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Surveillance of H7N9 avian influenza virus in farmers' markets in Beijing in 2019–2023

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Abstract

Avian influenza viruses (AIVs) present an ongoing threat of human infections. Continuous surveillance is important for detecting new infections and verifying prevention and control measures. Swabs of the external environment and throat swabs of employees were collected from six farmers' markets in Beijing to detect influenza A virus. Positive samples were sequenced, and their genetic characteristics analyzed. In total, 3251 environmental samples were collected from 2019 to 2023, 11 of which were positive for influenza A virus (positivity rate of 0.34%), including nine for H9N2 and two for H7N9. In a genetic analysis, all H7N9 samples showed low pathogenicity, and no mutations at highly pathogenic sites were detected. All 1135 throat swab samples from staff were negative for influenza A virus. At present, the detection rate of AIVs in farmers' markets is very low, and no adaptive mutations allowing cross-host transmission were found, indicating a low risk of AIV infection among the people of Beijing.

Keywords H7N9, Avian influenza virus, Genetic characterization, Low risk

Avian influenza viruses (AIVs) belong to the family *Orthomyxoviridae* and can be divided into 16 hemagglutinin (HA) subtypes and nine neuraminidase (NA) subtypes based on the major surface glycoproteins [1, 2]. Wild waterfowl are the natural hosts of AIVs, and all subtypes are detected in wild waterfowl [3, 4]. Under normal circumstances, these viruses do not cause disease symptoms in wild waterfowl. However, when AIVs infect other hosts, they can cause disease [5]. In chickens, AIVs are divided into high-pathogenicity AIVs (HPAIVs) and low-pathogenicity AIVs (LPAIVs) [6]. Currently, only the H5 and H7 subtypes are highly pathogenic. Since the first cross-species infection of humans by H5N1 AIV in 1997 [7] and subsequent human infections with subtypes H9N2, H5N6, H7N9, H10N3, and H3N8, the risk that AIVs pose to public health has attracted widespread attention [8, 9].

In 2013, the first human infection with H7N9 AIV occurred in the Yangtze River Delta region of China. The virus was produced by a rearrangement of H7 from waterfowl, N9 from wild birds, and an internal gene from H9N2 virus in chickens [10]. In 2013–2017, five waves of H7N9 infection caused more than 1700 severe human infections, with a patient mortality rate of 35% [11, 12].

Epidemiological surveillance showed that more than 80% of cases were caused by direct or indirect contact with live poultry, indicating that virus-carrying poultry is an important source of infection in humans [13]. In 2017, multiple basic amino acid sites were inserted into the HA cleavage site of the H7N9 virus, and the mutated virus became HPAIV. The use of an H7N9 avian influenza vaccine has reduced the prevalence of the virus in poultry and effectively controlled human infections. However, the antigenic drift caused by the high variability of the AIV genome and the consequent immunity to the

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vaccine that arises may override this block to the virus's spread in poultry. Indeed, there are still reports of H7N9 virus detected in poultry in China [14, 15]. Therefore, continuous monitoring of H7N9 AIV in live poultry markets is crucial to determining the risk of viral transmission and to the adoption of appropriate prevention and pre-emptive control measures.

In 2013, measures were taken to close live poultry markets to prevent the spread of AIVs, and farmers' markets became important sites of AIVs monitoring [16, 17]. The high population density at farmers' markets increases the risk of close contact between people during the sale of poultry products. In this study, AIVs surveillance was undertaken at six farmers' markets in Beijing from 2019 to 2023, to collect important data to guide the prevention of human AIV infections in Beijing.

Results

Surveillance results

In accordance with the Surveillance Plan for Highly Pathogenic Avian Influenza in the Environment of the Chinese Center for Disease Control and Prevention, six farmers' markets in the Tongzhou District of Beijing were chosen as monitoring points for AIVs: the Yunhetongyuan farmers' market (A), the Tongliconglin farmers' market (B), the Xinhai farmers' market (C), the Yangzhuangshunhang farmers' market (D), the Yangxinyuan farmers' market (E), and the Xinchengdongli farmers' market (F). Throat swab samples were also collected from employees and tested. All samples were analyzed with reverse transcription–real-time PCR.

From 2019 to 2023, a total of 3251 environmental samples and 1135 throat swabs from employees were collected and analyzed. The results are shown in Tables 1 and 2. All 1135 throat swabs were negative for influenza A virus. The median age of the 1135 employees tested was 51.0 years, and the sex ratio (male:female) was 4.88.

Molecular evolutionary analysis of HA and NA

Two H7N9-positive samples were sequenced on the Illumina platform. H7N9 reference sequences were downloaded from the GISAID database [18], and MEGA 6.0 software (Mega Software Solution Inc., Tallahassee, FL, USA) was used to construct phylogenetic trees for the HA and NA genes with the neighbor-joining method. The two viruses were designated A/Environment/Beijing/TZ055/2022 and A/Environment/Beijing/TZ084/2022. The phylogenetic analysis (Fig. 1) revealed that the HA

Table 1 Results of all environmental samples collected in the study

Sample types	Total tests	H9N2 positive	H7N9 positive	Total positive	Positive rate	Positive market	Positive year
Poultry meat	1701	6	1	7	0.41%	A	2023
Chopping board	653	2	0	2	0.31%	B	2023
Knife	244	0	0	0	0	-	-
Balance tray	210	0	0	0	0	-	-
Refrigerator door	289	1	0	1	0.35%	B	2023
Sink	154	0	1	1	0.65%	E	2023
Total	**3251**	**9**	**2**	**11**	**0.34%**	-	-

A: the Yunhetongyuan farmers' market; B: the Tongliconglin farmers' market; E: the Yangxinyuan farmers' market; -: represents no data.

Table 2 Samples collected and analyzed in different years

Sample types	Year					
	2019	2020	2021	2022	2023	Total
Poultry meat	491	185	254	269	502	1701
Chopping board	187	62	102	134	168	653
Knife	87	21	33	29	74	244
Balance tray	63	35	21	32	59	210
Refrigerator door	84	32	46	58	69	289
Sink	29	14	19	41	51	154
Throat swabs	216	151	196	126	446	1135
Total	**1157**	**500**	**671**	**689**	**1369**	**4386**

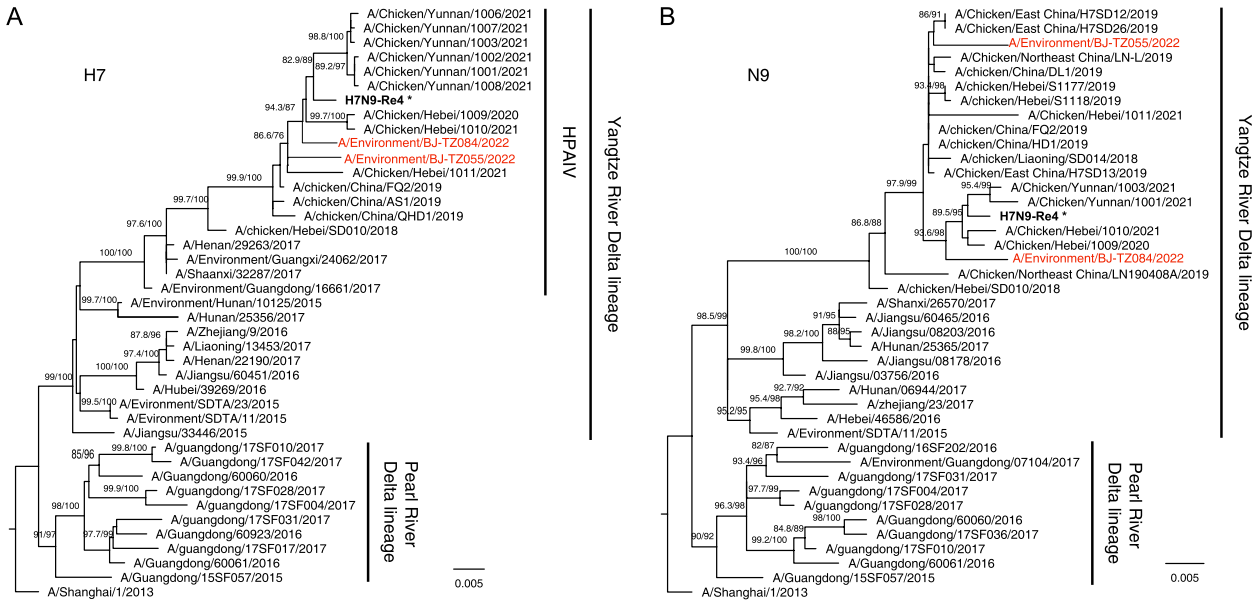

Fig. 1 Phylogenetic trees of the open reading frames encoding Hemagglutinin (HA) (**A**) and Neuraminidase (NA) (**B**) of subtype H7N9 influenza viruses isolated in Beijing. Viruses shown in red were characterized in this study. The trees were constructed with the neighbor-joining method in MEGA 6.0, with 1000 bootstrap replications to assign confidence to the groupings. H7N9-Re4*: recommended subtype H7N9 AIV vaccine strain from 2022. HPAIV: high-pathogenicity AIV

and NA genes of the two H7N9 viruses belonged to the Yangtze River Delta lineage.

Molecular characterization

The two H7N9-subtype influenza viruses contained multiple basic amino acids (PEVPKRKRTAR/GL) at the cleavage site between HA1 and HA2, indicating high pathogenicity in domestic poultry. Analysis of important amino acid sites in the HA protein revealed no mutations at G186, Q226L, or G228 in the receptor-binding sites of the two H7N9 strains. There were no mutations at key sites in the amino acid sequence of NA that confer resistance to neuraminidase inhibitors (E119V, R152K, H274Y, and R292K). Five amino acids (QISNT) were deleted from the NA stem region (Table 3). We also used the Prosite

Table 3 Key molecular markers of avian influenza A (H7N9) viruses in Beijing, 2023

Gene	Functional domain	Mutants	BJ-TZ055	BJ-TZ084	H7N9-Re4[a]
Hemagglutinin (HA)	Epitope A region	A122L	L	L	L
		T126K	K	K	K
		G186V	V	V	V
	Receptor binding sit	Q226L	L	L	L
		G228S	G	G	G
	Altered virulence	Cleavage sit	PEVPKRKRTA R/GL	PEVPKRKRTA R/GL	PEVPKGR/GL
Neuraminidase (NA)	Related to drug resistance (neuraminidase inhibitor)	E119V	E	E	E
		R152K	R	R	R
		H274Y	H	H	H
		R292K	R	R	R
	Related to virulence	QISNT	Del	Del	Del
Polymerase basic protein 2 (PB2)	Efficiency and increased virulence	E627K	E	E	E
		Q591K	Q	Q	Q

HA gene follows the H3 numbering system; NA gene follows the N2 numbering system; and PB2 gene is numbered from the start codon

[a] H7N9-Re4: recommended subtype H7N9 AIV vaccine strain from 2022

website [19] to analyze the *N*-glycosylation sites in HA. There were seven identical *N*-glycosylation sites in the HA genes of the two virus samples, located at 30NGTK33, 46NATE49, 141NGTT144, 167NATF170, 249NDTV252, 425NWTR428, and 497NNTY500.

Discussion

AIVs have been detected in live poultry markets worldwide, and human cases of avian influenza have mainly been contracted through contact with poultry [20]. Environmental transmission is an important way in which AIVs infect humans in live poultry markets. In a previous study, one human isolate and three environmental isolates collected within live poultry markets in Xiamen, China, were evaluated. The phylogeny, transmissibility, and pathogenicity of the four isolates were determined systematically. The overall efficiency of contact and aerosol transmissibility improved for the H9N2 virus, which evolved along the "chicken–environment–human" spreading chain in live poultry markets from the summer of 2019 to the summer of 2020, which may have contributed to the increasing probability of human infection [21].

Current research shows that the positivity rates of AIVs in environments outside live poultry markets are relatively high [22, 23]. Although detection conditions vary in different regions, live poultry markets are still reservoirs of AIVs and sites of their proliferation. In 2013, Beijing took the decisive measure of closing live poultry trading markets, which was an important and effective means of reducing environmental pollution with AIVs and controlling human infections, greatly reducing the risk of human infections [24]. To investigate whether there is still a risk of avian influenza in farmers' markets, a surveillance program by the Chinese Center for Disease Control and Prevention was established and is maintained in China. Six farmers' markets in the Tongzhou District were selected as monitoring points for AIVs in Beijing. This study summarizes the external environmental monitoring data and detection results from 2019 to 2023 (Tables 1 and 2). In total, 3251 environmental samples were collected, and nine strains of H9N2 viruses and two strains of H7N9 viruses were detected. H9N2 was the main subtype detected in the external environmental surveillance of AIVs, and most AIVs of the subtype are LPAIVs [21]. A phylogenetic analysis of the two H7N9 AIVs was undertaken based on the sequences of the HA and NA genes (Fig. 1). The HA genes of the two H7N9 virus strains and the vaccine strain H7N9-Re4 clustered on a single branch of the phylogenetic tree, indicating that the vaccine should have a very good immune effect. The positivity rate of the 3251 samples was sufficiently low to preclude any statistical analysis related to time or space. However, the 11 positive samples were all collected in 2023, which could be related to the frequent trade exchanges after the COVID-19 epidemic. Therefore, both H7N9 vaccine immunity and commodity monitoring must be strengthened further.

This research has shown that the genes encoding surface proteins HA and NA of the AIVs detected were relatively stable, and no reassortment had occurred. The mutation at amino acid 226 of the HA receptor-binding site is related to the ability of AIVs to bind to the SAα−2, 6 Gal receptors on the epithelial cells of the human upper respiratory tract. Mutation Q226L enhances the virus's ability to bind to these receptors, suggesting a higher likelihood of infection in humans. The G186V mutation at another key amino acid site for HA receptor binding is related to the virus's adaptation to a mammalian host [25]. The two H7N9 strains examined in this study showed no G186V mutation or Q226L mutation, indicating that poultry were still susceptible to these viruses. However, no significant resistance mutations were detected in the NA gene, indicating that neuraminidase inhibitors are still effective in treating infections with these strains (Table 1). However, the cleavage site sequence in the HA gene included a motif of multiple basic amino acids, PEVPKRKRTAR/GL, suggesting that the virus is highly pathogenic in poultry [6]. Simultaneously, the NA gene lacked five amino acids at positions 69–73 of the stem (QISNT). These results, combined with the results of the phylogenetic analysis (Fig. 1), indicate that vaccine Re-4 for H7N9 should still be effective against these strains. Moreover, all 1135 throat swabs from employees tested negative for AIVs, indicating that the risk of H7N9 was low. Although there are currently no critical site changes in the sequences of the viruses detected relative to that of the vaccine strain, because the virus mutates continually, it is still necessary to strengthen AIV surveillance and closely monitor changes at key genetic sites in the viral genome, to provide a basis for disease prevention, control, and risk assessment.

Materials and methods

Samples

Six farmers' markets in the Tongzhou District of Beijing were chosen as external environmental monitoring points for AIVs. Swabs from the external environment, including swabs of poultry meat, chopping boards, knives, balance trays, refrigerator doors, and sinks, as well as throat swabs from employees, were collected from 2019 to 2023 and stored at 4 °C for transportation. The samples were then sent to the Influenza Network

Surveillance Laboratory of Tongzhou District Center for Disease Prevention and Control in Beijing for influenza A virus detection.

Nucleic acid extraction and AIV detection

Viral RNA was extracted from the samples in a biosafety level 2 (BSL-2) laboratory using an automated magnetic bead method kit (CqEx-DNA/RNA Kit, Xi'an Tianlong Technology Co., Ltd., Xi'an, China). A 200 μL sampling solution was added to the lysate well in the reagent tube of the kit and placed in the equipment for nucleic acid extraction according to the instrument's instructions (GeneRotex 96 Nucleic Acid Extractor, Xi'an Tianlong Technology Co., Ltd., Xi'an, China). The eluent (50 μL) was used as the RNA template. Commercial kits were used to detect the viral nucleic acids of the influenza A virus. The HA-directed kit primarily detected H5, H7, and H9 influenza A viruses, whereas the NA-directed kit mainly detected the N1, N2, N6, and N9 subtypes. The reaction system was configured according to the reagent instructions and the amplification parameters. The thermal cycling procedure involved 45 cycles of 45 °C for 10 min, 95 °C for 5 min, 95 °C for 15 s, and 60 °C for 50 s with the fluorescent signal collection at 60 °C. Dual 6-carboxyfluorescein (FAM) and 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein (VIC) detection channels were used. The results were interpreted strictly according to the kit instructions.

Viral whole-genome sequencing

Samples positive for AIV nucleic acids were subjected to whole-genome sequencing. A primer combination and amplification system were prepared for the whole-genome amplification of the AIVs, and the abundance of the viral nucleic acid was amplified according to the reaction conditions. The amplification products were purified using the magnetic bead method, and enzymatically fragmented to construct nucleic acid libraries. After purification, the library products were quantified, and the libraries were loaded according to the requirements of the Illumina MiniSeq platform. The sequencing data were assembled with the CLC Genomics Workbench 23.0.2 software (QIAGEN, Hilden, German).

Virus sequence analysis

The MEGA 6.0 software was used to construct genetic evolutionary trees from the viral sequences and to analyze all mutation sites and key amino acids. The evolutionary trees were drawn using the neighbor-joining method, with 1000 bootstrap replications. The genome sequence of the reference strain was obtained from the Global Initiative on Sharing Avian Influenza Data (GISAID) database.

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Not applicable.

Authors' contributions

L.Z. contributed to the study design, virus detection, data analysis, and writing. C.Z., J.Z., J.W., and L.T. mainly collected the samples and analyzed the data. L.X., X.L., J.M., X.G., B.Z., and P.Z. mainly completed the detection work and analysis. J.L. participated in the data analysis. X.L. guided the study design and overall revision of the manuscript. All authors have read and approved the final manuscript.

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Data availability

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

Declarations

Ethics approval and consent to participate

This study was granted an exemption from requiring ethics approval by the Ethics Committee of Tongzhou District Center for Disease Control and Prevention. All participants were informed and verbally consented to participate in the research. There were no cases involving children under 18.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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