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SHORT COMMUNICATION

The Genetic Analysis of Broiler-origin H9N2 Influenza Virus with Internal Genes Highly Homologous to the Recent Human H3N8 Virus

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Abstract: The most prevalent subtype of avian influenza worldwide, H9N2, not only threatens human health and causes enormous financial losses in the poultry sector but also has the potential to be transmitted directly or indirectly between other species. This study discovered four H9 subtype avian influenza virus in Fujian in 2022. According to genetic analysis, these four viruses contain several binding sites for mammalian receptors (216 L and 217 M in HA gene). More crucially, the internal genes of the two isolates of XD-L2 and XD-L4 were closely related to those of the human H3N8 virus found this year, which could pose a threat to human health, especially that of poultry producers.

Keywords: *Cross-species transmission, Genetic analysis, H9N2 subtype avian influenza virus, Receptor binding sites*

İnsanlarda Son Dönem Saptanan H3N8 Virüsü İle Yüksek Homologluk Gösteren İnternal Genlere Sahip Broyler Orijinli H9N2 İnfluenza Virüsünün Genetik Analizi

Öz: Dünya genelinde en yaygın kuş gribi alt türü olan H9N2, yalnızca insan sağlığını tehdit etmekle ve kümes hayvancılığı sektöründe büyük mali kayıplara neden olmakla kalmaz, aynı zamanda diğer türler arasında doğrudan veya dolaylı olarak bulaşma potansiyeline de sahiptir. Bu çalışmada, 2022 yılında Fujian'da dört adet H9 alt tipli kuş gribi virüsü saptandı. Genetik araştırmalara göre, bu dört virus, memeli reseptörleri için çeşitli bağlanma bölgeleri içermekteydi (HA geninde 216 L ve 217 M). Daha da önemlisi, XD-L2 ve XD-L4 adlı iki izolatın internal genleri, bu yıl insanlarda saptanan H3N8 virüsünün genlerine çok benziyordu ve bu da insan sağlığı, özellikle de kümes hayvanı üreticileri için bir tehdit oluşturabilir.

Anahtar sözcükler: *Türler arası bulaş, Genetik analiz, H9N2 alt tipli avian influenza virüsü, Reseptör bağlanma bölgeleri*

INTRODUCTION

Except for H5 and H7 subtypes, H9N2 subtype of avian influenza virus (AIV) is considered one of the three major avian influenza (AIV) subtypes that severely endanger public health and poultry industry [1,2]. The single infection of H9 subtype AIV is usually of low pathogenicity to poultry [1], but it can cause immune suppression in poultry [1,3,4], cause secondary infection of pathogens such as infectious bronchitis virus, mycoplasma [3,5], and greatly increase the mortality of poultry. Hemagglutinin (HA) glycoprotein is an important determinant of influenza

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virus pathogenicity, but it is not the only determinant [4]. Whether there is neck deletion of the neuraminidase protein (NA) of AIV, changes in some key loci of its internal genes NS1 (nonstructural protein 1)-149, PA (polymerase acidic protein)-70, PB2 (polymerase basic protein 2)-627, and PB2-701). will affect the pathogenicity of virus [6,7].

Since 1998 [8], a few cases of human H9N2 infection have been documented in China. Research has depicted that H9N2 subtype AIV can cross the species barrier and infect people directly through human-poultry contact without needing an intermediate host [9]. To develop the ability to infect humans, H9N2 AIV can also donate part or even all internal genes and reassort with subtypes such as H5N6 and H7N9 [10,11]. The internal genes of human H3N8 subtype AIV found in the Chinese provinces of Hunan and Henan in 2022 were all derived from H9N2 subtype AIV [12]. Due to their frequent contact with chickens, ducks, and geese, poultry workers are considered to have a significant risk of infecting AIVs; approximately 2.3% of poultry workers have antibodies against H9N2 AIV [13].

The Chinese poultry business is well-established in Fujian. To understand whether the local epidemic H9N2 AIVs can spread across species and threaten the health of local population, four H9N2 subtype AIVs named A/Chicken/Fujian/XD-L1/2022 (H9N2) (XD-L1), A/Chicken/Fujian/XD-L2/2022 (H9N2) (XD-L2), A/Chicken/Fujian/XD-L3/2022 (H9N2) (XD-L3), and A/Chicken/Fujian/XD-L4/2022 (H9N2) (XD-L4) were isolated and identified in the H9N2 positive broiler samples from Fujian province.

Material and Methods

Source of the Virus

The test samples were collected from the farms in Fujian province in January 2022 and the H9N2 viruses isolated in this laboratory.

Genome-Wide Amplification of AIVs

By taking 200 μL of chicken embryo allantoic fluid containing virus, AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, China) was applied, and total viral RNA was extracted according to the manufacturer's instructions. A 50 μL volume of PCR reaction solution was prepared using the Super RT one-step RT-PCR kit (CWBIO, China) for PCR amplification according to the universal primers designed by Hoffmann et al.[14]. PCR product was subjected to 1% agarose gel electrophoresis, and the target band was purified using AxyPrep DNA Gel Extraction Kit (Axygen, China).

Cloning of PCR Products

A 20 μL system was prepared by adding 0.5 μL of pMDTM18-T Vector (TAKARA, China), 9.5 μL of purified DNA, and 10 μL of Solution I to a microcentrifuge tube. The mixture was then incubated at 16°C for 2 h.

According to the instructions of pMDTM18-T Vector Cloning Kit, the linked products were transformed into DH5α competent cells (TIANGEN, China) and coated in LB solid medium containing ampicillin for overnight culturing. A single colony was selected and inoculated into an LB liquid medium for culture. After 6 h, the liquid was identified by PCR.

Statistical Analysis

The bacteria containing the positive plasmids were sent to Tsingke Biotechnology Co., Ltd. for sequencing, and the sequencing results were spliced using SeqMan program in DNAstar software package. In NCBI database *(https://www.ncbi.nlm.nih.gov/),* to download the reference sequence, a phylogenetic tree was constructed using MAGA7 software. MegAlign program in DNAstar package was used for translation and sequence identity analysis.

Results

All isolates were members of h9.4.2.5 branch, according to genetic analysis of the HA genes *(Fig. 1).* The nucleotide sequence identity of isolates was less than 89%, and the amino acid sequence identity was less than 92% with domestic poultry vaccine strains A/chicken/Guangdong/SS/94 (H9N2) (SS), A/chicken/Shandong/6/96 (H9N2) (SD6/96), and A/chicken/Shanghai/F/98 (H9N2) (F/98) *(Table 1),* suggesting that the three vaccine strains may be unable to provide good protection against circulating H9N2 AIVs. Notably, HA of the isolates and mammalian strains share a higher nucleotide sequence identity. In particular, the HA nucleotide sequence identity of XD-L3 isolate and H9N2 subtype swine influenza virus A/swine/Shandong/TA009/2019 (H9N2) (TA009) was 96.6%, and the amino acid sequence identity was 97.5%. This is consistent with the sequence properties of the amino acids of low-pathogenic avian influenza because the cleavage sites of HA genes of XD-L1, XD-L2, XD-L3, and XD-L4 were all PSRSSRGLF, which did not contain multiple continuous basic amino acids [1]. All isolates carried Q216L and Q217M mutations, both of which potentially improve the ability to bind to mammalian receptors [6,15]. The four isolates also carried D148N mutation, which could improve virulence in mice and chickens [5], and N188T mutation, which boosts the virus replication and transmission in ferrets [5] (H9 mature HA numbering used throughout). All NA genes of isolates were closely related to those of avian-origin H9N2 AIV, according to the results of genetic evolution analysis. All four viruses were found to be 274H, 275V, and 294N in NA gene,

Table 1. *Nucleotide and amino acid sequence identities of HA gene*

Virus	Nucleotide and Amino Acid Sequence Identities of HA Gene (%)											
	XD-L1	XD-L2	XD-L3	XD-L4	BJ1/17	BJ1/16	MZ058	201501	TA009	SS	SD6/96	F/98
XD-L1	-	96.2	91.3	95.7	93.5	93.2	94.7	94.9	92.0	87.5	87.3	86.6
XD-L2	96.4	-	91.4	96.7	93.4	93.4	94.4	94.7	91.9	87.6	87.3	86.6
XD-L3	92.3	92.5	-	91.0	93.9	92.9	92.9	93.5	96.6	88.7	88.1	87.9
XD-L4	96.3	96.6	92.0	-	92.7	92.4	93.7	94.1	91.7	87.2	87.0	86.1
BJ1/17	94.1	94.7	93.8	93.6	-	98.8	95.5	96.0	93.7	89.2	89.0	88.5
BJ1/16	93.9	94.5	93.2	93.4	99.3	-	95.2	95.7	92.7	88.6	88.3	87.9
MZ058	95.2	95.0	93.9	94.1	96.3	96.4	-	98.3	93.6	89.3	88.9	88.1
201501	95.4	95.2	94.5	94.3	96.6	96.8	98.4	-	94.0	89.8	89.3	88.7
TA009	92.9	93.0	97.5	92.7	94.3	93.8	95.0	95.2	-	89.4	89.1	88.7
SS	88.9	88.8	91.6	88.6	90.4	90.6	90.7	91.3	91.8	-	97.9	96.5
SD6/96	88.6	88.6	90.9	88.2	90.4	90.6	90.6	90.7	91.1	97.0	-	96.4
F/98	88.8	88.8	90.7	88.6	90.0	90.2	90.0	90.6	91.1	96.6	95.4	-

The upper right corner is nucleotide sequence identities, and the lower left corner is amino acid sequence identities.
Reference strains: BJ1/17: A/Beijing/1/2017 (H9N2), BJ1/16: A/Beijing/1/2016 (H9N2), MZ058: A/Guangdong/MZ058/2016 (H9N2), 201501: A/Zhongshan/201501/2015 (H9N2), TA009: A/swine/Shandong/TA009/2019 (H9N2), SS: A/chicken/Guangdong/SS/94 (H9N2), SD6/96: A/chicken/Shandong/6/96 (H9N2), F/98: A/chicken/Shanghai/F/98 (H9N2)

suggesting that these viruses are sensitive to oseltamivir and enhance their binding ability to mammalian receptors (N2 numbering) [6,16].

Following internal gene analysis, it was found that all isolates had the mutations F103L and M106I in the NS1, which could increase the virulence of AIV in mammals and its capacity to replicate more readily [17]. The matrix protein (M) has 31N, which is amantadine resistant [18]. The three amino acids 70V, 224S, and 400P found in PA may increase the pathogenicity of mammal viruses [6]. The 588V mutation in PB2 increases its pathogenicity in mammals [6]. The fact that the isolates of XD-L2 and XD-L4 clustered closely into the same evolutionary branch as the recent H3N8 human AIV discovered this year raises particular concern based on the genetic evolution analysis of internal genes *(Fig. 1)*. The nucleotide sequence identities of internal genes were analyzed for the isolates, and the recent H3N8 viruses A/Changsha/1000/2022 (H3N8) (1000) and A/Henan/4-10/2022 (H3N8) (4-10) *(Table 2)*. The nucleotide sequence identities of M, nucleoprotein (NP), NS, PA, polymerase basic protein 1 (PB1), and PB2 between XD-L2/XD-L4 and A/Henan/4-10/2022 (H3N8) are 98.5%/98.6%, 98.4%/97.7%, 97.7%/98.1%, 96.8%/96.9%, 98.1%/97.3%, and 97.6%/96.9%, respectively. The amino acid sequence identities of M1, NP, NS1, PA, PB1, and PB2 of XD-L2/XD-L4 and A/Henan/4-10/2022 (H3N8) are 98.8%/99.6%, 100%/99.8%, 97.2%/96.8%, 99.4%/99.0%, 98.9%/98.7%, and 98.7%/98.7%. We must focus on the fact that all isolates contain amino acids of 363 K in HA and 672 L in PA, which play an important role in facilitating airborne transmission of the viruses [19].

Discussion

China is considered as the epicenter of the pandemic influenza virus and is an area where different AIVs co-circulate [20]. The first reported human infection with H9N2 virus in China was in 1998, and a complete genome sequence analysis of the isolates indicated that these human isolates probably originated from local chicken flocks [21]. Human infection with H9N2 virus occurred in Hong Kong in December 2003, in which all eight gene fragments came from poultry [1]. In 2016, there was even a case in which an H9N2 virus infection killed a patient with an underlying disease [1]. This suggests that surveillance of H9N2 virus in poultry should be strengthened to prevent the risk of H9N2 virus infection in humans.

In this study, we isolated four H9N2 subtype low pathogenic AIV from poultry farms in Fujian province. The HA gene sequence analysis demonstrated that the amino acid identity between the isolates and the known vaccine strains (three strains) was less than 92%, suggesting that these vaccine strains could not provide complete protection to the isolates. The results of the genetic study of isolates revealed that each gene has numerous mutations, some of which could enhance the pathogenicity of the virus (e.g., HA-148N, NS-149A, PA-70V, PA-224S, PA-400P) [5,6] and others could enhance its capacity to bind to mammalian receptors (HA-180E/V, HA-216L, HA-217M, NA-275V, and so on) [5-7,15]. N (XD-L1/XD-L3) and S (XD-L2/XD-L4) at position of 375 in PB1 gene plays a key role in adaptation and virulence in mammals [22]. The analysis of key loci of M and NA genes indicated that the isolated strains in this study may

Table 2. *Nucleotide and amino acid sequence identities of the internal genes*

Virus	Nucleotide and Amino Acid Sequence Identities of the Internal Genes (%)											
	M[a]						NP					
	XD-L1	XD-L2	XD-L3	XD-L4	1000	4-10	XD-L1	XD-L2	XD-L3	XD-L4	1000	4-10
XD-L1	-	97.1	95.4	97.8	95.4	97.6	-	95.1	94.8	95.3	96.0	95.3
XD-L2	97.6	-	95.9	98.9	95.4	98.5	99.8	-	95.1	97.5	94.7	98.4
XD-L3	.98.0	99.2	-	95.6	94.8	95.4	99.4	99.6	-	95.1	94.6	95.3
XD-L4	98.4	98.8	99.2	-	96.0	98.6	99.6	99.8	99.4	-	94.6	97.7
1000	98.4	98.4	98.8	99.2	-	95.7	99.4	99.2	99.2	99.0	-	94.8
4-10	98.4	98.8	99.2	99.6	99.2	-	99.8	100	99.6	99.8	99.2	-
	NS[b]						PA					
	XD-L1	XD-L2	XD-L3	XD-L4	1000	4-10	XD-L1	XD-L2	XD-L3	XD-L4	1000	4-10
XD-L1	-	97.0	96.9	97.4	96.4	97.4	-	94.6	95.0	94.7	94.0	94.8
XD-L2	98.2	-	95.6	97.9	97.9	97.7	99.2	-	96.8	96.7	93.8	96.8
XD-L3	97.7	97.2	-	96.2	95.3	96.2	99.0	98.9	-	96.7	94.3	96.8
XD-L4	98.2	98.2	96.8	-	97.4	98.1	98.7	98.6	98.5	-	94.0	96.9
1000	96.8	97.7	96.3	96.8	-	96.9	99.0	98.9	98.7	98.5	-	94.1
4-10	96.8	97.2	95.9	96.8	95.9	-	99.6	99.4	99.3	99.0	99.3	-
	PB1						PB2					
	XD-L1	XD-L2	XD-L3	XD-L4	1000	4-10	XD-L1	XD-L2	XD-L3	XD-L4	1000	4-10
XD-L1	-	94.0	93.6	93.7	93.6	93.8	-	97.4	95.7	96.2	94.2	96.2
XD-L2	98.3	-	92.5	97.6	92.2	98.1	98.7	-	94.1	97.7	94.5	97.6
XD-L3	98.0	97.5	-	92.7	94.8	92.9	97.9	97.5	-	93.7	93.3	93.9
XD-L4	98.0	98.7	97.2	-	91.9	97.3	98.6	98.6	97.5	-	93.7	96.9
1000	98.3	98.0	98.3	98.0	-	92.3	98.2	98.6	97.5	98.4	-	94.1
4-10	98.4	98.9	97.8	98.7	98.2	-	98.7	98.7	97.6	98.7	98.3	-

The upper right corner is nucleotide sequence identities, and the lower left corner is amino acid sequence identities.
[a] *The upper right corner is the nucleotide sequence identities of M gene, the lower left corner is the amino acid sequence identities of M1 protein.* [b] *The upper right corner is the nucleotide sequence identities of NS gene, the lower left corner is the amino acid sequence identities of NS1 protein.*
Reference strains: 1000: A/Changsha/1000/2022 (H3N8), 4-10: A/Henan/4-10/2022 (H3N8)

be amantadine-resistant but sensitive to oseltamivir. Notably, the isolates contain amino acids HA-K363 and PA-L672 [23], which are important for airborne transmission of the virus and may make the isolates more easily transmissible. The isolates XD-L2 and XD-L4 were found to be highly homologous to the most recent human H3N8 isolate based on genetic evolution analysis of their internal genes. This finding suggests that these two isolates may infect humans.

In conclusion, four H9N2 subtypes of low pathogenic AIVs were isolated from the Chinese province of Fujian in 2022. The isolates have mutations that increase virulence and enhance mammalian receptor binding and may pose a threat to humans. The concept that calls for preventing infectious diseases in animals to come before those in people is crucial for preventing and controlling zoonosis. Strengthening surveillance, prevention, and control of H9N2 subtype AIV is crucial, given the widespread prevalence of the virus in poultry in China.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (C.G. Liu) upon reasonable request.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Author Contributions

P.L., J.Y., and C.L. planned and designed the research. P.L., H.Z., and X.W. conducted the experiments. H.S., S.C., and L.M. analyzed the data. P.L. wrote the manuscript. T.Y., S.Z., Z.L., and C.L. revised the manuscript.

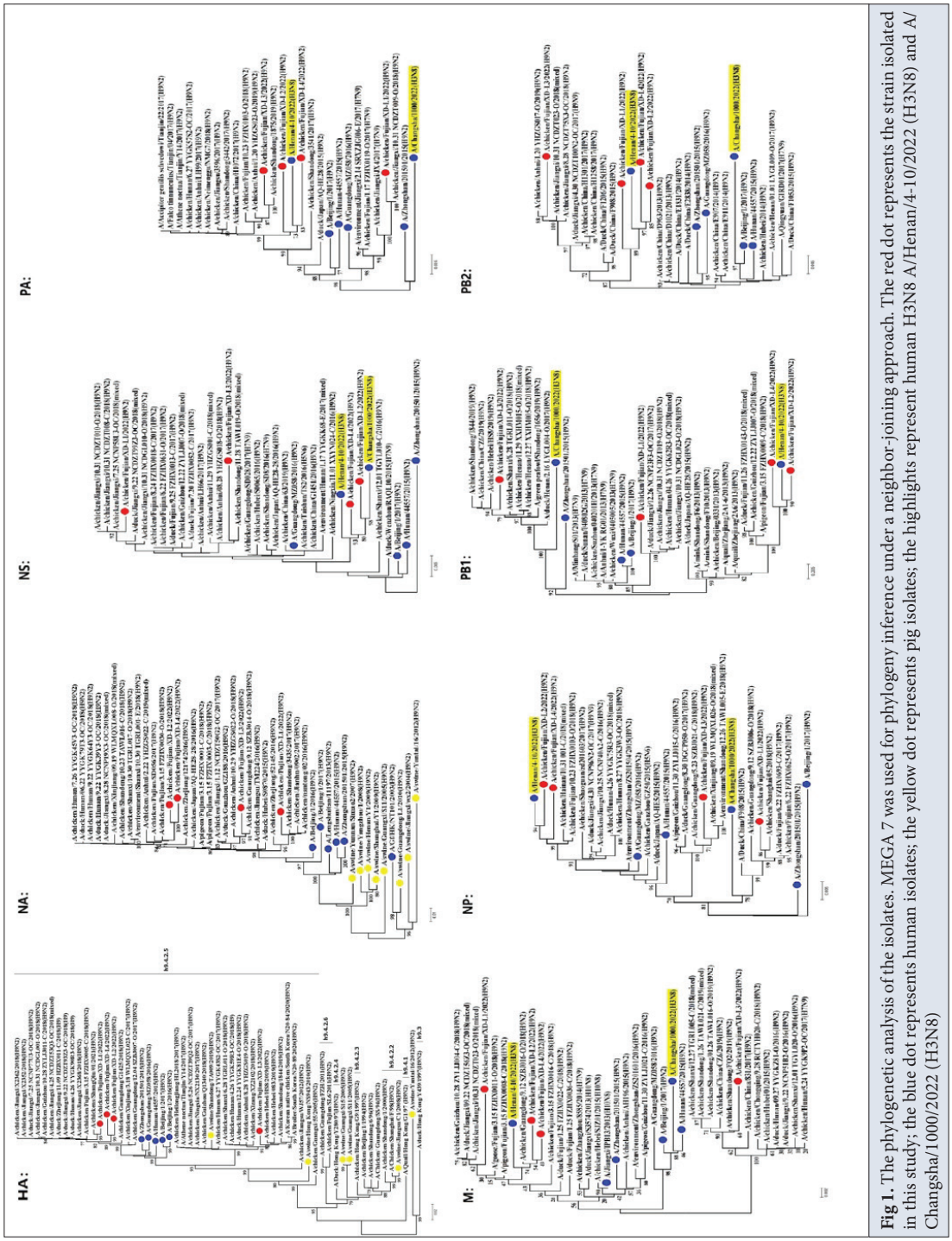

Fig 1. The phylogenetic analysis of the isolates. MEGA 7 was used for phylogeny inference under a neighbor-joining approach. The red dot represents the strain isolated in this study; the blue dot represents human isolates; the yellow dot represents pig isolates; the highlights represent human H3N8 A/Henan/4-10/2022 (H3N8) and A/Changsha/1000/2022 (H3N8)

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