Molecular Characteristics of H9N2 Avian Influenza Viruses in Live Poultry Markets in Anhui Province, China, 2013–2018

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Abstract

Objective In China, H9N2 avian influenza viruses were first reported in chickens in Guangdong province in the mid-1990s. Subsequently, the viruses spread to most regions nationwide, where they became panzootic and endemic. Based on environmental surveillance of live poultry markets in Anhui province from 2013 to 2018, a total of 33 representative environmental isolates were selected and studied systematically. Methods The genomic RNA of Anhui H9N2 isolates was subjected to RT-PCR amplification followed by sequencing analysis. Results Thirty-three strains were isolated from the embryonated eggs of specific-pathogen-free chickens. Phylogenetic analysis indicated that h9.4.2.5-like H9N2 viruses were predominant during 2013–2018 and acquired multiple specific amino acid mutations that may have increased their affinity for mammals and enhanced their infectivity and transmissibility. Additionally, six internal genes of H9N2 clustered together with the novel human-lethal reassortant viruses, such as low pathogenicity H7N9, H10N8 and Anhui H5N6 viruses, and even HPAI H7N9. Conclusion Because H9N2 viruses may be the donors of internal genes that lead to the generation of novel reassortant viruses with enhanced pathogenicity in Anhui province, continuous environmental surveillance of live poultry markets, a key source of reassorted H9N2 and other avian influenza viruses, is of great importance.

INTRODUCTION

Avian influenza viruses (AIVs) can be separated into two broad categories: highly pathogenic avian influenza viruses (HPAIV) and low pathogenic avian influenza viruses (LPAIV), based on their pathogenicity in chickens and molecular characterization of the hemagglutinin (HA) protein. H9N2 AIVs, the subject of this study, have become endemic in poultry across much of Asia, the Middle East, and West and North Africa[1,2]. In the mid-1990s, H9N2 AIV was first reported in chickens in Guangdong province of China, and subsequently became epidemic nationwide[3]. At present, the poultry-adapted H9N2 AIVs are not only a major threat to poultry health, but also human health, as some of the H9N2 lineage AIVs are zoonotic[1,4,5].

To prevent H9N2 AIV infection in chickens, over 20 different commercial vaccines are used in China, and these vaccines are frequently updated[3]. Unfortunately, H9N2 AIVs continue to persist in chickens, even in vaccinated chicken flocks, which is possibly due to antigenic drift[6-8]. Owing to the potential pressure of antigenic drift in poultry-adapted H9N2 AIVs, a large number of variant viruses evade the host's neutralizing antibodies.

Not only does antigenic drift lead to the emergence of variant viruses, but also the exchange of gene segments among different virus particles via gene reassortment generates a large number of novel viral descendants[9].

H9N2 AIVs are often found co-circulating in poultry with other AIV subtypes, such as H5 and H7 HPAIVs[10]. Like other AIVs, H9N2 is prone to gene reassortment that can affect host specificity, virulence and pathogenicity. Live poultry are host to co-circulating subtypes of AIVs, while live poultry trading may influence the transmission of AIVs[11]. Live poultry markets (LPMs), which are a key link in live poultry trading, require close monitoring. H9N2 AIVs may act as donors of genes to other AIVs, such as H5N1, H5N6, H7N9 and H10N8, which are responsible for high mortality rates in humans[1,12-14]. Based on this information, it was crucial to investigate the molecular characteristics and genetic reassortment of H9N2 AIVs isolated from LPMs to assess whether these viruses possess the ability to infect and transmit to a new host.

MATERIALS AND METHODS

Sample Collection

Between 2013 and 2018, AIV surveillance according to the China CDC guidelines was conducted in Anhui province. Environmental samples from LPMs were collected, which included poultry drinking water, feces, wipes from the surfaces of cages and boards used for the slaughtering of birds. These samples were stored in a viral transport medium composed of 15 μg/mL amphotericin B, 100 units/mL penicillin G, 50 μl/mL streptomycin and 1% bovine serum albumin in Hank's balanced salt solution (pH 7.4). Under refrigeration, these sample were transferred to the Laboratory of Influenza Surveillance immediately, where they were stored prior to further examination.

Virus Isolation and Identification

Viruses were propagated in specific-pathogen-free (SPF) chick embryonated eggs, then these eggs were incubated for 72–96 h at 37°C. The collected allantoic fluids were subjected to a hemagglutination assay with 1% turkey red blood cells (TRBCs). The HA-positive samples were further subtyped by real time RT-PCR using 16 sets of HA (H1–H16) primers and nine sets of NA (N1–N9) primers designed by the Chinese National Influenza Center (CNIC).

Viral RNA Extraction and RT-PCR

The HA-positive allantoic fluid was used for viral RNA extraction using a High Pure Viral Nucleic Acid Kit according to the manufacturer's instructions (Roche, Mannheim, Germany). Reverse transcription was performed with the Uni12 primer (5'-AGCAAAAGCAGG-3') and the RevertAid First-Strand cDNA Synthesis Kit (Fermentas, Canada). cDNAs of the eight segments were then amplified with gene-specific primers designed by the CNIC. All experiments were conducted in accordance with a previous published study[15].

Genome Sequencing and Phylogenetic Analysis

The PCR products were purified with a Wizard DNA Clean-Up System (Promega), then sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (AB) and analyzed with an ABI 3730XL DNA Analyzer. The BioEdit program (version 7.1.3.0) and ClustalW alignment algorithm (version 1.83) were initially used to compare and align nucleotide sequences. Phylogenetic trees were inferred using the neighbor-joining method with MEGA software (version 7.0.26), and bootstrap values were tested with 1000 replications[16]. The evolutionary distances were computed using the Maximum Composite Likelihood method and the units represent the number of base substitutions per site.

Molecular Analyses

The representative sequences used in this study for phylogenetic comparisons and molecular analyses were obtained from the Global Initiative on Sharing Avian Influenza Data (www.gisaid.org) and the GenBank database (www.ncbi.nlm.nih.gov). The Influenza Virus Sequence Annotation Tool (https://www.ncbi.nlm.nih.gov/genomes/FLU/annotation/) was used to predict protein sequences encoded by the input sequences. The potential N-glycosylation sites were predicted using the NetNGlyc 1.0 web server (http://www.cbs.dtu.dk/services/NetNGlyc/). By default, predictions were carried out only on the Asn-Xaa-Ser/Thr sequons.

RESULTS

Virus Isolation and Identification

Between 2013 and 2018, a total of 5110 environmental samples were collected from the sampling sites for poultry-related environmental surveillance in Anhui province. Analysis revealed that 582 environmental samples were positive for the H9N2 AIVs. From these samples, we obtained 33 strains using SPF chick embryonated eggs (the H9N2 isolation rate was 5.67%, Table S1). To further understand the molecular evolution of the H9N2 strains in different geographical locations, we selected all isolates from eight cities in Anhui province to construct a genetic evolutionary tree, including 16 isolates from Hefei, six isolates from Anqing, three isolates from Huaibei, two from Fuyang, two from Huainan, two from Maanshan, one from Chuzhou and one from Bengbu (Figure 1).

Table S1 . Basic information for the H9N2 avian influenza viruses isolated from the environmental samples in Anhui province from 2013 to 2018

Phylogenetic Analyses of the HA and NA genes

To better understand the genetic relationships between the H9N2 viruses in Anhui province, we sequenced the complete genomes of 33 representative strains. The genome sequences all shared high sequence similarity at the nucleotide level. For example, the HA and neuraminidase (NA) genes shared nucleotide identities of 93.2% to 96.4% and 93.3% to 99.3%, respectively.

As described in a previous report[17], four stem evolutionary clades of h9.1–h9.4 have been designated to map the HA gene phylogeny. All of the HA genes of the viruses in this study belonged to clade 9.4.2.5 represented by A/chicken/Guangxi/55/2005(H9N2), which differed from the A/quail/Hong Kong/G1/1997(H9N2)-like (G1-like, h9.4.1.1) and A/duck/Hong Kong/Y280/1997(H9N2) (Y280-like, H9.4.2.4) viruses prevailing in most Asian countries since 1994 (Figure 2A). The NA genes of all of the H9N2 viruses in this study belonged to the A/chicken/Beijing/1/1994(H9N2)-like (BJ/94-like) viruses and formed four branches together with the G1-like, G9-like and Y28-like viruses. All of the NA genes were closely clustered together with those of the Y280-like viruses, forming one group (Figure 2B).

Phylogenetic Analyses of the Internal Genes

The other six internal genes (polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), nucleocapsid protein (NP), matrix protein (MP) and non-structural protein (NS)) shared 94.4%–99.6%, 95%–98.7%, 96.1%–98%, 94%–99%, 96.6%–98.8% and 94.6%–98.9% nucleotide identity, respectively.

The topological structures of the phylogenetic trees of MP and PB2 were highly similar (Figure 2C and 2H). All of the MP and PB2 genes from the strains analyzed in this study belonged to the G1-like lineage, and clustered into two phylogenetic subgroups. The phylogenetic tree revealed that the PA, PB1, NP and NS genes of all of the strains clustered together with the A/chicken/Shanghai/F/98-like (F/98-like) viruses (Figure 2F, 2G, 2D and 2E). Taken together, these findings indicated that the six internal genes of the H9N2 viruses isolated in Anhui province from 2013 to 2016 had undergone dramatic evolution and reassortment.

To understand the relationship between these internal genes and some novel AIV infections in humans in recent years, we added the internal gene sequences of the early H7N9 (LP and HP), H5N6, H10N8 and H7N4 AIVs into the phylogenetic tree. Surprisingly, all of the internal genes of the early H7N9 and H10N8 AIVs, even HPAI H7N9, clustered together in one group with the strains analyzed in this study. Intriguingly, the early H5N6 AIVs from Guangdong, Changsha and Sichuan did not form one branch with these strains, but did cluster into one group with Anhui H5N6 AIVs. A similar phenomenon occurred for H7N4 AIVs, with only their PB2 genes belonging to the G1-like lineage forming a different subgroup.

Molecular Characterization

All of the HA genes contained 1742 nucleotides and most of the H9N2 strains possessed a conserved amino acid

sequence motif of*PSRSSR—GLF* at the cleavage site, except for EnAH 04713/18 (*PSKSSR—GLF* motif), which was a typical feature of LPAIVs. We also examined the amino acid substitutions at the receptor-binding site (RBS) of the HA protein, especially 183N and 190T. Among the 33 strains in this study, 29 isolates possessed an A190T mutation, whereas all isolates contained the H183N mutation. The amino acids at positions 226–228 were "LMG" in all strains. The potential N-linked glycosylation sites (PNLGSs) in the HA proteins of the 33 isolates were predicted by the NetNGlyc 1.0 web server. The results showed that positions 29, 141, 298, 305, 313, 492 and 551 were conserved. Non-conserved PNLGSs at positions 145 and 218 were located in the HA protein globular domain. Only EnAH09183/14 virus possessed a PNLGS at position 145, whereas the EnAH 08826/13, EnAH 08829/13, EnAH 09189/14 and EnAH 09180/14 viruses possessed a PNLGS at position 218. The seven PNLGSs in the H9N2 viruses in this study appeared to be relatively stable between 2015 and 2018 (Table 1).

Table 1. Molecular characteristics of the HA amino acid sequences in H9N2 AIVs in this study Dashes (-) indicate the absence of a potential glycosylation site

The NA gene from EnAH08829/13 virus did not cluster into one branch with other viruses in this study. The hemadsorbing sites (HBSs) of EnAH08829/13 virus contained "IRNGSRSG" and "DSENW" motifs at positions 366–373 and 399–403, whereas "PQE" was observed in position 431–433 in all of the strains. With the exception of EnAH08829/13, the other viruses in this study possessed the "IKNGSRSG" motif at position 366–373. Regarding the active center, the 33 strains possessed six different patterns, with mutations mainly evident at positions 141, 143, 149 and 155. All of the isolates in this study possessed an amino acid deletion in the stalk region, unlike viruses belonging to the G9-like lineage (Table 2).

Table 2. Molecular characteristics of the NA amino acid sequences in the H9N2 AIVs analyzed in this study

Some critical residues in the internal genes were associated with pathogenicity and infectivity, for example 627E and 701D in PB2 were characteristic of LPAIVs and were observed in 27 of the 33 strains in this study. Residues 100A, 356R and 409N in PA were human adaptation-related markers that were observed in 3 of the 33 strains. All of the strains possessed the I368V mutation in PB1 protein, but the H99Y substitution was not detected in any of the strains. The 588I and 591K mutations in PB2 were also not detected in these strains. Residues 95K, 224N and 242N were found in the M1 protein of 31 strains, and 37A was observed in this protein in all isolates. Residue 21G was detected in the M2 protein of 26 strains, all of which showed increased infectivity. The NS1-42S mutation was observed in all of the strains analyzed in this study, but 92E was only found in EnAH 19204/15 virus. The S31N mutation responsible for amantadine resistance was found in the M2 protein of all H9N2 isolates, but substitutions in the NA protein related to antiviral resistance to neuraminidase inhibitors were not detected (Table 3).

Table 3. Molecular characteristics of the internal amino acid sequences

in the H9N2 AIVs analyzed in this study

DISCUSSION

Though investigation of the prevalence of H9N2 virus in lower and middle income countries, either by poultry sero-surveys or by passive sampling, the virus was observed to be present at particularly high rates, especially in LPMs[10]. LPMs are not only a hub for poultry trading, but are also a major component of the disease transmission pathway[18], contributing to the spread of AIVs among poultry as well as facilitating zoonotic infections. The purpose of this study was to understand the evolution and molecular features of the H9N2 viruses, which were constantly circulating in LPMs in Anhui province between 2013 and 2018.

Phylogenetic analysis of the surface HA genes indicated that the lineage h9.4.2.5 was predominant in 2013–2018 in Anhui province. Although environmental strains belonging to h9.4.2.6 were not identified in this study, two strains isolated from chickens clustered into the 9.4.2.6 lineage, which is well established in southern China. The frequent live poultry trade aids the co-circulation of h9.4.2.5 and h9.4.2.6 viruses in Anhui. G1-like PB2 and M genes combined with the genetic backbone of F/98-like viruses was the predominant form of the six internal genes in the strains analyzed in this study. H9N2 subtype AIVs in

China have evolved into diversified clusters and genotypes, according to previous research[9], and viruses isolated from the environment in this study were designated as genotype S (generally equivalent to genotype G57). The genotype S H9N2 AIVs were mainly prevalent in the Yangtze River Delta region in eastern China, and Anhui province is located within this area. Evidence has been reported that genotype S exhibits greater infectivity than the other genotypes, and has been predominant since 2010 across China, causing a significant threat to the poultry industry[2].

To control and eradicate both the H7N9 low and highly pathogenic viruses, the H5/H7 bivalent inactivated vaccine was used to protect chickens and ducks from infection. This intervention decreased the prevalence of H7N9 viruses in poultry and humans, confirming that the vaccination of poultry played an important role in preventing the spread of H7N9 virus. However, from our research, it is clear that all internal genes of the early H7N9, H5N6(Anhui) and H10N8 AIVs, including HPAI H7N9, were clustered into one group together with the strains analyzed in the present study. These strains isolated from the environment originated from LPMs in Anhui province, where HPAI H7N9 and H10N8 outbreaks have not occurred. H9N2 AIVs exhibited wide antigenic variability, donating internal genes to other AIVs[19]. Therefore, if immune escape H5 or H7 AIVs emerge, it is possible that highly pathogenic H7N9 or H5Nx AIVs may reappear. Moreover, high genetic compatibility was found between H9N2 and H1N1pdm(2009) viruses, and reassortment with H3N2 is possible, potentially leading to the gain of respiratory transmissibility[3]. Our study showed that the absence or presence of PNLGSs and key amino acid mutations in RBSs, such as Q226L, may affect receptor affinity[20]. The N166D mutation in the HA that appeared in the strains isolated in 2018 in this study, may contribute to the failure of H9N2 vaccination in the field[21]. In this study, the 95K, 224N and 242N residues in the M1 protein and 21G in the M2 protein may all increase AIV infectivity[22]. Taken together, our findings indicate that most viral isolates from the environment in Anhui province possessed the ability to increase the affinity for mammals, and enhance infectivity and transmission.

In summary, we systematically analyzed H9N2 environmental isolates from Anhui province from 2013 to 2018. Phylogenetic analysis indicated that h9.4.2.5-like H9N2 viruses were predominant during 2013–2018. Additionally, these viruses had undergone dramatic reassortment and evolution, resulting in wide ranging genetic diversity. The acquired characteristics of increased affinity for mammals and enhanced infectivity and transmission among viral isolates are worthy of close attention.

supplement

Table S1. Basic information of H9N2 avian influenza viruses isolated from environmental samples in Anhui province from 2013 to 2018

Table 1. Molecular characteristics of HA amino acid sequences in H9N2 AIVs of this study

Table2. Molecular characteristics of NA amino acid sequences in H9N2 AIVs of this study

Table3. Molecular characteristics of internal amino acid sequences in H9N2 AIVs of this study

Figure 1. Geographical locations of H9N2 AIVs isolates in Anhui province.

Figure 2. Phylogenetic trees of HA (A), NA(B), MP(C), NP(D), NS(E), PA(F), PB1(G), PB2(H) genes of H9N2 subtype AIVs.

Full-length sequences with complete open reading frames were used for the phylogenetic analyses and neighbor-joining (NJ) trees were generated using MEGA 7.0.26. Estimates of the phylogenies were calculated by performing 1000 neighbor-joining bootstrap replicates. The phylogenetic trees of the genes were rooted to A/turkey/Wisconsin/1966 (A to H). The novel AIVs infected human like H7N9, H10N8, H5N6 and H7N4 were shown in light green and the novel HPAI H7N9 AIVs were shown in red. Our 33 isolates from environment samples in Anhui province between 2013 and 2018 were highlighted in pink. The bootstrap values[?]70 were shown.

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