major source of H7N9 infections in humans [12]. Our study indicates that the H7 subtype persists in the environment in these regions and may be associated with human infection and virus evolution. The geographical distribution of H5 was mainly located in Southern China. H5 has been widely circulating among poultry in China since 2004. The highly pathogenic avian influenza H5 subtype has evolved multiple clades and subclades[13]. Though control measures were carried out, birds and poultry still carried and transmitted the H5 virus. In Southern China, there are many poultry farms and LPMs. The environment provides the opportunity for H5 subtype transmission and evolution. Compared with the H5 and H7 subtypes, the H9 subtype geographic distribution was nationwide, and it was especially prevalent in the southern and western regions of China. The first H9N2 subtype was isolated in 1992, and since then, H9N2 has become the predominant subtype in poultry [14]. Live poultry trading and feeding patterns have caused H9N2[15] to become prevalent in different regions of China. The H9 subtype distribution characteristics provide the opportunity for avian virus reassortment.

Our results indicated that the prevalence of AIVs varied seasonally, with higher positivity rates of subtypes H5, H7, and H9 in late winter (December) and early spring (January) than in summer and autumn. Several studies have found that minor fluctuations in temperature, pH or salinity in aquatic habitats may enhance or diminish the persistence and infectivity of AIVs[16]. AIV transmission is be promoted under cool and dry conditions[17]. Our results were consistent with those of current studies and showed that AIVs can survive well in winter and early spring.

In our study, LPMs and sewage were proven to be environmental risk factors. The samples collected from LPMs displayed the highest positivity rates for the H5, H7, and H9 subtypes, which is consistent with previous studies. In China, a large amount of poultry is traded through LPMs [18]which are known to be major sources of AIVs causing significant public health concerns. Poultry market closure is still an effective measure when avian influenza infects humans or poultry [19]. We recommended that LPMs should be managed strictly. Some studies have reported that influenza viruses are waterborne pathogens that have the capacity to infect a wide variety of hosts and undergo genetic reassortment[20] In our study, we investigated five kinds of samples related to poultry feeding, sale, and slaughter. The results indicated that sewage may carry a large number of AIVs and transmit the virus among poultry.

Our studies demonstrated virus subtype diversity in environment-related avian influenza. Virus isolation indicated that the H5 subtype continuously existed in environmental samples in China, and the proportion of H5N1-subtype viruses decreased while the proportion of H5N6-subtype viruses increased dramatically during 2014–2017. Some studies have demonstrated that the H5 subtype circulating in China before 2012 was exclusively the H5N1 subtype, while H5N6, H5N8 and H5N2 emerged in China thereafter[21]. In accordance with these studies, our data reveal that the H5N6 subtype virus has already substituted H5N1 and became the dominant strain in the environment. The proportion of H7N9 virus in the environment increased significantly and became the main avian influenza virus subtype in the environment during 2014–2107. Five human epidemics of H7N9 were reported in China during 2013–2017[22]. Some studies reported that poultry-related environments were contaminated and poultry were infected with H7N9 in the regions where H7N9 emerged [23]. In our study, we also found that the H7N9 subtype was prevalent in the environment, especially in 2017. H7N9 in the environment should be controlled effectively to decrease

the human infection risk. H9N2 persisted in the environment based on our surveillance results. Currently, H9N2 is prevalent in chickens and wild birds in China. The poultry industry and traditional small-scale farming contribute to the H9N2 epidemiological situation. Though vaccination has been implemented, vaccination coverage is unsatisfactory. H9N2 in the environment allows the chance for zoonotic transmission in China.

Conclusion

Our findings indicate that avian-associated environments may contribute to the transmission of AIVs. The widespread and persistent circulation of avian influenza viruses in China increases the risk of zoonotic transmission and encourages the timely monitoring of changes in AIVs. Long-term control strategies and early interventions need to be developed for AIV outbreaks.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of supporting data

The datasets used and analysed 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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Tables

Table 1. Total influenza A and subtype H5, H7, and H9 nucleic acid positivity rates among environmental samples collected from different sites during 2014-2017.

Collection sites consisted of five locations: LPMs, slaughterhouses, backyards, poultry farms and wild bird habitats. Real-time PCR was used to screen for influenza A and the H5, H9 and H7 subtypes in the samples. The nucleic acid positivity rate of samples was defined as the number of positive samples/number of collected samples.

Type/subtype year	Positivity rate (%) (Number of positive samples/Number of samples collected)					
	Live poultry market	Slaughterhouse	Backyard	Poultry farm	Wild bird habitat	Mean (%)
Influenza A						
2014	30.61(12050/39365)	16.67(372/2232)	1.69(82/4847)	2.31(242/10458)	4.12(86/2086)	21.75(12832/58988)
2015	30.2(14245/47174)	19.16(396/2067)	4.23(185/4373)	2.85(270/9470)	0.53(11/2080)	23.18(15107/65164)
2016	35.24(17910/50882)	25.04(475/1897)	5.4(285/5274)	4.69(546/11649)	0.35(7/2003)	26.81(19223/71705)
2017	25.98(20620/79377)	23.70(720/3039)	2.83(341/12036)	3.33(508/15251)	0.33(10/3076)	19.68(22199/112779)
A/H5						
2014	7.2(2833/39365)	3.18(71/2232)	0.21(10/4847)	0.73(76/10458)	0(0/2086)	5.07(2990/58988)
2015	5.94(2802/47174)	3.87(80/2067)	0.8(225/4373)	0.29(27/9470)	0.1(2/2080)	4.81(3136/65164)
2016	6.01(3057/50882)	5.01(95/1897)	0.36(19/5274)	0.29(34/11649)	0.1(2/2003)	4.47(3207/71705)
2017	3.24(2572/79377)	2.47(75/3039)	0.19(23/12036)	0.19(29/15251)	0(0/3016)	2.39(2699/112779)
A/H9						
2014	15.68(6173/39365)	8.47(189/2232)	0.8(39/4847)	1.1(115/10458)	0.48(10/2086)	11.06(6526/58988)
2015	18.04(8509/47174)	9.82(203/2067)	2.74(120/4373)	1.19(113/9470)	0.24(5/2080)	13.73(8950/65164)
2016	19.51(9915/50882)	13.86(263/1897)	3.3(174/5274)	1.48(172/11649)	0.2(4/2003)	14.68(/1052871705)
2017	15.05(11950/79377)	14.61(444/3039)	1.37(165/12036)	1.24(189/15251)	0.2(6/3016)	11.31(12754/112779)
A/H7						
2014	5.43(2139/39365)	0.76(17/2232)	0.52(25/4847)	0.71(74/10458)	1.39(29/2086)	3.87(2284/58988)
2015	4.73(2231/47174)	2.18(45/2067)	0.8(35/4373)	0.04(4/9470)	0(0/2080)	3.55(2315/65164)
2016	5.57(2832/50882)	3.22(61/1897)	1.31(69/5274)	0.25(29/11649)	0.05(1/2003)	4.17(2992/71705)
2017	5.15(4090/79377)	2.57(78/3039)	0.75(90/12036)	1.36(207/15251)	0(0/3016)	3.96(4465/112779)

Table 2. Influenza A, H5, H7, and H9 nucleic acid positivity rates of different sample types.

Samples were collected from sewage, chopping boards, feeding troughs, cages and faeces. Real-time PCR was used to detect total influenza A and subtypes H5, H9 and H7 in the different sample types. The nucleic acid positivity rate for each different sample type was defined as the number of positive samples/number of collected samples.

Type/Subtype	Positivity rate (%) (Number of positive samples/Number of samples collected)					
year	Sewage	Chopping board	Feeding trough	Cage	Faeces	Mean (%)
Influenza A						
2014	37.31(2819/7556)	33.49(2373/7085)	26.15(2138/8175)	17.81(2538/14252)	12.92(2617/20259)	22.58(12485/61512)
2015	39.03(3025/7750)	33.74(3217/9535)	28.19(2300/8158)	19.76(3196/16174)	15.05(3333/22141)	23.45(15071/69148)
2016	40.17(3774/9394)	40.25(4558/11324)	27.64(2797/10121)	24.01(4013/16711)	18.79(4503/23962)	27.54(19645/78190)
2017	31.03(4559/14692)	27.03(4712/17433)	22.48(3450/15347)	18.19(4715/25919)	12.34(4398/35648)	19.79(21834/120142)
A/H5						
2014	14.56(1100/7556)	7.86(2373/7085)	7.38(2138/8175)	2.15(2538/14252)	1.9(385/20259)	5.06(3113/61512)
2015	12.12(939/7750)	7.07(674/9535)	6.06(494/8158)	2.35(380/16174)	1.89(418/22141)	4.54(3140/69148)
2016	9.59(901/9394)	9.04(1024/11324)	4.48(453/10121)	2.59(433/16711)	2.18(522/23962)	4.76(3721/78190)
2017	5.64(828/14692)	4.37(762/17433)	2.42(372/15347)	1.48(383/25919)	0.99(353/35648)	2.43(2917/120142)
A/H7						
2014	4.74(358/7556)	6.68(473/7085)	3.61(295/8175)	3.96(564/14252)	2.35(477/20259)	3.88(2388/61512)
2015	4.25(329/7750)	6.12(584/9535)	3.33(272/8158)	3.52(569/16174)	2.81(622/22141)	3.73(2576/69148)
2016	5.8(545/9394)	8.9(1008/11324)	2.93(297/10121)	4.3(718/16711)	3.19(765/23962)	4.78(3736/78190)
2017	5.73(842/14692)	6.31(1100/17433)	4.06(624/15347)	3.65(946/25919)	2.59(924/35648)	4.08(4902/120142)
A/H9						
2014	19.1(1443/7556)	17.12(1213/7085)	15.40(1259/8175)	9.12(1300/14252)	5.98(1212/20259)	11.06(6806/61512)
2015	24(1860/7750)	19.17(1828/9535)	19.48(1589/8158)	12.06(1951/16174)	8.05(1782/22141)	13.94(9642/69148)
2016	23.32(2191/9394)	21.3(2412/11324)	16.47(1667/10121)	13.44(2246/16711)	9.32(2234/23962)	15.23(11907/78190)
2017	18.48(2715/14692)	14.34(2505/17433)	13.12(2013/15347)	11.4(2954/25919)	6.36(2266/35648)	11.3(13580/120142)

Figures

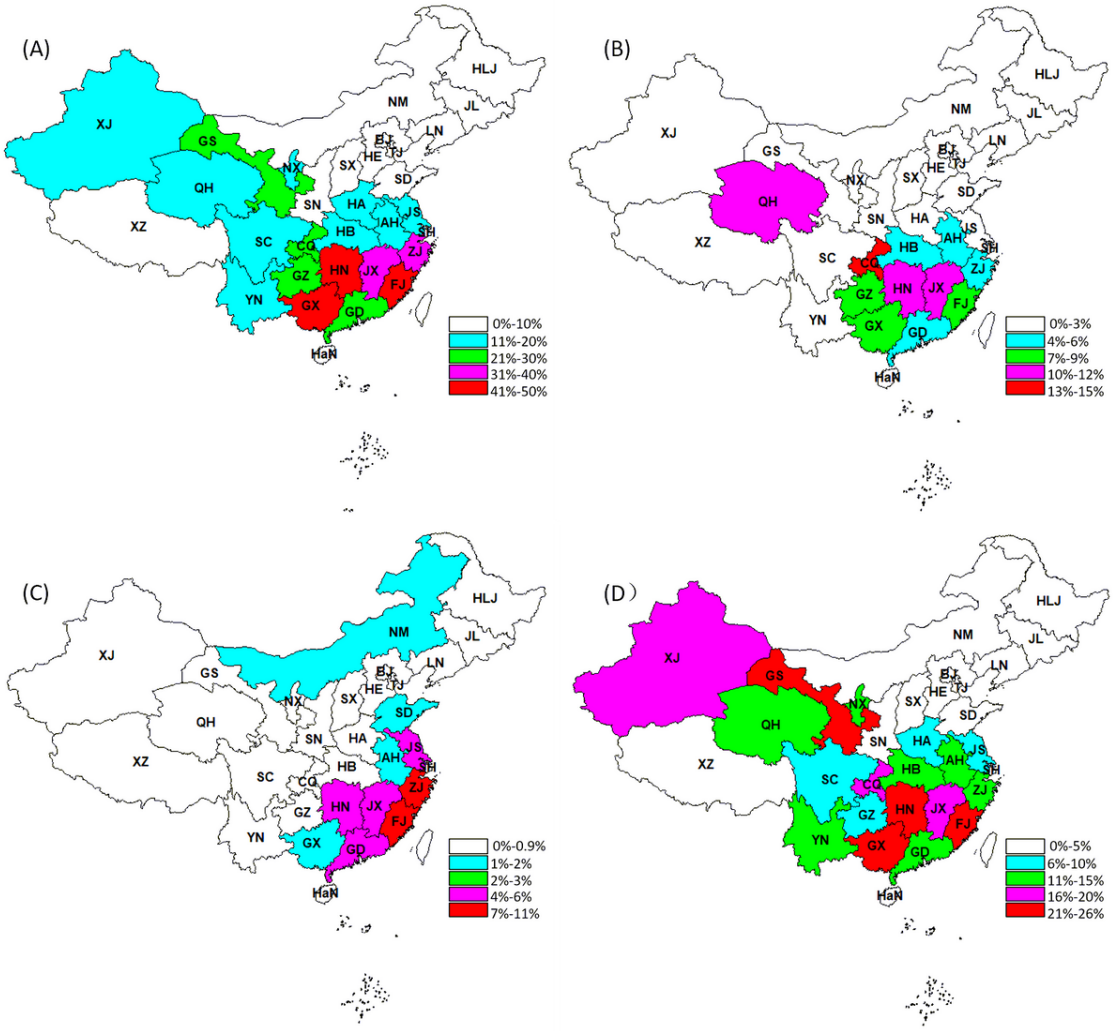

Figure 1

Geographical distributions of total influenza A and H5, H9, and H7 subtype nucleic acid positivity rates in the environmental samples during 2014-2017. Fig 1a-d indicates the total influenza A and H5, H7 and H9 subtype nucleic acid positivity rates, respectively. Different colours represent the range of positivity rates. MapInfo software version 7.0 was used to draw the maps. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

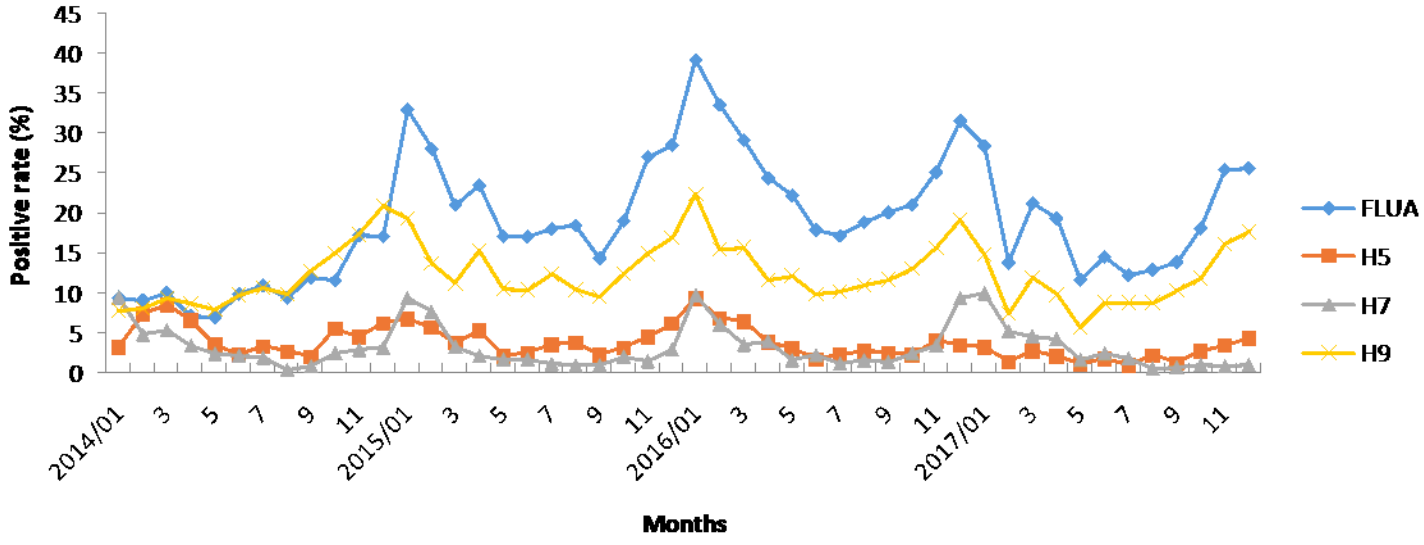

Figure 2

Monthly nucleic acid positivity rates of total influenza A and the H5, H7, and H9 subtypes during 2014-2017. The blue line represents the nucleic acid positivity rate of total influenza A; the orange line represents the nucleic acid positivity rate of subtype H5; the grey line represents the nucleic acid positivity rate of subtype H7; and the yellow line represents the nucleic acid positivity rate of subtype H9. MSOffice software (version 2007) was used to produce Fig 2.

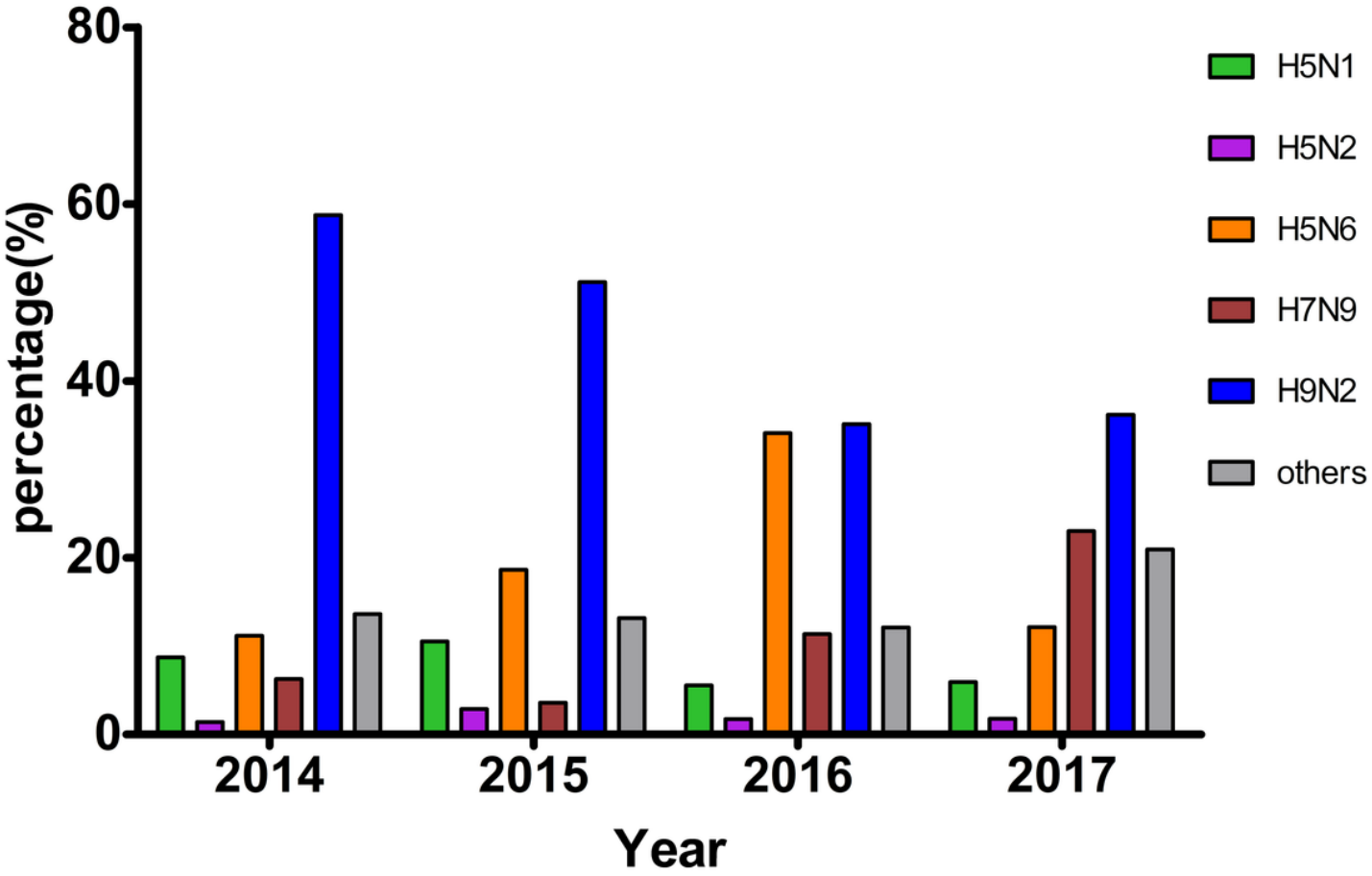

Figure 3

Proportions of H5N1, H5N6, H7N9, H9N2 and other virus subtypes during 2014-2017. The X axis represents different years, and the Y axis represents different subtype percentages (%). The percentage was defined as the number of different virus subtypes/number of viruses (%).

Supplementary Files

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- Supplimentary.docx