Gene Variation and Molecular Provenance Study on H9N2 Avian Influenza Virus

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Abstract. Avian Influenza (AI), a poultry infectious disease caused by type A influenza A virus, arouses worldwide attention. Although H9N2 influenza virus now widely prevalent around the world is a kind of low pathogenic AI, it can provide internal gene segment for H5N1, a highly pathogenic influenza virus. Therefore, the study, through understanding of the molecular evolution and gene variation pattern of H9N2 influenza virus, attempts to provide some theoretical basis for the prevention and treatment of infectious disease. The study acquires gene information of AI virus by adopting molecular virology and molecular cloning, analyzes the relation between crucial sites mutation of virus genes and virus properties as well as the source of virus, and builds up protein molecular structure by using bioinformatics method for presenting a more vivid picture of virus protein site mutation. The project has come up with an innovative research method of combining molecular virology and bioinformatics to interpret the variation pattern of virus.

Keywords: avian influenza, H9N2, variation, recombinant, evolution

1. Introduction

1.1. Background

In recent years, outbreak of influenza virus is witnessed worldwide, AI being included. Influenza Virus is a RNA filoviruses belonging to orthomyxoviridae [1], [2]. According to antigenicity of the influenza virus's Nucleoprotein (NP) and Matrix Protein (MP), it can be divided into A, B and C serotypes. Widely found in birds, humans and other animals, type A virus is vulnerable to antigenic variation, more easily transmitted, spread rapidly and easily pandemic. Influenza C virus is largely available to human and swine and type B to humans only. RNA of influenza A virus is composed of eight segments. Segments 1, 2 and 3 is encoding RNA polymerase, segment 4 hemagglutinin (HA), segment 5 NP, segment 6 neuraminidase (NA), segment 7 MP and segment 8 a kind of non-structural protein splicing RNA. Based on the HA and NA of virus particle surface, influenza A virus can be divided into 16 H subtypes and 9 N subtypes.

AI, depending on its pathogenicity, can be divided into highly pathogenic AI, low pathogenic AI and non-pathogenic AI [3]. Infected by low pathogenic avian influenza virus, mild respiratory and digestive tract symptoms are felt with lower mortality, while highly pathogenic AI is characterized by a serious systemic, hemorrhagic and septic symptoms and higher mortality.

The improved living standard and increased demand for poultry consumption promote the large-scale breeding industry. Meanwhile, to save costs, chickens, ducks and other poultry turn to polyculture, enhancing the recombinant of influenza virus among hosts and subtypes and accelerating the speed of influenza virus variation [4]. Chickens thus become important settlement for influenza virus after it breaks from its natural host, greatly increasing the spread and epidemicity of AI. H9N2, a low pathogenic virus, is often widely found in chickens, yet its related research is far from sufficient due to its hypovirulence [5]. It is reported that long-term latency of H9N2 is always found at the affected areas before highly pathogenic AI

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virus outbreaks there, therefore, H9N2 may provide evolution and the recombinant resources for highly pathogenic AI virus to impel its outbreak.

As long-term monitoring of poultry in Southern China reveals, since 2000, Ck/Bei-like subtype virus epidemic in chickens of southern China may be reverse transmitted back to domestic ducks, generating many new reassortant viruses [6], [7]. While according to the research findings of H9N2 subtype influenza virus prevalent in poultry of southern China from 2000 to 2005, Ck/Bei-like virus was spread from chickens to quail, duck, and other minor poultry such as pigeon, zebra finch, partridge, etc., which increases the likelihood of gene recombination and spread of influenza virus to mammals and even humans [8]-[10].

1.2. Problems

The virus found in middle school textbook is in simple structure, but it is the root of many diseases. The common cold in winter, for example, is mainly caused by influenza virus. In recent years, outbreaks of AI are repeatedly reported, and real-time epidemic monitoring was carried out in China due to H7N9 AI's lethality. All of these bring AI virus a great concern and make it awful as well. Through the study in Institute of Microbiology Chinese Academy of Sciences, it is informed that AI virus available to birds usually won't infect humans. Then how the AI virus infects humans in recent years, and what is its genetic variation and transmission path become several significant scientific problems facing AI virus research. Through efforts, we hope to address these problems by ourselves.

1.3. Content

Based on samples collected in poultry market from different regions of Hunan Province, H9N2 AI viruses are isolated and identified. Based on molecular biology techniques, gene amplification and sequencing are applied to the H9N2 AI viruses from poultry, analyzing virus vibration sites through multiple sequence alignment. Based on influenza virus molecular database, similar virus gene sequence information is retrieved, using sequence phylogenetic tree (evolutionary tree) to analyze and find related viruses. Through interpretation for the evolutionary tree of HA and NA gene segments of influenza virus, the source and genetic variation regularity of AI are traced; through homologous modeling for simulation of virus protein molecular structure, characteristics of the virus are determined via taxonomic character of important active sites.

2. Materials and Methods

Experiment plans are designed based on three principles of experimental design (randomization, control, replication), and all experiments are conducted in Institute of Microbiology CAS Key Laboratory of Pathogenic Microbiology and Immunology.

2.1. Experimental Materials

The hatched 9 to 11-day-old SPF chicken embryos are used for virus isolation and breeding. Experimental drug, reagent and instrument include 1% chicken red blood cells; RNA-extracted reagent Trizol purchased from Invitrogen; Reverse transcription kit purchased from TakaRa; reagents such as PCR related enzymes purchased from TransGen; antibiotics such as penicillin and streptomycin purchased from Sigma; sterile PBS (self-prepared), etc. Instrument were used such as chicken embryo incubator; Nucleic acid electrophoresis apparatus; UV gel imaging system; Bio-Tek PCR instrument; 4°C refrigerated high-speed centrifugator, etc.

2.2. Experimental Methods

2.2.1. Collection of pathological sample

Test papers of oropharynx and anus were collected by laboratory in poultry market from different regions of Hunan Province.

2.2.2. Virus isolation, breeding and preservation.

(1) Egg check: the hatched 9 to 11-day-old SPF chicken embryos are taken out from incubator with biological transfer box, placed in biosafety cabinet, detected with egg candler (lights in biosafety cabinet can

be turned off), and marked with the boundary of chamber and allantois and location of embryo via marker. If the chicken embryos are found to be dead, unfertilized, cracked, underdeveloped, or permeable on surface, they shall be put into high pressure sterilization bags.

(2) Sample treatment: The collected samples are melted from -70° C refrigerator, placed at 37° C for 1 hour after adding suitable amount of antibiotics, and then centrifuged at 7000rpm for 10 minutes.

(3) Inoculation of chicken embryos: chicken embryos are egg tray with chamber upward, marked, disinfected by 70%~75% alcohol wipes, punched at chamber with puncher, and made with a 10 x 6mm crack by tweezers. 3-5 chicken embryos are to be provided for each sample. Suck 100µl treated clinical sample by 1mL syringe with 16 needle; instill sterile liquid paraffin from the crack and gently shake the chicken embryo to make the liquid paraffin spread in the internal layer (visceral layer) of chicken embryo's shell membrane, during when the location of chicken embryo is clearly seen under egg candler; pierce into the allantoic cavity to inject 100µL virus solution with the needle; throw the needle into sharps container after inoculation and the injection into high pressure garbage bag; seal the chicken embryos with heat-melted paraffin.

(4) Cultivation of chicken embryos: chicken embryos are then put back to biological transfer box and transferred to 33 oC~37 oC incubator for cultivating three days. Check the growth on daily basis and abandon the chicken embryos dead within 24h (which deemed to be nonspecific death). When observing, chicken embryos will be taken out from incubator with biological transfer box to biosafety cabinet, observed with egg candler, and placed them back to incubator with biological transfer box upon completion of observation.

(5) Hemagglutination test: after three-day cultivation, the survived chicken embryos are placed at 4° C for the night. Extract the allantoic fluid of died chicken embryos within 24-72h and survived chicken embryos within 72h to undergo haemagglutination (HA) titer determination via chicken red blood cells. HA positive samples are judged to be virus positive. Virus titer record and relevant records shall be kept.

(6) Virus acquisition: chicken embryo allantoic fluid is drawn, subpackaged in microtubes, marked and preserved at -80 °C.

2.2.3. Extraction of virus nucleic acid

Virus solution cryopreserved is taken out at -80 °C, transferred to 1.5ml EP tube after it is melted at room temperature, added with RNA extraction reagent Trizol of 3 times of its volume, and placed at room temperature for 5min to enable full splitting of virus; add chloroform as per 200 µl per 1ml Trizol and put in quiescence at room temperature for 2 minutes after oscillation; after separation of oil phase and water phase through centrifugation for 15min at 12000rpm and 4oC, extract the upper water phase (without touching oil phase) to another centrifuge tube; add isopyknic isopropyl alcohol to be well mixed, and make RNA precipitation through quiescence at room temperature; after centrifugation for 15min at 12,000rpm and 4oC, discard supernatant and keep the RNA at tube bottom; add 0.5~1ml 75 % ethanol and mildly vibrate centrifuge tube to make precipitation suspended; after centrifugation for 5min at 8,000rpm and 4oC, maximize the removal of supernatant on the premise of avoiding the loss of RNA precipitation; repeat the previous step once, and then dry the precipitation to acquire virus sample RNA.

2.2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

(1) Reverse transcription: cDNA synthesis is conducted by referring to reverse transcription kit manual of Takara Biotechnology (Dalian) Co., Ltd. As for template RNA 1ng-1 μ g, 1 μ L Random Primer (25 μ M), add water up to 6 μ L and stay warm for 10min at 70°C after blending; after ice cooling for 2min, add 5xM-MLV buffer 2 μ L, dNTP mixture (10mM respectively) 0.5 μ L, RNA enzyme inhibitor (40U/ μ L) 0.25 μ L and RTase M-MLV (200U/ μ L) 0.5 μ L, and add water up to 10 μ L; then cDNA can be acquired by following the procedure of 30°C, 10 min and 42 °C, 1 h.

(2) PCR: follow 50ul PCR amplification system: rTaq (placed on ice), 0.4μ ; 10xRCR buffer, 4μ ; cDNA template, 1μ ; sense primer (Primer-s), 1μ ; reverse primer (primer-r), 1μ ; dNTPs, 4μ ; sterile H2O, 27.6 μ ; 95 °C pre-degeneration for 5min; 94 °C degeneration for 1min, annealing temperature of 58 °C for 30sec, 72 °C

extension for 1min, circulation of 36 times; 72° C extension for 10min, 4°C forever. Primer sequences are shown in Table 1.

(3) AGE (agarose gel electrophoresis): PCR products are examined by 1% AGE containing EB. 0.3g agarose added with 35ml electrophoresis buffer are heated by microwave until boiling, taken out for shaking and well mixing the liquid, heated for 5sec, taken out for shaking evenly, added with very little EB; then be poured into gel-carrying plate inserted with spotting comb, remove bubbles and take spotting comb out after the gel solidifies completely; PCR products are added with bromophenol blue upper sample and connected to 110V electrode for 25min. Amplification products are sent to biological company for sequencing.

| Gene Segment | Primer Name | Primer Sequence (5'-3') | Amplification | | | | |
|--------------|--------------|--------------------------------------|---------------|--|--|--|--|
| HA | Bm-HA-1-F | TATTCGTCTCAGGGAGCAAAAGCAGGGG | 1778bp | | | | |
| | Bm-HA-1778 | ATATCGTCTCGTATTAGTAGAAACAAGGGT GTTTT | | | | | |
| NA | Ba-NA-1-F | TATTGGTCTCAGGGAGCAAAAGCAGGAGT | 1413bp | | | | |
| | Ba-NA-1413-R | ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT | | | | | |

Table 1: Amplification Primer Sequences of HA and NA Genes of AIV

2.2.5. Gene sequence evolutionary tree analysis and protein sequence homology analysis

Sequencing results of amplification products are used for BLAST comparative analysis; MEGA4.0 software is used for analysis of evolutionary tree, protein homology, key gene functional sites on related strains; SWISS MODEL online tool (http://www.swissmodel.expasy.org) is utilized for protein structure homologous modelling.

3. Research Results and Analysis

3.1. HA Titer Determination of Virus

Extract chicken embryo allantoic fluid of inoculated samples collected for HA titer determination. Viruses of collected samples can coagulate with chicken red blood cell, namely hemagglutination. While this red blood cell coagulation can be inhibited by specific immune serum. As shown in Figure 1, the dilution factor of holes from left to right is the Nth power of 2. Results show that the four virus samples are larger than the 4th power of 2 to and all HA positive. The fifth row is served as blank control.

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|-----|---|---|---|---|---|---|---|---|
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Fig. 1: HA Titer Determination of Isolated Virus

3.2. HA and NA Genes Amplification and Sequencing

Extract virus nucleic acid from HA positive samples, and apply RT-PCR amplification for HA and NA genes in use of HA and NA specific primers, with specific amplified band. Estimate the amplified band size through comparison between amplified band and DNA marker, and determine the target band by brightness and size of the amplified bands. The target band with gel extraction is then sent to the company for HA and NA gene sequencing, with the obtained HA and NA genes sequence.

3.3. HA Sites Analysis

3.3.1. HA cleavage site analysis

The World Organization for Animal Health (OIE) has determined amino acid sequence of influenza virus hemagglutinin cleavage site (the amount of alkaline amino acid) to be one of the indicators for identifying the pathogenicity of influenza virus. Low pathogenic viruses, such as H1, H2, H3 human influenza virus, contain only one alkaline amino acid at HA cleavage sites and low pathogenic viruses in H5 and H7 AI viruses generally contain one to two alkaline amino acids, while highly pathogenic H5 and H7 viruses usually contain four or more alkaline amino acids. As the cleavage site comparison (Table 2) shows, 4 strains in this study comply with the characteristics of low pathogenic influenza virus, with the HA gene cleavage site sequence all being PSRSSR \downarrow G and only two alkaline amino acids contained, namely arginine (R or Arg). But amino acid composition at HA cleavage site differs with early isolates Dk/HK/Y280/97 and Ck/HK/G9/97.

| | 2 | U | 1 0 | | |
|-----------------------------------|---|---|---|---|--|
| | Amino acid Sequence at the Cleavage site | Amino acid residue at the receptor binding site | | | |
| Virus | | Receptor binding site (183,190,226) | Right-edge of binding Pocket(224- 229) | Left-edge of binding Pocket (134- 138) | |
| A/chicken/Beijing/1/1994 | PARSSR/GL | NVQ | NGQQGR | GTSKA | |
| A/Quail/Hong_Kong/G1/1997 | | HEL | .DL | .I.R. | |
| A/chicken/Hunan/04.22_XKY 46/2015 | .S | .TL | LM | T. | |
| A/chicken/Hunan/04.22_XKY69/2015 | .S | .TL | LM | T. | |
| A/Duck/Hong_Kong/Y280/97 | | .TL | L | | |
| A/duck/Hong_Kong/Y439/1997 | A.N | HE. | .D | R. | |
| A/chicken/Hunan/04.14_YY0501/2015 | .S | .TL | LM | T. | |
| A/chicken/Hunan/04.14 YY0506/2015 | .S | .TL | LM | T. | |

Table 2: Amino Acid Sequence Analysis at the Cleavage site and Receptor Binding Site

3.3.2. Analysis of HA receptor binding site

The pocket-shaped receptor binding site of influenza virus hemagglutinin located at the head end is associated with the host specificity of influenza virus. At a, In the comparison of receptor binding sites, the amino acid sites 183, 190 and 226 of HA protein are related to the host specificity of H9N2 influenza virus. As Table 2 shows, in the four strains under the experiment, site 183 is N, 190 is T, 226 is L. Left-edge binding site of HA amino acid is GTSKA (134-138, with the serial number corresponding to H3HA), and the right-edge is NGQQGR (224-229, with the serial number corresponding to H3HA). Four strains at site HA137 are I and L at 226 (see table 2). Generally, amino acid at site 226 of avian borne AIV is Q, while that of AIV infecting human will mutate to L. 226L will enhance the binding capacity of virus with human receptor thus increasing the risk of human infection. The four tested strains all present L at 226, suggesting the risk of human infection with the virus.

3.3.3. Analysis of HA antigenic site

The host immune system can kill virus by identifying the surface antigen of virus. As the major surface antigens of influenza virus are located in the HA gene, through building up immunity beforehand for poultry, poultry immune system can be trained in advance to identify AIV, thereby reducing pathogenicity. At the same time, virus may escape from the host immune system by changing its surface antigen. Through analysis of HA antigenic determinant region for 4 strains, it is found that, compared with the reference strain Ck/BJ/1/94, the four strains present significant variation at antigenic determinant regions B, C and D. This suggests that the four virus strains mutate in face of existing vaccine, thus weakening the protection effect of vaccine. It shall be noted that, the currently applicable vaccines may not fully protect poultry from virus infection as antigenicity of prevailing viruses is subject to great variation.

3.3.4. Analysis on evolutionary relationship of virus

To see characteristics of molecular evolution of these four strains of H9N2 influenza viruses with different origins, we made evolution analysis on different gene segments of each strain. Through comparison analysis on evolutionary relationship of virus, origins and characteristics of distribution of similar virus at different times can be identified. Generally, viruses that similar to the virus to be tested in the evolutionary

tree have relatively similar characteristics. As for evolution analysis, sequences that have high homology with these isolates of BLAST results and other H9N2 influenza viruses of different times and places and isolated from different animals are selected for comparison.

The evolution analysis shows that evolutionary tree of HA gene can be divided into North American and Eurasian branches, of which the latter one can be further divided into three sub-branches, namely A/Chicken/Beijing/1/94 (H9N2) (Ck/Bei-like for short), Quail/HongKong/G1/97 (H9N2) (G1-like for short) and Duck/Hong Kong/Y439/97 (H9N2) (Y439-like or Korean-like for short), which are main virus branches prevalent in China in recent years.

Four virus strains used in this study all belong to Ck/Bei-like sub-branch of Eurasian branch, which also includes Duck/Hong Kong/Y280/97 (H9N2) and isolates of most chicken-derived and duck-derived H9N2 subtype influenza viruses in Chinese Mainland. It is observed that H9N2 influenza viruses belonging to Ck/Bei-like remains the main genotype of H9N2 subtype isolates in China. Sub-branch I of this branch is represented by Duck/Hong Kong/Y280/97, and the four chicken-derived isolates used in the study all belong to this sub-branch.

Similar to HA gene, evolutionary tree of NA gene can also be divided into North American and Eurasian branches. Isolates used in this study all belong to the latter one and Duck/Hong Kong/Y280/97 branch.

It is noteworthy that these four virus strains isolated in 2015, seen from phyletic evolution tree of HA and NA, have suffered great variation compared to typical Y280 and certain variation compared to strains that has the closet homologous relationship in GenBank database prevalent between 2012 and 2014. This reminds us that it's necessary to update existing H9N2 viral vaccine according to new prevalent strains. These findings are approximately agreed with our study results on site mutations of virus HA.

Structural simulation assists to build up a more visual understanding of site mutations of viral protein. And structural simulation is to, taking existing protein structure as the model and through comparing the protein to be tested and the known one, roughly simulate structure of the protein to be tested and then make adjustments on details through differences of amino acid. The higher similarity the protein to be tested and the known one have, the higher fidelity the simulated structure will have. Hereon, we, through choosing hemagglutinin sequence of A/chicken/Hunan/XKY 46/2015/04.22 as the target sequence, taking known protein structure of HA (H9) as the model and by means of homologous modelling, built 3D structural model of the HA as shown on Fig. 2.



Fig. 2: 3D Structural Model of HA

Belonging to allo-hexamer, HA of influenza A viruses subtype consists of three HA1 subunits and three HA2 subunits. HA1 and HA2 are formed through enzymolysis of protein HA at cleavage site, therefore, efficiency of enzymolysis will directly influence the structure of mature protein HA. HA can be divided into two parts, namely the upper inflated bulb and bacilliform basilar part. The bulb is composed of three HA1 subunits; the inner structure is composed of several lamellas β arranged in reverse, forming an activity cavity, the part where influenza virus bonded with sialic acid receptor on the host cell surface; and the outer structure includes several highly variable loops, the main antigen site of influenza virus. The basilar part

mainly consists of HA2 subunits, and, from the structural point of view, mainly of alpha helix, relating to fusion of influenza virus envelope and host cell. Meanwhile, basilar part is also the part where conserved antigen of influenza virus located.

The results show that HA of new strains have high similarity and is most similar to strains isolated from Zhejiang district of China. Representative amino acid residue of new strains is mainly distributed on the surface of HA head.

4. Discussion

H9N2 AIV mainly infects the poultry, causing cough, sneeze and declining laying rate and hatchability to chicken. As reported by Guo et al., virus Ck/Beijing/1/94 can cause a lethality reaching 80% to experimental chicken. According to the monitoring on influenza in Chinese Mainland currently, H9N2 subtype influenza viruses epidemic among chicken, duck and other minor poultries at present in China mainly belong to sub-branch Ck/Bei-like. However, viruses of this branch involve a wide source, and recombinant of different viruses derived from different animals is quite probable. Moreover, as the natural reservoir of influenza virus, the travelling wild duck plays an extremely important role in transmission and recombinant of H9N2 subtype influenza viruses.

4.1. Relation between Evolution and Transmission of Gene Sequence of Influenza Virus

Four strains of AVIs subtype H9N2 belonging to Ck/Bei-like sub-branch with different sources and different isolated time preserved in the lab are selected as the experimental object, and whole genome sequencing and evolution analysis are conducted on these four strains, providing theoretical basis for studying its characteristics of pathogenicity and transmissibility. Evolution of the gene shows that HA gene and NA gene of the four strains all belong to sub-branch Ck/Bei-like, which suggests that, these viruses are the result of long-term epidemic and evolution in China without virus mixing or gene reassortment with other branches during the evolution process.

The four domestic isolated strains all belong to Ck/Bei-like, but different sub-branches. This shows that viruses of the branch will change during the process of natural selection and cause different variation; this can be known in detail from variation of protein HA at important sites.

Based on study of the evolutionary tree, we also finds out that viruses similar to these four virus strains are available in many cities of China, including Zhejiang, Jiangxi and Jiangsu, etc. This also indicates that viruses isolated and measured by us have wide presence in many regions of China, which is possibly caused by transportation and dealing of disease-carrying poultry. Meanwhile, since influenza virus of different regions involves different sources, gene re-assortment and exchange between different genes, leading to increased risks of virus infecting human.

4.2. Relationship between Gene Mutation at Important Sites and Properties of Virus

Analysis on gene mutation at important sites is conductive to perceiving changes of virus properties. Relative to AVI, mutation at HA cleavage site, important sites of receptor binding domain and important sites of S antigen domain is the main focus. The analysis shows that cleavage sites of the four viruses are different from the reference one, indicating that the virus is everlastingly changing. However, mutation at HA cleavage site didn't lead to obvious quantity increasing of alkaline amino acid (R or K), demonstrating that the virus still belong to low pathogenic AVI.

Among receptor binding domain of HA gene, especially at site 226, the four virus strains all appear to be L. It is well documented that, H9N2 strains which appears to be L at site 226 of HA protein molecule has the feature of infecting human beings. As found by Guo Yuanji et al, all H9N2 strains isolated from human and pigs appear to be leucine at site 226 of HA protein molecule, while the previous H9N2 AVI with avian origin appear to be Q. This change reminds us that the difference of the four strains at site 226 of HA protein may lead to infectious feature to humans. Although there is still no human being infected by H9N2 at present, it is worthy of our lasting attention in the future.

Through comparison and analysis on virus S antigen, we found great variation at antigenic determinants B, C and D. Changes of virus S antigen means that the virus can escape from the protection of vaccine.

Among the five antigenic determinants of AVI, three has suffered variation, meaning that protective effect of existing vaccine is declining and further reminding us that new evaluation on existing vaccines and development of new protective vaccines are necessary.

5. Conclusion

In this study, amplification, sequencing, genetic evolution and analysis on crucial functional sites are conducted on HA and NA genes of the four isolated H9N2 subtype AIV strains. The results show that HA and NA genes of the four strains all belong to branch Ck/Bei-like, but relatively great variation has occurred compared to the previous typical strains and epidemic strains. Through analysis on important sites of HA gene, variations of viruses are observed at HA cleavage site, receptor binding domain and S antigen domain. Such variation may lead to infectious feature to humans and viruses escaping from protection of existing vaccines. This reminds us to strengthen prevention, avoid infection of new epidemic viruses on humans and the necessity of updating vaccine for new epidemic viruses. Results of this study provide an important genetic clue for control and presentation of H9N2 subtype AIV.

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